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MINIREVIEW

Nitrite reduction by molybdoenzymes: a new class of nitric oxide-forming nitrite reductases

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Abstract Nitric oxide (NO) is a signalling molecule involved in several physiological processes, in both prokaryotes and eukaryotes, and nitrite is being recognised as an NO source particularly relevant to cell signalling and survival under challenging conditions. The "non-respiratory" nitrite reduction to NO is carried out by "non-dedicated" nitrite reductases, making use of metalloproteins present in cells to carry out other functions, such as several molybdoenzymes (a new class of nitric oxide-forming nitrite reductases). This minireview will highlight the physiological relevance of molybdenum-dependent nitrite-derived NO formation in mammalian, plant and bacterial signalling (and other) pathways. The mammalian xanthine oxidase/xanthine dehydrogenase, aldehyde oxidase, mitochondrial amidoxime-reducing component, plant nitrate reductase and bacterial aldehyde oxidoreductase and nitrate reductases will be considered. The nitrite reductase activity of each molybdoenzyme will be described and the review will be oriented to discuss the feasibility of the reactions from a (bio)chemical point of view. In addition, the molecular mechanism proposed for the molybdenum-dependent nitrite reduction will be discussed in detail.

Keywords Molybdenum · Nitrite reduction · Nitric oxide · Cell signalling · Moonlighting

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Abbreviations

AO	Aldehyde oxidase
AOR	Aldehyde oxidoreductases
CuNiR	Copper-containing nitrite reductase (one of the enzymes responsible for the respiratory nitrite reduction to NO)
DMSOR	Dimethylsulphoxide reductase
DPI	Diphenyleneiodonium chloride
EPR	Electron paramagnetic resonance spectroscopy
Fe/S	Iron–sulphur centre
mARC	Mammalian mitochondrial amidoxime-reducing component
MOSC	From molybdenum cofactor sulphurase C-terminal domain (proteins involved in pyranopterin cofactor biosynthesis)
NaR	Nitrate reductase (all types of nitrate reductase enzymes)
NiR	Nitrite reductases ("dedicated" and "non-dedicated" enzymes)
NO	Nitric oxide radical
NOS	NO synthases
ROS	Reactive oxygen species
SO	Sulphite oxidase
SOD	Superoxide dismutase
XD	Xanthine dehydrogenase
XO	Xanthine oxidase

Introduction: an outlook on molybdoenzymes and nitrite reduction

Molybdenum is essential to most of the living organisms [1, 2], from archaea and bacteria to higher plants and mammals, being part of the active site of enzymes that catalyse important redox reactions of the metabolism of

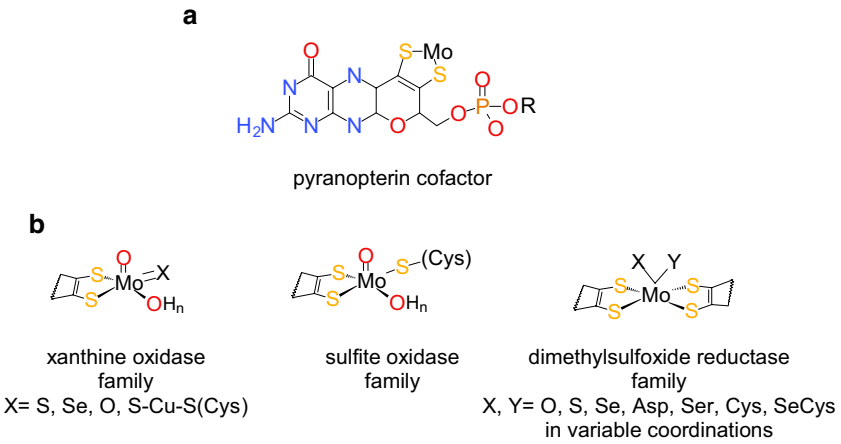


Fig. 1 Active site structures of the molybdoenzymes. **a** Structure of the pyranopterin cofactor. The cofactor is a pyranopterin-dithiolate moiety, which forms a five-membered ene-1,2-dithiolate chelate ring with the molybdenum atom; in eukaryotes, the cofactor is found in the simplest monophosphate form (R is a hydrogen atom), while in prokaryotes it is found esterified with several nucleotides (R can be one cytidine monophosphate, guanosine monophosphate or adenosine monophosphate). **b** Structures of the molybdenum centres of the three families of molybdoenzymes; for simplicity, only the dithiolate moiety of the pyranopterin cofactor is represented. The images were produced with Accelrys Draw 4.0 (Accelrys Software Inc.)

carbon, nitrogen, and sulphur [3–10]. Presently, more than 50 molybdenum-containing enzymes are known, many of which have been biochemically and structurally characterised, and several other are foreseen to be "discovered" in the near future based on genomic analyses [2, 11, 12]. Noteworthy, the great majority of the molybdoenzymes are prokaryotic, whereas only a restricted number of molybdoenzymes are found in eukaryotes [3–10].

Apart from nitrogenase, with its unique heteronuclear [$MoFe_7S_9$] cofactor[1] [12–16], all molybdoenzymes harbour one molybdenum atom coordinated by the *cis*-dithiolene group of one or two pyranopterin cofactor molecules (Fig. 1a) and by oxygen, sulphur or selenium atoms in a diversity of arrangements that determines the classification of the molybdoenzymes into three large families (Fig. 1b) [3–10]: xanthine oxidase (XO), sulphite oxidase (SO) and dimethylsulphoxide reductase (DMSOR) families. The active site of XO family enzymes holds the molybdenum atom coordinated, in a square-pyramidal geometry, by one apical oxo group and, in the equatorial plane, by the two sulphur atoms of one pyranopterin cofactor molecule, one labile –OH/OH_2 group and one terminal oxo, sulpho or seleno group. The CO dehydrogenase from *Oligotropha carboxidovorans*, with its unique binuclear Mo–S–Cu cofactor (with an –S–Cu–S(cysteine) instead of an equatorial terminal group), is also included in the XO family.[2] This family comprises enzymes such as mammalian XO and aldehyde oxidase (AO), *Desulfovibrio* aldehyde oxidoreductases (AOR), and prokaryotic nicotinate dehydrogenase, quinoline 2-oxidoreductase or 4-hydroxybenzoyl-CoA reductase. The active site of the SO family enzymes is closely related to the one of XO family, but with the distinctive feature of having the protein, through a cysteine residue, directly coordinated to the molybdenum. In these enzymes, the molybdenum centre displays the same square-pyramidal geometry, with the apical oxo group, but with the equatorial plane formed by two sulphur atoms of the pyranopterin, one oxo group and the cysteine sulphur atom. SO family enzymes include diverse prokaryotic sulphite dehydrogenases, plant, chicken and human SO[3] and eukaryotic assimilatory nitrate reductases (NaR; enzymes involved in nitrate assimilation in plants, algae and fungi) [17], as well as *Escherichia coli* YedY [18–22] or mammalian mitochondrial amidoxime-reducing component (mARC; enzymes involved in the reduction (dehydroxylation) of *S*- and *N*-hydroxylated compounds)[4] and the MOSC proteins homologues (involved in molybdenum centre sulphuration). The DMSOR family is the larger and more diverse family, structurally and functionally. The enzymes from this family are characterised by harbouring the molybdenum atom coordinated by two pyranopterin cofactor molecules (through four sulphur atoms), in a trigonal prismatic geometry completed by terminal oxo, sulpho groups and/or oxygen, sulphur and selenium atoms from

[1] See the contributions of both Hu and Ribbe and Bjornsson, Neese, Schrock, Einsle and DeBeer in this JBIC issue.

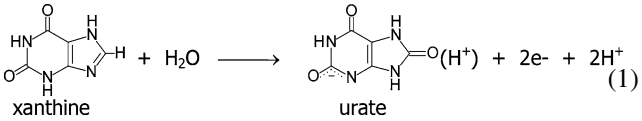
[2] See Hille et al.'s contribution in this J Biol Inorg Chem issue.

[3] See Kappler and Enemark's contribution in this J Biol Inorg Chem issue.

[4] See Ott et al.'s contribution in this J Biol Inorg Chem issue.

aspartate, serine, cysteine or selenocysteine residue side chains. This family is constituted by only prokaryotic enzymes of different functions, including DMSOR, formate dehydrogenase, arsenite oxidase and arsenate reductase, as well as dissimilatory NaR (membrane-bound and periplasmatic enzymes associated with the generation of a proton motive force or acting as an electron sink to eliminate excess of reducing equivalents) and assimilatory NaR (prokaryotic cytoplasmatic enzymes involved in nitrogen assimilation), among many others.

In general, the molybdoenzymes catalyse the transfer of an oxygen atom from water to the product (oxygen atom insertion) or from substrate to water (oxygen atom abstraction), in reactions that imply a net exchange of two electrons and in which the molybdenum cycles between Mo^{6+} and Mo^{4+} [3–10]. It is based on this catalytic feature that the molybdoenzymes are commonly, but inaccurately, referred to as oxotransferases (as will become evident from the exceptions described below). The XO family enzymes catalyse the hydrolysis of a C–H bond with formation of a novel C–O bond, in reactions of oxidative hydroxylation, as the prototype XO does during xanthine hydroxylation to urate (Eq. 1) [3, 23–28]. Although this is also the case of AO and AOR (Eq. 2), nicotinate dehydrogenase (Eq. 3) and quinoline 2-oxidoreductase (Eq. 4), there are at least two important exceptions: the CO dehydrogenase-catalysed carbon monoxide oxidation to carbon dioxide that does not involve hydrolysis of a C–H bond (Eq. 5) (see footenote 2), and the hydroxybenzoyl-CoA reductase that catalyses the irreversible dehydroxylation (a reduction) of the phenol ring (Eq. 6) [29, 30]. The members of the SO family, in contrast, are thought to be proper oxotransferases, as SO and NaR enzymes catalyse the simple transfer of an oxygen atom to, or from, a lone electron pair of the substrate (SO-catalysed sulphite oxidation to sulphate (Eq. 7) and NaR-catalysed nitrate reduction to nitrite (Eq. 8), respectively) (see footnote 3, [17). However, the recent identification of mammalian mARC (Eq. 9) and bacterial YedY, YcbX or YiiM, as well as several other MOSC proteins homologues (most of these are not yet characterised), demonstrated that SO family enzymes are also involved in the reduction of *S*- and *N*-hydroxylated compounds and in sulphuration reactions ([18–22], see footenote 4). Nevertheless, the catalytically more versatile family is undoubtedly the DMSOR family. These enzymes are able to catalyse diverse reactions types: (1) proper transfer of oxygen atom [e.g. DMSOR-catalysed DMSO reduction (Eq. 10) or NaR reaction (Eq. 8)], (2) cleavage of C–H bond [e.g. formate dehydrogenase-catalysed formate oxidation to carbon dioxide (Eq. 11)], (3) transfer of sulphur atom [e.g. polysulphide reductase-catalysed inorganic sulphur reduction to sulphide (Eq. 12)], (4) simultaneous oxidation and reduction [e.g. reductive dehydroxylation and concomitant oxidative hydroxylation catalysed by pyrogallol:phloroglucinol hydroxyltransferase (Eq. 13)] and (5) even hydration reactions [e.g. acetylene hydratase-catalysed hydration of acetylene to acetaldehyde, a non-redox reaction (Eq. 14)] [5, 31–33].



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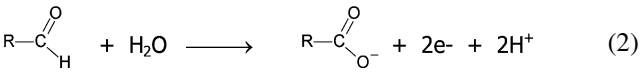

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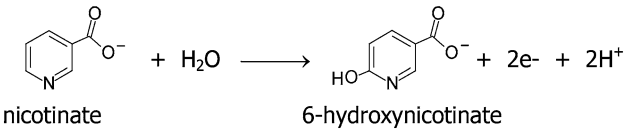

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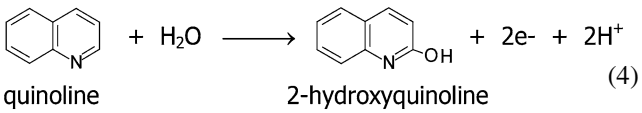
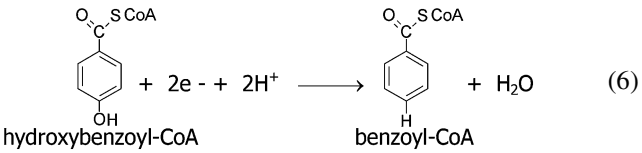


(4)

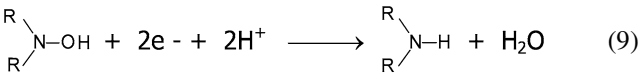
$$CO + H_2O \rightarrow OCO + 2e^- + 2H^+ \quad (5)$$



(6)

$$SO_3^{2-} + H_2O \rightarrow OSO_3^- + 2e^- + 2H^+ \quad (7)$$

$$ONO_2^- + 2e^- + 2H^+ \rightarrow NO_2^- + H_2O \quad (8)$$



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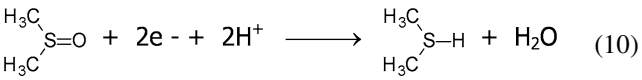
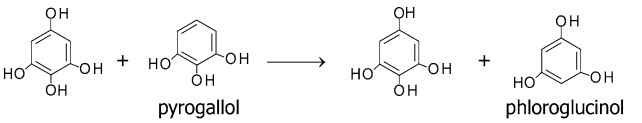


(10)

$$HCOO^- \rightarrow CO_2 + 2e^- + H^+ \quad (11)$$

$$(S_n)^{2-} + 2e^- \rightarrow S^{2-} + (S_{n-1})^{2-} \quad (12)$$



(13)

$$H - C \equiv C - H + H_2O \rightarrow H_3C - COH \quad (14)$$

In addition to that array of reactions, several molybdoenzymes, from the three families, are also being recognised for their ability to catalyse nitrite reduction to nitric oxide radical (NO) (Eq. 15), a signalling molecule involved in several physiological processes in both prokaryotes and eukaryotes. This is a novel catalytic capability and an unusual oxygen atom abstraction reaction assigned to a molybdenum site.

$$NO_2^- + 1e^- + 2H^+ \rightarrow {}^{\cdot}NO + H_2O \quad (15)$$

Nitrite is long known as one of the players of the biogeochemical cycle of nitrogen, participating in several respiratory and assimilatory pathways crucial to life on Earth and to the planetary nitrogen "recycling" [34–42]. More recently, however, nitrite has been also recognised as an important source of signalling NO, particularly relevant to cell signalling and survival under challenging conditions [42]. The nitrite-dependent signalling pathways have been described in mammals, plants and also bacteria, and are carried out by "non-dedicated" nitrite reductases (NiR), making use of metalloproteins present in cells to carry out other functions, such as numerous haemic proteins, and, of course, several molybdoenzymes from the three families. The nitrite-derived NO formation is, however, a complex subject, overshadowed by several biochemical constraints, of which the main ones are as follows: (1) In the case of haemic proteins, how can the formed NO avoid being rapidly trapped by the haem itself? (2) In the case of enzymes, how can nitrite compete with the "classic" oxidising substrates? (3) How can we reconcile the in vivo observed nitrite effects with the in vitro knowledge of nitrite reduction through those diverse pathways?

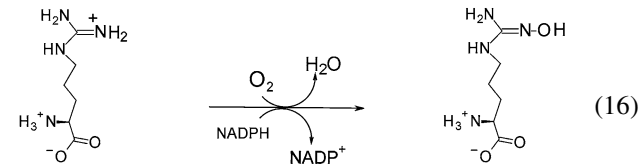
The nitrite-mediated signalling pathways are a recent, not yet generally recognised, and controversial subject. In this minireview, we will highlight the physiological relevance of molybdenum-dependent nitrite-derived NO formation in mammalian, plant and bacterial signalling (and other) pathways. In each case, the molybdoenzymes able to catalyse nitrite reduction will be described and the review will be oriented to discuss the feasibility of the reactions mainly from a chemical point of view. Finally, the molecular mechanism proposed for the molybdenum-dependent nitrite reduction will be discussed in detail.

Molybdenum-dependent nitrite-derived nitric oxide

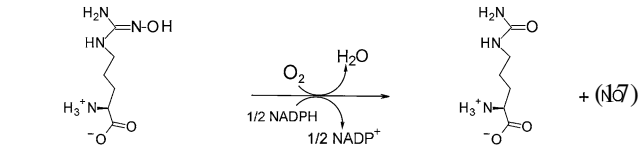
Mammals

Nitric oxide in mammals

In mammals, NO is involved in several physiological processes, 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 anti-tumour and anti-microbial activities [43]. To generate NO, mammals have NO synthases (NOS), complex enzymes constituted by one flavinic reductase domain and one haemic oxygenase domain, where the NO formation occurs [44–47]. These enzymes catalyse the NO formation from the guanidinium nitrogen atom of L-arginine, in a reaction that is dioxygen- and NADPH dependent (Eq. 16–17). Because of this dioxygen dependency, the onset of hypoxia/anoxia would hamper the NOS activity and the NO formation would be compromised.



(L-arginine) (Nω-hydroxyl-L-arginine) (16)



(Nω-hydroxyl-L-arginine) (L-citruline) + (17)

The NO biological effects are accomplished mainly by posttranslational modification of transition metal centres (mostly haems and labile [4Fe–4S] centres) and of cysteine residues and other thiols, to yield nitrosyl (–metal–N=O) and S-nitrosothiol (–S–N=O) derivates [48–57]. To control the specificity of NO signalling and to limit the NO reactivity and associated unwanted effects, the NOS activity is tightly regulated and the NO lifetime is controlled through its rapid oxidation to nitrite by dioxygen [57–60] or ceruloplasmin [61], and to nitrate through its reaction with, e.g. oxy-haemoglobin and oxy-myoglobin [43, 62–72].

With our growing knowledge of the NO physiological roles, nitrate and nitrite have been mainly overlooked and thought as "useless" end products of NO metabolism. From the end of the twentieth century/beginning of the twenty-first century, it has became clear that nitrite can be reduced back to NO under hypoxic/anoxic conditions (Eq. 15). Simultaneously, it was re-discovered [73] that nitrite can exert a significant protective action in vivo, during ischaemia and other pathological conditions [74–120]. Those findings triggered a novel concept and, presently, nitrite is considered as an NO "storage form" that can be made available to maintain the NO formation and ensure cell functioning under conditions of hypoxia/anoxia, precisely when the dioxygen-dependent NOS

activity is impaired. The physiological roles of nitrite-derived NO include vasodilation [121–125], regulation of gene expression [126], smooth muscle proliferation [104], angiogenesis [124], and, most important, regulation of mitochondrial respiration and energy production [127–132]. Thus, through the nitrite/NO "recycling" pathway, an organ under ischaemia can maintain (or even increase) the blood flow, modulate the dioxygen distribution and the reactive oxygen species formation and, at the same time, maintain an anti-inflammatory and anti-apoptotic environment.

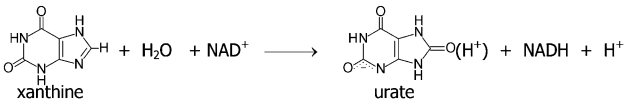
All the experimental evidence pointing towards a cytoprotective role for nitrite imposes the question of "who" reduces nitrite to NO in mammals. To date, no "dedicated" mammalian NiR has been identified. On the contrary, nitrite reduction to NO has been ascribed only to prokaryotic organisms that use d_1 haem-containing NiR and copper-containing NiR enzymes to derive energy, via denitrification, anaerobic ammonium oxidation and other related respiratory pathways [34–42]. However, in recent years, several mammalian metalloproteins, present in cells to carry out other functions, were shown to be able to reduce nitrite to NO ("non-dedicated" NiR): the molybdenum-containing enzymes, XO, AO, SO and mARC (here reviewed), a growing number of haem-containing proteins [127, 133–141], where haemoglobin and myoglobin stand out in a number of publications [122, 129, 142–150] and several other metalloproteins (e.g. [152–154]).

Molybdenum-dependent nitrite reduction in mammals

Xanthine oxidase/dehydrogenase and aldehyde oxidase Mammalian XO is a key enzyme in the catabolism of purines, where it catalyses the hydroxylation of both hypoxanthine and xanthine to urate, the terminal metabolite in humans and other mammals [3, 23–28]. The physiological function of mammalian AO remains a matter of debate, being a probable partner in the metabolism of neurotransmitters and retinoic acid [155–158]. In addition, the XO and AO ability to catalyse also the oxidation of a wide variety of aldehydes and substituted pyridines, purines, pteridines and related compounds, including NADH [159–169], has suggested their involvement in the xenobiotic metabolism. Furthermore, the enzymes' ability to catalyse the reduction of dioxygen has proposed their involvement in hydrogen peroxide-mediated signalling cascades [170–172] and, most pertinent, in several reactive oxygen species (ROS)-mediated diseases (when the cellular antioxidant defences cannot cope with the overproduction of ROS), accounting, in this way, for the extensively documented XO pathological role [166, 167, 173–197]. The proposed roles 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 both enzymes can also catalyse 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 theses enzymes are being thought: from deleterious ROS sources to beneficial NO generators.

Both enzymes are found in the cytoplasm of various tissues [198–206]. Noteworthy, besides the cytoplasm [199, 202], XO was described to be also present on the outer surface of the cell membrane of epithelial and endothelial cells [207–214] and of erythrocytes [95, 117]. In vivo, AO exists exclusively as an oxidase (reduces dioxygen, not NAD^+; Eq. 18) [157, 215], whereas XO exists predominantly as an NAD^+-dependent dehydrogenase, named xanthine dehydrogenase (XD; Eq. 19) [3, 23–28]. Yet, XD can be rapidly converted into a "strict" oxidase form that reduces dioxygen instead of NAD^+, the very well studied XO (Eq. 20). This conversion can be either reversible, through oxidation of Cys_{535} and Cys_{992}, or irreversible, by limited proteolysis after Lys_{551} or Lys_{569} (bovine enzyme numbering) [26, 216–223]. Accordingly, it has been suggested that, while XD is the predominant intracellular form, XO predominates extracellularly, due to the action of plasma proteases [210, 224].

$$\text{Aldehyde} + H_2O + O_2 \rightarrow \text{carboxylate} + n O_2^{\cdot -} + m H_2O_2 \quad (18)$$



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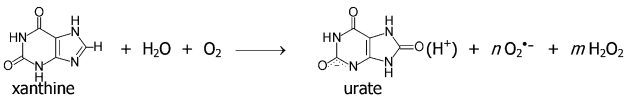

(20)

XO/XD and AO are structurally very similar. Both are complex homodimeric molybdoenzymes of the XO family (Fig. 1) that harbour (per monomer) one identical molybdenum centre, where the hydroxylation reactions occur, two [2Fe–2S] centres and one FAD, responsible for the reduction of dioxygen (XO, AO) or NAD^+ (XD) [3, 23–28, 220, 225–227]. Both molybdenum centres hold the molybdenum atom coordinated in the characteristic distorted square-pyramidal geometry, with an apical oxo group and with the four equatorial positions occupied by one essential sulfo group, one labile hydroxyl group and two sulfur atoms of the pyranopterin cofactor molecule (the cofactor

is found in the simplest monophosphate form characteristic of eukaryotes) (Fig. 2a). The molecular mechanism of XO[5] and AO-catalysed hydroxylation reactions is presently well established [3, 23–28, 225, 229]: (1) the hydroxylation catalysis is initiated with the activation of the molybdenum labile hydroxyl group (Mo–OH) by a neighbouring conserved deprotonated glutamate residue, to form an Mo^{6+}–O^{-}(=S) core (base-assisted catalysis); (2) it follows the nucleophilic attack of Mo–O^{-} on the carbon atom to be hydroxylated, with the simultaneous hydride transfer from the substrate to the essential sulfo group (Mo=S → Mo–SH), resulting in the formation of a covalent intermediate, Mo^{4+}–O–C–R(–SH) (where R represents the remainder of the substrate molecule); (3) the subsequent hydrolysis of the Mo–O bond releases the hydroxylated product and yields a Mo^{4+}–$OH_{(2)}$(–SH) core (oxidation half-reaction); (4) finally, the two electrons transferred from the substrate to the molybdenum are rapidly transferred, via the Fe/S centres, to the FAD, where the dioxygen or NAD^{+} reduction takes place (reduction half-reaction); (5) in the now oxidised molybdenum centre, the sulfo group is deprotonated and the initial Mo^{6+}–OH(=S) core is regenerated.

Besides the dioxygen and NAD^{+} reduction, XO/XD and AO catalyse also nitrite reduction, at their molybdenum centres, thus being able to contribute to the NO generation in mammals.

In vitro, under anaerobic conditions, XO [230–237] and AO [235, 237, 238] catalyse nitrite reduction to NO (Table 1). That NO is the product of nitrite reduction was independently confirmed by several methodologies (NO-selective electrode, EPR spectroscopy using different spin-trap types, chemiluminescence assays) [230, 231, 233, 236]. The nitrite reductase/NO synthase activity that has been demonstrated for enzymes purified from bovine milk, rat liver and also human liver is dependent on the simultaneous presence of enzyme, nitrite and a reducing substrate [230–238].

The nature and site of reaction of the reducing substrate do not alter the outcome of the reaction. NO generation can be triggered by aldehydes and heterocyclic compounds [such as xanthine (XO) and *N*′-methylnicotinamide (AO)], which react at the enzymes' molybdenum centre, and also by NADH that reacts at the FAD centre (Table 1). The XD-catalysed nitrite reduction was also recently demonstrated for the first time and it was confirmed that XD displays kinetic parameters similar to those of XO (Table 1) as expected (see footnote 1) [237].

Nitrite reduction occurs at the enzymes' molybdenum centre, as definitively demonstrated with a combination of spectroscopic and electrochemical methods (EPR to follow molybdenum oxidation and the environment and redox status of the other redox centres; a selective NO electrode to measure NO formation). Those studies were carried out using the XO/XD molybdenum-specific inhibitor allopurinol [236]. XO/XD hydroxylates allopurinol to oxypurinol (1*H*-pyrazolo [3,4-*d*]pyrimidine-4-ol to the corresponding 4,6-diol compound), which binds tightly to the reduced molybdenum, thus blocking it and inhibiting all reactions that occur at the molybdenum centre of XO/XD [239–241]. The formation of the oxypurinol–XO/XD complex, however, does not interfere with any reaction taking place at the Fe/S or FAD, as shown by (1) the NADH oxidation by molecular oxygen (that occurs at the flavin site) in the presence of the inhibitor [168] and (2) the EPR spectra of oxypurinol-inhibited NADH-reduced enzyme that display the characteristic Fe/S and FAD EPR signals (showing that those centres are not affected by inhibitor treatment) [236]. In view of this, if nitrite reduction occurs at the Fe/S or flavin sites, then the NADH-reduced XO/XD would be able to reduce nitrite in the presence of allopurinol. However, no NO formation is observed in the presence of the inhibitor [236]. Additional assays with deflavo-XO and deflavo-AO[6] and with native DPI-inhibited enzymes (a FAD-specific inhibitor), all of which displayed the same nitrite reductase activity as the native enzymes, further confirmed the exclusive participation of the molybdenum centre in nitrite reduction [237]. Also, the use of native enzymes with different AFR values[7] corroborated this conclusion [236, 237]. Simultaneously, using NADH-reduced desulfo-XO,[8] it was demonstrated that the molybdenum sulfo group is

[5] Mammalian XO and XD are two forms of the same protein (same gene product). Mammalian XO enzymes are synthesised as an NAD^{+}-dependent dehydrogenase form, the XD, and are believed to exist mostly as XD under normal physiological conditions [3, 27–32]. However, the XD form can be readily converted into a strict oxidase form, the XO. The only "functional" distinction between XD and XO lies in 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. During the XD into XO conversion process (through oxidation of cysteine residues or limited proteolysis), the protein conformation at the FAD centre is modified and this conformational alteration is responsible for the differentiated oxidising substrate specificity displayed by XO and XD [30, 230–237, 239, 242] (note that both dioxygen and NAD^{+} react at the FAD centre). On the other hand, the protein structure at the Fe/S and molybdenum centres is not changed during the conversion and, in accordance, the two enzyme forms, XO and XD, are virtually identical with respect to the binding and catalysis of substrates at the molybdenum centre [3, 27–30]. This is also the case of the nitrite reduction reaction that, as will be described, occurs at the molybdenum centre. For these reasons, XO and XD can be considered as one unique enzyme for the discussion of the overall structural organisation and molybdenum reactivity (reaction mechanism).

[6] Enzyme forms whose FAD centre was chemically removed.

[7] AFR, activity-to-flavin ratio, is a measure of the number of enzyme molecules with an intact molybdenum centre [3, 27–32, 230, 256].

[8] Enzyme form whose molybdenum sulpho group was chemically removed.

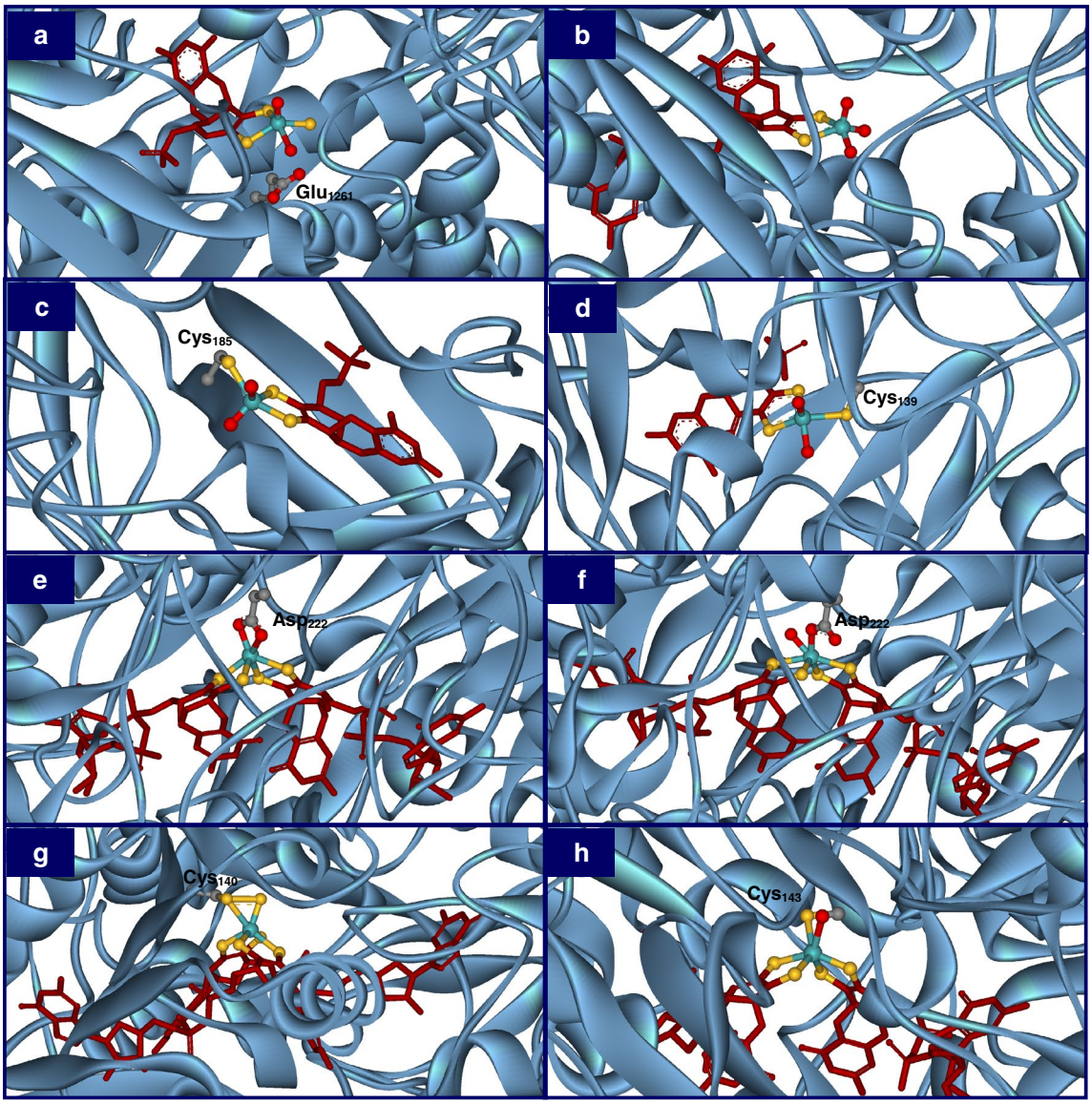

Fig. 2 Three-dimensional structure view of the molybdenum centre and neighbouring protein of **a** bovine XO, **b** *D. gigas* AOR, **c** chicken SO, **d** *P. angusta* NaR, **e** and **f** *E. coli* respiratory NaR, **g** *D. desulfuricans* periplasmatic NaR and **h** *E. coli* periplasmatic NaR. In the case of XO, it also explicitly represented the conserved glutamate residue. The structures shown are based on the PDB files 1FO4 (**a**), 1VLB (**b**), 1SOX (**c**), 2BIH (**d**), 1Q16 (**e**), 1R27 (**f**), 2NAP (**g**) and 2NYA (**h**); the pyranopterin cofactor is represented in dark red. The images were produced with Accelrys DS Visualizer, Accelrys Software Inc.

necessary for nitrite reductase activity (further discussed under "Mechanistic strategies for molybdenum-dependent nitrite reduction"), thus, providing another confirmation of the involvement of the molybdenum centre [236, 237]. Furthermore, it was also demonstrated that the NO formed during the catalytic cycle is not able to significantly react with the molybdenum sulfo group and inhibit the enzymes [236, 237].

The XO-, XD- and AO-catalysed NO formation is dependent on the pH, with the highest pseudo-first-order constants (k_{cat}/K_m) being observed at pH ≈6.3 (2.2 and 1.6 × 10^3 $M^{-1}s^{-1}$, for XO and AO), which corresponds to an increase of ≈8 times, relatively at pH 7.4 (Table 1) [237]. In addition, while the k_{cat} curves followed the same pH profile, indicating that also the highest rates of NO formation occur at pH values between 5.8 and 6.8, the K_m values for nitrite displayed an inverted bell-shaped pH curve, decreasing significantly, ≈5–6 times, for pH values lower than 6.8 (relatively to pH 7.4), with minima values of 600 and 1.8 mM, for XO and AO, respectively [237]. Hence, under pH 5.8–6.8, not only the pseudo-first-order rate constants reach their maxima values, but also the K_m values for

Table 1 Mammalian molybdoenzymes kinetic parameters for nitrite reduction/NO formation

Enzyme	Assay conditions		Kinetic parameters				
	Reducing substrate pH	Assay conditions	k_{cat} (s^{-1})	$K_m^{NO_2^-}$ (mM)	$k^{NO_2^-}$ ($M^{-1}s^{-1}$)	$K_m^{red\ subst}$ (μM)	$k^{red\ subst}$ ($M^{-1}s^{-1}$)
XO	Aldehyde pH 7.4	Rat liver XO Δ [nitrite], Δ [aldehyde], pH 7.4 [237]	0.99	3.6	275	43	23×10^3
		Bovine milk XO Δ [nitrite], Δ [aldehyde], pH 7.4 [236]	0.69	0.59	**1.2×10^3**	23	30×10^3
	Aldehyde pH 6.3	Rat liver XO Δ [nitrite], Δ [aldehyde], pH 6.3 [237]	1.5	0.67	**2.2×10^3**	78	19×10^3
AO	Aldehyde pH 7.4	Rat liver AO Δ [nitrite], Δ [aldehyde], pH 7.4 [237]	1.9	9.7	196	91	21×10^3
	Aldehyde pH 6.3	Rat liver AO Δ [nitrite], Δ [aldehyde], pH 6.3 [237]	3.4	2.1	**1.6×10^3**	204	17×10^3

Enzyme	Assay conditions		Apparent kinetic parameters for nitrite			Apparent kinetic parameters for reducing substrate		
	Reducing substrate	Assay conditions	$k_{cat}^{NO_2^-, app}$ (s^{-1})	$K_m^{NO_2^-, app}$ (mM)	$k^{NO_2^-, app}$ ($M^{-1}s^{-1}$)	$k_{cat}^{red\ subst, app}$ (s^{-1})	$K_m^{red\ subst, app}$ (μM)	$k^{red\ subst, app}$ ($M^{-1}s^{-1}$)
XO	Aldehyde pH 7.4	Human liver XO Δ [nitrite], 50 μM aldehyde, pH 7.4 [237]	0.41	2.2	186	–	–	–
		Rat liver XO Δ [nitrite], 50 μM aldehyde, pH 7.4 [237]	0.55	1.9	289	–	–	–
		Bovine milk XO Δ [nitrite], 50 μM aldehyde, pH 7.4 [233]	0.85	2.6	327	–	–	–
		Bovine milk XO Δ [nitrite], 50 μM aldehyde, pH 7.4 [236]	0.46	2.3	200	–	–	–
		Human liver XO Δ [aldehyde], 1 mM nitrite, pH 7.4 [237]	–	–	–	0.20	9.6	21×10^3
		Rat liver XO Δ [aldehyde], 1 mM nitrite, pH 7.4 [237]	–	–	–	0.21	9.4	22×10^3
		Bovine milk XO Δ [aldehyde], 1 mM nitrite, pH 7.4 [233]	–	–	–	0.34	35	9.7×10^3
		Bovine milk XO Δ [aldehyde], 1 mM nitrite, pH 7.4 [236]	–	–	–	0.37	89	4.2×10^3
	Aldehyde pH 6.3	Human liver XO Δ [nitrite], 750 μM aldehyde, pH 6.3 [237]	1.2	0.67	**1.8×10^3**	–	–	–
		Rat liver XO Δ [nitrite], 50 μM aldehyde, pH 6.3 [237]	0.581	0.25	**2.3×10^3**	–	–	–
		Rat liver XO Δ [aldehyde], 1 mM nitrite, pH 6.3 [237]	–	–	–	0.90	52	17×10^3
	NADH pH 7.4	Rat liver XO Δ [nitrite], 1 mM NADH, pH 7.4 [237]	0.19	2.1	90	–	–	–
		Bovine milk XO Δ [nitrite], 1 mM NADH, pH 7.4 [233]	0.28	2.3	122			
		Rat liver XO Δ [NADH], 1 mM nitrite, pH 7.4 [237]	–	–	–	0.074	259	286
		Bovine milk XO Δ [NADH], 1 mM nitrite, pH 7.4 [233]	–	–	–	0.17	878	194
	NADH pH 6.3	Rat liver XO Δ [nitrite], 1 mM NADH, pH 6.3 [237]	0.10	0.35	286	–	–	–
		Rat liver XO Δ [NADH], 1 mM nitrite, pH 6.3 [237]	–	–	–	0.19	1.5×10^3	127
	Xanthine pH 7.4	Rat liver XO Δ [nitrite], 10 μM xanthine, pH 7.4 [237]	2.3	3.0	**767**	–	–	–
		Bovine milk XO Δ [nitrite], 5 μM xanthine, pH 7.4 [233]	2.5	2.5	**1.0×10^3**	–	–	–
		Bovine milk XO Δ [xanthine], 1 mM nitrite, pH 7.4 [233]	–	–	–	0.68	1.5	453×10^3
XD	Aldehyde pH 7.4	Rat liver XD Δ [nitrite], 50 μM aldehyde, pH 7.4 [237]	0.51	2.0	255	–	–	–
		Rat liver XD Δ [aldehyde], 1 mM nitrite, pH 7.4 [237]	–	–	–	0.23	9.7	24×10^3
	Aldehyde pH 6.3	Rat liver XD Δ [nitrite], 750 μM aldehyde, pH 6.3 [237]	1.3	0.62	**2.1×10^3**	–	–	–

Table 1 continued

Enzyme	Assay conditions		Apparent kinetic parameters for nitrite			Apparent kinetic parameters for reducing substrate		
	Reducing substrate	Assay conditions	$k_{cat}^{NO_2^-, app}$ (s^{-1})	$K_m^{NO_2^-, app}$ (mM)	$k^{NO_2^-, app}$ ($M^{-1}s^{-1}$)	$k_{cat}^{red\ subst, app}$ (s^{-1})	$K_m^{red\ subst, app}$ (μM)	$k^{red\ subst, app}$ ($M^{-1}s^{-1}$)
AO	Aldehyde pH 7.4	Human liver AO Δ [nitrite], 50 μM aldehyde, pH 7.4 [237]	0.47	4.1	115	–	–	–
		Rat liver AO Δ [nitrite], 50 μM aldehyde, pH 7.4 [237]	0.67	3.6	186	–	–	–
		Rat liver AO Δ [nitrite], 50 μM aldehyde, pH 7.4 [238]		3.3		–	–	–
		Human liver AO Δ [aldehyde], 1 mM nitrite, pH 7.4 [237]	–	–	–	0.13	27	4.8×10^3
		Rat liver AO Δ [aldehyde], 1 mM nitrite, pH 7.4 [237]	–	–	–	0.17	11	15×10^3
		Rat liver AO Δ [aldehyde], 1 mM nitrite, pH 7.4 [238]	–	–	–		9.6	
	Aldehyde pH 6.3	Rat liver AO Δ [nitrite], 50 μM aldehyde, pH 6.3 [237]	0.66	0.43	**1.5×10^3**	–	–	–
		Rat liver AO Δ [aldehyde], 1 mM nitrite, pH 6.3 [237]	–	–	–	1.1	70	16×10^3
	NMN[a] pH 7.4	Rat liver AO Δ [nitrite], 400 μM NMN, pH 7.4 [237]	2.0	2.0	**1.0×10^3**	–	–	–
	NADH pH 7.4	Rat liver AO Δ [nitrite], 100 μM NADH, pH 7.4 [238]		2.7		–	–	–
		Rat liver AO Δ [NADH], 1 mM nitrite, pH 7.4 [238]	–	–	–		24	
mARC	NADH pH 7.4	Human mARC Δ [nitrite], 1 mM NADH, cyt. b_5 plus cyt. b_5 reductase, pH 7.4 [296]	0.1	9.5	11	–	–	–
		Human mARC Δ [NADH], 1 mM nitrite, cyt. b_5 plus cyt. b_5 reductase, pH 7.4 [296]	–	–	–	0.7×10^{-3}	6.5	108
SO	Sulfite pH 7.4	Human SO Δ [nitrite], 5 μM sulfite, pH 7.4 [316]	0.002	1.6	1.3	–	–	–
		Modified human SO harbouring only the molybdenum domain Δ [nitrite], 5 μM sulfite, pH 7.4 [316]	0.008	0.97	8.2	–	–	–
	Sulfite pH 6.5	Human SO Δ [nitrite], 5 μM sulfite, pH 6.5 [316]	0.004	1.7	2.4	–	–	–
		Modified human SO harbouring only the molybdenum domain Δ [nitrite], 5 μM sulfite, pH 6.5 [316]	0.014	0.50	28	–	–	–
	Phenos. pH 7.4	Human SO Δ [nitrite], 40 μM phenosafranine, pH 7.4 [316]	1.9	80	24	–	–	–

The highest values of nitrite specificity constant ($K^{NO_2^-}$) are presented in bold

[a] *N*′-methyl-nicotinamide

nitrite are minimised. These results are of major importance for the potential in vivo role of XO-/XD-/AO-dependent NO generation under ischaemia, when the pH can decrease to values as low as 6.0–5.5 (acidosis) [243–249] for two reasons. (1) The pseudo-first-order rate constant refers to the reaction rate when nitrite is present at a concentration much lower than its K_m. Because the in vivo nitrite concentration (<20 μM [53, 250–252]) is much lower than its K_m, the in vivo nitrite reduction would occur under conditions controlled by the pseudo-first-order rate constant (and not by the k_{cat}). In this scenario, it is significant that the highest pseudo-first-order rate constants are attained at the acidic pH values characteristics of ischaemia, precisely when a nitrite-dependent, NOS-independent, NO source would be needed. This pseudo-first-order rate constants' pH dependence would allow the enzymes to overcome the constraint imposed by the high K_m values/low nitrite availability. (2) Concomitantly, due to the pH-dependent decrease in the K_m for nitrite, lower nitrite concentrations would be needed to drive a similar rate of NO generation.

Nonetheless, and as can be foreseen, dioxygen and NAD^+, the "classic" oxidising substrates, act as strong competitive inhibitors of nitrite reduction, "stealing" the electrons needed to reduce nitrite [233, 234, 236, 237, 253]. Although the NAD^+ inhibition of the XD-dependent NO formation had not been yet studied, the dioxygen inhibition of XO/XD and AO reactions was characterised at pH 6.3 (Fig. 3a) [237]. The determined K_i values for dioxygen, ≈24 and ≈49 μM (for reducing substrate present at a concentration equal to its K_m and to 10 × K_m, respectively), are within the in vivo tissue dioxygen concentrations (≤50 μM, going from normoxia to hypoxia [254]). This suggests that the in vivo NO formation would not be completely abolished by dioxygen. Instead, the NO generation would be fine-tuned by the dioxygen availability, being amplified as the dioxygen concentration decreases towards the hypoxic and anoxic conditions. Furthermore, because the K_i values are modulated by the reducing substrate concentration (higher concentrations give higher K_i), the in vivo NO formation would also be controlled by the reducing substrates' availability. Hence, the ischaemia-induced reducing substrates accumulation could create enzyme "saturating" conditions, which would favour nitrite reduction and, at the same time, lead to lower dioxygen inhibitions.

The dioxygen inhibition was also studied in the presence of NADH. It has been argued that because NADH reacts at the enzymes FAD centre, the dioxygen inhibition would be inferior [234]. However, the K_i values for dioxygen obtained in the presence of NADH were within a similar range, ≈34 μM (for either 1 or 10 mM NADH), showing that the NO formation would not be favoured in the presence of NADH, compared to other reducing substrates as

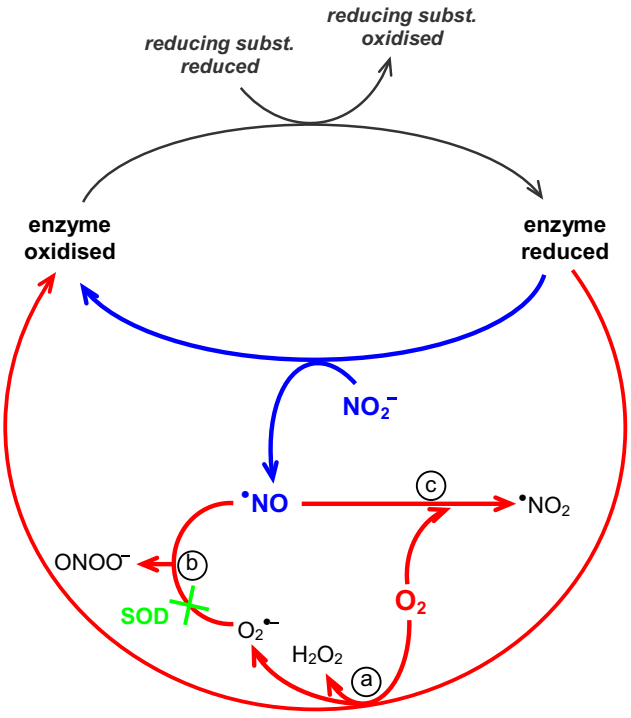

Fig. 3 Dioxygen effects on the NO status in XO/XD and AO systems. Dioxygen interferes with NO at different levels. *a* Dioxygen is efficiently reduced by the enzymes, consuming the electrons derived from the reducing substrates and, thus, reducing (inhibiting) NO formation. *b* Simultaneously, the superoxide anion radical formed reacts with NO to yield peroxynitrite. This dioxygen effect can be counteracted by the presence of SOD. *c* In addition, dioxygen can also react directly with NO to yield nitrogen dioxide radical and other products

aldehyde or xanthine [237]. From a physiological point of view, during an ischaemic event, the NO source would be dictated by the enzyme specificity for the different reducing substrates available, their concentrations and by the respective rate of NO formation. Although the NADH concentration would increase under ischaemia, the slow rates of NO generation in the presence of (patho)physiological NADH concentrations (<<3 mM [255–259] ≈K_m^{NADH} [233, 237]) suggest that the NADH-dependent NO formation would be smaller comparatively to hypoxanthine/xanthine that would also be accumulated (≤100 μM [260–263] > ≈$K_m^{xanthine}$ [233, 237]) and for which the NO generation rates are considerably higher (>20 times) [237].

Besides decreasing the amount of NO formed, dioxygen also decreases the amount of NO available to carry out the physiological functions (or to be detected in vitro), either through its direct reaction with NO (Fig. 3c) or indirectly through the reaction with superoxide radical anion (Fig. 3b). While the direct dioxygen reaction with NO is rather small, the NO consumption by superoxide radical in the absence of superoxide dismutase (SOD) is substantial, regardless of the dioxygen concentration present [237].

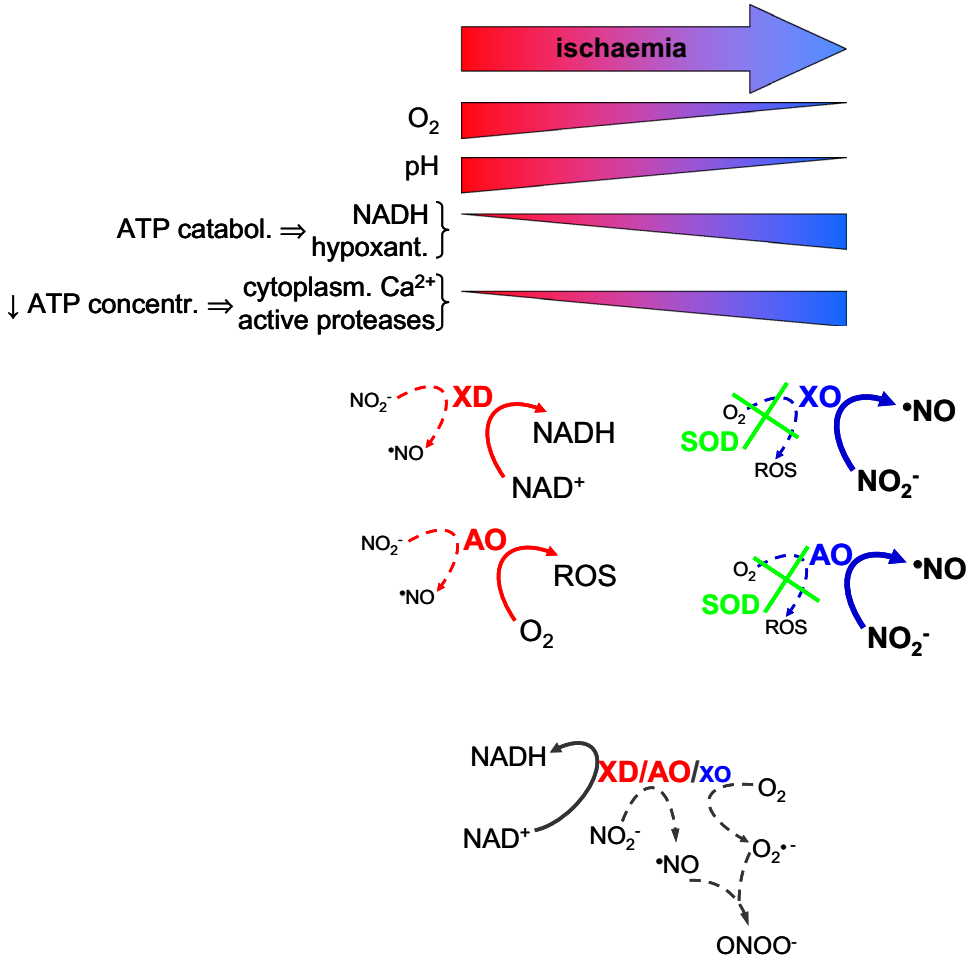

Fig. 4 Mechanism proposed for XO-/XD- and AO-dependent NO formation under ischaemia. See *text* for details. Modified from Ref. [46]

This "NO sink" is particularly relevant when the nitrite concentration is limiting, because the competition between nitrite and dioxygen is unfavourable to nitrite and more superoxide radical is formed. Most important, from the NO reaction with superoxide radical, the formation of the strong oxidising peroxynitrite results [48, 50, 264]. Those results highlight the critical role of SOD in achieving a net NO production in vivo and avoiding the formation of the deleterious peroxynitrite.

In summary, the in vitro studies suggest that the extent of XO/XD and AO-catalysed NO formation in vivo would be dependent on several factors. (1) Availability of reducing substrates—they provide the enzymes with the electrons needed to reduce nitrite and also modulate the extension of dioxygen inhibition. (2) Dioxygen availability—with K_i values within the physiological dioxygen concentrations, from normoxia to hypoxia, dioxygen would fine-tune the nitrite-dependent NO formation, being the probable factor that regulates and links the two NO sources, nitrite-dependent and NOS-dependent. (3) Presence of SOD—crucial to achieve a net NO production under non-anoxic conditions. (4) NAD^+—NAD^+ inhibition has not yet been studied, but it could have a marked impact on the XD-dependent NO formation. (5) Acidic conditions (pH ≤6.8)—greatly favour nitrite reduction (6) and, of course, nitrite availability.

In light of what is known from in vitro studies, during an ischaemic event, several phenomena occur that, in concert, can favour nitrite reduction by XO/XD and AO (Fig. 4): first, and obviously, the decrease in dioxygen concentration (hypoxia or even anoxia) results in acidosis (pH values as low as 6.0–5.5 [243–249]); second, in the course of ischaemia, as the mitochondrial electron transfer chain begins to be affected, ATP synthesis would be hindered and the subsequent ATP catabolism leads to an accumulation of hypoxanthine and NADH in tissues [255–263]. These reducing substrates' increase can "fuel" the enzymes with reducing equivalents to reduce nitrite; third, as the ATP concentration decreases, the transmembrane 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 [214, 265–271]. Hence, the formerly prevailing XD form (that reacts with NAD^+) would be converted into the "dioxygen-user" XO, by proteolysis; NAD^+ (regardless of its high

concentration) would be no longer a competitive substrate of nitrite reduction, because XO and AO do not react with it. Therefore, all the conditions seem to be gathered for XO- and AO-catalysed NO formation to be feasible and reasonable during in vivo ischaemia.

In accordance with the above reasoning, numerous in situ and in vivo studies suggested that XO/XD and AO-dependent NO formation can, in fact, occur in vivo, namely several studies with tissue homogenates (heart, aorta and liver) [234, 235] and with animal models of myocardial infarction [85], renal [86], cardiac [77] and liver [80] ischaemia/reperfusion injury, among many others [79, 89, 95, 97, 104, 113, 116, 117, 141, 231, 238, 272–276]. Those results 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 [77].

In spite of those in situ and in vitro studies, some authors argue that the high K_m values for nitrite [≈250 μM [236, 237]—2 mM [233], for XO, and ≈430 μM [237]—3 mM [238], for AO (Table 1)], 1–2 orders of magnitude higher than the nitrite concentration in tissues (<20 μM [53, 250–252]), are a major drawback for the relevance of these molybdenum-dependent pathways on the in vivo NO formation. However, the kinetic parameters indicate that these enzymes can produce NO, with reasonable rates [$k_{cat}/K_m \approx 2 \times 10^3$ $M^{-1}s^{-1}$, for rat liver enzymes at pH ≈6.3 [237] (Table 1)] that would be modulated by the availability of nitrite [233, 236, 237], i.e. by functioning in a concentration range well below the K_m value, the reaction rate is pseudo-first order on nitrite, thus allowing the NO generation to be directly controlled by the nitrite availability. In addition, in this way, the competitive inhibition carried out by dioxygen that displays K_i values in the range of its own physiological concentrations is more effective (the competition between nitrite and dioxygen, in vivo, is unfavourable to nitrite, because the reaction runs under nitrite-limiting conditions, but dioxygen-"sufficient" conditions). This means that the NO formation would be also fine-tuned by the dioxygen concentration and confers another level of regulation to the XO/AO-dependent NO generation.

It should be emphasised that the concentration of NO must be kept within the characteristics of a local signalling molecule and highly regulated. It is not conceivable to produce NO at micromolar or millimolar concentrations (the enzymes K_m order), when NO carries out its physiological functions at nanomolar concentrations. 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 as being formed [48, 50]). Thus, if these molybdoenzymes are to be physiologically relevant NO sources, they should not catalyse the formation of NO at the nitrite K_m concentration values [236, 237].

Another argument against the occurrence of these pathways in vivo is related with 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 artefact), to a small (20 %) and slow conversion [193, 198, 265–267, 269–271] and a conversion that is enhanced by hypoxic conditions and in vivo ischaemia [214, 268]. The issue here is the competition between nitrite and NAD^+ to react with reduced XD. The NAD^+ concentration (≈0.5–1 mM [255, 256, 277–280]), two to three orders of magnitude higher than the one of NADH, is not significantly decreased by the NADH accumulation during ischaemia [255–259]. 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 [281]) would prevail over nitrite reduction, and NO formation by this protein would be seriously compromised. Nonetheless, this would not hinder the NO generation by AO or by the XO present on the outer surface of the cell membrane of epithelial and endothelial cells [207–214] and of erythrocytes [95, 117], where plasma proteases were suggested to convert XD into XO [210, 224].

Finally, and as already extensively discussed, the competition between nitrite and dioxygen is certainly the critical limitation for the effective XD-/XO-and AO-dependent NO generation in vivo. On top of all those chemical and kinetic constraints, the proposed role of XO/XD as an NO source faces another obstacle: for long, countless studies have pointed towards a beneficial clinical outcome upon an ischaemic or related event through the inhibition of XO/XD (reduction of symptoms by treatment with allopurinol) [173, 174, 194, 282]. How can those numerous experimental evidences be reconciled with a beneficial XO-/XD-mediated role [283]?

Overall, in vitro, under anaerobic conditions, mammalian XO, XD and AO are able to reduce nitrite to NO. The NO formation can also be achieved in the presence of dioxygen, as long as SOD is also present. In vivo, however, the XO-/XD-/AO-catalysed NO formation would be dependent on the extent of ischaemia (extent of hypoxia), the co-presence of SOD and other antioxidants and by the availability of reducing and oxidising substrates, in particular dioxygen.

Mitochondrial amidoxime-reducing component mARC was first isolated in 2006 and identified as the fourth mammalian molybdoenzyme (after XO/XD, AO and SO) [284]. This SO family member, present in virtually all mammals as two isoforms, harbours only the molybdenum centre (with no additional redox centres) and is found anchored

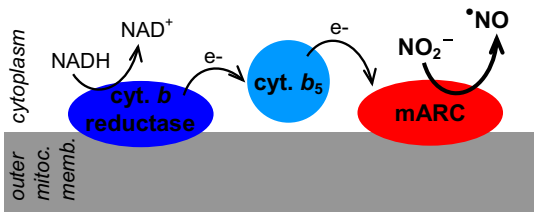

Fig. 5 Schematic representation of the mARC-containing enzymatic system, comprising mARC, cytochrome b_5 (cyt. b_5) and NADH-dependent cytochrome b_5 reductase

to the outer mitochondrial membrane, facing the cytoplasm [285–288]. mARC is the catalytic partner of a three-protein amidoxime-reducing chain that comprises also cytochrome b_5 and NADH-dependent cytochrome b_5 reductase, which are involved in electron transfer from NADH to the terminal oxidoreductase mARC (Fig. 5) [285, 291]. The mARC-containing enzymatic system is responsible for the aerobic reductive activation of several *N*-hydroxylated prodrugs such as amidoximes, *N*-hydroxy-guanidines or sulphohydroxamic acids [285, 289, 292]. However, its physiological function is not known, being probably involved in detoxification of mutagenic and toxic aromatic hydroxyl-amines, such as *N*-hydroxylated DNA base derivates [293, 294]. In addition, mARC has also been associated with lipid synthesis in adipocyte [295] and regulation of NOS-dependent NO synthesis, as N^{ω}-hydroxyl-L-arginine (Eq. 16, 17) can be reduced by mARC [287]. Very recently, the mARC-containing enzymatic system was also shown to catalyse the reduction of nitrite to NO, at the molybdenum centre, using NADH as reducing substrate, thus constituting an additional mammalian nitrite-dependent NO source [296].

The mARC-catalysed nitrite reduction/NO formation is remarkably similar to the XO/XD and AO ones, although it displays a considerably higher K_m value for nitrite (9.5 mM), associated with a lower pseudo-first-order rate constant ($k_{cat}/K_m = 11\ M^{-1}s^{-1}$) (Table 1) [296]. Yet, also mARC-dependent NO generation is dramatically decreased in the presence of dioxygen, probably due to (1) molybdenum oxidation by dioxygen (parallel to the competitive inhibition of XO/XD/AO, where dioxygen consumes the electrons needed to reduce nitrite[9]) and (2) to NO consumption by the superoxide radical formed at the FAD centre of cytochrome b_5 reductase (also parallel to what occurs in the XO/XD/AO systems). In addition, also nitrite reduction by mARC is favoured under acidic conditions, with the NO formation being increased (≈3-fold) when the pH is lowered from 7.5 to 6.5.

[9] Although in XD/XO/AO, the electrons consumption by dioxygen is made via the FAD centre.

Nitrite reduction to NO takes place at the mARC molybdenum centre, as demonstrated by mutation of the putative cysteine residue that coordinates to the molybdenum centre (characteristic of SO family enzymes (Fig. 1); Cys_{273} of human mARC1) [296]. Mutation of cysteine to an alanine residue would create an inactive tri-oxo molybdenum centre, as occurs in SO [297], and, in agreement, mutation abolished the NO formation, as well as the amidoxime reductase activity. Further confirmation was obtained with tungsten-substituted enzyme that displays no nitrite reductase activity [296].

The mARC-catalysed nitrite reduction pathway can contribute to the in vivo NO formation, under hypoxic conditions, when the dioxygen and pH are diminished and the increase in the NADH concentration "fuels" the enzyme with reducing equivalents—in a parallel situation to what was described above for XD/XO and AO. Hence, mARC can represent an additional pathway for the synthesis of signalling NO in the cytoplasm (note that mARC is located on the outer mitochondrial membrane, but facing the cytoplasm). However, because nitrite transport across membranes is limited, it is possible that mitochondria uses the cytoplasm-faced enzyme to synthesise NO that would be, subsequently, "internalised". More studies are needed to determine how mARC-dependent NO affects the mitochondrial function [296]. Yet, it is tempting for us to speculate that mARC is in an adequate location to be part of a signal transduction system, "transmitting" a mitochondrial signal to the cytoplasm.

Sulfite oxidase Mammalian SO[10] is a key enzyme in the catabolism of sulfur-containing amino acids and in the metabolism of xenobiotic sulfur-containing compounds, catalysing the oxidation of toxic sulfite to sulfate with the simultaneous reduction of cytochrome *c* (Eq. 21) [298].[11] Confirming its vital role in the detoxification of sulfite, human SO deficiency[12] causes severe neonatal neurological problems and early death [299–302]:

$$SO_3^{2-} + 2\text{cyt.}c\left(Fe^{3+}\right) + H_2O \rightarrow OSO_3^- + 2\text{cyt.}c\left(Fe^{2+}\right) + 2H^+. \quad (21)$$

Mammalian SO is found in the intermembrane space of mitochondria of virtually all mammalian tissues, being present in high concentrations in liver [303]. This is a homodimeric molybdoenzyme of the SO family (Fig. 1) that

[10] Because the enzyme does not catalyse the sulphite oxidation by molecular oxygen, a more appropriate name (Enzyme Nomenclature Commitee, IUBMB) would be sulphite oxidoreductase (SOR).

[11] See Kappler and Enemark's contribution in this JBIC issue.

[12] Caused by the inability to synthesise the pyranopterin cofactor or certain point mutations.

harbours (per monomer) one molybdenum centre, where sulfite oxidation reaction takes place, and one *b* haem responsible for cytochrome *c* binding and reduction [304]. The molybdenum centre[13] holds the molybdenum atom coordinated in the characteristic distorted square-pyramidal geometry, with an apical oxo group and with the equatorial positions occupied by one labile oxo group, one cysteine sulfur atom and two sulfur atoms from one pyranopterin cofactor molecule (the cofactor is found in the simplest monophosphate form, as is characteristic of eukaryotes) (Fig. 2b) [304]. Remarkably, the crystal structure of SO showed that the molybdenum and haem centres are more than 30Å apart [304]. Hence, it has been proposed that during catalysis a conformational alteration takes place (through a flexible polypeptide that links the two domains) that brings the two centres into greater proximity, to allow the rapid intramolecular electron transfer kinetics [305–307]. Furthermore, the haem domain can be hydrolysed from the protein by limited proteolysis, yielding a modified SO enzyme (harbouring only the molybdenum domain) that cannot transfer electrons to cytochrome *c*, but that is still able to catalyse the oxidation of sulfite in the presence of an artificial electron acceptor [308, 309].

SO-catalysed sulfite oxidation is believed to be a simple oxotransfer reaction, with the molybdenum centre acting as an oxygen atom donor [310–315]: (1) catalysis is initiated at the oxidised molybdenum centre (Mo^{6+}), with sulfite binding to the molybdenum equatorial labile oxo/hydroxyl group (Mo=O/Mo–OH), resulting in the two-electron reduction of the molybdenum atom ($Mo^{6+} \rightarrow Mo^{4+}$) and formation of a covalent intermediate, Mo^{4+}–O–SO_3; (2) the subsequent hydrolysis of the Mo–O bond releases the product (sulfate) and yields an Mo^{4+}–$OH_{(2)}$ core (oxidation half-reaction); (3) finally, the two electrons transferred from the substrate to the molybdenum are intramolecularly transferred, one at a time, to the haem, where cytochrome *c* will be reduced and the initial Mo^{6+}=O core is regenerated (reduction half-reaction).

Besides cytochrome *c* reduction, SO was recently described to catalyse also nitrite reduction [316], thus showing that all the four mammalian molybdoenzymes can contribute to NO generation. The SO-catalysed NO formation occurs at the enzyme molybdenum centre, using sulfite as the reducing substrate [316]. The NO generation by SO is also pH dependent, but the pseudo-first-order rate constants (k_{cat}/K_m) are noticeably small, 1.3 and 2.6 $M^{-1}s^{-1}$, at pH 7.4 and 6.5, respectively, as a consequence of very low k_{cat} values (0.002 and 0.004 s^{-1}, respectively) (Table 1). The issue is that in SO, contrary to the other mammalian molybdoenzymes, only the fully reduced molybdenum centre (Mo^{4+}) is able to reduce nitrite (SO Mo^{5+} does not reduce nitrite to NO). Hence, after a first nitrite reduction cycle, the SO molybdenum centre ends up in a "non-productive" state ($Mo^{4+} \rightarrow Mo^{5+}$) that cannot be further oxidised by a new nitrite molecule, or be reduced by sulfite (that is a strict two-electron donor).[14] If, instead of the physiological reducing substrate (sulfite), an artificial one-electron donor (phenosafranine) is used, higher NO formation rates are observed, resulting in a k_{cat} value of 1.9 s^{-1} (Table 1) [316]. Nevertheless, due to the very high K_m value for nitrite (80 mM), the pseudo-first-order constant (24 $M^{-1}s^{-1}$) is still considerably lower than the ones of XO and AO [2.2 and 1.6 × 10^3 $M^{-1}s^{-1}$, respectively (Table 1)]. Accordingly, for SO to efficiently catalyse nitrite reduction (either in vitro and in vivo), an appropriate one-electron donor (to reduce Mo^{5+} to Mo^{4+}) or acceptor (to oxidise Mo^{5+} to Mo^{6+}) must be available to regenerate the enzyme (and allow it to re-react with nitrite or sulfite, respectively) [316].

In addition, it was also argued that hypoxic and/or reductive conditions could favour the SO-catalysed NO formation [316]. Under hypoxic and/or reductive conditions, the concentration of reduced cytochrome *c* (the SO physiological oxidant substrate) would be high and the re-oxidation of the SO *b* haem would be slower. This would inhibit the SO intramolecular electron transfer from the molybdenum to the *b* haem and, eventually, favour nitrite reduction [316]. In fact, nitrite reduction to NO by a modified SO, harbouring only the molybdenum domain, displays pseudo-first-order rate constants six to ten times higher than the native SO [k_{cat}/K_m of 8.2 and 28 $M^{-1}s^{-1}$, at pH 7.4 and 6.5, respectively, with k_{cat} of 0.008 and 0.014 s^{-1} (Table 1)], even though greatly lower than the ones of XO and AO [316].

In spite of those in vitro results, SO was shown to play a dominant role in nitrite reduction and guanylate cyclase activation in human fibroblasts (by comparison of cells from normal and SO-deficient patients) [316]. Clearly, the SO-dependent NO formation is more complex than the XO-/XD-, AO- and mARC-mediated generation and future studies will shed light on how SO can contribute to the mammalian NO formation.

[13] Presently, the structure of human SO is not known. However, because the sequence identity among the eukaryotic SO is very high, with 68 % identity (85 % similarity) between the chicken and human enzymes, the structure of chicken liver SO (Fig. 2b) is a good template for the human counterpart [321].

[14] Because SO is not regenerated during the nitrite reduction reaction (SO catalyses a single turnover reaction), the values above indicated as k_{cat} and K_m are better described as $k_{electron\ transfer}$ and K_d values [333].

Higher plants

Nitric oxide in plants

Plants must perceive and respond to numerous external abiotic and biotic challenges, as well as internal signals, and NO has been shown to be involved in the response to temperature, salt and drought stresses and herbicide treatment; disease resistance pathways; germination, flowering, root development, leaf senescence and stomatal closure, among other processes [317–357]. Yet, the plant NO formation and signalling pathways are, by far, less well characterised than the mammalian counterparts.

As in mammals, plants can also synthesise NO through oxidative and reductive pathways. The oxidative pathways are believed to produce NO through the oxidation of organic compounds such as polyamines [358, 359], hydroxylamine [360] and arginine [361–365]. Nevertheless, no NOS homologous, gene or protein, has yet been found in higher plants [317, 345, 364, 366–368]. In the reductive pathways, apparently the predominant ones, NO is formed at the expense of nitrite reduction [341, 360, 369, 370]. However, as opposed to mammals, nitrite reduction to NO in plants occurs in a different "scenario": nitrate and nitrite, both precursors and end products of signalling NO, are also normal substrates of the plant nitrogen assimilation pathway (Eq. 22), when nitrate is the main nitrogen source available. This has two consequences. First, when plants use nitrite to synthesise signalling NO, they must do it in a controlled and parallel way to the assimilatory nitrite reduction to ammonium. Second, in plants nitrite can accumulate to very high (millimolar) concentrations, in particular under hypoxia/anoxia [371–375], a situation clearly different from the modest (nano- to micromolar) nitrite concentrations found in mammalian tissues. These circumstances should make the "signalling" nitrite/NO metabolism more complex in plants.

$$\text{nitrate} \xrightarrow{\text{molybdenum-containing NaR}} \text{nitrite} \xrightarrow{\text{sirohaem-containing NiR}} \text{ammonium} \quad (22)$$

Like mammals, plants do not have a "dedicated" NO-forming NiR and nitrite reduction/NO formation has been ascribed to metalloproteins present in cells to carry out other functions, including several haemic proteins and the molybdenum-containing cytoplasmatic NaR. Noteworthy, the sirohaem-containing NiR, responsible for the assimilatory nitrite reduction to ammonium (Eq. 22), is not able to reduce nitrite to NO [42].

Molybdenum-dependent nitrite reduction in higher plants

Nitrate reductase Nitrite-dependent NO formation in plants has been ascribed mainly to NaR, which has been hypothesised to play a role similar to the one of mammalian constitutive NOS. NaR is responsible for the first and rate-limiting step of plant nitrate assimilation pathway (Eq. 22), where it catalyses the nitrate reduction to nitrite, with the simultaneous oxidation of NAD(P)H (Eq. 23) [376–379]. In accordance with its key role in nitrogen metabolism, NaR is highly regulated by complex transcriptional, translational and posttranslational mechanisms that respond to nitrogen, carbon dioxide and dioxygen availabilities, pH, temperature and light [379–384]. Remarkable, NaR is rapidly degraded in darkness (half-life of 6 h [384]).

Plant NaR is a homodimeric molybdoenzyme, belonging to the sulfite oxidase family (Fig. 1) that holds the distinctive square-pyramidal molybdenum centre, with an apical oxo group and with the equatorial positions occupied by one labile oxo group, one cysteine sulfur atom and two sulfur atoms from one pyranopterin cofactor molecule (the cofactor is found in the simplest monophosphate form, as is characteristic of eukaryotes) (Fig. 2d) [356–379, 385–389]. Besides the molybdenum centre, where the nitrate reduction takes place, plant NaR holds (per monomer) one *b* haem and one FAD centre that is involved in the NAD(P)H binding and oxidation. NaR-catalysed nitrate reduction is believed to be a simple oxotransfer reaction, with the molybdenum centre acting as an oxygen atom acceptor [377–379, 389]: (1) the electrons provided by NAD(P)H are introduced at the FAD and transferred intramolecularly to the molybdenum centre (oxidation half-reaction); (2) in the reduced molybdenum centre, the now protonated labile oxo group ($Mo^{6+}{=}O \rightarrow Mo^{4+}{-}OH$) is displaced by nitrate; (3) nitrate binds through one of its oxygen atoms to the reduced molybdenum, which promotes the O–N bond cleavage and release of nitrite, with regeneration of the initial $Mo^{6+}{=}O$ group (reduction half-reaction):

$$NO_3^- + NADH + H^+ \rightarrow NO_2^- + NAD^+ + H_2O. \quad (23)$$

Besides this well-established role on the reduction of nitrate, NaR from different plant species were shown to also catalyse the subsequent nitrite reduction to NO (Eq. 24) in vitro [341, 390–392]. Furthermore, and most pertinent in the context of eukaryotic enzymes, also the NaR from the fungus *Aspergillus* was shown to be able to reduce nitrite to NO [393]. Based on the suggested nitrate reduction mechanism, it is not difficult to envisage the oxygen atom abstraction from nitrite to yield NO, with the NaR molybdenum atom accepting the nitrite oxygen atom—precisely the same mechanism that was proposed for the XO family members' reaction [236, 237] (discussed under "Mechanistic strategies for molybdenum-dependent nitrite reduction"). This suggestion is further supported by a recent theoretical study that showed that both nitrate and nitrite are easily reduced by plant NaR (to nitrite and NO, respectively), although, as expected, nitrate is the

thermodynamically preferred substrate [394]. Furthermore, and most important, the evidences of in vivo NaR-dependent NO generation are numerous, including studies with: (1) transgenic plants expressing a permanently active NaR[15] that accumulate nitrite and show a high NO emission rate [341, 402–404]; (2) NaR knockout (*nia1* and *nia2* genes) plants that fail to emit NO or display NO effects upon elicitation [336, 406–413]; (3) inactive NaR (e.g. plants with tungstate supply instead of molybdate) [407, 408, 414–419]; (4) and others [334, 356, 358, 393, 420–429]. NaR-dependent NO formation has been suggested to be involved in processes such as stomatal closure [336, 346, 408, 430, 431], onset of germination [369], phenylpropanoid metabolism [432] or immune defence mechanisms (because pathogen signals induce NaR and increase NO formation—strikingly similar to the mammalian-inducible NOS) [409, 411, 413, 418, 419, 433]:

$$NO_2^- + 1/2NADH + 3/2H^+ \rightarrow {}^{\cdot}NO + 1/2NAD^+ + H_2O. \quad (24)$$

With NaR displaying two apparently divergent activities: (1) formation of nitrite to be subsequently assimilated in the form of ammonium and (2) consumption of nitrite to form NO (Fig. 6), the plant cell needs an additional mechanism to regulate this enzyme. Remarkably, as discussed for mammals, the dioxygen concentration is one of the factors that seems to control the two NaR activities. Under normoxic conditions, the cytoplasmatic nitrate availability (in millimolar range [434]) "auto-controls" the nitrite reductase activity of NaR, because nitrate competitively inhibits nitrite reduction (K_i of 50 μM [341, 435]). Simultaneously, the available nitrite concentration,[16] one to two orders of magnitude lower than the respective K_m value (≈100 μM [341]), does not favour its reduction [341]. On the other hand, under hypoxic and acidic conditions, the NaR concentration and activity are increased [341, 371, 374, 375, 392, 436–442]. Simultaneously, nitrite reduction by assimilatory NiR is decreased, especially in hypoxic roots, due to decreased NAD(P)H generation through the pentose phosphate pathway [341, 371, 374, 375, 435, 437, 441, 443]. As a consequence, when nitrate is the main nitrogen source, nitrite accumulates in hypoxic tissues [371–375] and its reduction by NaR is progressively increased, leading to NO formation [341, 370, 371]. The same NaR "activity switch", from nitrate reductase to nitrite reductase, is observed upon nitrite accumulation triggered by inhibition of photosynthetic activity[17] [415, 446–448] or by the expression of an anti-sense assimilatory NiR with very low activity [406, 407, 449].

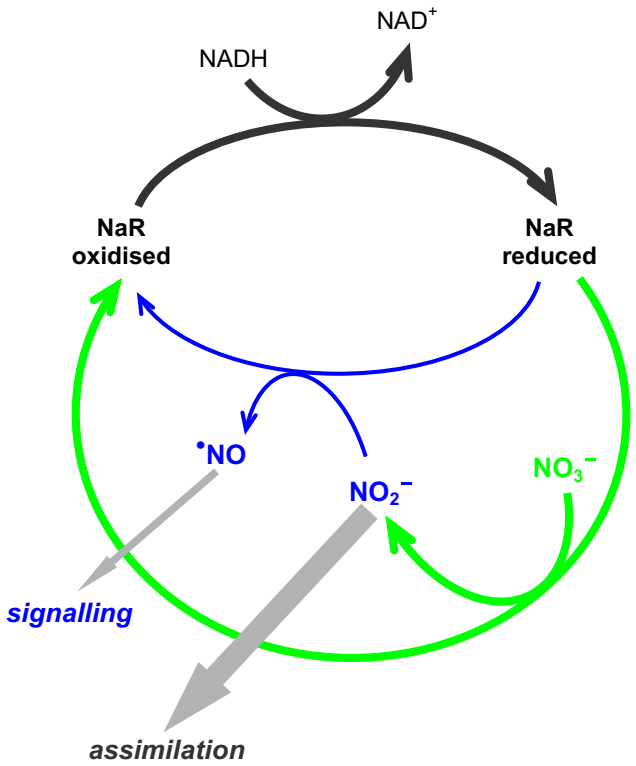

Fig. 6 Dual activity of plant NaR, nitrate reductase activity and nitrite reductase activity

In summary, when NaR activity is increased to an extent that nitrite formation exceeds its rate of consumption by assimilatory NiR and/or nitrite accumulates to an extent that NiR could not cope with it, the nitrite reductase activity of NaR would become significant and the formation of NO would be amplified (Fig. 6) [341]. This NaR

[15] NaR is highly regulated by complex transcriptional, translational and posttranslational mechanisms. The posttranslational regulation involves the phosphorylation of a serine residue in the linker region between the molybdenum and haem domains [413, 414]. The phosphorylation is catalysed by protein kinases, including AMP-activated [415] and calcium-dependent kinases [416]. This phosphorylation creates a recognition site that recruits a specific regulatory protein (one member of the 14-3-3 family), whose binding effectively inhibits the enzyme [417–419]. Thus, mutation of the key serine residue to an aspartate results in a plant that has the NaR always active [420–422]. In vivo, the NaR inactivation occurs rapidly in darkness or when carbon dioxide is removed. This posttranslational regulation is essential to lower the NaR activity at night, when photosynthetically generated reducing equivalents are not available to reduce nitrite to ammonia. In this way, the nocturnal nitrite levels would not increase to dangerous concentrations [423].

[16] Nitrite is promptly transported to the leaves' chloroplasts or roots' plastids, where it is rapidly reduced by SNIR, so that it does not accumulate.

[17] The inhibition of the photosynthetic electron flow causes nitrite accumulation, because its transport to the chloroplasts depends on the pH gradient across the chloroplast envelope (buildup by the photosynthetic activity). In addition, its reduction to ammonium is hindered in the absence of photosynthetically reduced ferredoxin [470, 471].

"activity switch" can be exploited by plant cells to rapidly and dynamically signalise and respond to different cellular challenges. For example, the NO-forming activity makes NaR an ideal cytoplasmatic nitrite sensor to "signalise" the presence of dangerous nitrite concentrations: NaR could "translate" the nitrite accumulation into an increased NO flux, which would be, subsequently, "translated" into a biological response through an NO-mediated reaction [405].

Clearly, the concentration of NaR-formed NO would be very low, predicted to be less than 1 % of the nitrate-reducing activity, due, mainly, to competitive inhibition by nitrate [341, 407]. However, as discussed for mammals, the NO concentration should be kept very low, within the characteristics of a local signalling molecule. Obviously, the plant NaR-dependent NO formation should be tightly controlled and it can be argued that the well-known complex NaR regulation serves not only to control the nitrogen assimilation, but also to regulate the formation of signalling NO.

Other molybdoenzymes The fact that the four mammalian molybdoenzymes, XD/XO, AO, SO and mARC, are able to reduce nitrite to NO anticipates similar roles for the homologous plant enzymes [42]. In fact, in vivo inhibition studies with allopurinol have revealed a probable role for XD in the NO formation in white lupin roots experiencing phosphate deficiency [450]. Moreover, the studies planned to assess the in vivo NaR-dependent NO formation through inhibiting with tungstate can also be interpreted as pointing to the involvement of the other molybdoenzymes, as in all of them tungsten can replace the molybdenum atom, producing inactive enzymes [451]. The same is true for the cyanide inhibition studies, since cyanide should remove the catalytically essential sulfo group of XD/XO and AO. Therefore, in addition to the in vivo studies, the definitive establishment of plant XD and/or XO,[18] AO, SO and potentially mARC, as NO sources, must wait for the kinetic characterisation of the nitrite reductase activity of the purified enzymes, which was not yet done [456].

Prokaryotes

Nitric oxide in prokaryotes

In prokaryotes, NO formation had for long been thought to occur only in denitrification, anaerobic ammonium oxidation and other related respiratory pathways [34–42, 457]. On those pathways, NO is a regular reaction product and substrate, being also a signalling molecule that regulates the genes required for its own anabolism/catabolism (a common regulatory strategy in biology). However, presently, it is clear that NO is also involved in "non-respiratory" pathways, including (1) cytoprotection against oxidative stress (in *Escherichia coli*, *Bacillus subtilis*, *Bacillus anthracis*, *Staphyloccuos aureus*) [458–464], (2) recovery from radiation-induced damage (*Deinococcus radiodurans*) [465] or (3) the biosynthesis of secondary metabolites, namely nitration of tryptophan (*Deinococcus radiodurans*) [466] and of the tryptophanyl moiety of thaxtomins (*Streptomyces turgidiscabies*) [369–371, 374, 375, 404–467].

The "non-respiratory" NO formation can be achieved through oxidative and reductive pathways. Several prokaryotes (such as, *Staphylococcus*, *Geobacillus*, *Bacillus*, *Rhodococcus*, *Streptomyces*, *Deinococcusus* and *Natronomonas* [463, 465, 469–476]) hold NOS enzymes, homologous to the oxygenase domain of the mammalian NOS, which catalyse the aerobic NO formation from arginine, using cellular redox equivalents that are not normally committed to NO production [467, 470–474, 477–485]. Yet, prokaryotes are also able to synthesise NO in an NOS-independent manner, through nitrite reduction. *Escherichia coli* and *Salmonella enterica* are two (long known) examples of bacteria that, not having an NOS enzyme, are able to form NO when growing under nitrate (anaerobic) conditions [486–491]. Also, the cyanobacterium *Microcystis aeruginosa* [492] and *Bacillus vireti* (whose genome indicates that it carries out dissimilatory nitrite reduction to ammonium) are able to generate nitrite-dependent NO. In addition, also the organisms that have NOS may rely on nitrite reduction to produce NO, as is the case of *Streptomyces* that can still produce a small amount of thaxtomin when the NOS gene is deleted [467–469].

Until recently, nitrite-dependent NO formation was assumed to arise from the "side" activity of the assimilatory sirohaem-containing NiR and dissimilatory *c*-haem-containing NiR (enzymes that catalyse nitrite reduction to ammonium), because studies with *E. coli* mutants suggested that both enzymes would be largely responsible for NO production [491, 493]. However, no NO generation could be observed with purified enzymes; on the contrary, both enzymes were proposed to catalyse instead NO consumption, as part of detoxification pathways [494–496]. Presently, the major source of nitrite-dependent non-respiratory NO is believed to be the molybdenum-containing respiratory NaR. Among other sources that remain to be identified, also haemic globins [497–502], AOR and other NaR probably contribute to this NO formation.

[18] The conversion of XD into an XO form is not common to all plants: while the enzyme from *Arabidopsis thaliana* does not have the two corresponding cysteine residues [479] involved in the conversion mechanism of the mammalian enzyme, the pea leaf peroxisomal enzyme was described to exist mainly (70 %) as an XO form [480–482].

Molybdenum-dependent nitrite reduction in prokaryotes

Nitrate reductases Prokaryotes use nitrate for dissimilatory and assimilatory processes and, for those purposes, these organisms hold three distinct types of NaR enzymes, present in different subcellular locations [503–516]: (1) respiratory membrane-bound NaR, associated with the generation of a proton motive force across the cytoplasmatic membrane; (2) periplasmatic NaR, involved in the generation of a proton motive force or acting as an electron sink to eliminate excess of reducing equivalents; and (3) cytoplasmatic assimilatory NaR, involved in nitrogen assimilation.[19]

All the prokaryotic NaR are molybdoenzymes, belonging to the DMSOR family (Fig. 1), which catalyse the two-electron reduction of nitrate to nitrite (Eq. 8) at their molybdenum centres. In spite of catalysing the same reaction and having the active site molybdenum atom coordinated by four sulfur atoms from two pyranopterin cofactor molecules (both present as a guanine dinucleotide, as is characteristic of the DMSOR family), the three NaR types display significant differences at the remainder of the molybdenum coordination sphere. In respiratory membrane-bound NaR, the molybdenum atom is further coordinated by the two oxygen atoms of one aspartate residue that is coordinated in a bidentate fashion (Fig. 2e) [506, 517] or by one terminal oxo group plus one oxygen atom from the aspartate residue coordinated in a monodentate mode (Fig. 2f) [507]; possibly, the monodentate and bidentate aspartate binding modes correspond to oxidised and reduced (by the synchrotron beam) proteins, respectively. However, in the periplasmatic NaR from *Desulfovibrio desulfuricans* or *Cupriavidus necator*, the molybdenum atom is coordinated instead by a cysteine sulfur atom plus one terminal sulfo group, forming a partial disulfide bond within each other (Fig. 2g) [518–521]. The *E. coli* [522] and *Rhodobacter sphaeroides* periplasmatic NaR, in turn, complete the molybdenum coordination with a terminal hydroxyl group plus the cysteine sulfur atom (Fig. 2h) [523]. The cytoplasmatic assimilatory NaR is the less studied one and, although clear structural insight awaits further investigation, it is probable that a cysteine residue coordinates the molybdenum atom [524]. Besides the diversity of their active sites, and in response to their different biological roles/subcellular locations, prokaryotic NaR also display different subunit compositions and quaternary structures. The respiratory enzyme from *E. coli* NaRGHI (product of the *narG*, *H* and *I* genes) is a dimer of heterotrimers, $(\alpha\beta\gamma)_2$, comprising [506, 507, 517]: (1) a cytoplasmatic nitrate-reducing NaRG subunit that holds the molybdenum centre and one [4Fe–4S] centre; (2) an electron transfer NaRH subunit that holds one [3Fe–4S] and three [4Fe–4S] centres; (3) and a membrane-bound quinol-oxidising NaRI subunit that holds two *b*-type haems. On the other hand, the *D. desulfuricans* periplasmatic NaR (product of the *napA* gene) is a monomeric enzyme, holding only one [4Fe–4S] centre, besides the molybdenum centre [518, 520], while the enzyme from *C. necator* (*napA* and *napB* genes) is a dimer harbouring in addition two more haems [521].

Several studies have suggested that the non-respiratory NO generation is due to the NaR-catalysed nitrite reduction [486–490, 492, 525–530], with the majority of NO being formed by the respiratory membrane-bound NaR [530–532] and a small contribution (less than 3 %) from the periplasmatic NaR [531, 532]; the potential contribution of the assimilatory cytoplasmatic NaR has not yet been investigated. The feasibility of respiratory NaR to catalyse nitrite reduction to NO was also demonstrated in a recent theoretical study [384].

NO formation by 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 enzyme expression [530, 532, 533]. Noteworthy, these conditions are similar to the ones described above for NO generation by plant NaR. Again in a similar way to the plant enzyme, nitrate also competitively inhibits the prokaryotic respiratory NaR-catalysed nitrite reduction (*e.g. S. enterica* specificity constant (k_{cat}/K_m) for nitrite is ≈150 times lower than the nitrate one [532]). Nitrite reduction is promoted only when the nitrate concentration decreases and nitrite builds up (K_m value for nitrite in the millimolar range [532]). 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, NO formation is very low [532]. Nevertheless, the extension of the prokaryotic NO synthesis seems to be dependent on the organism and, probably, on the role of NO in that organism. For example, while *E. coli* NO generation is estimated to be less than 1 % of the reduced [487, 531], the *S. enterica* NO formation can account for up to 20 % of the nitrate reduced [532].

The similarities in the nitrite-dependent NO formation by plant NaR (probably also fungus [393]) and bacterial respiratory NaR are noteworthy. This similar activity suggests that nitrite reduction/NO formation could be a general feature of all types of NaR enzymes. In this respect, it is intriguing why prokaryotic periplasmatic and assimilatory NaR would not be able to catalyse nitrite reduction to NO. Although with no theoretical support, it can be argued that nitrite reduction would be hampered by the sulfur-rich coordination of the molybdenum centre of periplasmatic NaR (Fig. 2g, h versus Fig. 2d, e, f). Undoubtedly, definitive conclusions must wait for kinetic and spectroscopic characterisation of the purified enzymes from different sources.

[19] It should be noted that the eukaryotic assimilatory cytoplasmatic NaR (discussed above), belonging to the SO family, is distinct from any type of prokaryotic NaR.

Aldehyde oxidoreductase The molybdoenzyme AOR can also contribute to the bacterial NO formation. AOR was first described by Moura et al. [534] and is believed to be an aldehyde scavenger (Eq. 25), acting in a complex chain of electron transfer proteins that links the oxidation of aldehydes to the reduction of protons in *Desulfovibrio* species [535]. AOR is an XO family member (Fig. 1), structurally similar to the mammalian XO and AO, but harbouring only two [2Fe–2S] centres (no FAD) and holding a slightly different molybdenum centre, apparently with an equatorial terminal oxo group instead of the sulfo group found in XO and AO, and with the pyranopterin cofactor esterified with cytidine monophosphate (Fig. 2b) [536–538],

$$\text{aldehyde} + \text{flavodoxin}_{\text{oxidised}} \rightarrow \text{carboxylate} + \text{flavodoxin}_{\text{reduced}}. \quad (25)$$

As the mammalian XO and AO enzymes, AOR was recently shown to catalyse nitrite reduction to NO [236]. The reaction was demonstrated to occur at the molybdenum centre employing a similar approach to the one described above for mammalian XO/XD and AO, but using ethylene glycol to specifically inhibit the AOR molybdenum centre [236]. In the presence of ethylene glycol, even though Fe/S is not affected (as shown by the presence of their characteristic EPR signals), no NO formation was observed, thus demonstrating that nitrite reduction occurs, as anticipated, at the molybdenum centre [236].

Once more, the amount of NO produced would be dependent on the accumulation of nitrite (K_m value in the millimolar range [236]) and is estimated to be low ($k_{cat}/K_m \approx 60\ M^{-1}s^{-1}$ [236]). In addition, the physiological relevance of this bacterial NO formation pathway would depend on the competition between nitrite and the expected oxidising substrate, which in the AOR case is flavodoxin [539]. Overall, the AOR-dependent NO formation would be controlled (1) by the availability of nitrite and (2) by the cellular redox status that determines the flavodoxin redox status, as well as the redox status of other proteins involved in the respiratory pathways. This suggests that the AOR-catalysed NO generation could be involved in cytoprotection against oxidative stress. It could be hypothesised that under normal conditions, the electron transporters involved in the respiratory pathways would be reduced and the aldehyde-oxidising activity of AOR would be coupled with the reduction of protons. But, in a situation of oxidative stress, as the proteins began to be oxidised and the respiratory pathways affected, nitrite would accumulate (absence of "respiratory" reducing equivalents to reduce it) and AOR would link aldehyde oxidation to nitrite reduction. The NO thus formed could, subsequently, participate in signalling cascades that would eventually protect the organism from oxidative damage. Although this hypothesis could be debatable, it illustrates how the bacterium could use the AOR-derived NO 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 protective (antioxidant) response.

Other molybdoenzymes The diversity of molybdoenzymes described to be able to reduce nitrite suggests that other prokaryotic molybdoenzymes could be involved in the generation of non-respiratory NO. Contrary to eukaryotes that possess only a restricted number of molybdoenzymes, prokaryotic organisms display a great diversity of these metalloenzymes [3–10] and it is worth investigating the possible role of other molybdoenzymes in nitrite reduction, namely those known for their ability to catalyse oxygen atom abstraction reactions and for which the reaction is thermodynamically favourable.

Mechanistic strategies for molybdenum-dependent nitrite reduction

Presently, it is clear that several molybdoenzymes, from the three families (Fig. 1), can catalyse nitrite reduction to NO. This suggests that the chemistry behind the reaction should not be much affected by the structural differences between the molybdenum centre characteristics of each family and, probably, a unifying mechanism can be envisaged. The different amino acid residues present at the substrate-binding pocket of each specific enzyme would necessarily modulate the nitrite-binding affinity to that active site and stabilise, more or less efficiently, the catalytic intermediates, thus, affecting the reaction kinetics. Yet, the reactivity or catalytic driving force to abstract one oxygen atom from nitrite seems to be conferred by a molybdenum centre with a minimum conserved structure being required.

The molecular mechanism of nitrite reduction catalysed by XO and related enzymes (XD, AO, AOR) was the first to be proposed based on kinetic and EPR spectroscopic data [236, 237] and it will be here used to illustrate how a molybdenum centre can carry out this reaction (Fig. 7, shadowed "inner" mechanism). A unifying mechanism is shown in Fig. 7 ("outer" mechanism) considering what we propose to be the minimum structure required for a molybdenum centre to carry out nitrite reduction, that is, a molybdenum with an oxo/hydroxyl/aqua ligand ($Mo–OH_{(n)}$, with $n = 0,1,2$) that is presumed to be exchanged by nitrite.

To catalyse nitrite reduction to NO, the molybdoenzyme has to bind nitrite, transfer one electron to it, cleave one of the nitrite N–O bonds and, ultimately, release the NO formed. To catalyse this reduction, the molybdenum must be reduced by a reducing substrate, during a first part of the catalytic cycle (oxidation half-reaction), e.g. XO reduction by xanthine or plant NaR reduction by NADH

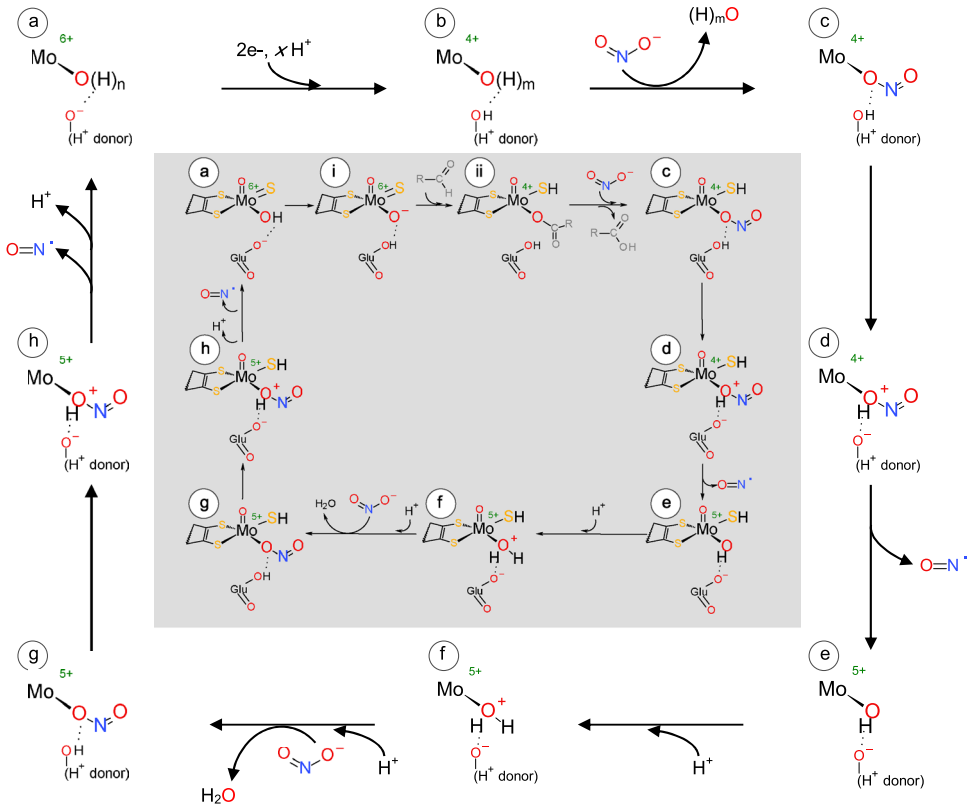

Fig. 7 Mechanism of nitrite reduction to NO catalysed by molybdoenzymes. Inside, shadowed in grey, the mechanism proposed for XO- and AO-catalysed nitrite reduction to NO is represented [236, 237]. Here, it is emphasised how the molybdenum centre is reduced by an aldehyde molecule ($a \rightarrow i \rightarrow ii \rightarrow c$). For simplicity, only the dithiolate moiety of the pyranopterin cofactor is represented. The "outer" mechanism aims to illustrate how a molybdoenzyme, from any of the three families, would carry out the reaction. In this "outer" mechanism, none of the molybdenum ligands were represented, except a "labile" oxo/hydroxyl/aqua ligand that is presumed to be exchanged by the nitrite molecule

[Fig. 7a → b → c; shadowed in grey, it is represented as the XO/AO reduction by an aldehyde (a → i → ii → c)]. Although nitrite binding could, in principle, occur before metal reduction, this is not likely and spectroscopic and kinetic data gave no evidence for nitrite interaction with oxidised molybdenum[20] [236, 237]. Furthermore, nitrite binding can take place before or after the release of the oxidised reducing substrate: nitrite can bind to molybdenum (1) displacing the bounded oxidised reducing substrate, through the formation of a ternary complex, as suggested for bovine milk XO and bacterial AOR [236]; or after product release, displacing a water molecule, via a "ping-pong" kinetic mechanism observed in rat liver XO and AO [237].

Nitrite is suggested to be bound to the molybdenum atom through one of its oxygen atoms (a "nitrito" binding mode) (Fig. 7c). 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 [540]; this chemistry makes the molybdenum cores excellent "oxygen atom exchangers", as long as the thermodynamics of the reactions is favourable [541]. In accordance, substrates or products of the XO family enzymes interact with the molybdenum atom through an oxygen atom and also nitrate interacts with the NaR molybdenum via one oxygen atom [3, 23–28]. Therefore, nitrite is proposed to bind only to reduced molybdenum and through one of its oxygen atoms—this is proposed to be the common and key complex, from which nitrite reduction is initiated in all molybdoenzymes (Fig. 7c).

Subsequently, the reduced molybdenum transfers one electron to nitrite, NO is formed and the molybdenum is oxidised to Mo^{5+} (yielding a paramagnetic centre that gives rise to the characteristic rapid type 1 EPR signal of XO) (Fig. 7c → e). Spectroscopic EPR assays demonstrated unequivocally that the reduced molybdenum centre of XO and AOR (enzymes reduced with compounds that interact at the molybdenum, Fe/S or the FAD centres) are oxidised in the presence of nitrite; assays with an NO-selective electrode showed the simultaneous NO formation, demonstrating that nitrite is concomitantly oxidised in the process [236].

To accomplish NO formation, the molybdenum centre has to promote N–O bond cleavage (O–N–O → N=O). This step (Fig. 7d → e) is suggested to be triggered by a protonation event. Nitrite reduction by mARC [296], plant NaR [341] and bacterial AOR [236] and NaR [489] is greatly accelerated under acid conditions and, for

[20] If nitrite was to bind to the oxidised molybdenum of XO, then its preliminary incubation in the enzyme reaction mixture, before the addition of xanthine or aldehyde, would modify the K_m value of the reducing substrate relatively to add nitrite after the reducing substrate addition, which was not observed (parallel to competitive inhibition).

mammalian XO and AO, it was shown to involve two protonation equilibriums with pK_a values of 5.9 and 6.8 (XO) and 6.0 and 7.0 (AO) [237]. The residues responsible for those protonation equilibria have not yet been identified. However, a kinetic pH effect study [237] put forward the hypothesis that the conserved glutamate residue (Glu_{1261} of bovine milk XO) is essential not only for the hydroxylation half-reaction (see description under Sect. 2 "Xanthine oxidase/dehydrogenase and aldehyde oxidase"), but also for nitrite reduction. Accordingly, it was proposed that [237]: (1) in the first part of the XO or AO catalytic cycle (oxidation half-reaction), the deprotonated glutamate functions as a base and assists the Mo–O^- nucleophilic attack to the carbon centre to be hydroxylated; (2) during the nitrite reduction part (reduction half-reaction), the same glutamate residue, but at this point protonated, functions as the proton donor required to reduce nitrite. In support of this hypothesis, it has to be noted that this glutamate residue has the adequate and best position inside the active site pocket to act as the proton donor. Nevertheless, this glutamate "dual" role must wait for experimental and theoretical confirmation.

Therefore, it is suggested that once the Mo^{4+}–O–N–O complex is formed (Fig. 7c), the reaction proceeds with the protonation of the nitrite oxygen atom bound to the molybdenum, at the expense of a neighbouring protonated residue, Glu_{1261} in bovine milk XO (Fig. 7d). This protonation step would trigger the electron transfer from the reduced molybdenum to the now protonated nitrite, causing the N–OH bond homolysis and subsequent NO release (Fig. 7e). Noteworthy, the previous protonation of nitrite would lead to the formation of a more stable "future" metal complex, that is, it would lead to the formation of Mo^{5+}–OH (Fig. 7e) instead of a Mo^{5+}–O^- complex. The pK_a values of the molybdenum coordinated ligands change dramatically with the oxidation state and the lower oxidation states hold highly protonated ligands [542, 543]. For this reason, in the XO Mo^{5+} complex, both terminal oxygen and sulfur atoms should end up protonated, and either the protonation event occurs before (as suggested) or after the NO release (this Mo^{5+}–OH(–SH) complex would produce the characteristic rapid type of EPR signal, with two interacting protons). So, if nitrite is protonated before it is converted to NO, the "future" metal complex would be in a more stable form (Mo^{5+}–OH).

A similar mechanism was proposed for nitrite reduction to NO by bacterial copper-containing nitrite reductase (CuNiR). This enzyme displays a similar pH dependence, with pK_a values of 5 and 7, and theoretical calculations have suggested that it is the proton transfer from a key neighbouring aspartate residue (pK_a of 5) that triggers the electron transfer from copper to nitrite (proton transfer triggering electron transfer) [544]. Moreover, also in CuNiR, the previous nitrite protonation results in the formation of a more stable metal complex, Cu–OH instead of Cu–O^-. Also the choice of the proton donor—if confirmed—seems to be similar: one aspartate in CuNiR and the glutamate residue in XO. The mechanism by which XO and AO promote the N–OH bond cleavage is presently not known. However, it is tempting to speculate that the strategy followed would be analogous to the CuNiR one,[21] 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 and copper, respectively [545–547]) [42].

At this stage (Fig. 7e), one molecule of NO is already formed and released. However, because the molybdenum centre reduction is a two-electron process, another nitrite molecule could be reduced. It should be here emphasised that, although this is certainly the case forf the XO-/XD- and AO-catalysed nitrite reduction [230–238], it is probable that in other enzymes, such as SO [316], the reaction stops at this step, at Mo^{5+}. The reaction is, then, suggested to proceed with the binding of a second nitrite molecule [236, 237]. To generate a good leaving group, water (Mo^{5+}–OH_2), the consumption of one proton is proposed (Fig. 7e → f). Subsequently, nitrite displaces the water molecule (Fig. 7f → g) and, after a second cycle of nitrite reduction/molybdenum oxidation, a second NO molecule is released (Fig. 7g → h → a). The molybdenum is now in a 6 + oxidation state, which would favour the deprotonation of its ligand(s) [542, 543] and readiness to start another catalytic cycle.

In summary, molybdenum-dependent nitrite reduction is suggested to be initiated with the nitrite binding to the reduced molybdenum centre, in a "nitrito" binding mode. After a protonation event, suggested to be mediated by a neighbouring protonated residue, the electron transfer from molybdenum to nitrite is triggered, the N–OH bond is homolytically cleaved and the NO is promptly released (Fig. 7).

The outlined mechanism can be applied to a molybdoenzyme of any of the three families, as long as the reduced molybdenum centre has an available coordination position to bind nitrite (Fig. 7, "outer" mechanism). This requisite is fulfilled by all the molybdoenzymes described herein: in XO/XD, AO and AOR, nitrite can bind displacing a water molecule or the bounded hydroxylated product; in mARC, SO and plant NaR, nitrite would displace the molybdenum labile hydroxyl group; in bacterial NaR, nitrite would bind as the "classic" substrate, nitrate, does, that is, probably via a carboxylate shift or sulfur shift [548]. Moreover, the presence of one or two pyranopterin molecules coordinating the

[21] In spite of the nitrite-binding mode in CuNiR being a "bidentate nitrito" mode, it is tempting not to follow all the similarities between the molybdenum- and copper-containing enzymes.

molybdenum atom (XO/SO or DMSOR families) does not seem to affect the ability to reduce nitrite.

Particularly interesting is the potential role of the molybdenum terminal sulfo group of XO family members. The mechanism proposed does not dictate any obvious catalytic role for this group in nitrite reduction. However, its presence was shown to be crucial for the XO-catalysed nitrite reduction, as desulfo-XO is not able to form NO [236]. A parallel phenomenon can be observed in XO inhibition by oxypurinol, where only the reduced sulfo-XO molecules are inhibited, in a process where the participation of the sulfhydryl group in the formation of the Mo—oxypurinol complex is not clearly understood [241]. The understanding of the role of the sulfo group in nitrite reduction is further complicated by the fact that AOR, which does not seem to have a sulfo group, is able to catalyse NO formation [236]. Furthermore, mammalian mARC, SO and plant NaR, with a cysteine sulfur in place of the terminal sulfo group, are able to reduce nitrite. Noteworthy, the cysteine sulfur coordination to molybdenum is essential for nitrite reduction, at least in mARC, where mutation of cysteine to an alanine residue abolishes nitrite reduction/NO formation [296]. Understanding the role of sulfo, oxo and amino acid ligands during molybdenum reduction and oxidation is of major importance to have a deeper knowledge of the molybdenum mechanistic strategies.

The suggested mechanism also highlights the ability of molybdoenzymes to catalyse both the oxygen atom abstraction and insertion, during the same catalytic cycle—at least in some of the enzymes. Several works performed with molybdenum model compounds [549–560] and with XO [561, 562] and DMSOR [563] led Holm and others, in the 1980s and 1990s, to propose the "oxotransfer hypothesis" (Fig. 8, blue arrows). Accordingly, the molybdoenzymes are commonly classified as oxotransferases to emphasise that these enzymes catalyse reactions involving substrates and products whose oxygen content differs by one atom (although this denomination cannot be generalised to all molybdoenzymes, as described in "Introduction: an outlook on molybdoenzymes and nitrite reduction"). The nitrite reductase activity of XO/XD, AO, AOR [236, 237] and SO [316] pushed that hypothesis into a "double oxotransfer hypothesis" (Fig. 8, red arrows), where: (1) one first substrate (xanthine, aldehyde or sulfite; 'R' in Fig. 8), acting as a reducing substrate and oxo group acceptor, is "oxygenated" (to yield urate, carboxylate or sulfate; 'RO'), (2) followed by a second substrate (nitrite; 'PO' in Fig. 8) that is "deoxygenated" (to NO; 'P') while functioning as the oxidising substrate and oxo group donor. This description does not intend to mean that it is compulsorily the oxygen atom of the second substrate that is inserted into the first substrate (because the molybdenum-labile hydroxyl group can be easily exchanged with solvent water), although it is possible that this is the case.

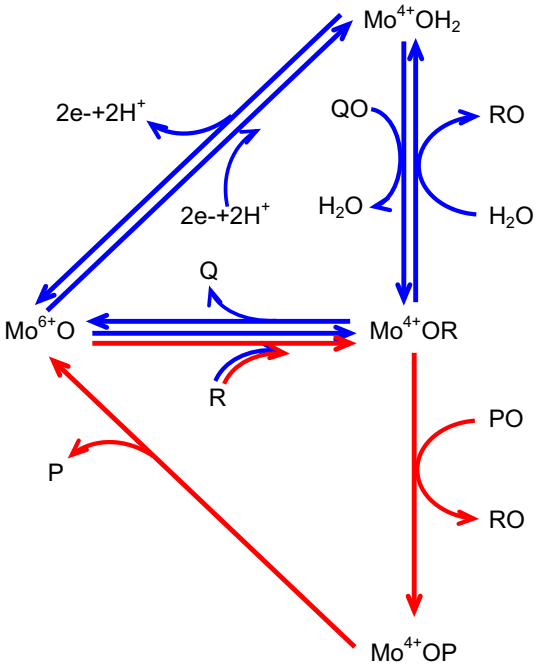

Fig. 8 Mono oxotransfer (*blue*) and double oxotransfer (*red*) hypothesis. The mono oxotransfer path (*blue arrows*) is represented with reversible reactions to account for "isolated" oxygen atom insertion and abstraction (e.g. sulphite oxidation and nitrate reduction reactions). The double oxotransfer path (*red arrows*) aims to illustrate the simultaneous oxygen atom insertion and abstraction that occurs during nitrite reduction to NO in the presence of, e.g. xanthine and XO, aldehyde and AO or AOR, and sulphite and SO. For simplicity, only the oxygen atom directly involved in the oxo transfer reactions is depicted

The schematic representation in Fig. 8 also aims to highlight that Mo^{6+} cores can be thought as competent oxo group donors, with the Mo^{4+} cores acting as oxo group acceptors, in accordance with the known chemistry of $Mo^{6+,5+,4+}$ [540, 541]. This allow us to suggest that, in the presence of two substrates, one oxo donor and the other an oxo acceptor, the molybdenum cores can catalyse the oxo group transfer between the two, provided that the thermodynamics of the reaction is favourable [541]. That is, the "double oxotransfer" reaction should be possible for substrates other than nitrite.

Conclusions

NO is a remarkable multi-task biomolecule. Its formation from nitrite constitutes the first committed step in denitrification and is an essential step in anaerobic ammonium oxidation and other primitive respiratory pathways, where nitrogen compounds are used to derive energy. For those respiratory functions, prokaryotes developed "dedicated" haem- and copper-containing NiR enzymes.

In mammals, NO is a crucial signalling molecule, involved in numerous physiological processes and also in some pathological conditions. For its synthesis, mammals use two distinct pathways that operate under opposite conditions: (1) an oxidative pathway that is mediated by specific haemic NOS enzymes and depends on dioxygen, (2) and a reductive pathway that is mediated by (apparently) several "non-dedicated" NiR, depends on nitrite and is favoured under low dioxygen concentrations and acidic conditions. With these two pathways, mammalian cells can maintain the vital NO formation under the entire dioxygen gradient, from normoxia to anoxia. Noteworthy, also plants and bacteria use similar nitrite-dependent pathways, mediated by "non-dedicated" NiR, to produce NO. Hence, biological nitrite reduction to NO can be seen as an ubiquitous universal reaction that was "invented" in a pre-aerobic past and has been "reinvented" and employed ever since to accomplish different biological functions in virtually all forms of life [564].

The "non-dedicated" NiR are metalloproteins, containing not only molybdenum, as the ones here reviewed, but also haem and copper [42] that are present in cells to accomplish other functions and whose activity is "switched" to a nitrite reductase/NO synthase when the cell needs to synthesise "non-respiratory" NO. From a chemical point of view, the cell just takes advantage of the redox chemistry of an already available redox system to generate NO. From a biological point of view, the activity "switch" allows the cell to create regulatory/signalling points from which the metabolism can be modulated/adapted to allow the cell to properly respond to the event that triggered the activity "switch". 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-recognised and common phenomenon—moonlighting—with important implications for systems biology and, in particular, for human physiology and pathology [565]. In the nitrite reduction/NO formation scenario, this phenomenon is carried out by several metalloproteins, of different physiological functions and cellular localisations, and is triggered (at least) by the dioxygen availability and/or cellular redox status.

The use of molybdoenzymes to catalyse nitrite reduction to NO is (to us) an obvious choice. The unique chemistry of molybdenum makes the molybdenum centres excellent '"oxygen atom exchangers" [540, 541], precisely that needed to convert nitrite into NO. In fact, molybdoenzymes are widely used for oxotransfer reactions, both abstractions and insertions, in carbon, sulfur and nitrogen metabolism (as described in "Introduction"). Of note, molybdenum is the only metal used in the nitrogen biochemical cycle to reduce nitrate and oxidise nitrite, being found in the active site of four NaR and two nitrite oxidoreductase enzymes [42]. In this context, it is surprising that no "dedicated" molybdenum-containing nitrite reductase is known to exist.

However, several molybdoenzymes that are associated with other cellular functions, with different molybdenum centre structures and substrate-binding pockets, are able to reduce nitrite to NO. The molecular mechanism to achieve nitrite reduction is not expected to be much different within molybdoenzymes of the three families: the reduced Mo^{4+} cores are proposed to function as oxo group acceptors, binding nitrite and abstracting one oxygen atom.

All this reasoning suggests that virtually all forms of life can use a molybdoenzyme, when necessary to produce NO for other purposes than respiration. Mammalian XO/XD, AO, SO, mARC, plant and fungus NaR and bacterial NaR and AOR may become the first—already numerous—examples of such utilisation to be described.

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