CHEMICAL REVIEWS

Review

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How Biology Handles Nitrite

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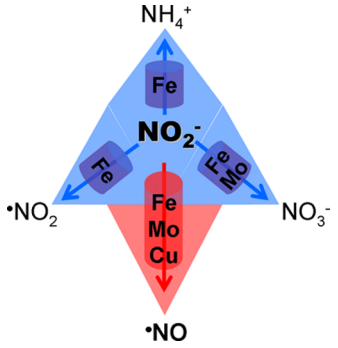

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1. INTRODUCTION

Nitrite is one of the players in the broad nitrogen biogeochemical cycle. This nitrogen oxo-anion is involved in key pathways crucial to life on Earth and to the planetary "recycling" of nitrogen. From a human perspective, nitrite (and nitrate) is an important food preservative that has been used for the last five millennia.[1] This successful nitrite utilization was, however, overshadowed in the 1970s, when it was suggested that nitrite might increase the incidence of cancer, through the formation of *N*-nitrosamines.[2−8] Recently, another twist took place, and nitrite is now being rediscovered as a beneficial molecule, endogenously formed or therapeutically added, involved in cell survival during hypoxic events, as will be here discussed.

In this Review, we will review the physiological role of nitrite in the biochemical cycle of nitrogen (section 2.1) and in mammalian and plant signaling pathways (sections 2.2.1 and 2.2.2). A very brief description of potential bacterial signaling (section 2.2.3) will also be included. In the "Nitrite in the Nitrogen Cycle" section, the main, well-established, pathways will be briefly described, with emphasis on the nitrite-mediated reactions. In the "Nitrite in Signaling Pathways" section, the nitrite-mediated reactions will be discussed with a deeper detail: the nitrite-mediated signaling and damaging pathways are a (comparatively) recent and controversial area, and the Review will be oriented to discuss the feasibility of these novel pathways mainly from the chemical point of view. As will be described in section 2, the living organisms use nitrite for remarkably different purposes, oxidizing and reducing it.

Subsequently (section 3), several key reaction mechanisms will be analyzed at a molecular level of detail, and structure/activity relationships will be, as much as possible, systematically explored to discuss the mechanistic strategies that biology developed to reduce and oxidize nitrite.

The global aim is to review the present functional, structural, and mechanistic knowledge of nitrite reduction/oxidation, to assess in what extent we understand how nitrite is handled by living organisms. Nitrite formation is outside the scope of this Review. This knowledge is essential for the comprehension of the global nitrogen biochemical cycle and, consequently, for the comprehension of the impressive changes the human activities

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are introducing in the cycle. Furthermore, the wealth of information gathered and discussed enables one to further evaluate the feasibility and physiological significance of the presently accepted "avenues" of NO formation in humans and plants and to foresee new potential pathways.

2. BIOLOGICAL FATE OF NITRITE

2.1. Biological Fate of Nitrite – Nitrite in the Nitrogen Cycle

Nitrogen is absolutely essential for life, being the fourth most abundant element in living organisms (behind hydrogen, oxygen, and carbon).[9] Nitrogen is used by all organisms for the biosynthesis of amino acids, nucleosides, and other fundamental compounds, and two nitrogen assimilatory pathways were developed to provide the necessary reduced nitrogen. This is then "recycled" in a universal "organic nitrogen pool". In addition, some organisms also use nitrogen compounds as substrates for "respiration", and for that purpose several nitrogen dissimilatory pathways have also evolved. The nitrogen biochemical cycle (Figure 1, Table 1) keeps this

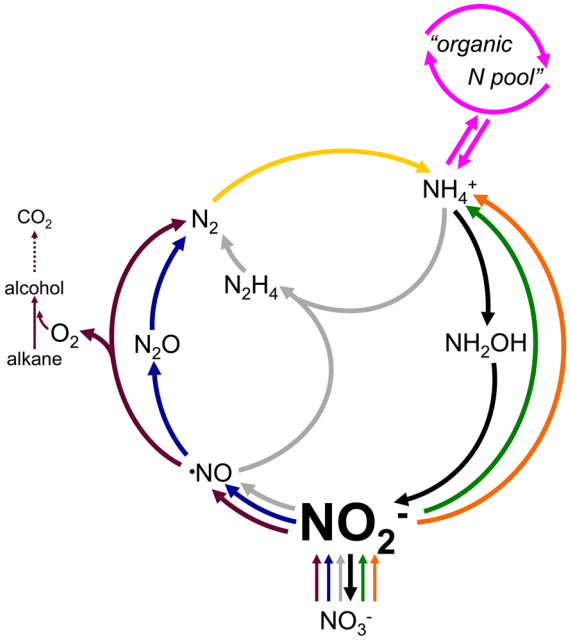
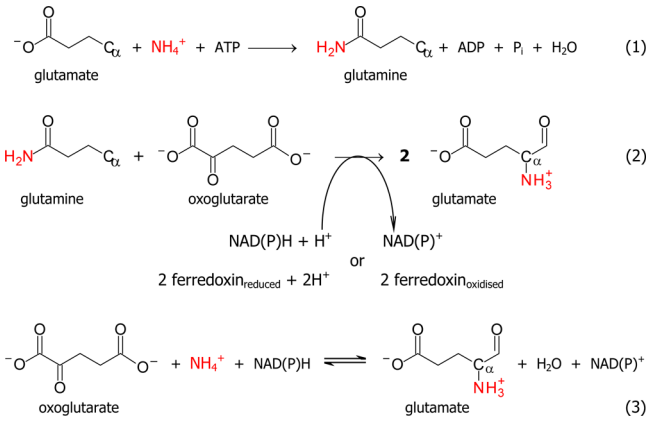


Figure 1. Biochemical cycle of nitrogen. Dinitrogen fixation, yellow arrow; assimilatory ammonification, orange arrows; "organic nitrogen pool", pink arrows; denitrification, blue arrows; dissimilatory nitrate reduction to ammonium (DNRA), green arrows; nitrification, black arrows; anaerobic ammonium oxidation (AnAmmOx), gray arrows; "denitrification/intra-aerobic methane oxidation", violet arrows.

element in forms available to support live on Earth, "starting" with fixation from the atmosphere (the largest nitrogen source) and "recycling" it through the dissimilatory pathways.[10−17]

2.1.1. Classic and New Pathways. Two nitrogen assimilatory pathways provide the reduced nitrogen (ammonium) essential for biosynthetic purposes (Figure 1, yellow and orange arrows, Table 1). The organisms capable of dinitrogen fixation (some free-living archea and bacteria, e.g., *Azotobacter*, and symbiotic bacteria, e.g., *Rhizobium*) possess a unique pathway, where the atmospheric dinitrogen is directly reduced to ammonium, in a reaction catalyzed by molybdenum/iron-dependent nitrogenases[18] (Figure 1, yellow arrow).[22−33] All of the other organisms, prokaryotic and eukaryotic, depend on environmentally (soils, oceans, and crust) available ammonium and nitrate/nitrite. The nitrate assimilation is dependent on two sequential reactions (assimilatory ammonification, Figure 1, orange arrows): first, the reduction of nitrate to nitrite, catalyzed by molybdenum-dependent nitrate reductases (NaR; described in refs 1667,1679), and then the reduction of nitrite to ammonium, catalyzed by sirohaem-containing nitrite reductases (CSNiR; described in section 3.1.2).[34−41] The electron source to carry out the nitrite reduction to ammonium is (i) the photosynthetically reduced ferredoxin, in photosynthetic organisms (e.g., cyanobacteria and chloroplasts of photosynthetic eukaryotes[36,39,42]), or (ii) the reduced pyridine nucleotide pool, in most heterotrophs, but also in some phototrophs (e.g., nonphotosynthetic tissues of higher plants[42] and *Rhodobacter capsulatus*,[43,44] respectively). For that reason, there are two types of assimilatory CSNiR: ferredoxin-dependent and NAD(P)H-dependent enzymes. Within each type, the enzymes share sequence and structural similarity, regardless of their prokaryotic or eukaryotic origin. Both types hold a sirohaem, where the nitrite reduction takes place, and an iron−sulfur center (Fe/S). Yet NAD(P)H-dependent enzymes contain an additional FAD domain that is involved in the NAD(P)H binding and oxidation.[34−44]

Ammonium then enters the "organic nitrogen pool" (Figure 1, pink arrows) in the form of two amino acids, glutamine and glutamate, through the concerted action of glutamine synthase (eq 1) and glutamate synthase (eq 2) of bacteria, fungi, and plants. Ammonium can also be directly incorporated into glutamate through the glutamate dehydrogenase enzyme present in all forms of life (eq 3). However, under most physiological conditions, glutamate dehydrogenase catalyzes instead the reverse reaction, yielding ammonium in amino acid catabolism. Hence, the key enzyme that controls the entrance of the "organic nitrogen pool" is the glutamine synthase, not surprisingly one of the most complex regulatory enzymes. Besides this crucial role, glutamine synthase is also of main importance in animals, where it is responsible for the removal of toxic ammonium.



The organic nitrogen, in the form of amino and amide groups, can now be transferred to other amino acids and several other nitrogen-containing biomolecules, supporting the vital biosynthetic needs. The organic nitrogen is then "recycled" between all living organism, through chain foods, waste products, and organic decay. Eventually, the organic nitrogen is converted back to ammonium and returned to the environment, in a process called mineralization, thus closing the "organic" part of the nitrogen cycle ((Figure 1, pink arrows).

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Table 1. Main Pathways of the Biochemical Cycle of Nitrogen

pathway	step	enzyme responsible for the step (organism where it is found; examples)	figure [a]	section [b]
dinitrogen fixation	$N_2 \longrightarrow NH_4^+$	molybdenum/iron-containing nitrogenase (free-living archea and bacteria and symbiotic bacteria; *Azotobacter*, *Rhizobium*) (small minority: iron only and vanadium/iron-dependent nitrogenases)	1, yelow arrow	2.1.1.
assimilatory ammonification	$NO_3^- \longrightarrow NO_2^-$	molybdenum-containing nitrate reductase (NaR) (prokaryotes and eukaryotes, phototrophs and heterotrophs; cyanobacteria, *Rhodobacter capsulatus*, chloroplasts, non-photosynthetic tissues of higher plants)	1, orange arrows	2.1.1., 2.2.3. note 1667 of 3.3.1.
	$NO_2^- \longrightarrow NH_4^+$	sirohaem-containing nitrite reductase (CSNiR) (see first step of assimilatory ammonification)	1, orange arrows, 23	2.1.1., 3.1.2.
denitrification	$NO_3^- \longrightarrow NO_2^-$	molybdenum-containing nitrate reductase (NaR) (archaea, eubacteria and some eukaryotes; *Pseudomonas aeruginosa*, *Alcaligenes xylosoxidans*, fungi, protozoa)	1, blue arrows, 2, 11	2.1.1., 2.2.3. note 1667, 1679 of 3.3.1.
	$NO_2^- \longrightarrow {}^{\bullet}NO$	d_1 haem-containing nitrite reductase (Cd_1NiR) (see first step of denitrification)	1, blue arrows, 2, 24,25	2.1.1., 3.2.1.
		copper-containing nitrite reductase (CuNiR) (see first step of denitrification)	1, blue arrows, 2, 26, 27	2.1.1., 3.2.2.
	${}^{\bullet}NO \longrightarrow N_2O$	haem iron/non-haem iron-containing nitric oxide reductase (see first step of denitrification)	1, blue arrows, 2	2.1.1., 2.1.2.
	$N_2O \longrightarrow N_2$	iron/copper-containing nitrous oxide reductase (see first step of denitrification)	1, blue arrows, 2	2.1.1.
dissimilatory nitrate reduction to ammonium (DNRA)	$NO_3^- \longrightarrow NO_2^-$	molybdenum-containing nitrate reductase (NaR) (prokaryotes; *Wolinella succinogenes*)	1, green arrows	2.1.1., 2.2.3. note 1667 of 3.3.1.
	$NO_2^- \longrightarrow NH_4^+$	*c* haem-containing nitrite reductase (CcNiR) (see first step of DNRA)	1, green arrows 21, 22	2.1.1., 2.1.2., 3.1.1.
nitrification	$NH_4^+ \longrightarrow NH_2OH$	iron/copper-containing ammonium monooxygenase (archaea and bacteria; *Nitrosomonas europaea*)	1, black arrows	2.1.1.
	$NH_2OH \longrightarrow NO_2^-$	P_{460} haem-containing hydroxylamine oxidoreductase (HAOR) (see first step of nitrification)	1, black arrows	2.1.1., 3.1.3.
	$NO_2^- \longrightarrow NO_3^-$	molybdenum-containing nitrite oxidoreductase (MoNiOR) (bacteria; *Nitrobacter*, *Nitrospira*)	1, black arrows	2.1.1., 3.3.1.
anaerobic ammonium oxidation (AnAmmOx)	$NO_2^- \longrightarrow {}^{\bullet}NO$	d_1 haem-containing nitrite reductase (Cd_1NiR) (prokaryotes; "*Candidatus* Kuenenia stuttgartiensis", "*Candidatus* Scalindua profunda")	1, blue arrows, 24, 25	2.1.1., 3.2.1.
	$NH_4^+ + {}^{\bullet}NO \longrightarrow N_2H_4$	*c* haem-containing hydrazine synthase (see first step of AnAmmOx)	1, gray arrows	2.1.1., 2.1.2.
	$N_2H_4 \longrightarrow N_2$	*c* haem-containing hydrazine oxidoreductase (see first step of AnAmmOx)	1, gray arrows	2.1.1.
"denitrification/ intra-aerobic methane oxidation"	$NO_2^- \longrightarrow {}^{\bullet}NO$	d_1 haem-containing nitrite reductase (Cd_1NiR) (prokaryotes; "*Candidatus* Methylomirabilis oxyfera")	1, blue arrows, 2, 24, 25	2.1.1., 3.2.1.
	$2{}^{\bullet}NO \longrightarrow N_2 + O_2$	hypothetical NO dismutase (see first step of "denitrification/intra-aerobic methane oxidation")	1, violet arrows, 2	2.1.1., 2.1.2.

[a]Figures where the reaction and/or enzyme is represented. [b]Sections where the reaction and/or enzyme is discussed.

The biochemical cycle of nitrogen continues with four main "classic" nitrogen dissimilatory pathways, where nitrogen compounds are used as electron donors/acceptors to derive energy (Figure 1, Table 1): (i) nitrate is anaerobically reduced to dinitrogen (denitrification − Figure 1, blue arrows), or (ii) to ammonium (dissimilatory nitrate reduction to ammonium (DNRA) − Figure 1, green arrows), (iii) while ammonium is aerobically oxidized to nitrate (nitrification − Figure 1, black arrows), or (iv) anaerobically oxidized to dinitrogen (anaerobic ammonium oxidation (AnAmmOx) − Figure 1, gray arrows).

Denitrification[45−47] is found in a wide range of organisms, from archaea to eubacteria and even in some eukaryotes (e.g., fungi,[48] protozoa, and benthic Foraminifera and *Gromiida*[54,56−60]). It occurs in both autotrophic and heterotrophic organisms, coupled to the anaerobic oxidation of organic carbon. Denitrification encompasses the anaerobic nitrate reduction to molecular dinitrogen, through four sequential steps (eq 4), involving three one-oxygen atom abstractions and one N−N bond formation ($NO \rightarrow N_2O$), catalyzed by specific metalloenzymes dependent on molybdenum, iron, and copper (Figure 1, blue arrows). The nitrite reduction to nitric oxide radical[61] (NO), in particular, is catalyzed by two different types of enzymes: iron-dependent and copper-dependent nitrite reductases. Denitrifier organisms have either one of these enzymes, but not both. The iron-dependent enzyme arbors *c* and d_1 haems (d_1 haem-containing nitrite reductase (Cd_1NiR),

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discussed in section 3.2.1).[46,47,62] This enzyme obtains the necessary electrons from a range of structurally different "soluble" electron carriers (pseudospecificity[63]), such as *c* type cytochromes (c_{550}, c_{551}, c_{554}) and copper proteins (azurins and pseudoazurins). The copper-containing nitrite reductases (CuNiR, discussed in section 3.2.2) are classified into two subgroups depending on their color being blue (e.g., from *Alcaligenes xylosoxidans*[64]) or green (e.g., from *Alcaligenes faecalis* or *Achromobacter cycloclastes*). The CuNiR are more widespread, although less abundant; they are less promiscuous with the electron donors than the Cd_1NiR, being pseudospecific in their interaction with azurin and cytochrome c_{551} (blue CuNiR) and pseudoazurin (green CuNiR).[66−68]

$$NO_3^- \rightarrow NO_2^- \rightarrow {}^{\bullet}NO \rightarrow N_2O \rightarrow N_2 \quad (4)$$

DNRA (Figure 1, green arrows) is a different strategy to reduce nitrate, employed to grow anaerobically. In this pathway, nitrite is directly reduced to ammonium (dissimilatory ammonification) with a multi-*c*-haem-containing enzyme (*c* haem-containing nitrite reductase (CcNiR), discussed in section 3.1.1). The necessary electrons are derived from the anaerobic oxidation of organic carbon through the membrane quinone pool.[69]

Conversely, nitrifiers use the aerobic oxidation of ammonium to nitrate, for the chemoautotrophic fixation of inorganic carbon (nitrification − Figure 1, black arrows).[70,71] Hence, DNRA and nitrification can be regarded as short-circuits that bypass the vast dinitrogen reservoir. Aerobic ammonium-oxidizing bacteria (e.g., *Nitrosomonas europaea*) use ammonium almost exclusively as the electron donor in "respiration" and oxidize it to hydroxylamine (catalyzed by ammonium monooxygenase), and then to nitrite (catalyzed by hydroxylamine oxidoreductase (HAOR)).[72] In addition, also archaea (from marine and soil environments) seem to be able to oxidize ammonium for chemolithoautotrophic growth.[92−96]

Subsequently, nitrite-oxidizing bacteria (e.g., *Nitrobacter* or *Nitrospira* species) are responsible for the final oxidation of nitrite to nitrate, through a reaction catalyzed by a molybdenum-containing nitrite oxidoreductase (MoNiOR, described in section 3.3.1). These bacteria are chemolithoautotrophs, phylogenetically heterogeneous, that derive energy from the nitrite oxidation to nitrate, in a strictly aerobic process, where all of the carbon needs can be satisfied with carbon dioxide assimilation (via Calvin cycle).[97−103] Nitrite oxidation "closes" the nitrification process and is believed to be the principal source of nitrate under aerobic conditions.

Ammonium can also be anaerobically oxidized in an exergonic pathway (AnAmmOx - Figure 1, gray arrows) used for the anaerobic chemoautotrophic growth of planctomycetes (e.g., "*Candidatus* Kuenenia stuttgartiensis" or "*Candidatus* Scalindua profunda").[104−113] In this pathway, ammonium is first oxidized by NO to yield hydrazine, in a reaction catalyzed by the dihaem-*c*-containing hydrazine synthase. Hydrazine is then oxidized to dinitrogen by the octa-haem-*c*-containing hydrazine oxidoreductase.[106,107,111,114,115] The necessary NO is formed via nitrite reduction by Cd_1NiR, an enzyme associated with the "classical" denitrification. In this way, the anaerobic ammonium oxidation is coupled to the nitrite reduction. In this context, it is worth mentioning that an AnAmmOx bacterium (strain KSU-1) was hypothesized to have a CuNiR enzyme, suggesting that the enzymatic "machinery" of the AnAmmOx organisms might be more "flexible" than initially thought.[116] According, AnAmmOx likely provided the first complete "recycling" of fixed nitrogen to dinitrogen and fulfilled this role until the emergence of the copper-containing nitrous oxide reductase, on the oxic era.[106,117] Operating in marine, freshwater, and terrestrial ecosystems, AnAmmOx is presently one of the major enigmas of the nitrogen biochemical cycle.

In addition to those well-recognized four nitrogen dissimilatory processes, several new processes are being identified (see, e.g., refs 118−121). The anaerobic phototrophic nitrite oxidation and the "denitrification/intra-aerobic methane oxidation" pathway represent two interesting examples, further described below.

Besides the well-known aerobic nitrite oxidation (part of nitrification), it was recently reported the occurrence of an anaerobic photosynthetically driven nitrite oxidation.[122] In this not yet characterized process, anoxygenic phototrophic bacteria (*Thiocapsa* and *Rhodopseudomonas*) use nitrite as electron donor for photosynthesis. This anaerobic nitrite oxidation pathway was found to be strictly light-dependent and, as foreseen, also molybdenum-dependent (metal obligatory for the MoNiOR activity of aerobic organisms; see section 3.3.1).[123] Although the utilization of inorganic nitrogen compounds as electron sources for anoxygenic photosynthesis had been predicted long ago,[124,125] only recently could it be demonstrated,[122] and this is the only known case of a photosynthetically driven oxidation in the nitrogen cycle.

Also remarkable is the novel "denitrification/intra-aerobic methane oxidation" pathway that links the nitrogen and carbon cycles (Figure 1, violet arrows). Recently, a new anaerobic bacterium was described, "*Candidatus* Methylomirabilis oxyfera", that couples the reduction of nitrite to dinitrogen (denitrification) with the oxidation of methane.[126−133] Methane is one of the least reactive organic molecules, and, so far, no known biochemical mechanism has been able to explain its activation in the absence of oxygen or reverse the last step of methanogenesis, which poses a problem for an anaerobic organism. Despite that, the anaerobic "M. oxyfera" encoded, transcribed, and expressed the well-established aerobic pathway for methane oxidation to carbon dioxide, the particulate methane monooxygenase complex.[132] On the other hand, "M. oxyfera" lacked the known gene cluster necessary to produce the enzyme that reduces nitrous oxide to dinitrogen, questioning how the organism produces the dinitrogen. Yet, "M. oxyfera" expresses a Cd_1NiR enzyme.[132] To solve this conflict, a new pathway was proposed in which Cd_1NiR-catalyzed NO is converted into dinitrogen and dioxygen by a NO dismutase enzyme (eq 5).[128] In this pathway, the last two steps of "classical" denitrification, NO and nitrous oxide reduction, would be replaced by NO dismutation, to yield, not only dinitrogen, but also dioxygen (Figure 2). The dioxygen formed intracellularly through this "version" of denitrification would be responsible for the intra-aerobic oxidation of methane, with the remaining dioxygen being used in "respiration" by terminal "respiratory" oxidases.[128,134] It was suggested that the "denitrification/intra-aerobic methane oxidation" pathway could have enabled the organisms to thrive on the abundant methane in the archaean atmosphere,[135] without direct dependence on oxygenic photosynthesis. However, it is debatable that the aerobic methane oxidation pathway would have evolved before a significant increase of the dioxygen in the atmosphere was attained.

$$2{}^{\bullet}NO \rightarrow N_2 + O_2 \quad (5)$$

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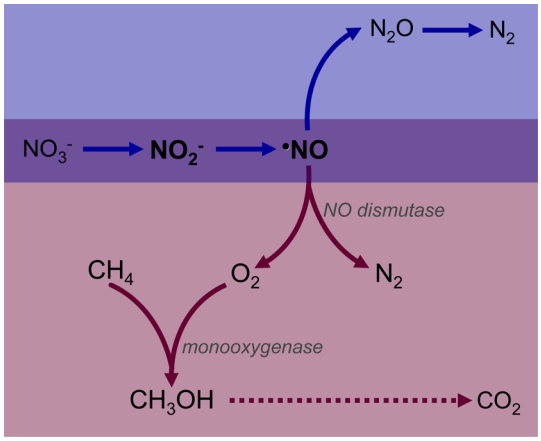

Figure 2. "Denitrification/intra-aerobic methane oxidation" pathway. Comparison of denitrification and "denitrification/intra-aerobic methane oxidation" pathways, shaded in blue and violet, respectively. See text for details.

It should be noted that the suggested NO dismutase was not yet identified, but two of the "M. oxyfera" most abundant proteins are potential candidates.[128,130] Genetic analysis revealed that those two proteins are the gene product of two quinol-dependent NO reductases paralogues with unusual features: (i) a different catalytic center, holding a glutamine instead of a glutamate residue and an asparagine instead of one of the conserved coordinating histidines residues of the "classic" NO reductases, (ii) probable absence of a proton channel, and (iii) probable absence of a quinol-binding site. Accordingly, these proteins would be unable to accept electrons or uptake protons from an external donor, two features that would compromise their function as NO reductases (eq 6), but would not hamper a NO dismutase activity (eq 5). Furthermore, despite the high expression of these two unusual NO reductase-like proteins, no appreciable nitrous oxide production is detected during nitrite-dependent methane oxidation. Therefore, it is tempting to speculate that these two highly expressed proteins could be responsible for the dismutation of NO.[128,130] According to this hypothesis, the NO dismutation would be achieved with a NO reductase-like active center, that is, with a haem/nonhaem iron center. To be confirmed, it would not be a novelty: chlorite dismutases (eq 7) are haemic enzymes,[136,137] and iron-dependent superoxide dismutases (eq 8) hold nonhaem iron.[138,139] In addition, a binuclear active center would be helpful to hold the reaction intermediates in place to cleave the N−O bond and rearrange the new N−N and O−O bonds. Nevertheless, the reaction mechanism of NO dismutation is presently difficult to envisage, either with haem/nonhaem or with other metal centers. In conclusion, the nature of the suggested NO dismutase must wait for the purification and rigorous characterization of the "M. oxyfera" proteins.[128,130]

$$2^{\bullet}NO + 2H^{+} + 2e^{-} \rightarrow N_2O + H_2O \quad (6)$$

$$OClO^{-} \rightarrow Cl^{-} + O_2 \quad (7)$$

$$2O_2^{\bullet -} + 2H^{+} \rightarrow H_2O_2 + O_2 \quad (8)$$

Remarkably, it was also suggested that a similar pathway is possibly used by the unrelated γ-proteobacterial strain HdN1 to synthesize oxygen, from nitrate or nitrite, for "intra-aerobic" hexadecane oxidation.[130,140,141] If confirmed, the "oxygenic denitrification" could be more widespread than initially thought and nitrite could became a relevant dioxygen precursor,[142] further highlighting the biological relevance of nitrite.

The old and new dissimilatory pathways "close" the nitrogen biochemical cycle (Figure 1, Table 1), with the fixed nitrogen being lost to the atmosphere, soils, crust, and oceans.

Over the last years, the discovery and addition of new organisms involved in the "classic" processes and the scrutiny of new ways to transform nitrite add more complexity to the system, revealing an intricate network of pathways. The regulation and control of these complex pathways is still a challenging problem, and future research will certainly change some of the presently established dogmas.

2.1.2. Nitrite and Nitrogen "Recycling". This section cannot be concluded without emphasizing the NO relevance to the biological nitrogen "recycling": the complete biological "recycling' of nitrogen requires the formation of a N−N bond, to ultimately produce dinitrogen, which is accomplished, as far as is presently known, only with the oxidizing power of NO.

The only recognized enzymes capable of bonding two nitrogen atoms are the NO reductase (eq 9; denitrification − Figure 1, blue arrows) and the hydrazine synthase (eq 10; AnAmmOx − Figure 1, gray arrows), and both use the oxidizing power of NO. In addition, also CcNiR (DNRA − Figure 1, green arrows) was described to catalyze the nitrous oxide formation from NO (eq 11; see section 3.1.1.2 for details). Moreover, the suggested NO dismutase (eq 12), crucial for the "denitrification/intra-aerobic methane oxidation" (Figure 1, violet arrows), is thought to use NO to form dinitrogen directly. This consensus supports the hypothesis that NO may have been the first deep electron sink on Earth, before the emergence of dioxygen.[111,151] Because NO is formed from the nitrite reduction, these NO roles, general oxidant and "maker" of N−N bonds, further emphasize the biological importance of nitrite. Additionally, as will be discussed in the following sections (sections 2.2.1.1 and 2.2.2.1), the nitrite reduction to NO is also employed by mammals and plants to sustain the NO-dependent signaling pathways under hypoxic/anoxic conditions.

$$2^{\bullet}NO + 2H^{+} + 2e^{-} \xrightarrow{\text{NO reductase } (b \text{ haem + non-haem iron})} N{\equiv}NO + H_2O \quad (9)$$

$$^{\bullet}NO + NH_4^{+} + 2H^{+} + 3e^{-} \xrightarrow{\text{hydrazine synthase } (c \text{ haem})} {}_2HN{\equiv}NH_2 + H_2O \quad (10)$$

$$2^{\bullet}NO + 2H^{+} + 2e^{-} \xrightarrow{\text{CcNiR } (c \text{ haem})} N{\equiv}NO + H_2O \quad (11)$$

$$2^{\bullet}NO \xrightarrow{\text{NO dismutase (haem + non-haem iron???)}} N{\equiv}N + O_2 \quad (12)$$

2.2. Biological Fate of Nitrite − Nitrite in Signaling Pathways

Apart from the most obvious molecules, like amino acids and nucleosides, nitrogen is also necessary for the biosynthesis of another essential molecule: the NO. NO is a signaling molecule involved in several physiological processes, in both prokaryotes and eukaryotes, and, as will be described, nitrite is an important source of NO (sections 2.2.1−2.2.3, Tables 2, 5, and 6). Nitrite oxidation to nitrate and nitrogen dioxide radical, in signaling and deleterious pathways, will be also addressed (sections 2.2.1.2 and 2.2.2.2, Table 4).

2.2.1. Nitrite in Signaling Pathways in Mammals. *2.2.1.1. Nitrite Reduction in Mammals.* In mammals, NO controls a plethora of functions, including vasodilation (through the well-known activation of guanylate cyclase), neurotransmission, immune response, platelet aggregation, apoptosis, and gene expression, and mediates a wide range of both antitumor and antimicrobial activities.[152] Nevertheless, the

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Table 2. Proteins Involved in Nitrite Reduction to NO in Mammals

protein	"classic" reaction "classic" functions	site of nitrite reduction reaction of nitrite reduction × major drawback [a]	figure [b]	section [c]
haemoglobin (Hb) (blood)	dioxygen transport	*b* haem (Fig. 19(a)), penta-coordinated by a histidine residue $NO_2^- + 2H^+ + (Hb)Fe^{2+} \longrightarrow (Hb)Fe^{3+} + {}^{\bullet}NO + H_2O$ × NO scavenging by haem	3, 4-9	2.2.1.1.1.
myoglobin (Mb) (cardiac, skeletal and smooth muscle)	dioxygen transport; recently, several *novel* functions were suggested: cardiac NO homeostasis, O_2 sensing, ROS scavenging, intracellular fatty acid transport	*b* haem (Fig. 19(a)), penta-coordinated by a histidine residue $NO_2^- + 2H^+ + (Mb)Fe^{2+} \longrightarrow (Mb)Fe^{3+} + {}^{\bullet}NO + H_2O$ × NO scavenging by haem	3, 4, 9	2.2.1.1.1.
neuroglobin (Nb) (nerve tissues)	no "classic" functions; it is a *new* protein, whose functions are still controversial: cellular redox state sensing, scavenging/sensing of ROS/RNS, O_2, CO, NO, inhibition of Cc-induced apoptosis	*b* haem (Fig. 19(a)), hexa-coordinated by two histidine residues hexa-coordination reversible $NO_2^- + 2H^+ + (Nb)Fe^{2+} \longrightarrow (Nb)Fe^{3+} + {}^{\bullet}NO + H_2O$ × NO scavenging by haem × mechanism of conversion of penta into hexa-coordinated molecules under hypoxia/anoxia	3, 4, 9, 10	2.2.1.1.2. (a)
cytoglobin (Cb) (apparently all tissue types)	no "classic" functions; it is a *new* protein, whose functions are still controversial: NADH oxidase, O_2 sensor, NO scavenger/formation	*b* haem (Fig. 19(a)), hexa-coordinated by two histidine residues hexa-coordination reversible $NO_2^- + 2H^+ + (Cb)Fe^{2+} \longrightarrow (Cb)Fe^{3+} + {}^{\bullet}NO + H_2O$ × NO scavenging by haem × mechanism of conversion of penta into hexa-coordinated ???	3, 4, 9	2.2.1.1.2. (a)
cytochrome *c* (Cc) (all tissue types)	electron transfer complex III ⟶ complex IV mitochondrial oxidative phosphorylation; "non-classic" functions include apoptotic signalling molecule, $O_2^{\bullet-}$ scavenger, lipid peroxidation catalyst, peroxidase-like enzyme, nitrating agent	*c* haem (Fig. 19(a)), hexa-coordinated by a histidine (proximal) and a methionine (distal) residues hexa-coordination reversible $NO_2^- + 2H^+ + (Cc)Fe^{2+} \longrightarrow (Cc)Fe^{3+} + {}^{\bullet}NO + H_2O$ × NO scavenging by haem	3, 9, 11	2.2.1.1.2. (b)
xanthine oxidase (XO) / xanthine dehydrogenase (XD) (liver, intestine, heart, mammal gland, small vessels endothelial and epithelial cells)	xanthine ⟶ urate catabolism of purines; "non-classic" functions include metabolism of xenobiotics, ROS-mediated signalling cascades	molybdenum centre of XO family type (Fig. 19(c)) $NO_2^- + 2H^+ + (XO/XD)Mo^{4+} \longrightarrow (XO/XD)Mo^{5+} + {}^{\bullet}NO + H_2O$ $NO_2^- + 2H^+ + (XO/XD)Mo^{5+} \longrightarrow (XO/XD)Mo^{6+} + {}^{\bullet}NO + H_2O$ × competition with O_2 × conversion of XD into XO under hypoxia/anoxia	3, 11, 12	2.2.1.1.3., 3.2.3.
aldehyde oxidase (AO) (liver, heart, lung, kidney, brain and eye)	aldehyde ⟶ carboxylate still controversial: metabolism of xenobiotics, neurotransmitters and retinoic acid, ROS-mediated signalling cascades	molybdenum centre of XO family type (Fig. 19(c)) $NO_2^- + 2H^+ + (AO)Mo^{4+} \longrightarrow (AO)Mo^{5+} + {}^{\bullet}NO + H_2O$ $NO_2^- + 2H^+ + (AO)Mo^{5+} \longrightarrow (AO)Mo^{6+} + {}^{\bullet}NO + H_2O$ × competition with O_2	3, 11	2.2.1.1.3.
carbonic anhydrase [d]	$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^- + H^+$ acid-base homeostasis and electrolyte balance	zinc (Zn^{2+}) $2NO_2^- + 2H^+ \rightleftharpoons 2HNO_2 \rightleftharpoons H_2O + N_2O_3$ $N_2O_3 \rightleftharpoons {}^{\bullet}NO + {}^{\bullet}NO_2$ × reaction mechanism in absence of a redox active metal is controversial × N_2O_3 dismutation to yield NO is controversial	3, 11	2.2.1.1.5.
protein-independent nitrite reduction [e]		$2NO_2^- + 2H^+ \rightleftharpoons 2HNO_2 \rightleftharpoons H_2O + N_2O_3$ $N_2O_3 \rightleftharpoons {}^{\bullet}NO + {}^{\bullet}NO_2$ × N_2O_3 dismutation to yield NO is controversial × a reducer is needed	3	2.2.1.1.4.

[a]Major drawback concerning the feasibility of nitrite reduction to release bioactive NO. [b]Figures where the protein is represented. [c]Sections where the protein is discussed. [d]Carbonic anhydrase is suggested to catalyze nitrous acid hydration and not nitrite reduction. It was here included to systematize all of the information provided under section 2.2.1.1. [e]Protein-independent nitrite reduction was here included to systematize all of the information provided under section 2.2.1.1.

overproduction of NO and other reactive nitrogen species (RNS), in particular peroxynitrite[153,154] (eq 13), has been implicated in several pathological conditions, such as chronic inflammation and infection conditions, septic shock syndrome,

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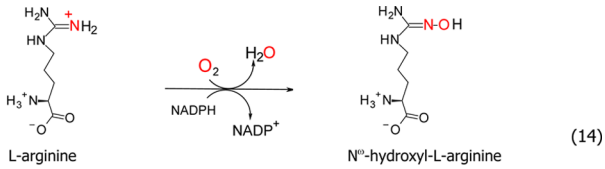
diabetes, and Parkinson's and Alzheimer's diseases.[155] All of these functions made the NO metabolism the focus of a huge interest by the medical scientific community. In 1992, NO was designated "Molecule of the Year" by *Science* magazine,[156] and, in 1998, the Nobel Prize in Physiology or Medicine awarded the discovery of NO as the "endothelium-derived relaxing factor". Since then, RNS never stopped being a "hot topic" in biomedical research.

$$O_2^{\bullet -} + {}^{\bullet}NO \rightarrow ONOO^- \quad (13)$$

Three tissue-dependent isoforms of NO synthases (NOS; neuronal, endothelial, and inducible NOS) catalyze the formation of NO from dioxygen and the guanidinium nitrogen atom of L-arginine (eqs 14, 15).[62,157−159] NOS are complex



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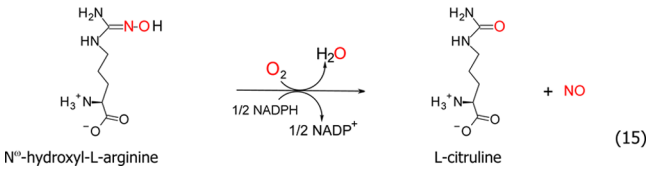

(15)

homodimeric enzymes, constituted by one flavinic reductase C-terminal domain and one haemic oxygenase N-terminal domain. During catalysis, the electrons from NADPH are transferred through the reductase domain to the *b* haem iron of the oxygenase domain. On the haem, the dioxygen is activated to hydroxylate L-arginine (eq 14); the N^{ω}-hydroxy-L-arginine formed is then oxidized to yield L-citrulline and NO (eq 15).The NO biological effects are accomplished, mainly, by posttranslational modification of transition metal centers (mostly haems and labile [4Fe−4S] centers) and of cysteine residues and other thiols, to yield nitrosyl (−metal−N═O) and *S*-nitrosothiol (−S−N═O, RSNO) derivates.[154,155,160−175] To control the specificity of NO signaling and to limit the NO toxicity, the NOS activity is tightly regulated. In addition, the NO lifetime is controlled through its rapid oxidation to nitrite, by dioxygen[175−178] or ceruloplasmin,[179] and to nitrate, by the well-known reaction with oxy-haemoglobin and oxy-myoglobin (see section 2.2.1.1.1 for details).

In fact, nitrate and nitrite were long regarded as "useless" end-products of the NO metabolism. As a result of this dogma, the human physiological role of nitrite was neglected until the end of the 20th century, when (i) it was realized that nitrite can be reduced back to NO under acidic and anaerobic conditions (eq 16) and (ii) it was rediscovered[180] that nitrite can be cytoprotective during in vivo ischaemia[181] and other pathological conditions (see references throughout the following subsections and, e.g., refs 182−204). Since then, a new concept began, and, presently, blood and tissue nitrite are thought as a NO "storage form" that can be made available to ensure cell functioning under conditions of hypoxia/anoxia,[205] precisely when the oxygen-dependent NOS activity is impaired.

$$NO_2^- + 1e^- + 2H^+ \rightarrow {}^{\bullet}NO + H_2O \quad (16)$$

The relevance of this nitrite-derived NO during hypoxia goes well beyond the obvious vasodilation.[186,206−209] It extends to other roles of the NOS-generated NO, such as the regulation of gene expression (e.g., haem oxygenase-1 or heat shock proteins expression),[210] smooth muscle proliferation,[202] angiogenesis,[208] or, most important, regulation of mitochondrial "respiration"[211] and energy production.[221−223] Through this nitrite "recycling" pathway, an organ under ischaemia can maintain (or even increase) the blood flow, modulate the dioxygen distribution and the reactive oxygen species (ROS) formation, and, at the same time, maintain an anti-inflammatory and antiapoptotic environment.

In this scenario, the question of "who" is reducing nitrite in mammals imposes itself. To date, no "dedicated" mammalian nitrite reductase was identified. On the contrary, the nitrite reduction to NO has been ascribed only to prokaryotic organisms, through Cd_1NiR and CuNiR enzymes (as described in section 2.1.1). Yet, while the absence of a "dedicated" nitrite reductase enzyme was interpreted as "mammals do not need to metabolize nitrite", the correct question was (long) disregarded: are other mammalian proteins reducing nitrite?

In recent years, several mammalian metalloproteins were shown to be able to reduce nitrite to NO (Figure 3, Table 2):

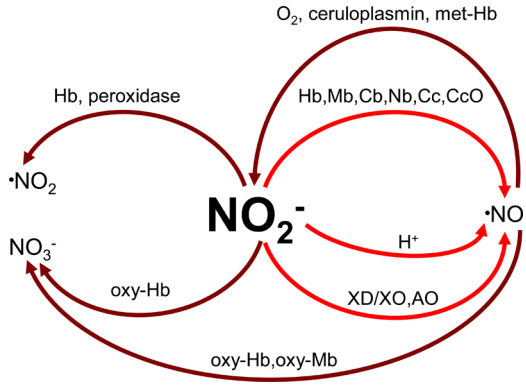

Figure 3. Nitrite in signaling pathways in mammals. See text for details and abbreviations.

the molybdenum-containing enzymes xanthine oxidase (XO) and aldehyde oxidase (AO) and a growing number of haem-containing proteins, where haemoglobin (Hb) and myoglobin (Mb) stand out by the number of publications, but including also neuroglobin (Nb), cytoglobin (Cb), cytochrome *c* (Cc), cytochrome P_{450},[224] cytochrome *c* oxidase (CcO),[225−227] NOS,[228,229] among several other proteins.[230,231]

Although outside the scope of this Review, it is very interesting that also nitrophorin 7, a salivary haemic protein from a blood-feeding insect, is able to reduce nitrite to NO.[232,233] Together with the haemic proteins of mammals and plants (discussed in the following section), this example strongly emphasizes the physiological relevance of haemic proteins on the nitrite reduction in higher organisms.

To restrict the scope of information presented to a manageable size, only some "nondedicated" nitrite reductases will be here discussed (Figure 3, Table 2): (i) Mb and XO, because they are the only ones that have been (so far) identified as crucial for the cytoprotective action of nitrite in vivo or ex vivo. They will be discussed in sections 2.2.1.1.1 and 2.2.1.1.3, along with Hb and AO, respectively; and (ii) Nb, Cb, and Cc (section 2.2.1.1.2), because they constitute promising examples of how the nitrite reduction could be allosterically regulated to create a "tunable" NO source. Moreover, only the reaction

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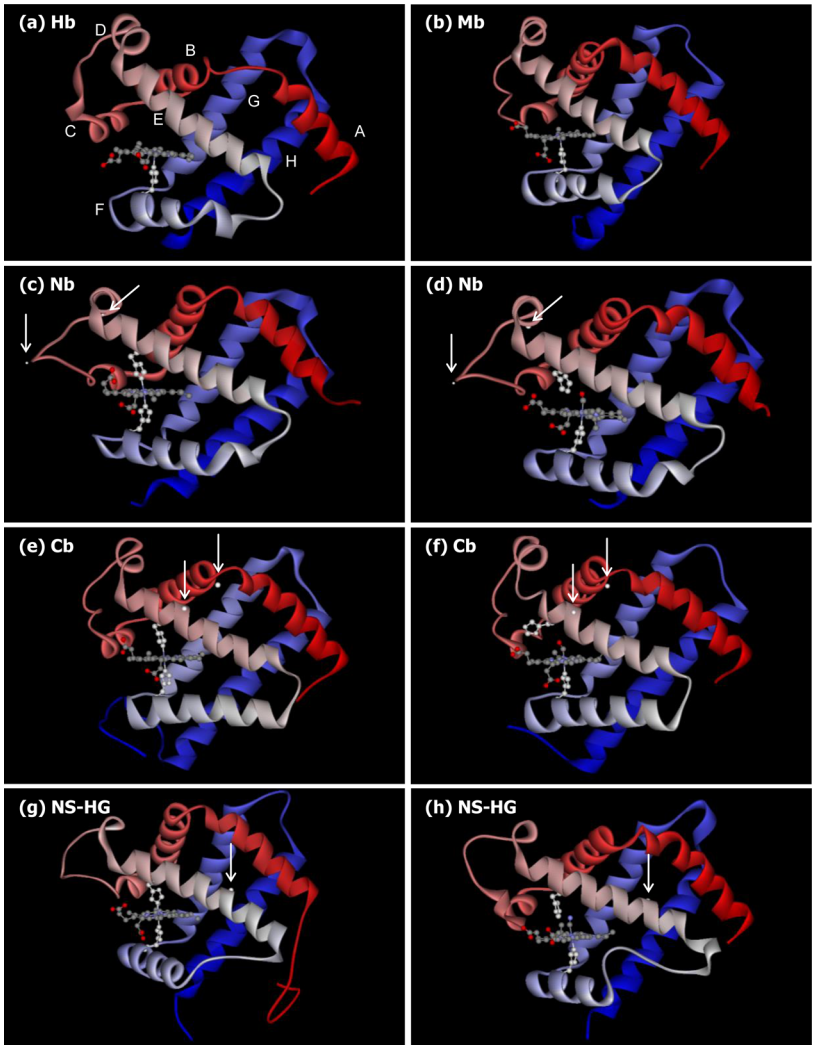

Figure 4. Haemic globins involved in nitrite reduction. Haemic globins are a large family of proteins that hold a haem and display the globin fold, typically, eight α-helices, named "A" to "H". These helices are shown in different colors (red/white/blue) and indicated in (a). The haem coordinating amino acid residues are shown in light gray. All images were produced with Accelrys DS Visualizer, Accelrys Software Inc. (a) Three-dimensional structure view of one monomer of human Hb. The proximal coordinating histidine F8, His_{92}, is shown. The image is based on PDB file 2DN1.[1695] (b) Three-dimensional structure view of horse heart Mb. The proximal coordinating histidine F8, His_{93}, is shown. The image is based on PDB file 1YMB.[1696] (c) Three-dimensional structure view of hexa-coordinated human Nb (protein mutated Cys_{46}Gly Cys_{55}Ser). The coordinating histidines F8, His_{96}, and E7, His_{64}, are shown. The positions of cysteine residues CD7, Cys_{46}, and D5, Cys_{55} are indicated by white dots/arrows. The image is based on PDB file 1OJ6.[415] (d) Three-dimensional structure view of CO-bounded murine Nb. Histidine F8, His_{96}, and a CO molecule are shown coordinated to the haem iron. Histidine E7, His_{64}, is also represented. The positions of cysteine residues CD7, Cys_{46}, and D5, Cys_{55} are indicated by white dots/arrows. The image is based on PDB file 1W92.[424] (e) Three-dimensional structure view of hexa-coordinated human Cb. The coordinating histidines F8, His_{113}, and E7, His_{81}, are shown. The positions of cysteine residues B2, Cys_{38}, and E9, Cys_{83} are indicated by white dots/arrows. The image is based on PDB file 2DC3.[1697] (f) Three-dimensional structure view of CO-bounded human Cb. Histidine F8, His_{113}, and a CO molecule are shown coordinated to the haem iron. Histidine E7, His_{81}, is also represented. The positions of cysteine residues B2, Cys_{38}, and E9, Cys_{83} are indicated by white dots/arrows. The image is based on PDB file 3AG0.[1698] (g) Three-dimensional structure view of one monomer of hexa-coordinated rice NS-HG. The coordinating histidines F8, His_{108}, and E7, His_{73}, are shown. The position of cysteine E16, Cys_{82} is indicated by white dots/arrow. The image is based on PDB file 1D8U.[438] (h) Three-dimensional structure view of barley CN-bounded NS-HG. Histidine F8, His_{105}, and a CN^- ion are shown coordinated to the haem iron. Histidine E7, His_{70}, is also represented. The position of cysteine E16, Cys_{79} is indicated by white dots/arrow. The image is based on PDB file 2OIF.[1106]

mechanism of XO-catalyzed nitrite reduction will be discussed (section 3.2.3). In addition, the protein-independent nitrite reduction and carbonic anhydrase-dependent NO formation will also be discussed (sections 2.2.1.1.4 and 2.2.1.1.5). As will be described, these pathways reduce nitrite to NO under acidic, hypoxic/anoxic conditions, precisely the conditions under which a "salvage" pathway is needed to accomplish the NOS role.

2.2.1.1.1. Haem-Dependent Nitrite Reduction – Haemoglobin and Myoglobin. A large number of studies have suggested that the mammalian nitrite reduction can be achieved by haemic proteins, with Hb (erythrocyte) and Mb (cardiac, skeletal,[234] and smooth[235] muscle) being two of the most studied proteins (Figure 4a,b, respectively).

To reduce nitrite to NO, the haem must be reduced and penta-coordinated to, in this way, provide the necessary electrons and have an "open site" to bind nitrite and carry

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Table 3. Possible Mechanisms To Explain Hb-Dependent Bioactive NO Formation

how	reaction	major drawback	figure
formation of an intermediate (possibly $^{\bullet}NO_2$) that oxidises (Hb)Fe^{2+}-NO and promotes NO dissociation	simultaneous reaction of nitrite with deoxy-Hb and oxy-Hb	low nitrite concentration to feed both reactions	5
formation of N_2O_3	catalytic cycle with deoxy-Hb and met-Hb	kinetic and thermodynamic constrains for N_2O_3 formation	6
formation of NO under allosteric control, at cell membrane within a metabolon	differentiated reactivity of nitrite/NO with R- and T-state Hb	NO trapping by membrane-bound deoxy-T-state Hb	7
formation NO under allosteric control, at cell membrane within a metabolon	NO "protected" by a high proportion of membrane-bound met-Hb	kinetic and thermodynamic constrains for N_2O_3 formation	8

out the oxygen atom abstraction and reduction (see section 3). In accordance, the ferrous deoxy-haemoglobin and deoxy-myoglobin (deoxy-Hb/Mb; (Hb/Mb)Fe^{2+}) do reduce nitrite to NO, under anaerobic conditions, in a reaction that is pH-dependent (eq 17; k(Hb, pH 6.5) ≈ 10 M^{-1} s^{-1},[236] k(Hb, pH 7.4) ≈ 0.1−1 M^{-1} s^{-1}, k(Mb, pH 7.4) ≈ 6−12 M^{-1} s^{-1}[206,222,237,238]).[237−242]

$$NO_2^- + 2H^+ + (Hb/Mb)Fe^{2+} \rightarrow (Hb/Mb)Fe^{3+} + {}^{\bullet}NO + H_2O \quad (17)$$

It is this in vitro anaerobic reaction that establishes the starting point for the proposed hypoxic Hb/Mb-dependent NO formation in vivo. Moreover, this reaction could contribute to the NO formation in all situations/localizations where significant Hb deoxygenation occurs: it should be noted that, even under normoxic conditions, the oxygen concentration at the precapillary arterioles and capillary is sufficiently low (<50 μM[243]) to promote significant Hb deoxygenation;[207,238,244,245] therefore, the Hb-dependent NO signaling could also occur locally, under nonischaemic conditions. This reasoning could explain why the in vivo administration of nitrite leads to a decrease in blood pressure, not only in hypertensive patients, but also in normal individuals.[193,195,197,206,246−251] The Mb-dependent NO formation, on the contrary, is expected to be significant only under hypoxia, because sufficient Mb deoxygenation only occurs when the oxygen concentration decreases to values lower than 3−4 μM.

Interestingly, nitrite also reacts with ferrous oxygenated Hb/Mb (oxy-Hb/Mb; (Hb/Mb)$Fe^{2+}-O_2$)), but to be oxidized to nitrate (eq 18; see section 2.2.1.2.1 for details). This oxidation reaction prevents the nitrite accumulation in vivo under normoxic conditions and is coresponsible for the NO lifetime control.[152,252,253]

The oxygen-controlled reactivity of nitrite, deoxy-Hb,Mb/reduction to NO versus oxy-Hb,Mb/oxidation to nitrate, could, thus, represent an ideal mechanism to control the NO formation throughout the entire physiological oxygen gradient, from normoxia to anoxia: (i) under normoxic conditions, NOS synthesizes NO, and the nitrite formed from the NO oxidation is oxidized to nitrate; and (ii) when the dioxygen concentration decreases, the deoxy-Hb/Mb reduces nitrite to NO to compensate the impairment of the NOS activity.

$$NO_2^- + H^+ + (Hb/Mb)Fe^{2+}-O_2 \rightarrow (Hb/Mb)Fe^{3+} + NO_3^- + 1/2H_2O_2 \quad (18)$$

Regardless of the "beauty" of this mechanism, the ability of the Hb,Mb/nitrite-dependent NO to perform its action in vivo is controversial, because the NO should (i) be rapidly (k_{on} ≈ 10^7 M^{-1} s^{-1}[254−263]) trapped in a stable complex with the deoxy-Hb/Mb (eq 19; K_d ≈ $10^{-12}-10^{-10}$ M[255,261,263−265])[237,238,254,266−268] and/or (ii) be rapidly oxidized (k ≈ 10^7-10^8 M^{-1} s^{-1}[152,252,254,257,262,269−275]) to nitrate by the oxy-Hb/Mb molecules still present under nonanoxic conditions[276] (eq 20). As a result of this well-documented in vitro haem chemistry, the in vivo physiological role of these haemic proteins in the formation of bioactive NO has been greatly questioned.

$$^{\bullet}NO + (Hb/Mb)Fe^{2+} \rightleftharpoons (Hb/Mb)Fe^{2+}-NO \quad (19)$$

$$^{\bullet}NO + (Hb/Mb)Fe^{2+}-O_2 \rightarrow (Hb/Mb)Fe^{3+} + NO_3^- \quad (20)$$

Despite these "chemical constrains", several in vivo, in situ, and in vitro experimental results indicate that red blood cells, isolated hearts, purified Hb, and Mb do generate NO and can stimulate the NO signaling, in the presence of nitrite, under hypoxia.[184−186,193−195,197,206,207,222,238,246−251,285−304] Particularly relevant are the studies showing that in vivo administration of nitrite generates NO, nitrosylates cardiomyocyte iron-containing proteins, and reduces (by 60%) myocardial infarction in mice; on the contrary, in Mb knockout mice, nitrite has no protective effects, and there is a decrease in NO formation.[222,293,296,304] These studies suggest that nitrite cytoprotection is exerted through its reduction to NO and implies a cytoprotective role for the pair Mb/nitrite in myocardial ischaemia-reperfusion injury.[305]

In light of all of the in vivo, in situ, and in vitro evidence, and to circumvent the NO scavenging by Hb/Mb, some authors argue that, even if the majority of NO is scavenged, enough NO would still be available to exert its role, because very low concentrations are needed to accomplish the physiological effects.[152,316] Concurrently, other authors focused on identify new mechanisms to explain how nitrite reduction can yield bioactive NO. The most relevant mechanisms so far suggested are summarized in Table 3 (Figures 5−8) and will be thoroughly discussed below.

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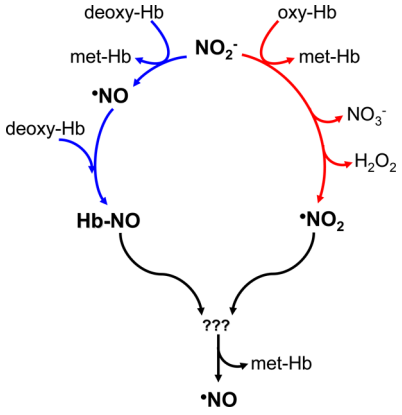

Figure 5. Possible mechanism for Hb-dependent NO formation. See text for details.

Before introducing those mechanisms that are expected to be essentially similar in both Hb and Mb, it is worth mentioning that Mb has important features that distinguish it from Hb: (i) Mb has a lower P_{50} value (1.5−3 μM for Mb versus ∼35 μM for Hb) that determines that Mb only becomes significantly deoxygenated at lower dioxygen concentrations; (ii) Mb has a lower reduction potential; (iii) it is a monomer without allosteric behavior; and (iv) it reduces nitrite faster than T-state Hb, at rates similar to those of R-state Hb. These properties must be kept in mind during the following mechanistic discussions that will be particularized for Hb for the sake of simplicity.

A possible mechanism to release NO involves the reactivity of nitrite with oxy-Hb itself (Figure 5). Several in vivo[285,317−319] and in vitro[320−324] observations suggest that, in the presence of oxy-Hb, the (Hb)Fe^{2+}−NO complex (eq 19) is not an irreversible trap of NO. On the contrary, it was suggested that an intermediate formed during nitrite oxidation by oxy-Hb, possibly nitrogen dioxide radical (•NO_2; see section 2.2.1.2.1), is able to oxidize (Hb)Fe^{2+}−NO and promote the NO dissociation[262,324] through an oxidative denitrosylation (eq 21).[324]

$$NO_2^- + 2H^+ + (Hb/Mb)Fe^{2+} \rightarrow (Hb/Mb)Fe^{3+} + {}^{\bullet}NO + H_2O \quad (17)$$

$${}^{\bullet}NO + (Hb)Fe^{2+} \rightleftharpoons (Hb)Fe^{2+}{-}NO \quad (19)$$

$$(Hb)Fe^{2+}{-}NO + {}^{\bullet}NO_2? \rightarrow ? \rightarrow (Hb)Fe^{3+}{-}NO \rightarrow ? \rightarrow (Hb)Fe^{3+} + {}^{\bullet}NO \quad (21)$$

In accordance with this proposal, the nitrite reduction and NO release would be accomplished by the simultaneous reaction of nitrite with deoxy-Hb and oxy-Hb.[324] Overall, nitrite is reduced much faster than it is oxidized, because reaction 21 consumes intermediate(s) of the propagation phase of the oxy-Hb-dependent oxidation (section 2.2.1.2.1, eqs 18,44−47). So, the simultaneous nitrite oxidation by oxy-Hb would be self-limited, and, at the same time, it would facilitate the NO release from the haem. The major conundrum of this mechanism is the in vivo small nitrite concentration (in the submicromolar range in the erythrocytes and plasma[171,325−328]) to feed all of the reactions; the experimental setup[324] used to build up the model employed a large nitrite/Hb ratio. Hence, to be feasible, this mechanism would require special spatial localization conditions (at the cell membrane, as will be discussed below) to control the local concentrations of reactants.[324] Moreover, in vivo, the formation of the key nitrogen dioxide (or other oxidative intermediate) should be inhibited by several antioxidants (as will be discussed in section 2.2.1.2.1). Therefore, while these reactions (eqs 17−19, 21) demonstrate that Hb has the intrinsic ability to, in vitro, form and release NO, the physiological relevance of these observations remains unclear and controversial.

Another possible mechanism for NO not to be scavenged by Hb is through the nitrite conversion to dinitrogen trioxide (N_2O_3), through eqs 17→22→23 or eqs 17→24→25, in a reaction model where Hb acts as a true catalyst (eq 26; Figure 6).[287,323,329−338] Both pathways (eqs 17→22→23 or eqs 17→24→25) are energetically feasible,[329,339] but, for the 22→23 branch, the "nitrito" binding mode of nitrite (Figure 20a) is essential for the successful production of dinitrogen trioxide.[339] Theoretical calculations showed that the complex (Hb)Fe^{3+}−NO_2^- has to have a "free" (unbound) nitrogen atom to react with NO and form the N−N bond of dinitrogen trioxide.[339] Interestingly, the "nitrito" binding mode is precisely the nitrite binding mode observed in crystals of the nitrite complex with ferric horse heart Mb[340] and human Hb,[341] which further supports the biologically feasibility of this pathway.

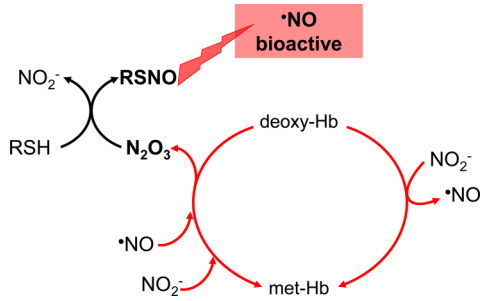

Figure 6. Possible mechanism for Hb-dependent NO formation. See text for details.

The dinitrogen trioxide is less reactive with the haem than the NO and is believed to be capable of crossing the cell membrane; thus, it would be, in principle, able to diffuse out of the red blood cell and reach the endothelium.[287,333,334] The bioactive NO is, subsequently, formed directly (eq 27) or indirectly (eq 28) from the dinitrogen trioxide.

$$NO_2^- + 2H^+ + (Hb)Fe^{2+} \rightarrow (Hb)Fe^{3+} + {}^{\bullet}NO + H_2O \quad (17)$$

and

$$NO_2^- + (Hb)Fe^{3+} \rightleftharpoons (Hb)Fe^{3+}{-}NO_2^- \quad (22)$$

$${}^{\bullet}NO + (Hb)Fe^{3+}{-}NO_2^- \rightarrow (Hb)Fe^{2+} + N_2O_3 \quad (23)$$

or

$${}^{\bullet}NO + (Hb)Fe^{3+} \rightleftharpoons (Hb)Fe^{3+}{-}NO \quad (24)$$

$$NO_2^- + (Hb)Fe^{3+}{-}NO \rightarrow (Hb)Fe^{2+} + N_2O_3 \quad (25)$$

Global reaction (eqs 17 + 22 + 23 or eqs 17 + 24 + 25):

$$2NO_2^- + 2H^+ \rightarrow N_2O_3 + H_2O \quad (26)$$

$$N_2O_3 \rightleftharpoons {}^{\bullet}NO + {}^{\bullet}NO_2 \quad (27)$$

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$$N_2O_3 + RSH \rightarrow RSNO + NO_2^- + H^+ \quad (28)$$

However, this model presents several drawbacks. First, the formation of the $(Hb)Fe^{3+}-NO_2^-$ complex (eq 22) in the presence of excess deoxy-Hb should not be favored, because the nitrite affinity for ferric haem is ca. 100 times lower than that for ferrous haem ($K_d \approx (1-5) \times 10^{-3}$ M[342−344] versus $(1-7) \times 10^{-5}$ M[343]). Even if the recently reassessed value of 5×10^{-5} M[345] is considered,[334,344,346] the presence of excess of ferrous Hb dictates that the actual concentration of the $(Hb)Fe^{3+}-NO_2^-$ complex would be in the nanomolar order. Second, this $(Hb)Fe^{3+}-NO_2^-$ complex (present at a very low concentration) has to compete with readily available $(Hb)Fe^{2+}$ (and $(Hb)Fe^{2+}-O_2$) for the reaction with NO (eq 23 versus eqs 19/20). Even if the $(Hb)Fe^{3+}-NO_2^-$ is considered to react as a $(Hb)Fe^{2+}-NO_2^{\bullet}$ radical species, at very rapid radical−radical reaction rates, the competition with the predominant $(Hb)Fe^{2+}$ (and $(Hb)Fe^{2+}-O_2$) may not be favorable. Third, a similar reasoning applies to the alternative pathway (eqs 24→25), because the formation of the $(Hb)Fe^{3+}-NO$ complex, in the presence of excess deoxy-Hb, is even more unfavorable (K_d(ferric complex, eq 24) $\approx 10^{-5}$ M versus K_d(ferrous complex, eq 19) $\approx 10^{-10}-10^{-12}$ M[236,262]). Regarding the competition for $(Hb)Fe^{3+}$ (eq 22 versus eq 24), it should be noted that, although the NO affinity is ca. 100 times higher than the nitrite one, both pathways are energetically feasible.[329] Fourth, the equilibrium 27 ($K \approx (2-7) \times 10^{-5}$ M[347,348]) is expected to be rapidly ($k \approx 10^9$ M^{-1} s^{-1}[347]) dislocated toward the dinitrogen trioxide formation, with subsequent hydrolysis to yield nitrite (reverse of eq 26).[155,348−350] These constrains suggest that, in vivo, the dinitrogen trioxide formation should not be favored or might require special spatial localization conditions (at the cell membrane; see next paragraph).[344] If formed, its reaction to yield RSNO (eq 28)[154,351−353] could constitute a probable way for NO to avoid being trapped by the haemic proteins, as will be described below.

It has also been suggested that NO escapes the red blood cell by being formed locally in the cell membrane, under allosteric control. It was shown that the kinetics of the nitrite reaction with Hb is more complex than previously thought and, most important, is controlled by the Hb conformation.[206,207,238,301,304,354−358] First, the nitrite reduction to form an electron delocalized intermediate (eqs 17a→17b)[286,359,360] is faster (5[236,301,358] to 50 times[238,324,354]) in the deoxy-haems of R-state Hb ($k \approx 10$ M^{-1} s^{-1}[238,301,358]), a conformation populated under normal dioxygen concentration. This behavior is consistent with the known higher affinity of the R-state for ligands and can be explained by the R-state haem having a more negative reduction potential (i.e., being a better electron donor) and/or having a more accessible haem pocket than the T-state.[207,337,355,357,361−363] Second, on the other hand, to proceed, reaction 17a needs the vacant ferrous deoxy-haems that predominate on the T-state. Third, although the intermediate formation is favored in the R-state, the NO release from the intermediate (eq 17c) is ca. 10 times faster in haems from the T-state ($k_{off} \approx 1$ s^{-1}[262,358]), a conformation populated under low oxygen concentration. This observation could be due to the conformational changes that take place during R to T conversion: modifications on the hydrogen bonds network of the distal haem pocket could affect the stability of NO on the haem[358,364] and facilitate its release. Therefore, the Hb conformation, which is dictated by the oxygen availability, affects all steps of nitrite reduction, but in opposite manners.

$$NO_2^- + 2H^+ + (Hb)Fe^{2+} \rightarrow (Hb)Fe^{2+}-NO^+ + H_2O \quad (17a)$$

$$(Hb)Fe^{2+}-NO^+ \rightleftharpoons (Hb)Fe^{3+}-NO \quad (17b)$$

$$(Hb)Fe^{3+}-NO \rightleftharpoons (Hb)Fe^{3+} + {}^{\bullet}NO \quad (17c)$$

The nitrite reduction rate as a function of oxygen fractional saturation results in a bell-shaped curve (Figure 7):[207,238,301,356]

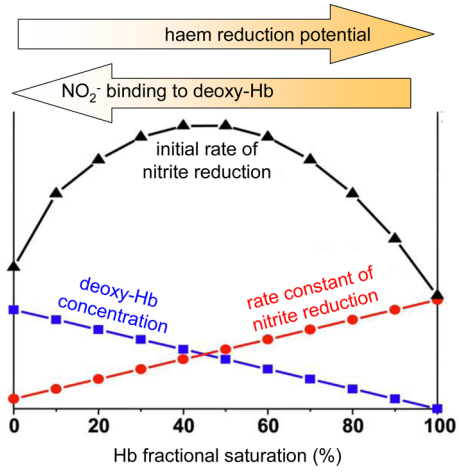

Figure 7. Mechanism for allosteric-regulated Hb-dependent NO formation. See text for details. Reproduced with permission from ref 207. Copyright 2006 American Society of Hematology.

(i) at a fractional saturation of zero, Hb is in the T-state and the reduction rate is low; (ii) as the oxygen fractional saturation increases, the rate rises in parallel with the increased R-state character of Hb (that reduces nitrite faster); and (iii) however, at higher fractional saturation, the T-state character and the concentration of deoxy-Hb (one substrate) decrease, and, consequently, the rate diminishes. At intermediate fractional saturations, Hb will be comprised of both R- and T-state molecules, and the reduction rate reaches maximum values around the Hb P_{50}, that is, around the oxygen concentration at which Hb is half-saturated (~35 μM; Figure 7).

The subsequent NO released out of the red blood cell, to diffuse to the vasculature, would be achieved by haems from the T-state, a conformation (i) populated under low oxygen concentration, (ii) favored at low pH, (iii) with an higher affinity for the cell membrane, within the context of a metabolon,[365] and (iv) with the highest NO release rates.[246,250,358]

Accordingly, as the Hb begins to be deoxygenated, but still retains the R-conformation, the increased rate of the intermediate formation (eqs 17a→17b) contributes to the formation of a pool of potentially bioactive NO. When Hb becomes further deoxygenated, the T-state is stabilized, its membrane-binding is enhanced, and the NO release out of the red blood cell is feasible.

Nevertheless, the localized NO release does not avoid the "problem" of NO being trapped by the membrane-bound deoxy-T-state Hb itself. In addition, because the NO diffusion is directed by its concentration gradient,[278,367,368] a significant fraction of this "membranar" NO should still diffuse toward the interior of the erythrocyte.

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Regarding the NO diffusion toward the interior of the red blood cell, it was suggested that a high proportion of membrane-bound met-haemoglobin[369] (met-Hb; (Hb)Fe^{3+}) would provide a diffusional barrier that would "shield" the NO (Figure 8).[370] If (Hb)Fe^{3+}–NO complex is formed to some extent[371] (eq 24), it would provide a way for NO to avoid scavenging:[322,323,338,359,372] the reaction of NO with met-Hb results in the formation of the nitrosylating complex (Hb)-Fe^{2+}–NO^+ (eqs 24→29), which either can be hydrolyzed to deoxy-Hb and nitrite (eq 30) or can transfer its nitrosyl group to nitrosylate nucleophilic compound such as thiols (RS^-) (eq 31).[260,330,373,374] In addition, as described above, the (Hb)-Fe^{3+}–NO complex can react with nitrite to eventually form RSNO as well (eqs 25→28). Furthermore, met-Hb could also promote the formation of RSNO via reactions 22→23→28. Moreover, the initial reductive nitrosylation[375] was shown to be faster (rate constant at least 5 times higher) in a subpopulation of β-subunit ferric haems, whose proportion is increased by T-state formation,[322,338] precisely the conformation with higher affinity for the cell membrane. So, in the presence of a high proportion of membrane-bound met-Hb, the NO formed by the low proportion of deoxy-Hb seems to have a chance to escape scavenging: formation of RSNO by locally concentrated met-Hb.

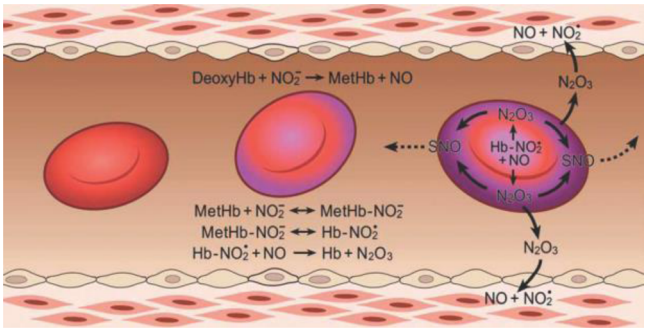

Figure 8. Mechanism for allosteric-regulated Hb-dependent NO formation, at cell membrane within a metabolon. See text for details. Reproduced with permission from ref 334. Copyright 2007 Macmillan Publishers Ltd.

Noteworthy, Mb does not seem to contribute to the formation of RSNO, under hypoxic acidic conditions, through this mechanism. The met-myoglobin (met-Mb) is nitrosylated only at pH values higher than 7.[374,377−379] At acidic conditions, met-Mb forms reversible complexes with NO ($K \approx 10^4\ M^{-1}$ for eq 24) and does not undergo intramolecular haem reduction (i.e., does not undergo reaction 29). This is in marked contrast to met-Hb that reacts readily and irreversibly with NO at pH lower than 6 to give the nitrosylating complex (Hb)Fe^{2+}–NO^+.

$$^{\bullet}NO + (Hb)Fe^{3+} \rightleftharpoons (Hb)Fe^{3+}\text{–}NO \quad (24)$$

$$(Hb)Fe^{3+}\text{–}NO \rightleftharpoons (Hb)Fe^{2+}\text{–}NO^{+} \quad (29)$$

$$(Hb)Fe^{2+}\text{–}NO^{+} + H_2O \rightarrow (Hb)Fe^{2+} + NO_2^{-} + 2H^{+} \quad (30)$$

$$(Hb)Fe^{2+}\text{–}NO^{+} + RS^{-} \rightarrow (Hb)Fe^{2+} + RSNO \quad (31)$$

$$(Hb)Fe^{3+}\text{–}NO + NO_2^{-} \rightarrow (Hb)Fe^{2+} + N_2O_3 \quad (25)$$

$$N_2O_3 + RSH \rightarrow RSNO + NO_2^{-} + H^{+} \quad (28)$$

$$NO_2^{-} + (Hb)Fe^{3+} \rightleftharpoons (Hb)Fe^{3+}\text{–}NO_2^{-} \quad (22)$$

$$^{\bullet}NO + (Hb)Fe^{3+}\text{–}NO_2^{-} \rightarrow (Hb)Fe^{2+} + N_2O_3 \quad (23)$$

$$N_2O_3 + RSH \rightarrow RSNO + NO_2^{-} + H^{+} \quad (28)$$

Concerning the formation of RSNO, note that their formation through reaction 31 could be initiated directly from ferrous Hb, via reaction 17a, without NO release. This mechanism was proposed for the self-*S*-nitrosylation of Hb, as a strategy for the release of NO:[206,321−323,333,380−384] the delocalized intermedi-

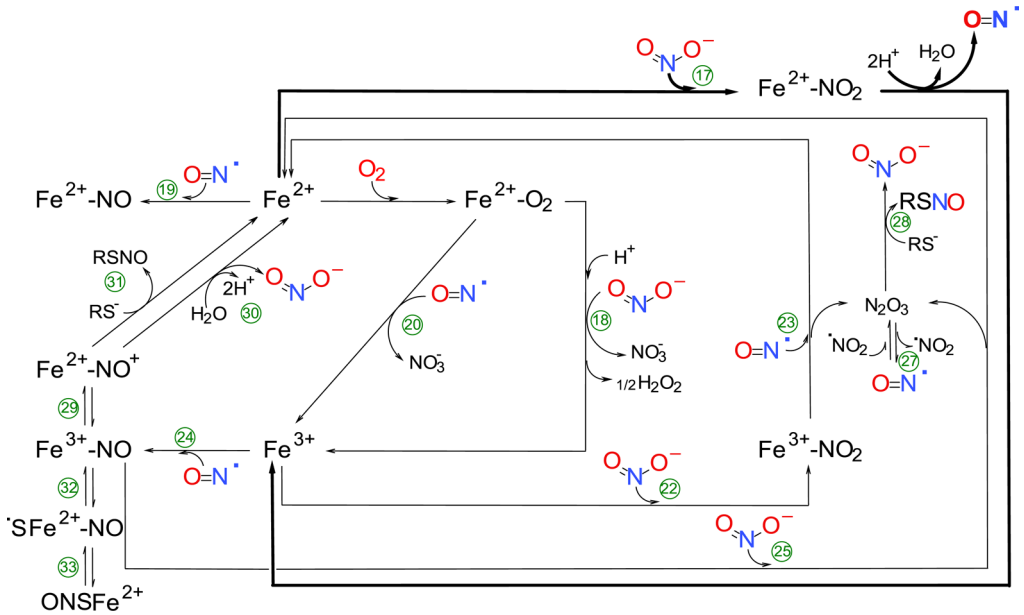

Figure 9. In vitro haemoglobin/myoglobin reactivity toward nitrite, nitric oxide, and related compounds. The green numbers (inside the circles) refer to the reaction numbering in the text. *Reaction 18 initiates an autocatalytic radical chain reaction responsible for nitrite oxidation (see section 2.2.1.2.1 for details). See text for details.

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ate (eqs 17a→17b) was suggested to be in equilibrium with a thiyl radical (eq 32)[385] to which the NO is transferred, yielding the well-known β-Cys_{93} *S*-nitrosylated Hb (eq 33). Subsequently, the Hb/membrane-bound NO would be transferred to the endothelial cells through transnitrosation of membrane thiol groups.

$$NO_2^- + 2H^+ + (Hb)Fe^{2+} \rightarrow (Hb)Fe^{2+}{-}NO^+ + H_2O \quad (17a)$$

$$(Hb)Fe^{2+}{-}NO^+ + RS^- \rightarrow (Hb)Fe^{2+} + RSNO \quad (31)$$

$$(Hb)Fe^{2+}{-}NO^+ \rightleftharpoons (Hb)Fe^{3+}{-}NO \quad (17b)$$

$$^-S(Hb)Fe^{2+}{-}NO^+ \rightleftharpoons {}^-S(Hb)Fe^{3+}{-}NO \rightleftharpoons^{\bullet} S(Hb)Fe^{2+}{-}NO \quad (32)$$

$$^{\bullet}S(Hb)Fe^{2+}{-}NO \rightleftharpoons ONS(Hb)Fe^{2+} \quad (33)$$

In this scenario of locally RSNO formation as a mechanism to explain the release of bioactive NO, it is noteworthy that nitrite administration leads to RSNO formation in vivo, not only in red blood cells, but also in other tissues.[171, 186, 206, 210, 293, 322, 323, 333, 334, 386−388]

RSNO are more stable than NO and less susceptible to be trapped by haem, two features that make them potential good NO reservoirs. Moreover, *S*-nitrosation is well recognized as a relevant cell signaling mechanism.[155,167,170−175,323,334,386] To elicit the NO-dependent effects, the RSNO would have to be, later, activated through homolytic or heterolytic decomposition or transnitrosation reactions (i.e., transfer of the NO^+ group). Yet, the mechanisms for their formation and NO release in vivo are still controversial.[154,166,389−391]

In summary, several in vivo, in situ, and in vitro studies indicate that hearts, red blood cells, Hb, and Mb do generate NO and stimulate NO signaling in the presence of nitrite, under hypoxia. However, the in vitro reactivity of haems with nitrite and NO involves a very intricate network of reactions (Figure 9), making it very difficult to foresee how the suggested in vivo outcome could be achieved. As a result, none of the mechanisms here discussed entirely explain how NO avoids the dogmatic scavenging by ferrous haems. In this respect, the formation of NO at the erythrocyte membrane, in the presence of a high proportion of membrane-bound met-Hb, represents the stronger mechanism.

In vivo, it is likely that a delicate balance between NO scavenging and NO formation takes place: while the scavenging occurs throughout the entire oxygen gradient (from normoxia to anoxia), the progressive deoxygenation promotes the nitrite reduction, eventually shifting the "scale" toward the NO generation. Taking the heart as an example to make this reasoning more clear: (i) Under normoxia, Mb would scavenge the NO[276] (through reaction 20). In this way, Mb could avoid the NO-dependent inhibition of CcO[280,281,308,392,393] or the inducible NOS-mediated nitrosative stress.[394,395] Hence, under normoxia, Mb would protect the mitochondrial "respiration" or the cell from the deleterious effects all NO. (ii) As the oxygen concentration decreases, a mismatch between the oxygen supply and consumption is "translated" into an increase of the deoxy-Mb fraction. The nitrite reductase activity of Mb could then become significant, even though concurrently with the omnipresent NO scavenging activity (eq 19). The NO formed would be responsible for a decrease in oxygen consumption, ROS formation, and down-regulation of the cardiac energy status,[222,293] thus protecting the heart during intense muscle exercise, acute myocardial hibernation, or infracted heart.[293,396]

This "activity switch" of Hb/Mb, from NO scavenger to NO generator, depending on the available oxygen concentration, reintroduces nitrite as a oxygen sensor molecule, but through a more sophisticated mechanism than the plain "deoxy-Hb,Mb/nitrite reduction versus oxy-Hb,Mb/nitrite oxidation" mentioned in the beginning of this section. It also emphasizes how mammals, which do not have a "dedicated" nitrite reductase, can reduce nitrite to NO: "reusing" proteins that we attribute to other functions and "switching" their activity when necessary. From a chemical point of view, mammals are just doing a "substrate adaptation" to an available redox system to fulfill their needs. In this case, taking advantage of the potential haem redox chemistry of proteins involved in oxygen transport. A haem is a perfect choice to reduce nitrite, as will become evident after comparing the mechanisms of reduction of several haemic proteins (sections 3.1 and 3.2.1).

One of the present major challenges is to discover how to connect the in vitro knowledge of nitrite reduction mechanisms to the in vivo observed nitrite effects: How can NO escape from the red blood cell? If RSNO are key NO reservoirs, which pathways can mediate nitrite-dependent *S*-nitrosation? Understanding the in vivo mechanisms by which Hb and Mb reduce nitrite to trigger the NO signaling remains, thus, an important goal for future research efforts. Moreover, the knowledge gathered from the Hb/Mb-mediated nitrite reduction would certainly be relevant to the understanding of other "non-dedicated" haem-containing nitrite reductases. It would be also relevant to the understanding of NO release from haemic receptors (e.g., guanylate cyclase) and transporters.

2.2.1.1.2. Haem-Dependent Nitrite Reduction − Neuroglobin, Cytoglobin, and Cytochrome c. Among the several haemic proteins proposed to act as nitrite reductases, the hexa-coordinated Nb, Cb, and Cc deserve to be here considered in more detail. To carry out catalysis, or simply react with a molecule, the haem has to have a free coordinating position to which the substrate or molecule should bind (see section 3 for more details). In this context, the nitrite reductase activity of penta-coordinated enzymes or metabolite transporters (as Hb and Mb in the absence of dioxygen) is not so surprising: that activity could arise from a "substrate adaptation" to the well-known redox chemistry of penta-coordinated haems. Yet the nitrite reductase activity of alleged hexa-coordinated haemic proteins raises two unavoidable questions: (i) Where does nitrite bind to be converted into NO? (ii) Why choose an hexa-coordinated protein?

2.2.1.1.2.a. Neuroglobin and Cytoglobin. Human Nb (Figure 4c,d) is a cytoplasmatic monomeric (~17 kDa) haemic globin (HG; see ref 1085) present in nerve tissues, as its name indicates, mainly in brain and retina.[397−402] Its amino acid sequence displays less than 25% of homology with other vertebrate Hb and Mb and reveals a very ancient origin: it was present long before the divergence of the genes encoding Mb and Hb and remained highly conserved throughout mammalian evolution, suggesting a strongly selected vital role.[397,403−408] Nb possesses a bis-histidinyl (His_{F8} and His_{E7}[409]) hexa-coordinated *b* haem, in both iron oxidation states,[397,404,412−420] as expected from its classification as a hexa-coordinated HG (Figure 4c). However, the distal histidinyl coordination is reversible (eq 34), and the penta-coordinated Nb is able to reversibly bind

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dioxygen, carbon monoxide, NO, or, as recently described, nitrite (Figure 4d). Remarkably, the hexa- to penta-coordination conversion is controlled through a redox mechanism that involves the formation (or cleavage, for reverse conversion) of an intramolecular disulfide bond, which decreases the distal histidine affinity[421] ($K \approx 3300$ versus 280[422,423,433]). Hence, the Nb affinity for "internal" (histidine) versus "external" ligands is expected to be directly controlled by the redox status of the cell.

$$(\text{protein})\text{Fe}^{2+}\text{–His}_{\text{distal}}(\text{protein}) \rightleftharpoons (\text{protein})\text{Fe}^{2+} + \text{His}(\text{protein}) \quad (34)$$

Physiologically, Nb was initially suggested to play a role similar to that of Mb: facilitate the dioxygen diffusion to mitochondria, to increase the oxygen availability to neurons.[397,437] However, given its high dioxygen affinity and low dioxygen dissociation rate,[397,404,413,438,439] Nb does not meet the equilibrium and kinetic requirements for functioning in oxygen transport by facilitated diffusion[440] under (the presently known) physiological conditions[404](Nb displays $k_{on} \approx 130–250\ \mu M^{-1}\ s^{-1}$ and $k_{off} \approx 0.3–0.8\ s^{-1}$;[404,413] values that compare with $k_{on} \approx 15\ \mu M^{-1}\ s^{-1}$ and $k_{off} \approx 13\ s^{-1}$ for sperm whale Mb). Furthermore, its (i) low tissue concentration (micromolar range, except in retina),[400,441] (ii) high auto-oxidation rate, (iii) low haem reduction potential,[413,435,442] and (iv) redox-controlled "internal" hexa-coordination support that Nb did not evolved to transport oxygen.[435,441] Instead, it is probable that Nb is involved in redox reactions, including (i) cellular redox status sensing or scavenging of ROS and RNS,[443–452] (ii) scavenging and/or sensing of dioxygen, carbon monoxide (another signaling molecule[453–456]), or NO,[397,408,414,438,443–445,457–466] (iii) inhibition of Cc-induced apoptosis[467–469] ("resetting the trigger level" for apoptosis; see ref 565 in section 2.2.1.1.2.b for details), (iv) among several other functions.[444,445,459,460,470–474] In addition, and as is suggested for Hb and Mb, there is much evidence (described below) that Nb can be involved in the generation of NO, through the nitrite reduction. In support of these roles, Nb has been linked to neuronal protection during ischaemia injury: Nb is induced by neuronal hypoxia in vitro and focal cerebral ischaemia in vivo, and its overexpression or knocking-down enhances or reduces, respectively, neuronal survival after hypoxia.[191,441,443–445,450,451,475–480]

The identification of Nb, associated with hypoxic conditions and with features similar to those of plant nonsymbiotic HG (see section 2.2.2.1.3 for details about these plant proteins) instead of the obvious Mb, triggered the search for other human globins, which culminated with the discovery of Cb.[439,457] Human Cb (Figure 4e,f) is a monomeric[481] (~21 kDa) hexa-coordinated HG.[484] Cb is present in the cytoplasm and nucleus of apparently all tissue types, although at varying concentrations (and, for that reason, was initially called histoglobin).[398,399,439,441,457,461,482,485–487] As Nb, it displays low amino acid sequence similarity with other vertebrate Hb and Mb,[405,439,457] but Cb is phylogenetically "younger", sharing a common ancestry with Mb.[457,471] The Cb distal histidine coordination is reversible (Figure 4f) and can be also under the control of a redox mechanism, involving, for example, the cysteine residues B2 and E9. However, the Cb distal histidine affinity ($K \approx 90–165$, with $k_{off} \approx 1–5\ s^{-1}$[439,484,488]) does not change significantly with disulfide bond cleavage or mutation of the Cys_{B2} and Cys_{E9} ($K \approx 90–120$, with $k_{off} \approx 2\ s^{-1}$[422,484]). Nevertheless, in vivo, other mechanisms could control the population of hexa- versus penta-coordinated Cb molecules. Noteworthy, although the distal histidine affinity of Cb is lower, the Cb dioxygen dissociation rate is equally low as in Nb ($k_{off} \approx 0.35–0.9\ s^{-1}$[439,484]). Accordingly, and as has been suggested for Nb, Cb is believed to be involved in cytoprotection under hypoxia, when it is up-regulated,[407,439,441,445,447,457,471,480,485–492] and under oxidative stress[212] conditions,[433,490,493–499] among other roles.[407,485,495,500,501] Cb has been proposed to act as a NADH oxidase,[483] a dioxygen sensor,[399] NO scavenger,[487,491] and as a nitrite reductase/NO synthase.

As expected from the previous discussion on Hb and Mb, the human ferrous deoxy-neuroglobin and deoxy-cytoglobin (deoxy-Nb and deoxy-Cb) do reduce nitrite to NO, under anaerobic conditions, in a reaction that is pH-dependent (equivalent to eq 17; k(Nb, pH 6.5) $\approx 1.2\ M^{-1}\ s^{-1}$, k(Nb, pH 7.4) $\approx 0.12–0.26\ M^{-1}\ s^{-1}$,[502] k(Cb, pH 6.0) $\approx 1.5\ M^{-1}\ s^{-1}$, k(Cb, pH 7.0) $\approx 0.14\ M^{-1}\ s^{-1}$[503]). However, contrary to the "open" (penta-coordinated) Hb and Mb, the Nb and Cb reactions are also dependent on the competition of nitrite with the distal histidine for binding to the haem. In the case of Nb, the nitrite reduction is further dependent on the redox state of the Cys_{CD7} and Cys_{D5}, whose binding controls the fraction of penta-coordinated haem-containing molecules. In accordance, the reduction of the disulfide bond or the mutation of the Cys_{CD7} and Cys_{D5} to alanine residues decreases the Nb reaction rate constant by a factor of 2 ($k \approx 0.06\ M^{-1}\ s^{-1}$ (pH 7.4)). Noteworthy, the replacement of the distal His_{E7} by a leucine or glutamine residue, which "locks" Nb in a penta-coordination state, leads to rates ~2000-fold higher than that of wild-type Nb (259 and 267 $M^{-1}\ s^{-1}$, respectively (pH 7.4)).[502] Furthermore, and remarkably, under acidic conditions (pH 6.5), the rate constants of the His_{E7}Leu and His_{E7}Gln mutants increase above 2500 and 2000 $M^{-1}\ s^{-1}$, respectively,[502] being the highest reaction rates of nitrite with a mammalian HG ever reported. In this respect, it should be noted that a similar mutation of the Mb leads to a marked decrease in the nitrite reduction (1.8 and $<0.5\ M^{-1}\ s^{-1}$ for His_{E7}Ala and His_{E7}Leu Mb mutants).[504,505] Although these high rates cannot reflect the physiological NO formation, they confirm that the Nb-dependent NO generation is feasible and controlled by the hexa- to penta-coordination conversion and, consequently, by (at least) the thiols redox state.

Unsurprisingly, the penta-coordinated deoxy-Nb/Cb also rapidly traps NO in a stable (Nb/Cb)Fe^{2+}–NO complex, as deoxy-Hb/Mb do (equivalent to eq 19; $k_{on} \approx 10^8\ M^{-1}\ s^{-1}$[445,459,488] and $k_{off} \approx 10^{-4}–10^{-3}\ s^{-1}$[445,459,506]). However, the competition of NO with the distal histidine for binding to the haem should result in a lower global NO affinity for Nb, comparatively to Hb/Mb. In addition, NO, as well as nitrite, can also be oxidized (scavenged) by oxy-neuroglobin (oxy-Nb)[461] and oxy-cytoglobin (oxy-Cb)[404,407,439,461,491] ($k \approx 10^7\ M^{-1}\ s^{-1}$[488]). Once more contrary to the Hb/Mb reactions 20 and 18, the global rate of these Nb and Cb oxidations is dependent on the competition between the dioxygen and the distal histidine (which controls the concentration of oxygenated protein). The higher auto-oxidation rate (comparatively to Hb/Mb) also contributes to further decrease the oxy-Nb/Cb concentration available to react with NO and nitrite.[413,435,442]

Regardless of those "side" reactions (eqs 18–20) and of all of the other possible haem reactions (Figure 9), the in vivo nitrite reductase/NO synthase activity of Nb and Cb is supported by two lines of evidence: (i) Nb[191,441,443–445,450,451,475–480] and Cb[407,439,441,445,447,457,471,484–492,503] have been shown to

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mediate cytoprotective responses to ischaemic stress, promoting cell survival, and (ii) in the presence of nitrite, both can stimulate the NO signaling and inhibit the mitochondrial "respiration", in a manner dependent on the pH, dioxygen concentration, cysteine redox state, and haem coordination state.[502,503]

Crucial for the in vivo significance of Nb and Cb reactions is the existence of regenerating systems that efficiently rereduce the oxidized met-neuroglobin and met-cytoglobin (met-Nb and met-Cb). This is particularly important, because these proteins are present at low concentrations (micromolar range). Comparatively, the high concentration of Hb and Mb allows them to effectively form and scavenge NO, despite the relatively slow rereduction rates.[275,507] Although presently there are no known specific reductases, met-Nb and met-Cb can be rapidly reduced in vitro using conditions that mimic those existing within living cells.[413,435,467] met-Cb, for example, can be rapidly reduced by cellular reductants such as cytochrome b_5 and cytochrome b_5 oxidoreductase,[487,488] cytochrome P_{450} reductase (NADPH; $k \approx 3 \times 10^5$ M^{-1} s^{-1}[488]), or even ascorbate ($k \approx 50$ M^{-1} s^{-1}[488]). These "recycling" reactions (ferric to ferrous) would greatly increase the rate of NO formation, as well as of NO scavenging, allowing Nb and Cb to act as catalysts of NO and nitrite metabolism at the low concentration that they are found in vivo.

In summary, the Nb and Cb-catalyzed nitrite reduction to NO presents the same features and "weaknesses" as discussed for Hb/Mb, but with an additional level of complexity: the nitrite reduction is modulated by the redox state of key surface cysteine residues and/or by any other hypothetical "modification" that decreases the distal histidine affinity, for example, phosphorylation, protein−protein interaction, protein−lipid interaction, nitration. In accordance, these HG have been suggested to be allosteric-regulated nitrite reductases. In particular for Nb, the following mechanism has been proposed (Figure 10): (i) Under normal conditions, the high cellular concentration of reduced thiols (e.g., GSH ≈ 5 mM, with GSH/GSSG > 500) keeps the Nb cysteine residues reduced and "locks" the protein mainly in a "closed" bis-histidinyl-hexa-coordinated configuration, with low nitrite reductase activity. (ii) As oxidative stress[212] conditions develop and the concentration of reduced thiols decreases, the population of disulfide-containing penta-coordinated Nb would increase, amplifying the NO formation.[502] Subsequently, the NO formed can, for example, inhibit the mitochondrial "respiration", limiting the dioxygen consumption and ROS formation, thus preventing further oxidative stress damage. Hence, the proposed mechanism allows the "translation" of changes in the cellular redox status into a differentiated NO flux that would be, subsequently, "translated" into a biological response (in the example, antioxidant protection). In a scenario of an ischaemic insult, the mechanism hypothesized predicts that the protective action of Nb would be exerted after the ischaemic phase, during reperfusion, when oxidative stress develops and dioxygen is already present. Nevertheless, the hexa- to penta-coordination conversion of either Nb or Cb could be, in principle, promoted by other regulators/mechanisms that are "triggered" during the hypoxic phase. Thus, the protective action of these proteins could, in principle, occur also under hypoxia (when the proteins are found to be up-regulated).

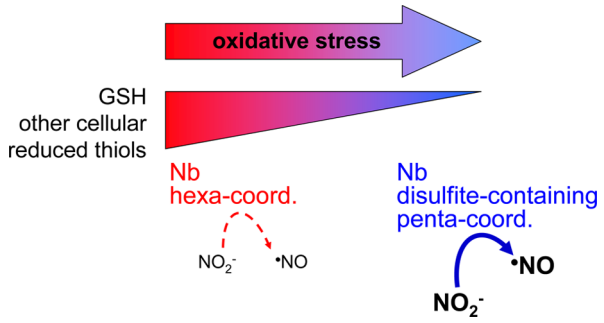

Figure 10. Mechanism for Nb-dependent NO formation as proposed in ref 502. See text for details.

The existence of the equilibrium 34 explains how an alleged hexa-coordinated haemic protein can bind nitrite to convert it into NO: the hexa-coordinated protein is not a "rigid body", and the "internal" sixth coordination position can be "open" to allow for the binding of an "external" ligand. The fact that this equilibrium can be modulated (changed in time) provides the answer for "why choose a hexa-coordinated protein?": to have a protein whose reactivity can be directly controlled by a selected stimuli, through a mechanism of the type "unready" versus "ready" (a regulatory strategy further discussed in section 3 and further exemplified with Cc and Cd_1NiR). A protein with such "tunable" reactivity would allow the tissue to quickly respond toward different cellular conditions.

In this scenario, Nb seems to present the ideal characteristics to be a nitrite reductase: (i) it is present at low concentrations, more in line with a catalyst role;[441] (ii) has a low haem reduction potential;[413,435,442] (iii) holds a large cavity (120 $Å^3$),[415] connecting the haem with the bulk, that has no counterpart in vertebrate Hb or Mb and that can function as a channel for substrates entrance/products release;[415] (iv) it is plausible to be involved in redox reactions, as discussed above; (v) it displays a redox-controlled "internal" hexa-coordination, linked with the cellular redox status; and (vi) it has been associated with hypoxia and oxidative stress conditions, when it is up-regulated. In addition, Nb is of ancient evolutionary origin, having remained highly conserved throughout mammalian evolution,[397,403−408] and it displays characteristics that are more in line with the bacterial and plant hexa-coordinated HG. The presence of these relatively similar proteins in so many different organisms suggests a strongly selected vital role. Nb may be a "molecular fossil" reminiscent of a redox catalytic function of the HG family that was vital to preserve throughout the evolution:[420] a nitrite reductase/NO synthase or/and a redox sensor.

The major challenges concerning the nitrite reductase activity of Nb and Cb are essentially the same as those discussed for Hb and Mb. Primarily, how can the NO formed avoid the dogmatic scavenging by oxy- and deoxy-Nb/Cb? The identification of regenerating systems that efficiently rereduce the oxidized met-Nb and met-Cb is also essential. Without this knowledge, the physiological significance of the Nb/Cb-mediated NO pathway could not be fully evaluated.

Also of major importance is the identification of other allosteric regulators of the hexa- to penta-coordination conversion of both Nb and Cb, for example, phosphorylation, protein−protein interactions, protein−lipid interactions, or nitration. This may reveal new mechanisms to control the nitrite reductase activity and, consequently, the NO signaling. In this respect, the interplay between nitrite reduction/thiols redox status/dioxygen should be further investigated: on one

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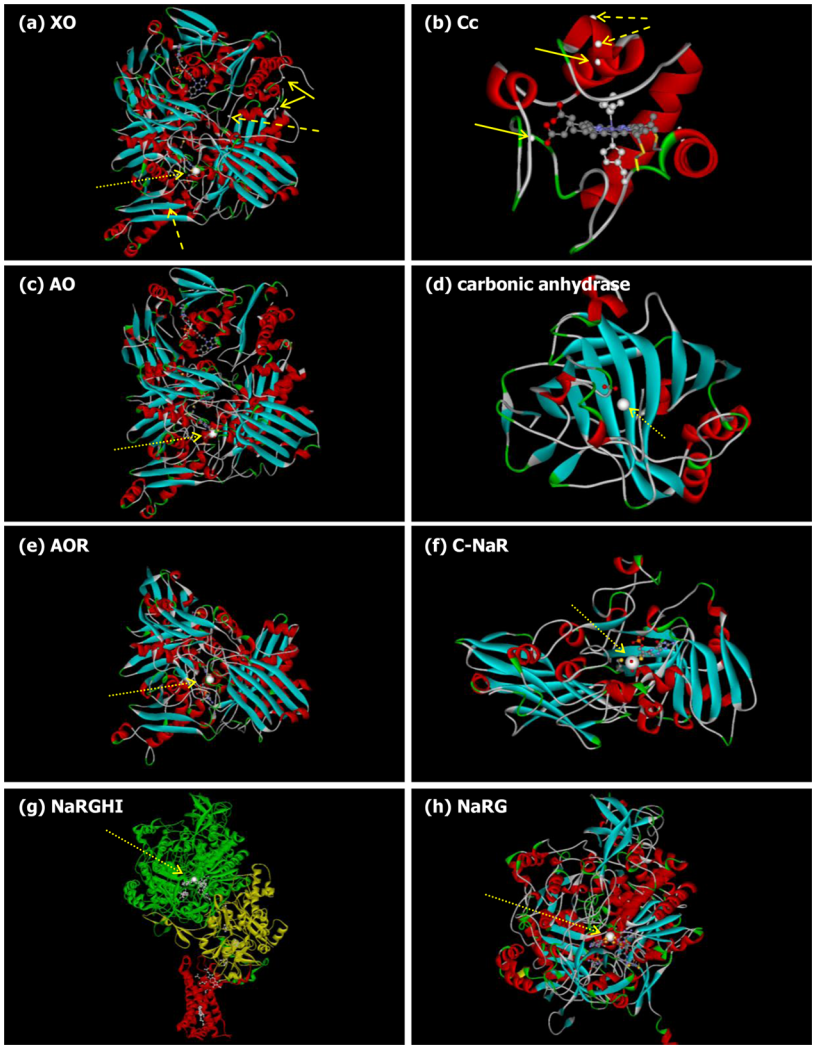

Figure 11. Some proteins involved in nitrite reduction. (a) Three-dimensional structure view of one monomer of bovine XO. The positions of residues involved in XD conversion into XO, Cys_{535}/Cys_{992} and Lys_{551}/Lys_{569}, are indicated by, respectively, solid and dashed yellow arrows. The molybdenum atom is colored in white and indicated by a dotted yellow arrow. The image is based on PDB file 1FO4.[1584] (b) Three-dimensional structure view of human Cc (protein mutated $Gly_{41}Ser$). The haem coordinating residues His_{18} and Met_{80} are shown in light gray. The positions of residues involved in Cc posttranslational modifications, Tyr_{48}/Tyr_{67} and Lys_{72}/Lys_{73}, are indicated by, respectively, solid and dashed yellow arrows. The image is based on PDB file 3ZCF.[1699] (c) Three-dimensional structure view of one monomer of mouse AO. The molybdenum atom is colored in white and indicated by a dotted yellow arrow. The image is based on PDB file 3ZYV.[1586] (d) Three-dimensional structure view of human carbonic anhydrase. This enzyme is believed to catalyze nitrous acid hydration and not nitrite reduction. The zinc atom is colored in white and indicated by a dotted yellow arrow. The image is based on PDB file 2VVA.[1700] (e) Three-dimensional structure view of one monomer of *Desulfovibrio gigas* AOR. The molybdenum atom is colored in white and indicated by a dotted yellow arrow. The image is based on PDB file 1VLB.[1583] (f) Three-dimensional structure view of the molybdenum-containing fragment of *Pichia Angusta* assimilatory NaR. The molybdenum atom is colored in white and indicated by a dotted yellow arrow. The image is based on PDB file 2BIH.[38] (g) Three-dimensional structure view of the *Escherichia coli* NaRGHI. NaRG, NaRH, and NaRI subunits are shown in green, yellow, and red, respectively. The molybdenum atom is colored in white and indicated by a dotted yellow arrow. The image is based on PDB file 1Q16.[1680] (h) Three-dimensional structure view of the *Escherichia coli* NaRG subunit. The molybdenum atom is colored in white and indicated by a dotted yellow arrow. The image is based on PDB file 1Q16.[1680] All images were produced with Accelrys DS Visualizer, Accelrys Software Inc. Except in (g), α helices and β sheets are shown in red and cyan, respectively; turns and coils are shown in green and white, respectively.

hand, nitrite reduction requires hypoxic conditions to increase the concentration of deoxy-Nb, but, on the other hand, those hypoxic conditions would not favor the formation of the disulfide-containing, penta-coordinated Nb that is able to reduce nitrite. Moreover, dioxygen is a probable inhibitor of nitrite reduction, because (i) both dioxygen and nitrite would compete to bind to Nb and (ii) the oxy-Nb formed would decrease the concentration of deoxy-Nb and simultaneously scavenge the NO formed.

2.2.1.1.2.b. Cytochrome c. Cc needs no introduction: discovered more than a century ago,[508] it is a small (~13 kDa) globular haemic protein (Figure 11b) present in the intermembranar space of the mitochondria; there, it transfers one electron from cytochrome bc_1 complex (Complex III) to cytochrome oxidase complex (Complex IV), in the electron transport chain responsible for the oxidative phosphorylation. However, besides this well-known "respiratory" function, Cc is also an apoptotic signaling molecule:[509−521] a variety of metabolic stimuli and insults trigger the Cc release to the

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cytoplasm, where it participates in signaling pathways underlying apoptotic cell death.[522] In addition, Cc is involved in oxidative and nitrosative stress responses: (i) it is an ideal antioxidant molecule to scavenge the superoxide anion radical[523] formed by the mitochondrial electron transport chain,[526] but it is also (ii) a lipid peroxidation catalyst, namely of mitochondria cardiolipin and of cytoplasm membrane phosphatidylserine (also associated with apoptosis),[527−530] (iii) a peroxidase-like enzyme, and (iv) a nitrating agent (roles discussed in section 2.2.1.2.3).

Cc holds a "closed" *c* haem, hexa-coordinated by His_{18} and Met_{80} (human numbering), as expected for an electron transfer protein (Figure 11b). Nevertheless, and as discussed for Nb and Cb, the Met_{80} coordination is reversible, and a penta-coordinated species is formed when Tyr_{67} is nitrated,[531−535] methionine is oxidized,[536,537] or Cc interacts with anionic lipids, such as the mitochondrial cardiolipin.[528,538−540] The Cc interaction with negatively charged lipids, membranes, and inorganic electrode surfaces has been subjected to numerous studies.[528,540−551] The interaction results, not only in protein conformational changes,[541−545,548,549,552,553] but also in a significant decrease (~300−400 mV[531,532,540,547,550,551]) of the reduction potential, which should facilitate the Cc participation in reduction reactions. Once the haem is "open" (becomes penta-coordinated), Cc can bind "external" ligands (substrates), and the haem redox chemistry can be explored to catalyze several reactions, including the reduction of nitrite and of hydrogen peroxide (peroxidase-like activity, discussed in section 2.2.1.2.3). In this way, the Cc activity is "switched" from simple electron transfer to catalysis, which is ideal for the Cc roles in stress and apoptotic responses.

The in vivo Cc "function switching", "respiration" into stress/apoptotic mediator, demands for regulatory mechanisms to control the "activity switching": hexa-coordinated/electron carrier into penta-coordinated/catalyst. Once more (as in Nb and Cb), the regulatory mechanisms (oxidation, nitration, interaction with lipids) consist of posttranslational structural modifications that facilitate the cleavage of the methionine-iron coordination.[527,528,553−556] In this respect, it is noteworthy that, besides the Met_{80}, also the Tyr_{48}, Tyr_{67}, and Lys_{72}−Lys_{73} (the lysine residues are involved in interactions with anionic phospholipids) (Figure 11b) are highly conserved in Cc from different species, suggesting the existence of multiple conserved biological "switches". In particular during apoptosis, it has been suggested that cardiolipin-binding is one of the main "switches".[540]

As it is by now expected, when under conditions that favor the haem penta-coordination, the Cc effectively catalyzes the anoxic and acidic nitrite reduction to NO; the nitrite reductase activity of hexa-coordinated Cc is negligible.[557] A comprehensive kinetic characterization was not yet undertaken, but 100 μM Cc (a reasonable physiological value for cytoplasmatic Cc[558,559]), in the presence of negatively charged liposomes, can catalyze the formation of NO at an appreciable rate of 0.18 nM s^{-1} (pH 6.4).[557] Also, the Cc mutant Met_{80}Ala, which harbors a "locked" penta-coordinated haem, displays an electrochemical catalytic response toward nitrite reduction that is similar to the bacterial Cd_1NiR one.[560] Somewhat surprising, the NO scavenging by Cc (equivalent to eq 19) is considerably slower than that by the HG, with a $k_{on} \approx 10$ and 10^3 M^{-1} s^{-1}, for hexa- and penta-coordinated Cc, respectively,[557,561−563] which could contribute to increase the bioavailable NO concentration. Nevertheless, once formed, the $(Cc)Fe^{2+}$−NO complex would be quite stable ($k_{off} \approx 10^{-5}$ and 10^{-7} s^{-1} for hexa- and penta-coordinated Cc, respectively).[561−563] Despite the expected NO trapping, the Cc-catalyzed NO formation can significantly inhibit the mitochondrial "respiration" through inhibition of CcO[557] and cause the guanylate cyclase activation (in vitro).[503]

The physiological significance of this Cc nitrite reductase activity would depend on the existence of regenerating systems that efficiently rereduce the oxidized Cc. Because Nb binds[450,451,467−469,564] and reduces ferric Cc very rapidly ($k = 2 \times 10^7$ M^{-1} s^{-1}[467,468,564]), this globin has been suggested to be one of potential Cc "cosubstrates".[565] If so, a Nb regenerating system must be in place to efficiently rereduce the Nb present in tissues in low concentrations (see section 2.2.1.1.2.a).

In summary, Cc is able to reduce nitrite and to produce bioactive NO, under the conditions found during ischaemia injury, apoptosis, and others, when its haem may become penta-coordinated due to protein nitration, oxidation, and interaction with anionic lipids. Hence, the nitrite reductase activity of Cc can be of physiological relevance not only for hypoxia- and redox-dependent signaling, but also for the apoptotic process: (i) on one hand, nitrite and low NO concentrations are known to prevent pore opening, lipid peroxidation, and Cc release, in isolated mitochondria after ischaemia;[557,567] (ii) but, on the other hand, high NO concentrations or high nitrite reduction rates can lead to Cc self-nitrosylation and nitration and inhibition of CcO, leading to membrane depolarisation, Cc release, and apoptosis.[557,568−573] One of the major challenges for future research is to understand how the posttranslational regulatory mechanisms are orchestrated to control the Cc "activity switch" from an electron transfer protein into a nitrite reductase (or other activities, like peroxidase). In particular, how are the nitrite reductase and nitrite oxidase (discussed in section 2.2.1.2.3) activities articulated?

Of major importance is also the identification of other regulators of the hexa- to penta-coordination conversion that should control the Cc action in apoptotic and nonapoptotic cells. Two examples follow: (i) It would be of interest to study the effect of partial proteolysis, which is known to increase the peroxidase and nitrating activities.[574] (ii) Because Tyr_{48} phosphorylation inhibits the electron transfer process and impairs the caspase activation (an antiapoptotic "switch"), it would be of interest to also study the phosphorylation effect.[575,576] Also pertinent would be (i) the identification of physiological relevant regenerating systems that efficiently rereduce the oxidized (Fe^{3+}) Cc, (ii) the comprehensive kinetic characterization of the nitrite reductase activity, (iii) the accurate description of the concentration and coordination state of cytoplasmatic and mitochondrial Cc, or (iv) the comparative study of the equivalent "side" reactions described in Figure 9.

To conclude, Cc is a "multitask" protein, certainly involved in complex regulator mechanisms that control its "activity switching". The allosteric/redox-regulated nitrite reductase activity could play a relevant role in the Cc-mediated signaling pathways. Precisely how this activity will affect signaling in apoptotic and nonapoptotic cells remains to be truly explored.[557]

2.2.1.1.3. Molybdenum-Dependent Nitrite Reduction − Xanthine Dehydrogenase/Oxidase and Aldehyde Oxidase. Several studies have suggested that the mammalian nitrite reduction can also be achieved with the molybdenum-containing XO and AO.

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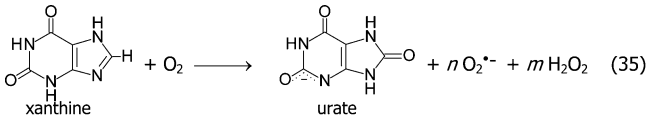
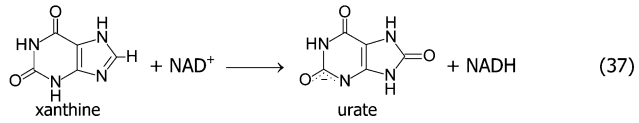
Mammalian XO (Figures 11a, 28, eq 35) and AO (Figure 11c, eq 36) are cytoplasmatic molybdoenzymes, belonging to the XO family (see section 3), that are present in various tissues.[577,596−602] Noteworthy, besides the cytoplasm,[603,604] XO was also described to be present on the outer surface of the cell membrane of endothelial and epithelial cells[605−612] and on the peroxisomes.[613,614] In vivo, XO exists predominantly as a NAD^+-dependent dehydrogenase, named xanthine dehydrogenase (XD, eq 37).[596−602] Yet, XD can be rapidly converted into a "strict" oxidase form that reduces dioxygen instead of NAD^+, the commonly studied and very well-documented XO. This conversion can be reversible, through oxidation of Cys_{535} and Cys_{992}, or irreversible, by limited proteolysis at Lys_{551} or Lys_{569} (conversion details will be described in section 3.2.3.1). Hence, XO/XD is the third protein type here described (after Nb and Cc) whose activity can be "switched" by a posttranslational conformational modification. AO is structurally similar to XO/XD, but exists exclusively as an oxidase (reduces dioxygen, not NAD^+).[615,616]



$$\text{xanthine} + O_2 \longrightarrow \text{urate} + n\,O_2^{\bullet-} + m\,H_2O_2 \quad (35)$$

$$\text{aldehyde} + O_2 \longrightarrow \text{carboxylate} + n\,O_2^{\bullet-} + m\,H_2O_2 \quad (36)$$



$$\text{xanthine} + NAD^+ \longrightarrow \text{urate} + NADH \quad (37)$$

Physiologically, mammalian XO/XD is a key enzyme in purine catabolism, where it catalyzes the hydroxylation of both hypoxanthine and xanthine to the terminal metabolite, urate, with the simultaneous reduction of dioxygen (XO, eq 35) or NAD^+ (XD, eq 37).[596−602] The physiological function of AO remains a matter of discussion, being a probable partner in the metabolism of xenobiotics, neurotransmitters, and retinoic acid.[616−619] However, XO/XD, as well as AO, catalyzes also the oxidation of a wide variety of substituted pyridines, purines, pteridines, related compounds, including the NADH,[578−584] and aldehydes.[596−602,620,621] Besides this broad specificity for oxidizing substrates, these enzymes are also promiscuous with the reducing substrates, being able to catalyze the reduction of several sulfoxides and *N*-oxides, including nitrate and nitrite.[622−634] This unusual broad specificity has suggested the enzymes participation in other physiological pathways, including the beneficial activation of pro-drugs (e.g., refs 616,618,635−637) or the deleterious activation of azo dyes used as colorants in food and cosmetics (e.g., ref 638). In addition, their ability to catalyze the reduction of dioxygen has suggested their involvement in signaling pathways and, most important, in some ROS-mediated diseases,[639−647] including ischaemia-reperfusion injury[648−653] and ethanol hepatotoxicity.[581,654−659] Furthermore, XO/XD is also the target of development of new drugs against hyperuricaemia and gout. The proposed roles of both XO/XD and AO in a range of physiological and pathological conditions have resulted in a considerable and increasing medical interest in these enzymes. More recently, the demonstration that XO/XD and AO can also catalyze the nitrite reduction with NO formation contributed to further stimulate the interest in the catalytic properties of these versatile enzymes. Interestingly, it also changed the way in which these enzymes are being thought: from damaging (ROS sources) to beneficial players (NO sources).

In vitro, under anaerobic conditions, XO/XD and AO catalyze the nitrite reduction to NO, in a reaction that is pH dependent (highly favored at pH <7).[626−634,660] The reaction mechanism will be discussed in section 3.2.3. However, and as can be foreseen, dioxygen and NAD^+, the "classic" oxidizing substrates, act as strong competitive inhibitors of the nitrite reduction, "stealing" the electrons needed to reduce nitrite.[629,631,661] This in vitro anaerobic reaction establishes the starting point for the suggested hypoxic XO/XD/AO-dependent NO formation in vivo.

During ischaemia, several events occur that, in concert, can favor the nitrite reduction by XO/XD and AO (Figure 12):

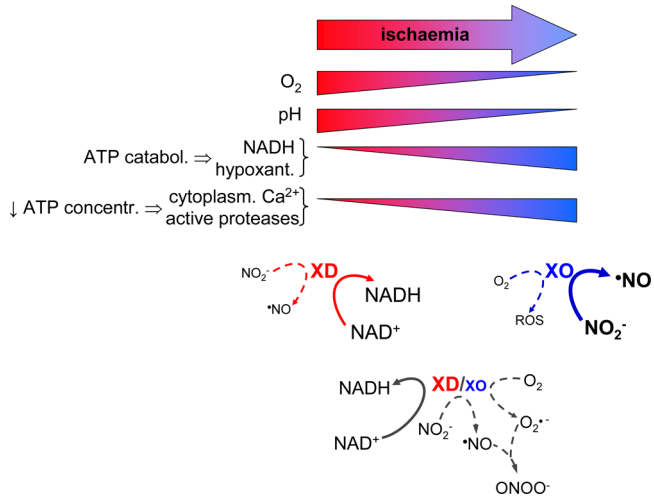


Figure 12. Mechanism proposed for XO-dependent nitrite reduction to NO under ischaemia. See text for details.

First, and obviously, is the decrease in dioxygen concentration (hypoxia or even anoxia) and the resulting acidosis (pH values of 6.5−5.5). Second, in the course of ischaemia, the mitochondrial electron transfer chain would be disrupted and the ATP synthesis hindered; the subsequent ATP catabolism leads to an accumulation of hypoxanthine and NADH in tissues.[662−668] This increase in the concentration of two reducing substrates can "fuel" the enzymes with reducing equivalents to reduce nitrite. Third, as the ATP concentration decreases, the transmembranar ion gradients are dissipated, causing elevated cytoplasmatic calcium concentrations, which, in turn, activate calcium-dependent proteases that would convert the XD into the XO form.[612,669−675] In summary, during ischaemia: (i) the pH values are lower enough to provide the acidic conditions required for the nitrite reaction; (ii) reducing substrates are available to supply the necessary electrons; (iii) the formerly prevailing XD form (that reacts with NAD^+) can be converted into the "dioxygen-user" XO, by proteolysis; (iv) the concentration of the competitive dioxygen is very low; and (v) NAD^+ (regardless of its high concentration) would be no longer a competitive substrate of the nitrite reduction, because XO and AO do not react with it. Therefore, all of the conditions seem to be gathered for nitrite to be reduced by XO and AO during in vivo ischaemia.

In accordance with the above reasoning, several in situ and in vivo studies suggested that these enzymes are acting as nitrite

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reductases, in models of ischaemia (and others) injury in heart, liver, lung, kidney, and vessels.[185−187, 192, 202, 203, 503, 627, 631−633, 676−681] Those studies were validated with employment of the XO and AO specific inhibitors allopurinol/oxypurinol and raloxifene, respectively, and also the general molybdoenzymes inhibitor tungstate. Particularly relevant is the demonstration of the XO/nitrite protective role within the context of cardiac ischaemia in an isolated heart model.[185]

Despite those in situ and in vitro studies, some authors argue that the high K_m values for nitrite (~0.5−2 mM[629,634] and ~3 mM,[633] for XO and AO, respectively), 1−2 orders of magnitude higher than the nitrite concentration in tissues (<20 μM[171,682,683]), are a major limitation for the in vivo relevance of these molybdenum-dependent pathways. However, the kinetic parameters indicate that these enzymes can produce NO, with reasonable rates ($k^{app} \approx 40\ M^{-1}\ s^{-1}$[634]), at conditions "fine-tuned" by the availability of nitrite and dioxygen.[629,634] That is, by functioning in a concentration range well below the K_m value, the reaction rate is first order on nitrite (k^{app}), thus allowing the NO formation to be directly controlled by the nitrite availability. In addition, the NO generation should be readily controlled by the strong competitor dioxygen, whose k_{cat}/K_m (pseudo-first-order rate constant) is 2−3 orders of magnitude higher.[684] In this way, the concentration of NO is kept within the characteristics of a local signaling molecule and controlled. In this respect, it should be emphasized that, in vivo, where NO performs its functions at nanomolar concentrations, it is not conceivable to produce NO at micromolar or millimolar levels (the enzymes K_m order). At micromolar concentrations, it would not be achievable to control the NO specificity and toxicity. In fact, it is in situations of NO overproduction that its deleterious effects began (e.g., in chronic inflammation, where 2−4 μM of NO was described to be formed[154,155]). Thus, if these molybdoenzymes are to be physiologically relevant NO sources, they should not catalyze the formation of NO at the nitrite K_m concentration values.[634]

Another point against the feasibility of these pathways in vivo is related to the conversion of the in vivo-predominant XD into XO. The extent and rate of this conversion are a matter of great controversy: from no conversion at all (with XO being considered as an experimental artifact), to a small (20%) and slow conversion[588,644,669−671,673−675,685,686] and a conversion that is enhanced by hypoxic conditions and in vivo ischaemia.[612,672] The issue here is the competition between nitrite and NAD^+ to react with reduced XD. The NAD^+ concentration (~0.5−1 mM[665,666,687−690]), 2−3 orders of magnitude higher than that of NADH, is not significantly decreased by the NADH accumulation during ischaemia.[664−668] As a result, if the conversion of XD into XO is not efficient (or does not occur at all), the NAD^+ reaction (with a k_{cat}/K_m 2−3 orders of magnitude higher[691]) would prevail over the nitrite reduction, and the NO formation by this protein would be seriously compromised. Nonetheless, the AO would still be able to achieve the NO formation, because it does not react with NAD^+.

In addition, also the competition between nitrite and dioxygen is critical, and these pathways require substantial hypoxia or even anoxia.[629,631,661] Moreover, in the presence of dioxygen, even at low concentrations (K_m for dioxygen is ~10^{-5} M[684,692]), the superoxide radical formed would react with NO, to yield the strong oxidizing peroxynitrite (eq 13).[153−155] Thus, under nonanoxic conditions, the copresence of superoxide dismutase is crucial for the NO signaling function.

Besides those chemical and kinetic constrains, the proposed role of XO/XD as a NO source faces another obstacle: for long, countless studies pointed toward a beneficial clinical outcome through the inhibition of XO (reduction of symptoms by treatment with allopurinol).[648,649,653,693] How can those results be reconciled with a beneficial XO-mediated role? Clearly, the old perspective has to be "broken" to allow new, more comprehensive, studies.[694]

In summary, in vitro, under anaerobic conditions, the molybdenum centers of XO/XD and AO are able to reduce nitrite and, contrary to the haemic proteins so far discussed, to promptly release the NO formed (fully discussed in section 3.2.3). With the molybdenum-containing proteins, the dilemma is related to the competition between nitrite and the "classic" oxidizing substrates, dioxygen and NAD^+, for the enzymes electrons. In vivo, the molybdenum-dependent NO formation would be determined by the extension of ischaemia (hypoxia), the copresence of superoxide dismutase and other antioxidants, and by the availability of oxidizing and reducing substrates. In the case of XD/XO, the NO formation is further dependent on an additional factor: its conversion to XO. Thus, a parallelism with Nb and Cc can be drawn, and the XD/XO can be suggested to be an allosteric-regulated nitrite reductase, controlled by limited proteolysis or by the redox state of key cysteine residues. Nevertheless, while the activity regulation by proteolysis is easily reconciled with an ischaemic event (Figure 12), the regulation through cysteines oxidation is subjected to criticism. As for Nb (Figure 10), it can be argued that, after the ischaemic phase, during reperfusion, as oxidative stress[212] conditions develop and the concentration of reduced thiols decreases, the fraction of XO increases and the NO formation would be promoted. However, under these conditions, the dioxygen present inhibits the nitrite reduction.

In light of the well-known molybdenum oxo-transfer chemistry (discussed in detail in section 3.2.3), the nitrite reductase activity of XO/XD and AO is not at all unexpected: once again, the mammalian cells are doing "substrate adaptations" to available redox systems and "switching" the systems activities in accordance with the cellular needs. In this context, the hypothesis of XD nitrite reductase activity being further dependent on the conversion into XO adds an additional level of complexity (allosteric regulation) to the "activity switching".

One of the major challenges concerning the molybdenum-dependent enzymes is to understand if, in vivo, the XD is converted into XO. Is the conversion an experimental artifact or a deliberated regulatory strategy? If it is not an artifact, how, to what extent, and when does the in vivo conversion take place? How are the posttranslational regulatory mechanisms orchestrated to control the "switching" between XD/XO activities? On the other hand, if the conversion does not occur in vivo, how can the XD avoid reacting with NAD^+ to be able to produce NO?

Another challenge is related to the development of novel in situ/in vivo experimental approaches. Most of the XO studies are validated with the employment of allopurinol/oxypurinol, which is a recognized ROS scavenger and is known to interfere with alternative purine catabolism pathways (in particular with adenosine).[695−698] Also, raloxifene (an estrogen receptor modulator) interferes with other vital pathways.[699] In this respect, the use of a new XO inhibitor, febuxostat (with a K_i value 3 orders of magnitude lower than the one of allopurinol),

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could in principle be useful. Also valuable would be the obtainment of viable XO/XD and AO mutants.

The in vivo relative relevance of these enzymes on the total nitrite-dependent NO formation also needs to be revaluated in a tissue-dependent manner. For example, the XO/XD interaction with the negatively charged glycosaminoglycans of vessel wall was poorly explored. It has been suggested that several pathologies (including liver and intestine ischaemia) cause the XO/XD release into circulation.[663,693,700−704] Once in circulation, XO/XD can bind to the endothelia glycosaminoglycans[605−612] to form a new complex, polysaccharide-XO/XD, which displays different kinetic properties toward xanthine and allopurinol.[705] How does this complex affect the nitrite activity of XO/XD? Is the enzyme reaching locations where it was not present before the injury and where the NO formation can be relevant?

2.2.1.1.4. Protein-Independent Nitrite Reduction. Remarkably, mammals still can count on another mechanism to produce NO: the protein-independent nitrite reduction under acidic and reducing conditions. The protein-independent NO formation has been demonstrated in the stomach,[706−713] skin surface,[714,715] infected urinary tract,[716] and oral cavity, even though the mouth higher pH (relative to the stomach one) dictates a lower NO formation.[717,718] This protein-independent NO generation may play a decisive role, not only on the (obvious) control of vasodilation,[719−721] but also in gastric mucosa formation[722] and in host defense (as bactericide in oral cavity,[718] stomach,[708,723−729] urinary tract,[716] and even in skin[715]). In this context, it is worth mentioning the commensal bacteria of the oral cavity that reduce nitrate to the necessary nitrite for NO generation in the stomach (through the swallowed saliva[730]).[713,726−729,732−734] Moreover, the microbial communities of human dental plaque are capable of carrying out the complete denitrification pathway, mediating the nitrate reduction to NO, and further to nitrous oxide and dinitrogen.[735] This constitutes an overlooked symbiotic interaction that may question the overuse of antibacterial mouthwash. On the other hand (and ironically), it is the nitrite production by some pathogenic nitrate-reducing bacteria that may cause their own destruction through the formation of cytotoxic concentrations of NO.[716]

The protein-independent NO formation relies on the nitrite decomposition, at acidic pH, to dinitrogen trioxide (eqs 38,39), which can then dismutate to NO and nitrogen dioxide radical (eq 27) or be converted to a RSNO (eq 28).[348,736,737]

$$2NO_2^- + 2H^+ \rightleftharpoons 2HNO_2 \quad (38)$$

$$2HNO_2 \rightleftharpoons H_2O + N_2O_3 \quad (39)$$

$$N_2O_3 \rightleftharpoons {}^{\bullet}NO + {}^{\bullet}NO_2 \quad (27)$$

Global reactions (eqs 38 + 39 + 27):

$$2NO_2^- + 2H^+ \rightarrow {}^{\bullet}NO + {}^{\bullet}NO_2 + H_2O \quad (40)$$

$$N_2O_3 + RSH \rightarrow RSNO + NO_2^- + H^+ \quad (28)$$

The extent of this NO generation, however, is expected to be very small and limited to conditions of profound hypoxia and/or acidosis, because it depends on (i) the formation of nitrous acid (pK_a 3.1−3.4 for eq 38) and (ii) the dismutation of dinitrogen trioxide, whose equilibrium (eq 27) is expected to be rapidly dislocated toward the NO consumption (as was discussed in section 2.2.1.1.1). Nevertheless, the rate and direction at which the reactions 38→39→27 would be driven (in vitro and in vivo) depend not only on the pH, but also on (i) factors that potentially shift the equilibriums by consuming NO, such as dioxygen (eq 41), haem-containing proteins, and thiol compounds, and (ii) the presence of reducing compounds.[738] In its turn, the aqueous redox chemistry of nitrite is, once more, highly pH-dependent: the nitrite reduction to NO is proton-coupled ($2H^+/e-$; eq 16), and its reduction potential drops from 1.00, at pH 1, to 0.37 V, at pH 7 (vs NHE).[738] Hence, on a laboratory scale, it is the reduction of acidified (pH 1) nitrite solutions with iodide that is used to rapidly and estequiometrically synthesized NO. In less harsh conditions, ascorbic acid, which acts as both a reducing and an acidifying agent, rapidly reduces nitrite to NO (eq 42).[7] In accordance, the NO generation in stomach and urinary tract was observed to be enhanced by the presence of vitamin C[7,716,721] and polyphenolic compounds.[711,712,739,740] In this respect, it is should be emphasized that reactions 38 plus 39 result in the global reaction 26, which was described to be catalyzed by ferrous Hb (in section 2.2.1.1.1). Hence, in both cases, the dinitrogen trioxide formation is dependent on the presence of a reducer. In summary, the protein-independent NO formation is expected to be highly pH and reducer-dependent.

$$2{}^{\bullet}NO + O_2 \rightleftharpoons 2{}^{\bullet}NO_2 \quad (41)$$

$$2HNO_2 + \text{ascorbic acid} \rightleftharpoons \text{dehydroascorbic acid} + 2{}^{\bullet}NO + 2H_2O \quad (42)$$

Although the protein-independent NO generation had been demonstrated in the few above-mentioned organs/localizations, it is worth mentioning that this pathway is likely to occur at any site where nitrite is present under acidic and reducing conditions,[741,742] in particular, in any tissue under ischaemia, where the pH decreases to values as low as 5.5 and the reducing equivalents accumulate.[741−746] In fact, it was shown that the reducing (nonenzymatic) compounds present in homogenates of hearts subjected to ischaemia greatly (40-fold, at pH 5.5) increase the rate of NO formation.[741,746,747] Even so, it should be noted that this protein-independent, reducers-dependent NO formation was observed to be lower (<15%[748]) than the protein-dependent one described in the previous sections.[629,748]

Presently, little is know about the regulation and physiological/pathological significance of this protein-independent NO formation pathway, in particular in the stomach, where its occurrence seems to be beyond any doubt. This is one of the major challenges for future research.

2.2.1.1.5. Nitrous Anhydrase. Besides the "nondedicated" nitrite reductases so far discussed, also the zinc-containing ubiquitous carbonic anhydrase (Figure 11d) was shown to readily produce NO from nitrite.[749] Because Zn^{2+}, with its d^{10} electronic configuration, does not support redox chemistry, the carbonic anhydrase was suggested to act as a nitrous anhydrase (eqs 38→39), in a reaction that would be equivalent to the reverse of the carbon dioxide hydration (eq 43). The subsequent dinitrogen trioxide dismutation would yield nitrogen dioxide radical and the aimed NO (eq 27).[749] The feasibility of this mechanism is, however, questionable, because (i) the formation of dinitrogen trioxide (eqs 38→39) and (ii) the direction and rate of the equilibrium 27 have been

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Table 4. Proteins Involved in Nitrite Oxidation in Mammals

protein	"classic" reaction "classic" functions	site of nitrite oxidation reaction of nitrite oxidation	figure [a]	section [b]
haemoglobin (Hb) (blood)	dioxygen transport	*b* haem (Fig. 19(a)), penta-coordinated by a histidine residue $(Hb)Fe^{2+}\text{-}O_2 + NO_2^- + H^+ \longrightarrow (Hb)Fe^{3+} + NO_3^- + 1/2H_2O_2$ $(^{\bullet}Hb)Fe^{4+}{=}O + NO_2^- \longrightarrow (Hb)Fe^{4+}{=}O + {}^{\bullet}NO_2$ $(Hb)Fe^{4+}{=}O + NO_2^- + 2H^+ \longrightarrow (Hb)Fe^{3+} + {}^{\bullet}NO_2 + H_2O$	4, 13, 14	2.2.1.2.1.
myoglobin (Mb) (cardiac, skeletal and smooth muscle)	dioxygen transport; recently, several *novel* functions were suggested: cardiac NO homeostasis, O_2 sensing, ROS scavenging, intracellular fatty acid transport	*b* haem (Fig. 19(a)), penta-coordinated by a histidine residue $(Mb)Fe^{2+}\text{-}O_2 + NO_2^- + H^+ \longrightarrow (Mb)Fe^{3+} + NO_3^- + 1/2H_2O_2$ $(^{\bullet}Mb)Fe^{4+}{=}O + NO_2^- \longrightarrow (Mb)Fe^{4+}{=}O + {}^{\bullet}NO_2$ $(Mb)Fe^{4+}{=}O + NO_2^- + 2H^+ \longrightarrow (Mb)Fe^{3+} + {}^{\bullet}NO_2 + H_2O$	4	2.2.1.2.1.
peroxidases (several tissue types)	antioxidant and immune defence	haem (Fig. 19(a)), penta-coordinated $(perox)R^{\bullet}\text{-}Fe^{4+}{=}O + NO_2^- \longrightarrow (perox)R\text{-}Fe^{4+}{=}O + {}^{\bullet}NO_2$ $(perox)R\text{-}Fe^{4+}{=}O + NO_2^- + 2H^+ \rightarrow (perox)R\text{-}Fe^{3+} + {}^{\bullet}NO_2 + H_2O$		2.2.1.2.2.
cytochrome *c* (Cc) (all tissue types)	electron transfer complex III ⟶ complex IV mitochondrial oxidative phosphorylation; "non-classic" functions include apoptotic signalling molecule, $O_2^{\bullet-}$ scavenger, lipid peroxidation catalyst, peroxidase-like enzyme, nitrating agent	*c* haem (Fig. 19(a)), hexa-coordinated by a histidine (proximal) and a methionine (distal) residues hexa-coordination reversible $(Cc)R^{\bullet}\text{-}Fe^{4+}{=}O + NO_2^- \longrightarrow (Cc)R\text{-}Fe^{4+}{=}O + {}^{\bullet}NO_2$ $(Cc)R\text{-}Fe^{4+}{=}O + NO_2^- + 2H^+ \longrightarrow (Cc)R\text{-}Fe^{3+} + {}^{\bullet}NO_2 + H_2O$	11	2.2.1.2.3.

[a]Figures where the protein is represented. [b]Sections where the protein is discussed.

subjected to controversy (as discussed in sections 2.2.1.1.1 and 2.2.1.1.4).

Despite those criticisms, the carbonic anhydrase-dependent NO was shown to induce the vasodilation of aortic rings.[749] Hence, this enzyme was suggested to contribute to the regulation of local blood flow in response to an increase in the tissue metabolic activity, through the following mechanism:[749] (i) increased metabolic activity results in an increased carbon dioxide formation; (ii) carbonic anhydrase very rapidly "translates" this higher carbon dioxide into a decreased pH (eq 43); (iii) the local transient acidosis would then favor the dioxygen delivery to the tissue, via the Bohr effect that decreases the Hb dioxygen affinity;[750] and (iv) in addition, the local transient acidosis would also favor the carbonic anhydrase-dependent NO formation (eqs 38→39→27), triggering the vasodilation, which further favors the dioxygen supply to the tissue.

Noteworthy, and contrary to all of the other pathways discussed, the carbonic anhydrase-dependent NO formation could occur before the dioxygen concentration decreases below critical values.[749]

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^- + H^+ \quad (43)$$

$$2NO_2^- + 2H^+ \rightleftharpoons 2HNO_2 \rightleftharpoons H_2O + N_2O_3 \quad (38,39)$$

$$N_2O_3 \rightleftharpoons {}^{\bullet}NO + {}^{\bullet}NO_2 \quad (27)$$

The major challenge concerning this nitrite anhydrase is, undoubtedly, the identification of the reaction mechanism responsible for the dinitrogen trioxide/NO formation in the absence of a redox active metal. Also, the competition between carbon dioxide and nitrite should be thoroughly explored.

2.2.1.1.6. Nitrite Reduction – Summary. In summary, the mammalian nitrite-derived NO formation is, theoretically, quite simple, noteworthy, much simpler than the NOS-catalyzed synthesis (eqs 14, 15). It involves the one-electron reduction of nitrite by a redox active metalloprotein and requires just protons (acidosis), a low dioxygen concentration, and an electron donor to reduce the metal. Therefore, from a chemical point of view, mammals that do not have a "dedicated" nitrite reductase can reduce nitrite by doing a "substrate adaptations" to an available redox active metalloprotein. From a physiological standpoint, mammals are "reusing" metalloproteins, present in cells to accomplish other functions, and "switching" the proteins activity when it is necessary to generate NO and the NOS activity is impaired. However, as was discussed, the in vivo nitrite "recycling" to NO is, actually, a very complex subject, further complicated by the (present) lack of knowledge to connect the in vitro understanding of nitrite reduction mechanisms with the in vivo observed nitrite effects.

Although the physiological relevance of each individual pathway (Figure 3, Table 2) is currently being (and will be) debated, the fact that nitrite can be reduced to NO (i) by diverse metalloproteins (enzymes, metabolite transporters, and electron transfers), with (ii) different cellular roles, (iii) tecidular and subcellular localizations, and (iv) molecular features, suggests that nitrite does play a critical role in the mammalian cellular homeostasis. From a physiological point of view, the existence of several NO formation pathways seems sensible. (i) It is not probable that biology developed only one specialized enzyme to generate such a crucial molecule. Certainly, it would be an advantage to have some "rescue" pathways to ensure the NO formation. (ii) These would be particularly relevant for mammals to be able to avoid the deleterious hypoxic/anoxic conditions, when the oxygen-dependent NOS activity would be impaired. (iii) In addition, the promiscuous function of an enzyme/protein can be reasoned as a "vestige" of the function of its ancestor.[751] The choice of nitrite as the NO source (instead of, e.g., another

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amino acid) can be thought as a "vestige" of the preaerobic pathways.[752,753]

Interestingly, some of those metalloproteins have a known oxygen-dependent or oxygen-related activity, but, under hypoxia, the proteins apparently "switch" from their "classic" activity to a nitrite reductase activity. Furthermore, in a few of these proteins, Nb, Cc, and possibly XD/XO, the "activity switch" is allosterically regulated. These "activity switches" put forward the hypothesis that nitrite is acting, not only as a NO source, but also as an oxygen or redox sensing molecule. According to this hypothesis, each individual nitrite-mediated pathway would be activated when the oxygen concentration decreases below its own threshold of oxygen-dependent activity. So, different pathways would be triggered by different oxygen concentrations/redox conditions. In this way, all pathways would act in a concerted and self-regulated manner, with each individual pathway being relevant under different conditions and in different tissues.[754,755]

The "activity switching" hypothesis adds another level of complexity to the intricate puzzle of nitrite-mediated pathways and catapults the nitrite relevance to the entire physiological oxygen gradient, from normoxia to anoxia.

2.2.1.2. Nitrite Oxidation in Mammals. When compared to its reduction, the nitrite oxidation (Figure 3, Table 4) and its potential role in mammalian physiology and pathology have been overlooked.

Although no "dedicated" mammalian nitrite oxidase was yet described, several haemic proteins seem to be used to carry out the oxidase function. Those proteins catalyze the nitrite oxidation, not only to nitrate (a two-electron oxidation), but also to nitrogen dioxide radical (one-electron reaction). As described in sections 2.2.1.1.1–2.2.1.1.2, the oxygenated HG are able to oxidase nitrite to nitrate (eq 18). In addition, nitrite can also be oxidized by several peroxidases, myeloperoxidase, lactoperoxidase, eosinophil peroxidase, and catalase, as well as by Cc and "inflammatory" oxidants, such as the hypochlorous acid (see references throughout the following subsections).

Unlike nitrate, the nitrogen dioxide is a powerfully oxidizing and nitrating agent that can nitrate, not only protein residues, but also fatty acids and guanine nucleotides.[756–764] Yet, while limited nitration of fatty acids and guanine nucleotides has been shown to elicit protective responses against inflammatory tissue injury,[765,766] protein nitration is considered to be a deleterious process that alters the protein function and targets it for degradation. Moreover, the nitration reactions have been evoked to explain the loss of protection observed when higher doses of nitrite are administered in ischaemia-reperfusion conditions.[186,191]

2.2.1.2.1. Haem-Dependent Nitrite Oxidation – Haemic Globins. The nitrite oxidation by oxy-Hb is known for more than a century, with the initial studies endeavored essentially to investigate nitrite poisoning.[767–779] From this reaction results the formation of nitrate and met-Hb (eq 18). However, the reaction kinetics is far more complex than just a simple bimolecular reaction (pH-dependent), with a single rate-limiting step. Instead, it was shown to be an autocatalytic radical chain reaction, described by reactions 18 and 44–47 (Figure 13).[779]

$$\text{initiation: } (\text{Hb})\text{Fe}^{2+}\text{–O}_2 + \text{NO}_2^- + \text{H}^+ \rightarrow (\text{Hb})\text{Fe}^{3+} + \text{NO}_3^- + 1/2\text{H}_2\text{O}_2 \quad (18)$$

$$\text{propagation: } (\text{Hb})\text{Fe}^{3+} + \text{H}_2\text{O}_2 \rightarrow (^{\bullet}\text{Hb})\text{Fe}^{4+}{=}\text{O} + \text{H}_2\text{O} \quad (44)$$

(where $(^{\bullet}\text{Hb})\text{Fe}^{4+}{=}\text{O}$ is ferryl-haemoglobin molecule with a radical on an amino acid residue, formed by the two electrons reduction of $(\text{Hb})\text{Fe}^{3+}$)

$$(^{\bullet}\text{Hb})\text{Fe}^{4+}{=}\text{O} + \text{NO}_2^- \rightarrow (\text{Hb})\text{Fe}^{4+}{=}\text{O} + {}^{\bullet}\text{NO}_2 \quad (45)$$

$$(\text{Hb})\text{Fe}^{4+}{=}\text{O} + \text{NO}_2^- + 2\text{H}^+ \rightarrow (\text{Hb})\text{Fe}^{3+} + {}^{\bullet}\text{NO}_2 + \text{H}_2\text{O} \quad (46)$$

$$(\text{Hb})\text{Fe}^{2+}\text{–O}_2 + {}^{\bullet}\text{NO}_2 \rightarrow (^{\bullet}\text{Hb})\text{Fe}^{4+}{=}\text{O} + \text{NO}_3^- \quad (47)$$

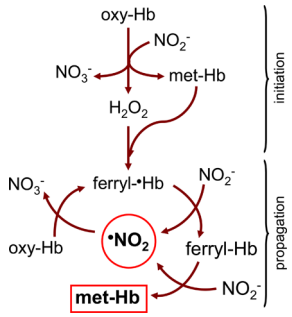

Figure 13. Mechanism of nitrite oxidation by oxy-Hb. See text for details. Figure modified from ref 779.

Global reaction of the propagation cycle (eqs 44 + 45 + 46 + 47):

$$(\text{Hb})\text{Fe}^{2+}\text{–O}_2 + 2\text{NO}_2^- + 2\text{H}^+ + \text{H}_2\text{O}_2 \rightarrow \text{NO}_3^- + 2\text{H}_2\text{O} + (^{\bullet}\text{Hb})\text{Fe}^{4+}{=}\text{O} + {}^{\bullet}\text{NO}_2 \quad (48)$$

The nitrogen dioxide and ferryl-haemoglobin radicals formed can initiate new propagation cycles, autocatalyzing the reaction until all oxy-Hb is consumed. In vivo, under normal conditions, however, the presence of antioxidants should limit the propagation phase: (i) catalase and glutathione peroxidase should consume the initiator hydrogen peroxide, (ii) the erythrocyte-NADH-cytochrome b_5 reductase system reduces the met-Hb to ferrous Hb, and (iii) small molecule antioxidants such as glutathione, urate, and ascorbate should reduce the radical species that propagate the cycle. Other termination reactions include (i) the nitrogen dioxide dimerization to dinitrogen tetraoxide (N_2O_4, eq 49; $K_d \approx 10^{-5}$ M[348]), (ii) its reaction with NO to yield dinitrogen trioxide (reverse eq 27 = eq 51), both of which could be hydrolyzed to yield nitrite and nitrate (eqs 50 and 52), and (iii) its participation in nitrating reactions.[756–763] Therefore, in vivo, the reaction is not expected to become autocatalytic, except for severe nitrite poisoning.[780] Under normal conditions, the rate of nitrite oxidation by oxy-Hb is suggested to be determined only by reaction 18 and to be slow ($k \approx 0.5$–$1\ \text{M}^{-1}\ \text{s}^{-1}$).[779]

$$2{}^{\bullet}\text{NO}_2 \rightleftharpoons \text{N}_2\text{O}_4 \quad (49)$$

$$\text{N}_2\text{O}_4 + \text{H}_2\text{O} \rightarrow \text{NO}_2^- + \text{NO}_3^- + 2\text{H}^+ \quad (50)$$

$${}^{\bullet}\text{NO} + {}^{\bullet}\text{NO}_2 \rightleftharpoons \text{N}_2\text{O}_3 \quad (51)$$

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$$N_2O_3 + H_2O \rightarrow 2NO_2^- + 2H^+ \quad (52)$$

Nevertheless, it should be noted that the reactions of the propagation phase are not triggered only by the previous nitrite oxidation (eq 18). The propagation phase (eq 44) is also driven by the presence of hydrogen peroxide originated from other sources, when the antioxidant defenses are not able to efficiently remove it. Actually, the peroxidase-like activity of Mb[763,784−788] and Hb[789−791] has long been known; it results in the formation of ferryl- (eq 53) and radical ferryl-haems (eq 44). These oxidized proteins, in a situation of oxidative stress,[212] could greatly amplify the oxidation of nitrite to nitrogen dioxide (eqs 45 and 46) and, thus, promote the deleterious protein nitration[759,792,793] observed, for example, during myocardial ischaemia-reperfusion injury.[794−798] (The presence of nitrated proteins in circulating erythrocytes is controversial.[759,799]) Moreover, the acidic conditions, characteristic of ischaemia, favor the nitration reactions and decrease the rate of nitrogen dioxide scavenging by thiols, therefore increasing effectively the efficiency of protein nitration.[793,800]

$$(Mb)Fe^{2+} + H_2O_2 \rightarrow (Mb)Fe^{4+}{=}O + H_2O \quad (53)$$

$$(Mb)Fe^{4+}{=}O + NO_2^- + 2H^+ \rightarrow (Mb)Fe^{3+} + {}^{\bullet}NO_2 + H_2O \quad (46)$$

$$(Mb)Fe^{3+} + H_2O_2 \rightarrow ({}^{\bullet}Mb)Fe^{4+}{=}O + H_2O \quad (44)$$

$$({}^{\bullet}Mb)Fe^{4+}{=}O + NO_2^- \rightarrow (Mb)Fe^{4+}{=}O + {}^{\bullet}NO_2 \quad (45)$$

Because nitrite can reduce the ferryl and radical ferryl species,[801,802] it was suggested that reactions 45 and 46 could function as "sinks" of those oxidizing species and, in this way, prevent the oxidation and peroxidation reactions they promote (Figure 14).[785,803−809] The nitrogen dioxide radical simultaneously formed in reactions 45 and 46 should be scavenged by the high concentration of thiols and other antioxidants present in cells, until a relatively high concentration is formed. Reactions 45 and 46 could also be regarded as "regenerators" of Mb (Figure 14). This "recycling" would allow a more efficient scavenging of hydrogen peroxide by Mb (eq 53). This reasoning implies that nitrite oxidation can be a mechanism of cytoprotection,[810] and it should not be thought of only as a source of deleterious nitrogen dioxide radical.[300]

Overall, it is clear that the reactions 45 and 46 have "two faces", and, in vivo, it is likely that a delicate balance takes place between the protective and deleterious role of nitrite/hydrogen peroxide/Mb (Figure 14).[300] The antioxidant capacity and the extent of the oxidative injury, in particular of ischaemic and inflammatory injury (where nitrite accumulates), could dictate whether the Hb/Mb peroxidase-like activity should be protective or deleterious.

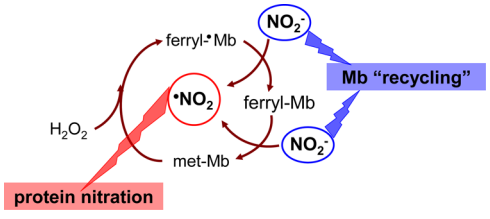

Figure 14. The "two faces" of nitrite oxidation: potential pathways to mediate deleterious and beneficial effects. See text for details.

In what concerns Nb (described in section 2.2.1.1.2), as expected, its oxidizing and nitrating activities are dependent on the conditions that favor the penta-coordination, with disulfide-containing Nb exhibiting the highest activities.[448] Because met-Nb does not react with hydrogen peroxide to form ferryl-haem species,[448,462] its nitrating activity has been ascribed to the following mechanism: nitrite binding to the haem iron, followed by reaction with hydrogen peroxide, to eventually yield an iron-peroxynitrite nitrating species[448] (see also ref 800). In this context, it is remarkable the apparent inability of Nb to form cytotoxic ferryl-haem species, which could be related and relevant to its observed role in cellular survival.[441,445]

2.2.1.2.2. Haem-Dependent Nitrite Oxidation − Peroxidases. It is remarkable that reactions 44−46, described for the autocatalytic nitrite oxidation by Hb, represent precisely a typical mechanism of a peroxidase-catalyzed reaction.[811−815] In general, a native ferric peroxidase reacts with hydrogen peroxide to form compound I (eq 54). The enzyme is then regenerated by two one-electron reduction steps, yielding compound II (equivalent to eq 55) and subsequently the ferric protein (equivalent to eq 56). Alternatively, the enzyme can be regenerated by one two-electron reaction, oxidizing halides to the respective hypohalous acids (as, e.g., in myeloperoxidase), or oxidizing a second molecule of hydrogen peroxide to dioxygen (as in catalase). Hence, taking as model the mechanism above-described for oxy-Hb, it is not difficult to envisage how a peroxidase enzyme can account for the nitrite oxidation (eqs 54→55→56 or global reaction in eq 57), as long as nitrite can bind to the active site and the reaction is kinetically feasible.

$$\text{(peroxidase)R-Fe}^{3+} + H_2O_2 \longrightarrow \underbrace{\text{(peroxidase)R}^{\bullet}\text{-Fe}^{4+}{=}O}_{\text{Compound I}} + H_2O \quad (54)$$

$$\underbrace{\text{(peroxidase)R}^{\bullet}\text{-Fe}^{4+}{=}O}_{\text{Compound I}} + NO_2^- \longrightarrow \underbrace{\text{(peroxidase)R-Fe}^{4+}{=}O}_{\text{Compound II}} + {}^{\bullet}NO_2 \quad (55)$$

$$\underbrace{\text{(peroxidase)R-Fe}^{4+}{=}O}_{\text{Compound II}} + NO_2^- + 2H^+ \longrightarrow \text{(peroxidase)R-Fe}^{3+} + {}^{\bullet}NO_2 + H_2O \quad (56)$$

Global reaction (eqs 54 + 55 + 56):

$$H_2O_2 + 2NO_2^- + 2H^+ \rightarrow 2{}^{\bullet}NO_2 + 2H_2O \quad (57)$$

For instance, nitrite is readily oxidized by myeloperoxidase at acidic pH (<6), in the presence of hydrogen peroxide, with a rate that is limited by the reaction with compound II.[816−821] In fact, nitrite is a good substrate for myeloperoxidase compound I (eq 55; $k \approx 2 \times 10^6$ M^{-1} s^{-1} (pH 7) or 10×10^6 M^{-1} s^{-1} (pH 5)), but it reacts more slowly with myeloperoxidase compound II (eq 56; $k \approx 6 \times 10^2$ M^{-1} s^{-1} (pH 7) or 900×10^2 M^{-1} s^{-1} (pH 5)).[820,821] Therefore, if a "fast substrate" is available to reduce compound II and regenerate the enzyme, the myeloperoxidase can rapidly oxidize nitrite to nitrogen dioxide.[821,822] The subsequent nitrogen dioxide-mediated nitration reactions could constitute a feasible protective response at the onset of inflammatory injury, through limited nitration of fatty acids and guanine nucleotides.[765,766] The nitrogen dioxide reactions could also constitute a defensive mechanism against pathological microorganisms (e.g., *P. aeruginosa*[823]). However, under chronic inflammatory conditions, when nitrite accumulates as a consequence of the induction of inducible NOS,[827−829] myeloperoxidase could

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catalyze a burst of nitrogen dioxide. Hence, myeloperoxidase could be responsible for the observed biomolecules nitration in a wide range of inflammatory diseases involving activated neutrophils and macrophages.[822] A parallel situation is thought to occur in asthma and other allergic inflammatory disorders characterized by activation of eosinophils.[816,817,822,830−836]

The eosinophils, however, are more efficient (at least 4-fold) at promoting nitration than the neutrophils, because the nitrite oxidation by compound II is faster ($k \approx 6 \times 10^3$ M^{-1} s^{-1} at pH 7.4) than in myeloperoxidase.[830,836]

With lactoperoxidase a different situation occurs, because nitrite rapidly reduces compound I by two electrons directly to the ferric state ($k \approx 2 \times 10^7$ M^{-1} s^{-1} (pH 7.2)[837]), with no observable nitrogen dioxide formation,[816, 817, 822, 830, 833−835, 837, 838] as catalase does.[769, 834, 839, 840] However, the one-electron reduction of compound I can be carried out by a small molecular mass phenolic substrate (Ph−OH; eq 58), followed by nitrite reduction of compound II to yield the nitrogen dioxide (eq 56) and subsequent phenol nitration (eq 59).[841] In addition, at least in vitro, in the presence of a high nitrite concentration, lactoperoxidase is able to promote nitration via an iron-peroxynitrite species, as was suggested for Mb (section 2.2.1.2.1).[841]

$$\underbrace{\text{(peroxidase)R}^{\bullet}\text{-Fe}^{4+}\text{=O}}_{\text{Compound I}} + \text{Ph-OH} \longrightarrow \underbrace{\text{(peroxidase)R-Fe}^{4+}\text{=O}}_{\text{Compound II}} + \text{Ph-O}^{\bullet} + \text{H}^{+} \quad (58)$$

$$\underbrace{\text{(peroxidase)R-Fe}^{4+}\text{=O}}_{\text{Compound II}} + \text{NO}_2^{-} + 2\text{H}^{+} \longrightarrow \text{(peroxidase)R-Fe}^{3+} + {}^{\bullet}\text{NO}_2 + \text{H}_2\text{O} \quad (56)$$

$$\text{Ph}-\text{O}^{\bullet} + {}^{\bullet}\text{NO}_2 \rightarrow \text{HO}-\text{Ph}-\text{NO}_2 \quad (59)$$

In summary, it is presently widely accepted that peroxidases-mediated oxidation of nitrite contributes to the observed deleterious nitration in several pathologies, in particular, in inflammatory conditions.[822]

2.2.1.2.3. Haem-Dependent Nitrite Oxidation − Cytochrome c. The peroxidase-like mechanism of nitrite oxidation can be further generalized, anticipating that other haemic proteins with peroxidase-like activity[842] can also promote the nitrogen dioxide radical formation, contributing to cellular oxidative damage.[844−846] In this respect, the "multitask" Cc stands out. (See section 2.2.1.1.2.b for the description of this hexa/penta-coordinated haemic protein, its several proposed activities/roles, including the peroxidase-like activity, and suggested "activity switching" mechanisms.)

In the presence of hydrogen peroxide, Cc catalyzes the oxidation of various reducers, including endogenous antioxidants, such as glutathione or ascorbate.[844,847−852] This peroxidase-like reaction is carried out through the formation of a compound I-like intermediate, in which one oxidizing equivalent is present as an oxoferryl-haem species and the other as a protein tyrosyl radical (equivalent to reactions 44 plus 45). The subsequent nitrite oxidation by the oxoferryl-haem yields nitrogen dioxide radical, in a reaction similar to the peroxidases one.[853] The nitrogen dioxide would then nitrate Cc itself and proximal molecules.

As expected, the peroxidase and nitrating activities of Cc are greatly increased by the presence of cardiolipin,[527,530,540,843] oxidation by peroxynitrite[532] or reactive halogen species,[536] nitration,[531,532] and also by partial proteolysis.[853−857]

In this context, it is of note that the presence of nitrated proteins and of cytoplasmatic Cc are two characteristics of cells undergoing apoptosis; moreover, mitoplasts depleted versus repleted with Cc display significant differences in nitration yields.[853] This "coincidence" could be due (at least in part) to the nitrite oxidase activity of Cc, which would constitute a plausible mechanism for the cell to control the molecular and spatial specificity of the nitration reactions.[853]

The Cc peroxidase-like activity can also constitute an important antioxidant defense against hydrogen peroxide-dependent oxidative damage, in mitochondria.[858] Besides phagocytes, mitochondria are a relevant source of hydrogen peroxide. Under normal conditions, mitochondria oxidize up to 1−2% of the oxygen consumed to hydrogen peroxide and superoxide radical.[859] Under "non-normal" conditions that proportion is higher, for example, during ischaemia-reperfusion injury or in the presence of redox cycling drugs.[860−863] Hence, Cc could be an antioxidant molecule that scavenges the hydrogen peroxide formed. In this scenario, the presence of nitrite could contribute to "regenerate" Cc, as was discussed for Hb/Mb, exerting, in this way, a beneficial (antioxidant) effect.

Yet, Cc has also been associated with deleterious effects, such as peroxidation of mitochondrial cardiolipin[528,529] or burst nitrations.[853] Overall, the beneficial and deleterious effects of Cc are probably in a delicate balance, as was above-discussed for Hb/Mb (section 2.2.1.2.1).

2.2.1.2.4. Nitrite Oxidation − Summary. One of the mechanisms that prevents the in vivo accumulation of nitrite is its oxidation. While the formation of the relatively "inert" nitrate is usually associated with the nitrite reaction with oxygenated Hb and Mb, it should not be forgotten that the same reaction produces, besides hydrogen peroxide, the reactive nitrogen dioxide radical. On the other hand, the formation of that oxidizing and nitrating radical has been linked with the nitrite reaction with proteins with peroxidatic activity.

In summary, to oxidize nitrite, either by one or by two electrons, mammals are doing "substrate adaptations" to the haem redox chemistry of haemic proteins already present in cells to accomplish other functions. However, contrary to its reduction, the nitrite oxidation seems to have two quickly identifiable "faces": (i) the beneficial, controlled formation of the signaling/defensive nitrogen dioxide and (ii) the deleterious, pathological, nitrogen dioxide overproduction. The "ugly face" of this nitrite handling arises from the accumulation of nitrite and hydrogen peroxide under conditions of oxidative and nitrosative stress, when both can be responsible for biomolecules modifications (oxidation and nitration) and, consequently, for injury.

Following the identification of nitrated proteins under a variety of pathological conditions, in vivo, the mechanisms of nitrotyrosine and nitrotryptophan formation became the focus of interest by the medical scientific community. For long, the observation of nitrotyrosine residues was considered as an unequivocal fingerprint of the peroxynitrite formation. However, the occurrence of nitrated proteins in cells would reflect not only the peroxynitrite formation or the NO trapping by tyrosyl radicals, but also the nitrite oxidation by "inflammatory" oxidants[756,816,864] or by haemic proteins such as those here described. The existence of these different nitrating pathways supports the relevance of this nitrite-dependent posttranslational protein modification for normal cellular signaling, but also in injury.[853]

Regardless of its physiological and pathological significance, the mechanisms of formation of nitrogen dioxide radical are only beginning to be studied at a molecular level of detail. Concerning the nitrite oxidation, the future goals include not

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only the mechanistic aspects, but mainly the understanding of its in vivo interplay and relative relevance.

2.2.1.3. Mammalian Nitrite Handling – Concluding Remarks. To conclude the discussion on the mammalian nitrite handling, two last points must be addressed.

First, in a review of the mammalian nitrite roles, it must be mentioned that nitrite itself has been suggested to be a signaling molecule, that is, with no need to be converted into the "active" molecule.[210,865] Although the nitrite stability and in vivo abundance would make it an interesting signaling molecule, it is difficult to prove that it is nitrite itself, and not NO or nitrogen dioxide, that is responsible for the observed effects.[866] If confirmed, this mechanism may overcome the impasse of the rapid NO scavenging.

Second, if nitrite is a key molecule for cellular homeostasis, how is its concentration controlled to respond to daily changes in dietary intake and in NO metabolism (NOS activity/NO consumption; Figure 15)? In humans, nitrite is obtained from diet sources such as vegetables (e.g., spinach, lettuce, or beetroot), curried meat,[6,733,867−869] or drinking water,[870] either directly[869] or indirectly through the nitrate reduction by commensal bacteria in the mouth[730] and gastrointestinal tract.[713,726,727,732,734] Most of this exogenous nitrite (which it is not converted in the stomach) diffuses to the systemic circulation, where it is transported to resistance vessels and tissues.[726] The second main source of nitrite is the NO oxidation itself,[152,871,872] with 70% of plasma nitrite being probably derived from endogenously produced NO.[326,873] To this pool of nitrite contributes the oxidation of NO through (i) reaction 30, (ii) reaction with dioxygen[874] (eqs 41→51→52), and (iii) ceruloplasmin.[179]

$$^{\bullet}NO + (Hb)Fe^{3+} \rightleftharpoons (Hb)Fe^{3+}{-}NO \quad (24)$$

$$(Hb)Fe^{3+}{-}NO \rightleftharpoons (Hb)Fe^{2+}{-}NO^{+} \quad (29)$$

$$(Hb)Fe^{2+}{-}NO^{+} + H_2O \rightarrow (Hb)Fe^{2+} + NO_2^{-} + 2H^{+} \quad (30)$$

$$2\,^{\bullet}NO + O_2 \rightleftharpoons 2\,^{\bullet}NO_2 \quad (41)$$

$$^{\bullet}NO + {}^{\bullet}NO_2 \rightleftharpoons N_2O_3 \quad (51)$$

$$N_2O_3 + H_2O \rightarrow 2NO_2^{-} + 2H^{+} \quad (52)$$

All of the nitrite sources are, thus, potentially subjected to great daily variation (Figure 15). Hence, how does a cell control the nitrite concentration to cope with its oxidation and reduction reactions? Presently, the mechanisms of nitrite transport and intracellular accumulation/export are poorly understood and mainly restricted to the erythrocyte, where the anion exchanger AE1 of band 3 is probably involved in (see ref 365).[875−879] Yet it would be very interesting if the nitrite transport across cell membranes was modulated by the oxygen concentration, allowing the nitrite transport to be directed to cells that need it to produce NO.[878,879] Future work will dictate if this is a reasonable hypothesis.

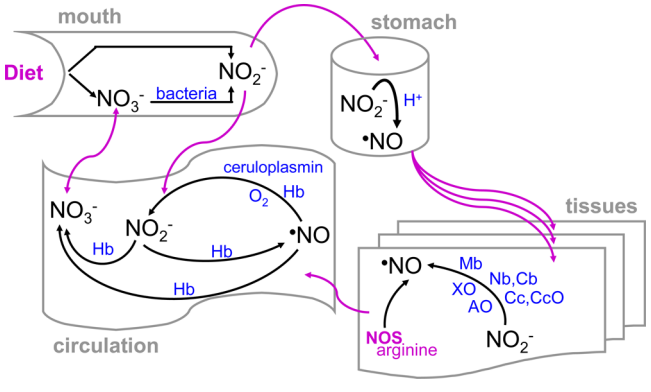

Figure 15. Nitrate/nitrite/NO sources in humans. See text for details.

Undoubtedly, the knowledge of nitrite physiological roles in mammals has evolved considerably over the last two decades, giving a novel relevance to the formerly "irrelevant" nitrite. This knowledge offers/will create new therapeutic approaches for the management of several pathological conditions,[880−885] including ischaemia injury, cardiovascular dysfunctions, myocardial infarction, stroke, or pulmonary hypertension.[185, 186, 191, 194, 198, 200, 206, 247, 285, 886−899] Also, in infection, nitrite is being used as a bactericide/fungicide in stomach and airways (see ref 823).[900−903] Thus, the mammalian nitrite metabolism research will certainly reserve many surprises for the future.

2.2.2. Nitrite in Signaling (and Other) Pathways in Plants. As all other organisms, plants must perceive and respond to a plethora of external stimulus and internal signals. NO is one of the signaling molecules used by plants to respond to abiotic and biotic challenges, as well as for their survival: NO has been shown to be involved in response to temperature, salt, or drought stresses, in disease resistance pathways, germination, flowering, root development, leaf senescence, or stomatal closure.[904−944] However, the plant NO formation and signaling pathways are, by far, less well characterized than the mammalian counterparts.

Plants have several potential NO-generating proteins, localized in different subcellular compartments, but only a few of them have been thoroughly studied. Presently, it is accepted that NO can be formed through oxidative and reductive pathways.

The oxidative pathways are believed to produce NO, aerobically, through the oxidation of organic compounds such as polyamines,[945,946] hydroxylamine,[947] and arginine.[948−952] Surprisingly, although arginine-dependent NO formation, inhibited by mammal NOS inhibitors, can be measured and localized in plant tissues and organelles, no homologous NOS (gene or protein) was yet found in higher plants.[904,932,951,953−955] In fact, the two previous NOS candidates for higher plants, a variant of the P protein of the glycine decarboxylase complex[956] and AtNOS1,[953,957] were found not to be NOS enzymes.[958,959] Nevertheless, the green algae *Ostreococcus tauri* was shown to hold a NOS (45% similar to human NOS[960,961]), and *Symbiodinium bermudense*[962,963] and *Chattonella marina*[964] were shown to have NOS-like activity. This raises the intriguing question of why higher plants do not have/need a NOS, contrary to animals, algae, or prokaryotes.

In the reductive pathways, apparently the predominant ones,[928,947,965,966] NO is formed through the (by now familiar) nitrite reduction and is favored by low dioxygen concentrations and acidic conditions (see references throughout the following sections). However, in plants, the nitrite reduction occurs in a different "scenario": nitrate and nitrite, both precursors and end-products of signaling NO, are also normal substrates of the plant nitrogen assimilation pathway (see section 2.1.1; Figure 1, orange arrows; Table 1). When nitrate is the main nitrogen source available, plants must assimilate it under conditions that range from normoxia to anoxia; nitrate and nitrite can, thus,

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accumulate to very high (millimolar) concentrations, in particular under hypoxia/anoxia (further discussed below). This situation is clearly different from the modest (nano- to micromolar) nitrite concentrations found in mammalian tissues, where nitrite is not involved in any primary biosynthetic pathway. As a consequence, when plants use nitrite to synthesize signaling NO, they must do it in a controlled and parallel way to the nitrogen assimilation. Is this the reason plant nitrate reductase is one of the possible nitrite reductases/NO synthases? These circumstances make the "signaling" nitrite/NO metabolism more complex in plants, but also allow nitrite to have roles not possible in mammals, like the maintenance of the cellular redox status under hypoxia (as will be discussed in section 2.2.2.1.4).

The nitrite reduction/NO formation in plants has been ascribed to proteins such as the molybdenum-containing cytoplasmatic nitrate reductase (C-NaR) or the haem-containing Cc, CcO, cytochrome bc_1 (Complex III), or nonsymbiotic HG (see references in the following subsections). Unexpectedly, the CSNiR, responsible for the assimilatory nitrite reduction to ammonium, does not reduce nitrite to NO (see sections 2.1.1 and 3.1.2 for details about CSNiR; Figure 1, orange arrows, Table 1).

To restrict the scope of information presented to a manageable size, only a few proteins will be here discussed (Figure 16, Table 5). Some proteins were chosen due to their

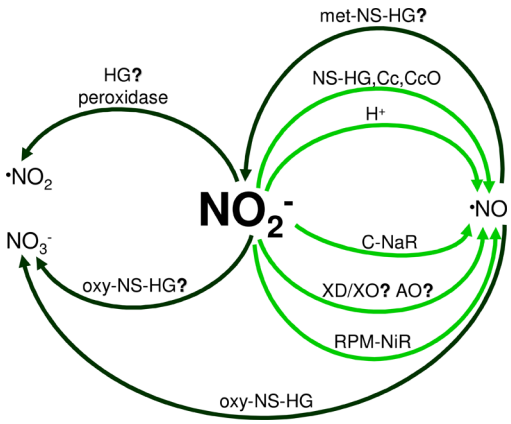

Figure 16. Nitrite in signaling pathways in plants. See text for details and abbreviations.

parallelism with the mammals and others because of their novelty. Therefore, only C-NaR, XO/XD, AO (molybdoenzymes), nonsymbiotic HG (haemic proteins), and the plasma membrane-bound nitrite reductase and nitrate reductase (the novel "players") will be discussed (sections 2.2.2.1.1−2.2.2.1.4). The protein-independent nitrite reduction will also be included (section 2.2.2.1.5). In addition, also the nitrite oxidation by plants will be briefly mentioned (section 2.2.2.2).

2.2.2.1. Nitrite Reduction in Plants. 2.2.2.1.1. Molybdenum-Dependent Nitrite Reduction − Cytoplasmatic Nitrate Reductase. The nitrite-dependent NO formation in plants, either in vivo or in vitro, has been ascribed mainly to the C-NaR. This enzyme is involved in the first and rate-limiting step of plant nitrate assimilation pathway, where it catalyzes the nitrate reduction to nitrite, with the simultaneous oxidation of NADH (eq 60; see section 2.1.1, Figure 1, orange arrows, Table 1).[601,967−973] The C-NaR is a homodimeric molybdoenzyme (Figure 11f), belonging to the sulfite oxidase family (see section 3; Figure 19c). Besides the molybdenum center, where the nitrate reduction takes place, C-NaR holds one b_5 haem and one FAD center, which is involved in the NADH binding and oxidation. In accordance with its key role in the nitrogen metabolism, C-NaR is highly regulated by complex transcriptional, translational, and posttranslational mechanisms that respond to nitrogen, carbon dioxide, and dioxygen availabilities, pH, temperature, and light.[967,969−971,973,974] Noteworthy, C-NaR is rapidly degraded in darkness (half-life of 6 h).

$$NO_3^- + NADH + H^+ \rightarrow NO_2^- + NAD^+ + H_2O \quad (60)$$

Besides this well-established role in the reduction of nitrate, C-NaR from different species were shown to also catalyze the subsequent nitrite reduction to NO (eq 61), not only in vitro,[928,975−977] but also in vivo. The in vivo evidences for C-NaR-dependent NO generation were provided by studies with (i) transgenic plants expressing a permanently active C-NaR,[978,928,984−986] (ii) C-NaR knockout mutants (*nia*1 and *nia*2 genes),[923,988−995] (iii) inactive C-NaR (e.g., plants with tungstate supply instead of molybdate),[989,990,996−1001] and (iv) others.[921,943,945,1002−1012] The nitrite reduction by C-NaR was also studied in silico, and it was found that both nitrate and nitrite are easily reduced (to nitrite and NO, respectively), although, as expected, nitrate is the preferred substrate.[1013]

$$NO_2^- + 1/2NADH + 3/2H^+ \rightarrow {}^{\bullet}NO + 1/2NAD^+ + H_2O \quad (61)$$

These two C-NaR activities, nitrate reductase and nitrite reductase, seem to be controlled by the dioxygen concentration. (a) Under normoxic conditions, the cytoplasmatic nitrate availability (in millimolar range[1014]) "autocontrols" the C-NaR nitrite reductase activity, because nitrate competitively inhibits the nitrite reduction (K_i of 50 μM[928,1015]). Simultaneously, the available nitrite concentration,[1016] 1−2 orders of magnitude lower than the respective K_m value (~100 μM[928]), does not favor its reduction.[928] (b) Under hypoxic and acidic conditions,[1017] the C-NaR concentration and activity are increased.[928,977,1032−1041] Simultaneously, the nitrite reduction by CSNiR is decreased, especially in hypoxic roots, due to the decreased NAD(P)H generation through the pentose phosphate pathway.[928,1015,1032,1034−1036,1040,1042] As a consequence, when nitrate is the main nitrogen source, nitrite accumulates in hypoxic tissues,[1032,1034,1035,1043,1044] and its reduction by C-NaR is progressively increased, leading to the NO formation.[928,966,1032] The same C-NaR "activity switch", from nitrate reductase to nitrite reductase, is observed upon nitrite accumulation triggered by inhibition of the photosynthetic activity[997,1045,1047−1049] or by the expression of an antisense CSNiR with very low activity.[988,989,1050]

In summary, when C-NaR activity is increased to an extent that nitrite formation exceeds its rate of consumption by CSNiR and/or nitrite accumulates to an extent that CSNiR could not cope with it, the nitrite reductase activity of C-NaR would become significant and the formation of NO is amplified.[928] The concept of doing "substrate adaptations" in plants is obvious and opportune: if plants are using C-NaR to abstract one oxygen atom from nitrate, why not to use the same redox system to continue the reaction and abstract the second oxygen atom?

Clearly, the amount of NO produced would be very low, predicted to be much less than 1% of the nitrate reducing activity (mainly due to competitive inhibition by nitrate).[928,989]

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Table 5. Proteins Involved in Nitrite Reduction to NO in Plants

protein	"classic" reaction "classic" functions	site of nitrite reduction reaction of nitrite reduction × major drawback [a]	figure [b]	section [c]
cytoplasmatic nitrate reductase (C-NaR)	$NO_3^- + NADH + H^+ \longrightarrow NO_2^- + NAD^+ + H_2O$ nitrate assimilation	molybdenum centre of sulfite oxidase family type (Fig. 19(c)) $NO_2^- + 1/2NADH + 3/2H^+ \longrightarrow {}^{\bullet}NO + 1/2NAD^+ + H_2O$ × competition with nitrate	11, 16	2.2.2.1.1.
non-symbiotic haemic globins (NS-HG)	no "classic" functions; are *new* proteins, whose functions are still controversial and speculative: NO scavenging/formation, O_2 sensing, maintenance of cellular redox status under hypoxia/anoxia, preventing nitrite accumulation during hypoxia/anoxia	*b* haem (Fig. 19(a)), hexa-coordinated by two histidine residues hexa-coordination reversible $NO_2^- + 2H^+ + (NS\text{-}HG)Fe^{2+} \longrightarrow (NS\text{-}HG)Fe^{3+} + {}^{\bullet}NO + H_2O$ × high global affinity for $O_2 \Rightarrow$ very low deoxy-NS-HG concent. × NO scavenging by haem × mechanism of conversion of penta into hexa-coordinated ???	4, 16	2.2.2.1.3.
root specific plasma membrane-bound nitrite reductase (RPM-NiR)	no "classic" functions; is a *new* protein, whose functions are still controversial and speculative: nitrite/nitrate sensing, O_2 sensing, maintenance of cellular redox status under hypoxia/anoxia	site of nitrite reduction ??? reaction of nitrite reduction???	16,17	2.2.2.1.4.

[a]Major drawback concerning the feasibility of nitrite reduction to release bioactive NO. [b]Figures where the protein is represented. [c]Sections where the protein is discussed.

However, as was discussed for mammals, the NO concentration should be kept very low, within the characteristics of a local signaling molecule. Obviously, and again as in mammals, the plant NO formation should be tightly controlled. Hence, it can be argued that the well-known complex C-NaR regulation serves not only to control the nitrogen assimilation, but also to regulate the formation of the signaling NO.

The C-NaR-dependent NO formation has been suggested to be involved in (i) stomatal closure,[923,933,990,1051,1052] (ii) onset of germination,[965] (iii) phenylpropanoid metabolism,[1053] or (iv) immune defense mechanisms, because pathogen signals induce the C-NaR and increase the NO formation (strikingly similar to the mammalian inducible NOS).[991,993,995,1000,1001,1054] This enzyme is also suited to play a role as a cytoplasmatic nitrite sensor, to "signalize" the presence of toxic nitrite concentrations.[987] In addition, C-NaR/nitrite may be acting not only as a NO source, but also as an oxygen sensor: it is intriguing that an enzyme that is rapidly degraded in darkness to avoid nitrite accumulation[987] is increased during hypoxia, which also leads to nitrite accumulation. Is this a coincidence or a strategy?

Before finishing this section, another C-NaR activity must be discussed: the C-NaR-catalyzed dioxygen reduction.[977,1055,1056] This reaction not only consumes the electrons needed to reduce both nitrate and nitrite, but also produces superoxide that scavenges NO to form the strong oxidizing peroxynitrite (eq 13). However, the NO formation by purified C-NaR was described to be rather insensitive to the presence of air,[989] suggesting that the reduction of dioxygen would not compete with the nitrite reduction. In addition, the dioxygen reduction would also compromise the in vivo nitrate reduction under normoxia, which was not yet reported to occur. Surely, the relative extension of nitrate reduction versus nitrite reduction versus dioxygen reduction will depend on the respective in vivo concentrations and kinetic specificity constants, whose values remain to be determined. If the dioxygen reductase activity is significant in vivo, then dioxygen would control the C-NaR at two time scales: (i) short-term, determining the nature of the reaction products, and (ii) long-term, increasing/decreasing C-NaR activity and concentration.

2.2.2.1.2. Molybdenum-Dependent Nitrite Reduction – Xanthine Dehydrogenase/Oxidase and Aldehyde Oxidase. The molybdoenzyme XD is a relevant candidate to reduce nitrite to NO in plants. As the mammalian enzyme (see sections 2.2.1.1.3 and 3.2.3), plant XD is involved in the purine catabolism and in the generation of reactive oxygen species,[1057−1059] in the cytoplasm[1060] and in peroxisomes.[1061−1065] However, the conversion of XD into a XO form is not common to all plants: while the enzyme from *Arabidopsis thaliana* does not have the two corresponding cysteine residues[1057] involved in the conversion mechanism of the mammalian enzyme[1066] (see section 3.2.3), the pea leaf peroxisomal enzyme was described to exist mainly (70%) as a XO form.[1063−1065] Besides the purine catabolism, plant XD has been suggested to be involved in a variety of challenging environmental conditions, where increased XD activities and ROS production were observed, namely upon drought stress,[1058] plant−pathogen interactions,[1067−1069] hypersensitive response,[1070] and natural senescence.[1057,1071]

The fact that mammalian XD/XO is able to reduce nitrite to NO (sections 2.2.1.1.3 and 3.2.3) anticipates a similar role for the homologous plant enzyme. In accordance, in vivo inhibition studies with allopurinol have revealed a probable role for the enzyme in the formation of NO upon phosphate deficiency, in white lupin roots.[1072] In this context, other studies, planned to assess the in vivo C-NaR-dependent NO formation by inhibiting C-NaR with tungstate, or using cyanide, should be revaluated. Besides interfering severely with the metabolism, both tungstate and cyanide should inhibit XD/XO, by replacing the molybdenum atom and removing the sulfo group of the catalytic center, respectively (see section 3.2.3).[1073] Thus, the use of these and other nonspecific inhibitors should be done with care: the decreased NO formation attributed to C-NaR might be due also to the inhibition of XD/XO. Nevertheless,

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the definitive establishment of plant XD/XO as a NO source must wait for the characterization of the nitrite reductase activity of purified XD/XO, which was not yet accomplished.[1074]

Another relevant candidate to contribute to the plant NO formation is AO. Like the mammalian enzyme, the plant AO is a "strict" oxidase that catalyzes only the reduction of dioxygen (not NAD^+).[1058,1075] Initially, it was described to catalyze the formation of only hydrogen peroxide,[1058] but a recent work demonstrated the expected superoxide radical formation.[1076] The plant AO isoenzymes (*Arabidopsis thaliana*, e.g., contains four isoforms[1077−1080]) are responsible for the oxidation of the abscisic aldehyde into the absicisic acid,[1079,1081] one plant hormone involved in development processes and in a variety of abiotic and biotic stress responses.[1082−1084] Plant AO isoforms are also implicated in the biosynthesis of indole-3-acetic acid, an auxin phytohormone, during early stages of plant development.[1077] The AO involvement in plant NO formation is still speculative, although promising.

2.2.2.1.3. Haem-Dependent Nitrite Reduction − Haemic Globins. Haemic globins (HG)[1085] are a large family of ancient haemic proteins, with the globin fold,[1086] which are widely distributed in all kingdoms of life. The HG family includes the well-known dioxygen transporters Hb, Mb, and leghaemoglobin,[1087] which use a penta-coordinated *b* haem to reversibly bind and transport dioxygen. This family also comprises several hexa-coordinated haemic proteins, including the recently discovered mammalian Nb and Cb (described in section 2.2.1.1.2), the cyanobacterium *Synechocystis* HG (section 2.2.3), and the plant nonsymbiotic HG (NS-HG), whose physiological functions remain a matter of debate.

The discovery of NS-HG was triggered by the search for HG in nonleguminous plants, where leghaemoglobin had been previously found in root nodules. The search culminated with the identification of new globins, named (not very originally) "nonsymbiotic haemoglobins". NS-HG, contrary to leghaemoglobins, are believed to be present in the entire plant kingdom, in low concentrations, although with different expressions in different tissues and in response to different types of stress.[1092,1094−1103]

Presently, plant HG can be divided into four groups:[1103−1106] the penta-coordinated leghaemoglobins and three classes of NS-HG that show some character of bis-histidinyl-hexa-coordination, via reversible intramolecular coordination. Herein, only class 1 NS-HG will be addressed, because it is the most studied one (a brief description of classes 2 and 3 can be found in ref 1107).

Class 1 NS-HG are homodimers (~35 kDa; Figure 4g,h)[1096,1120−1122] expressed in low concentrations (5−20 μM in hypoxic tissues, 2 orders of magnitude lower than leghaemoglobin[1123,1124]). A conserved cysteine residue, located in the same α-helix that holds the distal histidine, seems to be involved in the dimer formation,[1120,1125] as well as in the increased rate of haem iron reduction.[1126] These proteins have the lowest affinity for the distal histidine coordination ($K \approx 1-2$[1103,1108,1127]) and display a low dioxygen dissociation rate constant ($k_{off} \approx 0.03-0.2$ s^{-1} and $k_{on} \approx 60-70$ μM^{-1} s^{-1}[1096,1097,1104,1120,1128,1129]) (Figure 11g,h). This results in a global oxygen affinity (2−3 nM) 2 orders of magnitude higher than the CcO one and suggests that class 1 proteins remain oxygenated at extremely low oxygen concentrations.[1121,1128,1129] These characteristics suggest that class 1 proteins do not meet the equilibrium and kinetic requirements for functioning in oxygen transport,[1092,1096,1103,1117,1130] as was described for Nb (section 2.2.1.1.2; also ref 440). Instead, it has been suggested that class 1 NS-HG are involved in NO scavenging and in the maintenance of the cellular redox status, contributing to the cell survival under hypoxia (further discussed below and in section 2.2.2.1.4). In this respect, possible mechanisms of action of NS-HG involve sensing of oxygen and other gaseous ligands, NO scavenging, and formation (further discussed below).[1117,1131]

Following the discussion on the mammalian hexa-coordinated HG, it is expected that plant NS-HG would also be able to reduce nitrite to NO upon conversion to a penta-coordinated state. Furthermore, as was described for mammalian Nb and Cb, also plant NS-HG have been found to be induced by low dioxygen concentrations, suggesting a cellular protective role during hypoxia.[1095,1097,1104,1119,1120,1131−1136] However, and surprisingly, this potential plant nitrite reductase only recently began to be explored with the discovery that rice deoxygenated NS-HG (deoxy-NS-HG) reduces nitrite to NO with a remarkable rate constant of 83 M^{-1} s^{-1}, at pH 7, under anaerobic conditions (equivalent to eq 17).[1137] The pH effect was not yet thoroughly explored, but it is expected that the rate further increases as the pH value decreases.

This nitrite reductase/NO synthase activity of plant NS-HG is supposed to be of physiological relevance, because (i) the fraction of "open" penta-coordinated NS-HG molecules is high (affinity constant for coordination by the distal histidine of ~1−2[1108,1127]), (ii) plants can be often subjected to extreme hypoxia/anoxia (see ref 1017), (iii) during which nitrite accumulates, (iv) the pH values decrease, and (v) NS-HG are induced and can become deoxygenated.

Nevertheless, and as expected, the NO formed can be rapidly trapped by deoxy-NS-HG (equivalent to eq 19)[1137] or be oxidized by oxygenated NS-HG (oxy-NS-HG; equivalent to eq 20). In fact, in the literature, plant NS-HG are mentioned mainly as NO scavengers responsible for the NO oxidation to nitrate.[1007,1092,1103,1110,1119,1124,1126,1134−1136,1138−1150] The rate constants toward dioxygen and the resulting high global oxygen affinity[1104,1120,1128,1129] suggest that these NS-HG remain oxygenated even at extremely low oxygen concentrations,[1128,1129] thus supporting their role as NO scavengers (and not as NO sources). Also, the observation of reduced NO formation in plants overexpressing NS-HG supports this role.[1134,1141] In this respect, it is interesting that NS-HG are also induced by conditions where the NO formation might be increased, namely by nitrate, nitrite, or NO itself.[1144,1145,1149,1151] This up-regulation is more in line with a scavenger role, that is, defense against the deleterious excessive NO formation, than with a NO synthase role. Noteworthy, also the hypoxia-mediated induction of NS-HG could be related to the NO accumulation that is known to occur under low dioxygen stress.

According to these evidences, NS-HG would be key players of NO homeostasis, preventing the unwanted NO effects in a manner similar to that described for mammalian oxy-Hb and oxy-Mb (see ref 276, section 2.2.1.1.1). Yet, as reasoned above, the nitrite reductase/NO synthase activity of NS-HG can also be of physiologically relevance. Hence, it is plausible that, in vivo, a delicate balance takes place between NO scavenging and NO formation, as was exemplified for Mb (end of section 2.2.1.1.1): NS-HG might be responsible for "translating" a mismatch between oxygen supply and consumption into an increased NO flux. However, in the NS-HG case, the reaction

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should be shifted toward lower dioxygen concentrations, because NS-HG become deoxygenated at lower oxygen concentrations. Concurrently, the fluxes of NO should be higher, as the rate constant of nitrite reduction and available nitrite concentrations are higher.

In addition to (i) controlling the NO homeostasis and (ii) generating NO under extreme hypoxia, plant NS-HG can (iii) contribute to consume the nitrite that can accumulate to dangerous concentrations during hypoxia/anoxia and (iv) maintain the cellular redox status under hypoxia/anoxia (the last point will be addressed in section 2.2.2.1.4).

Crucial for all of the above catalytic roles of plant NS-HG is the protein conversion to an "open" penta-coordinate state. In accordance, it is of major importance to characterize potential mechanisms that control the conversion of hexa- into penta-coordinated states. Those mechanisms would dictate if NS-HG are posttranslationally redox/allosteric-regulated nitrite reductases or, on the contrary, if the equilibrium penta/hexa could not be modified (regulated) by a posttranslational modification.

Also decisive for the in vivo significance of the NS-HG reactions is the existence of regenerating systems that efficiently rereduce the oxidized proteins. In plant roots, one of the most plausible systems comprises the enzyme monodehydroascorbate reductase and NADH (at least in barley, with K_m (NS-HG) = 0.3 μM).[1126,1146] Nevertheless, this system is believed to be slow to account for a "catalytic" rereduction of NS-HG,[1137] and the identification of more efficient systems is one of the challenges for future research. It is also essential to study and compare the nitrite reductase activity of the other plant HG, not only of classes 2 and 3 NS-HG, but also of leghaemoglobins, that are penta-coordinated, present in higher (millimolar) concentrations, and could display a behavior more in line with the Mb one. To conclude, also the question of how the nitrite-dependent NO avoids the dogmatic haem scavenging, to be able to fulfill its signaling role, has to be answered in the plant kingdom.

In the context of the plant haem-dependent nitrite reduction, it is noteworthy that Cc should also be a physiologically relevant nitrite reductase in plants.

2.2.2.1.4. New Pathways – Plasma Membrane-Bound Nitrite Reductase. Plants are also able to produce nitrite-dependent NO in root apoplast, through the recently identified root-specific, plasma membrane-bound nitrite reductase (RPM-NiR). This novel enzyme catalyzes the formation of NO from nitrite using a not yet identified physiological electron donor.[1152,1153] Its activity is maximal under the acidic conditions (pH 6) characteristic of hypoxia and is reversibly inhibited by dioxygen.[1152,1154] Most important, it can account (with 500 nmol/g FW/h[1136]) for the NO formation rates observed under hypoxic conditions (10–50 nmol/g FW/h[928,1134]). The RPM-NiR was suggested to act in concert and in tight association with the root-specific, plasma membrane-bound, succinate-dependent, nitrate reductase (RPM-NaR).[1038,1152–1158] The RPM-NaR, localized on the apoplastic side of the plasma membrane, is assumed to catalyze the in vivo reduction of apoplastic nitrate to nitrite.

The root apoplastic nitrite-dependent NO formation might, thus, be one of the primary signals that report the presence of nitrate in roots.[1153] For the same reasoning, the toxicity cause by high nitrate concentrations might be the result of increased NO formation. In addition, it may also act as an oxygen sensor, because RPM-NiR-dependent NO formation is reversibly inhibited by dioxygen.[1152,1154] Furthermore, a role in root developmental[1159] or in regulation of mycorrhizal inoculation[1160] was also envisaged.

Nonetheless, the root apoplastic nitrite might have another function. Root, the key organ that provides nutrients and water to the whole organism, is commonly subjected to hypoxia (see ref 1017). To ensure cell viability under hypoxic conditions, plants promote the glycolysis and fermentation pathways to synthesize ATP and regenerate NAD^+. However, there is evidence that an additional pathway, mediated by nitrate, may be important for plant survival under hypoxic conditions.[1007,1134,1135,1140] In fact, it has long been known that the presence of nitrate increases the tolerance to flooding.[1161–1164] The nitrate-mediated protection has been ascribed to the nitrate reduction to ammonium, a pathway that consumes 4 mol of NAD(P)H and six protons, being, in this way, more efficient at regenerating NAD^+ and consuming protons than any of the fermentation reactions (see nitrate assimilation under section 2.1.1; Figure 1, orange arrows; Table 1).[1024,1033,1040,1136] Yet the NO formation by the sequential activity of RPM-NaR and RPM-NiR puts forward another hypothesis for the beneficial role of nitrate: the operation of a cycle that actively "recycles" NADH using nitrate, nitrite, and NO (Figure 17).[1136] The C-NaR is also suggested to contribute

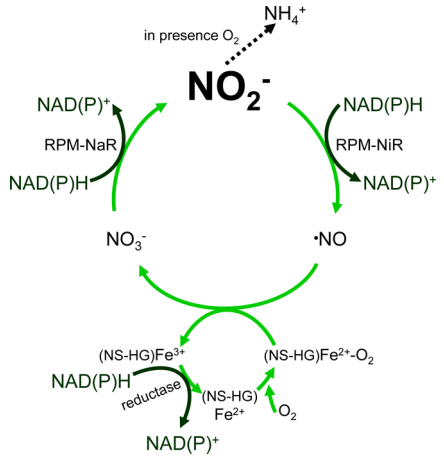

Figure 17. Suggested cycle for the regeneration of $NAD(P)^+$ during hypoxia – potential new role for nitrite. See text for details. Figure modified from ref 1136.

to this cycle, although its activity in the roots is believed to be lower than that of the RPM-NaR.[1038,1136] The cycle is proposed to be closed by a hypoxia-induced oxygenated class 1 NS-HG, which, due to its high oxygen affinity, remains oxygenated even at extremely low oxygen concentrations (details about these proteins in section 2.2.2.1.3). The NS-HG would oxidize NO to nitrate (equivalent to eq 20) and would be, subsequently, "recycled" by a reductase (Figure 17).[1136,1143] Because both the RPM-NiR activity and the induction of NS-HG are controlled by dioxygen concentration, the metabolic flux through the cycle would be limited to hypoxic conditions and would not compromise the nitrogen assimilation (nitrate reduction to ammonium) under normoxia. This cyclic pathway is suggested to oxidize 2.5 NADH molecules per nitrate molecule "recycled", helping, in this way, to maintain the redox status of the cell along with the fermentation pathways.[1136,1143] Hence, in plants, nitrite may play a new role: contribute to the oxidation of NAD(P)H under hypoxia and ultimately maintain the cellular redox and energy status.

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In this context, it is noteworthy that nitrite might also be involved in another alternative pathway that would enable plants to maintain the ammonium production and/or ATP generation even under anoxia (<1 nM).[1165] In accordance with a recent suggestion,[1165] under anoxia, plants would be able to reduce nitrite to ammonium through three individual steps (eq 62) that would replace the normoxic one-step CSNiR-catalyzed reaction (details in section 3.1.2). The last of these three steps (eq 62) was suggested to be catalyzed by deoxy-NS-HG that were shown to reduce not only nitrite to NO (section 2.2.2.1.3), but also hydroxylamine to ammonium (25 mM^{-1} s^{-1}).[1165] The reasonability of an haem to catalyze the hydroxylamine reduction to ammonium is supported by the known reactivity of the *c* haem of CcNiR, sirohaem of CSNiR (sections 3.1.1 and 3.1.2), and *b* haem of Hb and Mb.[1165−1167] In addition, also several model complexes have been shown to electrochemically reduce NO to ammonia, via hydroxylamine-like intermediates.[1168−1170] Nevertheless, this alternative pathway raises two important questions: how NS-HG transfers the two electrons needed to reduce hydroxylamine and how is the three-electrons reduction of NO carried out? In summary, this alternative nitrite-mediated pathway would remove toxic nitrogen metabolites, at the same time as it would act as an electron sink by coupling the reducing power produced during anaerobic glycolysis to the ammonium production.[1165]

$$NO_2^- - (1e\text{-}) \rightarrow {}^{\bullet}NO - (3e\text{-}) \rightarrow NH_2OH - (2e\text{-}) \rightarrow NH_4^+ \quad (62)$$

Globally, both proposals suggest that, under hypoxia, nitrogen oxides and oxo-anions are essential metabolites to decrease the NAD(P)H concentrations and maintain the ATP/ADP ratio sufficiently high to enable plants to survive.

The increasing amount of evidence that root apoplastic nitrite/NO may play important roles in vivo demands for a deeper knowledge of the two root-specific RPM-NaR and RPM-NiR enzymes. Unfortunately, the present data are still very limited (and raise more questions than answers). The RPM-NiR is believed to be different from CSNiR and to share some features with RPM-NaR: (i) succinate as electrons source, probably via the quinone pool of plasma membrane, and (ii) the regulatory mechanisms, inhibition by dioxygen and activity dependent on nitrate supply.[1154] The RPM-NaR has also been described to be different from the known NaR of higher plants.[1158] Instead, there is evidence that it might be similar to the prokaryotic "respiratory" membrane-bound nitrate reductases NaRGHI (see refs 1667,1679 for details about these enzymes and Figure 11g,h).[1171] Recently, the successful RPM-NaR extraction from *Hordeum vulgare* indicated that this protein is remarkably similar in sequence to the prokaryotic NaRH subunit. Interestingly, NaRH is not the subunit responsible for nitrate reduction. Furthermore, a database study suggested that *Populus trichocarpa* might hold homologous NaRH (75% identity) and NaRG (68% identity) peptides, although the existence of NarI-related peptides in plants remains questionable.[1171] This alleged similar structure and localization of RPM-NaR puts forward the hypothesis of a similar function, that is, the hypothesis that higher plants are able to carry out part of the denitrification pathway (Figure 1, blue arrows; section 2.1.1, Table 1).[1171] This polemic hypothesis raises the possibility that nitrate/RPM-NaR/nitrite/RPM-NiR might be able to contribute to the ATP synthesis under hypoxic/anoxic conditions. If confirmed, this will be an unprecedented role for nitrite in higher organisms, as a "respiratory" substrate! Such hypothesis, however, must wait for the future evaluation of the hypothetical ability of nitrate/nitrite reduction to create a proton motive force at root plasma membranes.[1171]

2.2.2.1.5. Other Nitrite Reduction Reactions. In addition to the nitrite reduction reactions so far discussed, plant could also rely on a protein-independent NO source. As previously discussed (section 2.2.1.1.4), the protein-independent NO formation depends on the nitrite decomposition to dinitrogen trioxide, in an overall reaction that is highly pH and reducer-dependent. The acidic pH,[1172−1174] the availability of nitrite, and the presence of reducing antioxidants, like ascorbate and phenolic compounds,[1175,1176] suggested that the apoplast could be a relevant source of protein-independent NO on roots and seeds.[922,1175,1177] In addition, the protein-independent NO formation could occur at microlocalized acidic environments, as in the chloroplast.[1176]

Besides the apoplast, plasma membrane, and cytoplasm, also mitochondria, peroxisomes, and chloroplasts have been found to generate nitrite-dependent NO, although the proteins responsible for the catalysis were not yet identified.[966,1010,1074,1178−1181]

2.2.2.2. Nitrite Oxidation in Plants. Plants also handle nitrite to oxidize it to nitrate and nitrogen dioxide radical using haemic proteins with peroxidase-like activity. Nevertheless, this oxidative chemistry has been (comparatively) poorly studied.

As it is expected following the discussion on mammalian nitrite oxidation, plant peroxidases can behave as "non-dedicated" nitrite oxidases.[817,819,834,835,1182−1185] In addition, also a number of plant HG with peroxidase-like activity were shown to be able to oxidize nitrite and mediate protein nitration, at least in vitro.[1186]

2.2.2.3. Plant Nitrite Handling − Concluding Remarks. In summary, nitrite reduction to NO in plants could be accomplished by diverse metalloproteins, with different cellular roles and different tecidular and subcellular localizations (Figure 16, Table 5), suggesting that nitrite-dependent NO performs specific functions in the various subcellular compartments and tissues. Despite the diversity, all of the pathways reviewed depend on hypoxic/anoxic conditions to operate. As was discussed for mammals, it is possible that each individual pathway is activated when the dioxygen concentration decreases below its own threshold of oxygen-dependent activity. In this way, all pathways would act in a concerted and self-regulated manner, with each pathway being relevant under different conditions and in different tissues. Thus, in plants, like in mammals, nitrite could act not only as a source of signaling NO, but also as an oxygen sensor. Nevertheless, due to the nitrite specific function in plants, as an ordinary metabolite of nitrogen assimilation, the control of its CSNiR-independent reduction has to be more complex (or at least different from) than the mammalian one.

To reduce nitrite to NO, higher plants and mammals share some strategies: both do "substrate adaptations" to available redox systems, depending on proteins that are not "normally" committed to synthesize NO. The C-NaR is a remarkable example of how plants can obtain a "new" NO synthase from an "old" (i.e., previously existent) protein. For this reason, it would be very interesting to understand if plant XD/XO and AO are also able to generate NO. Yet plants use also different, unique, strategies to handle nitrite: the RPM-NaR and RPM-NiR enzymes constitute a new and promising pathway, not

only for NO synthesis, but also for nitrite-dependent NAD(P)H "recycling" under hypoxia/anoxia.

The major challenge in plants is, undoubtedly, to attain the same level of knowledge we now have about mammalian NO formation and signaling, focusing on (i) characterization of the pathways so far identified and on potential new ones (inspired by the mammalian ones) and (ii) how those different pathways could act in a concerted manner to produce the signaling network observed in plants.

Regarding the first point (pathways characterization), the study of NO formation by plant proteins (both nitrite-dependent and independent) is still a new field. Most of the enzymatic reactions were not yet characterized, neither kinetically nor at molecular level. Moreover, the great majority of studies relied on the use of inhibitors, most of them not specific at all, and on genetic manipulation that, besides silencing the target enzyme, also extensively alters the metabolism, as is observed with C-NaR knockout mutants. The "bottleneck" here is the capacity to obtain the purified proteins in sufficient quantities and the development of new, more specific, in vivo assays. In this point, the most imperative questions are related to the molecular composition and chemistry behind the RPM-NaR and RPM-NiR reactions and the expected ability of XD/XO and AO to form NO. Also, the characterization of the reaction mechanism of nitrite reduction by C-NaR (a sulfite oxidase family member) is crucial to our understanding of the molybdenum-dependent reactivity toward nitrite. In addition, although outside of the scope of this Review, it is clear that the search for a higher plant NOS will continue. Presently, it seems that higher plants have lost the specific NOS in the course of evolution. Is its activity carried out using a different chemistry and, consequently, a protein not related to NOS?

Concerning the second point (concerted action of different pathways to produce the signaling network observed in plants), the questions are more numerous and exciting. How do plants regulate the nitrite-dependent NO signaling in the presence of a variable nitrate supply? Why do they have two NO forming nitrate reductases, one in the cytoplasm and another facing the apoplasm? Are plants really doing denitrification?

Nitrite/NO metabolism in humans is becoming increasingly important, offering innovative therapeutic approaches. The plant nitrite/NO metabolism is also promising. The future world will be characterized by increasing concentrations of carbon dioxide, global warming, and occurrence of extensive flooding (with consequent root anoxia), drought, and other extreme meteorological phenomena. In such a scenario, the knowledge of plant nitrite/NO metabolism would certainly be essential for understanding and managing crop productivity.

The influence of carbon dioxide on nitrate (and nitrite) assimilation is an interesting example. Since the Industrial Revolution, the atmospheric carbon dioxide has increased ca. 40%, and predictions are that it may double by the end of the 21st century.[1187] This carbon dioxide increase could be mitigated through its photosynthetic assimilation, but many plants are unable to sustain rapid growth under elevated carbon dioxide, because, at least, nitrate assimilation into organic compounds is inhibited[1188] by elevated carbon dioxide.[1187,1189−1193] Nitrate is the most abundant form of inorganic nitrogen in agricultural (temperate well aerated) soils,[1199] and, if plants are unable to assimilate it, crops will become depleted of organic nitrogen compounds and thus compromised.[1191] Therefore, in the near future, the relative availability of soil nitrate and ammonium will be crucial to determine the crops productivity and the food quality, as well as to achieve the desirable biological sequestration of carbon dioxide. However, if the decline in nitrate assimilation is already being studied in several plants,[1188] the effects on the generation of NO have been overlooked, and, plausibly, NO is a key molecule in the response to increased carbon dioxide. Furthermore, to understand how plants respond to the soil available nitrogen form, the NO metabolism has certainly to be considered.

Another remarkable example of the potential relevance of plant nitrite/NO metabolism would be the managing of crop productivity under extensive flooding, because the oxygen availability influences nitrite-dependent NO formation, which, in turn, controls "respiration" in plant mitochondria.[1200−1202]

2.2.3. Nitrite in Signaling (and Other) Pathways in Bacteria. In prokaryotes, the NO formation had for long been thought to occur only in denitrification (section 2.1.1; Figure 1, blue arrows; Table 1), where the anaerobic reduction of nitrogen compounds is used to derive energy. More recently, NO was found to take part in other dissimilatory pathways that "copy" the first steps of denitrification (Figure 1, gray and violet arrows; Table 1). Therefore, on the organisms that carry out those pathways, NO is an ordinary intermediate metabolite, that is, a reaction product and substrate of a "respiratory" pathway. In addition, NO is also as a signaling molecule that activates the genes required for its own anabolism/catabolism (a common regulatory strategy in biology) through, for example, regulators of the FNR family (in *Pseudomonas*, *Paracoccus*, and *Rhodobacter*).

However, presently, it is clear that the prokaryotic NO is also involved in "nonrespiratory" pathways. Two examples are the recovery from radiation-induced damage (in *Deinococcus radiodurans*)[1203] and the biosynthesis of secondary metabolites, namely nitration of tryptophane (*Deinococcus radiodurans*)[1204] and of the tryptophanyl moiety of thaxtomins (*Streptomyces turgidiscabies*[1205−1207]). In addition, "nonrespiratory" NO is also involved in cytoprotection against oxidative stress, through different mechanisms, such as activation of catalase, inhibition of the Fenton chemistry by *S*-nitrosation of reduced thiols, and through transcription factors like OxyR, SoxR, Fur, or FNR (in *Escherichia coli*, *Bacillus subtilis*, *Bacillus anthracis*, *Staphyloccuos aureus*).[1208−1213] In this scenario, it is intriguing that NO is important for the pathogen survival[1213,1214] and, simultaneously, for the host defense (phagocytes produce ROS and RNS to damage pathogens and protect themselves from infection). Clearly, there is still much to be learned about host/pathogen interactions.

In prokaryotes, the aerobic NO formation is catalyzed by enzymes homologous to the oxygenase domain of the mammalian NOS (see section 2.2.1.1), but lacking the reductase domain.[1205,1215−1225] These prokaryotic enzymes successfully reduce arginine to NO, using cellular redox equivalents that are not normally committed to the NO production.[1220,1225,1226] The *Sorangium cellulosum* NOS is an exception, because the enzyme holds a "fused" reductase domain (even though with a different domain organization).[1227,1228] Interestingly, the prokaryotic NOS is found only in a subset of bacteria, mostly gram-positive (*Exiguobacterium*, *Staphylococcus*, *Geobacillus*, *Bacillus*, *Rhodococcus*, *Streptomyces*, and *Deinococcusus*), but also gram-negative (*Sorangium cellulosum*[1227]), and in an archeon (*Natronomonas*).[1203,1207,1213,1219,1220,1223,1225,1229,1230] This distribution rai-

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Table 6. Proteins Involved in Nitrite Reduction to NO in Prokaryotes

protein	"classic" reaction "classic" functions	site of nitrite reduction reaction of nitrite reduction × major drawback [a]	figure [b]	section [c]
"respiratory" membrane-bound nitrate reductase (NaRGHI)	$NO_3^- + 2e^- + 2H^+ \longrightarrow NO_2^- + H_2O$ "respiration"	molybdenum centre of dimethylsulfoxide reductase family type (Fig. 19(c)) $NO_2^- + 2H^+ + (NaR)Mo^{4+/5+} \longrightarrow (NaR)Mo^{5+/6+} + {}^{\bullet}NO + H_2O$ × competition with nitrate	11	2.1.1., 2.2.3., note 1667, 1679 of 3.3.1.
haemic globin (HG)	no "classic" functions; is a *new* protein, whose functions are still controversial and speculative: NO scavenging/formation, antioxidant defence	haem, hexa-coordination reversible $NO_2^- + 2H^+ + (HG)Fe^{2+} \longrightarrow (HG)Fe^{3+} + {}^{\bullet}NO + H_2O$ × NO scavenging by haem × mechanism of conversion of penta into hexa-coordinated ???		2.2.3.
aldehyde oxidoreductase (AOR)	aldehyde ⟶ carboxylate aldehyde scavenger linked to reduction of protons	molybdenum centre of XO family type (Fig. 19(c)) $NO_2^- + 2H^+ + (AOR)Mo^{4+} \longrightarrow (AOR)Mo^{5+} + {}^{\bullet}NO + H_2O$ $NO_2^- + 2H^+ + (AOR)Mo^{5+} \longrightarrow (AOR)Mo^{6+} + {}^{\bullet}NO + H_2O$ × competition with physiological oxidising substrates	11	2.2.3. 3.2.3.

[a]Major drawback concerning the feasibility of nitrite reduction to release bioactive NO. [b]Figures where the protein is represented. [c]Sections where the protein is discussed.

ses the question of why are not prokaryotic NOS widespread and what is the evolutionary relationship between the animal and prokaryotic proteins? (Note that plants do not seem to have a NOS enzyme.)

Nevertheless, bacteria are able to synthesize NO in a NOS-independent manner, through (once more) the nitrite reduction, therefore expanding the formation of NO to other prokaryotic organisms. *Escherichia coli* and *Salmonella enterica* are two (long known) examples of bacteria that, not having a NOS enzyme, are able to produce NO when growing under nitrate "respiring" (anaerobic) conditions.[1231−1236] Also, the cyanobacterium *Microcystis aerugrinosa*[1237] and *Bacillus vireti* (whose genome indicates that it carries out DNRA; Figure 1, green arrows) are able to generate nitrite-dependent NO. In addition, also known NOS holder organisms may rely on the nitrite reduction to produce NO. For example, in the NOS-deficient *Streptomyces*, a small amount of thaxtomin is still produced.[1205−1207] However, this prokaryotic nitrite-dependent, "nonrespiratory" NO formation should be carried out in a tightly controlled and parallel way to the nitrogen assimilation and/or dissimilation (as was discussed for plants).

Until recently, the nitrite-dependent NO formation was assumed to arise from the activity of CSNiR and CcNiR (see sections 2.1.1, 3.1.1, 3.1.2), because studies with mutants suggested that both enzymes would be largely responsible for the NO production.[1236,1238] However, no NO generation could be observed with purified enzymes. As will be discussed in sections 3.1.1 and 3.1.2, both enzymes catalyze the nitrite reduction to ammonium without releasing any intermediates. On the contrary, both enzymes were proposed to catalyze the NO reduction, as a detoxification process[1239−1241] (see also section 3.1.1.2).

Concurrently, other studies suggested that the nitrite-dependent NO formation is due to the nitrite reduction by NaR under anaerobic, nitrate-rich conditions, upon nitrite accumulation (Table 6).[1231−1235,1237,1242−1247] Prokaryotes use nitrate for dissimilatory and assimilatory processes (section 2.1.1), and, for those purposes, prokaryotes hold three types of NaR enzymes, "respiratory" membrane-bound NaR, periplasmatic NaR, and assimilatory cytoplasmatic NaR (described in refs 1667,1679). Presently, there is an increasing number of studies showing that most of the NO is formed by the "respiratory" membrane-bound NaR (Figure 11g,h),[1247−1249] with the periplasmatic NaR contributing very little to the bacterial NO (less than 3%).[1248,1249] Furthermore, an in silico study supported the feasibility of "respiratory" NaR to catalyze the nitrite reduction to NO.[1013] The hypothetical contribution of the assimilatory cytoplasmatic NaR was not yet investigated.

The NO formation by the "respiratory" NaR would depend on a combination of anaerobic, nitrate sufficiency, and nitrite accumulating conditions, which would not only promote the reaction, but also induce the enzyme expression.[47,1247,1249] Remarkably, these conditions are similar to those described for the NO generation by plant cytoplasmatic C-NaR (in the previous section). As in C-NaR, nitrate competitively inhibits the "respiratory" NaR-catalyzed nitrite reduction (e.g., *S. enterica* specificity constant for nitrite is ∼150 times lower than the nitrate one[1249]). The nitrite reduction is promoted only when the nitrate concentration decreases and nitrite builds up (K_m value for nitrite in the millimolar range[1249]). In accordance, under nitrate-limited growth conditions, when both nitrate and nitrite are present at low micromolar concentrations and the expression of "respiratory" NaR is repressed, the NO formation is very low.[1249] Nevertheless, the extension of the prokaryotic NO synthesis seems to be dependent on the organism and, probably, on the role of NO on that organism. While *E. coli* NO generation is estimated to be less than 1%,[1232,1248] the *S. enterica* NO formation can account for up to 20% of the nitrate reduced.[1249,1250]

The similarities in the nitrite-dependent NO formation by plant C-NaR and bacterial "respiratory" NaR are noteworthy. Furthermore, also the fungus *Aspergillus* NaR was shown to be able to reduce nitrite to NO.[1005] This similar activity suggests that the nitrite reduction/NO formation could be a general feature of all types of NaR enzymes. In this respect, it is intriguing why bacterial periplasmatic and assimilatory NaR would not be able to catalyze the nitrite reduction. It can be argued that the nitrite reduction is hampered by the sulfur-rich coordination of the molybdenum center of periplasmatic NaR (see sections 3 and 3.3.1, Figure 19c; also ref 1667). However, definitive conclusions must wait for kinetic and spectroscopic characterization of the purified enzymes (the studies cited

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above were carried out with NaR mutants; the hypothetical NO formation by purified periplasmatic and cytoplasmtic NaR was not yet kinetically characterized nor studied in silico).

Although the "respiratory" NaR is presently believed to be the major source of nitrite-dependent signaling NO, other sources remain to be identified. Because of the parallelism with mammals, two NO sources will be described below, an HG and a XO family enzyme member (Table 6).

Deoxygenated HG are believed to be relevant NO sources (details about these proteins were given in sections 2.2.2.1.3 and 2.2.1.1.2.a). In accordance, an HG from the cyanobacterium *Synechocystis*[1251−1255] was shown to be able to reduce nitrite to NO with a remarkable rate constant of 68 M^{-1} s^{-1}, at pH 7, under anaerobic conditions.[1137] Together with NS-HG plant, these are the fastest HG-dependent nitrite reductases described so far. The significant rate of nitrite reduction, combined with a high available nitrite concentration (millimolar range), makes this HG a potentially relevant NO source under hypoxic/anoxic conditions. Nonetheless, as the other HG here described and, for example, the well-documented bacterial flavohaemoglobin,[1256] this bacterial protein is also a potential NO scavenger.[1137] In accordance, its physiological function could be related to the defense against the oxidative and nitrosative bursts from the immune systems of their hosts.[507,1257,1258]

To sum, the reactivity of prokaryotic HG toward nitrite, NO, and dioxygen, as well as the associated physiological functions, are under the same debate as other HG. For the sake of simplicity, the discussion of those "strengths" and "weaknesses" will not be here duplicated, and readers should refer to sections 2.2.2.1.3 and 2.2.1.1.1.

The XO family member aldehyde oxidoreductase (AOR) can also contribute to the bacterial NO formation. AOR was first described by Moura et al.[1259] and is believed to be an aldehyde scavenger (eq 63), acting in a complex chain of electron transfer proteins that links the oxidation of aldehydes to the reduction of protons.[1260] AOR is a molybdoenzyme belonging to the XO family (Figure 11e, Figure 19c), being structurally similar to mammalian XO and AO (see section 3.2.3 for details).

$$\text{aldehyde} + \text{flavodoxin}_{\text{oxidized}} \rightarrow \text{carboxylate} + \text{flavodoxin}_{\text{reduced}} \quad (63)$$

As the mammalian enzymes, AOR was recently shown to catalyze the nitrite reduction to NO.[634] Once again, the amount of NO produced would be dependent on the accumulation of nitrite (K_m value in the millimolar range[634]) and is estimated to be low ($k^{app} \approx 13$ M^{-1} s^{-1}[634]). In addition, the physiological relevance of this bacterial NO formation pathway would depend on the competition between nitrite and the expected oxidizing substrate, flavodoxin.[1261] Overall, the AOR-dependent NO formation would be controlled (i) by the availability of nitrite and (ii) by the cellular redox status that determines the flavodoxin redox status, as well as the redox status of other proteins involved in "respiratory" pathways. Accordingly, the following cytoprotective pathway against oxidative stress could be suggested: (i) under normal conditions, the electron transporters involved in "respiratory" pathways would be reduced and the aldehyde oxidizing activity of AOR would be coupled with the reduction of protons; (ii) in a situation of oxidative stress, as the "respiratory" pathways began to be disrupted and the proteins began to become oxidized, nitrite would accumulate and AOR could link the aldehyde oxidation to nitrite reduction. The NO thus formed could, subsequently, participate in signaling cascades that would eventually protect the organism from oxidative stress damage. In this way, this hypothetical mechanism would allow the bacteria to "translate" a situation of oxidative stress (a change in the cellular redox status) into a differentiated NO flux that would be, subsequently, "translated" into a biological defensive (antioxidant) response.

The nitrite reduction by bacterial NaR and AOR, fungus NaR, plant C-NaR, and mammalian XD/XO and AO suggests that all forms of life can use a molybdoenzyme when they need to produce NO for purposes other than "respiration". This consensus emphasizes the relevance of the molybdenum chemistry to catalyze the oxygen atom abstraction from nitrite and suggests that it is worth investigating the possible role of bacterial XD in nitrite reduction.

3. BIOLOGICAL MECHANISTIC STRATEGIES TO HANDLE NITRITE

Nitrite participates in several different metabolic pathways, with remarkably different biological purposes (section 2). From a mechanistic point of view, these pathways can be better thought of as a series of nitrogen compounds, with oxidations states ranging from 5+ (nitrate) to 3− (ammonium), that are interconverted, oxidized and reduced, by several metalloenzymes (Figure 18). The nitrite oxidation involves the abstraction of one electron (to form nitrogen dioxide radical) or the addition of one oxygen atom (to form nitrate), while the reduction can be a "simple" abstraction of one oxygen atom (to yield NO), or a "complex" abstraction of both oxygen atoms with the addition of four protons (to ammonium). To carry out these distinct reactions, biology developed several strategies, exploring different nitrite binding modes (Figure 20) and using, in the great majority of cases, haemic iron and molybdenum, but also copper (Figure 19).

Haem is one of the most employed cofactors in biology, participating in electron transfer, catalysis, sensing, and transport of small molecules.[9,1262,1263] One haem consists of an iron atom coordinated by four equatorial nitrogen atoms of a porphyrin ring (Figure 19a) and by one or two axial ligands. The number of the axial ligands is dictated by the haem function. For electron transfer (e.g., "respiratory" Cc), haem has two axial ligands from the protein, typically histidine and/or methionine residues, leaving no vacant coordinating position ("closed" hexa-coordinated haem).[1264,1265] For small molecules transport, haem is coordinated by only one amino acid residue ("open" penta-coordinated haem), creating an "open" position for binding (e.g., the monohistidinyl-coordinated haem of Hb that transports dioxygen).[9,1262] The same situation is (usually) found in catalysis, where a water/substrate molecule occupies the sixth coordination position (water is a weak ligand that can be easily replaced by the substrate). These are the "ready" enzymes, according to the nomenclature of Moura et al.[1265] However, there are some enzymatic active centers where haems are hexa-coordinated and need an activation step to lose an axial ligand to become penta-coordinated, the "unready" enzymes.[1265] Some examples worth mentioning of "unready" proteins are Cd_1NiR (see section 3.2.1), bacterial cytochrome *c* peroxidase,[1266−1268] Nb, or Cc (section 2.2.1.1.2). As discussed, "unready" proteins are ideal regulatory points to control the cellular metabolism ("unready" versus "ready" as a mechanism of "on/off"). The nature of the fifth protein-derived haem axial

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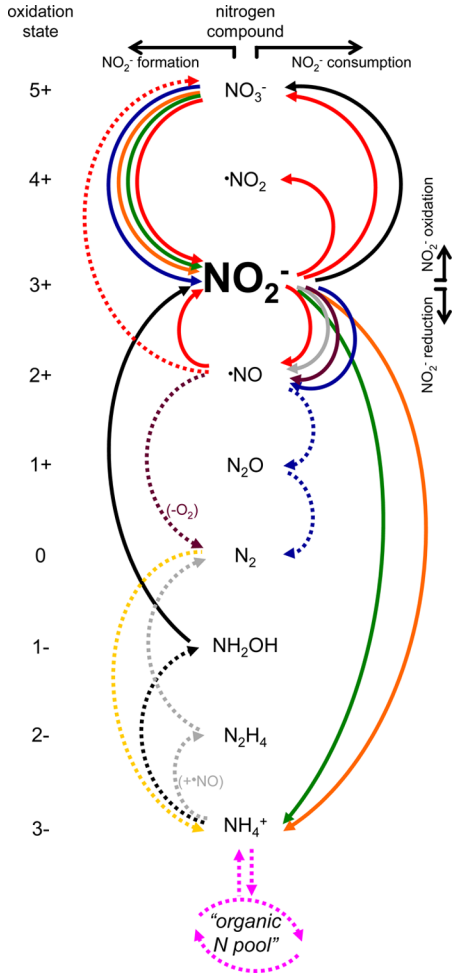

Figure 18. Chemical view of the nitrite reduction/oxidation in the nitrogen biochemical cycle and signaling pathways. The pathways represented in Figures 1, 3, and 16 are here depicted as a whole series of nitrogen compounds, with oxidations states ranging from 5+ (nitrate) to 3− (ammonium), to emphasize the redox chemistry involved in each step. Signaling pathways, red arrows; dinitrogen fixation, yelow arrow; assimilatory ammonification, orange arrows; "organic nitrogen pool", pink arrows; denitrification, blue arrows; dissimilatory nitrate reduction to ammonium (DNRA), green arrows; nitrification, black arrows; anaerobic ammonium oxidation (AnAmmOx), gray arrows; "denitrification/intra-aerobic methane oxidation", violet arrows. Dotted lines represent reactions that do not involve nitrite handling.

ligand seems to be dependent on the activity type of the haemic enzyme: cytochromes P_{450}[1269] and NOS[1270] have a cysteine, whereas peroxidases have a histidine[1271] and haemic catalases have a tyrosine residue.[1272] Yet there are also some enigmas to solve, like the unusual lysine coordination present on CcNiR (see section 3.1.1). The haem great versatility is also conferred by the structure of the porphyrinic ring (Figure 19a), as is well exemplified by the reactivities with nitrite of CcNiR (*c* haem: forms and releases NH_4^+), Cd_1NiR (d_1 haem: forms and releases NO), and Mb (*b* haem: forms and bounds NO).

Copper is not so widely used, but it also plays crucial roles for survival of organisms (e.g., CcO, in oxidative phosphorylation, or superoxide dismutase, in antioxidant defense). It is employed in one of the nitrite reductase enzymes, the CuNiR (section 3.2.2). This metal participates in redox reactions, either in simple electron transfers (e.g., plastocyanin or pseudoazurin) or in catalysis (e.g., activation and reduction of dioxygen, superoxide radical, nitrous oxide, and nitrite). A copper center consists of a copper atom coordinated by nitrogen, oxygen, or sulfur atoms from different amino acid residues. According to their geometry and electronic structure, the copper centers have been divided into several groups: (i) type 1 (T1; Figure 19b), where copper is coordinated by two histidines, one cysteine, and a variable axial ligand (e.g., a sulfur atom of a methionine residue, as will be described for the CuNiR T1 centers); (ii) type 2 (T2), with no sulfur atom coordination (e.g., the three histidines and water/nitrite molecule of the CuNiR T2 centers); (iii) type 3, with coupled binuclear copper centers (e.g., haemocyanin);[1273−1276] (iv) trinuclear copper clusters (containing type 2 and 3 centers);[1277−1279] (v) mixed-valence binuclear Cu_A center;[1280−1284] (vi) heteronuclear Cu_B-$haem_A$ center (CcO);[1285] and (vii) tetranuclear Cu_Z center (nitrous oxide reductase).[1286−1289]

Molybdenum is essential to most organisms, including humans, catalyzing important redox reactions of the metabolism of carbon, nitrogen, and sulfur, many of which constitute critical steps in the global biogeochemical cycles of those elements.[597,1290−1295] With the exception of the iron/molybdenum cofactor of nitrogenase, involved in dinitrogen fixation (section 2.1.1; Figure 1, yellow arrow, Table 1), and a few other heteronuclear centers, whose physiological function is not yet fully understood,[1296−1298] molybdenum is found in a mononuclear form, hereafter designated as molybdenum center. In these centers, molybdenum is coordinated by the *cis*-dithiolene group of one or two pyranopterin cofactor molecules (Figure 19c (i)) and by oxygen, sulfur, or selenium atoms in a diversity of arrangements that determinates the classification of the molybdoenzymes into three families (Figure 19c (ii)):[597] XO, sulfite oxidase, and dimethylsulfoxide reductase families. In addition, a fourth family might be created to hold a molybdenum-containing enzyme recently described in mammals, the mitochondrial amidoxime-reducing component (mARC),[1299−1301] whose classification is presently unknown.

The mechanistic strategies of biological nitrite reduction/oxidation will be discussed in the following sections (3.1−3.3). The starting point will be the protein structure (under "Enzymatic Machinery"). A brief description of the enzyme promiscuity[1302] (under "Promiscuity") will also be included. A detailed discussion of the reaction mechanism will follow ("Mechanism"), where we will confine our considerations only to the active centers. The structure−activity relationships will be, as much as possible, systematically explored to discuss the mechanistic strategies biology developed to reduce/oxidize nitrite.

3.1. Nitrite Reduction to Ammonium

The great majority of biological redox reactions involve one or two-electron reduction/oxidation steps. When higher reductions/oxidations are needed, the reaction is divided into several individual reactions of one or two-electron reduction/oxidation, each catalyzed by a specific enzyme. The denitrification pathway (Figure 1, blue arrows, Table 1) is an example where nitrate is reduced by five electrons to dinitrogen in four individual steps (eq 4). The uncommon multielectron reactions, where several electrons are transferred through a single enzyme, without the release of any intermediate, are devoted to key steps of the metabolism. Oxidative phosphorylation (dioxygen reduction by four electrons to water, catalyzed by CcO), "respiration", and assimilation of sulfur

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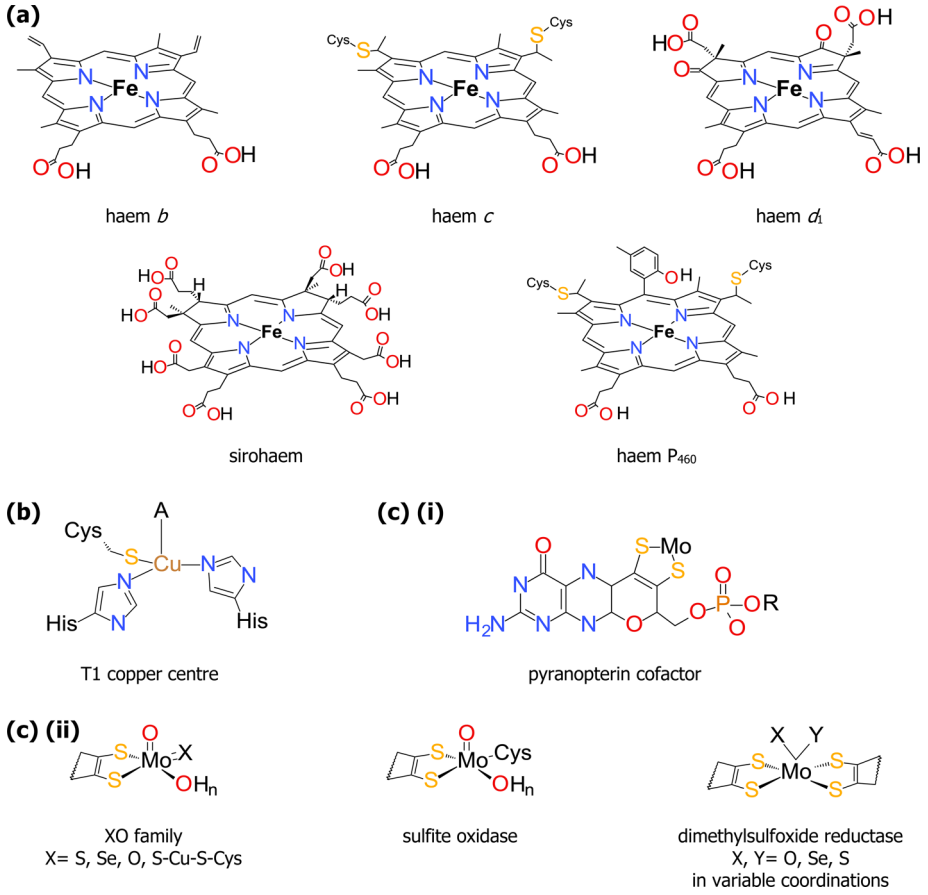

Figure 19. Structures relevant to nitrite reduction/oxidation. (a) Haem structures. The structure of the porphyrin ring determines the haem classification as a *b*, *c*, d_1, sirohaem, P_{460}, among other types here not shown. (b) Structure of type 1 copper center. The T1 single copper is coordinated by two histidines and one cysteine residue, in a trigonal planar geometry, and by a variable axial ligand (A), while the T2 copper center is coordinated in a similar geometry by nitrogen and/or oxygen atoms-containing ligands, but with no sulfur atom coordination. (c) Molybdenum centers structures. (i) Structure of the pyranopterin cofactor. The cofactor is a pyranopterin-dithiolate moiety, which forms a five-membered ene-1,2-dithiolate chelate ring with the molybdenum atom; in eukaryotes, the cofactor is found in the simplest monophosphate form (R is an hydrogen atom), while in prokaryotes it is found esterificated with several nucleotides (R can be one cytidine monophosphate, guanosine monophosphate, or adenosine monophosphate). (ii) Structures of the molybdenum centers of the three families of molybdoenzymes; for simplicity, only the dithiolate moiety of the pyranopterin cofactor is represented. The xanthine oxidase family enzymes hold a L−Mo═X(−OH/OH_2)(═O) core, where L stands for the pyranopterin cofactor and X represents terminal ═O, ═S, ═Se, or −S−Cu−S−cysteine residue; the sulfite oxidase family has a L−Mo−S−cysteine residue (−OH/OH_2)(═O) core; the dimethylsulfoxide reductase family comprises a L_2−Mo−X(−Y) core, where X and Y represent terminal ═O, −OH, ═S, and −SH groups and/or oxygen, sulfur, or selenium atoms from cysteine, selenocysteine, serine, or aspartate residue side chains.

(sulfite reduction by six electrons to sulfide, catalyzed by sulfite reductases), assimilation of molecular nitrogen (dinitrogen reduction by six electrons to ammonium, catalyzed by nitrogenases) are some of the few examples found in biology.

The assimilation and "respiration" of nitrite constitute two remarkable examples of what was stated above (Figure 1, Table 1). In both pathways, nitrite is reduced by six electrons to ammonium, in a reaction catalyzed by a single enzyme, without the release of any intermediate. The metal chosen for this catalysis is iron, probably a reminiscence of the prebiotic ammonium formation on the early Earth, where nitrite is believed to have been readily reduced to ammonium by reduced iron.[1303]

However, assimilation and "respiration" serve different cellular purposes and evolved to be two distinct metabolic pathways, located in different cellular compartments (cytoplasm/chloroplasts strom and periplasm/cytoplasmatic membrane, respectively), catalyzed by structurally different enzymes, containing different redox centers (here discussed separately in sections 3.1.1 and 3.1.2).

3.1.1. Dissimilatory Nitrite Reduction to Ammonium.

The dissimilatory nitrite reduction to ammonium (eq 64) is achieved within the *c* haem of multi-*c*-haems-containing nitrite reductase (CcNiR) enzymes (EC 1.7.2.2).

$$NO_2^- + 6e^- + 8H^+ \xrightarrow{c\,\text{haem}\ [Fe]} NH_4^+ + 2H_2O \qquad (64)$$
$$E^{o\prime}(NO_2^-/NH_4^+) = +0.75\ V$$

3.1.1.1. Enzymatic Machinery. CcNiR (also known as NrfA[1304]) are part of larger complexes. In the periplasm of *Wolinella succinogenes*,[69,1305,1306] *Desulfovibrio desulfuricans*,[1307] or *D. vulgaris*,[1308,1309] CcNiR forms a stable membrane-associated complex with NrfH, most likely $NrfA_4NrfH_2$.[69,1306,1308] NrfH is a small (~20 kDa), membrane-anchored, tetra-haemic quinol oxidase that provides electrons to CcNiR directly from the membrane quinone

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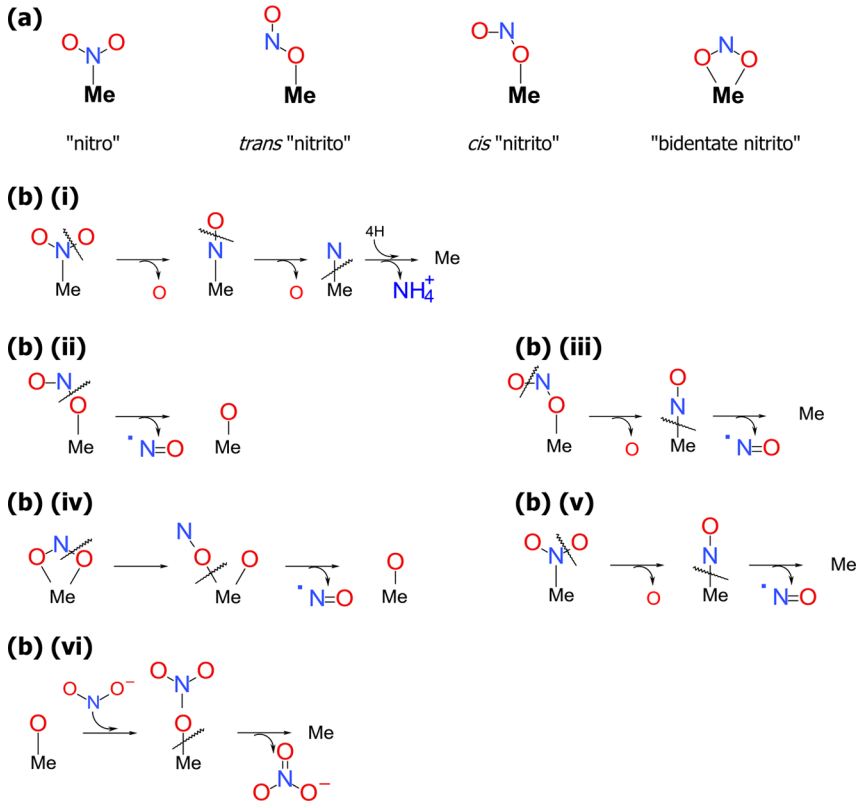

Figure 20. Nitrite binding modes. (a) Possible binding modes of nitrite to the metal atom (Me). Nitrite can bind the metal atom through the nitrogen atom, the so-called "nitro" mode, via one of the oxygen atoms, in a trans or cis conformation of the "nitrito" mode, or through both oxygen atoms, in a "bidentate nitrito" mode. (b) Binding modes for each product of nitrite reaction expected a priori; schematic representation (it is not intended to be a mechanistic representation). (i) To form ammonium, it would be expected that only the "nitro" binding mode is productive, to "remove" both oxygen atoms and "add" several protons without releasing intermediates. (ii)−(v) To synthesize NO, all of the binding modes seem feasible, although the simplest possible mechanism would be the (ii), via a "nitrito" binding mode, involving only one N−O bond cleavage with "automatic" release of NO (the abstracted oxygen atom remain bound to the metal, probably as a water molecule). (vi) To produce nitrate it is necessary to "add" one oxygen atom, and the simplest mechanism would be with a "nitro" binding mode to an oxo group of the metal (which, in the course of the reaction, would be "added" to the product molecule).

pool, in a fast and efficient way.[1305,1306,1310−1312] *Escherichia coli* CcNiR, on the contrary, uses a "soluble" periplasmatic penta-haem cytochrome (NrfB) as a direct redox partner ($NrfA_2NrfB_2$), which, in its turn, acts in conjunction with a ferredoxin (NrfC) and a putative membrane quinol oxidase (NrfD).[1312−1316]

All CcNiR structurally characterized to date host penta-haemic or octa-haemic subunits. Penta-haem CcNiR are homodimers (~120 kDa), with each monomer folded as a single domain (*Wolinella succinogenes* (Figure 21),[1305] *Escherichia coli*,[1314] *Sulfurospirillum deleyianum*,[1317] *Desulfovibrio desulfuricans*,[1307,1318,1319] *Desulfovibrio vulgaris*,[1308,1320,1321] *Shewanella oneidensis*[1322]). These CcNiR contain five covalently bound *c* haems (Figure 19a) per monomer, arranged in near-parallel and near-perpendicular haem pairs (Figure 21b). Four of the haems (#2−#5, Figure 21b) are bis-histidinyl-coordinated (CysXXCysHis binding motif) and, together, form a "wire" that facilitates the fast and effective electron transfer from the physiological partner (likely through haem #2[1314]) to the active site.[1323] The fifth haem (#1) constitutes the enzyme active site and is coordinated by a water/hydroxyl group in the distal position and by a lysine residue in the proximal position ($CysXXCysLys_{134}$ motif, *W. succinogenes* numbering; Figure 21c).[1317,1324,1325] The active site also comprises conserved histidine (His_{277}) and arginine (Arg_{114}) residues, which undergo hydrogen-bonding to nitrite and play a key role in the reduction process (as will be described). The active site pocket is completed with one tyrosine (Tyr_{218}) and one glutamine (Gln_{276}) residue that forms a conserved calcium binding site, localized at ~10 Å from the iron atom. The presence of calcium is essential for the enzyme activity,[1326] possibly due to a structural and/or catalytic roles: to keep the distal histidine away from the iron atom, conformational stabilization through electrostatic interactions[1319,1326,1327] and/or to facilitate the proton transfer steps.[1328] In the *D. desulfuricans* enzyme, a second calcium ion was identified coordinated to the propionates of the noncatalytic haems #3 and #4, whose main role is believed to be structural.[1319] Furthermore, two channels allow the nitrite entrance and the ammonium release.[1305,1317] The first one is a funnel-like entrance, with a significantly positive electrostatic surface potential that stabilizes the negatively charged substrate and presumably supplies the necessary protons (eq 64);[1319] the second channel, localized on the opposite site of substrate entry, has a predominantly negative electrostatic surface potential to assist the efflux of the cationic product.

The octa-haem CcNiR (*Thialkalivibrio nitratireducens*[1329−1331] and *Thioalkalivibrio paradoxus*[1332]) is a homo-hexameric enzyme containing eight haems per monomer. The monomer of the octa-haem CcNiR consists of two domains: one N-terminal domain, with three haems in a unique fold, and one catalytic C-terminal domain, with five haems in an

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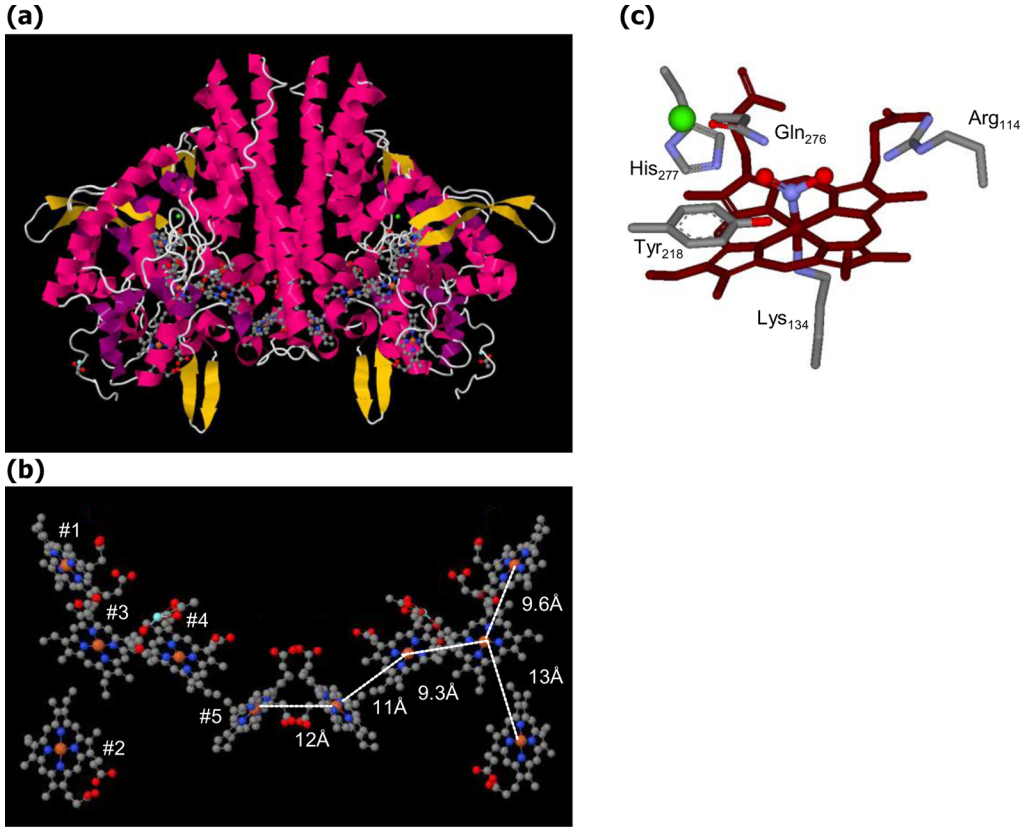

Figure 21. Cytochrome *c*-containing nitrite reductase − penta-haemic enzyme. (a) Three-dimensional structure view of the *Wolinella succinogenes* CcNiR homodimer (α helices and β sheets are shown in pink and yellow, respectively). The protein folds into one compact domain, with α helices as the predominant secondary structural motif, ranging from short helical turns to four long helices at the C-terminal end of the peptide chain. (b) Haems arrangement in the same orientation as in (a). The five haems are numbered according to their attachment to the protein chain (shown on the monomer on the left); haem #1 is the catalytic center. The distances (Fe-to-Fe) between adjacent centers (expressed in Å) are shown on the monomer on the right. (c) Catalytic haem with the nitrite molecule bounded (haem is represented in dark red and the calcium atom in green). The structures shown in (a) and (b) are based on the PDB file 1FS7[1305] (the images were obtained from www.rcsb.org/pdb/explore/jmol); the structure shown in (c) is based on the PDB file 2E80[1352] (the image was produced with Accelrys DS Visualizer, Accelrys Software Inc.).

arrangement similar to that found in the penta-haem CcNiR. Despite the low (20%) sequence homology with known penta-haem CcNiR, the catalytic haem of octa-haem CcNiR comprises (i) the lysine residue at the proximal position (CysXXCysLys motif), (ii) the histidine, arginine, and tyrosine residues at the distal side, and (iii) two conserved calcium binding sites. However, octa-haem CcNiR has special structural features, such as an unusual topography of the product channels that opens into the void interior space of the protein hexamer.

3.1.1.2. Promiscuity. In addition to nitrite, CcNiR also catalyzes the reduction of NO and of hydroxylamine to ammonium, although with lower specific activities.[1324,1326,1333−1337] Surprisingly, CcNiR is also able to catalyze the reduction of NO to nitrous oxide (eq 11),[1334,1338,1339] a reaction typical of denitrifiers (Figure 1, blue arrows, Table 1). Although the NO reductase activity of CcNiR has been known for long, only recently has it been re-evaluated, when it was realized that CcNiR is a key player of the oxidative and nitrosative stress defense network of *W. succinogenes* and *E. coli*.[1339−1344] Noteworthy, CcNiR mediates resistance, not only to hydroxylamine and NO, but also to hydrogen peroxide-induced stress.[1345] Moreover, CcNiR catalyzes the six-electron reduction of sulfite to sulfide and with a specific activity higher[1346−1349] than that reported for the true dissimilatory sulfite reductase. In this way, CcNiR connects the sulfur and nitrogen cycles.[1326,1347−1351] CcNiR is, therefore, one more "multitask" protein, involved in at least two distinct functions, anaerobic "respiration" and stress defense.

3.1.1.3. Mechanism. CcNiR catalyzes the reduction of nitrite to ammonium. To catalyze this reaction, CcNiR has to remove two oxygen atoms from nitrite (cleavage of two N−O bonds), at the same time as it has to add six electrons and eight protons (eq 64), and do all of these without releasing any intermediates, with no doubt a remarkable reaction.

To achieve this purpose, the properties of the catalytic haem are certainly crucial, starting from the back-bonding interaction between iron and nitrite that, as will be described below, is quite important for the substrate binding mode and strength and for the N−O bond activation. Also, the unusual lysine coordination present on CcNiR should have a decisive functional role, although theoretical calculations have not yet shown any striking electronic reasoning for lysine choice over the more conventional histidine ligand.[1328,1352,1353] Nevertheless, this odd coordination should represent an evolutionary advantage, because it requires the additional biological effort of producing a specialized haem lyase (coevolution).[1354−1357] In addition to the direct iron coordination, the second sphere coordination should play an essential role to define the catalytic properties of the haem. In CcNiR, the active site arginine and histidine residues seem to be important in directing the nitrite binding mode, which, in turn, would condition the reaction product: because CcNiR abstracts both oxygen atoms from

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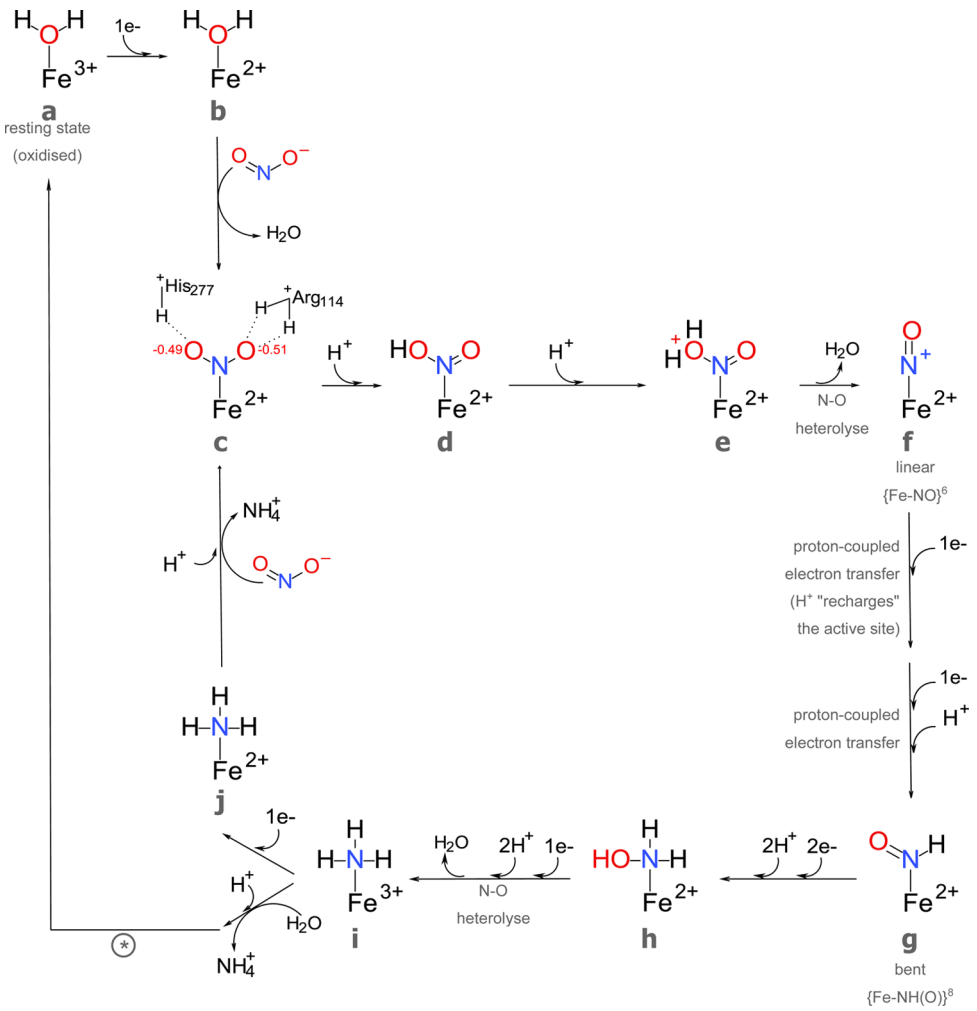

Figure 22. Mechanism of nitrite reduction to ammonium catalyzed by cytochrome *c*-containing nitrite reductase. See text for details. *When all of the reducing substrate or nitrite is consumed, the enzyme returns to the oxidized resting state.

nitrite, it can be anticipated that the "nitro" binding mode is the productive one (Figure 20a,b(i)). This proposal is supported by theoretical studies that point toward the "nitro" coordination as the energetically more favorable one,[1352,1353] and by the crystallographic structures showing the "nitro" binding mode in nitrite and hydroxylamine complexes of oxidized CcNiR.[1330,1352]

The catalytic cycle is believed to begin from a $Fe^{2+}-OH_2$ complex (Figure 22b), because the reduced enzyme binds nitrite much more strongly than the oxidized form.[1352,1353,1358−1362] Despite the less favorable electrostatic interaction ($Fe^{3+}\cdots NO_2^-$ versus $Fe^{2+}\cdots NO_2^-$), the back-bonding interaction with reduced iron (an effect described below) makes nitrite affinity for Fe^{2+} higher (stronger Fe−N bond) than that for Fe^{3+}.

After the reduction of $Fe^{3+}-OH_2$ (the enzyme resting state (Figure 22a)), the Fe−O bond is weakened, as a consequence of the increase in the Fe−O distance, and the axial water molecule is readily displaced by nitrite, resulting in a low spin $Fe^{2+}-NO_2^-$ complex (Figure 22c).[1352,1353,1361] When nitrite is approaching the iron, the positively charged Arg_{114} and protonated His_{277} would establish strong hydrogen bonds with the nitrite oxygen atoms, "anchoring" the nitrite molecule in the "nitro" mode (Figure 22c).[1353] These hydrogen bonds would cause an asymmetric charge distribution in nitrite, with the oxygen atoms gaining additional negative charge and thus becoming more susceptible to electrophilic attack. Simultaneously, the N−O bonds became longer and more single bond-like (1.25 instead of 1.5 bond order) and thus weaker, which should facilitate the cleavage.[1353]

In addition to these interactions with the positively charged residues, a back-bonding interaction between the nitrogen atom and the iron is of key importance for the Fe−N bond strength and for the initial activation of the N−O bond:[1352,1353,1359,1360] in the nitrite−iron complex, the nitrite LUMO, which has π-antibonding character, interacts with the iron HOMO, d_{xz} orbital, receiving electron density from the iron. Moreover, theoretical calculations[1353] suggest that nitrite is positioned with its plane perpendicular to the iron d_{xz} orbital, in a way that the π-acceptor capability of the nitrite nitrogen atom is maximized.[1353,1363,1364] This back-bonding interaction makes two effects: (i) a stronger Fe−N bond (responsible for the strong binding of substrate to reduced active site) and (ii) a weaker N−O bond, that is, longer and with a lower order, due to the electron density transfer into an orbital that is antibonding with respect to the N−O bond.

Upon nitrite binding with the concomitant N−O bond activation, the reaction proceeds with the abstraction of the first oxygen atom, through heterolytic cleavage (Figure 22c→f). The first oxygen atom to be abstracted is the one closest to the

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His_{277}, as suggested by the crystal structure of oxidized CcNiR complexed with the putative reaction intermediate hydroxylamine.[1352] To do this abstraction, this oxygen has to be converted into a good leaving group, which is achieved with the addition of two protons (Figure 22c→e). In fact, the protonated His_{277}, positioned at a short distance from the nitrite oxygen atom (1.56 Å[1353]) and with a "fine-tuned" acidity,[1365] is well suited for this role.[1353,1366] Remarkably, His_{277} is located to facilitate the donation of protons and at the same time to "anchor" the nitrite in the correct position to maximize the back-bonding interaction with the iron atom.[1353] At high pH values (>8, where His_{277} is most likely deprotonated) is the Arg_{114} (with a $pK_a \gg 7$) that probably acts as a proton donor.[1353] After the protonation steps, the $\{FeNO\}^6$ intermediate[1367] is formed (Figure 22f), and the first water molecule is released.

The reaction is proposed to proceed toward the formation of a $\{Fe-NH(O)\}^8$ complex (Figure 22g). To accomplish it, the enzyme active site must be "recharged" with the necessary protons and electrons.[1328] At this point, the enzyme must either overcome or avoid the formation of a $\{FeNO\}^7$ complex. Such complex (prepared reacting reduced CcNiR with NO) has been well characterized spectroscopically, kinetically,[1352,1369−1372] and at a theoretical level,[1328] and it is proposed experimentally to represent a thermodynamic sink and confirmed theoretically to represent a deep potential energy minimum. Moreover, in model complexes[1170,1373−1375] and also in Mb,[1376] the reduction potentials of $\{FeNO\}^7$ complexes were found to be truly low (<−1 V[1170,1373−1375] and −650 mV,[1376] respectively).

To avoid the formation of $\{FeNO\}^7$ complexes, biology has developed several strategies, including hydride transfer (as is the case of the eukaryotic NO reductase of *Fusarium oxysporum*[1377]) and proton-coupled electron transfer. In CcNiR, thermodynamic and kinetic theoretical analysis suggested that two proton-coupled electron transfer steps are the probable mechanism to achieve the formation of the $\{Fe-NH(O)\}^8$ complex (Figure 22f→g):[1328] (i) the first step proceeds rapidly (activation barrier of 1.6 kcal/mol) and is highly exothermic (−46 kcal/mol), leading to the probable protonation of the active site Arg_{114}; (ii) the subsequent proton-coupled electron transfer results in the formation of the $\{Fe-NH(O)\}^8$ complex through a rate-limiting equilibrium step (activation barrier of 6.5 kcal/mol, in an almost thermoneutral (0.7 kcal/mol) step). Possible alternative mechanisms, involving individual reduction and protonation steps, were also analyzed.[1328] Noteworthy, the calculations revealed that, while the first electron can be added either via simple reduction or coupled with proton transfer, the second electron can only be supplied upon addition of a proton (a simple second reduction requires more than 40 kcal/mol).[1328] Nevertheless, the simulation of reaction kinetics showed that the mechanism involving two proton-coupled electron transfers is the more probable one, leading to the formation of only trace quantities of the $\{Fe(NO)\}^7$ intermediate.

The resultant double occupation of the NO π-antibonding orbital leads to the splitting of one of the N−O bonds and to the formation of two separate lone electron pairs on the NO nitrogen and oxygen atoms.[1328] These two lone electron pairs should make the NO more easily protonable.[1168,1328,1352,1378−1381] However, contrary to what happens with the first oxygen atom to be abstracted (Figure 22c→e), at this reaction point it is the nitrogen atom that is thought to be protonated,[1328,1352] resulting in the formation of a $\{Fe-NH(O)\}^8$ complex (Figure 22g). This suggestion is supported by much evidence: (i) free HNO is much more stable than NOH (∼23 kcal/mol),[1352] (ii) the formation of Fe−NOH versus Fe−N(H)O is not energetically favorable (by ∼19 kcal/mol),[1352] (iii) HNO is formed in a model system of ferrous reduction of NO,[1382] (iv) HNO was observed in a biologically relevant system (Mb),[1376,1383] and (v) the Mb−NH(O) formation (over Mb−NOH) was confirmed by theoretical calculations.[1384] To achieve the $\{Fe-NH(O)\}^8$ formation, also the protonation of the Tyr_{218} was shown to be needed.[1328] This protonation may be necessary to maintain a high redox potential of the active site, to facilitate the next reduction step.[1328] The proton supply process can be modulated by the conserved calcium biding site, present at close proximity of the iron atom (see the "Enzymatic Machinery"):[1328] a proton localized on the calcium site would be more favorably transferred, because its energy is higher (∼6 kcal/mol) than the one of a proton in a pure water solution. As a result, the calcium site could be involved in facilitating the proton transfer steps.

In a few words, the proton-coupled electron transfer mechanism efficiently "recharges" the enzyme active site: the electron transfer haems donate the electrons; the change in the reduction state is immediately accompanied by proton transfer from a well-organized network of proton donors in the inlet channel, part of which is the calcium site.[1328] Each reduction increases the proton affinity of the previous intermediate, and each protonation increases its electrophilicity. The "recharged" enzyme then promptly reduces $\{FeNO\}^6$ to the reactive $\{Fe-NH(O)\}^8$ intermediate (Figure 22f→g), thus overcoming the formation of the stable $\{FeNO\}^7$ intermediate. In this context, it can be speculated that the reasoning for the presence of four noncatalytic haems in CcNiR is the requirement for the timely electron transfer. This speculation, however, is questioned by CSNiR that, despite catalyzing the same reaction, has only one Fe/S center to transfer electrons to the active site (see section 3.1.2).

At this stage (Figure 22g), the enzyme still has one oxygen atom to remove and several protons and electrons to add (eq 64). Unfortunately, the mechanism of this last part of the reaction is not yet so well characterized. The reaction should continue to form an iron−hydroxylamine complex (Figure 22h). The formation of such intermediate is suggested by the known hydroxylamine reductase activity of CcNiR that reduces this compound with one-half of the specific activity observed with nitrite. The oxidized form of this complex (Fe^{3+}−hydroxylamine) was, in fact, observed in crystals of oxidized CcNiR, where the oxygen atom was found hydrogen-bonded to the N_ε atom of the Arg_{114}.[1335,1352] To form this complex, the reaction is suggested to proceed with the alternating transfer of two electrons and two protons, possibly as two proton-coupled electron transfers (with no critical energetic barriers, regardless the sequence of transfers[1352]) to yield the Fe−NH(H)(OH) complex (Figure 22h).

Now, the reaction is almost completed. The addition of one more proton converts the hydroxyl moiety into a water molecule (the second one), which leaves the enzyme (Figure 22h→i). After the addition of one electron, the oxidation of Fe^{2+} coupled to a proton transfer would yield the Fe^{3+}−ammonium complex (Figure 22i). Theoretical calculations indicate that the crucial role of the active site Tyr_{218}, revealed by the almost complete inactivation of the enzyme when this residue is mutated to a phenylalanine,[1349] is probably carried

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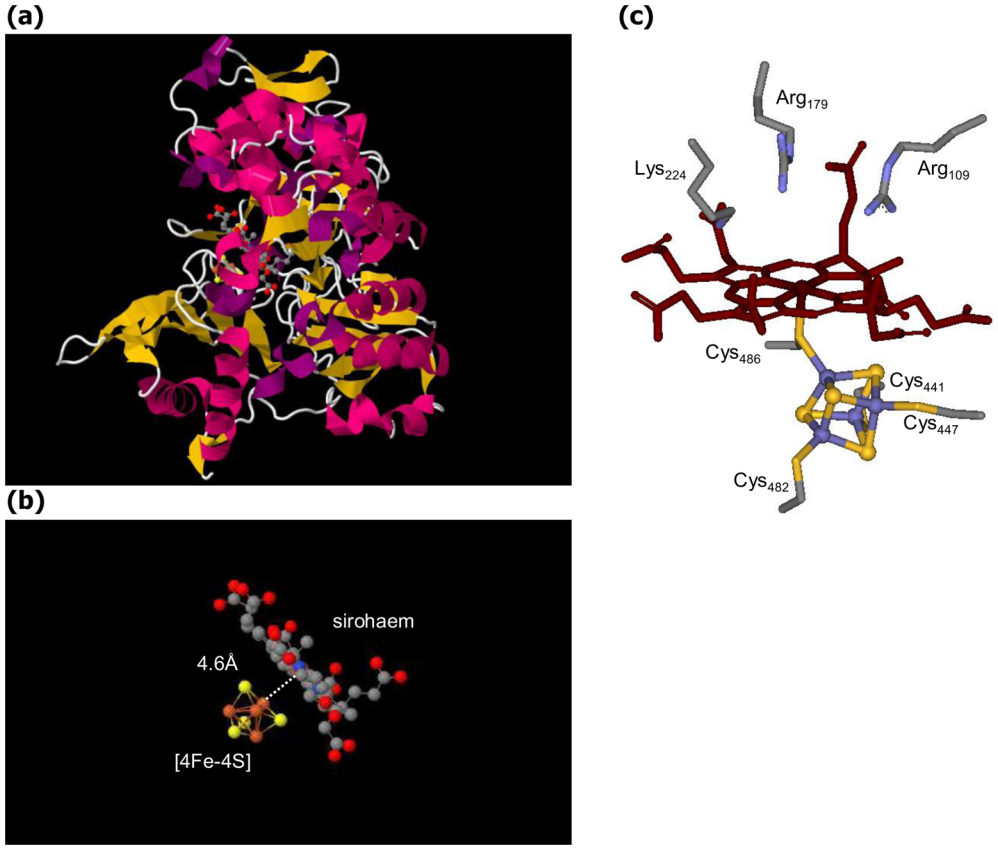

Figure 23. Sirohaem-containing nitrite reductase. (a) Three-dimensional structure view of the *Spinacia oleracea* CSNiR monomer (α helices and β sheets are shown in pink and yellow, respectively). The protein folds into three domains, in a fairly compact structure. The sirohaem and [4Fe−4S] center are localized at the interface of the three domains, in a wide (>8 Å) tunnel. (b) Sirohaem and [4Fe−4S] center arrangement in the same orientation as in (a). The distance (sirohaem)Fe-to-(Fe/S)Fe of 4.6 Å is indicated. (c) Catalytic sirohaem (haem is represented in dark red). The structures shown are based on the PDB file 2AKJ[1383] (the images in (a) and (b) were obtained from www.rcsb.org/pdb/explore/jmol; the image in (c) was produced with Accelrys DS Visualizer, Accelrys Software Inc.).

out at this final stage, possibly in a radical step involving hydrogen atom transfer.[1353]

The Fe^{3+}−ammonium complex can then be readily dissociated.[1352] This suggestion is supported by model ammonium−iron porphyrins complexes that show a strong tendency to hydrolyze,[1385] unless a steric protective group is included.[1386] Alternatively (energetically similar[1352,1362]), the Fe^{3+}−ammonium complex is first reduced to Fe^{2+}−ammonium (Figure 22j), after which the ammonium is displaced by a new nitrite molecule (Figure 22j→c), closing the catalytic cycle. Note that, until this point, all of the reaction steps were proposed to take place with reduced iron, which is made possible due to the efficient electron transfer to the active site. Ammonium is, subsequently, electrostatically guided to the protein surface through the product channel.

In summary, the CcNiR-catalyzed reaction is presently suggested to start from a ferrous−nitrite complex. The nitrite reduction proceeds with the initial cleavage of the first N−O bond, to form an $\{FeNO\}^6$ complex (Figure 22c→f). The success of the first N−O bond cleavage (Figure 22c→f) is due (at least) to the back-bonding with iron and to the hydrogen bonds with second sphere amino acid residues. The back-bonding interaction (i) strengths the Fe−N bond and (ii) weakens the N−O bond. The hydrogen bonds (i) direct the nitrite molecule to the correct position to allow the back-bonding interaction; (ii) enhance this back-bonding (stabilizing the nitrite π-antibonding orbital, bringing it energetically even closer to the iron *d* orbitals); (iii) cause an asymmetric charge distribution that activates the N−O bond (make it more single bond-like) for reductive cleavage; and (iv) supply the necessary protons.

The $\{FeNO\}^6$ intermediate is subsequently reduced and protonated to eventually form an iron−hydroxylamine complex (Figure 22f→h). This reduction is made possible through two proton-coupled electron transfers that overcome the formation of the stable $\{Fe(NO)\}^7$ complex. Each reduction step increases the proton affinity of the previous intermediate, and each protonation increases its electrophilicity. Once the hydroxylamine intermediate is formed, further reduction and protonation lead to lysis of the last N−O bond, and the product is released (Figure 22h→c).

3.1.2. Assimilatory Nitrite Reduction to Ammonium. The assimilatory nitrite reduction to ammonium (eq 65) is achieved within the sirohaem of the sirohaem-containing nitrite reductase (CSNiR) enzymes. As described in section 2.1.1, both ferredoxin- and NAD(P)H-dependent enzymes hold a sirohaem and an Fe/S. The NAD(P)H-dependent enzymes (EC 1.7.1.4) contain, in addition, a FAD domain, bound to an extended N-terminus, which is involved in the NAD(P)H binding and oxidation. This Review will focus only on the ferredoxin-dependent nitrite reductases (EC 1.7.7.1).

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$$NO_2^- + 6e^- + 8H^+ \xrightarrow{\text{sirohaem Fe}} NH_4^+ + 2H_2O \quad (65)$$
$$E^{0\prime}(NO_2^-/NH_4^+) = +0.75\ V$$

3.1.2.1. Enzymatic Machinery. CSNiR have been studied from a number of higher plants, algae, and cyanobacteria. The spinach chloroplast enzyme is the most extensively characterized CSNiR (also the first 3D structure known[1387]), whereas much less is known about the enzymes from cyanobacteria[1388] or from algae.[1389−1391] Recently, the tobacco enzyme became the focus of increasing interest.[1392−1394]

CSNiR (product of *nasB*, *nirB*, and *Nii* genes, in, e.g., *Paracoccus denitrificans*,[1395] *E. coli*,[1396] and tobacco,[1397] respectively) are monomers (~65 kDa), folded into three domains, containing one [4Fe−4S] center and one sirohaem (Figure 23).[1387,1394,1398,1399] The [4Fe−4S] center is coordinated by four cysteine sulfur atoms, with the characteristic $Cys_{441}XXXXXCys_{447}X_{(n)}Cys_{482}XXXCys_{486}$ motif (spinach enzyme numbering), and transfers the electrons from the physiological electron donor (reduced ferredoxin) to sirohaem (Figure 23c).[1400,1401] Sirohaem (Figure 19a) is found in nitrite and sulfite reductases, where it constitutes the enzymes active site.[1398,1402] The sirohaem is connected to the Fe/S through the Cys_{486} residue that also coordinates the haem iron (Figure 23c). Despite being coupled by the bridging sulfur, the Fe/S and sirohaem act as independent one-electron carriers in catalysis[1402] and in titrations.[1387,1400] The haem sixth coordination position is occupied by a water molecule.[1403] The active site also comprises conserved Arg_{109}, Arg_{179}, and Lys_{224} residues, which are thought to facilitate the binding and subsequent reduction of nitrite (hydrogen-bonding/proton donation).[1387,1392,1402] In addition, several other potentially positively charged residues seem to be essential to stabilize the sirohaem eight carboxylate groups,[1387] some of which are also conserved in sulfite reductases.[1404−1408] CSNiR has a wide (>8 Å) tapered channel, where the Fe/S and sirohaem are located, and through which nitrite, ammonium, and solvent have a clear path between the protein surface and the haem distal side. It is also at the surface of this channel that the reduced ferredoxin is proposed to bind, to deliver one electron at a time to the CSNiR Fe/S, "closing" the channel from the solvent in the binding process.[1387,1399,1409,1410]

3.1.2.2. Promiscuity. Like CcNiR, CSNiR also catalyzes the reduction of hydroxylamine to ammonium[1390,1411−1413] and of sulfite to sulfide, but with lower affinity comparatively to nitrite.[1414,1415] Remarkably, in tobacco enzyme, the mutation of the active site residue Asn_{226} to a lysine results in an increase of the sulfite reductase activity of 1 order of magnitude.[1393]

3.1.2.3. Mechanism. CSNiR-catalyzed nitrite reduction to ammonium also takes place without the release of detectable intermediates, and several biochemical, spectroscopic, and crystallographic studies support that the reaction mechanism is similar to the CcNiR one and to the dissimilatory sulfite reductase one[1416] on its key aspects: (i) The reaction is believed to start from a low spin (sirohaem)Fe^{2+}−nitrite complex.[1400,1403,1413,1414,1417−1422] (ii) The nitrite molecule bounds through the "nitro" mode,[1403,1420−1422] stabilized through hydrogen bonds with positively charged Lys_{244}, Arg_{179}, and Arg_{109} residues.[1387,1392] (iii) The first oxygen atom to be abstracted is the one closest to the Lys_{224}, in a similar way to what was proposed for the dissimilatory sulfite reductase[1416]). (iv) Lys_{224} and Arg_{179} are the probable proton donors to form the first water molecule and the Fe^{2+}−NO intermediate.[1392,1403,1422−1424] (v) The Fe^{2+}−NH(H)OH intermediate[1390,1402,1422] was proposed to be formed with the protons derived from "recharged" Arg_{179} and Arg_{109} residues and from a water molecule hydrogen-bonded to the "recharged" Lys_{224}.[1392]

Despite catalyzing the same reaction, with a mechanism that it is suggested to be similar, there are some remarkable differences. The first obvious distinctive features are the haem type, *c* haem versus sirohaem, and its axial coordination, a nitrogen atom from a lysine versus a sulfur atom from the cysteine-coupled Fe/S center. Thus, contrary to what may be expected, CSNiR is not structurally related to CcNiR; instead, it is related to the other enzyme known to have a sirohaem, the assimilatory sulfite reductase.[1404,1415] Furthermore, it must be bore in mind that the utilization of different haem types requires a higher biological effort to synthesize all molecules.[1425−1428] The rationalization for these two choices, c versus sirohaem, must wait for theoretical studies on the different haem types and coordinations.

Another distinctive feature is the CSNiR ability to "store" electrons. Unlike CcNiR, with its four *c* haems, CSNiR has only one Fe/S to hold one electron. It has also only a single ferredoxin binding site[1387,1402,1429,1430] to receive one electron at a time, from the ferredoxin [2Fe−2S] center (i.e., the physiological partner is a one-electron donor). Those facts mean that the CSNiR must receive the necessary six electrons in one-electron steps and make the elucidation of the electron transfer particularly challenging.

Remarkably, these two characteristics (catalytic haem and electron transfer centers) are not important to determine the nature of the reaction product. The two active site structures represent two "solutions" to solve the same "problem", but within distinct biological purposes, "respiration" versus assimilation. A similar phenomenon will be discussed for the "respiratory" nitrite reduction to NO, where two truly different "solutions" were found for the same reaction, iron- and copper-dependent enzymes.

3.1.3. Nitrite Reduction to Ammonium by Other Enzymes. Tetrathionate reductase (TTR) and hydroxylamine oxidoreductase (HAOR) are two multihaemic enzymes that were shown to also catalyze the reduction of nitrite to ammonium.[1431−1436]

TTR catalyzes the reduction of tetrathionate ($S_4O_6^{2-}$) to thiosulfate ($S_2O_3^{2-}$) (eq 66). The *Shewanella oneidensis* enzyme is a perisplamatic protein (~55 kDa) that holds eight *c* haems, seven of which are bis-histidinyl-coordinated and one, the catalytic site, that has the unusual lysine coordination[1437] characteristic of CcNiR.[1432,1433]

HAOR catalyzes the oxidation of hydroxylamine to nitrite (eq 67), in the second step of nitrification (section 2.1.1; Figure 1, black arrows, Table 1).[1431,1434−1436,1438−1440] The enzyme from *Nitrosomonas europaea* is a homotrimer (~210 kDa) of octa-haemic monomers. Seven of the haems are typical bis-histidinyl-coordinated *c* haems, whose role is only electron transfer, while the active site is constituted by a histidinyl-coordinated P_{460} haem (Figure 19a).

$$S_4O_6^{2-} + 2e^- \xrightarrow{c\ \text{haem Fe}} 2S_2O_3^{2-} \quad (66)$$

$$NH_2OH + H_2O \xrightarrow{P_{460}\ \text{haem Fe}} NO_2^- + 4e^- + 5H^+ \quad (67)$$

The structures of these two enzymes and of CcNiR show significant similarities in haem "architecture". Five of the TTR

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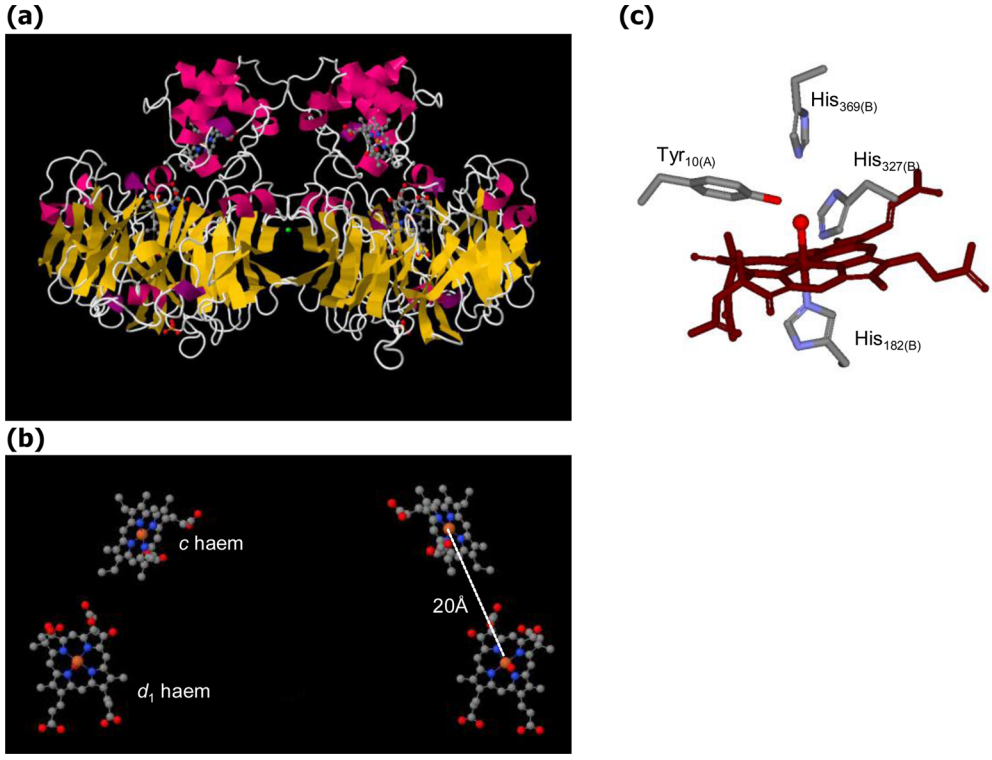

Figure 24. Cytochrome d_1-containing nitrite reductase. (a) Three-dimensional structure view of oxidized *Pseudomonas aeruginosa* Cd_1NiR homodimer (α helices and β sheets are shown in pink and yellow, respectively). The protein folds into two domains: (i) one typical bacterial α-helical cytochrome *c* N-terminal domain (on top), containing one *c* haem, and (ii) one eight-bladed β-propeller C-terminal domain (on bottom), comprising one d_1 haem. The N-terminal Ala_7-Pro_{29} loop is "swapped" between the two monomers; as a result, the Tyr_{10} of one monomer is found close to the d_1 haem of the partner monomer (i.e., the Tyr_{10} of monomer "A" is hydrogen-bonded to the hydroxyl group that coordinates the d_1 haem of monomer "B"). (b) Haems arrangement in the same orientation as in (a), *c* haem on top and d_1 haem on bottom. The distance between the *c* and d_1 haem within a monomer (Fe-to-Fe of 19.7 Å; edge-to-edge of 9.0 Å) is shown on the monomer on the right. (c) Catalytic haem with a hydroxyl moiety bounded (haem is represented in dark red). The structures shown in (a) and (b) are based on the PDB file 1NIR[1443] (the images were obtained from www.rcsb.org/pdb/explore/jmol); the structure shown in (c) is based on the same PDB file (the image was produced with Accelrys DS Visualizer, Accelrys Software Inc.).

haems are almost overlapped with the CcNiR haems, including the substrate binding site and the position of the coordinating lysine at the active site. Four of the HAOR haems are superposable with the noncatalytic haems of CcNiR, with the catalytic haems positioned flipped with respect to each other. In addition, both TTR[1432,1433] and HAOR[1434−1436] are able to catalyze the reduction of nitrite and hydroxylamine to ammonium as CcNiR does. Actually, the TTR catalytic specificities toward nitrite and hydroxylamine, comparable to the CcNiR ones,[1433] suggest that this enzyme may have a role in the biochemical cycle of nitrogen.

On the other hand, it is surprising how HAOR, an enzyme that is "tuned" to catalyze the oxidation of hydroxylamine, is able to catalyze reduction reactions. In fact, the HAOR specificity constants toward nitrite and hydroxylamine reduction are 2 orders of magnitude lower than the CcNiR ones.[1434,1435] However, in vitro, in the presence of sufficient reducing power, HAOR and CcNiR reactions probably proceed through comparable intermediates (eq 68). The occurrence of those reductase activities under more physiological conditions, where the P_{460} haem should be mostly oxidized,[1436] is, nevertheless, controversial.

$$\mathrm{Fe{-}NH_4^+} \leftarrow \mathrm{Fe{-}N(H_2)OH} \rightleftharpoons \mathrm{Fe{-}N(H)O} \rightleftharpoons \mathrm{Fe{-}NO} \rightleftharpoons \mathrm{Fe{-}NO_2^-} \quad (68)$$

It was hypothesized that the multihaemic enzymes of the "oxidative branches" of nitrogen cycle, namely HAOR (nitrification) and hydrazine oxidoreductase (AnAmmOx), had evolved from the more ancient penta-haemic nitrite reductase enzymes, under a variety of environmental pressures that triggered the function changing from reduction to oxidation.[1350,1441]

3.2. Nitrite Reduction to Nitric Oxide

NO is a remarkable biomolecule. Its formation from nitrite constitutes the first committed step in denitrification and is an essential step in the primitive AnAmmOx and other "respiratory" pathways (section 2.1.1; Figure 1, Table 1). NO is also a crucial mammalian signaling molecule, and, for its synthesis, biology developed a "dedicated" enzyme, NOS, and several nitrite "recycling" pathways to ensure the NO formation when the NOS activity is impaired (section 2.2.1.1). Remarkably, also plants and bacteria are using similar "rescue" nitrite-dependent pathways to produce NO (sections 2.2.2.1 and 2.2.3). Therefore, the biological nitrite reduction to NO is an ubiquitous, universal reaction that was "invented" for the anaerobic world and has been "reinvented" and employed ever since.

To carry out this "old" reaction, biology developed different "solutions", using three metals, iron, copper, and molybdenum. For the "respiratory" function, two distinct classes of enzymes were developed, haem-dependent and copper-dependent nitrite

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reductases (Cd_1NiR, section 3.2.1, and CuNiR, section 3.2.2, respectively). Remarkably, the first "solution", the iron-dependent enzyme developed in the preoxic era, was so successful that it was used in different strategies of survival (denitrification, AnAmmOx, "denitrification/intra-aerobic methane oxidation", and possibly others). This wide use further emphasizes the in vivo importance of NO as the alleged first deep electron sink on Earth, before the emergence of dioxygen.[151,1442] For signaling pathways, biology "reuses" iron and molybdenum-based redox systems, present in cells to accomplish other functions, to carry out the nitrite reduction to NO (section 2.2).

3.2.1. Dissimilatory Nitrite Reduction to Nitric Oxide by an Iron-Dependent Enzyme. The dissimilatory iron-dependent nitrite reduction to NO (eq 69) is achieved with the d_1 haem of the *c* and d_1 haems-containing nitrite reductase (Cd_1NiR) enzymes (EC 1.7.2.1).

$$NO_2^- + 1e^- + 2H^+ \xrightarrow{d_1\ \text{haem}\ [Fe]} {}^{\bullet}NO + H_2O \qquad (69)$$
$$E^{o\prime}\ (NO_2^-/{}^{\bullet}NO) = +0.34\ V$$

3.2.1.1. Enzymatic Machinery. Cd_1NiR (product of *nirS* genes) are periplasmatic homodimers (~120 kDa), with each monomer folded into two domains: (i) one typical bacterial α-helical cytochrome *c* N-terminal domain, containing one *c* haem, and (ii) one eight-bladed β-propeller C-terminal domain, comprising one d_1 haem (*Pseudomonas aeruginosa* (Figure 24)[1443−1446] and *Paracoccus pantotrophus*[1447−1451]). The *c* haem is responsible for the electron transfer from different electron carriers (section 2.1.1) to the d_1 haem (Figure 19a), which constitutes the enzyme active site.[1444,1450,1452−1457] The active site also comprises two conserved histidine residues (His_{327} and His_{369}, in *P. aeruginosa*, and His_{345} and His_{388}, in *P. pantotrophus*), which are essential for catalysis (Figure 24c) as will be discussed.[1443−1451,1458,1459]

P. aeruginosa Cd_1NiR has the *c* haem coordinated by Met_{88} and His_{51}, while the d_1 haem is coordinated by His_{182}, in the proximal position, and by a hydroxyl group, in the distal position (Figure 24c).[1443] When the d_1 haem is oxidized, regardless of the *c* haem state,[1460] this hydroxyl group is hydrogen-bonded to the Tyr_{10} of the partner monomer (this occurs because the 7−29 loop is "swapped" over the two monomers).[1443] Accordingly, the active site haem is in a "closed" hexa-coordinated state. After d_1 haem reduction, the 56−62 loop is displaced (6 Å), and the Tyr_{10} is (4.2 Å) shifted away from the d_1 haem iron.[1444,1460] This concerted movement in the N-domain of one monomer "opens" the active site haem present in the C-domain of the partner monomer, allowing nitrite to bind to the haem iron. For the following mechanism discussion, it should be here emphasized that it is the electron transfer to d_1 haem, and not the *c* haem reduction, that is responsible for these conformational changes.[1460]

In *P. pantotrophus* Cd_1NiR, the structural changes are more complex. Despite having a similar overall structure, in *P. pantotrophus*, the N-terminal segment is not "domain-swapped", but "wrapped" around the same monomer, at the interface between the two domains.[1447] In addition, the as-isolated, oxidized enzyme has the *c* haem His_{17}-His_{69}-coordinated and the d_1 haem His_{200}-Tyr_{25}-coordinated (proximal and distal positions, respectively).[1447,1449,1450] This haems coordination is surprising: first, because neither His_{17} nor Tyr_{25} are conserved in other Cd_1NiR, apart from the very closely related *P. denitrificans* enzyme;[1461] and, second, because Tyr_{25} is coordinating the iron directly. The presence of a His_{200}-Tyr_{25}-coordinated active site haem, with no vacant position for substrate binding, indicates that the *P. pantotrophus* Cd_1NiR is isolated in an inactive form, an "unready" enzyme (as denominated in section 3). However, in a situation parallel to that of *P. aeruginosa* Cd_1NiR, reduction of the *P. pantotrophus* enzyme triggers a concerted movement in a N-domain loop that changes the coordination sphere of both haems: the *c* haem becomes Met_{106}-His_{69}-coordinated, and the tyrosine residue of the d_1 haem is displaced to yield an "open" penta-coordinated active site, now a "ready" enzyme.[1443,1450,1462,1463] Accompanying the structural changes, the prereduction of *P. pantotrophus* Cd_1NiR leads to a catalytically more active enzyme, which exhibits higher k_{cat} values than the as-isolated enzyme.[1456,1464,1468]

The conformational changes needed to activate Cd_1NiR could be relevant to the in vivo regulation of the enzyme (and not an experimental artifact that has to be overcome). Actually, the *P. pantotrophus* Tyr_{25}Ser mutant, which has a "locked" penta-coordinated d_1 haem iron, is an active enzyme without needing the reductive activation step.[1469] Similarly, the *P. aeruginosa* Tyr_{10}Phe mutant is functionally and spectroscopically identical to the wild-type.[1470,1471] Therefore, the presence of the tyrosine residue, which is not essential for catalysis, could be an intentional biological regulatory strategy: having an enzyme whose reactivity can be controlled through a mechanism of the type "unready versus ready" ("on/off") to allow the organism to quickly respond toward different cellular/environmental conditions/stimuli[1463,1469] (as was previously discussed for Nb, Cc, XD/XO).

The Cd_1NiR structural changes, however, are not driven only by reduction, but also by the protonation state of the residues at the active site. In fact, it is remarkable how comparable are the structures of the oxidized *P. aeruginosa* His_{369}Ala and His_{327}Ala mutants at pH 5.5−6.5[1445,1458,1463] and of the reduced wild-type *P. pantotrophus* Cd_1NiR crystallized under anaerobic conditions at pH 9.0.[1463] These three structures (i) preserve the individual *c* and d_1 coordination and folding characteristic of reduced enzymes and (ii) show the *c* domain ~60° rotated, ~20 Å displaced, relatively to the d_1 domain. The structural organization observed in the mutants was initially interpreted as suggesting that the two conserved histidines are crucial for the enzyme conformation.[1445] Yet, the fact that a parallel structure was observed in the wild-type *P. pantotrophus* enzyme at pH 9, where the histidine residues are likely deprotonated and thus uncharged and electrostatically equivalent to the His-Ala mutants of *P. aeruginosa*, points toward another hypothesis: it is the protonation state of those histidines that is determinant for the conformation adopted by Cd_1NiR. Therefore, it is probable that not only the localized iron charge (oxidized versus reduced), but also the overall charge in the active site control the enzyme conformation.

These remarkable Cd_1NiR structural changes, driven by oxidation/reduction and also protonation/deprotonation, further support that the conformational changes are a deliberate mechanistic strategy of Cd_1NiR.

3.2.1.2. Promiscuity. Besides the nitrite reduction to NO, Cd_1NiR catalyzes the two-electron reduction of hydroxylamine to ammonium and the four-electron reduction of dioxygen to water, in the presence of any of its electron donors.[1456,1468,1472] In fact, the enzyme was long thought to be an oxidase and classified as a cytochrome oxidase (EC 1.9.3.2).

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Contrary to CcNiR, the Cd_1NiR does not catalyze the reduction of NO to hydroxylamine (eq 70). This is a very interestingly observation, because that reaction would prevent the primitive "recycling" of nitrogen through the AnAmmOx (eq 71) suggested in section 2.1.1.

$$NO_2^- \xrightarrow{Cd_1NiR} {}^{\bullet}NO \xrightarrow{X} NH_2OH \xrightarrow{Cd_1NiR} NH_4^+ \quad (70)$$

$$^{\bullet}NO + NH_4^+ \xrightarrow{\text{hydraz. synthase}} N_2H_4 \xrightarrow{\text{hydraz. oxidoreduct.}} N_2 \quad (71)$$

3.2.1.3. Mechanism. Comparatively to the nitrite reduction to ammonium, the formation of NO is considerably simpler. As will be discussed, the Cd_1NiR-catalyzed reaction involves the nitrite binding to the reduced d_1 haem iron, followed by the abstraction of one oxygen atom and the addition of one electron. In this way, the Cd_1NiR reaction can be considered as a "copy" of the first part of the CcNiR reaction illustrated in Figure 22b→f. However, the well-known high affinity of NO to haem iron raises an intriguing question: how is the NO released from the active site haem of the Cd_1NiR?

The Cd_1NiR catalytic cycle begins after the reductive activation of the resting (inactive) enzyme, with the concomitant tyrosine displacement from the d_1 reduced iron (Figure 25a→b). In addition to "opening" the active site haem, the enzyme reduction provides the reduced iron, for which the nitrite affinity is higher.[1445,1451] For NO formation, the "nitrito" binding mode would be a priori expected (Figure 20a), although all of the binding modes are feasible (Figure 20b(ii)–(v)). However, crystal structures showed that nitrite binds to Cd_1NiR in the "nitro" mode (Figure 25c), with the nitrite oxygen atoms forming hydrogen bonds with the two conserved histidine residues (His_{327} and His_{369}, in *P. aeruginosa*, and His_{345} and His_{388}, in *P. pantotrophus*).[1443,1444,1447,1450,1458,1473,1474] This Cd_1NiR–nitrite interaction mode is clearly a "copy" of the CcNiR one, and the initial nitrite binding and activation is believed to be ruled by the same principles that were proposed for CcNiR: back-bonding and hydrogen bond. However, it

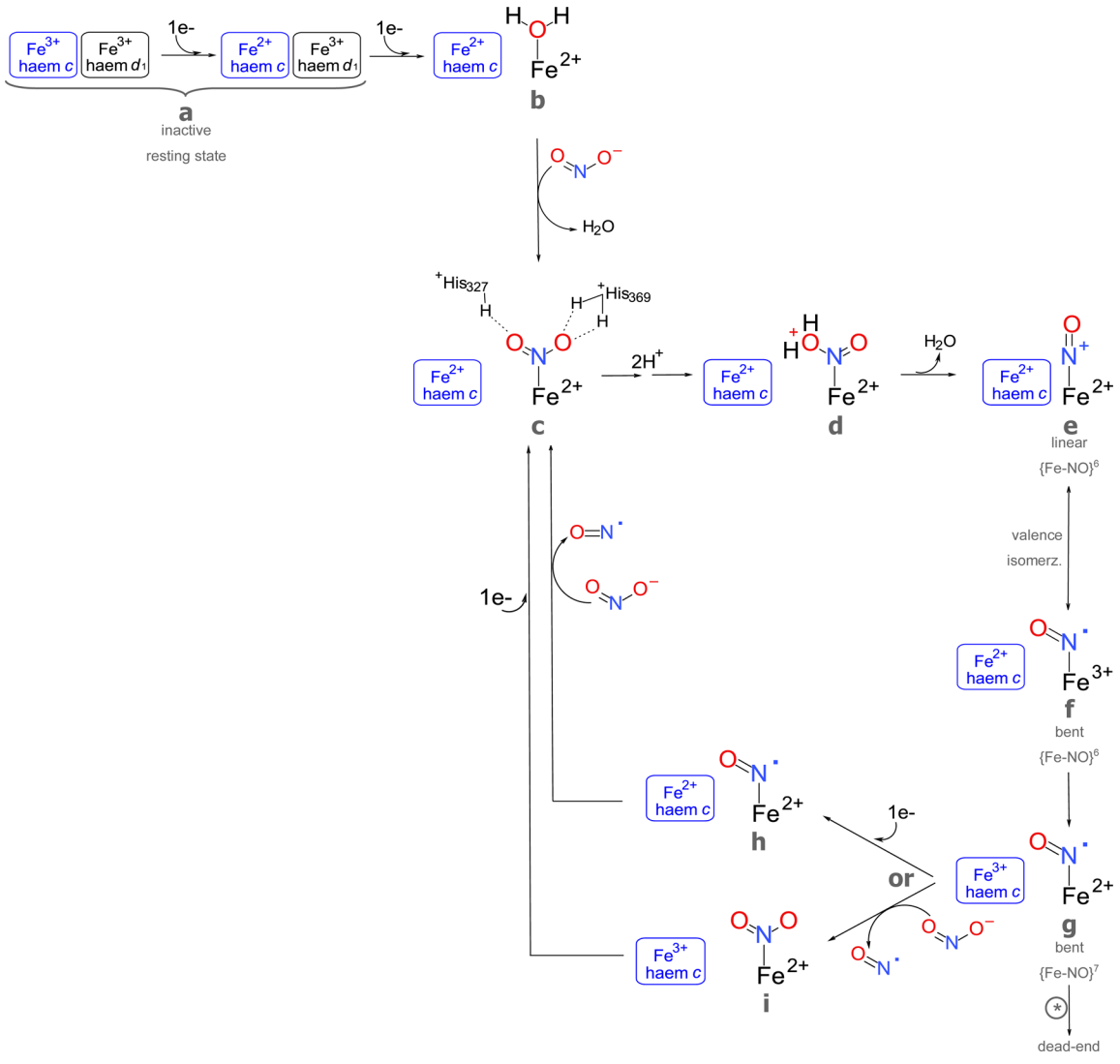

Figure 25. Mechanism of nitrite reduction to NO catalyzed by cytochrome d_1-containing nitrite reductase. See text for details. The *c* haem is represented as a blue box. The nitrite reduction reaction, taking place at the d_1 haem, is explicitly represented (except in "a", where the d_1 haem is represented as a black box). The fast NO release is triggered by the electron transfer from *c* to d_1 haem, but the present data do not allow one to discriminate if the NO is released from the "mixed-valence" protein (g→i), or from the fully reduced enzyme (h→c). *In the absence of reducing substrate or nitrite, the enzyme is trapped in a "dead-end" species (g).

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should be here emphasized that it is plausible that the d_1 haem unique features (two electron-withdrawing carbonyl groups (Figure 19a) and inversion of the iron *d* orbitals energy levels, both discussed below) made the d_1 haem less effective at such back-bonding than the *c* haem of CcNiR.[1475]

Subsequent to the nitrite binding, and again as in CcNiR, the oxygen atom to be abstracted is protonated, the N−O bond is heterolytically cleaved, and a water molecule is released (Figure 25c→e).[1445,1459,1473,1474] The two conserved histidine residues are well positioned to act as proton donors,[1443−1451,1458,1459] and their key role in this step is supported by the abolishment of the nitrite reductase activity on the His_{327}Ala and His_{369}Ala mutants[1445] and by theoretical calculations.[1473] Nonetheless, the two histidine residues are not equivalent, and the His_{369} (located at a shorter distance from both nitrite oxygen atoms) seems to be essential to control the stability of the enzyme−substrate complex, possibly via the formation of two hydrogen bonds.[1445,1446,1473] These steps (Figure 25c→e) culminate with the formation of a ferrous $\{FeNO\}^6$ complex, as was described for CcNiR (Figure 22f).

From this point, the similitude ends and the CcNiR and Cd_1NiR reactions follow separate strategies. While the CcNiR "objective" is to retain the NO bound to be further reduced, the Cd_1NiR should promote the rapid NO release (the purpose of an NO synthase enzyme).

In the $\{FeNO\}^6$ complex, the NO is already formed, and it is "waiting" to be released from the active site. Initially, due to the very slow dissociation of ferrous−NO complexes in haemic proteins (see, e.g., the discussion about the haemic proteins in section 2.2, and also refs 254−268,1459), the Cd_1NiR Fe^{2+}−NO^+ complex (Figure 25e) was considered to be a "dead-end" product. For that reason, the NO was suggested to be released from the Fe^{3+}−$^{\bullet}$NO complex (Figure 25f).[1369,1444,1445,1452,1478] Such ferric complex is formed by intramolecular iron oxidation (equivalent to eq 29), with the transfer of one electron from the Fe^{2+} into the bound NO (Figure 25e→f, "valence isomerization"); the NO π-antibonding orbital would readily accommodate this electron. Theoretical calculations[1479] showed that the low-spin Fe^{3+}−$^{\bullet}$NO state exists as a stable energy minimum, located just 1−3 kcal/mol above the Fe^{2+}−NO^+ ground state and with a Fe−N bond only 0.05−0.1 Å longer than the Fe^{2+}−NO^+ one. As a result, a small elongation of the Fe−N bond, caused, for example, by an interaction of the protein with the bound NO, would trigger the critical electron transfer from Fe^{2+} into NO^+. The ferric complex thus formed would have weaker Fe−NO and N−O bonds, due to the reduction of back-bonding and to the electron transfer into a π-antibonding.[1479] Further elongation of the Fe−N bond (0.2 Å) would lead to a high-spin Fe^{3+}−$^{\bullet}$NO state, further lowering the thermodynamic stability of the Fe−N bond (10 to 4 kcal/mol).[1479] Accordingly, once the system has entered the ferric high-spin state, the dissociative nature of this potential energy surface would force the NO dissociation, providing an explanation for the observed higher dissociation rate constants of NO in ferric comparatively to ferrous complexes.[1479]

Although reasonable, the initial proposal of NO release from the Fe^{3+}−$^{\bullet}$NO complex (Figure 25f) was never proven. In fact, several studies failed to show kinetically competent NO dissociation from the ferric complex.[1465,1478] In addition, experimental evidence suggested that the last step of NO formation should be different: (i) nitrite reduction is not inhibited after preincubation of reduced enzyme with a large excess of added NO, showing that the fully reduced enzyme−NO complex is not a "dead-end" species;[1459,1480,1481] (ii) the NO release is triggered by the intramolecular *c* to d_1 haem electron transfer;[1480,1482] (iii) the presence of the electron donor (that transfers electrons to the *c* haem) must contribute to effecting NO release;[1456,1478,1483,1484] and (iv) the Cd_1NiR mutant (*P. pantotrophus* Met_{106}His mutant) with the *c* haem "locked" in the oxidized state (with a reduction potential of −60 mV[1465]) and reduced d_1 haem, is able to reduce nitrite to NO, but unable to release the NO from the ferric d_1 complex thus formed.[1465] Together, these data indicate that (i) the iron−NO complex has to be first reduced, with one electron transferred from the *c* haem (Figure 25f→g), and that (ii) NO is released from the ferrous $\{FeNO\}^7$ complex thus formed. However, this new via of NO release is, once more, contrary to the well-known properties of the ferrous−NO complexes (see above).

After much debate about the iron−NO complexes dissociation, this impasse was recently overcome with the demonstration that NO can be rapidly released from the ferrous d_1 haem: k_{off} of 200 and 65 s^{-1}, from *P. pantotrophus* Cd_1NiR,[1481] and of 35 and 6 s^{-1}, from *P. aeruginosa* Cd_1NiR[1459,1477] (biphasic dissociations in both cases), values that compare with $k_{off} \approx 10^{-5}$−10^{-3} s^{-1} from Mb and Hb or guanylate cyclase.[163,255,261] Moreover, and most important, these $(d_1)Fe^{2+}$−NO dissociation rates are sufficiently high to be catalytically relevant, with the NO dissociation being the probable rate-limiting step in *P. pantotrophus* Cd_1NiR ($k_{off} \approx k_{cat}$ = 72 s^{-1}[1456]) and, possibly, in *P. aeruginosa* Cd_1NiR ($k_{off} \approx k_{cat} \approx$ intramolecular electron transfer from *c* to $d_1 \approx$ 3−6 s^{-1}).[1445,1452,1456,1459,1482,1485] The agreement between the catalytic kinetic parameters and the rates of $(d_1)Fe^{2+}$−NO dissociation strongly supports the NO release from the ferrous $\{FeNO\}^7$ complex during the catalytic cycle.[1459,1474,1481,1486] The present data, however, do not allow one to discriminate if the NO is released from the "mixed-valence" protein, $(d_1)Fe^{2+}$−$(c)Fe^{3+}$ (Figure 25g→i), or from the fully reduced enzyme, $(d_1)Fe^{2+}$−$(c)Fe^{2+}$ (Figure 25g→h→c).[1459,1481]

Once the ferrous $\{FeNO\}^7$ complex is formed, a new nitrite molecule is able to react with the enzyme at a rate limited by the NO dissociation, showing that the high affinity of ferrous d_1 haem for nitrite actively contributes to the NO dissociation during the catalytic cycle.[1477] Thus, after nitrite displacement of the bound NO, the reduced d_1 haem can immediately start a new catalytic cycle, not being inhibited between (Figure 25g→h→c or g→i→c).

Interestingly, the release of NO from a ferrous $\{FeNO\}^7$ complex can rationalize why an enzyme that catalyzes a one-electron reduction has two one-electron redox centers: nitrite reduction oxidizes the d_1 haem, and the *c* haem is needed to rapidly rereduce the d_1 haem to trigger the NO release.

The surprising rapid NO dissociation from a $(d_1)Fe^{2+}$−NO complex is largely controlled by the unique features of the d_1 haem structure. An elegant and definitive demonstration of the relevance of haem was the observation that apomyoglobin (that has a different binding pocket), when reconstituted with d_1 haem, releases NO at a rate 4 orders of magnitude greater than the native *b* haem-containing Mb (2 s^{-1}[1481] versus 1,2 × 10^{-4} s^{-1}[255,256,259,260]). The mechanism by which the d_1 haem achieves this fast NO release is, presently, not well understood, but two of the d_1 features should be important. First is the peculiar ordering of the *d* orbitals energy levels of the d_1 haem iron: $(d_{xz},d_{yz})^4(d_{xy})^1$, instead of the common $(d_{xy})^2(d_{xz},d_{yz})^3$.[1449,1487] The fact that the d_1 iron HOMO lies

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in the haem plane, instead of lying above and below the haem plane (d_{xz},d_{yz}), would not favor its interaction with the NO SOMO/LUMO (π-antibonding), and thus the NO would be less strongly bound to the d_1 than to a *b* type haem.[1449] Second, the presence of two electron-withdrawing carbonyl groups in the d_1 structure (Figure 19a) would weaken the electron donation of the iron orbitals to the NO nitrogen atom, contributing to weaken the Fe−N bond.[1481] Future spectroscopic and theoretical studies will undoubtedly contribute to a better understanding of the properties of this unique haem ring.

In addition to the unique properties given by the singular d_1 haem structure, the Cd_1NiR catalysis seems to be also dependent on deliberate conformational changes, allosteric control of catalysis. The remarkable Cd_1NiR conformational changes, driven by oxidation/reduction and/or protonation/deprotonation (described under "Enzymatic Machinery"), can, in fact, represent an additional mechanism to control the catalysis, and they were recently evoked to explain the negative cooperativity in the intramolecular electron transfer:[1485] this decrease in the intramolecular electron transfer rate as the level of enzyme reduction increases may, in principle, arise from a decrease in the electronic coupling between the *c* and d_1 haems, which, in turn, may be caused by conformational changes. Accordingly, the relocation of Tyr_{10}, displacement of the 56−62 loop, and the large rotation/sliding of the *c* haem domain would disrupt the hydrogen-bond network, thus decreasing the electronic coupling between the *c* and d_1 haems.[1485] In the same way, the decreased intramolecular electron transfer rate as the pH is increased toward basic values (5.8 to 8.0)[1488] could be attributed to conformational changes.

Yet the allosteric control may have other implications. It is tempting to speculate that the *c* and d_1 domains are acting as "rigid bodies", linked by flexible loops that propagate the changes in the oxidation state and protonation state occurring in one haem pocket to the other one. In this "modular" structure, it can be speculated that the *c* haem oxidation/d_1 haem reduction (Figure 25f→g) and/or the deprotonation of the conserved histidines (Figure 25c→e) would initiate a conformational alteration that modifies the position of the residues involved in the stabilization of the (d_1)Fe−NO complex and/or affects the d_1 affinity for ligands, facilitating, in this way, the NO release. After NO release, the enzyme would have to undergo another conformational change, to return to the "initial" conformation that would favor nitrite binding. This step could be triggered by the reduction of the *c* haem (by the external electron donor) or by the reprotonation of the histidine residues. Obviously, future work will be needed to evaluate the possibility of these conformational changes being part of the catalytic mechanism.

To finish this discussion, it should be mentioned that a dynamic and cooperative network of bonds (not controlled by the conformational changes) was also proposed to be responsible for the strong nitrite binding and fast NO release.[1474] A combination of high field electron−nuclear double resonance techniques (to detect the hydrogen bonds) and density function theory calculations (to correlate the experimental results with the structure)[1474] puts forward the following hypothesis: (i) in the beginning of the catalytic cycle, the positively charged histidine residues "attract" the nitrite, hold it in place, and donate the necessary protons to do oxygen atom abstraction; (ii) at the same time, the Tyr_{10} establishes a hydrogen bond with the nitrite nitrogen atom; and (iii) after the iron−NO complex formation, the now deprotonated His_{369} forms a hydrogen bond with the Tyr_{10}, removing it from the proximity of the still bound NO, that is, preventing it from forming a hydrogen bond with the NO and, in this way, facilitating the NO release. Alternatively, if the His_{369} is rapidly reprotonated and forms a hydrogen bond with the NO oxygen atom, it was proposed that the simultaneous hydrogen bond between the tyrosine and the NO nitrogen atom ((Tyr)O−H···N(O)−Fe) would populate the (partial sp^2) nitrogen nonbonding orbital, weakening the Fe−N bond, and, therefore, facilitating the NO release.[1474] With this proposal, the nitrite reduction is facilitated by a dynamic and cooperative network of hydrogen bonds that, first, keeps the nitrite in the haem and then, after the oxygen abstraction step, changes and no longer holds the NO in the active site.[1489] The role ascribed to His_{369} has been difficult to prove. The fast NO dissociation displayed by the His_{369}Ala mutant (only 2 times slower than the wild-type[1459]) seems to indicate that this residue is not involved in the NO release. However, in the mutant, the Tyr_{10} is displaced such that it cannot form a hydrogen bond with the NO nitrogen atom.[1474] Also, the role of the tyrosine residue has been criticized, because the Tyr_{10}Phe mutant is catalytically active.[1470,1471] Nevertheless, it can be argued that, in the absence of Tyr_{10}, its role can be played by His_{327}.[1474] A more definite assignment of the tyrosine role must wait for measurement of the rate of NO release from the Tyr_{10}Phe mutant, or from *Pseudomonas stutzeri* Cd_1NiR, which does not have an equivalent tyrosine.

In summary, as presently suggested, the catalytic cycle of Cd_1NiR can be considered as a "copy" of the first part of the CcNiR reaction, until the $\{FeNO\}^6$ complex is formed (Figure 25b→e versus Figure 22b→f). Once this complex is formed, the CcNiR keeps the NO bound to be further reduced by two electrons, yielding an $\{FeNO\}^8$ complex. The Cd_1NiR, on the other hand, reduces that complex by only one electron (to $\{FeNO\}^7$), and, after that, the rapid NO dissociation is promoted (Figure 25f→g→h). The dissociation mechanism has to be fast, to compete with the hypothetical reduction by a second electron, and efficient, to effectively promote the NO release. The nature of this mechanism is the most interesting aspect of the Cd_1NiR-catalyzed reaction: what happens after the electron transfer from *c* to d_1 haem that triggers the fast NO release? Undoubtedly, the unique d_1 haem has a leading role in controlling the Cd_1NiR reactivity with NO. Its exceptionally high NO dissociation rate, in conjunction with the probable allosteric control of catalysis and the probable dynamic network of hydrogen bonds, act synergistically to efficiently synthesize and release NO.

Cd_1NiR uses the same basic machinery, haem, as CcNiR and CSNIR, but "tuned" by a different ring structure to yield a different product, NO. The d_1 haem evolved specifically to form and release NO: (i) it is present only in the Cd_1NiR enzyme,[1490] where it is "tailored" to meet the challenging mechanistic requirements of NO formation and release, and (ii) this "tailored" structure required the development of huge and specific machinery to be synthesized (d_1 synthesis requires a substantial biological effort, met by the *nirECFD-LGHJN* genes products[1491]). The evolutionary success of the d_1 haem structure is put in evidence by the presence of the Cd_1NiR enzyme in so many organisms still today.

Despite all of the successful efforts to create the d_1 haem, and due to the importance of the nitrite reduction/NO formation, biology made other "experiments" that culminate with the

development of a different, de novo, invention of the oxic era: the CuNiR.

3.2.2. Dissimilatory Nitrite Reduction to Nitric Oxide by a Copper-Dependent Enzyme. The dissimilatory copper-dependent nitrite reduction to NO (eq 72) is achieved with the copper center of the copper-containing nitrite reductase (CuNiR) enzymes (EC 1.7.2.1).

$$NO_2^- + 1e^- + 2H^+ \xrightarrow{\text{Cu centre}} {}^{\bullet}NO + H_2O \qquad (72)$$

$$E^{o\prime}(NO_2^-/{}^{\bullet}NO) = +0.34\ V$$

3.2.2.1. Enzymatic Machinery. CuNiR (product of *nirK* genes) are periplasmatic homotrimers (~110 kDa), with each monomer folded into two eight-stranded β-barrel domains, called I and II (*Alcaligenes faecalis*,[1492−1495] *Achromobacter cycloclastes* (Figure 26),[1496−1498] *Alcaligenes xylosoxidans*,[1499−1505] *Rhodobacter sphaeroides*[1506]). The three monomers are tightly associated around a central channel of 5−6 Å, with domains I positioned at the corners of the trimer and domains II forming the core of the molecule. An extensive network of hydrogen bonds (within and between monomers) maintains the rigidity of these complex structures, where ~1/3 of the monomer surface has to be used in the trimer formation.[1497]

The CuNiR enzymes contain two different copper centers per monomer, one T1 and one T2 (see section 3; Figure 19b): the T1 center is responsible for the electron transfer from the physiological partners to the T2 center, which constitutes the enzyme active site. Each T1 center is located within domain I of each monomer, while the T2 is found bound in a cleft formed by apposition of domain II of one monomer and domain I of the adjacent monomer (Figure 26a,b). The T1 center copper is

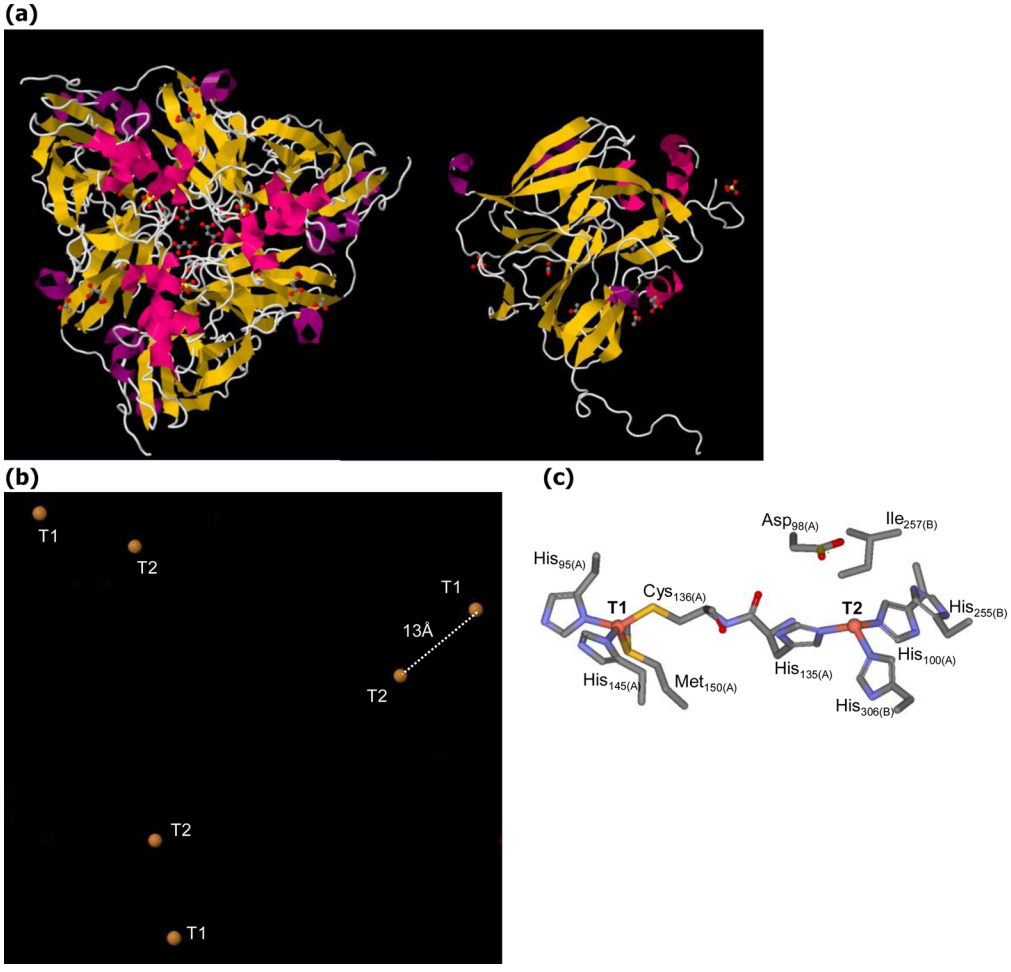

Figure 26. Copper-containing nitrite reductase. (a) Three-dimensional structure view of the *Achromobacter cycloclastes* CuNiR homotrimer and monomer (on the left and right, respectively; α helices and β sheets are shown in pink and yellow, respectively). Each monomer folds into two eight-stranded β-barrel domains (domains I and II), stacked on each other, with one long and two short α-helical regions. The three monomers are tightly associated around a central channel of 5−6 Å, with domains I positioned at the corners of the trimer and domains II forming the core of the molecule. Each monomer contains two different copper centers, T1 and T2. The T1 center is located within domain I, buried 7 Å beneath the protein surface. The T2 is found bound at the interface of adjacent monomers, in a cleft formed by apposition of domain II of one monomer and domain I of the adjacent monomer, at the bottom of a ~12 Å deep solvent channel. (b) Copper centers arrangement in the same orientation as in the homotrimer in (a); the three T1 and three T2 centers are identified. The distance (T1)Cu-to-(T2)Cu of 12.5 Å is indicated in one of the three "catalytic pairs". (c) T1 and T2 copper centers. The T2 copper center (the active site) is coordinated by three histidines: His_{100} and His_{135}, from domain I of monomer "A", and His_{306}, from domain II of the adjacent monomer "B". The structures shown in (a) and (b) are based on the PDB file 2BW4[1498] (the images were obtained from www.rcsb.org/pdb/explore/jmol); the structure shown in (c) is based on the PDB file 1NIA[1497] (the image was produced with Accelrys DS Visualizer, Accelrys Software Inc.).

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coordinated by two histidine (His_{95} and His_{145}) and one cysteine (Cys_{136}) residues, in a distorted trigonal planar geometry, and by one methionine residue (Met_{150}) forming a weaker interaction in an axial position (*A. cycloclastes* numbering (Figure 26c)). The T2 center copper (the active site) is coordinated by three histidines (His_{100}, His_{135}, and His_{306}) and by a water molecule, in a distorted tetrahedral geometry (Figure 26c). The two centers are connected through the (T1)Cys_{136}−(T2)His_{135} bond, which enables a path for rapid electron transfer across the ∼12.5 Å that separates the copper atoms.[1492,1504,1507,1508] The CuNiR active site also comprises conserved aspartate, histidine, and isoleucine residues (Asp_{98}, His_{255}, and Ile_{257}, the last two provided by the domain II of the adjacent monomer, Figure 26c) that are essential for catalysis.[1495,1503,1504,1509,1510]

Historically, CuNiR were classified into two subgroups, depending on their color being blue or green in the as-isolated oxidized state (e.g., the *A. xylosoxidans* blue CuNiR and the *A. faecalis*, *A. cycloclastes*, or *Sinorhizobium meliloti* green CuNiR[1511]). The color of the enzyme is given by the T1 center: while the methioine sulfur ligand of the *A. xylosoxidans* blue enzyme deviates only slightly from the axial position, that of the *A. faecalis* green enzyme is in a considerably tilted position (relatively to the His-His-Cys plane), resulting in a distorted tetrahedral and flattened tetrahedral geometries, respectively.[1500] The perturbed geometry of the green enzymes modifies the copper−ligands interactions, resulting in the redistribution of absorption intensity in the charge transfer and ligand field transitions:[1512] the absorption at ∼600 nm decreases, the intensity of the absorption envelope at ∼450 nm increases, and the EPR signal change from axial to rhombic symmetry. Besides the small differences in the geometry of the T1 center, green and blue CuNiR show a marked difference in the overall surface charge distribution. This is probably responsible for the different specificity toward the redox partner, pseudoazurin for green CuNiR and azurin for blue CuNiR (thus supporting the electron transfer via protein−protein complex formation).[1497,1500,1513,1514]

More recently, the resolution of the structure of a CuNiR isolated from the *Hyphomicrobium denitrificans*[1515] led to the definition of a novel family of CuNiR, characterized by an homohexameric structure containing an additional T1 copper center per monomer.[1516] The *H. denitrificans* CuNiR monomer is folded into three domains: one N-terminal β-barrel domain (∼15 kDa), containing one T1 copper center, and two C-terminal domains (∼35 kDa), containing the T1 and T2 centers in the characteristic organization of "classic" trimeric CuNiR. The N-terminal and C-terminal T1 copper centers are blue and green, respectively, given the enzyme its greenish-blue color in the oxidized form.[1516−1519] A genome analysis points toward the wide occurrence of this new N-terminal extended CuNiR, which probably exists also in two distinct subclasses as the "classic" family, that is, blue and green enzymes.[1520] The *H. denitrificans* CuNiR molecule is a dimer of trimers (a trigonal prism-shaped hexameric structure) containing 12 T1 and 6 T2 copper centers, for a total of 18 copper atoms per molecule.[1515] However, and surprisingly, the additional copper center was found to be too far away (>24 Å) from the catalytic core to effectively participate in electron transfer.[1515,1521] Nevertheless, that distance can be shortened to an appropriate value for electron transfer by a simple rotation of the "extra" domain, a movement that could take place, hypothetically, when the redox partner protein binds.[1521,1522]

In addition to these N-terminal extended CuNiR, genome analysis identified the occurrence of another type of CuNiR with a C-terminal extension containing a *c* type haem.[1520,1523] In accordance, the *Ralstonia pickettii* enzyme was shown to be a trimeric protein (∼50 kDa), with each monomer holding, besides the two copper centers, a single *c* haem.[1521,1524] The enzyme folds as the "classic" (only copper-containing) CuNiR, with the additional haem domain of one monomer localized in close proximity to the T1 center of the adjacent monomer, with a copper-to-haem edge distance of 10.1 Å (an electron transfer compatible distance).[1521] The observed folding clearly defines an electron transfer path between the haem domain and the catalytic core, supporting the functional importance of the haem during turnover.[1521] In this context, the new *c* haem-copper-containing enzyme can be thought of as a "fused version" of the electron transfer complex of a "classic" CuNiR with cytochrome c_{551}, whose structure was recently elucidated.[68] In this "fused version", the enzyme may have captured the partner gene to construct an effective self-sufficient electron transfer system, where the additional domain acts as a physiological electron donor: electron donor and acceptor proteins fused together by genomic acquisition for functional advantage.[1521]

The enzyme from *Pseudoalteromonas haloplanktis* constitutes a different example of a "fused" *c* haem-copper-containing nitrite reductase. This protein folds as a unique trimeric "domain-swapped" structure, with the haem domain localized at the surface of the T1 center from the adjacent monomer at a haem-to-copper distance of 10.6 Å.[1522] Therefore, the conformation of the *P. haloplanktis* enzyme shows a self-sufficient electron transfer system. However, the structural aspects of the domain−domain interface and the electron transfer kinetics indicate that the haem−copper domain interaction should be highly transient, that is, similar to the interaction of two noncovalently bound proteins in an electron transfer complex.[1522] In addition, and as mentioned above for the additional copper center of the *H. denitrificans* enzyme, the haem does not seem to be involved in the electron transfer from the cognate redox partner protein to the catalytic core.[1522] Clearly, there is still much to be learned about the biological function of the "extra redox domains" of the extended CuNiR enzymes. The occurrence of several types of "extensions" supports that they are of biological importance; otherwise, why were not the additional domains evolutionally split or lost?[1522]

Another example of how the "classic" CuNiR structure can be transformed is provided by the *Neisseria gonorrheae* enzyme: its structure revealed a C-terminal extension that is glycosylated, as well as the deletion of specific surface loops and the inclusion of a lipid modification site in a N-terminal extension (this enzyme is the major anaerobically induced outer membrane lipoprotein).[1525,1526]

The "classic" and "new" N- and C-terminal extended CuNiR enzymes constitute, thus, fascinating examples of how biology once had found a "solution", reuses it recurrently, introducing only minor adjustments to respond to specific cellular demands.

3.2.2.2. Promiscuity. In addition to the nitrite reductase, CuNiR has significant superoxide dismutase activity (∼56% of the bovine enzyme activity)[1500,1502] and is also able to catalyze the reduction of dioxygen to hydrogen peroxide (being inactivated in the process).[1527]

3.2.2.3. Mechanism. Although it was initially thought that the CuNiR reaction mechanism would be a "copy" of the Cd_1NiR one,[1369] the most recent results point toward a

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different strategy. One of the key differences between CuNiR and Cd_1NiR is the nitrite binding mode: nitrite binds to the CuNiR T2 center, either oxidized or reduced, in an asymmetric "bidentate nitrito" mode (Figure 20a). Several crystal structures and theoretical calculations[1494,1497,1498,1510,1528−1534] show that nitrite is bound through its two oxygen atoms, in a distorted square pyramidal geometry, with the two oxygen atoms of nitrite plus the two nitrogen atoms of the histidine residues in the distorted base; the longest Cu−O bond is with the oxygen atom closest to the conserved aspartate residue (Figure 27c).

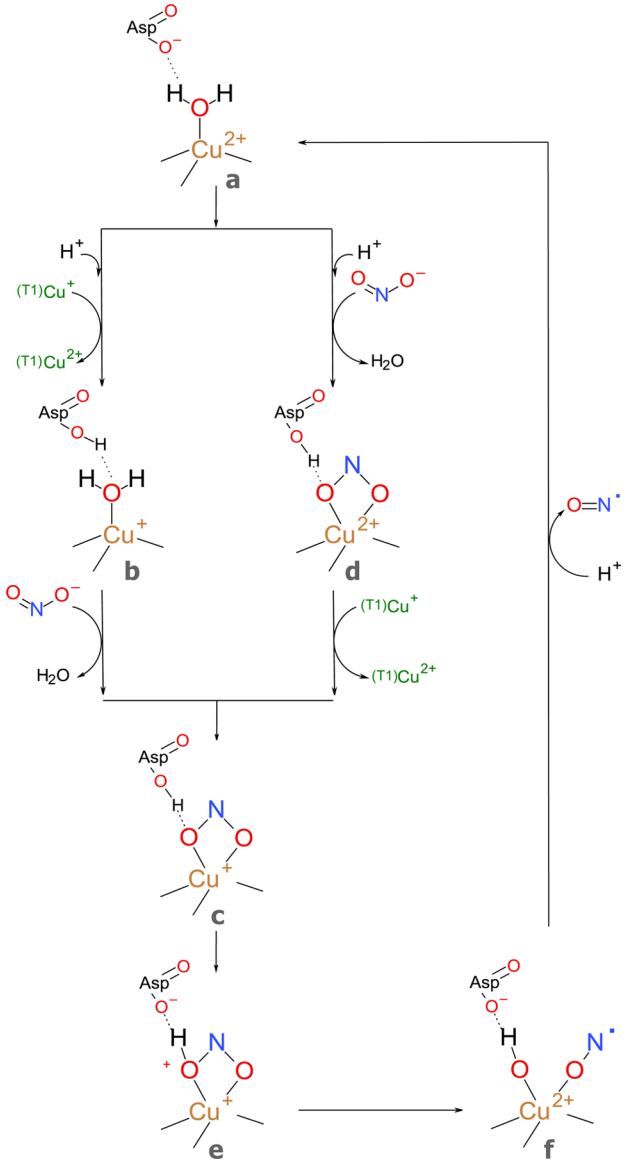

Figure 27. Mechanism of nitrite reduction to NO catalyzed by copper-containing nitrite reductase. See text for details.

The observation of the "bidentate nitrito" binding mode was unpredicted, because $Cu^+-NO_2^-$ model complexes bind nitrite, generally, through the nitrogen atom[1535−1538] (exceptions in, e.g., refs 1539−1541). Nevertheless, cupric copper compounds are known that bind nitrite through the three binding modes ("nitro",[1542,1543] "nitrito",[1543−1545] and "bidentate nitrito"[1543,1546−1552]). Theoretical calculations[1552] showed that the three possibilities are energetically close, with "bidentate nitrito" and "nitrito" displaying the lowest total energy (just 0.1 kcal/mol apart), but only 5.5 kcal/mol lower than the "nitro" mode. Consequently, the CuNiR nitrite binding mode should be directed by the conserved active site amino acid residues, the aspartate and histidine residues, but also by the hydrophobic isoleucine residue present on top of the nitrite binding site.[1494,1495,1498,1510,1534,1553,1554]

The mechanism of nitrite reduction to NO by CuNiR is less well characterized than the Cd_1NiR one, with several details not known yet.[1555] To begin, there is a debate about which specie binds the nitrite: the oxidized or the reduced T2 center? Both hypotheses are supported by several crystallographic and kinetic studies.[1369,1494,1495,1497,1502,1533,1534,1556−1569] In solution, CuNiR follows a random sequential steady-state mechanism, with two alternative routes: (i) T2 center reduction followed by nitrite binding (Figure 27a→b→c) or (ii) nitrite binding to the oxidized T2 followed by T2 reduction (Figure 27a→d→c).[1559,1562,1567,1569] The prevailing route is determined by the nitrite concentration and pH (e.g., the second route prevails at high nitrite concentration or high pH, when the T1 to T2 electron transfer is the rate-limiting step). Moreover, the kinetic studies suggest that the reduced T2 center can exist in two interconvertible forms, where only one form is catalytically active. Although the inconsistent results may be ascribed to different assay conditions and/or to the presence of CuNiR molecules with inactive reduced T2 centers, a definitive consensus is far from being established, and both routes are here considered (i.e., a random sequential mechanism). Accordingly, in Figure 27, the beginning of the catalytic cycle is depicted with the T2 center reduction, followed by nitrite binding (Figure 27a→b→c) and with the nitrite binding to the oxidized T2 center, after which an electron is transferred from the T1 center (Figure 27a→d→c).

Several elegant combinations of experimental and theoretical studies confirmed that the T2 center reduction is triggered by a protonation step (Figure 27a→b or d→c).[1499,1508,1533,1558,1566,1570−1574] Theoretical calculations[1531,1533] suggested that it is protonation of the conserved aspartate residue that increases (electrostatically) the reduction potential of the T2 center, and thus drives the electron transfer from T1 to T2 and eventually the nitrite reduction. In addition, nitrite binding has also been suggested to induce a favorable shift in the reduction potential of the T2 center.[1534] Therefore, the nitrite-bound reduced T2 center, with a neighboring protonated aspartate residue (Figure 27c), is suggested to be the key complex that initiates the nitrite reduction,[1533] regardless of the order of the events that leads to its formation.

Once the square pyramidal $(T2)Cu^+-O(N)O$ complex (Figure 27c) is formed, the reaction is believed to proceed with the protonation of the oxygen atom to be abstracted. Steady-state kinetic studies show the existence of two protonation equilibriums that were attributed to the only two ionizable conserved residues present in the T2 center, the aspartate (to which is attributed a pK_a of ∼5) and histidine residues (pK_a ∼7).[1495,1498,1503,1508,1528,1553,1557,1558,1575,1576] Both residues were found to be essential for the CuNiR activity,[1495,1503,1509,1528,1553,1558,1572] but theoretical calculations suggested that the proton is transferred from the protonated aspartate residue[1533] (Figure 27c→e). The subsequent N−OH bond cleavage is thought to be facilitated by an emergent back-bonding interaction between the copper and the nitrite oxygen atoms:[1533] (i) on the initial nitrite−copper complex (Figure 27e) there is no back-bonding;[1577] (ii) along the reaction coordinate, as the N−OH bond is elongated, its σ-antibonding

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orbital is lowered in energy and becomes mixed with the copper HOMO, the d_{x2-y2}, receiving electron density from the copper. This back-bonding is made possible by the "bidentate nitrito" binding mode of nitrite, which allows for an efficient interaction between the copper and the nitrite oxygen atoms.[1533] The back-bonding interaction would strengthen the Cu−OH bond and weaken the N−OH bond, leading to the N−O bond homolysis and, eventually, to the NO dissociation from the copper complex (Figure 27e→a).[1533]

Unfortunately, the details of the NO release from the copper complex are still elusive. The catalytic cycle would continue with the addition of a second proton, probably transferred from the histidine residue,[1495,1503,1509,1528,1553,1558,1572] to yield a bound water molecule (Figure 27a), but the order of the protonation/NO release was not yet definitively established.

Nevertheless, the side-on Cu^+−NO complex observed in some crystal structures[1498,1530,1532] does not seem to be part of the catalytic cycle. In-depth spectroscopic characterization coupled with theoretical calculations[1578] demonstrated that, in solution, the complex formed by reacting reduced CuNiR either with nitrite[1579] or with exogenous NO[1561,1579] is in a strongly bent (~135°) end-on conformation, but likely not in a side-on conformation. Actually, additional theoretical calculations showed that the side-on binding mode corresponds only to a local energy minimum, the global minimum being attained with the end-on conformation (localized 8.4 kcal/mol below the former).[1580] Moreover, the side-on conformation is thought to be largely due to steric interactions with Ile_{257}, which were suggested to destabilize the end-on species.[1580] Accordingly, in solution, a small conformational change of the active site would modify the position of Ile_{257} and would allow the formation of the end-on binding mode. On the other hand, in a crystal, where the orientation of the amino acid residues must be strongly restricted, the repositioning of the Ile_{257} would not be feasible, and the side-on conformation would be formed. Therefore, the existence of the side-on Cu^+−NO complex and its involvement in the catalytic cycle is subject to criticism.

In summary, the key steps for the CuNiR-catalyzed NO formation are the proton transfer from the aspartate residue to the nitrite molecule, followed by the electron transfer from the reduced copper to the now protonated nitrite, that is, proton transfer triggering electron transfer.[1533] According to the suggested mechanism, the success of the N−O bond lysis by the CuNiR is due to the unusual nitrite binding mode and to the presence of a proton donor amino acid residue:[1533] (i) the "bidentate nitrito" binding mode of nitrite enables an effective back-bonding interaction that lowers the activation barrier for the N−OH bond cleavage and simultaneously stabilizes the Cu−OH complex to be formed; and (ii) the presence of a proton donor residue (the protonated aspartate) is essential for the stabilization of the copper complex that will be formed after the N−O bond cleavage (Cu−OH). It should be noted that, if the N−O bond was cleaved without the proton being transferred, it would result in the formation of a Cu^{2+}−O^- complex, where the charge density is not stabilized (energetically very unfavorable, >50 kcal/mol versus <16 kcal/mol[1533]). In this point, the choice of an aspartate, a residue with a low pK_a value that is not usually associated with proton donation, is intriguing. In the CcNiR, CSNiR, and Cd_1NiR-catalyzed reactions, the proton donors were found to be the "expected" positively charged residues, histidine, arginine, and lysine residues. The strategies to reduce nitrite to NO followed by Cd_1NiR (an old enzyme) and CuNiR (a de novo invention of the oxic era) are thus remarkably different.

3.2.3. Signaling Nitrite Reduction to Nitric Oxide by a Molybdenum-Dependent Enzyme. The mammalian signaling molybdenum-dependent nitrite reduction to NO (eq 73) can be achieved with the molybdenum center of the xanthine oxidase (XO) enzyme (EC 1.17.3.2).

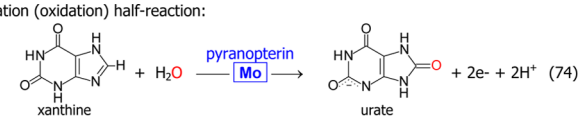
$$NO_2^- + 1e^- + 2H^+ \xrightarrow{\text{pyranopterin Mo}} {}^{\bullet}NO + H_2O \qquad (73)$$

$$E^{o\prime}(NO_2^-/{}^{\bullet}NO) = +0.34\ V$$

As described under "Nitrite in Signaling Pathways", besides mammalian XO, also mammalian AO (section 2.2.1.1.3) and bacterial AOR (section 2.2.3) catalyze the nitrite reduction to NO. Mammalian XO and AO are structurally very similar, both comprising one identical molybdenum center (described below), two Fe/S, and one FAD center. Bacterial AOR holds a slightly different molybdenum center, with the pyranopterin cofactor esterificated with cytidine monophosphate (Figure 19c (i)), and only two Fe/S (no FAD center). *Desulfovibrio gigas* AOR was the first XO family member for which the crystal structure was determined,[1581−1583] with the first mammalian XO and AO structures being reported only in 2000[1584] and in 2012,[1585,1586] respectively. To restrict the information presented to a manageable size, for the sake of simplicity, this Review will focus only on the mammalian XO, the benchmark of this family of molybdoenzymes.

Physiologically, mammalian XO is a key enzyme in purine catabolism, where it catalyzes the hydroxylation of both hypoxanthine and xanthine to the terminal metabolite urate, with the simultaneous reduction of dioxygen to superoxide anion radical and hydrogen peroxide (eq 35).[596−602] In this sense, the XO is quite different from the previously described enzymes: XO has two "classic" substrates, hypoxanthine/xanthine and dioxygen, and two active sites to react with each substrate, molybdenum and FAD centers (eqs 74, 75), respectively.

hydroxylation (oxidation) half-reaction:



(74)

reduction half-reaction:

$$O_2 + 2e^- + 2H^+ \xrightarrow{\text{FAD}} n\,O_2^{\bullet-} + m\,H_2O_2 \qquad (75)$$

3.2.3.1. Enzymatic Machinery. Mammalian XO is a cytoplasmatic (see also section 2.2.1.1.3) homodimer (~290 kDa), with each monomer folded into three domains:[1584,1587] (i) one small (~20 kDa) N-terminal domain, constituted by two subdomains (characterized by α-helical and β-sheet structures), each holding one [2Fe−2S] center (named Fe/S I and II, respectively); (ii) a second domain (~40 kDa), holding one FAD; and (iii) one large (~85 kDa) C-terminal domain, also constituted by two subdomains that bind the molybdenum center at their interface (Figure 28a,b). Although the two monomers contact each other through the molybdenum domain, their molybdenum centers are ~50 Å apart, suggesting that the two monomers act independently.

The molybdenum center, solvent-accessible at the bottom of a 14.5 Å hydrophobic channel, is the XO active site responsible for the hydroxylation half-reaction (eq 74).[1584,1587] It holds the molybdenum atom coordinated in a distorted square pyramidal

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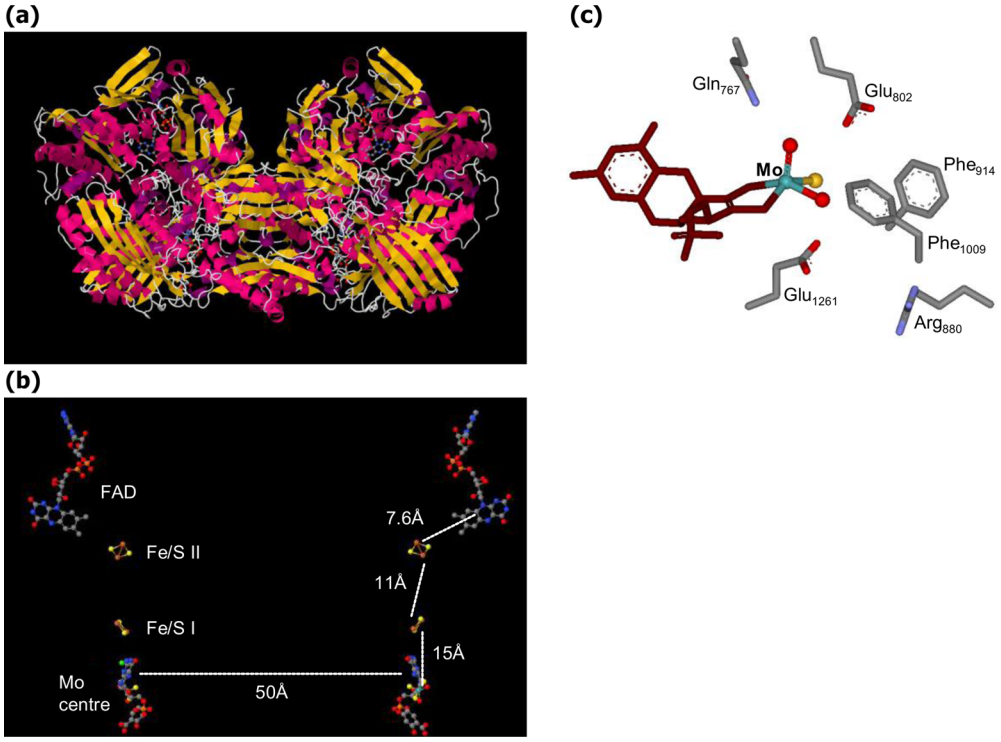

Figure 28. Molybdenum-containing mammalian xanthine oxidase. (a) Three-dimensional structure view of the bovine milk XO homodimer (α helices and β sheets are shown in pink and yellow, respectively). (b) Arrangement of the four redox centers shown in the same orientation as in (a). The four redox centers are identified on the monomer on the left (molybdenum, Fe/SI, Fe/SII, and FAD centers), and the distances between adjacent centers (expressed in Å) are shown on the monomer on the right. (c) Molybdenum catalytic center (pyranopterin cofactor is represented in dark red). The structures shown are based on the PDB file 1FO4[1584] (the images in (a) and (b) were obtained from www.rcsb.org/pdb/explore/jmol; the image in (c) was produced with Accelrys DS Visualizer, Accelrys Software Inc.).

geometry, with an apical oxo (=O) group and with the four equatorial positions occupied by one essential sulfo (=S) group, one labile hydroxo (−OH) group, and two sulfur atoms of the *cis*-dithiolene (−S−C=C−S−) group of the pyranopterin cofactor molecule (Figure 19c; Figure 28c).[1584,1587,1588] This active site also comprises four conserved glutamate (Glu_{802}, Glu_{1261}), glutamine (Gln_{767}), and arginine residues (Arg_{880}, bovine enzyme numbering), essential for the hydroxylation reaction (Figure 28c). In addition, two phenylalanine residues (Phe_{914}, Phe_{1009}) seem to be important to position the substrate (hypoxanthine/xanthine) in front of the equatorial labile Mo−OH group, in a plane parallel to the apical Mo=O group of the molybdenum center (Figure 28c). Closest to the molybdenum center is the Fe/S I, coordinated by the $Cys_{113}XXCys_{116}X_{(n)}Cys_{148}XCys_{150}$ motif (Figure 28b). Further away, in the proximity of the FAD center, the Fe/S II is coordinated by $Cys_{43}XXXXCys_{48}XXCys_{51}X_{(n)}Cys_{73}$, in a folding that resembles that of plant [2Fe−2S] ferredoxins.[1589,1590] At last, FAD, the active site responsible for the reduction half-reaction (eq 75), is bound in an extended conformation in a deep cleft of the FAD domain, with the *si*-side of the isoalloxazine ring accessible to the solvent. On the whole, the four redox centers (molybdenum, Fe/S I, Fe/S II, and FAD centers) are aligned in an almost linear fashion (Figure 28b), defining an intramolecular electron transfer pathway that rapidly delivers the electrons from the molybdenum center (where the hydroxylation takes place, eq 74) to the FAD (to reduce dioxygen, eq 75).

In the context of the XO enzymatic machinery, it is pertinent to mention here the structural changes responsible for the interconversion of XD into XO.

The mammalian XO enzymes are synthesized as a NAD^+-dependent dehydrogenase form, the XD, and, under normal physiological conditions, exist mostly as such in the cell.[596−602] However, the XD form can be readily converted into a "strict" oxidase form, the XO.[1591] The distinction between XD and XO is based on the electron acceptor used by each form: while XD transfers the electrons preferentially to NAD^+, XO fails to react with NAD^+ and uses exclusively dioxygen (as the oxidase classification indicates).[1603−1607,1613,1614] At this point, it should be mentioned that both dioxygen and NAD^+ react at the FAD center. That is, the electrons introduced at the molybdenum center, during xanthine hydroxylation, are transferred to the FAD in both enzyme forms; once in the FAD center, the electrons react with dioxygen or NAD^+, depending on the affinity of each enzyme form.

The dehydrogenase into oxidase conversion can be reversible, through oxidation of the Cys_{535} and Cys_{992}, or irreversible, by proteolysis after Lys_{551} or Lys_{569}.[600,1584,1602−1609,1611,1613,1615−1619] The substrate "switching", dioxygen versus NAD^+, is achieved by the following mechanism:[600,1584,1619] the disulfide bond formation or the proteolysis drive a movement of the 423−433 loop, on the *si*-face of the FAD isoalloxazine ring, that shifts the Asp_{429} away and bring the Arg_{426} guanidinium group closer. In this way, the loop displacement changes the electrostatic potential in the vicinity of the FAD (from negatively to positively charged), increasing the FAD midpoint potential. In addition, the novel

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loop position obstructs the access to the FAD binding site, blocking the NAD^+ binding to FAD. In concert, these conformational changes, that occur only at the FAD center, are responsible for the different substrate specificity of XD and XO. The fact that the global fold of XD and XO is not significantly changed at the Fe/S and molybdenum centers[600,1584,1619] is consistent with the kinetic studies that demonstrate that the two enzyme forms are virtually identical with respect to the binding and catalysis of substrates at the molybdenum center.[596−602] This is also the case of the nitrite reduction reaction (that, as will be described, occurs at the molybdenum center).

The concerted conformational changes responsible for the dehydrogenase into oxidase conversion suggest that the conversion could play a role in vivo, in a situation similar to that discussed for Nb, Cc, or Cd_1NiR. Indeed, the dehydrogenase into oxidase conversion could be the basis of several cellular regulatory strategies: (i) Proteolysis, triggered, for example, by an hypoxic event, would contribute to the formation of XO, an enzyme form that favors the nitrite reduction/NO formation, instead of the NAD^+ reduction by XD, as discussed in section 2.2.1.1.3. (ii) Under oxidative stress conditions, for example, during the reperfusion phase, the decreased concentration of reduced thiols would increase the population of disulfide-containing XO molecules; the ROS formed by this enzyme form would then be responsible for signaling cascades (see, e.g., refs 1620,1621). The same mechanism would contribute to some ROS-mediated diseases,[639−647] including ischaemia-reperfusion injury[648−653,1622−1625] and ethanol toxicity,[581,654−659] in situations where the cellular antioxidants could not cope with the overproduction of ROS (accounting, in this way, for the well accepted XO pathological role). Therefore, XD/XO could be another protein type with posttranslational allosteric control of catalysis.

3.2.3.2. Promiscuity. To no other of the enzymes here described does the term promiscuity make more sense: XO has a "broad specificity" for both reducing and oxidizing substrates. Besides the well-known hydroxylation of hypoxanthine and xanthine, XO catalyzes the oxidation of a wide variety of aldehydes and substituted pyridines, purines, pteridines, and related compounds.[578−584,596−602,620,621,1626,1627] XO can also catalyze the reduction of several compounds apart from dioxygen (or NAD^+ by XD), including several sulfoxides, *N*-oxides, and nitrate and nitrite.[622−634] This promiscuity has suggested the XO involvement in xenobiotic metabolism.[1628]

3.2.3.3. Mechanism − Xanthine Hydroxylation. Before discussing the nitrite reduction mechanism, it is useful to briefly review the xanthine hydroxylation reaction mechanism (eq 74).

In general, the molybdoenzymes (of all families) catalyze the transfer of an oxygen atom from water to product or from substrate to water, in reactions that imply a net exchange of two electrons and in which the molybdenum cycles between Mo^{6+} and Mo^{4+}.[596−602] It is based on this catalytic feature that these enzymes are commonly referred to as oxo-transferases. Most of the enzymes of the XO family, and XO in particular, catalyze the cleavage of a C−H bond, with the subsequent oxygen atom insertion in a novel C−O bond, in reactions of oxidative hydroxylation. Yet there are exceptions, like the hydroxybenzoyl-CoA reductase, that catalyzes the irreversible dehydroxylation (an oxygen abstraction reaction) of the phenol ring.

In its reaction with xanthine, the XO molybdenum center catalyzes the insertion of its equatorial oxygen atom into the xanthine molecule to produce urate (Figure 29a→b).[596−602]

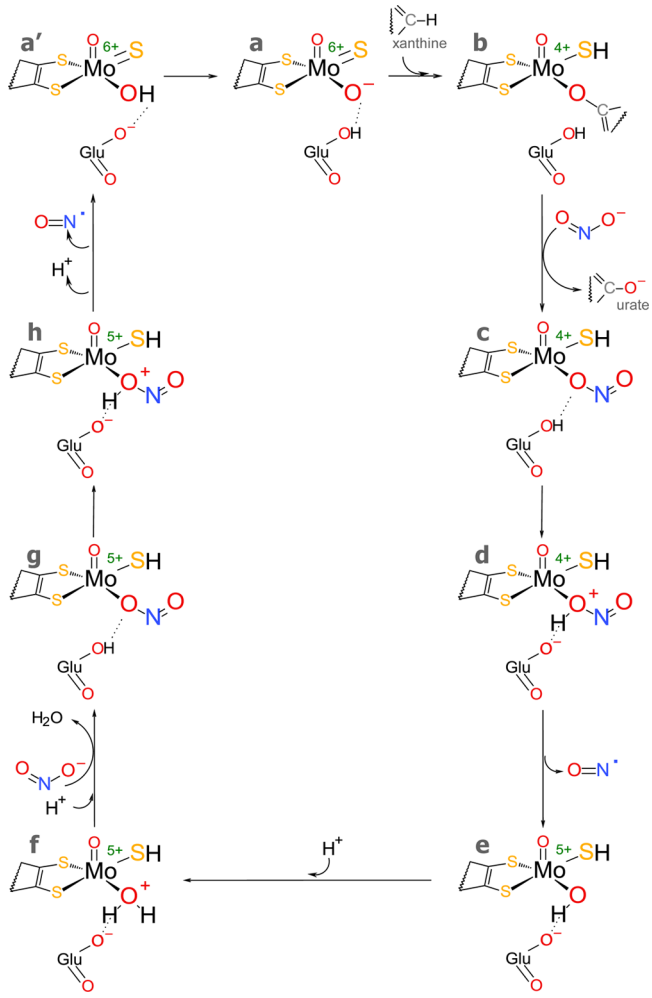

Figure 29. Mechanism of nitrite reduction to NO catalyzed by xanthine oxidase. See text for details.

The two electrons (eq 74) thus introduced into the molybdenum (Mo^{6+}→Mo^{4+}) are then rapidly distributed throughout the Fe/S and FAD centers, according to their redox potentials. At the FAD center, the electrons are finally transferred to dioxygen (or NAD^+, in XD), to give superoxide anion radical and hydrogen peroxide (or, in XD, NADH). The intramolecular electron transfer (Mo→Fe/S I→Fe/S II→FAD) is, therefore, an integral aspect of the XO catalysis (eqs 74→75). The XO-catalyzed hydroxylation reaction is, in this way, quite different from the monooxygenases reaction, as XO generates (rather than consumes) reducing equivalents and uses dioxygen as an oxidant and not as the source of oxygen atoms, which in the XO case is, ultimately, water.

Before resuming to the nitrite reduction mechanism itself, two remarks should be made: (i) nitrite reduction is virtually identical in both XO and XD,[634] and (ii) electrons obtained from the reducing substrate must reduce nitrite and cannot be deviated to dioxygen, NAD^+, or other electron acceptor. It is because of this last point that the XO-dependent nitrite reduction/NO formation is thought to occur only under hypoxic/anoxic conditions (as described in section 2.2.1.1.3).

3.2.3.4. Mechanism − Nitrite Reduction. To catalyze the nitrite reduction to NO, XO, like Cd_1NiR and CuNiR, has to bind nitrite, transfer one electron, cleave a N−O bond, and release the NO thus formed. However, the mechanism by

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which the XO molybdenum center carries out this reaction still has some lacunae to fill. Nevertheless, an outline of the mechanism can easily be drawn (Figure 29) taking into account the following points.

First, nitrite is believed to be bound through one of its oxygen atoms ("nitrito" binding mode (Figure 20a)). The $Mo^{6+,5+,4+}$ chemistry is dominated by the formation of oxides and sulfides, but the strong tendency of molybdenum to bind oxo groups is balanced by its ability to easily lose a single oxygen atom;[1637] this chemistry makes the molybdenum cores excellent "oxygen atom exchangers", as long as the thermodynamics of the reactions is favorable.[1638] In accordance, substrates or products of XO family enzymes interact with the molybdenum atom through an oxygen atom.[596−602]

Second, nitrite should bind to the reduced molybdenum center. Kinetic and spectroscopic (EPR) studies showed that the NO formation only occurs after molybdenum reduction and suggested a role for nitrite in the displacement of urate from the active site.[634]

Third, the reduced molybdenum transfers one electron to nitrite, being oxidized in this process. Spectroscopic (EPR) assays demonstrated unequivocally that the reduced molybdenum center (enzyme reduced with compounds that interact at the molybdenum, Fe/S, or the FAD centers) is oxidized in the presence of nitrite.[634] Moreover, the simultaneous NO formation showed that nitrite was concomitantly oxidized.

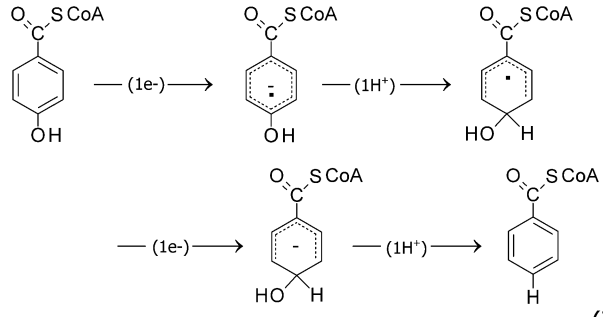
Fourth, the molybdenum center catalyzes the N−O bond cleavage and releases the NO thus formed. That the molybdenum is, in fact, the center responsible for the NO formation was definitively demonstrated with a combination of spectroscopic (EPR to follow the molybdenum oxidation) and electrochemical (to measure the NO formation) methods, using the molybdenum specific inhibitor allopurinol.[634] In addition, the nature of the product of the nitrite reduction (i.e., NO) was independently confirmed by several other methodologies.[629,630,634] In this context, it should be noted that one XO family enzyme catalyzing a reduction, oxygen abstraction, reaction is not a novelty, because the hydroxybenzoyl-CoA reductase catalyzes the irreversible dehydroxylation of the phenol ring (to yield benzoyl-CoA).[1639−1641]

Fifth, the reaction is thought to be triggered by a protonation event. The nitrite reduction/NO formation is greatly accelerated at acid conditions (pH < 7) and involves two protonation equilibriums with pK_a values of 5.9 and 6.8.[1642] The residues responsible for the protonation equilibriums, however, were not yet identified. This pH dependency is also observed on Cd_1NiR[1459,1643] and CuNiR enzymes[1495,1498,1503,1508,1528,1553,1557,1558,1575,1577] and must be due to the protons necessary to carry out the reaction (eq 73).

Sixth, the NO formation and release is expected to involve only one bond cleavage. Bearing in mind that nitrite is believed to be bound via the "nitrito" binding mode, the simplest way to release NO would be through one bond cleavage (MoO−NO (Figure 20b (ii))). Although it is possible that NO release requires two bond cleavages (MoON−O and then Mo−ON (Figure 20b (iii))), this is improbable: besides involving an extra "unnecessary" step, that step is not consistent with the known molybdenum chemistry, because it would leave the molybdenum atom without any oxo group (not consistent with the known molybdenum chemistry mentioned in the first point).

In view of these considerations, it is suggested that the XO-catalyzed nitrite reduction follows the mechanism depicted in Figure 29.[634,1644] After the molybdenum reduction by xanthine (Figure 29a→b), nitrite binds to the reduced molybdenum, via the "nitrito" binding mode, displacing the urate (Figure 29b→c).[634] Once the Mo^{4+}−O−N−O complex is formed, the reaction is suggested to proceed with the protonation of the oxygen atom bound to the molybdenum (Figure 29c→d).[1644] The conserved Glu_{1261} residue is well positioned to act as the proton donor, but this key role must wait for theoretical studies to be confirmed. This protonation step could trigger the electron transfer from the reduced molybdenum to the now protonated nitrite, as was described for CuNiR (section 3.2.2), causing the N−OH bond homolysis and subsequent NO release (Figure 29d→e).[1644] The mechanism by which the N−OH bond cleavage is undertaken is presently not known. However, it is tempting to speculate that the strategy followed by XO would be analogous to the CuNiR one, because both metals share the same square pyramidal geometry and have a redox active HOMO on the *xy* plane (d_{xy} and d_{x2-y2}, for molybdenum[1645−1647] and copper, respectively).[1644] Furthermore, also in XO, the protonation of nitrite leads to the formation of a more stable "future" metal complex: in this case, Mo^{5+}−OH (Figure 29e) instead of Mo^{5+}−O^- (similar to the CuNiR Cu−OH versus Cu−O^-). The pK_a values of the molybdenum coordinated ligands change dramatically with the oxidation state, with the lower oxidation states holding highly protonated ligands.[1648,1649] For this reason, in the Mo^{5+} complex (Figure 29e), both the oxygen and the sulfur atoms should end up protonated; the protonation event occurs either before (as suggested) or after the NO release. Finally, also the choice of the proton donor, if it is confirmed, seems to be similar: one aspartate in CuNiR and the Glu_{1261} residue in XO.[1644]

At this point, it is instructive to compare the nitrite reduction with the reaction of hydroxybenzoyl-CoA reductase, another XO family member. This enzyme catalyzes the reductive dehydroxylation of hydroxybenzoyl-CoA to yield benzoyl-CoA (also an oxygen atom abstraction reaction; eq 76). The reaction is proposed to follow a Birch-like reduction mechanism, involving single electron and proton transfer steps to the phenol ring, with formation of radical intermediates.[1640] Moreover, for the reaction to proceed, it is considered essential to stabilize the ketyl radical intermediate, which is accomplished by the presence of the carbonyl moiety on the phenol ring. On the suggested nitrite reduction mechanism, the reduction of nitrite also involves single electron and proton transfer steps and the formation of radical "intermediates". But, in the nitrite case, the radicals formed, NO and the Mo^{5+} core, are stable enough to have independent existence.



(76)

At this stage (Figure 29e), one molecule of NO is already formed and released. However, because xanthine oxidation is a two-electron process (Mo^{6+}→Mo^{4+}; eq 74), the molybdenum

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center still has one electron (Mo^{5+}) to reduce another nitrite molecule. Thus, the reaction is proposed to proceed with the binding of a second nitrite molecule.[634] To generate a good leaving group, water ($Mo^{5+}-OH_2$), the consumption of one proton is suggested (Figure 29e→f). Subsequently, nitrite displaces the water molecule (Figure 29f→g), and, after a second cycle of nitrite reduction/molybdenum oxidation, a second NO molecule is released (Figure 29g→h→a). The molybdenum is now in a 6+ oxidation state (Figure 29a), which would favor the deprotonation of its ligands,[1648,1649] and ready to start another catalytic cycle.

In summary, although the mechanism of XO-catalyzed nitrite reduction is not yet fully defined, it seems probable that a strategy similar to that of the CuNiR is employed. Therefore, in XO, nitrite is suggested to bind to the reduced molybdenum center, in a "nitrito" binding mode, and, after a protonation event, the N−OH bond is believed to be homolytically cleaved and the NO promptly released.

The molybdenum unique chemistry makes the molybdenum centers excellent "oxygen atom exchangers", precisely what is needed to catalyze the nitrite conversion into NO (eq 73). In fact, the molybdenum centers are widely used for oxo-transfer reactions, both abstractions and insertions, in the carbon, sulfur, and nitrogen metabolism.[596−602,1290−1295] In this context, it is surprising that no "dedicated" molybdenum-containing nitrite reductase is known to exist. This reasoning suggests that is highly probable that living organisms are using molybdenum to synthesize NO, but employing enzymes that we attribute to other functions. Mammalian XO and AO, plant C-NaR, and bacterial AOR and NaR may become the first examples of such utilization to be described.

3.3. Nitrite Oxidation to Nitrate

Nitrite oxidation has received far less attention than its reduction.

On the eukaryotic side, nitrite oxidation began to be studied only recently, and no "dedicated" nitrite oxidase was yet described to exist. Instead, eukaryotes seem to oxidize nitrite to nitrate and/or to nitrogen dioxide, using different haemic proteins present in cells to accomplish other functions (sections 2.2.1.2 and 2.2.2.2).

On the prokaryotic side, nitrite oxidation constitutes the last step of nitrification (section 2.1.1; Figure 1, black arrows, Table 1). The nitrifier nitrite oxidation is carried out by chemolithoautotrophs that derive energy from the oxidation of nitrite to nitrate, in a strictly aerobic process, where all of the carbon needs can be satisfied with carbon dioxide assimilation (through the Calvin cycle).[97−100,102,103,1650−1652] Nevertheless, nitrite oxidation is thermodynamically unfavorable, and the lithotrophic growth is slow and inefficient.[1651−1653] These nitrite-oxidizing bacteria are thus hard to maintain, and not many groups are trained to keep them in culture,[103] the reason these bacteria and their key enzyme nitrite oxidoreductase have been poorly studied.

Noteworthy, nitrite oxidation is also being found in some other processes, including in anaerobic pathways (see section 2.1.1). Interestingly, the AnAmmOx bacteria "*Candidatus* Kuenenia stuttgartiensis" (Figure 1, gray arrows, Table 1) also seem to oxidize nitrite to nitrate, but anaerobically. It was recently suggested that the gene product initially annotated as a nitrate reductase (nitrite forming) may be involved in the nitrite oxidation (nitrite consuming).[111,1654] This oxidation would produce electrons that could be transferred to the quinone pool, through a membrane-bound subunit, or be used in different reduction reactions, via putative copper- and haem-containing proteins.

Presently, it is not possible to discuss the molecular mechanism of nitrite oxidation, but only review the (still limited) knowledge on the bacterial MoNiOR enzymatic machinery and to speculate about its mechanism (section 3.3.1).

3.3.1. Dissimilatory Nitrite Oxidation to Nitrate. The dissimilatory nitrite oxidation to nitrate (eq 77) is achieved with the molybdenum center of the molybdenum-containing nitrite oxidoreductase (MoNiOR) enzymes (tentatively classified as EC 1.7.99.4).

$$NO_2^- + H_2O \xrightarrow{\text{pyranopterin}\ [\text{Mo}]} NO_3^- + 2e^- + 2H^+ \qquad (77)$$
$$E^{o\prime}\ (NO_3^-/NO_2^-) = +\ 0.43V$$

3.3.1.1. Enzymatic Machinery. The known MoNiOR (product of *nxr* genes) are membrane-bound proteins that can be divided into two groups depending on their subcellular localization:[1655] (i) enzymes anchored on the periplasmatic side of the cytoplasmatic membrane (*Nitrospira*, *Nitrospina*, and "*Candidatus* Nitrotoga" MoNiOR)[1651,1656,1658−1663] and (ii) enzymes anchored on the cytoplasmatic side of the intracytoplasmatic and cytoplasmatic membranes (*Nitrobacter* and *Nitrococcus* MoNiOR).[103,1652,1658,1657,1664,1665] Very recently, a new bacterium was added to the list of the few known nitrifying nitrite-oxidizing organisms: *Nitrolancetus hollandicus* that belongs to the widespread phylum *Chloroflexi* and is believed to hold a cytoplasm-faced MoNiOR similar to those of the *Nitrobacter* and *Nitrococcus*.[1666]

Presently, there is no known structure of a MoNiOR, but there is evidence that MoNiOR must share several structural features with a NaR enzyme, more precisely with the "respiratory" NaR (details about these enzymes in refs 1667 and 1679).[1682] The *Nitrobacter hamburgensis* MoNiOR, a cytoplasm-faced enzyme, is a heterotrimer constituted by a catalytically active $\alpha\beta$-complex (~115 and 65 kDa) that interacts with a membranar *c* type haemic γ-subunit (~32 kDa).[1652,1658,1683,1684] The *Nitrospira moscoviensis* MoNiOR, a periplasm-faced enzyme, is also a catalytically active $\alpha\beta$-complex (~130 and 46 kDa),[1661] but the hypothetical transmembranar γ-subunit, responsible for the electron transfer between the β-subunit and the electron transport chain, was not yet identified and awaits experimental clarification.[1656,1661] However, for a periplasm-faced enzyme, the electrons from quinol oxidation do not need to pass back across the membrane, and, perhaps, a membranar haemic subunit would not be needed.[1681]

The MoNiOR β-subunit (product of *nxrB* gene) of *Nitrobacter hamburgensis* displays a cysteines distribution identical to that of the *E. coli* NaRH (with a total sequence identity of 45%[100]), and, accordingly, it probably holds three [4Fe−4S] and one [3Fe−4S] centers.[100] Four cysteine-rich binding motifs of Fe/S were also identified in *Nitrococcus* and *Nitrospira* β-subunits.[1656] Because of the similarities with NaRH, the MoNiOR β-subunit is believed to be responsible for the electron transfer from the α-subunit (where the nitrite oxidation should occur) to the γ-subunit or directly to the membrane electron transport chain. The α-subunit (product of *nxrA* gene) shows also a significant similarity to the C-terminal sequences of the *E. coli* NaRG, and the biochemical

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characterizations carried out point toward the presence of one Fe/S and one molybdenum center in both MoNiOR groups.[100,1656,1658,1661,1684,1685] The molybdenum center, where the nitrite oxidation should occur (the enzyme active site), is assumed to be coordinated by two pyranopterin cofactor molecules, as in NaRG. The molybdenum center should also hold an oxo group (again as NaRG) that, in the course of the reaction, would be transferred to the nitrite molecule to yield nitrate.

3.3.1.2. Mechanism. Little is known about the MoNiOR-catalyzed reaction. As in other molybdenum-containing enzymes, the "new" oxygen atom of nitrate is derived from water and not from dioxygen (eq 77),[1686−1689] with the molybdenum center probably intermediating the oxygen atom transfer (see the description of a molybdoenzyme catalytic mechanism in section 3.2.3). The elucidation of the structure and mechanistic strategies followed by cytoplasm- and periplasm-faced MoNiOR must wait for future experimental work.

It is interesting to note that nitrite-oxidizing bacteria are versatile organisms that catalyze both nitrite oxidation and nitrate reduction. These bacteria can "switch" from aerobic nitrite oxidation to anaerobic growth by dissimilatory nitrate reduction, using pyruvate (*Nitrobacter*[1651,1652,1658,1690−1692]) or hydrogen (*Nitrospira*[1651]) as electron donors. If the bacteria employ the same enzyme or synthesize de novo a different protein is not known. However, the *Nitrobacter hamburgensis* MoNiOR is able to catalyze the nitrate reduction, at least in vitro,[1658,1683,1684] while the *Nitrospira* enzyme is not.[1656]

Presently, the similarities between (i) "respiratory" cytoplasm-faced NaRGHI, (ii) periplasmatic NaR, (iii) cytoplasmatic anabolic NaR, and (iv) cytoplasm- and (v) periplasm-faced MoNiOR suggest that biology had found one perfect "solution" to interconvert nitrate and nitrite: the pyranopterin-coordinated molybdenum center.

4. OUTLOOK

Nitrite is long known as one of the players of the biogeochemical cycle of nitrogen, participating in key pathways crucial to life on Earth and to the planetary "recycling" of nitrogen. More recently, nitrite is also being recognized as a molecule relevant to cell signaling and survival, virtually in all forms of life. Despite all of those different biological functions, nitrite reduction/oxidation seems remarkably similar in all cases.

Iron, more specifically haem iron, is probably the most used metal to reduce nitrite. As discussed, prokaryotes reduce nitrite for assimilatory and dissimilatory purposes, using different haem types (*c*, sirohaem and d_1), with diverse axial coordinations (lysine, cysteine, and histidine residues), in several protein arrangements (dimers/monomers, with several more haems, with only one additional haem or one Fe/S). Nevertheless, despite the differences, all of the enzymes discussed (i) bind nitrite to ferrous iron, through the "nitro" mode, (ii) activate the nitrite molecule through iron back-bonding and hydrogen bonding to positively charged residues at the active site, (iii) promote the double protonation of one of the nitrite oxygen atoms (releasing a water molecule), and (iv) form an iron−NO complex. Subsequently, the iron−NO complex is cleaved and the NO is released, or the complex is retained and further protonated and reduced to yield ammonium.

The prokaryotic haemic strategy to form NO was so successful that we can find several "vestiges" of the earlier, preaerobic, pathways on nowadays mammalian and plant cells:[753] for example, under hypoxia, HG or Mb are taking advantage of the "old" haem redox chemistry, thoroughly explored on the prokaryotic haemic nitrite reductases, to form NO. The "lessons" from the ancestral prokaryotic world become more clear when it is realized that the chemistry behind an anaerobic activity is a perfect solution for the contemporary hypoxic/anoxic generation of NO. However, we cannot fail to ask, if the aim was to generate NO, why do mammals lose the unique d_1 haem or the CuNiR enzyme and develop a complex oxygen-dependent *b* haem-containing NOS enzyme? As discussed, the strong binding of NO to haems imposes severe mechanistic constraints to haem-containing nitrite reductases/NO synthases, NO transporters, and NO receptors (e.g., guanylate cyclase), all of which have to avoid the dogmatic scavenging by haem iron. The d_1 haem ability to promptly release NO would have allowed the organism to overcome this "dilemma".

Remarkably, the mammalian cells are also using haemic proteins to oxidize nitrite under normoxic conditions. In this case, it is the haem peroxidatic activity that is being "copied". Noteworthy, the usage of this chemistry in prokaryotes is not recognized, and only a nitrite oxidoreductase enzyme is presently known to exist, the MoNiOR.

Besides haem iron, also molybdenum can be widely used to reduce/oxidize nitrite. Its unique chemistry makes the enzymatic molybdenum centers excellent "oxygen atom exchangers", precisely what is needed to abstract/insert one oxygen atom from/into nitrite or even to remove one oxygen atom from nitrate (to form nitrite). In fact, the molybdenum "solution" to interconvert nitrate and nitrite was so successful that three types of molybdenum-containing NaR and two types of MoNiOR were developed.

As was discussed, several molybdoenzymes, with different molybdenum center structures (Figure 19c) and active site pockets, are able to reduce nitrite to generate signaling NO. However, despite the differences, the reaction mechanism "outline" is not expected to be much different within different molybdoenzymes, or even within different reaction types:[1693] (i) Mo^{6+} cores are believed to be oxo group donors, producing "oxygenated" molecules, for example, nitrate (eq 77) or urate (eq 74); (ii) reduced Mo^{4+} cores are proposed to act as oxo group acceptors, binding an oxo-molecule and abstracting one oxygen atom, for example, binding nitrite to produce NO (eq 73). In this mechanism, the molybdenum atom would intermediate the oxygen atom transfer from one substrate to the second substrate ("oxygen atom exchange"), as long as the thermodynamics of the reactions is favorable. According to this "double oxo transfer" hypothesis, the nitrite reductase activity of the several molybdoenzymes here described, bacterial AOR and NaR, fungus NaR, plant C-NaR, and mammalian XO/XD and AO, is not at all unexpected: these proteins generate NO using the molybdenum redox chemistry already "tested" in diverse oxo-transfer reactions of the carbon, sulfur, and nitrogen metabolism. On the contrary, it is surprising that no "dedicated" molybdenum-containing nitrite reductase is known to exist. Are the molybdenum-containing nitrite reductases "disguised" under proteins that we attribute to other functions?

Biology also uses copper to reduce nitrite, although, comparatively, its utilization is not so diversified. Nonetheless, the copper-dependent nitrite reduction probably shares many

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features with the molybdenum handling: nitrite is bound through the "nitrito" mode, and protonation of one of its oxygen atoms yields a metal–hydroxo complex and releases NO.

In summary, to reduce/oxidize nitrite, biology developed several strategies, exploring different protein structures, metals, and nitrite binding modes, but using a comparable "blueprint". Those strategies are the result of different "evolutionary assays": (i) CcNiR and CSNiR, two "solutions" based on the same basic machinery (a haem) to solve the same "problem", form ammonium, but with distinct biological purposes, "respiration" and assimilation; (ii) Cd_1NiR and CuNiR, two truly different "solutions", developed in different geologic eras, to solve the same "problem", the "respiratory" NO formation; (iii) CcNiR and Cd_1NiR, two "solutions" based on the same basic machinery (a haem) to solve two different "problems", "respiratory" formation of ammonium and NO; and (iv) penta- and octa-haemic CcNiR, "classic" and extended CuNiR, AOR, and FAD-containing XO/XD/AO, CSNiR, and FAD-containing CSNiR. All represent fascinating examples of how biology once had found a "solution", and reuses it, introducing only minor adjustments to respond to specific cellular demands. However, in the context of nitrite reduction, the most striking aspect is the evolutionary convergence, through which virtually all forms of life are using a myriad of different metalloproteins to achieve the same objective: reduce nitrite to signaling NO.

To generate nitrite-dependent signaling NO, the organisms "reuse" different metalloproteins, present in the cells to accomplish other functions, and "switch" their activities to a nitrite reductase/NO synthase, when it is necessary. From a chemical point of view, the organisms are just using the redox chemistry of an available redox system and doing a "substrate adaptation" to generate NO. The biological use of a single protein to accomplish more than one function is not a new concept introduced with the nitrite/NO metabolism. This is a well recognized and common phenomenon, moonlighting, with important implications for systems biology and, in particular, for human physiology and pathology (see, e.g., ref 1694). In the nitrite reduction scenario, this phenomenon is carried out by several proteins and is triggered by the oxygen availability and/or cellular redox status, and, in some cases, by sophisticated (and very interesting) posttranslational modifications that regulate the new nitrite reductase activity of the protein. In accordance, it can be hypothesized that the "nonrespiratory" nitrite reduction to NO is part of a conserved regulatory mechanism that "translates" the dioxygen availability/cellular redox imbalance into a differentiated flux of NO, and then into a biological response that would overcome/repair the cellular changes. As F. Cutruzzolà et al. as pointed out, nitrite reduction to NO is a ubiquitous function, from a preaerobic past,[753] that has been "reinvented" and employed ever since.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

AnAmmOx	anaerobic ammonium oxidation
AO	aldehyde oxidase
AOR	aldehyde oxidoreductase
Cb	cytoglobin
Cc	cytochrome *c*
CcNiR	cytochrome *c*-containing nitrite reductase (multi-*c*-haems-containing nitrite reductase)
CcO	cytochrome *c* oxidase (mitochondrial)
Cd_1NiR	cytochrome d_1-containing nitrite reductase (*c* and d_1 haems-containing nitrite reductase)
C-NaR	cytoplasmatic nitrate reductase of plants
CSNiR	cytochrome sirohaem-containing nitrite reductase (Fe/S and sirohaem-containing nitrite reductase)
CuNiR	copper-containing nitrite reductase (T1 and T2-containing nitrite reductase)
deoxy-Cb	deoxy-cytoglobin; $(Cb)Fe^{2+}$
deoxy-Hb	deoxy-haemoglobin; $(Hb)Fe^{2+}$
deoxy-Mb	deoxy-myoglobin; $(Mb)Fe^{2+}$
deoxy-Nb	deoxy-neuroglobin; $(Nb)Fe^{2+}$
deoxy-NS-HG	deoxygenated nonsymbiotic haemic globin of plants; $(NS\text{-}HG)Fe^{2+}$
DNRA	dissimilatory nitrate reduction to ammonium
e^-	electron(s)
EPR	electronic paramagnetic resonance spectroscopy
Fe/S	iron/sulfur center
HAOR	hydroxylamine oxidoreductase
Hb	haemoglobin
HG	haemic globin
Mb	myoglobin
met-Cb	met-cytoglobin; $(Cb)Fe^{3+}$
met-Hb	met-haemoglobin; $(Hb)Fe^{3+}$
met-Mb	met-myoglobin; $(Mb)Fe^{3+}$
met-Nb	met-neuroglobin; $(Nb)Fe^{3+}$
MoNiOR	molybdenum-containing nitrite oxidoreductase
NaR	nitrate reductase (all types of enzymes that reduce nitrate to nitrite)
NaRGHI	"respiratory" nitrate reductase, after the name of the encoding genes, *narG*, *H*, and *I*
NaRG	NaRH and NaRI, each of the three NaRGHI subunits
Nb	neuroglobin
NO	nitric oxide radical
NOS	NO synthase
NrfA	another designation for CcNiR, after the name of the encoding gene *nrf* (nitrite reduction with formate)
NrfB	NrfC, NrfD, NrfH, other proteins that interact with NrfA, belonging to the same encoding *nrf* gene
NS-HG	nonsymbiotic haemic globin of plants
oxy-Cb	oxy-cytoglobin; $(Cb)Fe^{2+}-O_2$
oxy-Hb	oxy-haemoglobin; $(Hb)Fe^{2+}-O_2$
oxy-Mb	oxy-myoglobin; $(Mb)Fe^{2+}-O_2$
oxy-Nb	oxy-neuroglobin; $(Nb)Fe^{2+}-O_2$
oxy-NS-HG	oxygenated nonsymbiotic haemic globin of plants; $(NS\text{-}HG)Fe^{2+}-O_2$
P_{50}	the oxygen concentration at which Hb or Mb are half-saturated
RNS	reactive nitrogen species
ROS	reactive oxygen species
RPM-NaR	root-specific plasma membrane-bound, succinate-dependent, nitrate reductase of plants
RPM-NiR	root-specific plasma membrane-bound nitrite reductase of plants
RSH	thiol compound
RSNO	*S*-nitrosothiol
T1 and T2	type 1 and 2 copper centers of CuNiR, respectively
TTR	tetrathionate reductase
XD	xanthine dehydrogenase
XO	xanthine oxidase

Note: The abbreviation of the enzymes name indicates the function and, in some cases, includes the type of center or metal present at the active site. A capital C stands for cytochrome and is followed by the letter of the haem type, c, d_1, and S for haem *c*, haem d_1, and sirohaem, respectively (Cc, Cd_1, CS). The chemical symbols Cu and Mo were used for the respective metals. Ni and Na were used to discriminate between nitrite and nitrate. At the end of the abbreviation, R, O, OR, and D stand for reductase, oxidase, oxidoreductase, and dehydrogenase, respectively. To distinguish between the cytoplasmatic and the root-specific plasma membrane-bound enzymes of plants, the prefixes C- and RPM- were introduced (C-NaR, RPM-NaR, RPM-NiR). Because Hb and Mb are well-recognized abbreviations for haemoglobin and myoglobin, the same scheme was followed for neuroglobin (Nb) and cytoglobin (Cb). To discriminate between haemoglobin and haemic globins in general, the abbreviation HG was introduced, giving, consequently, NS-HG for the nonsymbiotic haemic globins of plants.

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complexes termed "metabolons", like band 3. Band 3 comprises an anion transporter, various glycolytic enzymes, the presumptive carbon dioxide transporter, and carbonic anhydrase. Together, these proteins seem to play a key role in regulating the cell metabolism and its ion and gas transport function. In addition, deoxygenated Hb binds to the band 3 cytoplasmatic face, in the same region as the glycolytic enzymes.

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(1017) Like the ischaemic events of mammals (see section 2.2.1.1), higher plant tissues can also be subjected to a decrease in the dioxygen concentration. Roots can be subjected to hypoxia or even anoxia after a strong rainfall, because the soil usually becomes flooded, for a short or long period of time, depending on its drainage capacity.[1018] Thus, hypoxia of root systems occurs frequently in nature,[1019] affecting the majority of plants at some time during their life cycle.[1020] In addition, also the aerial photosynthetic tissues can be subjected to hypoxia, for example, upon stomatal closure triggered by an increase in temperature (inhibiting the photosynthetic activity). As in other aerobic organisms, when the dioxygen concentration decreases, the plant mitochondrial oxidative phosphorylation is diminished/ceased, ATP decreases, and NADH increases. Glycolysis and fermentation are then promoted to synthesise ATP and regenerate NAD^+, leading to the accumulation, in plants, of ethanol, lactate, and alanine.[1021,1022] Subsequently, acidification of the cytoplasm occurs[1023,1024] and the photosynthetic activity is reduced.[1025,1026] However, the lactic fermentation is transient, as acidification of the cytoplasm inhibits the lactate production[1027] and stimulates the ethanol production.[1027,1028] The ethanolic fermentation, in its turn, enables, besides the generation of ATP, a more efficient (on a molar basis) regeneration of NAD^+ and consumption of protons.[1029,1030] In this way, in plants, the energy production can proceed during several hours of hypoxia, without severe cytoplasmatic acidosis.[1028,1031] This explains (at least partially) why plants survive far longer under hypoxia than does any higher animal that uses exclusively lactic fermentation.
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(1188) The inhibition of nitrate assimilation by elevated carbon dioxide may occur, at least, at three levels (use Table 1, Figure 1, green arrows to follow).[1187,1189−1193] (i) Inhibition may take place at the first step of assimilation, the C-NaR-catalyzed nitrate reduction to nitrite, due to lack of reducing power: increased carbon dioxide concentrations decrease photorespiration and, consequently, decrease malate export from chloroplasts, which, in turn, could decrease the amount of available cytoplasmatic NADH to reduce nitrate.[1193−1196] The differentiated behavior of C_3 and C_4 plants toward increased carbon dioxide supports this hypothesis. C_4 plants developed a mechanism to circumvent the wasteful photorespiration and have a high NADH concentration in the bundle-sheath cells. Concomitantly, the nitrate assimilation by C_4 plants is relatively independent of carbon dioxide concentration and limited to the mesophyll cells. (ii) Inhibition may also occur during nitrite translocation into chloroplast, where it is further reduced to ammonium: it has been shown that hydrogen carbonate inhibits the nitrite influx into isolated chloroplast, probably because nitrite transporters transport both nitrite and hydrogen

carbonate.[1191,1197] (iii) In addition, inhibition may be caused by lack of reducing power to carry out the final reduction to ammonium: ammonium formation may proceed only if the availability of reduced ferredoxin exceeds that needed for the formation of the NADPH,[1198] and, for most plants, this occurs when carbon dioxide availability limits C_3 carbon fixation.[1191] If carbon dioxide assimilation is increased, the concomitant competition for reducing equivalents might inhibit the nitrite reduction to ammonium. Inhibition of nitrate assimilation by elevated carbon dioxide is an important phenomenon, common among C_3 species (studied in barley, tomato, wheat, and *Arabidopsis*).[1187,1193] Nevertheless, ecosystems display a broad range of responses toward increased carbon dioxide concentrations, possibly as a result of the seasonal and spatial fluctuations in the relative availabilities of ammonium and nitrate. Noteworthy, in ecosystems where ammonium is the dominant nitrogen form, the net primary productivity increases (as much as 25%) under elevated carbon dioxide.

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(1655) The existence of these two types of enzyme is, probably, the response to different environmental pressures. *Nitrospira*, *Nitrospina*, and "*Candidatus Nitrotoga*" bacteria, with a periplasm-faced MoNiOR, grow only under nitrite limitation (0.29−2.9 mM), while *Nitrobacter* and *Nitrococcus*, with a citoplasm-faced enzyme, are adapted to high nitrite concentrations (up to 29 mM).[103] Therefore, it can be speculated that a periplasm-faced MoNiOR would allow the bacteria to grow with low nitrite concentrations and, at the same time, would be responsible for the inhibitory effects of high nitrite concentration on bacteria growth. On the other hand, a cytoplasm-faced enzyme would depend on a nitrite/nitrate antiport system and would necessitate/tolerate a higher nitrite concentration.[1656,1657]
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(1667) Prokaryotes use nitrate for assimilatory and dissimilatory processes (see section 2.1.1) and, for those purposes, hold three types of NaR: (i) "respiratory" membrane-bound cytoplasm-faced NaR (Figure 11g,h), associated with the generation of a proton motive force across the cytoplasmatic membrane; (ii) periplasmatic NaR, involved with the generation of a proton motive force or acting as an electron sink to eliminate excess of reducing equivalents; and (iii) cytoplasmatic assimilatory NaR, involved in nitrogen assimilation.[1668−1671]All of the prokaryotic NaR are molybdoenzymes of the dimethylsulfoxide reductase family (see section 3; Figure 19c) and catalyze the two electrons reduction of nitrate to nitrite (reverse of eq 77) at their molybdenum centee. Apart from the four sulfur atoms from the two pyranopterin molecules (Figure 19c), the molybdenum atom is coordinated by: (i) in "respiratory" membrane-bound NaR, by two oxygen atoms (both from an aspartate residue or one from a terminal oxo group plus another one from an aspartate residue[1672]); (ii) in periplasmatic NaR, by two sulfur atoms (from a terminal sulfo group and another from a cysteine residue) or one terminal hydroxo group plus a sulfur atom from a cysteine residue;[1673−1677] and (iii) in cytoplasmatic assimilatory NaR, probably by a sulfur atom from a cysteine residue (although clear structural insight awaits further investigation).[1678] It should be noted that, despite catalyzing the same reaction, the eukaryotic assimilatory cytoplasmatic NaR, C-NaR (section 2.2.2.1.1), is distinct from any type of prokaryotic NaR. Belonging to the sulfite oxidase family, its molybdenum atom (responsible for the nitrate reduction) is coordinated by only one pyranopterin molecule (two sulfur atoms), plus one sulfur atom from a cysteine residue, one oxo, and one hydroxo groups (Figure 19c).

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(1679) "Respiratory" NaR are membrane-bound cytoplasm-faced molybdoenzymes and, as the name indicates, are used by the organisms to generate a proton motive force across the cytoplasmatic membrane.[1668,1672,1680] They are also called NaRGHI, because they are the product of the *narG*, *H*, and *I* genes. These enzymes of the dimethylsulfoxide reductase family are heterotrimers, comprising (Figure 11g,h): (i) a cytolasmatic nitrate-reducing NaRG subunit (~125 kDa) that holds one molybdenum center (with the molybdenum atom coordinated as described in ref 1667, the active site) and one [4Fe−4S] center; (ii) an electron-transfer NaRH subunit (~60 kDa) that holds one [3Fe−4S] and three [4Fe−4S] centers; and (iii) a membrane-bound quinol-oxidizing NaRI subunit (~22 kDa) that holds two *b*-type haems. Interestingly, and in harmony with the MoNiOR dual localization, it was recently concluded that there is evidence for the occurrence of both archaeal and bacterial periplasm-faced NaRGHI-type nitrate reductases, that is, NaRGHI enzymes with the active site on the outside of the cytoplasmatic membrane.[1681] This exciting hypothesis, however, waits for experimental confirmation.
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(1693) In general, the molybdoenzymes of all families (Figure 19c) catalyze the abstraction or insertion of one oxygen atom into a substrate molecule, in reactions that imply a net exchange of two electrons and in which the molybdenum cycles between Mo^{6+} and Mo^{4+}.[596−602] It is based on this catalytic feature that these enzymes are commonly referred to as oxo-transferases. However, there is at least one important exception that does not involve an oxygen atom transfer reaction: the formate (HCO_2^-) oxidation to produce carbon dioxide (CO_2) catalyzed by formate dehydrogenase (dimethylsulfoxide reductase family).
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NOTE ADDED IN PROOF

After this Review was accepted, a new work on the reaction mechanism of CcNiR was published: Bykov, D.; Plog, M.; Neese, F. *J. Biol. Inorg. Chem.*, **2014**, *19*, 97.