CHAPTER 1

Molybdenum and Tungsten-Containing Enzymes: An Overview

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1.1 Introduction

Molybdenum and tungsten are heavy metallic elements, belonging to the sixth group of the "d-block" of the periodic table, with electronic configurations [Kr] $4d^5\ 5s^1$ and [Xe] $4f^{14}\ 5d^4\ 6s^2$, respectively (atomic numbers 42 and 74). They are trace elements, either in the Universe or in Earth crustal rocks and oceans (Table 1.1). In spite of that scarcity, molybdenum is essential to most organisms,[1,2] from archaea and bacteria to higher plants and mammals, being found in the active site of enzymes that catalyze oxidation–reduction reactions involving carbon, nitrogen and sulfur atoms of key metabolites.[3–10] Some of the molybdenum-dependent reactions constitute key steps in the global biogeochemical cycles of carbon, nitrogen, sulfur and oxygen, with particular emphasis on the atmospheric dinitrogen fixation (reduction) into organic ammonium (nitrogen cycle/nitrogenase enzyme).

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Table 1.1 Abundances of molybdenum, tungsten and some other elements with biological relevance in different environments[675]

Location	Abundance (ppb by atoms)						
	Mo	W	Fe	H	C	N	O
Universe	0.1	0.003	20×10^3	930×10^6	500×10^3	90×10^3	800×10^3
Crustal rocks	230	120	23×10^6	31×10^6	3.1×10^3	29×10^3	600×10^6
Oceans	0.64	0.004	0.33	662×10^6	14.4×10^3	220	331×10^6
Human body	7	—	6.7×10^3	620×10^6	120×10^6	12×10^6	240×10^6

Presently, more than 50 molybdenum-containing enzymes are known. The great majority are prokaryotic, with eukaryotes holding only a restricted number of molybdoenzymes.[4–10] Noteworthy, while all higher eukaryotic organisms use this element, many unicellular eukaryotes, including *Saccharomyces* and most other yeasts, have lost the ability to use molybdenum.[1,2] Tungsten, probably because of its limited bioavailability (Table 1.1),[11] is far less used, being present predominantly in thermophilic anaerobes,[3,12,13] although it is also found in some strictly aerobic bacteria (*e.g.* certain methylotrophs[14–19]).

This chapter provides an overview on the molybdo- and tungstoenzymes. Their physiological context and significance will be described in Section 1.2, where the recent hypothesis that the lack of molybdenum could have been the limiting factor for the life evolution and expansion on early Earth will receive special attention (Section 1.2.1). A brief introduction to the chemical properties that shape the catalytically competent molybdenum/tungsten centres will be made in Section 1.3. In Section 1.4, the enzymes will be grouped in five main families (Sections 1.4.1 to 1.4.5), according to their metal/cofactor structure, and a general view on the structural (section (a)) and mechanistic (section (b)) versatility of each family will be presented. A brief account of novel heteronuclear centres containing molybdenum, whose physiological function is not yet fully understood, will be made in Section 1.4.6. A final outlook on our present knowledge about these enzymes will conclude this chapter.

1.2 Living with Molybdenum and Tungsten

The *human history* of molybdenum began in the 18th century, when Carl Wilhelm Scheele isolated molybdic acid ($MoO_3 \cdot H_2O$) and Peter Jacob Hjelm subsequently found a dark metallic powder that he named "molybdenum".[20] Nevertheless the successful and widespread use of molybdenum only took place in the 20th century and nowadays it is used in bridges and buildings (I.M. Pei's steel pyramid entrance for the Musée du Louvre is an elegant example), pipes and power plants, cars and computers, paints, plastics, catalysts and medical procedures.[21–23] By contrast, the *biological history* of molybdenum is almost as old as life on Earth.

When we think about the elements that are essential for life on Earth, we hardly ever consider molybdenum. The biological role of molybdenum

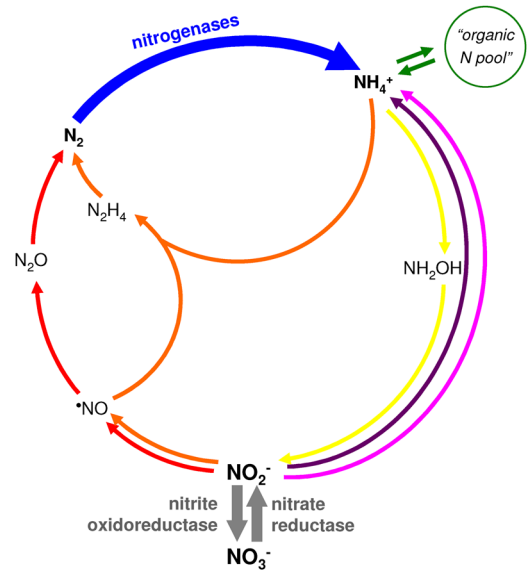


Figure 1.1 Biochemical cycle of nitrogen. Dinitrogen fixation, blue arrow; "organic nitrogen pool", green arrows; assimilatory ammonification, pink arrow; dissimilatory nitrate reduction to ammonium, violet arrow; nitrification, yellow arrows; denitrification, red arrows; anaerobic ammonium oxidation (AnAmmOx), orange arrows. The steps catalyzed by molybdenum-containing enzymes are highlighted with thick arrows, nitrogenase (blue), nitrate reductase and nitrite oxidoreductase (grey). Adapted with permission from ref. 62. Copyright 2014 American Chemical Society.

can only be appreciated when put in perspective. Nitrogen is the fourth most abundant element in living organisms (only behind hydrogen, oxygen and carbon) and life on Earth depends on the nitrogen biogeochemical cycle to keep this element in forms that can be used by the organisms.[24–33] Noteworthy, the "closing" of the nitrogen cycle, with the atmospheric dinitrogen fixation into ammonium[30,34–36] (Figure 1.1, blue arrow), depends virtually entirely on the trace element molybdenum : nitrogenase, a prokaryotic enzyme responsible for dinitrogen reduction to ammonium, requires one molybdenum atom in its active site[†] (Figure 1.3b; see Section 1.4.5 and ref. 55). The few organisms possessing this enzyme are capable of producing their own reduced ("fixed") nitrogen forms, using directly the atmospheric dinitrogen, the largest nitrogen source (biological nitrogen fixation is the main route by which nitrogen enters the biosphere).[56–58] All other organisms, the vast majority of life on Earth, depend on the availability of ammonium and nitrate (from soils, oceans and other organisms).[30,36,59–62]

[†]Note that, besides the molybdenum/iron-dependent enzyme, there are also other nitrogenases that depend on vanadium/iron and only on iron, but they exhibit different catalytic efficiencies and products stoichiometry.[37–54].

With this wide perspective in mind, the molybdenum biological role certainly assumes another dimension. In fact, it was recently proposed that the lack of molybdenum, while hampering the existence of an efficient nitrogenase, could have been the limiting factor for life evolution and expansion on early Earth, as described below (Section 1.2.1). However, the involvement of molybdenum in the nitrogen cycle is not restricted to the dinitrogen fixation, as the element is also essential for the reduction of nitrate to nitrite and for the oxidation of nitrite to nitrate (Figure 1.1, grey arrows), both processes being exclusively dependent (as far as is presently known) on the molybdenum-containing enzymes nitrate reductases (from both prokaryotic and eukaryotic sources) and nitrite oxidoreductases (from prokaryotes only).[62]

Noteworthy, molybdenum has also been suggested to be essential for nitrite reduction to nitric oxide for biological signalling purposes. Nitric oxide is a signalling molecule involved in several physiological processes, in both prokaryotes and eukaryotes, and nitrite is presently recognized as a nitric oxide source particularly relevant to cell signalling and survival under challenging conditions.[62,63] Nitrite-dependent signalling pathways have been described in mammals, plants and also bacteria, and are carried out by proteins present in cells to carry out other functions, including several molybdoenzymes (which thus form a new class of "non-dedicated" nitric oxide-forming nitrite reductases): mammalian xanthine oxidoreductase, aldehyde oxidase,[64,65] sulfite oxidase[66] and mitochondrial amidoxime reducing component,[67] plant nitrate reductase[62] and bacterial aldehyde oxidoreductase[64] and nitrate reductases.[62]

Molybdenum is also involved in the carbon cycle. The first example that comes to mind is provided by the formate dehydrogenases that are used by acetogens to fix carbon dioxide (reduce it) into formate and eventually form acetate; but molybdenum is also present in carbon monoxide dehydrogenases (catalyzing the oxidation of carbon monoxide to carbon dioxide), aldehyde oxidoreductases (catalyzing the interconversion between aldehydes and carboxylic acids) and in other formate dehydrogenases (that are involved in physiological pathways where formate is oxidized to carbon dioxide). The primitive carbon cycle would have also been dependent on molybdenum, as the metal (together with tungsten) would have been essential for the earliest, strictly anaerobic, organisms to handle aldehydes and carboxylic acids, catalyzing their interconversion.[68]

Molybdenum also plays several other "carbon-related" roles in modern higher organisms. The aldehyde oxidase of higher plants is responsible for the oxidation of abscisic aldehyde to abscisic acid (a plant hormone involved in development processes and in a variety of abiotic and biotic stress responses)[69,70] and has been implicated in the biosynthesis of indole-3-acetic acid (an auxin phytohormone).[71] The mammalian aldehyde oxidases have been suggested to participate in the formation of retinoic acid (a metabolite of retinol (vitamin A) that is involved in growth and development) and in the metabolism of xenobiotic compounds, where they would catalyze the hydroxylation of carbon centres of heterocyclic aromatic compounds and the oxidation of aldehydic groups.[72–76]

The dependence of higher plants and animals on molybdenum is also observed in purine catabolism, where xanthine oxidoreductase is involved in

the hydroxylation of hypoxanthine and xanthine into urate.[77–79] Noteworthy, involvement of molybdenum in purine metabolism is common to virtually all forms of life and only a small number of organisms use other mechanisms to oxidize xanthine (*e.g.* some yeasts[80]), thus confirming the essential role of molybdenum for life on Earth.

Another important aspect of molybdenum in biology can be seen in sulfite-oxidizing enzymes, which are used by almost all forms of life in the catabolism of sulfur-containing amino acids and other sulfur-containing compounds, oxidizing sulfite to sulfate. Certainly, sulfite oxidase is one of the most striking examples of the human dependence on molybdenum.[81–86] Sulfite (derived not only from the catabolism of sulfur-containing amino acids, but also from sulfur-containing xenobiotic compounds) is toxic and its controlled oxidation to sulfate is critical for survival. Underscoring this vital role, human sulfite oxidase deficiency results in severe neonatal neurological problems, including attenuated growth of the brain, mental retardation, seizures and early death.‡[81–86] Molybdenum-dependent sulfite-oxidizing enzymes are also important for some prokaryotes that are able to generate energy from the respiratory oxidation of inorganic sulfur compounds[87–90] – hence, extending the role of molybdenum to the sulfur cycle.

Tungsten was likely an essential element for the earliest life forms (see Section 1.2.1 below for some details about Earth's history). Under euxinic conditions (sulfidic and anoxic conditions), tungsten forms relatively soluble salts (WS_4^{2-}) and it was therefore probably more available in the euxinic ocean than molybdenum (which would have been present as the water-insoluble MoS_2). The same reasoning explains the higher tungsten availability in today's marine hydrothermal vent waters, precisely where most of the hyperthermophilic organisms were discovered that were found to possess tungstoenzymes.[91] As with molybdenum, it is believed that tungsten would have carried out much the same chemistry as it does today in the enzymes of contemporary organisms. The reversible handling of aldehydes and carboxylic acids by primitive strict anaerobes is plausibly matched by the aldehyde : ferredoxin oxidoreductase of today's *Pyrococcus furiosus* (one of the benchmark tungstoenzymes). Still, today only relatively few organisms utilize tungsten, which might seem puzzling if one considers the chemical similarities between tungsten and molybdenum and the fact that both metals are coordinated by the same organic cofactor (Figure 1.3a; described below). Indeed, it seems that for each tungstoenzyme there is a homologous molybdoenzyme, either in the same or in different organisms, and there are several examples of molybdo- and tungstoenzymes that catalyze the same reaction (*e.g.* aldehydes, oxidoreductases and formate dehydrogenases that can contain molybdenum or tungsten). Could the modern scarcity of tungstoenzymes reflect the early Earth scenario? Were the tungstoenzymes widespread in early Earth and subsequently lost

‡Sulfite oxidase deficiency can be caused by protein point mutations, but also by the inability to synthesize the cofactor that holds the molybdenum atom in the active site (Figure.1.3a; described below); the last case results in deficiency in all four human molybdoenzymes. However, only the sulfite oxidase deficiency is a serious life threat.

with the "pollution" of the atmosphere by dioxygen, forcing organisms to use molybdenum (available as the highly water-soluble MoO_4^{2-}) instead? That is, did molybdenum become dominant only later in the Earth's history, due to its availability and properties? This is a plausible scenario if one takes into account the higher availability of tungsten under euxinic conditions and the chemical singularities of tungsten (instead of the similarities between the two metals): tungsten compounds exhibit lower reduction potentials, higher bond strengths and enhanced thermal stability compared to iso-structural molybdenum counterparts, but are more sensitive to dioxygen.[3,12,92–96] These differences support the idea that tungsten would have been a better choice for anaerobic low reduction potential reactions carried out under euxinic conditions. As the environmental conditions were changing and the Earth became increasingly oxygenated, tungsten could have been replaced by molybdenum: the chemical similarities between the two metals could have been exploited by the surviving organisms to evolve enzymes that enabled them to continue catalyzing the same old reactions and new reactions dictated by the needs imposed by the new environment.[§]

Regardless, both molybdo- and tungstoenzymes probably existed in the last universal common ancestor (LUCA).[106,107] Therefore, the two cofactors that hold the metals in the enzymes active site would also have to have been present. This is particularly remarkable when we realize how elaborated the two cofactors are (particularly the nitrogenase one; Figure 1.3) and how "limited" their utilization compared to, for instance, porphyrin-related structures. Why do living organisms expend so much effort to use these metals in a (comparatively) small number of reactions? This effort (including synthesizing the protein machinery to scavenge the metals from the environment, producing and inserting the specialized cofactors and regulating the whole process) underscores how important both metals would have been, and still are to extant organisms, particularly in the case of molybdenum.

1.2.1 The Nitrogen-to-Molybdenum Bio-to-Inorganic Bridge Hypothesis

The atmosphere of early Earth held no dioxygen (if present, it would be less than 10^{-5} times the current atmospheric level). Only in the second half of the Earth's 4560 million years (Myr) history, between 2450 and 2220 Myr ago, did dioxygen levels rise in the oceans and atmosphere as a consequence of the

[§]A note of caution in this simplistic scenario, where molybdenum "simply" took the place of tungsten: the differences between molybdenum and tungsten are sufficient to interfere with the properties of the vast majority of today's enzymes. In fact, tungsten is regarded as an antagonist and inhibitor of molybdoenzymes and the substitution of molybdenum by tungsten results in metal-free and tungsten-substituted enzymes, both with no enzymatic activity.[97–105] This outcome arises from differences in the metals' uptake and/or incorporation into the enzymes, but also from differences in the properties of the enzymes themselves. However, it should be noted that there are some prokaryotic enzymes that are active with either molybdenum or tungsten, as will be discussed in Section 1.4.4.

"invention" of oxygenic photosynthesis by cyanobacteria[108–111] – the so-called "Great Oxidation Event". Recent geochemical data[112,113] are changing that time frame, however, suggesting that small amounts of dioxygen were present in the environment more than 50 Myr before the start of the Great Oxidation Event, supporting the hypothesis that primitive organisms had learned to produce dioxygen much earlier than previously thought[112,114] – future work will determine if the dioxygen biogenesis is even more ancient than presently thought. Presently, several geoscientists defend the idea that the Earth oxygenation proceeded in two broad steps, near *ca.* 2500 and *ca.* 540 Myr ago.[109,115–121] (Readers not familiar with geochemical studies are referred to note ¶ for a brief explanation.)

During the first oxygenation phase, probably only the ocean surface was affected by photosynthesizing bacteria. Although the dioxygen would have started to increase, only *ca.* 2150 Myr ago, more than 300 Myr after the initial

¶The history of Earth oxygenation is written in the geological record of redox–sensitive transition metal elements preserved in ancient authigenic sediments. The principle is that the amount of those elements present in sedimentary rocks is determined by the dioxygen availability during formation of the sediments.

On early, anoxic (strongly reducing) Earth, molybdenum would have been largely retained in crustal sulfide minerals (it would have not been weathered, solubilized) and its presence would have been small in the oceans and sediments. Under low dioxygen pressures, the rate of dissolution of submarine and sub-aerial sulfide minerals (such as molybdenite, MoS_2) would have been enhanced. Hence, after the rise of dioxygen, oxidative weathering of molybdenum-containing sulfide minerals in crustal rocks would have led to the molybdenum accumulation in oceans (molybdenum dissolution, in the form of the soluble molybdate ion, MoO_4^{2-}).

In fact, today, molybdenum is the most abundant transition metal element in the oceans (present at a concentration of ≈110 nM). Under oxygenated conditions, the oceanic organic-rich sediments would, consequently, show a high authigenic molybdenum enrichment (today, typically values are >100 ppm in sediments *versus* <1 ppm in average crust). Under euxinic, *i.e.* sulfidic and anoxic, conditions (conditions created after the rise of dioxygen; see below), the hydrogen sulfide would have reduced the dissolved molybdenum to Mo^{4+} and the highly insoluble molybdenum sulfide, MoS_2, would have been formed; thus molybdenum would have been removed from the sea water solution. Noteworthy, under euxinic conditions, tungsten and vanadium form relatively soluble salts and they were probably more available in the euxinic ocean. Following this reasoning, the amount of molybdenum in oceanic sedimentary rocks rich in organic matter (black shales) should be a good indicator of the amount of dioxygen in sea water and atmosphere.

A similar analysis would apply to sulfur. On early, anoxic Earth, sulfur would have been largely retained in minerals, and sulfate (and, consequently, hydrogen sulfide) would have been sparse in pre-oxic oceans (before the appearance of dioxygen). The rise of dioxygen would have weathered the minerals (solubilized and oxidized them to release sulfate) and sulfate would have been accumulated in the oceans (where, today, it is the second most abundant anion). In a post-oxic era (after the appearance of dioxygen), under anoxic conditions, the mobilized sulfate would have been reduced to hydrogen sulfide by sulfate-reducing bacteria, creating, in this way, euxinic conditions.

On the contrary, iron would have behaved in an opposite way. Iron would have been easily mobilized during anoxic weathering, thus enriching the sulfur-poor oceans (as aqueous ferrous) and the sediments, leading to banded iron formations. Oxygenation of surface environments would have oxidized the aqueous ferrous iron to insoluble ferric oxides, thus reducing the availability of this element. Euxinic conditions would have also reduced the availability of iron, but in the form of insoluble iron sulfides. Hence, both dioxygen and hydrogen sulfide may have pulled the dissolved iron from solution and be responsible for the disappearance of banded iron formations.

rise in atmospheric dioxygen, the dioxygen pressure would have been sufficiently high to cause the persistent and vigorous oxidative weathering of the molybdenum-containing sulfide minerals in crustal rocks (note that sulfide minerals weather rapidly, and a very low pressure of dioxygen would be enough to account for this effect). Molybdenum, released in this way, would have accumulated (dissolved) in oceans, eventually resulting in the enrichment of the authigenic ocean sediments.[109,115,121] However, the greater oxidative weathering would have also increased the delivery of sulfur to the ocean. The consequent increase in the oceanic hydrogen sulfide would have, in its turn, removed the molybdenum from solution (as the insoluble MoS_2).[116,121–123] Accordingly, expansion of the euxinic (sulfidic and anoxic) conditions, after *ca.* 1800 Myr ago, would have kept molybdenum availability below 10–20% of the modern value. Those same conditions could have promoted the removal of dissolved iron from the sea water (as insoluble iron sulfides) and kept the iron availability low.[116] (In the classic model, the disappearance of banded iron formations is explained invoking oxygenation of the deep ocean in this early time frame, as a consequence of the formation of insoluble ferric oxides;[124,125] according to this new hypothesis, the disappearance of banded iron formations was due to the precipitation of iron under euxinic conditions (anoxic) and not to deep ocean oxygenation, which is suggested to have taken place only more than 1000 Myr later).

The second oxygenation phase is suggested to mark the time when the entire ocean became oxygenated. By *ca.* 660–550 Myr ago, the sediments content suggests an extreme molybdenum presence in the oceans, pointing to the oxygenation of the deep ocean and to the corresponding decrease in sulfidic conditions.[119–121] The process responsible for this sudden change in the molybdenum record and dioxygen presence, however, is not yet fully understood.[126,127] Thus, according to this hypothesis, atmospheric dioxygen levels did not increase monotonically to their modern value but rather proceeded in two phases, separated by ≈2000 Myr. Interestingly, the same time frame is suggested to separate the emergence and subsequent expansion of eukaryotes.[114,123,128] This coincidence has raised the hypothesis that the molybdenum oceanic bioavailability could have played a major role in the ≈2000 Myr delay in the development of early life.[121,123,129]

Plausibly, early forms of life would have employed ferrous iron, abundant on the anoxic oceans, to handle abiotic ammonium, nitrite and nitric oxide and also to fix atmospheric dinitrogen through a primitive iron-containing nitrogenase.[130,131] The rise of dioxygen, with subsequent oxidation and precipitation of iron, would have turned the oceanic dissolved iron into a scarce element. Concurrently, the dioxygen-triggered mobilization of molybdenum would have allowed the evolution of a molybdenum-containing nitrogenase, a new enzyme able to efficiently fix (reduce) dinitrogen into ammonium. However, the subsequent onset of deep ocean euxinia would have acted to remove the dissolved molybdenum (and iron), maintaining the oceanic molybdenum (and iron) concentration low. This molybdenum scarcity would have hampered the expansion of molybdenum-containing nitrogenase,[131] limiting the availability of "bio-nitrogen" for the early organisms (limiting the rate

of dinitrogen fixation and the supply of fixed nitrogen inter-organisms). Ultimately, the molybdenum scarcity could have restricted the evolutionary path of eukaryotes[121,123,129,132] – *the nitrogen-to-molybdenum bio-to-inorganic bridge hypothesis*.

Nevertheless, the molybdenum shortage would have also contributed to limit, spatially and temporally, the extent of sulfidic conditions, because molybdenum would be also essential (directly or indirectly) for the bacteria that carry out the reduction of sulfate to sulfide, and because organic matter is required for the exhaustion of dioxygen.[121] This negative feedback mechanism could explain the apparent decline in euxinic deposition after 1400 Myr ago. By *ca.* 660–550 Myr ago, the deep ocean oxygenation and the increased molybdenum availability would have favoured the diversification of dinitrogen fixing organisms; this would have boosted the photosynthetic dioxygen production in the oceans and, in this way, the ocean-atmosphere thorough oxygenation (like a vicious cycle).[‖] With a more efficient respiratory substrate and "bio-nitrogen" source, the stage was set for the subsequent evolution and proliferation of structurally complex forms of life, leading to the Cambrian explosion of metazoan life – remarkably, only in the last tenth of the Earth's history.

In this scenario, Earth and life on it would have evolved together, with different key events being closely interrelated:[134–136] the accumulation of (biologically produced) dioxygen triggered the geological molybdenum release, but the subsequent environmental removal of molybdenum retarded its biological utilization and, eventually, delayed the evolution of complex life for *ca.* 2000 Myr. This scenario exemplifies how biology, geology and the atmosphere might have interacted and the knowledge gathered may be helpful to understand the environmental and climate issues we are facing today (*e.g.* the greenhouse effect gas carbon dioxide scavenging by an iron-starved ocean). More important in the context of this book, the hypothesis described above emphasizes the critical biological role of molybdenum for the life on Earth. At the same time, this hypothesis also raises the question as to why there is (as far as is presently known) no tungsten-dependent nitrogenase. If the organisms were able to develop iron and vanadium-/iron-dependent nitrogenases, why did they not also use tungsten?

1.3 Chemistry Relevant to Molybdenum and Tungsten Biochemistry

Organisms use molybdenum and tungsten for the most part to catalyze oxidation–reduction reactions, most of which involve oxygen atom transfer to/from a carbon, nitrogen and sulfur atom of key metabolites.[3–10] Certainly, both metals exhibit the chemical properties appropriate for redox biochemistry:[26]

[‖]But the "revolution" in dinitrogen fixation initiated by the dioxygen rise had not been finished yet: the dioxygen that triggered (*indirectly*) the evolution of molybdenum-dependent nitrogenase also inhibits (*directly*) the *new* enzyme. This forced the nitrogen-fixing organism to evolve mechanism to protect the enzyme from dioxygen.[133] The structural protection in aerobic organisms continues to the present.

they are redox-active under physiological conditions and their oxidation state can range from 6+, 5+ and 4+, and even 3+, in the molybdenum of nitrogenase. This versatility allows molybdenum- and tungsten-containing enzymes to catalyze either two-electron ($M^{6+} \leftrightarrow M^{4+}$) or one-electron ($M^{6+} \leftrightarrow M^{5+}$, $M^{5+} \leftrightarrow M^{4+}$) oxidation-reduction reactions, and thus couple obligatory two-electron and one-electron systems, *e.g.* the reduction of a two-electron respiratory substrate with a one-electron transfer protein. In addition, their chemistry is dominated by the formation of oxides and sulfides and a very versatile first coordination sphere. The strong tendency of molybdenum to bind oxo groups is balanced by its ability to easily lose a single oxygen atom, a property that makes molybdenum complexes excellent "oxygen atom exchangers",[137–156] as long as the thermodynamics of the reaction of "oxygen exchange" is favourable (eqn (1.1) (M stands for metal))[139,143,152] – the "oxo transfer hypothesis" coined by Holm and others in the 1980s.

$$M{-}O + X \rightleftharpoons M + X{-}O \tag{1.1}$$

Organisms explore this rich chemistry to carry out oxo transfer reactions: typically, the molybdo- and tungstoenzymes catalyze the transfer of an oxygen atom from water to product – oxygen atom insertion (eqn (1.2); Figure 1.2, blue arrows) – or from substrate to water –oxygen atom abstraction (eqn (1.3); Figure 1.2, green arrows) – in reactions that entail a net exchange of two electrons, in which the molybdenum/tungsten atom cycle between Mo^{6+}/W^{6+} and Mo^{4+}/W^{4+}, and, most importantly, where the metal is the direct oxygen atom acceptor or donor.[3–10,154–159] (The detailed mechanisms through which the enzymes catalyze these reactions will be discussed in Sections 1.4.1–1.4.5.) It is based on these catalytic features that these enzymes are commonly, although inaccurately, referred to as oxotransferases, since there are several noteworthy exceptions to the oxo transfer activity. The versatile chemistry of molybdenum and tungsten has allowed the evolution of enzymes that catalyze reactions, for example, of (i) proton abstraction (formate dehydrogenase-catalyzed formate oxidation to carbon dioxide; eqn (1.25) in Section 1.4.3), (ii) sulfur atom transfer (polysulfide reductase-catalyzed inorganic sulfur reduction to sulfide (eqn (1.26) in Section 1.4.3) or MOSC-catalyzed sulfurations) or (iii) even non-redox hydration reaction (acetylene hydratase-catalyzed hydration of acetylene to acetaldehyde; eqn (1.28) in Section 1.4.3).

$$M^{6+} + R + H_2O \rightarrow M^{4+} + R{-}O + 2H^+ \tag{1.2}$$
$$M^{4+} + Q{-}O + 2H^+ \rightarrow M^{6+} + Q + H_2O \tag{1.3}$$

Another interesting exception is the group of enzymes able to catalyze both oxygen atom insertion and abstraction during the same catalytic cycle. This is the case of the prokaryotic molybdenum-containing pyrogallol : phloroglucinol transhydroxylase (eqn (1.27) in Section 1.4.3)). This enzyme catalyzes the "simple" hydroxyl transfer between two hydroxylated benzene compounds:[160] the reduced molybdenum core accepts one hydroxyl group

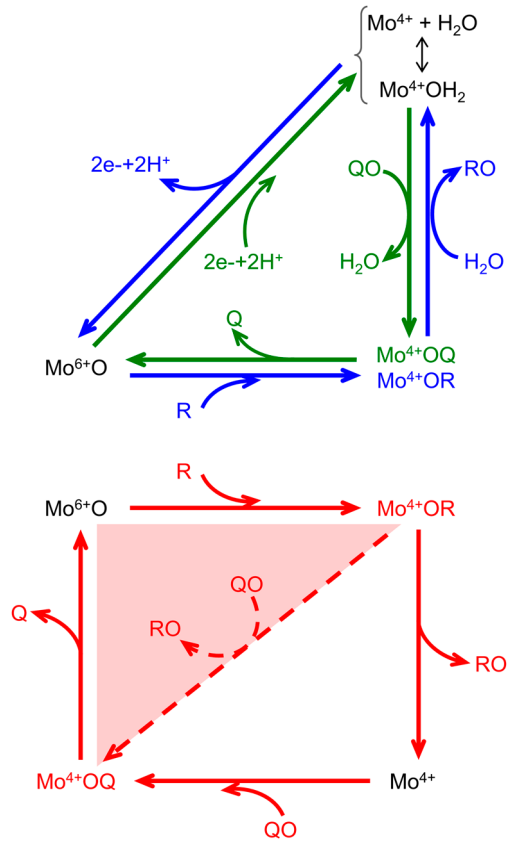


Figure 1.2 Mono oxo transfer (blue and green) and double oxo transfer (red) hypothesis. This schematic representation highlights that Mo^{6+} cores can be thought of as competent oxo donors, while the Mo^{4+} cores would act as oxo acceptors. The mono oxo transfer path for oxygen atom insertion reactions is represented in blue (*e.g.* for the sulfite oxidase reaction (eqn (1.16)), R is sulfite and RO is sulfate). The mono oxo transfer path for oxygen atom abstraction reactions is represented in green (*e.g.* for the nitrate reductase reaction (eqn (1.17)), QO is nitrate and Q is nitrite). The double oxo transfer path is represented in red (*e.g.* for the simultaneous oxygen atom insertion and abstraction reaction of the pyrogallol : phloroglucinol transhydroxylase (eqn (1.27)), R represents pyrogallol that is hydroxylated to tetrahydroxybenzene, represented by RO, and tetrahydroxybenzene, QO, is reduced to phloroglucinol, represented by Q. The shaded triangle illustrates the possibility that substrate QO displaces the product RO, without the formation of a reduced "free" molybdenum centre.

from one of the substrates, to become itself oxidized and hydroxylated; subsequently the molybdenum core transfers the hydroxyl group to the second substrate in an oxidative hydroxylation (thus becoming reduced and "dehydroxylated"). Therefore, this enzyme uses its molybdenum centre to directly transfer the hydroxyl group from one substrate to the second one,

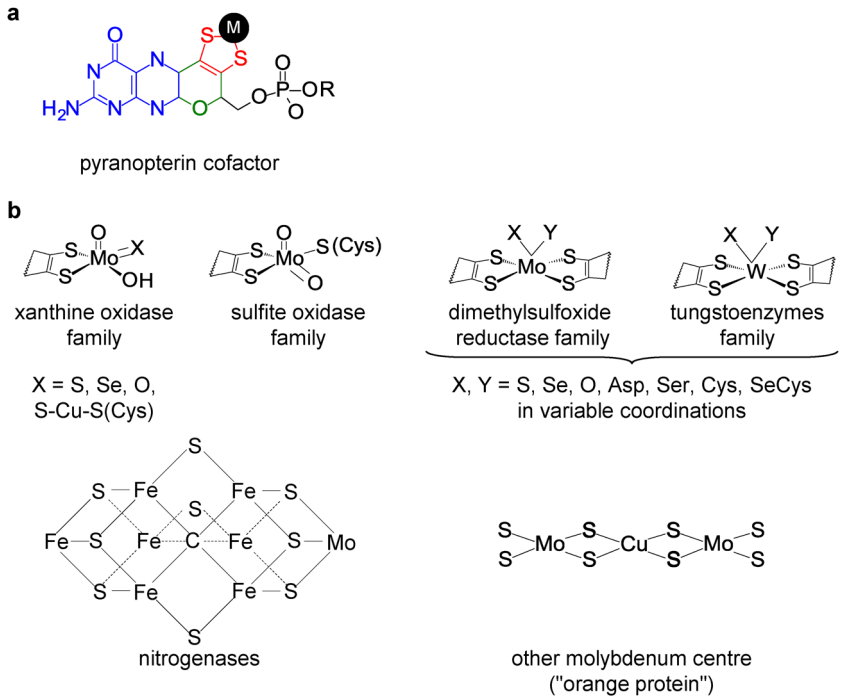


Figure 1.3 Schematic representation of the structures of the pyranopterin cofactor (a), the active site of molybdo- and tungstoenzymes and the "orange protein" (b). (a) The pyranopterin cofactor molecule is formed by pyrano(green)-pterin(blue)-dithiolene(red)-methylphosphate(black) moieties. The dithiolene (–S–C=C–S–) group forms a five-membered ene-1,2-dithiolate chelate ring with the molybdenum/tungsten atom. In eukaryotes, the cofactor is found in the simplest monophosphate form (R is a hydrogen atom), while in prokaryotes it is most often found esterified with several nucleotides (R can be one cytidine monophosphate, guanosine monophosphate or adenosine monophosphate). M, stands for metal, molybdenum or tungsten. (b) Structures of the molybdenum and tungsten centres of the five families of molybdo- and tungstoenzymes in the oxidized form and of the "orange protein". For simplicity, only the *cis*-dithiolene group of the pyranopterin cofactor is represented in the xanthine oxidase, sulfite oxidase, dimethylsulfoxide reductase and tungstoenzymes families. In carbon monoxide dehydrogenase, where X represents S–Cu–S(Cys), the terminal hydroxyl (Mo–OH) is replaced by an oxo group (Mo=O). The images were produced with Accelrys Draw 4.0 (Accelrys Software Inc.).

but without using water as the ultimate oxygen acceptor/donor** (eqn (1.4); Figure 1.2, red arrows).

$$R + Q\text{–}O \rightarrow R\text{–}O + Q \qquad (1.4)$$

**There are other mechanistic proposals, more complex, for this enzyme,[161] but all must explain the hydroxyl transfer activity, with the hydroxyl group not being derived from solvent.

Another example of the ability to catalyze oxygen atom insertion and abstraction in the same catalytic cycle is provided by the nitric oxide-forming nitrite reductase activity (eqn (1.5)) of some molybdoenzymes (see Section 1.2 for the physiological context of this activity).[62,63] Mammalian xanthine oxidoreductase, aldehyde oxidase[64,65] and sulfite oxidase[66] and bacterial aldehyde oxidoreductase[64] catalyze the oxygen atom insertion into their "classic" substrates (xanthine (eqn (1.6) in Section 1.4.1)), aldehyde (eqn (1.7) in Section 1.4.1)), sulfite (eqn (1.16) in Section 1.4.2)) and aldehyde (eqn (1.26) in Section 1.4.3)), respectively); the resulting reduced molybdenum centres are then able to catalyze the abstraction of the oxygen atom of nitrite to yield nitric oxide (eqn (1.4); Figure 1.2, red arrows). Hence, nitrite, the second substrate, acts as oxidizing substrate and oxo group donor, while the "classic" substrate functions as a reducing substrate and oxo group acceptor.[63] This description does not intend to mean that it is necessarily the oxygen atom of the second substrate that is inserted into the "classic" substrate (which was not yet confirmed experimentally, because the molybdenum labile oxo/hydroxyl group can be easily exchanged with solvent water), although, as represented in Figure 1.2, red arrows, it is possible that this is the case.

$$NO_2^- + e^- + 2H^+ \rightarrow {}^{\bullet}NO + H_2O \qquad (1.5)$$

These molybdenum-containing enzymes[††] demonstrate that, in the presence of an oxo donor and oxo acceptor, the molybdenum centre can catalyze the oxygen transfer between the two as long as the thermodynamics of the reaction is favourable.[139,143,152] In other words, the nitrite reductase activity can be common to other molybdoenzymes and, more interestingly, the "double oxo transfer" reaction should be possible for substrates other than nitrite.

The versatile chemistry displayed by the molybdenum- and tungsten-containing enzymes is also a consequence of the "environment" (first and second coordination spheres) of the metals inside the protein, which would shape the final catalytically competent metal centre. As will be discussed in Section 1.4, with very few exceptions, these enzymes hold the molybdenum or tungsten atom coordinated by a unique cofactor: a pyranopterin moiety modified with a *cis*-dithiolene group (–S–C=C–S–) and a methylphosphate (which can be further esterified) (Figure 1.3a), herein designated as the pyranopterin cofactor.[‡‡] The pyranopterin cofactor is not thought to be an "innocent scaffold",[162–166] but has been suggested to facilitate intramolecular

[††]Of note, the nitric oxide-forming nitrite reductase activity was also described in other molybdoenzymes, namely in mammalian mitochondrial amidoxime reducing component (eqn (1.21))[67] and plant and bacterial nitrate reductases[62] (eqn (1.16)). However, the "classic" activity of those enzymes is already an oxygen atom abstraction reaction and, hence, no concomitant oxygen insertion was demonstrated.

[‡‡]The pyranopterin cofactor molecule is still commonly referred to as "molybdopterin", because, historically, the cofactor was first identified in molybdenum-containing enzymes. However, because the same cofactor molecule is used to coordinate tungsten in tungstoenzymes (described under Section 1.4.1), this denomination is not clear.

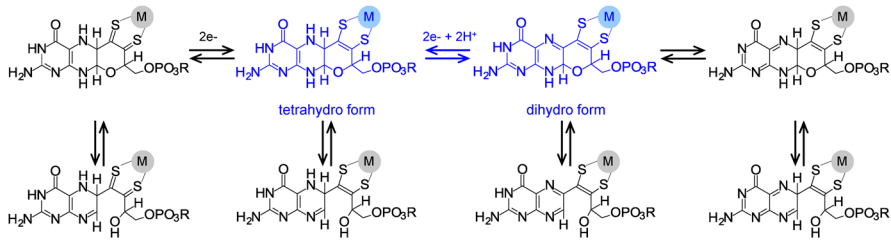


Figure 1.4 Schematic representation of some isomers and redox forms of the pyranopterin cofactor. The dihydro and tetrahydro forms are highlighted in blue. The structures represented below correspond to the scission reaction of the pyran ring (the "open pyran ring forms"). M, stands for metal.

electron transfer, acting like a "wire" to conduct the electrons to other redox-active centres that are most often found in these enzymes. Moreover, because the pyranopterin cofactor has several potential structural isoforms and oxidation states (Figure 1.4),[153,167–172] each adopting a different geometry, it has recently been suggested that each enzyme holds a binding pocket that selectively controls the pyranopterin conformation, mainly in the dihydro and tetrahydro reduced forms, in order to tune the metal centre oxidation state and facilitate electron transfer.[173] Of note, two crystal structures have been reported in which the pyran ring of the cofactor is in the open form, that of the respiratory *E. coli* NaR and that of *Aromatoleum aromaticum* ethylbenzene reductase.[174–176] Although it can be suggested that the opening and closing of the pyran ring might provide protons for the reaction,[170,177,178] its physiological relevance remains to be established. In fact, it has recently been suggested that the as-isolated periplasmatic nitrate reductase from *Rhodobacter sphaeroides* holds its proximal cofactor molecule in a fully oxidized and ring-opened form; this enzyme form has been suggested to require an activation mechanism that involves both the cyclization of the pyran ring and the reduction of the pterin to yield a catalytically competent tricyclic tetrahydropyranopterin cofactor and recovery of the physiologic intramolecular electron transfer between the molybdenum centre and its [4Fe–4S] centre.[179]

Besides the pyranopterin cofactor, the protein and the other atoms of the metals' coordination sphere can influence reactivity. In fact, even small alterations of the metal centre geometry can alter the energy level of the metal d orbitals, in particular of the d_{xy} orbital in the ground state, thus modulating the reduction potential of the molybdenum centre.[180–185] The influence of the protein can be envisaged, for example, in enzymes where the metal is coordinated by two pyranopterin cofactors: the trigonal prismatic coordination geometry observed in the enzymes contrasts with the octahedral geometry found in model compounds, suggesting that the geometry in the enzymes is imposed by the polypeptide chain,[186–188] apparently with the objective of creating an entatic state that would labilize the oxo group and facilitate its dissociation.[189] The role of the first coordination sphere in modulating enzyme reactivity can be appreciated when the effects of sulfo or thiolate

groups are compared: in xanthine oxidoreductase, the presence of a highly covalent terminal sulfur atom (Mo=S) with an available S π-bond, instead of thiolate (Mo–S(Cys)), allows the enzyme to accept a proton plus two electrons (hydride), thus facilitating the cleavage of a C–H bond. On the other hand, in sulfite oxidase, which holds a thiolate (Mo–S(Cys)), it is an apical terminal oxygen, with a formal triple bond (Mo≡O), that would act to labilize the oxo group to facilitate its dissociation and the metal re-oxidation.[190–196] In the following Section (1.4), the description of the structural and mechanistic properties of an array of molybdenum- and tungsten-containing enzymes will illustrate how the organisms exploit the versatile chemistry of these two metals.

1.4 Molybdenum- and Tungsten-Containing Enzymes

The biological relevance of molybdenum was demonstrated in the early 1950s–1960s, by Bray, Beinert, Lowe, Massey, Palmer and others, with ground-breaking studies performed by electron paramagnetic resonance spectroscopy that demonstrated the reduction of molybdenum to Mo^{5+} under different conditions, using mainly the molybdoenzymes xanthine oxidase and sulfite oxidase.[197–212] Noteworthy, xanthine oxidase had been already purified in 1924.[213] Tungsten only attracted the attention of the "bio-community" much later. Although it had been known since the 1970s that tungsten stimulated the growth of some acetogens and methanogens,[14,15,214–217] only in the 1980s was the first tungsten enzyme purified, one formate dehydrogenase,[218] and only in the 1990s was interest in these enzymes really boosted,[219–221] in particular after the first crystal structure determination in 1995, of the aldehyde : ferredoxin oxidoreductase from *Pyrococcus furiosus*.[222] The simultaneous determination of the first crystal structure of a molybdoenzyme, the aldehyde oxidoreductase from *Desulfovibrio gigas*,[223] and, subsequently, of dimethylsulfoxide reductase from *Rhodobacter sphaeroides*,[224] allowed the direct comparison between molybdo- and tungstoenzymes and highlighted how these enzymes are strikingly related.

Presently, more than 50 molybdoenzymes are known, many of which have already been biochemically and structurally characterized, and the number is increasing every year, with several more being foreseen to be identified in the near future based on genomic analyses.[2,225,226] With the exception of the unique heteronuclear [$MoFe_7S_9C$] cofactor of nitrogenase, and a few other heteronuclear centres whose physiological function is not yet fully understood (Figure 1.3b; described under Sections 1.4.5 and 1.4.6), molybdenum is found in the enzymes active sites in a mononuclear form, [§§] hereafter designated as molybdenum centre.[4–10] In these centres, one molybdenum atom

[§§]The carbon monoxide dehydrogenase from *Oligotropha carboxidovorans* or *Hydrogenophaga pseudoflava*, with its unique binuclear Mo/Cu cofactor, is another exception. Nevertheless, this enzyme is grouped under the mononuclear molybdoenzymes classification, as a member of the xanthine oxidase family (discussed below).

is coordinated by the *cis*-dithiolene (–S–C=C–S–) group of one or two pyranopterin cofactor molecules (Figure 1.3a) and by oxygen and/or sulfur and/or selenium atoms in a diversity of arrangements that established the innovator classification of the mononuclear molybdoenzymes into three large families, denominated after one benchmark enzyme (Figure 1.3b):[4] the xanthine oxidase family, the sulfite oxidase family and the dimethylsulfoxide reductase family (described under Sections 1.4.1–1.4.3).

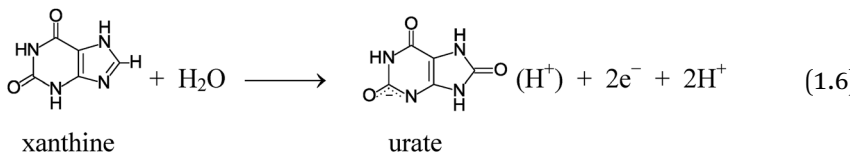
Tungsten is also found in the active site of enzymes in a mononuclear form (herein denominated tungsten centre), where it is coordinated by the *cis*-dithiolene group of two molecules of the same pyranopterin cofactor found in molybdoenzymes (Figure 1.3a).[3,5,227] The tungsten coordination sphere is completed with oxygen and/or sulfur and/or selenium atoms in a diversity of arrangements, most often in the same geometry as found in members of the dimethylsulfoxide reductase family of molybdenum enzymes (Figure 1.3b). The classification of tungstoenzymes has been a matter of some controversy. Some authors pool all the tungsten-containing enzymes into a single family, characterized by the presence of a tungsten-bis-pyranopterin system. Others prefer to classify the tungsten-containing aldehyde : ferredoxin oxidoreductases separately, in a distinct family, with the other tungstoenzymes in the molybdenum-containing dimethylsulfoxide reductase family. This latter classification is based on the fact that the great majority of the prokaryotic molybdo- and tungstoenzymes harbour an active site with the metal coordinated by two pyranopterin molecules esterified to guanosine monophosphate, forming a pyranopterin guanosine dinucleotide (PGD) (which is not the case of the aldehyde : ferredoxin oxidoreductases, whose pyranopterin cofactor molecules are in the mononucleotide form) (Figure 1.3a). As such, some authors place the molybdenum-containing dimethylsulfoxide reductase family enzymes and those tungstoenzymes containing PGD together in a unique super-family denominated "molybdenum/tungsten-bis pyranopterin guanosine dinucleotide-containing enzymes", with the acronym Mo/W-bis PGD[228] (where the family name is not misleading, the name stressing the presence of both molybdo- and tungstoenzymes). This classification is particularly useful when one aims to highlight the structural/functional similarities between, for example, the homologous molybdenum-containing and tungsten-containing formate dehydrogenases.

We suggest that a systematic organization, based on the metal/cofactor structure, should be followed and all tungsten–pyranopterin-containing enzymes should be grouped together. This tungstoenzymes family can then be sub-divided to account for differences between members, as some authors do with other families (the dimethylsulfoxide reductase family is sometimes organized in subfamilies based on the amino acid residue that coordinates the molybdenum atom; also the sulfite oxidase family has become extremely heterogeneous, with the identification of new members). Following the above criteria based on the metal/cofactor structure, the molybdo- and tungstoenzymes will be here organized into five families (Figure 1.3b), described in Sections 1.4.1 to 1.4.5: xanthine oxidase family,

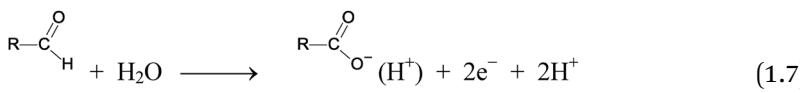
sulfite oxidase family, dimethylsulfoxide reductase family, tungstoenzymes family and nitrogenases.

1.4.1 The Xanthine Oxidase Family

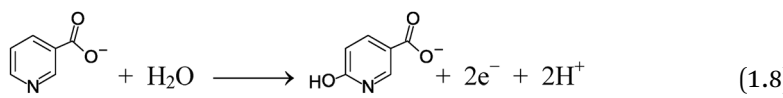
The active site of the xanthine oxidase (XO) family enzymes (in its oxidized form) has a molybdenum ion coordinated in a square-pyramidal geometry by an apical oxo group (Mo=O) and, in the equatorial plane, by the two sulfur atoms of the *cis*-dithiolene group of one pyranopterin cofactor molecule,[¶¶] one catalytically labile –OH group (in most cases) and one terminal sulfo (Mo=S; in most cases) or seleno (Mo=Se) or oxo (Mo=O) group (Figure 1.3b). This family comprises enzymes such as mammalian XO (eqn (1.6)) and aldehyde oxidase (AO; eqn (1.7)), prokaryotic *Desulfovibrio* aldehyde oxidoreductases (AOR, eqn (1.7)), *Eubacterium barkerii* nicotinate dehydrogenase (eqn (1.8)), *Pseudomonas putida* quinoline 2-oxidoreductase (eqn (1.9)) and *Thauera aromatica* 4-hydroxybenzoyl-CoA reductase (eqn (1.10)) (Table 1.2). The prokaryotic *Oligotropha carboxidovorans* carbon monoxide dehydrogenase (eqn (1.11)), with its unique binuclear Mo/Cu cofactor coordinated to the polypeptide chain by one cysteine residue through the Mo–S–Cu–S(Cys) motif, is also included in the XO family.[229,230] Although the active site of this enzyme is not mononuclear and its sulfur atom is not terminal (as is the case in other XO family enzymes), its classification under the XO family is supported by the considerable sequence and structural homology with the bovine XO and common reactivity, and also its sensitivity to inhibition by cyanide.[‖‖,229–233]



xanthine urate (1.6)



(1.7)



nicotinate 6-hydroxynicotinate (1.8)

[¶¶]The pyranopterin cofactor molecule in prokaryotic enzymes is often found esterified, typically with a cytidine monophosphate (Figure 1.3a).

[‖‖]Cyanide reacts with the equatorial Mo=S group of the molybdenum centre, abstracting its sulfur atom in the form of thiocyanide and leaving a Mo=O group in its place. The desulfo forms thus formed are non-functional.

Table 1.2 Structural characteristics of some molybdoenzymes and tungstoenzymes.

Enzyme	Molybdenum/tungsten coordination[a]	Subunit composition[b]	Notes (ref.)
Xanthine oxidase family			
Mammalian xanthine oxidase	PMN, =O, =S, –OH	α_2, α: Mo, 2 [2Fe–2S], FAD	Eqn (1.6), (1.12) and (1.13), Figures 1.5, 1.6, see text for details
R. capsulatus xanthine oxidoreductase	PMN, =O, =S, –OH	$(\alpha\beta)_2$, a: Mo, b: 2 [2Fe–2S], FAD	Eqn (1.6) ([257])
Mammalian aldehyde oxidase	PMN, =O, =S, –OH	α_2, α: Mo, 2 [2Fe–2S], FAD	Eqn (1.7), (1.14), Figure 1.6, see text for details
Desulfovibrio aldehyde oxidoreductase	PCD, =O, =O, –OH	α_2, α: Mo, 2 [2Fe–2S]	Eqn (1.7), (1.15), Figures 1.5, 1.6, see text for details
E. coli aldehyde oxidoreductases	PCD, =O, =S, –OH	αβγ, α: Mo, β: FAD, [4Fe–4S], γ: 2 [2Fe–2S]	Eqn (1.7), Figure 1.6, ([310])
E. barkerii, nicotinate dehydrogenase	PCD, =O, =Se, –OH	$(\alpha\beta\gamma\delta)_2$, α, β: Mo, γ: 2 [2Fe–2S], δ: FAD	Eqn (1.8), see text for details
P. putida quinoline 2-oxidoreductase	PCD, =O, =S, –OH	$(\alpha\beta\gamma)_2$, α: Mo, β: 2 [2Fe–2S], γ: FAD	Eqn (1.9), see text for details
T. aromatica, 4-hydroxybenzoyl-CoA reductase	PCD, =O, =O, –OH	$(\alpha\beta\gamma)_2$, α: Mo, β: 2 [2Fe–2S], γ: FAD, [4Fe–4S]	Eqn (1.10), Figure 1.5, see text for details
Oligotropha carboxidovorans, carbon monoxide dehydrogenase	PCD, =O, –S–Cu–S(Cys), =O	$(\alpha\beta\gamma)_2$, α: Mo, β: 2 [2Fe–2S], γ: FAD	Eqn (1.11), Figure 1.5, see text for details
Sulfite oxidase family			
Chicken sulfite oxidase	PMN, =O, –S(Cys), =O	α_2, α: Mo, haem	Eqn (1.16), (1.18), Figures 1.7, 1.8, see text for details
Plant sulfite oxidase	PMN, =O, –S(Cys), =O	α_2, α: Mo	Eqn (1.16), (1.19), Figures 1.7, 1.8, see text for details
S. novella sulfite dehydrogenase	PMN, =O, –S(Cys), =O	αβ, α: Mo, β: Haem	Eqn (1.16) Figures 1.7, 1.8, see text for details
Sinorhizobium meliloti sulfite dehydrogenase	PMN, =O, –S(Cys), =O	α_2, α: Mo	Eqn (1.16), see text for details

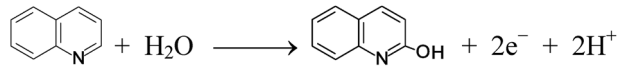
Enzyme	Molybdenum/tungsten coordination[a]	Subunit composition[b]	Notes (ref.)
Eukaryotic assimilatory nitrate reductases	PMN, =O, –S(Cys), =O	α_2, α: Mo, haem, FAD	Eqn (1.17) and (1.20), Figures 1.7, 1.8, see text for details
Mammalian mitochondrial amidoxime reducing component	PMN, =O, –S(Cys), =O	Three protein chain complex with cyt. b_5 plus cit. *b* reductase	Eqn (1.21), Figure 1.7, see text for details
E. coli YedY	PMN, =O, –S(Cys), =O	αβ, α: Mo, β: Haem	([396])
Dimethylsulfoxide reductase family			
R. sphaeroides dimethylsulfoxide reductase	PGD, –O(Ser), =O	α, α: Mo	Eqn (1.22), Figures 1.9, 1.10, see text for details
E. coli dimethylsulfoxide reductase	PGD, –O(Ser), =O?	(αβγ)?, α: Mo, [4Fe–4S]?, β: 4 [4Fe–4S]?, γ: none?	Eqn (1.22), Figures 1.9, 1.10, see text for details
D. desulfuricans periplasmatic nitrate reductase	PGD, –S(Cys), =S	α, α: Mo, [4Fe–4S]	Eqn (1.17), Figures 1.9, 1.10, see text for details
C. necator periplasmatic nitrate reductase	PGD, –S(Cys), =S	αβ, α: Mo, [4Fe–4S], β: 2 haem	Eqn (1.17), see text for details
E. coli periplasmatic nitrate reductase	PGD, –S(Cys), –OH	α, α: Mo, [4Fe–4S]	Eqn (1.17), see text for details
E. coli, respiratory nitrate reductase (NaRGHI)	PGD, –O(Asp), =O or PGD, –O(Asp)O–	$(\alpha\beta\gamma)_2$, α: Mo, [4Fe–4S], β: 3 [4Fe–4S], [3Fe–4S], γ: 2 haem	Eqn (1.17), Figure 1.9, see text for details
E. coli formate dehydrogenase H	PGD, –Se(Cys), =S	α, α: Mo, [4Fe–4S]	Eqn (1.25), Figures 1.9, 1.11, see text for details
E. coli formate dehydrogenase N	PGD, –Se(Cys), =S	$(\alpha\beta\gamma)_3$, α: Mo, [4Fe–4S], β: 4 [4Fe–4S], γ: 2 haem	Eqn (1.25), Figures 1.9, 1.11, see text for details
E. coli formate dehydrogenase O	PGD, –Se(Cys), =S	$(\alpha\beta\gamma)_3$, α: Mo, [4Fe–4S], β: 4 [4Fe–4S], γ: 2 *b* haems	Eqn (1.25), [455,469,470]
D. desulfuricans formate dehydrogenase	PGD, –Se(Cys), S?	αβγ, α: Mo, [4Fe–4S], β: [4Fe–4S], γ: 4 *c* haems	Eqn (1.25), [471 and 472]
R. eutropha formate dehydrogenase	PGD, –S(Cys), S?	αβγδ?, α: Mo, 2 [2Fe–2S], 3 [4Fe–4S], β: [4Fe–4S], FMN, γ: 2 [2Fe–2S]	Eqn (1.25), [473 and 475]
R. capsulatus formate dehydrogenase	PGD, –S(Cys), =S	$(\alpha\beta\gamma)_2$, α: Mo, [2Fe–2S], 4 [4Fe–4S], β: [4Fe–4S], FMN, γ: [2Fe–2S]	Eqn (1.25), ([476])

(*continued*)

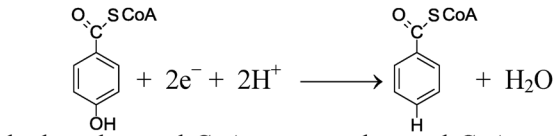
Table 1.2 (*continued*)

Enzyme	Molybdenum/tungsten coordination[a]	Subunit composition[b]	Notes (ref.)
M. formicicum formate dehydrogenase	PGD, –S(Cys), S?	αβ, FAD, several Fe/S, Zn	Eqn (1.25), ([676–681])
T. thermophilus polysulfide reductase	PGD, –S(Cys), –OH	$(\alpha\beta\gamma\delta)_2$, α: Mo, [4Fe–4S], β: 4 [4Fe–4S], γ: none	Eqn (1.26), see text for details
A. faecalis arsenite oxidase	PGD, =O, –OH	αβ, α: Mo, [3Fe–4S], β: [2Fe–2S]	Eqn (1.23), [404–407]
P. acidigallici pyrogallol:phloroglucinol transhydroxylase	PGD, –O(Ser), –OH/–OH_2?	αβ, α: Mo, β: 3 [4Fe–4S]	Eqn (1.27), ([161])
A. aromaticum ethylbenzene dehydrogenase	PGD, –O(Asp), =O?	αβγ, α: Mo, [4Fe–4S], β: 3 [4Fe–4S], [3Fe–4S], γ: haem	([176,682–685])
Shewanella massilia trimethylamine-*N*-oxide reductase tungstoenzymes family	PGD, –O(Ser), =O	α, α: Mo	([686,687])
P. furiosus aldehyde:ferredoxin oxidoreductase	PMN, =O, =O/–OH?	α_2, α: W, [4Fe–4S]	Eqn (1.7), Figure 1.12, see text for details
P. furiosus formaldehyde:ferredoxin oxidoreductase	PMN, =O, =O/–OH?	α_4, α: W, [4Fe–4S]	Eqn (1.7), see text for details
D. gigas formate dehydrogenase	PGD, –Se(Cys), =S	αβ, α: W, [4Fe–4S], β: 3 [4Fe–4S]	Eqn (1.25), Figure 1.12, see text for details
Methylobacterium extorquens formate dehydrogenase	PGD, –S(Cys), S?	αβ, α: W, ≥1 Fe/S, β: [4Fe–4S], FMN	Eqn (1.25), ([18])
G. metallireducens benzoyl-CoA reductase	PMN, –S(Cys)?	Multimeric, α: W, [4Fe–4S]	Eqn (1.29), Figure 1.12, see text for details
P. acetylenicus acetylene hydratase	PGD, –S(Cys), –OH_2	α, α: W, [4Fe–4S]	Eqn (1.28), Figure 1.12, see text for details

[a]PCD, pyranopterin cytidine dinucleotide; PGD, pyranopterin guanosine dinucleotide; PMN, pyranopterin mononucleotide (see Figure 1.3a).
[b]Here, the chemical symbols Mo and W stand for molybdenum and tungsten centre, respectively.



quinoline 2-hydroxyquinoline (1.9)



hydroxybenzoyl-CoA benzoyl-CoA (1.10)

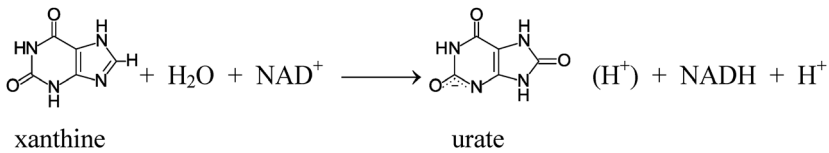
$$CO + H_2O \rightarrow OCO + 2e^- + 2H^+ \quad (1.11)$$

a) The history of bovine milk XO, the prototype enzyme of this family, began more than a century ago, with the Schardinger observation (in 1902) of the decolourization (reduction) of methylene blue by formaldehyde in the presence of fresh milk.[234] This was followed by the discovery that milk could also oxidize hypoxanthine and xanthine to urate[235] and the partial purification of XO in 1924.[213] Being one of the most studied enzymes, our present understanding of its structure and function allows us to discuss its reaction mechanism with atomic and electronic details for each catalytic intermediate, as well as the complex mechanism by which posttranslational conformational modifications promote an "activity switch".

Mammalian XO enzymes are cytoplasmatic proteins synthesized as NAD^+-dependent dehydrogenases (denominated xanthine dehydrogenase (XD), eqn (1.12)), and are believed to exist under normal physiological conditions mostly in this form *in vivo*.[154–159] XD can be rapidly converted into a "strict" oxidase form, however, that reduces dioxygen rather than NAD^+ – the commonly studied and very well-documented XO (eqn (1.13)). This conversion can be reversible, through oxidation of key cysteine residues, or irreversible, by limited proteolysis.***,[157,236–252] However, the extent and

***It is to be emphasized that mammalian XO and XD are two forms of the same protein (same gene product) that arise as the result of posttranslational conformational modifications (oxidation of key cysteine residues or limited proteolysis);[157,236–252] to designate the two forms, without regard to the oxidizing substrate, it is preferable to use the denomination "xanthine oxidoreductase". 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^+ (but it can also reduce dioxygen), XO fails to react with NAD^+ and uses exclusively dioxygen. During the XD into XO conversion process, the protein conformation at the FAD centre is modified and this conformational alteration is responsible for the differentiated oxidizing substrate specificity displayed by XO and XD (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.[154–159] Hence, XO and XD can be considered as one unique entity for the discussion of the overall structural organization and molybdenum reactivity (reaction mechanism). Noteworthy, the enzyme from other sources, *e.g.* turkey and chicken,[253–255] insects[256] and bacteria[257] exists solely as a dehydrogenase form.

rate of conversion of XD into XO *in vivo* have been a matter of great controversy.[237,240,241,258–266] Physiologically, mammalian XD (XO) is a key enzyme in purine catabolism, where it catalyzes the hydroxylation of both hypoxanthine and xanthine to the terminal metabolite, urate (eqn (1.6)).[154–159] It has also been suggested to be involved in the xenobiotic compounds metabolism and in ROS-mediated signalling pathways and ROS-mediated diseases.[265–292]



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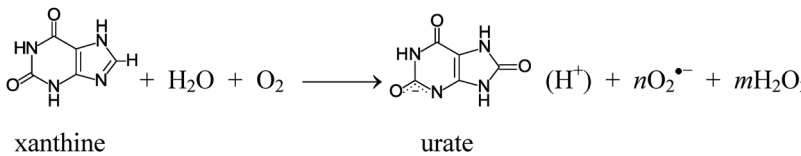


(1.13)

In its oxidized form, the molybdenum centre of mammalian XO has the characteristic catalytically labile –OH group and an essential terminal sulfo group (Mo=S). Interestingly, some prokaryotic enzymes have instead a terminal seleno group (Mo=Se) that is also essential for the activity[293,294] (*e.g.* nicotinate dehydrogenase, another member of the XO family[295–297]). Besides the molybdenum centre, mammalian XO possesses three additional redox-active centres: two [2Fe–2S] clusters and one FAD. Structurally, mammalian XOs are homodimeric enzymes (α_2) with each monomer folded into three domains, which can be thought of as pseudo $\alpha'\beta'\gamma'$ subunits, each holding one type of redox-active centre (N-terminal domain holds the Fe/S centres, central domain, the FAD site, and, C-terminal, the molybdenum centre) (Figure 1.5).[248,298] The four redox-active centres are aligned in an almost linear fashion that defines an intramolecular electron transfer pathway delivering electrons from the molybdenum centre to the FAD, the sites where the hydroxylation and dioxygen reduction take place, respectively (with Fe/S centres intermediating the electron transfer; discussed below).

Mammalian AO enzymes are structurally very similar to XO, holding one identical molybdenum centre, as well as two Fe/S centres and one FAD centre, in the same α_2 structure. They exist exclusively as an oxidase form (reducing dioxygen, not NAD^+; eqn (1.14)). While humans have only one AO, some

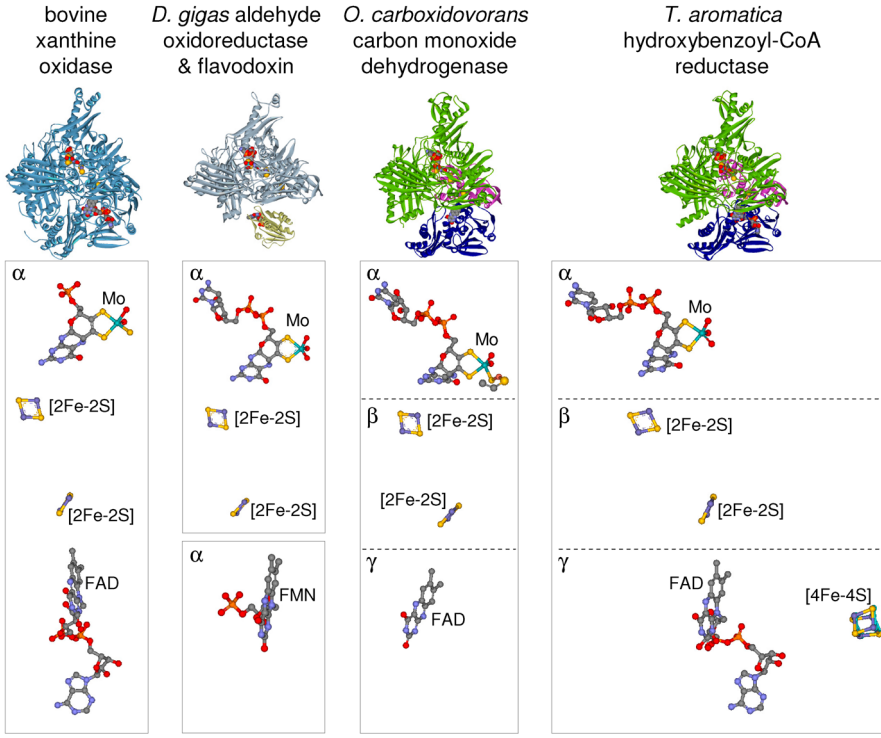
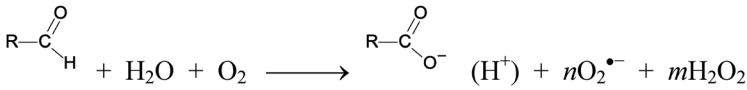


Figure 1.5 Three-dimensional structure view of the bovine xanthine oxidase, *D. gigas* aldehyde oxidoreductase plus flavodoxin, *O. carboxidovorans* carbon monoxide dehydrogenase and *T. aromatica* hydroxybenzoyl-CoA reductase (top) and of the arrangement of their redox cofactors (bottom). Only one subunit in XO and AOR (α_2) or one αβγ group in the other two enzymes ($(\alpha\beta\gamma)_2$) is represented. The number of colours reflects the number of subunits of each enzyme and its cofactor composition: XO α – blue; AOR α – light blue plus flavodoxin α – yellow; carbon monoxide dehydrogenase and hydroxybenzoyl-CoA reductase αβγ – green, pink, dark blue. The structures shown are based on the PDB files 1FO4 (XO), 1VLB (AOR), 1FX1 (flavodoxin), 1N5W (carbon monoxide dehydrogenase) and 1RM6 (hydroxybenzoyl-CoA reductase), and were produced with Accelrys DS Visualizer, Accelrys Software Inc.

mammals encode multiple tissue-specific isoforms (the mouse genome, for example, encodes four AOs).[75,299–302] Bacterial AOR from *Desulfovibrio* species holds a slightly different molybdenum centre, with a second equatorial oxo group in place of the more frequent sulfo group, and it harbours only two Fe/S centres besides the molybdenum centre (no FAD centre), in an α_2 structure.[223,303,304] In spite of this, when the AOR structure is represented with its putative physiological partner, the flavin-containing flavodoxin (eqn (1.15)), which can be regarded as a pseudo γ′ subunit, it becomes apparent that the structural homology with XO is preserved (Figure 1.5).[305]



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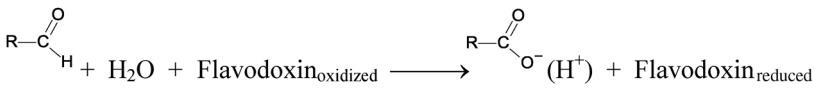


(1.15)

The same structural arrangement is observed for the molybdenum, two Fe/S and one FAD centres of carbon monoxide dehydrogenase ($(\alpha\beta\gamma)_2$ structure), which has a binuclear active site with a non-terminal sulfur (Mo–S–Cu–S(Cys)) and a second equatorial oxo group (Mo=O) in place of the usual labile Mo–OH.[229–233,306,307] The 4-hydroxybenzoyl-CoA reductase from *Thauera aromatica* has a characteristic XO molybdenum centre and displays considerable structural homology with XO, but possesses an additional fifth redox active centre, one [4Fe–4S] centre in a $(\alpha\beta\gamma)_2$ structure (the fifth centre is found in the FAD subunit).[308,309] The periplasmatic AOR from *E. coli* similarly possesses an additional [4Fe–4S] centre, in a heterotrimeric structure.[310] The structural similarity of homologous domains/subunits of XO family enzymes thus suggests an evolutionary path through which older αβγ trimeric structures (with linked or free γ subunit) evolved into the newer eukaryotic monomeric structures with pseudo α′β′γ′ subunits: three "building blocks" (polypeptides/proteins) fused into a single polypeptide.[305]

b) XO family enzymes typically catalyze, at their molybdenum centres, the hydroxylation of a C–H bond in aromatic heterocyclic compounds and aldehydes.[154–159,311] Such oxidative hydroxylations are seen in the XO-catalyzed conversion of xanthine to urate (eqn (1.6)), as well as in the reactions catalyzed by AO and AOR (eqn (1.7)), nicotinate dehydrogenase (eqn (1.8)) and quinoline 2-oxidoreductase (eqn (1.9)). There are, however, at least two important exceptions: the carbon monoxide dehydrogenase-catalyzed oxidation of carbon monoxide to carbon dioxide, which does not involve hydrolysis of a C–H bond (eqn (1.11)),[229–233,306,307] and the reaction of hydroxybenzoyl-CoA reductase, which involves the irreversible dehydroxylation of the phenol ring, an oxygen atom abstraction/reduction reaction (eqn (1.10)).[312,313]

The molecular mechanism of XO hydroxylation reaction is presently well established and believed to be essentially similar in other members of this family, namely in AO and AOR enzymes (Figure 1.6):[154–159,248,298,314,315] (1) the catalysis is initiated with the activation of the molybdenum catalytically labile hydroxyl group (Mo–OH) by a neighbouring conserved deprotonated glutamate residue, to form an Mo^{6+}–O– core (base-assisted catalysis); (2) the now deprotonated oxygen undertakes nucleophilic attack on the carbon atom to be hydroxylated, with the simultaneous transfer of hydride from

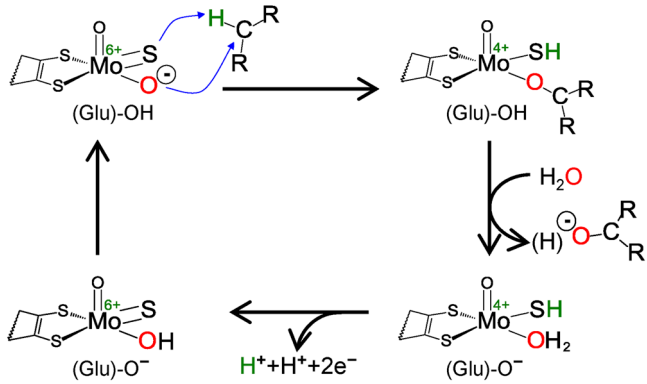


Figure 1.6 Simplified mechanistic proposal for the reaction catalyzed by mammalian xanthine oxidase. See text for details.

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) hydroxide then displaces the hydroxylated product from the molybdenum coordination sphere to yield a Mo^{4+}–$OH_{(2)}$(–SH) core (oxidation half-reaction); (4) the two electrons transferred from the substrate to the molybdenum during the reductive half-reaction (Mo^{6+} → Mo^{4+}) are rapidly transferred, *via* the Fe/S centres, to the FAD (Mo → Fe/S → FAD), where the reduction of dioxygen (or NAD^+, in the case of the XD form) takes place (oxidative half-reaction); and (5) in the now oxidized molybdenum centre (Mo^{4+} → Mo^{6+}), the sulfo group is deprotonated and the initial Mo^{6+}–OH(=S) core is regenerated. Intramolecular electron transfer through XO (Mo → Fe/S → FAD) is, therefore, an integral aspect of the XO catalysis. Of note, (i) water is the ultimate source of the oxygen atom incorporated into the hydroxylated product, as is characteristic of molybdoenzymes (Section 1.3) (the molybdenum labile hydroxyl group (Mo–OH) that ends up in the product of the reaction (urate) is regenerated from a solvent water molecule when the catalytic cycle is closed)[316,317] and (ii) dioxygen is only the oxidizing substrate. The hydroxylation reactions catalyzed by these molybdoenzymes is, in this way, quite different from the reaction catalyzed by monooxygenases as it generates, rather than consumes, reducing equivalents and uses dioxygen as an oxidant rather than as the source of the oxygen atom incorporated into product.

1.4.2 The Sulfite Oxidase Family

The molybdenum centre of the enzymes of the sulfite oxidase (SO) family is closely related to that of the XO family, but with the distinctive feature of having the polypeptide chain, through a cysteine residue, directly coordinated to the molybdenum. In these enzymes, the molybdenum centre displays (in

its oxidized form) the same square-pyramidal geometry, with the apical oxo group (Mo=O), but with the equatorial plane formed by two sulfur atoms of the pyranopterin cofactor, one oxo group (Mo=O) and the cysteine thiolate ligand (Mo–S(Cys)) (Figure 1.3b). The SO family includes enzymes involved in sulfite oxidation (eqn (1.16)), namely diverse prokaryotic sulfite dehydrogenases and plant, chicken and human SO, but also enzymes that catalyze other reactions, such as (i) the eukaryotic assimilatory nitrate reductases (NaR; eqn (1.17); enzymes involved in nitrate assimilation in plants, algae and fungi[†††]), (ii) the *Escherichia coli* YedY or the mammalian mitochondrial amidoxime reducing component (mARC; enzymes involved in the reduction (dehydroxylation) of *S*- and *N*-hydroxylated compounds), (iii) the MOSC protein family (involved in molybdenum centre sulfuration) (Table 1.2) and (iv) other archaeal and bacterial enzymes of unknown function, identified on the basis of genomic analyses.

$$SO_2^{2-} + H_2O \rightarrow OSO_2^{2-} + 2e^- + 2H^+ \quad (1.16)$$

$$ONO_2^- + 2e^- + 2H^+ \rightarrow NO_2^- + H_2O \quad (1.17)$$

a) Chicken liver SO,[‡‡‡] the benchmark enzyme of this family, as well as the SO from humans and other vertebrates, is a homodimeric enzyme (α_2), with each monomer folded into two domains, one comprising one *b*-type haem and the other the molybdenum centre, both of which can (once more) be thought of as pseudo α′β′ subunits (Figure 1.7).[318] Physiologically, the vertebrate SO, located in the mitochondrial intermembrane space, is a key enzyme in the catabolism of sulfur-containing amino acids and other compounds, catalyzing the oxidation of the toxic sulfite to sulfate (at the molybdenum centre), with the simultaneous reduction of cytochrome *c* (at the *b*-type haem) (eqn (1.18)).[319] Interestingly, SO contributes to the generation of cellular energy, through its cytochrome *c* reduction activity[320,321] – a relic of a prokaryotic respiratory process? Remarkably, the molybdenum and haem centres of chicken SO have been found to be more than 30 Å apart in the crystal structure.[318] Hence, during catalysis, a conformational alteration would have to take place, through the flexible polypeptide that links the two domains, to bring the two centres into sufficiently close proximity as to allow rapid intramolecular electron transfer observed, (sulfite)Mo → Fe(cytochrome).[322–324]

[†††]It should be noted that the eukaryotic assimilatory NaR is distinct from any type of prokaryotic NaR enzymes, which are classified as members of the dimethylsulfoxide reductase family.

[‡‡‡]Although this enzyme can reduce both cytochrome *c* and dioxygen, its physiological (and preferred) partner is thought to be the cytochrome *c*. Hence, instead of sulfite oxidase (oxidase means an enzyme that reduces dioxygen), a more appropriate name according to the Enzyme Nomenclature Committee (IUBMB) would be sulfite oxidoreductase. A similar reasoning would apply to the prokaryotic sulfite dehydrogenases, whose physiological partners are most often cytochromes. Only the plant enzymes are true oxidases.

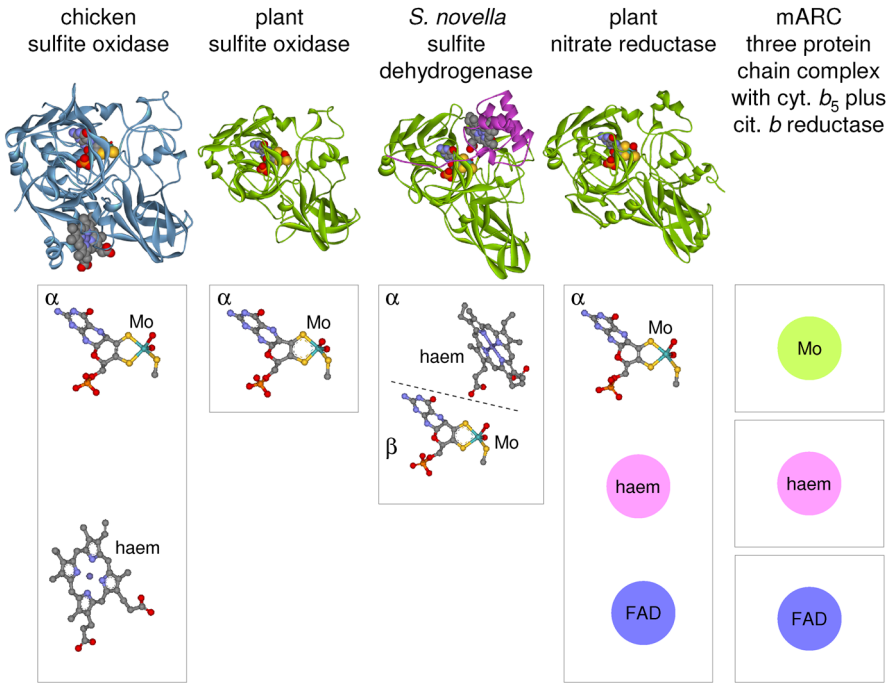


Figure 1.7 Three-dimensional structure view of chicken and plant (*A. thaliana*) sulfite oxidase, *S. novella* sulfite dehydrogenase and plant (*P. angusta*) nitrate reductase (top) and of the arrangement of their redox cofactors (bottom). Only one subunit in chicken and plant SO and plant NaR (α_2) is represented. The number of colours reflects the number of subunits of each enzyme and its cofactor composition: chicken SO α – blue; plant SO and NaR α – green; bacterial sulfite dehydrogenase αβ – green, pink. In the case of plant NaR, only the 3D structure of the domain holding the molybdenum centre is represented. The three protein chain complex with mARC is represented only schematically (its structure is not known yet). The structures shown are based on the PDB files 1SOX (chicken SO), 1OGP (*A. thaliana* SO), 2BPB (*S. novella* sulfite dehydrogenase) and 2BIH (NaR molybdenum domain), and were produced with Accelrys DS Visualizer, Accelrys Software Inc.

Contrary to vertebrate SO, the plant enzymes are peroxisomal and use (reduce) dioxygen as the physiological partner (eqn (1.19)). Most significantly, these enzymes hold only the molybdenum centre (no haem) in an α_2 structure that nevertheless shows the same basic architecture and fold structure as the molybdenum domain of vertebrate SO.[325–328] The "non-uniformity" of sulfite-oxidizing enzymes continues with the prokaryotic counterparts (sulfite dehydrogenases): these are typically periplasmatic enzymes that display different structural organizations and redox cofactor compositions, and whose physiological electron acceptor is most often a

cytochrome.[87–90,329–331] While most of the sulfite dehydrogenases appear to contain only the molybdenum centre in a homodimeric or monomeric organization,[88,90,320,330] significantly different enzymes also exist. The sulfite dehydrogenase from *Starkeya novella* is a heterodimeric (αβ) enzyme, containing a *c*-type haem (in its β subunit) in addition to the molybdenum centre (in the α subunit).[332–334] However, contrary to the crystal structure of chicken SO, the two redox-active cofactors are in this case in close proximity to each other (16 Å). Moreover, while the basic structure of the molybdenum domain is, once more, maintained, the *S. novella* β subunit is found wrapped around the α subunit. In this way, in spite of not being covalently linked to the molybdenum domain (as the haem domain of the chicken enzyme is), the *S. novella* folding ensures that the haem is "locked" in the "right place", with no need of a conformational change to form a competent intramolecular electron transfer pathway. The *S. novella* enzyme is thus crystallized as a stable dimer, with the cofactors in the "correct positions", in clear contrast to the chicken SO with its mobile molybdenum and haem domains that would have to move relatively to each other during the catalytic cycle. Interestingly, the *S. novella c* haem is found in a position similar to that which can be modelled for the *b* haem of chicken enzyme,[334] thus suggesting that there is an ideal molybdenum/haem interaction mode that is reproduced in different forms of life, with different haem types and in different physiological contexts. The question now is what other structural variations in sulfite oxidizing enzymes are yet to be discovered? And, more intriguing, why have vertebrate SO evolved so as to require the flexible linker movement to reposition the two cofactors? This is particularly interesting (and significant) in light of the fact that the structure and fold of the molybdenum domain is maintained in all sulfite oxidizing enzymes whose structure is presently known.

$$SO_2^{2-} + H_2O + 2\text{cyt. } c\,(Fe^{3+}) \rightarrow OSO_2^{2-} + 2\text{cyt. } c\,(Fe^{2+}) + 2H^+ \quad (1.18)$$

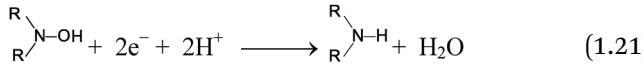
$$SO_2^{2-} + H_2O + O_2 \rightarrow OSO_2^{2-} + 2O_2^{\bullet-} + 2H^+ \quad (1.19)$$

Eukaryotic assimilatory nitrate reducing enzymes**** also belong to the SO family. These enzymes are involved in the first and rate-limiting step of nitrate assimilation in plants, algae and fungi (Figure 1.1, grey arrow), where they catalyze the nitrate reduction to nitrite, at their molybdenum centre, with the simultaneous oxidation of NAD(P)H (eqn (1.20)).[158,335–343] They are cytoplasmatic homodimeric enzymes (α_2), containing, besides the characteristic molybdenum centre, one *b*-type haem and one FAD centre that is responsible for the NAD(P)H binding and oxidation (Figure 1.7).[335,344,345] Consistent with their classification as SO family members, the NaR molybdenum domain is strikingly similar to one of chicken and plant SO. The FAD domain has been suggested to contribute to the correct positioning of the haem domain for intramolecular electron transfer. Interestingly, the phosphorylation of a serine residue in the linker region between the molybdenum and haem domains,[346,347]

followed by the binding of a specific regulatory protein, effectively inhibits the enzyme through the inhibition of the intramolecular electron transfer.§§§,[353–356]

$$ONO_2^- + NADH + H^+ \rightarrow NO_2^- + H_2O + NAD^+ \quad (1.20)$$

Another member of the SO family is the recently described mARC (the fourth mammalian molybdoenzyme, after XO, AO and SO).[357] Its physiological function is not known, but it is probably involved in the detoxification of mutagenic and toxic aromatic hydroxyl-amines, such as *N*-hydroxylated DNA base derivates,[358,359] through the catalysis of the reduction of *N*- and *S*-hydroxylated compounds at the molybdenum centre (eqn (1.21)),[360–363] among other possible roles.[67,364,365] This mitochondrial enzyme (outer membrane-bounded, facing the cytoplasm) is monomeric and contains only the molybdenum centre (with no additional redox-active centres)[361,364,366,367] that coordinates the conserved cysteine residue[368] and an apical oxo group (Mo=O).[369] However, mARC functions in concert with cytochrome b_5 and a NADH-dependent cytochrome b_5 reductase, which are involved in electron transfer from NADH to the mARC (Figure 1.7), thus forming a three-protein electron transfer chain resembling the arrangement of redox-active cofactors seen in NaR enzymes: NAD(P)H → FAD → haem → Mo → *N*- and *S*-hydroxylated compounds.[357,360,366,367,370,371]



(1.21)

b) The members of the SO family are thought to be proper oxotransferases, as exemplified by SO and NaR, that catalyze the "simple" transfer of one oxygen atom to, or from, a lone electron pair of the substrate, with their molybdenum atom mediating the transfer: (i) SO-catalyzed sulfite oxidation to sulfate (eqn (1.16)), where the $Mo^{6+}{=}O_{(equatorial)}$ centre is the direct oxygen donor (oxygen insertion/oxidation reaction), and (ii) NaR-catalyzed nitrate reduction to nitrite (eqn (1.17)), where the Mo^{4+} is the direct oxygen acceptor (oxygen abstraction/reduction reaction). As in the XO family, water is the ultimate oxygen atom donor or acceptor (the oxygen atom from nitrate ends up eventually in solvent). Also, as seen in XO, the two half-reactions of chicken/human SO and plant NaR are physically separated, with one of the half-reactions being carried out at the molybdenum centre and the other elsewhere

§§§As expected from its key role on the 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.[337,339–341,343,348] The posttranslational regulation involves the phosphorylation of a serine residue in the linker region between the molybdenum and haem domains.[349,350] The phosphorylation is catalyzed by protein kinases, including AMP-activated[351] and calcium-dependent kinases.[352] 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.[353–356]

(*i.e.* at the haem of SO or the FAD of NaR); therefore, intramolecular electron transfer (Mo → haem or FAD → haem → Mo) is, once again, an integral aspect of catalysis.

Chicken/human SO-catalyzed sulfite oxidation is presently well understood (Figure 1.8):[195,318,372–379] (1) catalysis is initiated at the oxidized molybdenum centre, initiated by nucleophilic attack of the sulfite lone-pair of electrons on the catalytically labile equatorial oxo group of the molybdenum (Mo^{6+}=O), which can be thought of as an electrophilic oxygen; ¶¶¶ this results in the formation of a covalent Mo^{4+}–O–SO_3 intermediate, where the molybdenum atom has become reduced by two electrons with a formal triple bond to the apical oxo group (Mo=O_{apical});[190–196] (2) the presence of this apical "spectator oxo" (so named because it is not directly involved in the oxygen atom transfer) facilitates the subsequent cleavage of the Mo–O(substrate)$_{equatorial}$ bond (weakens it); product (sulfate) is then released to yield a Mo^{4+}–$OH_{(2)}$ core (the reductive 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* (the physiological partner) will be reduced, and the initial Mo^{6+}=O core is regenerated (oxidative half-reaction). In these enzymes with an obligatory one-electron cofactor (haem), a transient Mo^{5+} species is an obligate intermediate formed as the two electrons of Mo^{4+} are sequentially transferred to the haem, which must wait for the physiological oxidizing partner to be re-oxidized.

In the plant SO that is devoid of additional redox cofactors, the oxidative half-reaction is also carried out at molybdenum centre, with the one-electron reduction of dioxygen to superoxide radical anion, which, then, spontaneously dismutates to hydrogen peroxide,[387] again implicating the formation of a Mo^{5+} intermediate. It should be emphasized that this reactivity of a molybdenum centre towards dioxygen is not common, although haem-deficient variants of mammalian SO exhibit a dioxygen reduction/sulfite oxidation activity similar to that exhibited by the plant enzyme,[388] suggesting that the absence of a second redox-active centre (haem) allows the enzyme to transfer the electrons to the dioxygen *via* the molybdenum centre. However, this is not the case of the sulfite dehydrogenase from *Sinorhizobium meliloti* (SorT), which, in spite of having only the molybdenum centre in a similar active site, does not react with dioxygen[389] – which leaves the intriguing reactivity of molybdenum centres toward dioxygen yet to be fully understood.

The molecular mechanism of NaR-catalyzed nitrate reduction to nitrite is believed to be the reverse of that seen with SO, with the abstraction of an oxygen atom and the intramolecular electron flow occurring in the opposite direction. Hence (Figure 1.8):[337,340,343,344,390–393] (1) catalysis is initiated with the reduction of FAD by NAD(P)H (the reductive half-reaction); (2) the two

¶¶¶This labile oxo group is doubled bonded to the molybdenum in the 6+ oxidation state, which is an electron sink, and, thus, it can be thought of as electrophilic. Supporting the proposal of the nucleophilic attack to this electrophilic oxygen, there are several experimental evidences with model complexes[142,380–386] and theoretical data.[374,376]

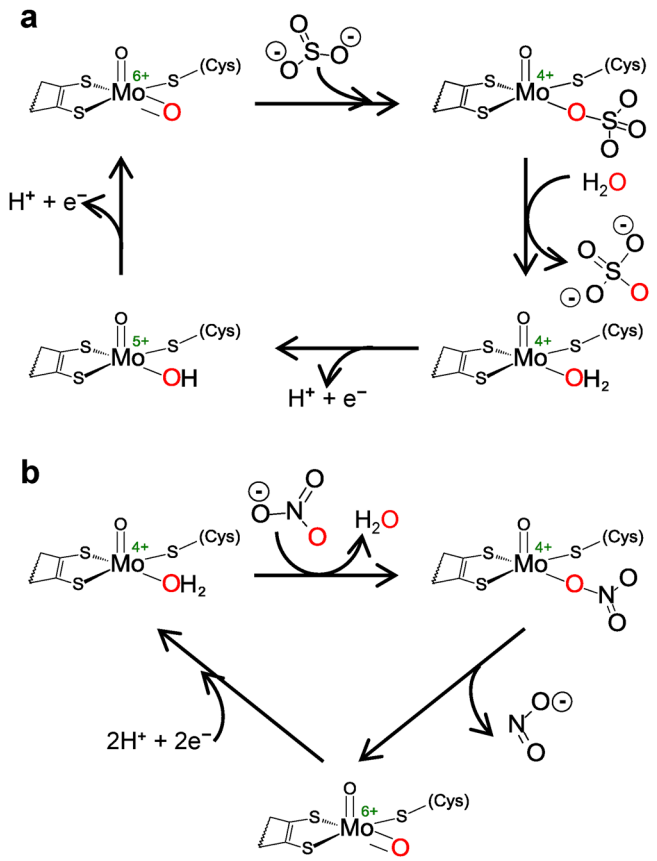


Figure 1.8 Simplified mechanistic proposal for the reaction catalyzed by vertebrate sulfite oxidase (a) and plant nitrate reductase (b) See text for details.

electrons are transferred intramolecularly, through the haem, to the molybdenum centre (FAD → haem → Mo); in the now reduced molybdenum centre, the equatorial labile oxo group is protonated (Mo^{6+}=O → Mo^{4+}–OH); (3) nitrate binds to the molybdenum, displacing the labile group; this results in the formation of the covalent intermediate Mo^{4+}–O–NO_2; (4) the subsequent O–N bond cleavage releases the product (nitrite) and regenerates the Mo^{6+}=O core (the oxidative half-reaction).

The similarities between the molybdenum-binding domains and molybdenum centres of SO and NaR, together with the complementarity of their reaction mechanisms, raise the intriguing question of whether a SO enzyme might efficiently catalyze the nitrate reduction (and *vice versa*)? The answer seems to reside in two amino acid residues that are found in the SO active site, but not in NaR: the SO substrate binding pocket comprises five residues that are conserved in all known eukaryotic SO, three arginines, one tyrosine and one tryptophan; three of these residues are also conserved in NaR enzymes,

but the tyrosine is replaced by an asparagine in NaR, and one of the arginines (arginine 450 and 472 in chicken and human SO, respectively) is replaced by a methionine. Noteworthy, mutagenesis of the arginine and tyrosone to methionine and asparagine, respectively, in SO enables the human SO enzyme to reduce nitrate with a $k_{cat} \approx 1\ s^{-1}$ and $K_m \approx 25\ \mu M$ ($k_{cat}/K_m \approx 10^4\ M^{-1}s^{-1}$), while decreasing the kinetic parameters toward sulfite oxidation ($k_{cat} \approx 25\ s^{-1}$ and $1\ s^{-1}$ and $K_m \approx 3\ \mu M$ and 5 mM, for wild-type and variant SO proteins, respectively).[394] When these values of nitrate reduction activity are compared with ones of true NaR enzymes, it becomes apparent how remarkable is the modification promoted by the change of only two residues (*e.g.* $k_{cat} \approx 159\ s^{-1}$ and $K_m \approx 30\ \mu M$ for *Pichia pastoris* NaR[395]). It has been suggested that the SO arginine/NaR methionine could mediate conformational changes of the active site upon substrate binding, with one positively charged arginine hampering the nitrate binding to molybdenum through one of its oxygen atoms.[394] In addition, the NaR methionine might change the oxidation–reduction properties of the molybdenum centre, favouring the nitrate reduction.

The newly recognized members of this family clearly show that the chemistry carried out by the enzymes of the SO family is more diverse than "just" the oxidation of sulfite or the reduction of nitrate. The recent identification of mammalian mARC and bacterial YcbX, YiiM or YedY, as well as of several MOSC proteins homologues (most of these not yet characterized), demonstrated that the active sites of SO family enzymes are also able to catalyze the reduction (dehydroxylation) of *S*- and *N*-hydroxylated compounds and sulfuration reactions.[368,396–403]

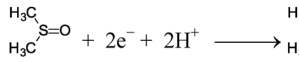
1.4.3 The Dimethylsulfoxide Reductase Family

The enzymes in this family are characterized by having four sulfur atoms of two pyranopterin cofactor molecules[‖‖‖‖] coordinated to the molybdenum atom (in its oxidized form) in a trigonal prismatic geometry completed by oxygen and/or sulfur and/or selenium atoms in a diversity of arrangements (Figure 1.3b); the molybdenum atom is most often directly coordinated by the polypeptide chain *via* aspartate, serine, cysteine or selenocysteine residue side chains.[****] The dimethylsulfoxide reductase (DMSOR) family consists only of prokaryotic enzymes, but having remarkably different activities, including DMSOR (eqn (1.22)), arsenite oxidase (eqn (1.23)) and arsenate reductase (eqn (1.24)), dissimilatory NaR (eqn (1.17); respiratory mem-

[‖‖‖‖]The pyranopterin cofactor molecule is apparently always found esterified with a guanosine monophosphate (Figure 1.3a).

[****]There is at least one exception: the arsenite oxidase (eqn (1.23)) from *Alcaligenes faecalis*, whose crystal structure showed a molybdenum centre coordinated by the two characteristic pyranopterin molecules plus an apical oxo group (Mo=O), in a square-pyramidal coordination geometry, with no coordination to the polypeptide chain.[404–406] X-ray absorption data showed the presence of two oxo groups (MoO_2) or of one oxo plus a hydroxyl group (Mo=O(–OH)),[407] suggesting that the molybdenum centre in the crystal could have been reduced in the synchrotron beam.

brane-bound and periplasmatic enzymes associated with the generation of a proton motive force (Figure 1.1) or acting as an electron sink to eliminate excess of reducing equivalents) and assimilatory NaR (eqn (1.17); prokaryotic cytoplasmatic enzymes involved in nitrogen assimilation (Figure 1.1)), as well as enzymes that do not catalyze oxygen atom transfer reactions, such as formate dehydrogenase (FDH; eqn (1.25)) and polysulfide reductase (eqn (1.26)), among many others (Table 1.2).



$$+ 2e^- + 2H^+ \longrightarrow + H_2O \quad (1.22)$$

$$AsO_3^{3-} + H_2O \rightarrow OAsO_3^{3-} + 2e^- + 2H^+ \quad (1.23)$$

$$OAsO_3^{3-} + 2e^- + 2H^+ \rightarrow AsO_3^{3-} + H_2O \quad (1.24)$$

$$HCOO^- \rightarrow CO_2 + 2e^- + H^+ \quad (1.25)$$

$$(S_n)^{2-} + 2e^- + 2H^+ \rightarrow (2H^+)S^{2-} + (S_{n-1})^{2-} \quad (1.26)$$

a) The DMSOR family is the most diverse of the three families, comprising enzymes with widely varying subunit composition and makeup of additional redox-active cofactors.[228,408,409] To restrict the information presented to a manageable size, only a limited number of enzymes will be described, namely two DMSOR, one polysulfide reductase and several NaR and FDH enzymes.

Rhodobacter sphaeroides DMSOR, the benchmark enzyme of this family, as well as its homologue from *R. capsulatus*, is a periplasmatic monomeric protein containing only the molybdenum centre as a redox-active group, with the molybdenum atom coordinated (in its oxidized form) by the characteristic two pyranopterin molecules (in the form of the dinucleotide of guanine) plus one catalytically labile oxo group (Mo=O) and one serine side chain oxygen atom (Mo–O(Ser)) (Figure 1.9).[224,410–412] Physiologically, this DMSOR is a dissimilatory enzyme that catalyzes the reduction of DMSO to dimethylsulfide (DMS) (eqn (1.22)), but without contributing to the transmembrane proton gradient (thus acting as an electron sink to eliminate excess of reducing equivalents).[413–415]

Contrary to the "simple" enzyme from *Rhodobacter*, the "complex" DMSOR from *E. coli* contributes to the transmembrane proton gradient, through the oxidation of menaquinol coupled to DMSO reduction.[416–420] This *E. coli* respiratory enzyme is a membrane-bound periplasm-facing heterotrimeric (αβγ) enzyme, which is suggested to be comprised of (with no crystal structure available yet): (i) a periplasmatic DMSO-reducing subunit that holds the molybdenum centre, similar to the *Rhodobacter* one, and one [4Fe–4S] centre; the amino acid residue sequence of the molybdenum-binding domain of this subunit is similar to the corresponding part of the *Rhodobacter* enzyme, with the small domain containing the Fe/S centre inserted in an N-terminal domain of the subunit; (ii) a second periplasmatic subunit containing four [4Fe–4S] centres that is thought to be involved in intramolecular electron transfer (from the menaquinol to the DMSO-reducing molybdenum centre); and (iii) a membrane-integral subunit that has no

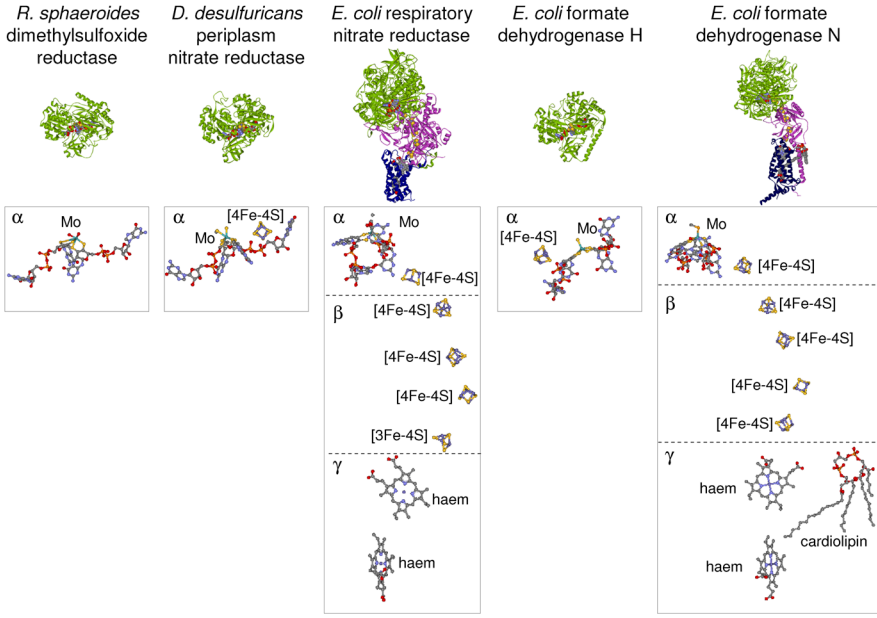


Figure 1.9 Three-dimensional structure view of *R. sphaeroides* dimethylsulfoxide reductase, *D. desulfuricans* periplasmatic nitrate reductase, *E. coli* respiratory nitrate reductase, *E. coli* formate dehydrogenase H and *E. coli* formate dehydrogenase N (top) and of the arrangement of their redox cofactors (bottom). Only one αβγ group of the *E. coli* respiratory NaR ($(\alpha\beta\gamma)_2$) and *E. coli* FDH N ($(\alpha\beta\gamma)_3$) is represented. The number of colours reflects the number of subunits of each enzyme and its cofactor composition: DMSOR, *D. desulfuricans* periplasmatic NaR and *E. coli* FDH H α – green; *E. coli* respiratory NaR and *E. coli* FDH N αβγ – green, pink, dark blue. The structures shown are based on the PDB files 1EU1 (DMSOR), 2NAP (*D. desulfuricans* periplasmatic NaR), 1Q16 (*E. coli* respiratory NaR), 2IV2 (*E. coli* FDH H) and 1KQF (*E. coli* FDH N), and were produced with Accelrys DS Visualizer, Accelrys Software Inc.

redox-active cofactors and that presumably binds the menaquinol necessary for enzyme reduction.

The respiratory, membrane-bound periplasm-faced, polysulfide reductase from *Thermus thermophilus*, involved in the reduction of inorganic sulfur to sulfide, a sulfur atom transfer reaction (eqn (1.26)), has a similar subunit organization and cofactor composition, despite being organized as a dimer of trimers ($(\alpha\beta\gamma)_2$).[421–423] Its crystal structure shows that the cofactorless, membrane-integral subunit does bind the menaquinol and in a position near one of the Fe/S centres (named FS3) of the electron transfer β subunit, as expected (and suggested for the *E. coli* DMSOR). The molybdenum centre of the polysulfide reductase, however, is coordinated by a cysteine residue (Mo–S(Cys)) rather than the serine found in the DMSORs and probably by a catalytically labile hydroxyl group (Mo–OH) (the pyranopterin cofactors are also in the form of a guanine dinucleotide).

The NaR and FDH enzymes can have similarly "simple" and "complex" subunit organizations and cofactor compositions, most often correlated to their physiological function. For example, the "simple" monomeric periplasmatic *Desulfovibrio desulfuricans* NaR and monomeric cytoplasmatic *E. coli* FDH-H, both of which contain only the molybdenum centre and one [4Fe–4S] centre, contrast with the "complex" $(\alpha\beta\gamma)_2$ membrane-bound respiratory *E. coli* NaR and $(\alpha\beta\gamma)_3$ membrane-bound respiratory *E. coli* FDH-N, both of which contain seven additional redox-active cofactors (Fe/S and haems) in addition to their molybdenum centres (all described below).

Prokaryotes use nitrate for dissimilatory and assimilatory processes (Figure 1.1) and encode three distinct NaR enzymes[††††] present in different subcellular locations:[174,175,228,424–434] (a) membrane-bound cytoplasm-faced respiratory NaR, associated with the generation of a proton motive force across the cytoplasmatic membrane; [‡‡‡‡] (b) periplasmatic NaR, involved in the generation of a proton motive force or acting as an electron sink to eliminate excess of reducing equivalents; and (c) cytoplasmatic assimilatory NaR, involved in nitrogen assimilation.[§§§§] In response to their different biological roles/subcellular locations, these enzymes have different subunit organizations and cofactor compositions (Figure 1.9). The respiratory enzyme from *E. coli* NaRGHI (product of the *narG*, *H* and *I* genes) is a dimer of heterotrimers $(\alpha\beta\gamma)_2$, made up of:[175,435] (a) a cytoplasmatic nitrate-reducing NaRG subunit that holds the molybdenum centre and one [4Fe–4S] centre; (b) an electron transfer NaRH subunit that holds one [3Fe–4S] and three [4Fe–4S] centres, responsible for the intramolecular electron transfer from the membrane quinol pool to the nitrate-reducing molybdenum centre; and (c) a membrane-bound quinol-oxidizing NaRI subunit that holds two *b*-type haems. On the other hand, the periplasmatic NaR from *D. desulfuricans* (product of the *napA* gene) is a monomeric enzyme with only one [4Fe–4S] centre in addition to the molybdenum centre,[436,437] while the enzyme from *C. necator* (*napA* and *napB* genes) is a dimer harbouring two haems in addition to the molybdenum centre.[438]

All these prokaryotic NaR catalyze the two-electron reduction of nitrate to nitrite (eqn (1.17)) at their molybdenum centre. Interestingly, in spite of catalyzing the same reaction and having the molybdenum atom coordinated

[††††]In the "molybdenum-community" or the "prokaryotic field", these three enzymes are commonly referred to as Nar (membrane-bound, respiratory), Nap (periplasmatic) and Nas (cytoplasmatic, assimilatory). However, for readers outside those fields, this denomination is not intuitive, because the letters "r", "p" and "s" do not specify the differences between the three types of enzymes (differences in function, subcellular location or even in active site structure). As such, herein the abbreviation NaR, from nitrate reductase, will be used, specifying the substrate and the type of reaction catalyzed, as is recommended by the Enzyme Nomenclature Committee (IUBMB) for the trivial names of enzymes.

[‡‡‡‡]Interestingly, evidence has been recently described for the occurrence of both archaeal and bacterial periplasm-faced NaRGHI-type nitrate reductases, *i.e.* enzymes similar to the ones described above, but with the active site on the outside of the cytoplasmatic membrane.[428,430,432] This exciting hypothesis awaits confirmation.

[§§§§]As indicated above, the eukaryotic assimilatory cytoplasmatic NaR, belonging to the SO family, is a distinct enzyme.

by the characteristic four sulfur atoms from two pyranopterin cofactor molecules (in the form of the guanine dinucleotide), the three types of NaR enzymes display significant differences in the remainder of the molybdenum coordination sphere. In the respiratory membrane-bound NaR, the molybdenum atom is further coordinated by an aspartate residue that is coordinated in a bidentate fashion,[174,435] or alternatively by one terminal oxo group plus one oxygen atom from the aspartate residue coordinated in a monodentate mode; the monodentate and bidentate aspartate binding modes correspond, possibly, to oxidized 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.[436–439] The *E. coli* and *Rhodobacter sphaeroides* periplasmatic NaR, on their turn, complete the molybdenum coordination with the cysteine sulfur atom plus a terminal hydroxyl group.[440,441] The cytoplasmatic assimilatory NaR is the least studied one of the NaRs, but it is likely that a cysteine residue is coordinated to the molybdenum atom in this enzyme.[442]

As with the NaRs, the prokaryotic FDH enzymes (eqn (1.25)) are also involved in different biochemical pathways, operate in different subcellular locations and use diverse physiological redox partners (membrane quinols, cytoplasmatic and periplasmatic cytochromes, ferredoxins, NAD or coenzyme F_{420} (an obligate two-electron acceptor, flavin derivative)).[228,443,444] As a result, FDHs are also structurally heterogeneous proteins, displaying diverse subunit organizations and cofactor compositions,[¶¶¶¶] and again only a few examples will be here described.

E. coli expresses three FDHs: (a) a cytoplasmatic enzyme designated formate dehydrogenase H (FDH-H)[445] that is part of the formate-hydrogen lyase system and involved in the oxidation of formate and generation of molecular hydrogen formation under fermentative (anaerobic) growth conditions;[228,446] (b) a membrane-bound periplasm-facing FDH designated formate dehydrogenase N (FDH-N) that is part of the anaerobic nitrate-formate respiratory pathway (catalyzed by a supermolecular formate : nitrate oxidoreductase system formed with the NaRGHI) involved with the generation of a proton motive force;[423,447–453] and (c) a second membrane-bound periplasm-faced FDH, designated formate dehydrogenase O (FDH-O), that operates under aerobic conditions in another nitrate-formate respiratory pathway (this with the NaRZWV enzyme).[454–458]

The *E. coli* FDH-H is a monomeric enzyme with only one [4Fe–4S] centre in addition to the molybdenum centre (Figure 1.9).[459–462] The molybdenum centre is the site of formate oxidation, and the Fe/S centre is responsible for the subsequent intramolecular electron transfer to the physiological acceptor,

[¶¶¶¶]Of note, besides molybdenum- and tungsten-containing enzymes (the latter ones described in Section 1.4.4.), there are also metal-independent FDH enzymes, dependent on NAD^+, that belong to the D-specific dehydrogenases of the 2-oxyacid family.[444]

probably a ferredoxin protein; the crystal structure shows the Fe/S centre in the N-terminal domain, as is suggested for the subunit of *E. coli* DMSOR. The molybdenum is coordinated by four sulfur atoms of the two pyranopterins (in the form of guanine dinucleotide), characteristic of this family, and by a conserved essential selenocysteine residue (Mo–Se(Cys)) and a labile hydroxyl group.[462] However, a subsequent reinterpretation of the original electron density map has revealed a different coordination sphere for the formate-reduced FDH-H, with a terminal sulfur atom rather than oxygen, and with the selenocysteine residue not bound to the molybdenum, but shifted away (9 Å).[463] Accordingly, the oxidized molybdenum centre of FDH-H is presently being thought of as a trigonal prismatic structure coordinated by the selenocysteine residue and one terminal sulfur atom, in addition to the four sulfur atoms from the two pyranopterins.[‖‖‖‖‖‖463]

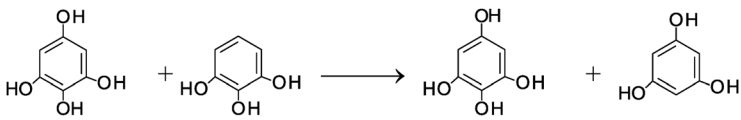
The *E. coli* FDH-N is a complex trimer of trimers ($(\alpha\beta\gamma)_3$), with eight redox-active cofactors in an overall organization of the αβγ unit that is quite similar to that seen in the respiratory NaRGHI (Figure 1.9).[449,450,465–468] The FDH-N αβγ unit consists of:[450] (i) a periplasmatic formate-oxidizing subunit that holds one molybdenum centre and one [4Fe–4S] centre, with the domains involved in binding the Fe/S and molybdenum centres structurally similar to the FDH-H monomer; (ii) a periplasmatic electron transfer subunit that harbours four [4Fe–4S] centres and is responsible for the intramolecular electron transfer from the formate-oxidizing molybdenum centre to the membrane quinone pool; and (iii) a membrane-bound quinone-reducing subunit that has two *b*-type haems. The trimer of αβγ units, $(\alpha\beta\gamma)_3$, is very tightly packed and a cardiolipin molecule is maintained at the trimer interface, thus suggesting that this complex arrangement is physiologically meaningful. The molybdenum of FDH-N is coordinated by selenocysteine and a terminal sulfur atom, as seen in its FDH-H counterpart, in addition to the characteristic four sulfur atoms of the two pyranopterins (in the form of guanine dinucleotide).[464]

Noteworthy, the *E. coli* FDH-O is believed to be structurally similar to the homologous FDH-N (for which no crystal structure is yet available).[455,469,470] The FDH from *D. desulfuricans* is also a complex periplasmatic enzyme, with a heterotrimeric structure (αβγ), but possesses only two [4Fe–4S] centres (one in each of the α and β subunits) with four *c* haems (γ subunit) and the molybdenum centre (the last coordinated by a selenocysteine and, probably, a terminal sulfur; no crystal structure is yet available).[471,472] The enzymes from *Ralstonia eutropha*[473–475] and *Rhodobacter capsulatus*[476] are even more complex, holding seven Fe/S centres plus one FMN per αβγ protomer in addition to the molybdenum centre (which, in these two enzymes, is expected to be coordinated by a cysteine residue, and a terminal sulfur atom; again, no crystal structure is as yet available).

[‖‖‖‖‖‖]Besides the crystallographic data, also the recent identification of a sulfurtransferase that would insert the sulfur atom into the FDH molybdenum centre[464] supports that all *E. coli* FDH could have a terminal sulfur atom.

To summarize, the DMSOR family is the most diverse of the molybdenum-containing enzymes families, consisting of enzymes with different structural organization and composition of redox-active cofactors (Fe/S centres, haems and flavins), ranging from, *e.g.*, (i) the monomeric *Rhodobacter* DMSOR with only the molybdenum centre, to (ii) the monomeric *Desulfovibrio desulfuricans* periplasmatic NaR that contains the molybdenum centre plus one Fe/S centre, and ending in (iii) the multimeric respiratory *E. coli* FDH-N, with several Fe/S and haems, besides the molybdenum centre, or in other FDH enzymes that further hold one FMN or FAD.

b) Matching its structural diversity, the DMSOR family is also catalytically extremely versatile, including enzymes that catalyze a variety of reaction types, including several not involving oxygen atom transfer, *e.g.*: (i) cleavage of a C–H bond as carried out by different FDHs that catalyze the reversible formate oxidation to carbon dioxide (eqn (1.25)); (ii) sulfur atom transfer as seen in the polysulfide reductase-catalyzed inorganic sulfur reduction to sulfide (eqn (1.26)); (iii) hydroxyl transfer with *simultaneous* oxidation and reduction (where water is not the oxygen atom acceptor or donor), such as the reductive dehydroxylation and concomitant oxidative hydroxylation catalyzed by pyrogallol : phloroglucinol transhydroxylase (eqn (1.27)); (iv) and even a hydration reaction, as observed in the acetylene hydratase-catalyzed hydration of acetylene to acetaldehyde, a non-redox reaction (eqn (1.28))); (v) besides the more common oxygen atom transfer to, or from, a lone electron pair of the substrate, as can be observed in arsenite oxidase-catalyzed oxidation of arsenite to arsenate (eqn (1.23)) or in dimethylsulfide dehydrogenase-catalyzed oxidation of DMS to DMSO (reverse of eqn (1.22)) (oxygen insertion/oxidation reaction) and in NaR (eqn (1.17)), arsenate reductase (eqn (1.24)) or DMSOR reactions (eqn (1.22)) (oxygen abstraction/reduction reaction).[161,409,423,477]



pyrogallol phloroglucinol (1.27)

$$H–C \equiv C–H + H_2O \rightarrow H_3C–COH \quad (1.28)$$

The electrons derived from (or necessary to carry out) these reactions are intramolecularly transferred to the electron acceptor (or from the electron donor) through the different redox-active centres of each enzyme (Fe/S centres, haems and flavins); therefore, the oxidative and reductive half-reactions are physically separated and the intramolecular electron transfer is an integral aspect of catalysis – the obvious exception is the monomeric, molybdenum-only, *Rhodobacter* DMSOR and related enzymes (Table 1.2). The reaction mechanism of *Rhodobacter* DMSOR is now well established and believed to

be essentially similar in other DMSOR enzymes (Figure 1.10a):[478–481] (1) catalysis is initiated with enzyme reduction by the pentaheme DorC protein (the reductive half-reaction); upon reduction of the molybdenum, the catalytically labile oxo group is protonated and released in the form of a water molecule, resulting in the formation of a penta-coordinate, square-pyramidal core, with the serine residue occupying the apical position ((Ser)O–Mo^{6+}=O → (Ser)O–Mo^{4+}), *via* a (Ser)O–Mo^{5+}–OH intermediate; (2) DMSO binding to the molybdenum yields a hexa-coordinate (Ser)O–Mo^{4+}–O–S(dimethyl) intermediate; (3) the subsequent O–S bond cleavage releases the product (DMS) and regenerates the initial (Ser)O–Mo^{6+}=O core (the oxidative half-reaction).

The outline of the mechanism of the oxygen abstraction reaction catalyzed by DMSOR shares some aspects with the eukaryotic NaR reaction (from the SO family): in both enzymes a catalytically labile oxo ligand of the molybdenum is lost to allow the binding of the substrate, *via* the oxygen atom to be transferred, resulting in the formation of a Mo^{4+}–O(substrate) complex; following

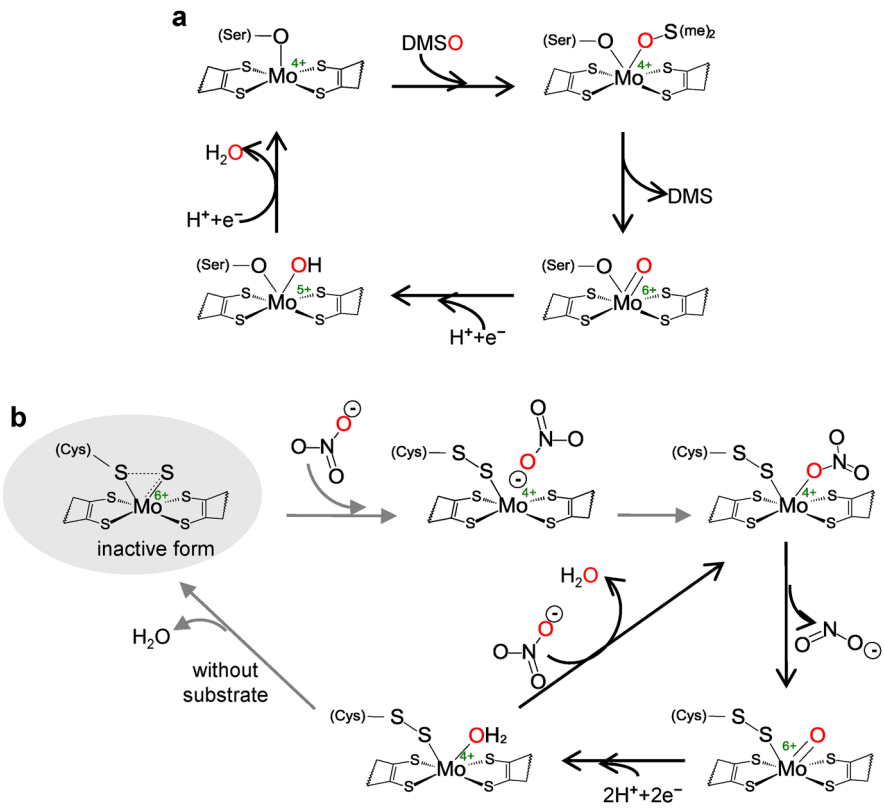


Figure 1.10 Simplified mechanistic proposal for the reaction catalyzed by dimethylsulfoxide reductase (a) and *D. desulfuricans* periplasmatic nitrate reductase (b). See text for details.

the O-substrate bond cleavage, the product is released and the initial molybdenum core, with a "fresh" labile oxo group, is regenerated. Moreover, in both cases water is the ultimate destination of the oxygen atom abstracted from the substrate. However, to catalyze these oxygen atom abstraction reactions, the DMSOR enzyme cycles between a hexa-coordinated mono-oxo Mo^{6+} core and a penta-coordinated *des*oxo Mo^{4+} core, while the eukaryotic NaR does it between a penta-coordinated di-oxo Mo^{6+} core and a mono-oxo Mo^{4+} core. Therefore, in spite of using different "enzymatic machineries", both enzymes carry out a similar chemical outcome: direct oxygen atom transfer from the substrate to the metal, followed by "recycling" with the solvent.

A similar mechanism has been suggested for the periplasmatic NaR from *E. coli*, but with nitrate binding to the Mo^{5+} oxidation state (rather than Mo^{4+}):[440] (1) reduction of the molybdenum centre by one electron results in a penta-coordinated *des*oxo Mo^{5+} core ((Cys)S–Mo^{6+}–OH → (Cys)S–Mo^{5+}; this is parallel to the DMSOR penta-coordinated desoxo (Ser)O–Mo^{4+} core); nitrate binds to this complex, through the oxygen atom to be abstracted, to form the hexa-coordinated intermediate with bound substrate; (2) after reduction by the second electron, the O–N bond is cleaved, the product (nitrite) is released and the initial hexa-coordinated mono-oxo Mo^{6+} core regenerated upon protonation. A comparable mechanistic strategy has been proposed for the sulfur atom transfer reaction of the polysulfide reductase, with the terminal sulfur atom of the polysulfide displacing the catalytically labile hydroxyl group to become directly coordinated to the reduced molybdenum atom ((Cys)S–Mo^{6+}–OH → (Cys)S–Mo^{4+}–S(polysulfide)).[482,483]

The enzymes lacking an exchangeable oxygen ligand, however, must follow a different mechanistic strategy. This is the case of the *D. desulfuricans* and *C. necator* periplasmatic NaR, which have a terminal sulfur atom, forming a partial disulfide bond with the sulfur atom of the cysteine residue side chain, rather than an oxo/hydroxyl group. Starting from the principle that the sulfo ligand would have to remain bound to the molybdenum throughout the catalytic cycle (*i.e.* that it is not be released as an oxo/hydroxyl group can), it has been proposed, based on theoretical calculations, that the coordinating cysteine residue dissociates from the molybdenum atom through a "sulfur shift" mechanism (Figure 1.10b):[484,485] catalysis is initiated when nitrate reaches the (inactive) oxidized hexa-coordinated molybdenum centre ((Cys)S–Mo=S); the repulsive environment generated would trigger the insertion of the sulfur atom into the (Cys)S–Mo bond, to yield an active (Cys)S–S–Mo core. As a result of this sulfur shift, the molybdenum is formally reduced to Mo^{4+} and a *new* binding position is created to bind nitrate through one of its oxygen atoms and yield a hexa-coordinated (Cys)S–S–Mo–ONO_2 intermediate. The cleavage of the O–NO_2 bond leads to product release, leaving a hexa-coordinated mono-oxo Mo^{6+} core, with a "fresh" oxo group; this core can then be reduced and, after displacement of the oxo labile group by a new molecule of nitrate, a new catalytic cycle can start. This mechanism can be rationalized using the same generalization introduced above with the comparison between the eukaryotic NaR and DMSOR reactions: direct oxygen atom transfer from the substrate to the metal, followed by "recycling" with

the solvent, involving, in this case, hexa-coordinated intermediates created by an activating initial sulfur-shifting.

The sulfur shift is an elegant mechanism to explain how a metal with no available coordination position can bind a substrate, converting an "unready enzyme" (with a complete coordination sphere) into a "ready enzyme". The complete coordination, with a ligand that is not exchangeable (Mo=S) plus another ligand that could be exchanged (Mo–S(Cys)), could provide a mechanism to protect the active site from "unwanted" compounds (inhibition) or may be a regulatory strategy to control the enzyme's activity (mechanism "on/off") to respond to the cellular needs. Still, high-resolution structures coupled with spectroscopic and kinetic data are needed to support the existence of the two polypeptide alternating conformations and the identity of the different catalytic intermediates.

The FDH enzymes also have molybdenum coordinated by a terminal sulfur atom plus a selenocysteine or cysteine residue and a similar sulfur shift mechanism has been proposed to create a vacant position for formate binding, with formate triggering the formation of the active hexa-coordinated (Cys)Se–S–Mo–OCO(H) or (Cys)S–S–Mo–OCO(H) intermediate (in selenocysteine or cysteine-containing FDH, respectively) (Figure 1.11a).[486,487] In this case, however, the substrate (formate) has one proton to be abstracted (formate oxidation; **** eqn (1.25)). Therefore, based on theoretical calculations, it has been proposed that:[486,487] (1) the (Cys)Se–S (or (Cys)S–S) bond is cleaved; (2) the selenol anion formed ((Cys)Se^-), stabilized by a hydrogen bond with a key histidine residue, abstracts the formate Cα proton to yield a carbon dioxide moiety coordinated in a bidentate mode to molybdenum ($C_{carbon\ dioxide}$–S–Mo–O–$C_{carbon\ dioxide}$); (3) carbon dioxide is eventually released, leaving a penta-coordinated Mo^{4+}=S centre; (4) the catalytic cycle would be closed with the oxidation of Mo^{4+} to Mo^{6+}, *via* intramolecular electron transfer, and deprotonation of the selenocysteine/cysteine residue. The molybdenum centre can, then, bind a new formate molecule and start a new catalytic cycle; in the absence of formate, the selenocysteine/cysteine-containing loop is reoriented, the (Cys)Se–Mo=S (or (Cys)S–Mo=S) bond reformed and the enzyme returns to the inactive hexa-coordinate form.

Other reaction mechanisms have been suggested for FDH,[463,490,491] but all rely on the dissociation of the selenocysteine/cysteine residue and assume the mandatory requirement of formate to bind directly to the molybdenum atom. However, there is no clear-cut experimental evidence for formate binding to the molybdenum atom. Moreover, because the formate oxidation is not an oxygen atom transfer reaction, the direct formate binding to the molybdenum might not even be necessary. In accordance, a very recent proposal pointed to a different chemical strategy, through which formate oxidation occurs *via* hydride transfer and not proton plus two electrons transfer

****The FDH-catalyzed reaction is not an oxygen-atom transfer reaction; the reaction product is carbon dioxide ($HCOO^- \rightarrow CO_2 + 2e^- + H^+$) and not hydrogencarbonate ($HCOO^- + H_2O - X \rightarrow HOCOO^- + 2e^- + 2H^+$).[488] In fact, oxidation of ^{13}C-labelled formate in ^{18}O-enriched water demonstrated clearly the formation of $^{13}CO_2$ gas with no ^{18}O atoms.[488,489]

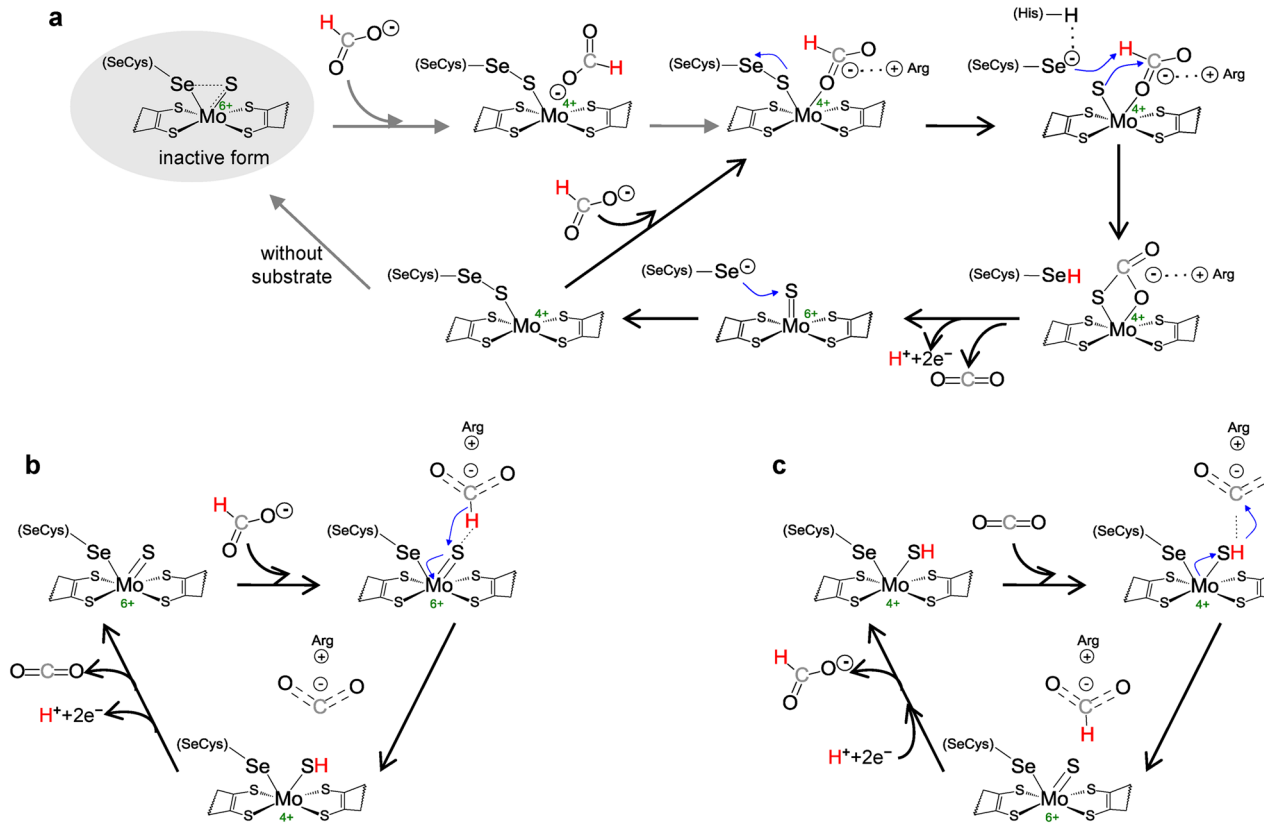


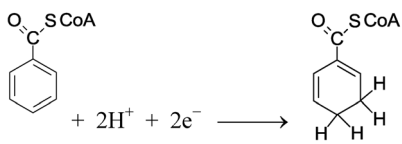
Figure 1.11 Simplified mechanistic proposals for the reaction catalyzed by formate dehydrogenase. Formate dehydrogenase-catalyzed formate oxidation (a, b) and carbon dioxide reduction (c). See text for details.

(Figure 1.11b).[492] The direct hydride acceptor is the terminal sulfo group of the oxidized molybdenum centre (Mo^{6+}=S) and formate does not bind directly to the molybdenum atom; the selenocysteine/cysteine residue was suggested to remain bound to the molybdenum atom throughout the all catalytic cycle, even though it is not a requirement in this mechanistic hypothesis.[492] The same chemical strategy has also been suggested to explain the reverse reaction of carbon dioxide reduction to formate, with the terminal sulfo group of the reduced molybdenum centre (Mo^{4+}-SH) being thought of as the hydride donor (Figure 1.11c).[493] Certainly, much more research is essential to critically evaluate the diverse mechanistic proposals.

To conclude, it should be emphasized that the DMSOR family comprises many other enzymes in addition to those described here; the array of reactions catalyzed is extensive (Table 1.2) and well beyond the oxygen, hydrogen and sulfur atom transfer reactions considered here.

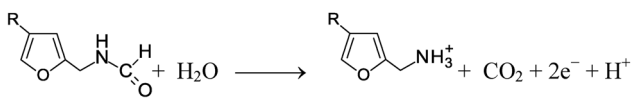
1.4.4 The Tungstoenzymes Family

The active site of tungstoenzymes harbours one tungsten atom coordinated by four sulfur atoms of two pyranopterin cofactor molecules, plus oxygen and/or sulfur and/or selenium atoms in a trigonal prismatic geometry (as observed in the DMSOR family of molybdoenzymes) or in a distorted octahedral coordination geometry (Figure 1.3b). This family consists of prokaryotic enzymes of diverse functions, including (i) aldehyde oxidoreductases (eqn (1.7)), as the enzymes from *Pyrococcus furiosus* aldehyde : ferredoxin oxidoreductases (W-AOR), formaldehyde : ferredoxin oxidoreductase and glyceraldehyde-3-phosphate : ferredoxin oxidoreductase, or the *Clostridium formicoaceticum* carboxylic acid reductase, (ii) FDHs (eqn (1.25)) from, *e.g.*, *Moorella thermoacetica* (*Clostridium thermoaceticum*), *Desulfovibrio gigas* and others, (iii) *Pelobacter acetylenicus* acetylene hydratase (eqn (1.28)), (iv) *Geobacter metallireducens* benzoyl-CoA reductase (eqn (1.29)) and (v) *M. thermoautotrophicum N*-formylmethanofuran dehydrogenase (eqn (1.30)) (Table 1.2). Interestingly, except for the last two, all these enzymes have a known molybdenum-containing counterpart (which seems to have some evolutionary significance, as noted in Section 1.2).



(1.29)

benzoyl-CoA cyclohexa-1,5-diene-1-carboxyl-CoA



(1.30)

formyl-methanofuran methanofuran

a) Compared to the molybdenum-containing enzymes, our knowledge about the structural and mechanistic properties of the tungstoenzymes is more limited. Nevertheless, the first crystal structure of a pyranopterin-containing enzyme was that of the W-AOR from the hyperthermophilic archaeon *Pyrococcus furiosus*[222] – one of the benchmarks of tungstoenzymes.

W-AOR enzymes are involved in handling aldehydes, catalyzing the reversible oxidation of aldehydes to the respective carboxylic acids, with the simultaneous reduction of ferredoxin. The *P. furiosus* W-AOR is a homodimeric enzyme (α_2) with one tungsten centre, the site of substrate oxidation, and one [4Fe–4S] centre that is involved in intramolecular electron transfer to the physiological partner (a [4Fe–4S] ferredoxin) (Figure 1.12).[222] The tungsten centre is coordinated by the characteristic two pyranopterin cofactor molecules, in this case in the mononucleotide form; their phosphates groups interact with a Mg^{2+} ion that intervenes between them. The first coordination sphere of tungsten, in its oxidized form, is completed with two oxo groups (W=O) or, possibly, with one oxo and one hydroxyl groups (W=O(–OH); as

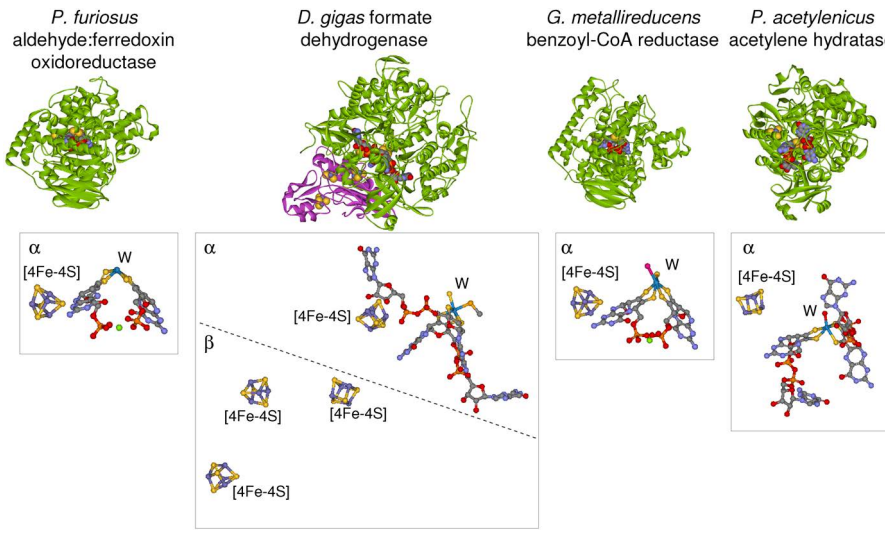


Figure 1.12 Three-dimensional structure view of *P. furiosus* aldehyde : ferredoxin oxidoreductase, *D. gigas* formate dehydrogenase, *G. metallireducens* benzoyl-CoA reductase and *P. acetylenicus* acetylene hydratase (top) and of the arrangement of their redox cofactors (bottom). Only one subunit in W-AOR (α_2) is represented. The number of colours reflect the number of subunits of each enzyme and its cofactor composition: W-AOR, benzoyl-CoA reductase and acetylene hydratase α – green; FDH αβ – green, pink. In the case of the benzoyl-CoA reductase, only the 3D structure of the subunit holding the tungsten centre is presented; the sixth unknown ligand is represented in pink. The structures shown are based on the PDB files 1AOR (W-AOR), 1H0H (FDH), 4Z3Z (benzoyl-CoA reductase) and 2E7Z (acetylene hydratase), and were produced with Accelrys DS Visualizer, Accelrys Software Inc.

observed in the molybdenum centre of arsenite oxidized), but with no ligand from the polypeptide chain. The reduced form is probably penta-coordinate, with only one terminal oxygen atom. The closely related formaldehyde : ferredoxin oxidoreductase displays a homotetrameric structure, but with each monomer comprising a similar active site architecture and also one [4Fe–4S] centre.[494–496]

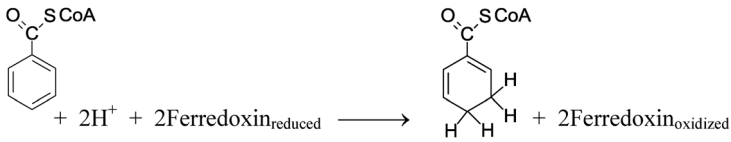
Several FDH enzymes contain tungsten and, in spite of the structural/functional similarities with the molybdenum-containing FDH, they belong to the tungstoenzymes family, according to our organization. This is also the case of the *N*-formylmethanofuran dehydrogenase, which together with the FDH contributes to carbon dioxide fixation (reduction) into *N*-formylmethanofuran and formate (acetate), respectively. The FDH from *D. gigas* is a periplasmatic heterodimer (αβ) that harbours four [4Fe–4S] centres and the tungsten centre (Figure 1.12).[497–499] As anticipated, the tungsten centre is the active site, where formate oxidation to carbon dioxide takes place (eqn (1.25)); the Fe/S centres are responsible for the subsequent intramolecular electron transfer to the physiological acceptor, a periplasmatic *c*-type cytochrome. Noteworthy, the α subunit (folded into four domains) is homologous to the *E. coli* molybdenum-containing FDH-H and contains one Fe/S centre (bound by the N-terminal domain) and the tungsten centre (bound mainly through the other three domains). Also the β subunit, folded into two domains, one containing one Fe/S and the other two Fe/S centres (total of three), displays a similar arrangement to the one found in the *E. coli* FDH-N β subunit (although in FDH-N both domains contain two Fe/S centres each (total of four)). The tungsten centre is coordinated by the characteristic pair of pyranopterin molecules, in this case in the form of dinucleotide of guanine, plus one selenocysteine (Mo–Se(Cys)) and one terminal sulfur atom (Mo=S), again similar to the *E. coli* molybdenum-containing FDH enzymes (also the conserved histidine and arginine residues are present in the active site pocket).

Other bacteria from the *Desulfovibrio* genus encode both molybdenum- and tungsten-containing FDH enzymes (but with no crystal structure available yet). *D. alaskensis* expresses three FDH,[500] two of which have been characterized and one found to be a tungsten-containing FDH (the product of the *W-fdh* genes). The second enzyme has been shown to incorporate either molybdenum or tungsten (product of *Mo/W-fdh* genes), thus being the first FDH known to be able to do this. However, this enzyme should preferentially harbour molybdenum, as suggested by the slight upregulation of the *Mo/W-fdh* genes when the bacterium is grown under molybdenum supplementation and strong downregulation under tungsten supplementation.[500] Both enzymes have the characteristic tungsten centre coordinated by two pyranopterin molecules, in the form of dinucleotide of guanine, and several Fe/S centres.[500,501] Analysis of the *D. vulgaris* genome has also suggested the presence of three periplasmatic FDHs,[502,503] one of which is a dimeric FDH that can also incorporate either molybdenum or tungsten.[503,504]

Noteworthy, there are also tungsten-containing NAD(P)-dependent FDHs, including enzymes from the acetogenic *Moorella thermoacetica* (*Clostridium thermoaceticum*),[218,505] *Clostridium formicoaceticum*[215,506] or *C. carboxidivorans*,[507] but also of the methylotrophic *Methylobacterium extorquens*.[18] One of the FDH of the aerobic *M. extorquens*[19] is a heterodimeric (αβ) tungsten-containing/NAD(P)-dependent FDH.[18] The α subunit displays ≈35% identity with *E. coli* molybdenum-containing FDH-H and has been suggested to harbour at least one Fe/S centre in addition to a tungsten centre coordinated by a cysteine residue. The β subunit contains the putative binding motifs for an Fe/S centre and the FMN (responsible for the NAD(P) binding). The NADP-dependent tungsten-containing FDH from *M. thermoacetica* – the first enzyme shown to have tungsten – is an $(\alpha\beta)_2$ heterotetramer, containing several Fe/S centres (at least two [2Fe–2Fe] and two [4Fe–4S]) in addition to the selenocysteine-coordinated tungsten centre.[13–15,218] The presence of a tungsten-containing FDH in strictly aerobic bacteria may indicate that tungstoenzymes are not restricted to anaerobic organisms and are probably more widespread than previously thought.

Tungsten is also essential for the benzoyl-CoA reductase of the "Fe^{3+}-respiring" *Geobacter metallireducens*, a key enzyme in the microbial anaerobic degradation of monocyclic aromatic compounds. This complex enzyme, suggested to consist of eight subunits,[508] catalyzes the reduction of benzoyl-CoA to cyclohexa-1,5-diene-1-carboxyl-CoA by ferredoxin (eqn (1.31)), a remarkable reaction in terms of its extremely low reduction potential ($E^{\circ\prime}$ = −622 mV) and in terms of the chemistry carried out, because it does not involve the "classic" oxygen atom abstraction/insertion.[509,510] The benzoyl-CoA reductase subunit containing the active site was recently crystallized.[511] Like W-AOR, with which it shares 33% sequence identity,[222,512] it has one [4Fe–4S] centre, involved in intramolecular electron transfer from the other redox centres of the enzyme, and the tungsten centre, which in benzoyl-CoA reductase is accommodated in a highly hydrophobic pocket (Figure 1.12). The tungsten atom is coordinated by the two characteristic pyranopterin cofactor molecules, in the mononucleotide form, plus a cysteine sulfur atom (W–S(Cys)) and a six ligand whose nature has not been not established. While analysis of the electron density excludes hydroxyl or oxo ligands and suggests an electron-rich species, such as a sulfur atom or a diatomic group, X-ray absorption fine structure spectroscopy (EXAFS) has excluded the scenario of a coordination sphere with six sulfur ligands and suggested instead a linear diatomic ligand such as a CN or CO. As cyanide incubation results in activation of the enzyme, whereas CO has no effect, the unambiguous identification of the sixth ligand must wait for crystal structures with resolutions below 1.5 Å. Of note, the tungsten centre of benzoyl-CoA reductase displays a distorted octahedral coordination geometry, rather than the characteristic trigonal prismatic geometry. Another noteworthy feature of the benzoyl-Co A reductase is the presence of zinc[508] and of a Zn^{2+}-binding site, where the metal is tetrahedrally coordinated by two histidine and two glutamate residues, 11.5 Å away from the tungsten atom.[511] The invariance of these four amino acid residues

and the proximity to the tungsten centre suggest that the zinc may have a key role in enzyme activity, possibly to protect the tungsten centre from bulk solvent and to stabilize the region surrounding the cofactor molecules.[511]



(1.31)

benzoyl-CoA cyclohexa-1,5-diene-1-carboxyl-CoA

The tungsten-containing acetylene hydratase from the anaerobe *Pelobacter acetylenicus* also catalyzes a remarkable reaction in terms of the chemistry carried out: the non-redox hydration of acetylene to acetaldehyde (eqn (1.28)) as part of an anaerobic degradation pathway of unsaturated hydrocarbons.[477,513,514] Acetylene hydratase is a monomeric enzyme, harbouring a [4Fe–4S] centre in addition to the tungsten centre, which is adjacent to a hydrophobic acetylene-binding pocket (Figure 1.12).[515,516] The tungsten is coordinated by the two characteristic pyranopterin cofactor molecules, in the form of dinucleotide of guanine, plus a cysteine sulfur atom (W–S(Cys)) and a tightly coordinated water molecule as the sixth ligand. Of note, although the acetylene hydratase-catalyzed reaction is non-redox (its tungsten and Fe/S centres do not change their oxidation states during catalysis), the tungsten centre must be reduced to W^{4+} for the enzyme to be active.[517] As with the benzoyl-CoA reductase, the tungsten centre of acetylene hydratase has a geometry closer to the octahedral (or trigonal antiprismatic) due to a slight rotation of one of the pyranopterin cofactor molecules.[516]

b) Contrary to the reaction mechanisms of XO, SO or DMSOR, which are presently well understood, the tungstoenzymes were less thoroughly studied; still, there are several recent remarkable breakthroughs.[508,511,514,516,518–520] On the other hand, the formate oxidation mechanism is believed to be, overall, similar to the molybdenum-containing enzymes.

The presently known tungstoenzymes raise some puzzling questions: (i) why are the vast majority of these molybdo- plus tungstoenzymes active only with molybdenum (in other words, why do the vast majority of organisms use only molybdenum) and (ii) why do so few organisms have enzymes that function only with tungsten (*i.e.* what turns those few tungsten-dependent organisms into "exceptions" to the "rule" of molybdenum-users)?

Tungsten was, until the 1980s, regarded as an antagonist and inhibitor of the molybdoenzymes (see note §, in Section 1.2),[97–105] which delayed the recognition of its own biological relevance.[13,216] Subsequently, after the identification of the first tungstoenzymes, it became apparent that for almost all of the known tungstoenzymes there is a homologous molybdoenzyme, either in the same or in different organisms.[12] While this general observation makes sense in terms of the chemical similarities between tungsten and

molybdenum, and given that both metals are coordinated by the same cofactor molecule, it did not help to provide a clear answer to the above questions. A plausible explanation can, however, be made if one takes into account the chemical singularities of tungsten, relatively to molybdenum, and its different bioavailability, as was discussed in Section 1.2, suggesting that tungsten is a better choice for low reduction potential reactions carried out anaerobically at higher temperatures, in a sulfidic environment.[3] This hypothesis is supported by the tungsten-dependence of hyperthermophilic archaea, the large number of tungstoenzymes found in hyperthermophilic organisms and by the fact that most of the prokaryotes that contain tungstoenzymes are obligate anaerobes[521,522] (although notably there are exceptions, in some aerobic methylotrophic organisms). In addition, this hypothesis is consistent with the reduction potentials of the reactions catalyzed by the tungsten-dependent benzoyl-CoA reductase, W-AOR/carboxylic acid reductase, *N*-formylmethanofuran dehydrogenase and FDH, that have values of −622 mV, −580 mV, −500 mV and −430 mV, respectively.[523,524] Also the non-existence, as far as is presently known, of a tungsten-containing NaR (catalyzing a reaction with a reduction potential of +420 mV) is in full agreement with this hypothesis. Still, the *Pyrobaculum aerophilum* NaR can incorporate and be active with tungsten, when grown in the absence of molybdenum (although in the presence of both metals, the enzyme was reported to contain mostly molybdenum).[525,526] A tungsten-containing form of the *E. coli* trimethylamine *N*-oxide reductase, which catalyzes a reaction with a reduction potential of +130 mV, is formed when this metal is more available in the medium.[527] On the other hand, the above hypothesis dictates that molybdenum would be a good choice for reactions with higher reduction potentials, under either anaerobic or aerobic conditions, but not at high temperatures.[3,12,92–96] In agreement, it seems that prokaryotes that grow at low or moderate temperatures are able to express tungstoenzymes and homologous and/or different molybdoenzymes. This is the case, *e.g.*, of the sulfate reducer bacterium *Desulfovibrio alaskensis* that expresses both tungsten- and molybdenum-containing FDH and one molybdenum-containing AOR. Yet, as this example illustrates, molybdoenzymes are also able to catalyze reactions with low reduction potential values (namely the aldehyde/carboxylic acid reaction). Therefore, to reconcile the obligatory tungsten-dependence of the different aldehyde oxidoreductases of *P. furiosus*,[519,528,529] it may be that it is only under high-temperature conditions that the unique chemical properties of tungsten are most advantageous – future work will determine if this is universally correct.

A third intriguing question regards why some – indeed very few and only prokaryotic – enzymes can function with either molybdenum or tungsten. This is the case of one of the FDH enzymes from *D. alaskensis* and from *D. vulgaris*, which are active with either molybdenum or tungsten. It is also the case of the molybdenum-containing *R. capsulatus* DMSOR[530] and *E. coli* trimethylamine *N*-oxide reductase,[527] whose tungsten-substituted forms are active. Obviously, in these cases, one is tempted to suggest that the chemical similarities between molybdenum and tungsten and the fact that both metals are

coordinated by the same cofactor is the basis for the utilization of both metals. However, as far as is presently known, these cases are "rare exceptions" and not the "rule". In fact, the substitution of molybdenum by tungsten,[97–105] and *vice versa*,[3,12,528,529] typically results in metal-free and tungsten-/molybdenum-substituted enzymes, all with no enzymatic activity. In this scenario of "metal selectivity", the question arises as to why the above-mentioned enzymes are not selective. It can be argued that it would be an advantage to incorporate both metals. This could be particularly relevant in sulfate-reducing bacteria, for which molybdenum bioavailability can be limited as a result of sulfide production by these organisms (whose environmental accumulation would lead to the molybdenum precipitation). But, if this were the case, why would other molybdo- and tungstoenzymes from these organisms be selective for one or the other metal? *D. gigas*, *e.g.*, has a tungsten-containing FDH and a molybdenum-containing AOR, both of which are active only with the respective metal. Clearly, the incorporation of both or of only one of the two metals in active prokaryotic enzymes is far from being understood.

1.4.5 The Nitrogenases

The active site of the molybdenum-containing nitrogenases holds a complex high-nuclearity centre, comprising one molybdenum atom, several iron and sulfur atoms plus a carbon atom, [$MoFe_7S_9C$] (Figure 1.3b). (The molybdenum-independent nitrogenases will not be here discussed (see note †, in Section 1.2) and the term "nitrogenase" will be here used to refer only to the molybdenum-dependent enzyme.) Nitrogenase enzymes have been purified from a variety of bacteria, including *Azotobacter vinelandii*, *Klebsiella pneumoniae* and *Clostridium pasteurianum*.[531–533] The enzymes from these diazotrophs catalyze the remarkable reaction of dinitrogen reduction (fixation) to ammonium, with the cleavage of the exceptionally stable N≡N triple bond (eqn (1.32)), a key step in the biogeochemical cycle of nitrogen (Figure 1.1, blue arrow; see Section 1.2). Of note, nitrogenases are also able to catalyze the reduction of the isoelectronic carbon monoxide to hydrocarbon,[47,49,50,534–536] cyanide or acetylene, as well as of carbon dioxide,[537,538] ethylene, nitrous oxide, nitrite, azide and hydrazine.[45,538–548]

$$N{\equiv}N + 8H^+ + 8e^- + 16MgATP \rightarrow 2NH_3 + H_2 + 16MgADP + 16P_i \qquad (1.32)$$

a) The history of nitrogenase is quite old, with the first mention of the organism's ability to fix dinitrogen occurring in the middle of the 19th century;[549] the term "nitrogenase" was introduced in 1934 by Burk and collaborators.[550,551] Since then, the progress of our knowledge about nitrogenases has been extraordinary.

The nitrogenase from *Azotobacter vinelandii* is comprised by of two proteins (Figure 1.13):[44,54,531,532,540,552–579] (i) the "iron protein" (NifH), a homodimer containing one ATP-binding site per monomer and one [4Fe–4S] centre that bridges the two monomers of the dimer; and (ii) the "molybdenum–iron protein"

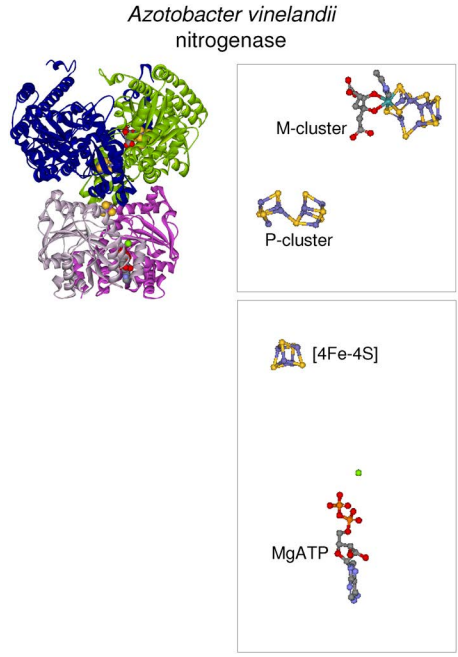


Figure 1.13 Three-dimensional structure view of *A. vinelandii* nitrogenase (top) and of the arrangement of its redox cofactors (bottom). Top – Only one αβ unit of the "molybdenum–iron protein" ($(\alpha\beta)_2$) is represented, in green and dark blue; both α subunits of the "iron protein" (α_2) are represented, in pink and light pink. Bottom – For the "molybdenum–iron protein", the M-cluster of one α subunit and the P-cluster that is found in the α/β subunit interface are represented. For the "iron protein", the MgATP of one monomer and the [4Fe–4S] centre that bridges the two monomers of the dimer are represented. The structures shown are based on the PDB files 1N2C, and were produced with Accelrys DS Visualizer, Accelrys Software Inc.

(NifDK), an $(\alpha\beta)_2$ heterotetramer consisting of one $[Fe_8S_7]$ "P cluster" that bridges the αβ pair of each dimer, and one $[MoFe_7S_9C$–homocitrate] "M-cluster", in each α subunit. Both P- and M-clusters, with their high nuclearity and unusual structures, are unique to the nitrogenase enzymes. The P-cluster can be viewed as two [4Fe–3S] cubanes bridged by a μ_6-sulfide, with the six sulfur atoms coming from six cysteine residues;[553,554,566,577,580–583] it can exist in a fully reduced, all-ferrous, form and in a two-electron oxidized form.[555,558,559,572,576,584–586] The M-cluster can be viewed as a [4Fe–3S] and [Mo–3Fe–3S] cubane bridged by three μ_2-sulfides, with only one sulfur coming from a cysteine residue, the one that coordinates the terminal iron ion.[554,566,576,577,587,588] A histidine residue coordinates the molybdenum, whose coordination sphere is completed by the 2-hydroxyl and 2-carboxyl groups of homocitrate and the three bridging sulfurs. While it has been assumed that molybdenum is in the 4+ oxidation state (Mo^{4+}),[589–596] a recent reassignment suggests an unprecedented 3+ state

(Mo^{3+}) instead.[597,598] The central cavity of the M-cluster holds a recently identified μ_6-coordinated carbide ion (C^{4-});[566,576,577,599–604] it is thought to play a role in stabilizing the M-cluster structure[601,602] and/or in modulating the reactivity of the M-cluster, or even in the interaction with the substrate.[605,606] The M-cluster can undergo a reversible one-electron oxidation–reduction process.[540]

The "iron protein" acts as an ATP-dependent reductase for the molybdenum–iron protein (eqn (1.32)). It has been proposed that upon ATP binding/hydrolysis, a conformational rearrangement takes place that allows the "iron protein" to bind to the "molybdenum–iron protein", forming a pseudo γ_2 electron transfer subunit that would transfer electrons from its [4Fe–4S] centre to the P-cluster of the "molybdenum–iron protein".[44,559,563,567,572,607–612] The electrons are subsequently transferred from the P-cluster to the M-cluster, the active site where reduction of dinitrogen takes place. Nitrogenase-catalyzed substrate reduction thus requires nucleotide-dependent electron delivery from the "iron protein" to the "molybdenum–iron protein". After substrate reduction, products are released and the complex between the "iron protein" and the "molybdenum–iron protein" dissociates.

b) Unravelling the mechanism by which the exceptionally stable triple bond of dinitrogen, N≡N, is cleaved by nitrogenase is an exciting and highly challenging task. This is particularly obvious when one compares the biological process to the harsh industrial conditions used for ammonia production from dinitrogen in the Haber–Bosch process.[613–615] For our society, the relevance of the dinitrogen reduction can be appreciated when one realizes that the factor that most frequently limits agricultural production is the nitrogen availability and that nearly half of the existing human population could not be fed without the use of industrially produced nitrogen fertilizers.[30] Understanding of biological dinitrogen reduction could thus help in the development of improved "greener" synthetic catalysts or of genetically modified organisms with the capacity to fix their own nitrogen. However, while the structure of the nitrogenase components and of their metal centres is presently well known, there are still many uncertainties regarding the metals' oxidation states, the electronic structure of the M-cluster and how this controls the centre's reactivity; this knowledge is essential to fully understand the mechanism of dinitrogen reduction.

Reflecting the interest in this enzyme, numerous experimental[538,545,558,562,574,616–646] and theoretical[593,596,647–663] studies have been carried out to shed light on several aspects of the nitrogenase reaction mechanism. A recent mechanistic proposal suggests that the activation and reduction of dinitrogen depends on the prior reductive elimination of iron-based hydrides in the form of dihydrogen from the M-cluster, it having long been recognized that evolution of dihydrogen is obligatory in dinitrogen reduction.[53,643,664–666] Molybdenum, as anticipated, plays a critical role in the mechanism of dinitrogen reduction, as is demonstrated by enzymes formed in molybdenum-deficient environments and by tungsten-substituted inactive enzymes.[667] Although it is not the substrate binding site, which is thought to be one of the irons,[575,668] molybdenum may be essential to tune the reduction potential of the cofactor or of the neighbouring

iron, or alternatively be involved in modifying the electronic structure of the cofactor to facilitate the substrate binding or protonation.

1.4.6 A Novel Heterometallic Cluster Containing Molybdenum Found in Biology

Besides the molybdenum and tungsten centres that form the active site of several enzymes (described in the previous sections), there are also a few other heteronuclear centres whose physiological function is not yet fully understood.[669–673] X-ray absorption fine structure (EXAFS) studies have revealed the presence of a novel linear cluster formulated as $[S_2MoS_2CuS_2MoS_2]$ (Figure 1.3b), a unique heterometallic cluster in biological systems.[669,670] The cluster is contained in a small monomeric "orange protein" isolated from sulfate reducing bacteria, and is non-covalently bound to the protein. Native "orange protein" has only been isolated from *Desulfovibrio gigas*, although all sequenced *Desulfovibrio* complete genomes have putative open reading frames encoding similar proteins. The metal cluster reconstitution of recombinant apo-"orange protein" has been obtained for *D. gigas* protein through a protein metal cluster assisted synthesis and NMR data sustained that the cluster is stabilized inside the protein by both electrostatic and hydrophobic interactions.[672,674] In different sulfate reducing bacteria the genes encoding putative "orange proteins" are located in operons that also encode other proteins, such as putative iron–sulfur ATPases.

1.5 Outlook

Our knowledge of molybdenum- and tungsten-containing enzymes has progressed considerably since the observation of Schardinger in 1902 of the decolourization (reduction) of methylene blue by formaldehyde in presence of fresh milk (through the action of the presently well-known molybdoenzyme xanthine oxidase). During the last years, the structural, spectroscopic and kinetic data, complemented by theoretical information (computational chemistry), about model compounds and enzymes, have provided an extraordinarily detailed picture of molybdenum- and tungsten-containing enzymes. The metabolic pathways responsible for the pyranopterin cofactor synthesis, metal insertion and subsequent modification (when this is the case) and their regulation are also presently well understood.

For many enzymes, crystallographic and spectroscopic studies have provided atomic and electronic details about the active site centres, the other redox-active centres present and the overall structure. Studies carried out with model compounds, together with the computational chemistry and kinetics, has enabled us to discuss enzyme reaction mechanism in atomic and electronic detail for each catalytic intermediary, and establish key structure-activity relationships for several enzymes. Genome analysis and microbiological work have revealed the phylogenetic relationships between different enzymes and anticipated the discovery of new enzymes.

Still, there are numerous open questions for the future. The deeper we go into a topic, the more questions arise and, particularly for the newly discovered enzymes, much work remains to be done regarding their structural characterization and reactivity and mechanistic probing. This introductory chapter on the general topic of molybdenum and tungsten in biology is an "appetizer" for the overall book, where more detailed information will be discussed in the different chapters. Relevant aspects of the chemistry of molybdenum and tungsten in terms of biochemistry are described and an overview of the different families (xanthine oxidase, sulfite oxidase, dimethylsulfoxide reductase, tungstoenzymes families, including nitrogenases) given with emphasis on structures and mechanistic aspects. We hope that we have managed to transmit that, because of its impact on the biogeochemical cycles, in particular of nitrogen and carbon, and on the present life existence and survival, our planet would be quite different without molybdenum!

Abbreviations

AO, aldehyde oxidase;
AOR, bacterial molybdenum-containing aldehyde oxidoreductases
DMS, dimethylsulfide
DMSO, dimethylsulfoxide
DMSOR, dimethylsulfoxide reductase
FDH, formate dehydrogenase (all types of formate dehydrogenase enzymes)
FDH-H, *E. coli* formate dehydrogenase H, from the formate-hydrogen lyase system
FDH-N, *E. coli* formate dehydrogenase N, from the anaerobic nitrate-formate respiratory pathway
FDH-O, *E. coli* formate dehydrogenase O, from the aerobic respiratory pathways
Fe/S, iron–sulfur centre
M, metal, molybdenum or tungsten
mARC, mitochondrial amidoxime reducing component
MOSC, from molybdenum cofactor sulfurase C-terminal domain (proteins involved in pyranopterin cofactor biosynthesis)
Mo/W-bis PGD, molybdenum/tungsten-bis pyranopterin guanosine dinucleotide-containing enzymes
Myr, million years
NaR, nitrate reductase (all types of nitrate reductase enzymes, prokaryotic and eukaryotic ones)
NaRGHI, respiratory nitrate reductase (prokaryotic), after the name of the encoding genes, *narG*, *H* and *I*
NaRZWV, respiratory nitrate reductase (prokaryotic), after the name of the encoding genes, *narZ*, *W* and *V*
PCD, pyranopterin cytidine dinucleotide
PGD, pyranopterin guanosine dinucleotide
PMN, pyranopterin mononucleotide

RO, Sreactive oxygen species
SO, sulfite oxidase
XD, xanthine dehydrogenase
XO, xanthine oxidase
W-AOR, tungsten-containing aldehyde:ferredoxin oxidoreductases (to distinguish from the molybdenum-containing aldehyde oxidoreductases).

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