Mechanistic aspects of molybdenum-containing enzymes

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1. Introduction

In the past several years, the number of enzymes known to possess mononuclear molybdenum centers in their active sites has increased significantly, and well over 50 such enzymes have been identified that catalyze a variety of hydroxylation, oxygen atom transfer and other oxidation-reduction reactions. Many of these enzymes have been isolated from either obligate anaerobes or facultative anaerobes grown under anaerobic or microaerobic conditions, and are involved in a variety of anabolic, catabolic and energy-conserving metabolic pathways. There are now several enzymes having known crystal structure, and this new structural information has provided the basis for an increasingly detailed understanding of the mechanisms of action of these enzymes. In the present review, an overview of our present understanding of the mechanism of action of these enzymes will be presented, and those of a few selected enzymes will be considered in greater detail.

Several alternative classification schemes have been suggested for these molybdenum-containing enzymes, but for the purposes of the present discussion they will be considered to fall into three families, based on the structures of their molybdenum centers in their oxidized Mo(VI) state (Fig. 1) [1]. These families include: (i) the molybdenum hydroxylases, a large and broadly dispersed family of enzymes that possess an MoOS unit and catalyze the hydroxylation of a broad range of aldehydes and aromatic

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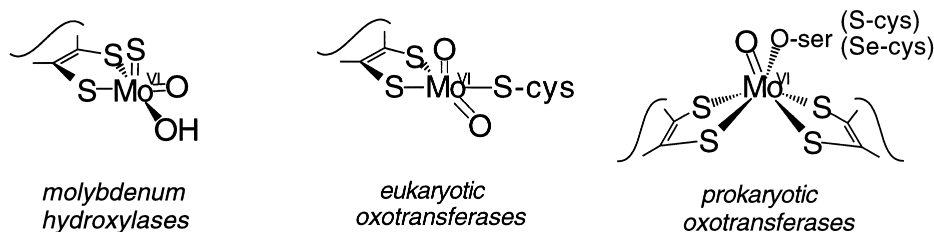


Fig. 1. Active site structures of the mononuclear molybdenum enzymes. From left to right, the molybdenum hydroxylases, the eukaryotic oxotransferases and the prokaryotic oxotransferases (and related enzymes).

heterocycles; (ii) the eukaryotic oxo transferases, a family that at present includes only sulfite oxidase and the assimilatory nitrate reductases, enzymes which possess an MoO_2 unit in their active sites and which catalyze oxygen atom transfer to or from a substrate; and finally (iii) a diverse group of prokaryotic enzymes that catalyze either oxo atom transfer or other oxidation-reduction reactions. As will be seen below, however, some may catalyze significantly more complex (and interesting) reactions. Enzymes of this last group have a common molybdenum center structure in which the metal is coordinated by a pair of dithiolene ligands contributed by an unusual pterin cofactor [2]. This cofactor is common to all the mononuclear molybdenum (and tungsten) enzymes, but whereas the first two families possess only a single equivalent bound to the metal, in members of the third family two equivalents of the cofactor coordinate to the metal. It should be emphasized that this final group is structurally more diverse than the first two, and can be subdivided into enzymes that possess an Mo-O(Ser), Mo-S(Cys) or Mo-Se(Se-Cys) group contributed by the polypeptide; in addition, some of these enzymes appear to possess an Mo = S group rather than the more commonly encountered Mo = O (see [1] for a review).

The pterin cofactor that is common to all these enzymes has the structure shown in Fig. 2. In addition to the pterin itself, there is an additional pyran ring that possesses an exocyclic dithiolene moiety by which the cofactor coordinates the metal, plus a short phosphorylated side chain. In eukaryotic systems the cofactor possesses the structure shown, but in prokaryotic systems the cofactor is usually found elaborated as the guanine, adenine, cytidine or hypoxanthine dinucleotide [2]. The cofactor has been called ‘molybdopterin’ or ‘moco’ (for ‘molybdenum cofactor’), although this nomenclature is misleading for two reasons: (i) the term refers to the organic cofactor itself without molybdenum bound to it; and (ii) the identical cofactor is found in tungsten-containing enzymes (where it has occasionally been referred to as ‘tungstopterin’). The term pyranopterin has also been suggested, and this has the advantage of lacking the confusion associated with either of the above terms.

With this general background concerning the active site structures of these enzymes, we will now turn to a consideration of their mechanisms of action. We focus here on work done since 1990, referring the reader to recent reviews for work done prior to this time [1,3].

2. The molybdenum hydroxylases

As indicated above, the molybdenum hydroxylases constitute a large and widely dispersed group of enzymes. These enzymes are unique among the many biological systems that catalyze hydroxylation of either aromatic or aliphatic substrates in that water rather than dioxygen is the source of the oxygen

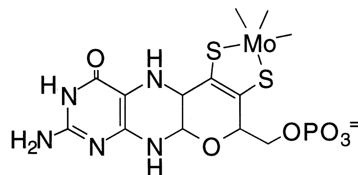


Fig. 2. Structure of the pterin cofactor associated with molybdenum- and tungsten-containing enzymes. In prokaryotic enzymes, the cofactor exists as the dinucleotide of guanine, adenine, cytidine or hypoxanthine.

incorporated into the product, and reducing equivalents are generated rather than consumed in the course of the reaction. In humans, there is a xanthine oxidoreductase (or dehydrogenase) and several aldehyde oxidases that have specific tissue distributions. In bacteria there are a large number of enzymes that are expressed when organisms are grown on various heterocyclic compounds as sole carbon source, and other enzymes that act on aldehyde substrates (taking these to the corresponding carboxylic acid). The sole member of this family that has been examined crystallographically is the aldehyde oxidoreductase[2] from *Desulfovibrio gigas* [4,5], an enzyme that is involved in the generation of H_2 using reducing equivalents obtained from aldehyde substrates. The structure of the molybdenum center of this enzyme (in the oxidized Mo(VI) form) is that shown in Fig. 1, which represents a composite of the X-ray crystal work in conjunction with the results of X-ray absorption spectroscopy (XAS) studies of other members of this family (most notably the xanthine oxidase from cow's milk). The coordination geometry inferred from the crystallographic work is that of a distorted square pyramid, with an apical Mo = S and the two sulfurs of the pterin cofactor, an Mo = O and a fifth oxygen ligand defining the basal plane [4]. This last ligand has been formulated as a water molecule on the basis of an Mo-O distance of ~2.2 Å, which is in the range for a metal-bound water (as opposed to hydroxide). As has been pointed out recently, however, this distance must be regarded as an upper limit for the true Mo-O distance, owing to series truncation artifacts around the heavy metal ion that occur in generation of the electron density map for the protein – these artifacts may in some cases lead to an overestimation of the true Mo-O distance by as much as 0.3 Å [6]. It remains to be seen whether this is a concern in the case of the molybdenum center of the aldehyde oxidoreductase. A hydroxide ligand is preferred here on the following additional grounds: (i) the XAS work with xanthine oxidase indicates a (weak) Mo-O (or N) scattering at a shorter distance of 1.98 Å rather than the crystallographically observed 2.2 Å; (ii) in small inorganic complexes of Mo(VI) oxygen ligands are most frequently found as the fully deprotonated Mo = O, occasionally as the singly protonated Mo-OH, but only extremely rarely as the fully protonated Mo-OH_2; and (iii) computational studies have shown that an Mo-OH is expected to be considerably more stable from a thermodynamic standpoint than an Mo-H_2O in the specific coordination geometry found in the active site of the aldehyde oxidoreductase.

The mechanistic significance of the degree of protonation of this oxygen ligand lies in recent work indicating quite strongly that it is this oxygen which is incorporated into the hydroxyl group of the product [7–9], with the site subsequently regenerated by hydroxide/water from the solvent for the next catalytic cycle. It has been known for some time that these enzymes (as represented by xanthine oxidase) possess such a catalytically labile oxygen, but in the absence of specific knowledge of an Mo-OH(2) ligand to the metal, the Mo = O has been considered the likely candidate for this oxygen. In light of this more recent work, however, a mechanism can be formulated in which the substrate becomes hydroxylated by nucleophilic attack of a metal-bound hydroxide on the substrate, followed by hydride transfer from the carbon to become hydroxylated to the Mo = S group (known from earlier XAS work to become protonated upon reduction of the molybdenum center) (Fig. 3).

An active site glutamate (Glu-869 in the aldehyde oxidoreductase, conserved among molybdenum hydroxylases), has been proposed to act as a base catalyst in the formation of the C-O bond of product, and possibly also in the displacement of the molybdenum-bound product that is generated by this step of the reaction [5]. Indeed, the pH dependence of the reaction of the bovine xanthine oxidase provides

[2] This molybdenum-containing hydroxylase is frequently referred to as AOR in the literature, but is not to be confused with the tungsten-containing aldehyde:ferredoxin oxidoreductase from archaeal sources such as *Pyrococcus furiosus*, which is also referred to by the same abbreviation. The tungsten-containing enzyme bears some structural relationship to the third family of molybdenum enzymes considered here, in that the active site metal is coordinated to two equivalents of the pyranopterin cofactor, but does not resemble the molybdenum hydroxylases. When there is danger of confusion between these two enzymes, we suggest using the atomic symbol as a prefix, viz. 'Mo-AOR' or 'W-AOR', to clearly delineate which enzyme is meant. The molybdenum-containing enzyme has occasionally been referred to by the trivial designation 'Mop', which is not recommended.

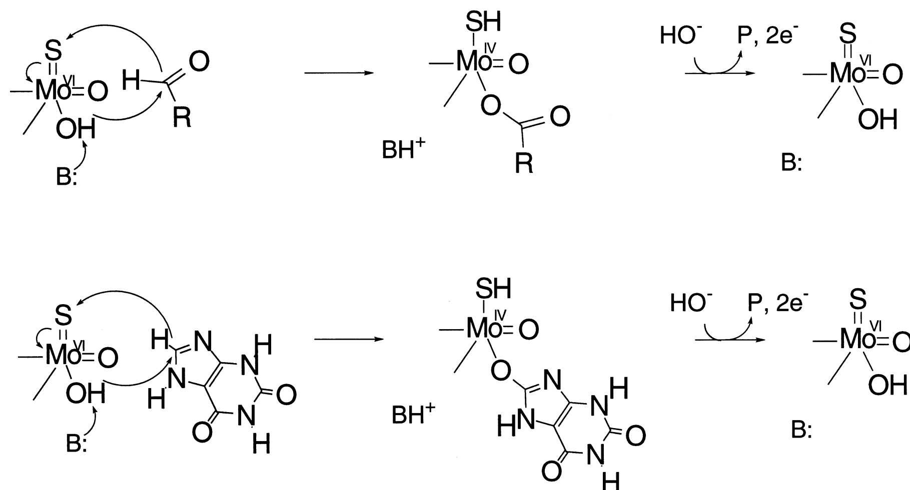


Fig. 3. Proposed mechanisms for the molybdenum hydroxylases. The reaction with aldehydes (top) and xanthine (bottom).

clear evidence for the participation of an active site base in this portion of the catalytic sequence with both heterocyclic substrates [10] and simple aldehydes [9]. It thus appears that these enzymes catalyze hydroxylation of both general classes of substrate, aldehydes and aromatic heterocycles, by this same basic mechanism.

Another aspect of catalysis that has been much informed by the crystal structure of the aldehyde oxidoreductase has to do with the manner in which reducing equivalents, once introduced at the molybdenum center, are removed from the metal to complete the catalytic sequence. In the structure of the aldehyde oxidoreductase, the amino group of the pterin cofactor that lies distal to its dithiolene group is found to be hydrogen-bonded to a cysteine residue that is a ligand to one of the two 2Fe/2S iron-sulfur clusters found in the enzyme [4]. On the basis of the physical disposition of the cofactor, it seems very likely that it is involved in facilitating electron transfer from the molybdenum to the iron-sulfur center, the physiological direction of electron flow in the enzyme. A loosely similar arrangement is found in other molybdenum enzymes, but the situation is not a general one: thus the cofactor is found to intervene between the molybdenum and 4Fe/4S center of the *Escherichia coli* formate dehydrogenase H (a bis-dithiolene enzyme) [11] but not between the molybdenum and heme of chicken sulfite oxidase [12].

3. The eukaryotic oxo transferases

The molybdenum-containing oxo transferases from eukaryotic sources, sulfite oxidase from vertebrate liver or the assimilatory nitrate reductase from higher plants, algae, yeast and fungi, catalyze a chemistry that is fundamentally more straightforward than that of the molybdenum hydroxylases. The enzyme from this group that has been structurally characterized by X-ray crystallography is the sulfite oxidase from chicken liver [12]. As indicated in Fig. 1, the coordination geometry of the molybdenum center of this enzyme is distorted square pyramidal, with an apical Mo = O and a basal plane defined by the two dithiolene sulfurs of the pterin cofactor, the remaining Mo = O and a cysteine thiolate contributed by the polypeptide[3]. The present evidence suggests quite strongly that these enzymes alternate between Mo(VI)O_2 and Mo(IV)O centers in the course of their catalytic sequences, directly

[3] The crystal structure actually indicates one apical Mo = O group and a longer basal Mo-OH. The enzyme in the crystal presumably was of the reduced Mo(IV)-containing form of the enzyme, generated either by contaminating sulfite in the mother liquor or by the X-ray beam itself in the course of data acquisition. XAS studies of oxidized and reduced sulfite oxidase have unambiguously demonstrated that the oxidized enzyme has two short Mo = O bonds, while reduction leads to protonation of one of these, with corresponding elongation of the Mo-OH bond.

analogous to the now well-characterized chemistry of small molybdenum complexes thought to represent functional models of these enzymes [13,14] (Fig. 4).

These model systems are found to be quite robust and able to turn over repeatedly in the presence of a suitable oxygen atom donor/acceptor pair. In light of the large negative entropy of activation associated with the reaction of the $Mo(VI)O_2$ model with a phosphine acceptor, an associative reaction mechanism having a highly ordered transition state has been proposed, with a phosphine lone electron pair undertaking nucleophilic attack on one of the Mo = O groups to initiate the reaction. Indeed, this chemistry has been successfully examined computationally, and the reaction is understood in considerable detail [15]. Briefly, the phosphine is thought to approach the MoO_2 unit at 90°, thereby maximizing orbital overlap between the phosphine lone pair and a π^* orbital of the Mo = O group, followed by rotation about the Mo-O bond. This rotation results in formation of an O-P σ bond and reduction of the Mo-O bond order to 1; at the same time the stability of the remaining Mo = O bond becomes significantly stronger due to increased π-backbonding to the metal, with the bond order increasing to 3. It is this increase in bond order of the remaining Mo = O group – as the first is lost in the course of the reaction – that makes the overall process thermodynamically favorable, as much of the cost of losing one of the quite stable Mo = O bonds is recovered in the stronger bond that remains. This 'spectator oxo' effect plays an important role in determining the chemical reactivity of these MoO_2 centers.

A comparable mechanism is likely for the reaction of sulfite oxidase with sulfite (Fig. 3), in which the key element is attack on an Mo = O group by a substrate lone pair. It is found, for example, that methylation of the two oxyanions of sulfite results in a significant increase in K_d (obtained from the substrate-concentration dependence of the rate of enzyme reduction upon reaction with substrate), but the limiting rate of the reaction is essentially unchanged from that observed with sulfite [16]. The

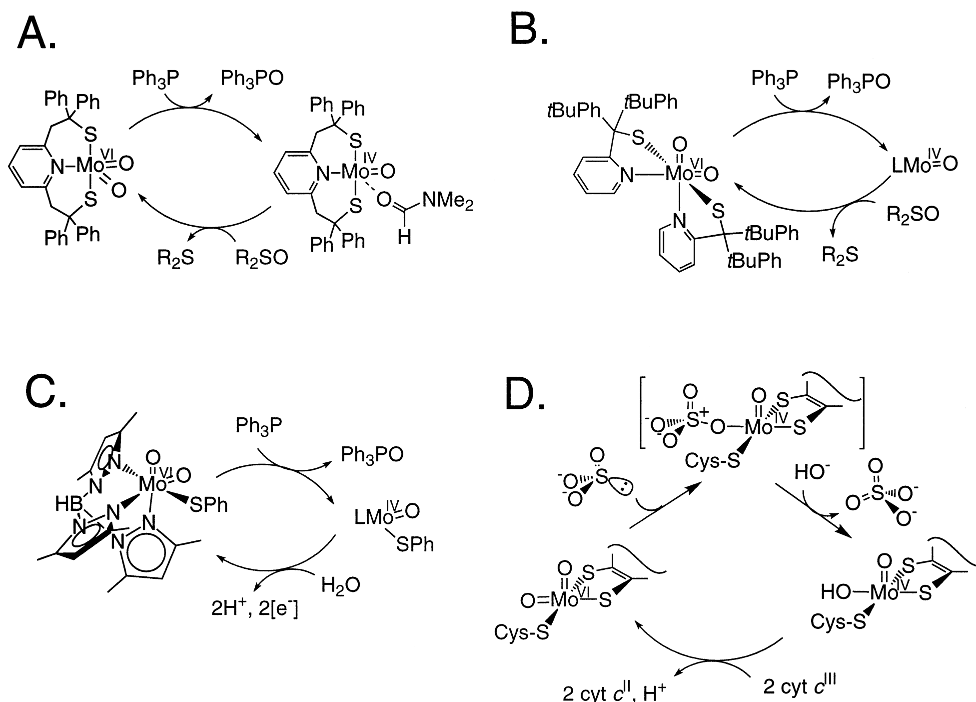


Fig. 4. Catalytic cycles for molybdenum model complexes (A–C) and sulfite oxidase (D). In all cases, the system cycles between dioxo Mo(VI) and mono-oxo Mo(IV) states.

results indicate that the oxyanion groups contribute significantly to substrate binding, but are not required for breakdown of the Michaelis complex; the implication is that the substrate need not coordinate directly to the molybdenum center in order for the reaction to proceed. This is consistent with the known substrate binding site of the protein, which consists of a set of three positively charged arginine residues around a solvent-accessible cavity of appropriate size to accommodate sulfite (in the actually determined crystal structure this position was found to be occupied by sulfate from the mother liquor) [12]. It is to be emphasized, however, that the dimethylsulfite substrate does exhibit saturation kinetics, and that a kinetically discrete Michaelis complex also forms with this substrate. Evidently, interactions in addition to salt bridges between the substrate oxyanions and enzyme arginines must occur between substrate and active site in stabilizing the Michaelis complex.

On the basis of the present evidence, it is likely that the reaction mechanism of nitrate reductase operates essentially in the reverse of that for sulfite oxidase. The fact that the physiological direction for the sulfite/sulfate couple is toward sulfate (making the enzyme an oxidase) while that for the nitrite/nitrate couple is toward nitrite (making the enzyme a reductase) has principally to do with the relative stabilities of the S = O and N = O bonds, which determine the effectiveness of the participating species as oxo donors and acceptors. This has been placed on a quantitative basis, and a thermodynamic scale established by which the direction of reaction for a given pair of couples can be predicted [17]. While there clearly must be stringent substrate selectivity between sulfite oxidase and nitrate reductase (neither is able to function as a sulfite:nitrate oxidoreductase, although this reaction is thermodynamically favorable), it is known from XAS studies that the molybdenum centers of the two enzymes are very similar and the molybdenum-binding domains of the proteins exhibit considerable amino acid sequence homology [1,3]. Having said this, neither sulfite oxidase nor nitrate reductase has been examined from a mechanistic standpoint in anything like the detail as, say, xanthine oxidase, and the extent to which their reaction mechanisms are closely related remains to be established experimentally.

4. The prokaryotic oxo transferases and related enzymes

Many of the proteins from bacterial sources that constitute the third family of molybdenum-containing enzymes catalyze oxygen atom transfer reactions that are nominally similar to those catalyzed by sulfite oxidase and the (assimilatory) nitrate reductases. Thus DMSO reductase, the dissimilatory nitrate reductases and biotin-*S*-oxide reductase all catalyze reactions that clearly involve oxygen atom transfer. Given that these enzymes likely possess only a single Mo = O group in their oxidized forms, however (see below), the implication is that they alternate between mono-oxo Mo(VI) and *des*-oxo Mo(IV) forms and lack any 'spectator oxo' aspect to their reaction mechanisms (Fig. 5). It has been demonstrated that DMSO reductase is able to catalyze oxygen atom transfer between ^{18}O-labeled DMSO and a water-soluble phosphine oxo acceptor via a catalytically labile oxygen site [18], and the label found to be present in the Mo = O group on the basis of resonance Raman work [19]. Furthermore XAS results with both oxidized and reduced DMSO reductase [20] are consistent with the enzyme cycling as indicated in Fig. 5.

The X-ray crystal structure of DMSO reductase has been determined by three different laboratories (one working with the enzyme of *Rhodobacter sphaeroides*, and the other two with the closely related *R. capsulatus* enzyme) and the results convey a considerably more complicated picture than that in-

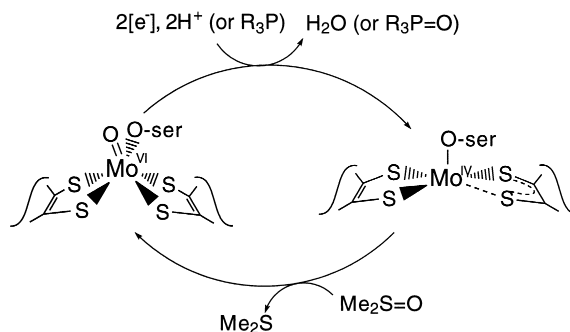
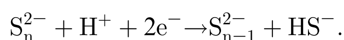


Fig. 5. A proposed reaction mechanism for DMSO reductase. The enzyme is proposed to cycle between mono-oxo Mo(VI) and desoxo Mo(IV) states.

dicated above for the reaction mechanism of the enzyme. Although the structures of the polypeptides in all three structures are virtually identical, the molybdenum centers are quite different. In the first structure reported by Rees and colleagues [21], the molybdenum center of the oxidized enzyme has a structure basically as shown in Fig. 1, with a distorted trigonal prismatic coordination geometry. Upon reduction the Mo = O group is lost, consistent with the chemistry shown in Fig. 5. Coordination of one of the two dithiolene groups is seriously perturbed in the reduced enzyme, however. One of the Mo-S bonds is quite long at 2.9 Å (by comparison, the Mo-S bonds of the other dithiolene are 2.5 Å) and the other has been lost altogether, with a non-bonding Mo-S distance of 3.7 Å. These changes have been taken to reflect a keto-enol tautomerization in the dithiolene in which the sulfur at long distance from the molybdenum possesses considerable thioketo character. In the second crystal structure of oxidized enzyme published by Huber and coworkers (working with the *R. capsulatus* protein) [22], the first dithiolene is present in essentially the same position as in the structure of the *R. sphaeroides* protein, but the second is found to have dissociated completely from the molybdenum: the Mo-S distances for the dithiolene sulfurs are 3.5 and 3.9 Å, with the vacated ligand coordination positions being occupied by a second Mo = O group at 1.7 Å. In the final structure published by Bailey and coworkers and again of the *R. capsulatus* enzyme [23], the ligand coordination sphere of the molybdenum in the oxidized enzyme is expanded from six to seven in a complex overall geometry. The additional ligand is described as a long Mo = O, implying that the oxidized enzyme is dioxo Mo(VI) rather than mono-oxo. At present it is difficult to ascertain how such structures relate to one another in an overall reaction scheme, and clearly much work remains to be done to unambiguously establish the relationship of these structures to the catalytic sequence of the enzyme.

The other enzyme from this family that is of known crystal structure is the formate dehydrogenase H of *E. coli* [11]. This enzyme is of the selenocysteine-containing subfamily, and unlike the DMSO reductases (which possess only a molybdenum center) also has a 4Fe/4S center. The oxidized enzyme has basically the structure shown in Fig. 1, with a distorted trigonal prismatic coordination geometry. The terminal oxygen was found at 2.2 Å, however, and has been ascribed to an Mo-OH rather than Mo = O group. This distance has recently been confirmed by XAS, and it seems likely that one effect of the substitution of Se for O in the protein ligand to the metal is to weaken the Mo = O bond. This is not entirely surprising, as the increased covalency of the Mo-X bond in the series O → S → Se would reasonably be expected to have the effect of weakening other metal-ligand bonds as the formal oxidation state of the metal is reduced. Formate dehydrogenase H catalyzes the oxidation of formate to CO_2, shunting the reducing equivalents thus generated into the production of H_2. Although the enzyme could conceivably catalyze the reaction by hydroxylation of formate to give bicarbonate, followed by dehydration to yield CO_2, it appears instead to operate by direct oxidation of formate [24], and is thus an example of a molybdenum enzyme that does not catalyze oxygen atom transfer. On the basis of the crystal structure of both the oxidized and the reduced enzyme, a mechanism has been proposed in which formate binds to the metal by displacing the Mo-OH, with base-assisted reduction of the metal taking place with the participation of an active site histidine residue [11]. Transient protonation of the selenocysteine has been suggested to take place in the course of the reaction, prior to protonation of the histidine.

Another molybdenum enzyme that does not catalyze oxygen atom transfer is the polysulfide reductase of *Wolinella succinogenes* [25,26]. This enzyme utilizes reducing equivalents obtained from formate dehydrogenase to reduce polysulfide according to the following scheme:



$$S_n^{2-} + H^+ + 2e^- \rightarrow S_{n-1}^{2-} + HS^-.$$

This reaction clearly does not involve oxygen atom transfer from the standpoint of its overall stoichiometry, and it is unlikely that transient formation of an S-O bond occurs in the course of the reaction.

5. Chemistry of oxo transfer reactions

As discussed above, in most prokaryotic enzymes

the oxygen atom of the transferred group originates from water. The chemical challenge in these reactions is the fact that both reactants, i.e. water (or an OH group) and the substrate are nucleophiles which would not react readily with each other. Obviously a polarization change ('Umpolung') of one of the reactants is required.

One possibility for such an 'Umpolung' is that the molybdenum coordinates a water molecule or a hydroxyl ion and delivers it to the substrate as a formal ^{+}OH. Alternatively, 'Umpolung' of the substrate could occur by oxidation (e.g. hydroquinone → quinone) which could then react with water either directly or by mediation of the molybdenum cofactor.

In the case of xanthine oxidase which is one of the most thoroughly studied molybdenum-containing enzymes (see Section 2 and Fig. 3), about half a dozen mechanisms have been proposed [1,5,8,9,27]. In some of these the molybdenum-bound H_2O (or OH group) is transferred as a nucleophile; in others the oxygen is transferred from an Mo = O species as an electrophile and, again in others, first a molybdenum-carbon bond is formed with the substrate, followed by addition of molybdenum-coordinated water. Experimental details in favor of or against these mechanisms have been discussed in recent reviews [1,28].

While the exact role of molybdenum in enzymic hydroxylations is a matter of debate, there are some molybdenum-containing enzymes that catalyze redox reactions without hydroxyl or oxo transfer. Simon and coworkers [29–31] described a (2*R*)-hydroxycarboxylate viologen oxidoreductase containing molybdopterin mononucleotide and iron sulfur clusters but no other cofactors. The enzyme reduces a large number of 2-oxo carboxylates to the corresponding (2*R*)-hydroxy acids. More recently, Khangulov et al. [24] showed that a selenium-containing formate dehydrogenase H from *E. coli* converts formate to carbon dioxide without hydroxyl transfer (see Section 4). The role of the molybdenum cofactor in these reactions is unknown, but it is obvious that molybdenum-containing enzymes can mediate redox reactions similar to those catalyzed by NAD- or FAD-dependent enzymes.

6. Pyrogallol-phloroglucinol transhydroxylase of *Pelobacter acidigallici*

Pyrogallol-phloroglucinol transhydroxylase was studied in detail with *Pelobacter acidigallici*. This strictly anaerobic bacterium ferments gallic acid, pyrogallol, phloroglucinol, and phloroglucinol carboxylic acid to three molecules of acetate (plus CO_2). The carboxylates are first decarboxylated, and pyrogallol is converted into phloroglucinol which undergoes reductive dearomatization [32,33] and subsequent hydrolytic cleavage to 3-hydroxy-5 oxohexanoate which is oxidized and thiolytically cleaved to three acetyl CoA molecules [34].

The transhydroxylase reaction converting pyrogallol to phloroglucinol represents a chemical challenge. It was shown that 1,2,3,5-tetrahydroxybenzene was required in cell-free extracts at stoichiometric amounts to make the reaction run. The enzyme activity was maximal (and equivalent to physiological reaction rates) in the presence of 1 mM tetrahydroxybenzene. Stimulation by DMSO required far higher additions (up to 1 M) and was obviously far less specific [33]. Combinations of various reactants demonstrated that this enzyme activity could transfer hydroxyl groups also from other hydroxybenzenes, and transfer them to other phenols; from these comparisons, a reaction mechanism was suggested which explained the reaction as a hydroxyl transfer between two phenolic substrates, e.g. pyrogallol and 1,2,3,5-tetrahydroxybenzene, to form phloroglucinol and a new molecule of tetrahydroxybenzene which could react again as a co-substrate with a new pyrogallol molecule, according to Fig. 6. This is an ingenious solution to the problem but is not unique in enzyme chemistry: also the enzymes phosphoglucomutase and phosphoglycerate mutase take advantage of their co-catalysts, i.e. glucose-1,6-bisphosphate and glycerate-2,3-bisphosphate, respectively. It was shown recently by an incubation experiment with $^{18}OH_2$ that there is no uptake of oxygen from water in the transhydroxylase reaction, and that the hydroxyl groups are transferred only between the phenolic substrates (Reichenbecher and Schink, manuscript submitted).

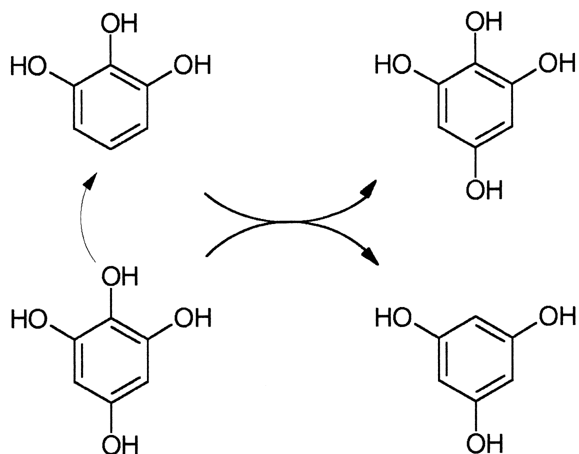


Fig. 6. Proposed role of 1,2,3,5-tetrahydroxybenzene as co-catalyst in the pyrogallol-phloroglucinol transhydroxylase reaction.

From a look at the reaction scheme, it is obvious that a hydroxyl transfer from the tetrahydroxybenzene to pyrogallol cannot occur directly. One of us (J.R.) suggested a cyclic catalytic mechanism as depicted in Fig. 7. Here, as a first step an oxidation of pyrogallol to the corresponding *ortho*-quinone is postulated, the latter now being subject to a nucleophilic attack by an OH-group of tetrahydroxybenzene. Going through chemically plausible intermediates and reactions, the covalent adduct between substrate and co-catalyst can be cleaved to form the product phloroglucinol and the quinone form of tetrahydroxybenzene. Reduction of the latter with simultaneous oxidation of a molecule pyrogallol would close the catalytic cycle.

To examine this hypothetical mechanism we synthesized the two hexahydroxy diphenylethers **1** and **2** (Fig. 8). Although these were previously synthesized

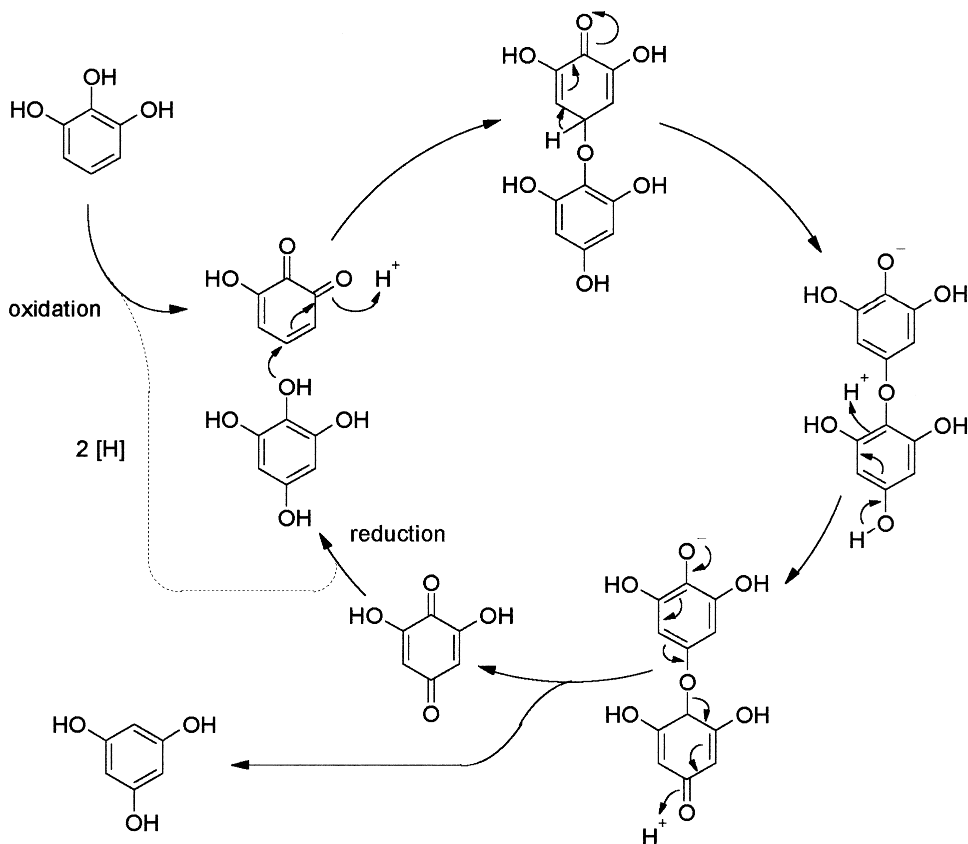


Fig. 7. Putative mechanism for the pyrogallol-phloroglucinol transhydroxylase reaction through a diphenylether intermediate.

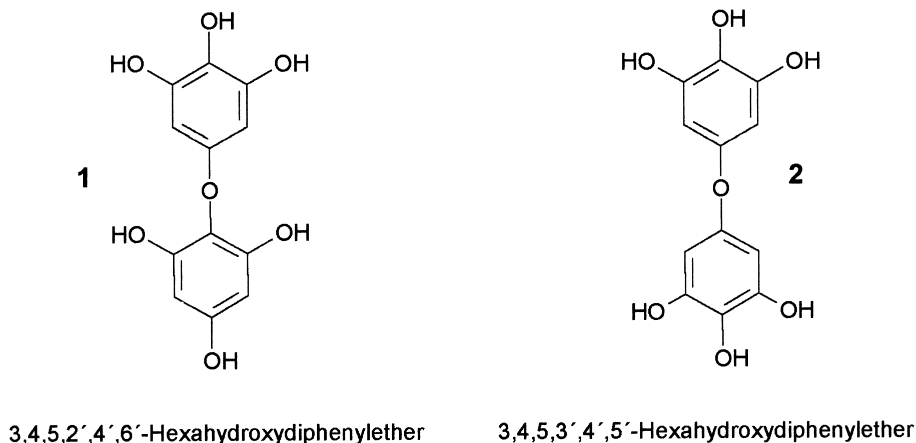


Fig. 8. Structures of the synthesized hexahydroxydiphenylethers.

in a protected form [35], the unprotected compounds are too sensitive to oxygen and could not be obtained in pure form with the techniques existing in those days. In our hands, both hexahydroxy diphenylethers were successfully purified by HPLC and the pure compounds were characterized by ^{1}H- and ^{13}C-NMR and mass spectroscopy (U. Bartlewski-Hof and J. Rétey, to be published).

Pure transhydroxylase in the absence of the co-catalyst tetrahydroxybenzene converted 0.5 mM pyrogallol in the presence of 1 mM hexahydroxy diphenylether during 50 min incubation into three new compounds (ca. 1% of the pyrogallol), one of which was identified by its retention time and UV spectrum as phloroglucinol (U. Bartlewski-Hof, J. Rétey, unpublished). This and another compound with longer retention time were formed only in the presence of transhydroxylase, while the third one is formed non-enzymatically and is probably an oxidation product of the asymmetrical hexahydroxy diphenylether **1**. The latter may have undergone slow enzyme-catalyzed cleavage to phloroglucinol and the quinone form of tetrahydroxybenzene (Fig. 8). Three explanations can be offered for the sluggish reaction: (i) the hexahydroxy diphenylether **1** is not an intermediate of the catalytic cycle; (ii) there is a barrier for its transfer to the active site; or (iii) the enzyme is blocked in the oxidized state. In another experiment, the reaction was conducted in the presence of dithiothreitol as a reducing agent (U. Bartlewski-Hof, J. Rétey, unpublished). In the first 3 h, conversion of pyrogallol into phloroglucinol was very slow, but overnight (18 h) the conversion was complete. Moreover, a small but significant amount of tetrahydroxybenzene was formed, with concomitant decrease of the concentration of compound **1**. Obviously, the lag period was overcome once enough co-catalyst was formed. A similar result was obtained with the symmetric hexahydroxy diphenylether **2** in the presence of glutathione as reducing agent. These results show that the diphenylethers **1** and **2** do interact with transhydroxylase, but their slow conversion renders a possible function as intermediates questionable.

More recently it was found that transhydroxylase contains a molybdopterin dinucleotide cofactor and FeS clusters [36,37], and belongs to the DMSO reductase family [38]. The reaction mechanism above (Fig. 7) is in conflict with the widely accepted role of molybdenum-containing enzymes according to which the molybdenum center is the acceptor and donor of hydroxyl or oxo groups. Therefore, an alternative mechanism implying such a role for the molybdenum cofactor is depicted in Fig. 9. According to this mechanism, an electrophilic substitution by the Mo-oxo group occurs at position 5 of pyrogallol. The following steps are again chemically plausible which does not mean that they occur on the enzyme. Though tetrahydroxybenzene is an intermediate it is not obvious why it is needed to start the reaction cycle.

One could also suggest a further mechanism in which the pyrogallol substrate undergoes 'Umpo-

lung' by oxidation to the *ortho*-quinone, and thus may be attacked by the nucleophilic OH group coordinated to the molybdenum center (Fig. 10). This leads to tetrahydroxybenzene as intermediate which may turn 180° at the active site and react with the molybdenum center in a reverse way as described in the first half of the cycle. One explanation for the need of a relatively high concentration of the co-catalyst may be that its turning is only possible by a dissociation/reassociation process. After release from the enzyme it may compete with pyrogallol for the active site. Although a direct involvement of the molybdenum center in the transhydroxylation is likely, analogous to the xanthine dehydrogenase reaction, further experiments are required to establish either one of the reaction mechanisms suggested here.

Nevertheless, pyrogallol-phloroglucinol transhydroxylase is unique because it does not catalyze a net hydroxylation of phenols, as do the hydroxylases discussed above (Section 2). Furthermore, the question is still open as to how the pool of tetrahydroxybenzene that is required as co-catalyst to enable the reaction to run (Fig. 6) is generated in the growing cell. Experiments with DMSO, other *S*-oxides, *N*-oxides, and also molecular oxygen have provided evidence that they all can act as primary hydroxyl donors to initiate the transhydroxylation of pyrogallol to phloroglucinol, probably by formation of a catalytic amount of tetrahydroxybenzene (Reichenbecher and Schink, unpublished results). However, since neither one of these oxidized precursors is available to the cells growing under strictly anoxic conditions these experiments do not offer a convincing answer to this open question.

Other oxygen-independent hydroxylations have been discovered recently that are involved in anaerobic degradation of aromatic compounds by nitrate-reducing bacteria, e.g., the hydroxylation of resorci-

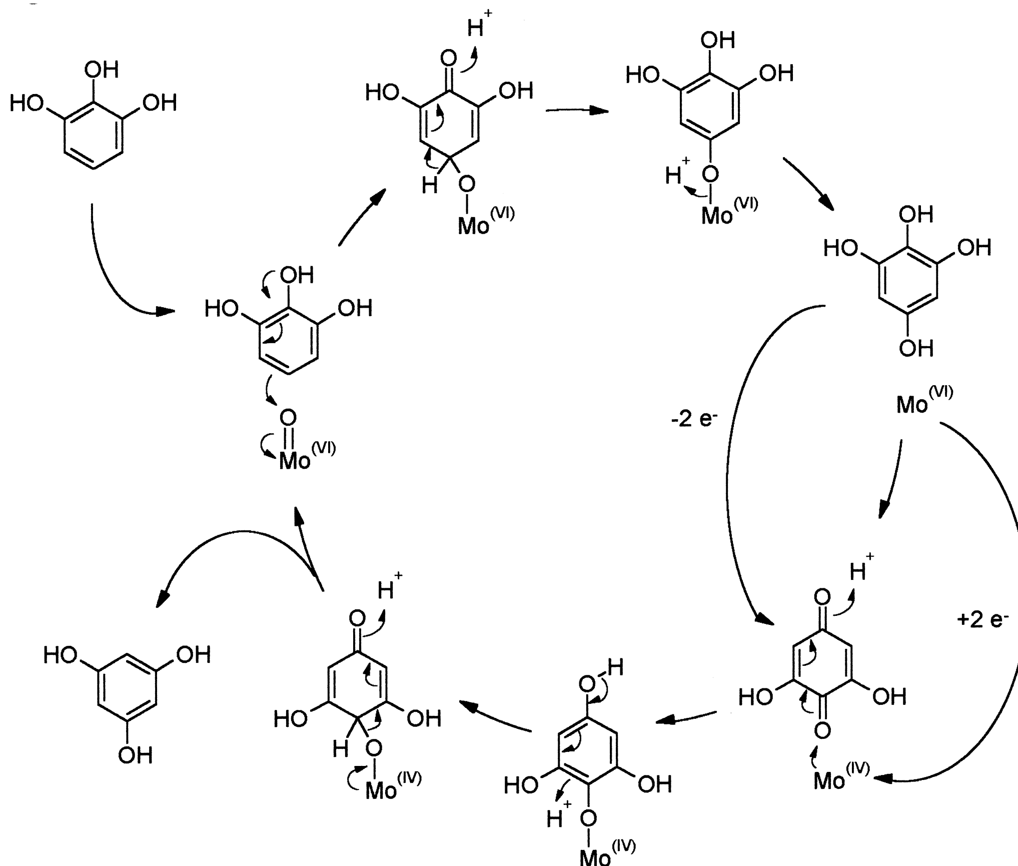


Fig. 9. Putative mechanism for the transhydroxylase reaction via 'Umpolung' of the hydroxyl group by the molybdenum cofactor.

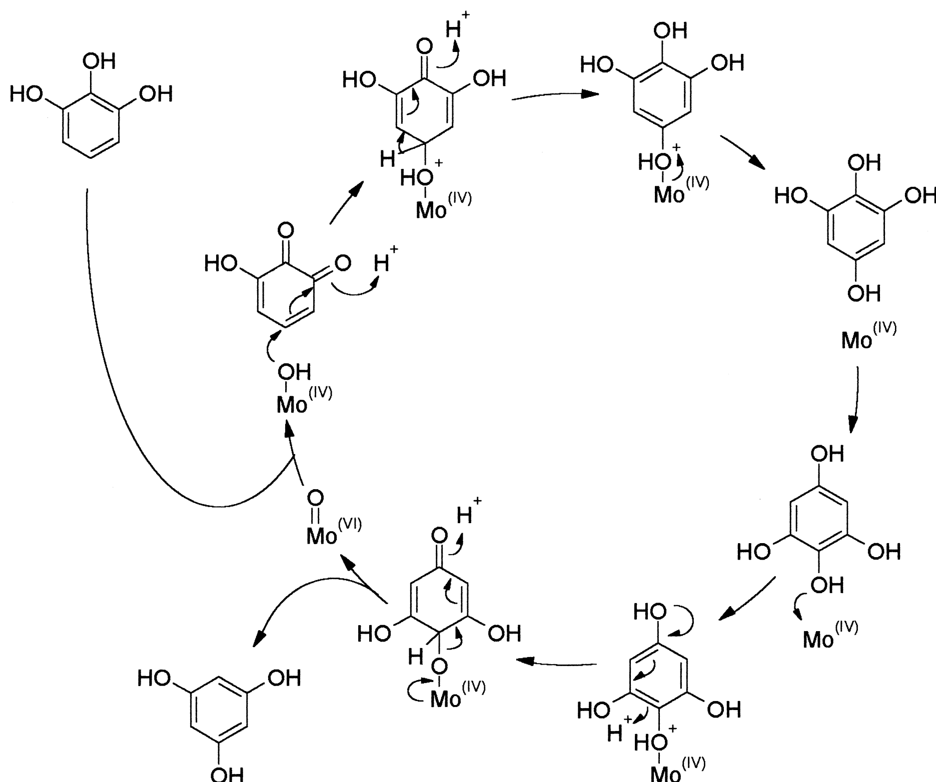


Fig. 10. Putative mechanism for the transhydroxylase reaction via 'Umpolung' of the substrate by oxidation to an *ortho*-quinone and nucleophilic hydroxylation by a molybdenum-coordinated OH group.

nol [39] or of α-resorcylic acid [40] to hydroxyhydroquinone. These reactions may be catalyzed by molybdo enzymes, but since these enzymes have not yet been purified a possible involvement of molybdenum remains a matter of speculation.

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