A crystallographic view of the molybdenum cofactor †

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The molybdenum cofactor (Moco) has been found to be associated with a diverse set of redox enzymes and contains a mononuclear molybdenum or tungsten ion co-ordinated by the dithiolene sulfurs of one or two molybdopterin {a pterin [2-amino-4(1*H*)-pteridinone] derivative} ligands. The remaining co-ordination sites on the metal are occupied by non-protein oxygen or sulfur species and, occasionally, amino acid side chains. The molybdopterin ligand can exhibit oxidation-state-dependent changes in structure and metal co-ordination, and may also interact with other redox groups in the enzyme. These observations suggest that the molybdopterin may participate in the various electron-transfer reactions associated with the catalytic mechanism of Moco containing enzymes.

Molybdenum and tungsten are found to be associated with a diverse range of redox active enzymes that catalyze basic reactions in the metabolism of nitrogen, sulfur and carbon.[1] With the exception of nitrogenases that usually contain an iron–molybdenum–sulfur cluster, molybdenum and tungsten are incorporated into proteins as the molybdenum cofactor, or Moco, that contains a mononuclear Mo (or W) atom co-ordinated to the sulfur atoms of a pterin [2-amino-4(1*H*)-pteridinone] derivative named molybdopterin. The past two years have witnessed an explosion in the crystallographic characterization of Moco containing enzymes, beginning with the structure of the tungsten containing enzyme aldehyde ferredoxin oxidoreductase (AOR) from *Pyrococcus furiosus*.[2] Subsequently, the structures of three enzymes with the molybdenum containing form of Moco have been published, aldehyde oxidoreductase (or Mop for molybdenum protein) from *Desulfovibrio gigas*;[3,4] dimethyl sulfoxide (dmso) reductase from *Rhodobacter sphaeroides*,[5] followed by the structures of the homologous protein from *Rhodobacter capsulatus*;[6,7] and formate dehydrogenase H (FDH) from *Escherichia coli*.[8] In addition, the structures of the molybdoenzyme sulfite oxidase (SO) from chicken liver[9] and the tungstoenzyme formaldehyde ferredoxin oxidoreductase (FOR) from *Pyrococcus furiosus*[10] are nearing completion. Rather than providing a comprehensive survey of Moco enzymes, this Perspective will emphasize structural aspects of Moco, highlighting the AOR and dmso reductase systems. More detailed discussions of the biochemistry, spectroscopy and mechanisms of Moco enzymes may be found in several excellent recent reviews.[11–20]

Structure of the Mo Cofactor

Molybdenum cofactor containing enzymes are identified by the presence of two components: (*a*) mononuclear Mo or W ions and (*b*) molybdopterin. The Mo or W ion in Moco is found co-ordinated to three types of ligands: sulfur atoms provided by the molybdopterin; non-protein oxygen or sulfur species, such as oxo, water or sulfido; and (optionally) amino acid side chains. These interactions are described in turn.

The current model for the structure of molybdopterin is illustrated in Fig. 1. This model is based on the original proposal for the molybdopterin structure that was established through the efforts of Rajagopalan and co-workers.[12,14] Rajagopalan pro-
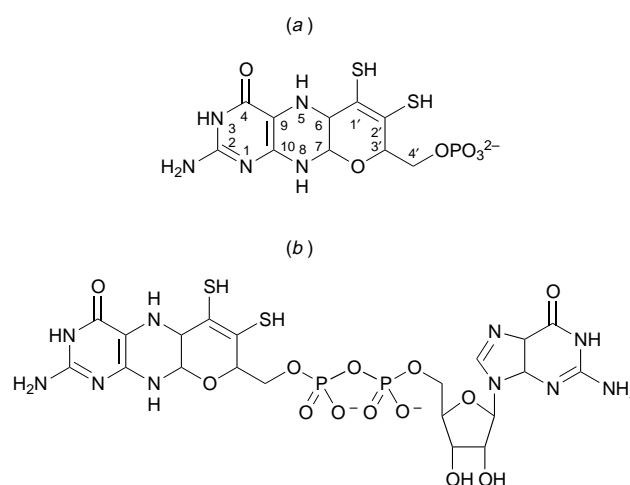
Fig. 1 (*a*) Structure of molybdopterin, as observed in *P. furiosus* AOR and other Moco containing enzymes. The atom numbering scheme of the molybdopterin is indicated. (*b*) Structure of the pterin guanosine dinucleotide cofactor present in *R. sphaeroides* dmso reductase

posed that Moco contains a bicyclic pterin derivative, termed molybdopterin, with the pterin ring substituted at position 6 by a phosphorylated dihydroxybutyl side chain containing a *cis*-dithiolene group. The sulfur atoms of the dithiolene group were proposed to co-ordinate to Mo, with a stoichiometry of one molybdopterin per Mo. In bacteria, additional variability of the Moco is achieved by conjugation of one of the nucleotides guanosine- (GMP) (Fig. 1), adenosine- (AMP), inosine- (IMP) or cytidine-5'-monophosphate (CMP) to the phosphate group of the molybdopterin. The AOR crystal structure[2] established the general validity of this model, but with the unanticipated observation that the molybdopterin is tricyclic. The third ring, a pyran, is formed by the attack of the 3' hydroxyl group from the dihydroxybutyl side chain on the C[7] atom of the pterin. Pterins with this tricyclic structure had actually been synthesized and characterized prior to the identification of this species in the AOR structure.[21,22] The tricyclic form of the pterin has been subsequently observed in all available crystal structures of Moco containing enzymes.[2–10] As demonstrated by these structures, the molybdopterin system is distinctly non-planar (Fig. 2) in both the central pyrazine and the pyran rings. In particular, the pyran ring adopts a half-chair conformation that deviates significantly from the plane of the pterin system. In the enzyme bound molybdopterins studied so far, the best plane defined by the pyran ring is tilted ≈40° from the plane of the pterin ring.

Table 1 Distribution of hydrogen-bond donors and acceptors in the pterins of Mo cofactor containing enzymes *

Atom	Consensus	AOR			dmso reductase		FDH	
		Pterin 1	Pterin 2	Mop	P Pterin	Q Pterin	Pterin 1	Pterin 2
N^1	A	A	—	A	A	—	A	A/D
N^2	D	D	D	D	D	D	D	A/D
N^3	D	D	D	—	D	D	D	D
O^4	A	A	Cation	A	A	A	A	A
N^5	D	—	—	A/D	D	?	D	A
N^8	D	D	D	—	D	D	D	D

* Abbreviations are: dmso reductase (*Rhodobacter sphaeroides* dmso reductase), FDH (*Escherichia coli* formate dehydrogenase H), Mop (*Desulfovibrio gigas* aldehyde oxidoreductase) and AOR (*Pyrococcus furiosus* aldehyde oxidoreductase), A for hydrogen-bond acceptor, D for hydrogen-bond donor, '—' if this atom is not involved in any hydrogen-bonded interaction and '?' if this atom could be either the donor or the acceptor in the hydrogen bond. Atom numbers for the pterin are as defined in Fig. 1(*a*). Contacts in AOR are only given if they occur in both monomers of the dimer. Pterin 2 of FDH and pterin 1 of AOR are likely involved in electron transfer to their respective 4Fe-4S clusters.
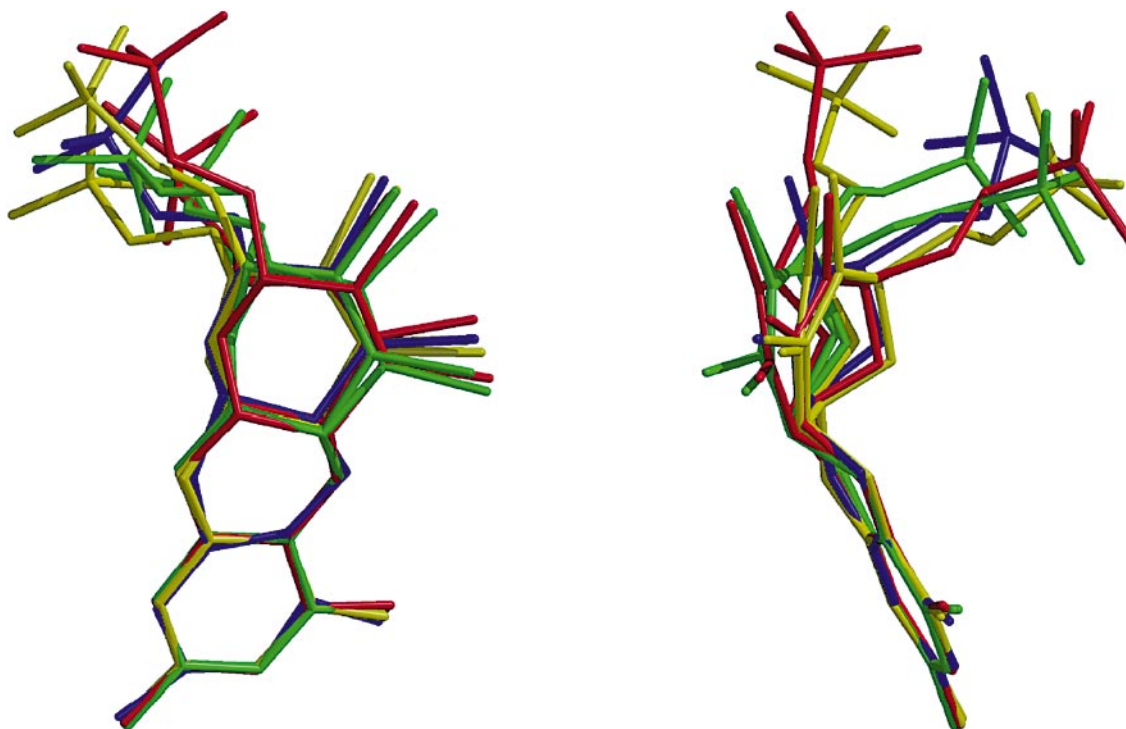

Fig. 2 Perpendicular views of a superposition of the pterin cofactors observed in AOR,[2] Mop,[3] oxidized *R. sphaeroides* dmso reductase[5] and reduced FDH.[8] All figures of molecular structures have been prepared with MOLSCRIPT,[23] utilizing Brookhaven Protein Data Bank coordinate sets 1AOR for AOR; 1ALO for Mop; 1CXS and 1CXT for oxidized and reduced *R. sphaeroides* dmso reductase, respectively; and 1FDO and 1FDI for oxidized and reduced FDH, respectively

However, this relationship between rings is not precisely fixed, as a least-squares superposition of the pterins reveals conformational flexibility in the way the pyran ring is tilted out of the plane of the conjugated part of the pterin (Fig. 2). Additional conformational flexibility is introduced through the torsion angles of the phosphorylated hydroxymethyl side chain that leads to a wide distribution of positions for the phosphate group with respect to the pterin system.

As implied by the stereochemistry at positions 6 and 7, the molybdopterin is structurally equivalent to the fully reduced tetrahydropterin oxidation state. Additional support for this conclusion arises from the observation that both N^5 and N^8 are likely to be protonated, since they are generally used as hydrogen-bond donors in the crystal structures analyzed so far (Table 1). This behavior argues against the existence of dihydropterin states such as 5,6-dihydropterin and 7,8-dihydropterin, as well as the various quininoid forms of the dihydropterin, since they are not compatible with the observed pattern of hydrogen-bond donors and acceptors observed in the known crystal structures. The tetrahydropterin state for the tricyclic structure would likely be equivalent to a dihydropterin (possibly the 5,6-dihydropterin or the 5,8-dihydropterin) in the ring-opened form of the bicyclic pterin.

The crystallographic analyses have confirmed that the Mo/W ion is co-ordinated by the dithiolene sulfurs of the molybdopterin ligand, as anticipated,[12,14] although alternative co-ordination modes utilizing the pterin ring system had been considered.[24] Typical values for the Mo/W–dithiolene sulfur bond distance are ≈2.4 Å (although see discussion below). The Mo/W may be co-ordinated by the dithiolene groups of either one or two molybdopterins; indeed, an unexpected feature of the AOR structure[2] was the presence of two molybdopterins since it had been generally assumed that only one molybdopterin was present in Moco.

In addition to the molybdopterin ligand, the metal center is also co-ordinated by a second type of non-protein group that contains oxygen or sulfur. The identity of these species is sensitive to the pH and metal center oxidation state, and can include one or more oxo, hydroxo, water, sulfido and sulfhydryl groups. Mechanistically, these species are extremely important, as they can be intimately involved in the oxidation–reduction reactions catalyzed by Moco containing enzymes. It can be difficult to

distinguish between these groups in a macromolecular crystallographic analysis, however, since the metal–ligand distances differ by only a few tenths of an Ångstrom, which is typically within experimental error. Consequently, assignment of these ligands should be viewed with the appropriate degree of caution.

Three different types of amino acid side chains have been observed to co-ordinate Mo: serine in dmso reductase,[5] selenocysteine in FDH[8] and cysteine in sulfite oxidase.[25] However, an amino acid side chain is not an obligatory component of the Mo/W co-ordination environment; for example, no amino acid side chains are observed to co-ordinate to the metal in the crystallographically observed structures of AOR and Mop, although it has also been proposed that a glutamic acid residue may transiently co-ordinate to the Mo during the catalytic mechanism of Mop.[3,4]

Families of Mo Cofactor containing Enzymes

Molybdenum cofactor containing enzymes are broadly defined by the presence of mononuclear Mo or W co-ordinated to one or two molybdopterins. On the basis of amino acid sequences and spectroscopic properties, Moco containing enzymes may be further divided into four general families, designated by the name of one of the better characterized members of each family: dmso reductase, sulfite oxidase, xanthine oxidase and aldehyde ferredoxin oxidoreductase. To a first approximation, members of the first three families utilize the molybdenum form of Moco, while the AOR family utilizes the tungsten form of Moco, but exceptions are known.[18] New families will undoubtedly be recognized in the future as more molybdenum and tungsten containing enzymes are isolated and characterized.

Molybdenum cofactor containing enzymes typically catalyze the transfer of an oxygen atom, ultimately derived from or incorporated into water, to or from a substrate in a two-electron redox reaction. (It is worth noting, however, that for certain enzymes, such as formate dehydrogenase, hydride transfer provides a plausible alternative to oxygen transfer.[8]) On the basis of the type of oxygen transfer reaction that is catalyzed, Moco containing enzymes may be divided into two categories[17] that each include two of the families identified from sequence and spectroscopic properties. The first category of enzymes is represented by the xanthine oxidase and aldehyde ferredoxin oxidoreductase families that catalyze oxidative hydroxylation reactions of aldehydes and aromatic heterocyclic compounds as shown in equation (1). Enzymes in this category appear not to
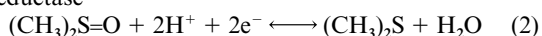
Aldehyde oxidoreductase
$$R\text{–}CHO + H_2O \longleftrightarrow R\text{–}COOH + 2H^+ + 2e^- \quad (1)$$
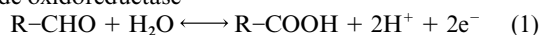
have a permanent Mo/W ligand provided by an amino acid side chain, although significant differences in Moco are evident, including the nature and number of the non-protein O/S ligands, the number of molybdopterins, and the presence or absence of the nucleotide form of the cofactor.

The second reaction category is exemplified by the remaining two families, dmso reductase and sulfite oxidase, that catalyze oxygen atom transfer to or from an available electron lone pair of a substrate [equation (2)]. In contrast to the first category,

dmso reductase
$$(CH_3)_2S\text{=}O + 2H^+ + 2e^- \longleftrightarrow (CH_3)_2S + H_2O \quad (2)$$

enzymes in this group do appear to have a permanent Mo/W ligand provided by an amino acid side chain, although, again, significant differences in Moco are evident, including the nature and number of the non-protein O/S ligands, the number of molybdopterins, and the presence or absence of the nucleotide form of the cofactor.

It is convenient to consider the overall reaction mechanism catalyzed by Moco containing enzymes as consisting of a coupled pair of reductive and oxidative half-reactions. Since these enzymes catalyze a two-electron redox reaction to or from the substrate, it seems plausible that the Mo undergoes an overall two-electron change in oxidation state during the appropriate half-reaction. However, as detailed in the discussion on dmso reductase, the possibility that the molybdopterin ring and/or dithiolene group may also participate in the redox reaction of some Moco enzymes cannot be excluded. The return to the resting state of the enzyme must involve the appropriate addition or removal of electrons from Moco, by means of electron transfer with a second redox center. In many, but not all, Moco containing enzymes, the second center is typically a one-electron redox group such as a heme or iron–sulfur cluster, that is also present in the protein.

To illustrate specific aspects of these more general observations, the structures of the Moco containing enzymes AOR and dmso reductase are described in more detail.

Aldehyde Ferredoxin Oxidoreductase

Aldehyde ferredoxin oxidoreductase from the hyperthermophilic archaeon *P. furiosus* catalyzes the oxidation of aldehydes to carboxylates and may play a role in peptide fermentation in this organism. This enzyme is a dimer of two identical 605 residue (66 kDa $\approx 1.096 \times 10^{-25}$ kg) subunits. Three different types of metal sites are found in the AOR protein dimer, including two copies of the tungsten center, two copies of a 4Fe-4S cluster, and a single metal atom located at the dimer interface. The tungsten cofactor and 4Fe-4S cluster are positioned in close proximity within each subunit (closest distance ≈ 8 Å between metals). The mononuclear tetrahedral metal center is most likely Fe, and is positioned on the dimer two-fold axis ≈ 25 Å from the other metal centers. The 4Fe-4S clusters and tungsten cofactors in different subunits of the dimer are separated by ≈ 50 Å. Each subunit of AOR folds into three domains, with the binding sites for the tungsten cofactor and 4Fe-4S cluster located at the interfaces of these domains. The polypeptide fold of domain 1 exhibits a pseudo-two-fold axis that coincides approximately with the two-fold axis of the tungsten cofactor (see below). A channel, formed at the interface between domains 2 and 3 that straddles this two-fold axis, is likely to provide substrate access to the tungsten center. Although strong sequence similarities are evident among members of the AOR family, differences do exist; for example, although the homologous FOR has a similar active site environment and polypeptide fold, it exhibits a different quaternary structure (tetrameric) and lacks the mononuclear Fe site found at the dimer interface in AOR.[10,26]

The tungsten ion in AOR is co-ordinated by the four dithiolene sulfurs from the two molybdopterins bound to each subunit (Fig. 3) in an arrangement that may be best described as a distorted square pyramid. No protein ligands are co-ordinated to the metal. The bond distances between the tungsten and sulfur atoms average 2.34 Å. The base of the pyramid is defined by pairs of sulfurs separated by ≈ 3.0 Å on each edge, both for sulfurs in the same and different dithiolene groups. The tungsten is positioned ≈ 1 Å above the least-squares plane defined by the four sulfurs. The presence and location of oxo groups co-ordinated to the tungsten has not yet been definitely established crystallographically in AOR, perhaps due to the heterogeneous nature of the tungsten center in as-isolated AOR,[27] or crystallographic problems associated with locating light atoms in the vicinity of heavy metals. However, one oxo group has been recently observed co-ordinated to the W in the FOR crystal structure.[10]

In addition to interactions between the dithiolene sulfurs and tungsten, the two molybdopterin ligands are also linked through their phosphate groups, which co-ordinate axial sites of the same magnesium ion. The two molybdopterin ligands are approximately related by a two-fold rotation about an axis
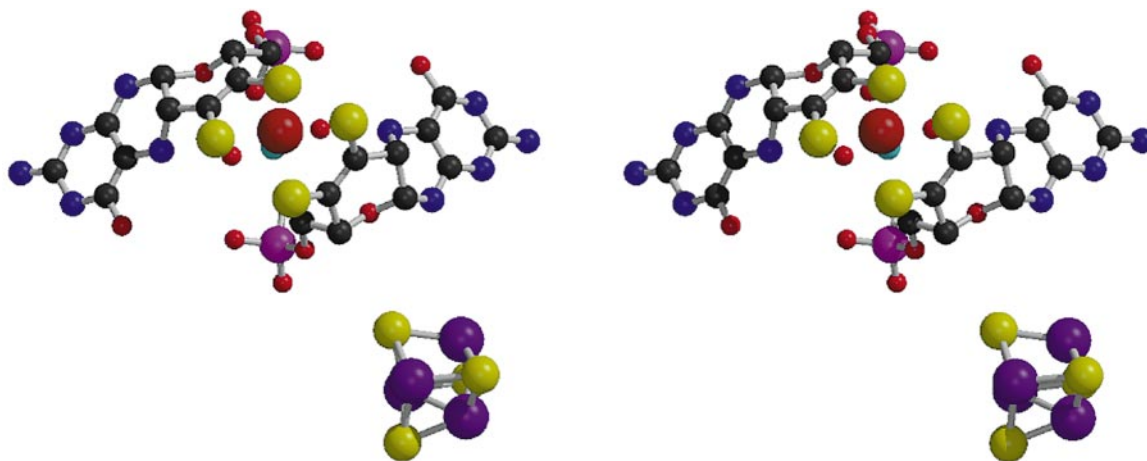
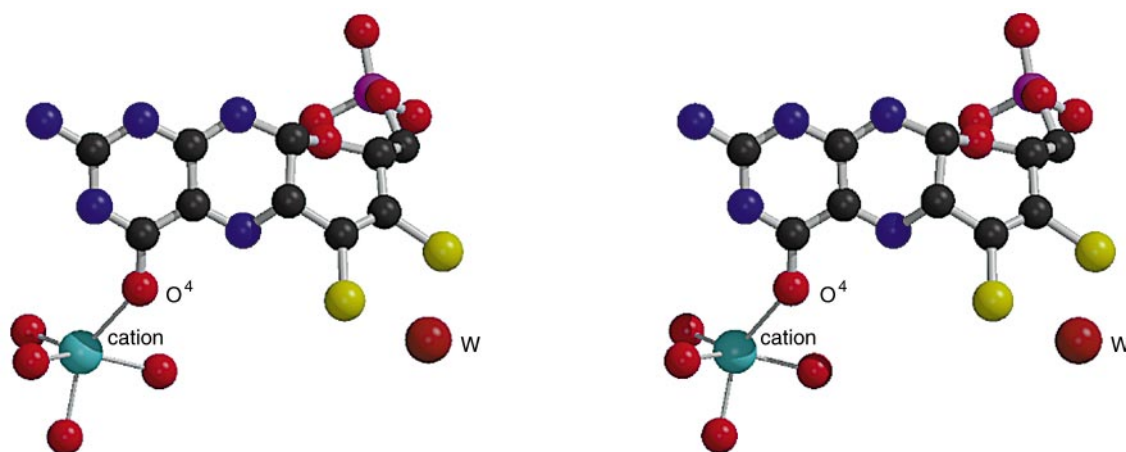**Fig. 3** Stereoview of the AOR Moco, with the tungsten, molybdopterin and 4Fe-4S cluster

Fig. 4 Stereoview of the co-ordination of a cation, tentatively identified as sodium or magnesium, to the O^4 oxygen of one of the molybdopterins in AOR
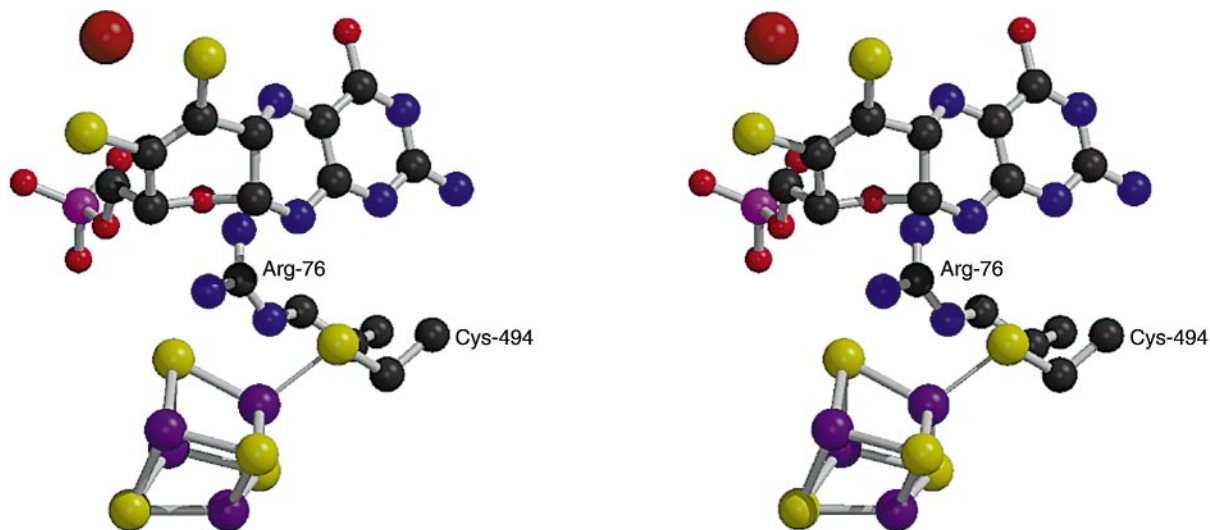

Fig. 5 Stereoview illustrating interactions between a molybdopterin and the 4Fe-4S cluster of AOR, mediated by hydrogen-bonding interactions involving the side chains of Arg-76 and Cys-494

that passes through both the tungsten and magnesium sites. An additional interaction between a group on the molybdopterin and a cation involves the pterin ring oxygen O^4 on one of the two molybdopterins. This oxygen appears to co-ordinate a sodium or magnesium ion in AOR[2] (Fig. 4) and a calcium ion in FOR.[10] Although the identity of these ions has not been conclusively established, these assignments are consistent with the electron density, co-ordination geometry and the distances to the surrounding ligands.

The 4Fe-4S cluster is positioned approximately 10 Å from the

tungsten atom in AOR, and is buried $\approx$6 Å below the van der Waals surface of the protein. This arrangement is consistent with the postulated role of the 4Fe-4S cluster as an intermediary for electron transfer between the tungsten cofactor and ferredoxin, the physiological electron acceptor of AOR. The 4Fe-4S cluster is linked to one of the two molybdopterins of the tungsten cofactor by two distinct sets of interactions (Fig. 5). The molybdopterin closest to the 4Fe-4S cluster is the one that does not exhibit the interaction with the cation described above. The sidechain of Arg-76 (Arg = arginine) bridges the molyb-
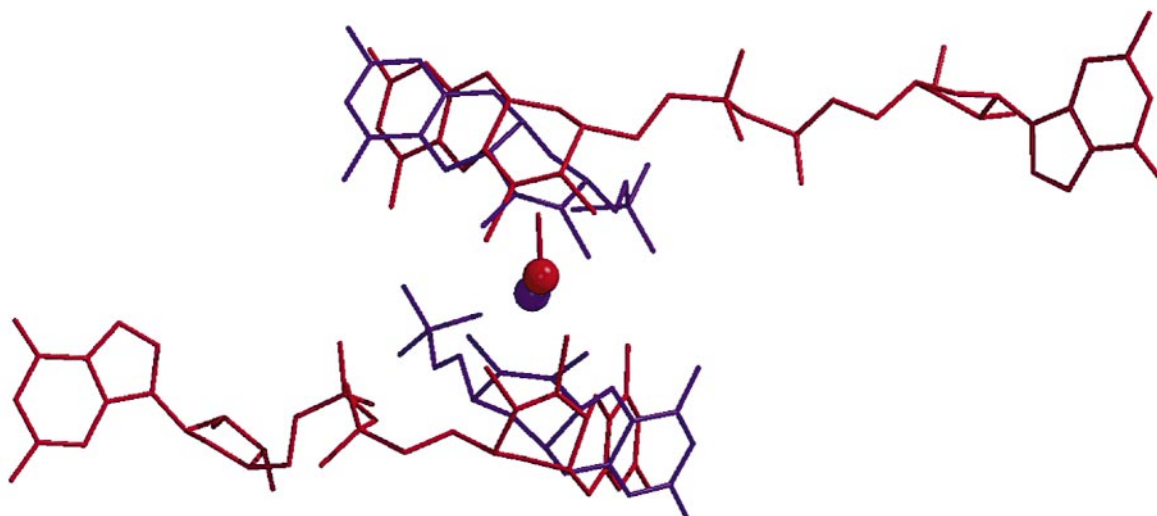
Fig. 6 Superposition of the Moco molybdopterins and metal ions from AOR (blue lines) and dmso reductase (red lines)
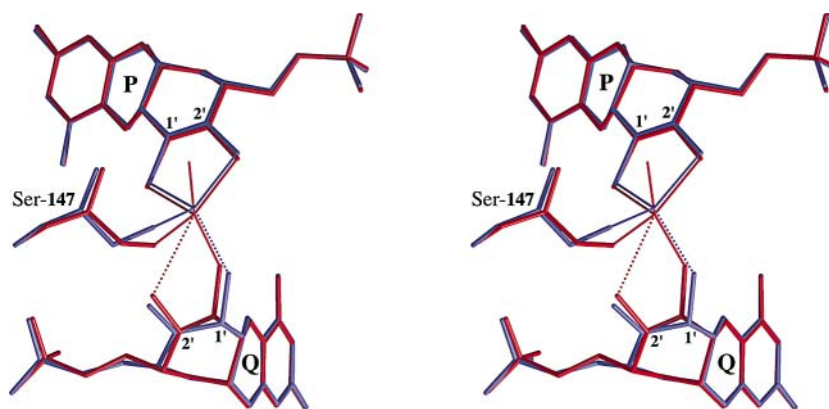

Fig. 7 Stereoview comparing the oxidized (red) and reduced (blue) forms of *R. sphaeroides* dmso reductase. Short co-ordinating interactions to Mo are shown with thin solid lines, whereas longer ones are shown with dotted lines

dopterin and 4Fe-4S cluster by forming hydrogen bonds to an inorganic sulfur in the cluster, and to three sites on the molybdopterin: N^8, the pyran oxygen and a phosphate oxygen. In addition, S_γ of Cys-494 (Cys = cysteine), a 4Fe-4S cluster ligand, is positioned to accept a hydrogen bond from the pterin ring nitrogen N^8. These interactions could provide electron-transfer pathways between the metal and the iron–sulfur cluster.

dmso Reductase

The dmso reductase from *R. sphaeroides* is a water soluble, single subunit protein with 780 residues that contains no cofactor other than Moco. Under anaerobic conditions, this enzyme can function as a terminal reductase that reduces dimethyl sulfoxide to dimethyl sulfide, to provide a more efficient energy metabolism for the organism. The structure of the dmso reductase from *R. sphaeroides* has been crystallographically characterized in both its oxidized and reduced forms at resolutions of 2.2 Å and 2.4 Å resolution, respectively,[5] and more recently the oxidized structure has been refined at 1.4 Å resolution.[28] Subsequently, the crystal structure of the highly homologous dmso reductase from *R. capsulatus* (77% sequence identity) was solved by molecular replacement using *R. sphaeroides* dmso reductase as search model.[6,7] An important recent development has been the structure determination of the homologous selenocysteine containing enzyme formate dehydrogenase H from *E. coli* which, in addition to Moco, also contains a 4Fe-4S cluster.[8]

The polypeptide chain of dmso reductase folds into four domains (designated I, II, III and IV) that form a slightly elongated molecule with overall main chain dimensions of

$75 \times 55 \times 65$ Å. The spatial arrangement of domains I to III creates a large depression on one side of the molecule resembling a funnel, with the active site located at the bottom of the funnel. The active sites of the dmso reductases and *E. coli* formate dehydrogenase were found to contain two copies of the GMP dinucleotide form of molybdopterin that co-ordinate to Mo with an approximate two-fold axis of symmetry passing through the Mo. While there are general similarities in the overall Moco structure between AOR and dmso reductase (Fig. 6), differences are evident, particularly in the details of the metal co-ordination by the dithiolene sulfurs (see below). In dmso reductase, residues interacting with the cofactor are scattered throughout the linear sequence and are located in domains II, III and IV. Domains II and III interact primarily with each of the guanosines and share structural similarity, despite the lack of any detectable sequence similarity. A stretch of highly conserved residues forming a polypeptide loop in domain IV is crucial for binding the two pterin moieties of the cofactor. In addition to the residues interacting with the pterins, the dmso reductase family of Moco containing enzymes is also characterized by a protein ligand binding to the Mo. This ligand may be either a serine, such as Ser-147 in dmso reductase, a cysteine or a selenocysteine as in *E. coli* formate dehydrogenase H.

In the oxidized, Mo^{VI} form of dmso reductase from *Rhodobacter sphaeroides*, the four dithiolene sulfur atoms of the two molybdopterins (arbitrarily designated P and Q) co-ordinate the Mo atom in an asymmetric fashion (Fig. 7). The two sulfur atoms in the P pterin and $S^{1'}$ in the Q pterin are 2.5 Å from the metal, whereas $S^{2'}$ is 3.3 Å away. The sulfur–sulfur distances in the dithiolene groups of the P and Q pterins are 3.2 and 2.6 Å, respectively, which suggests that there is some disulfide bond character to the Q pterin dithiolene. An oxo

group, at 1.7 Å distance, forms an additional ligand to the Mo atom, and the co-ordination sphere is completed by the side chain of Ser-147, with a Mo−O distance of 1.75 Å. Thus, the Mo atom is fully co-ordinated by five ligands and weakly co-ordinated by a sixth ligand arranged in distorted trigonal-bipyramid geometry. The ligands are positioned such that the sulfur atoms of the P pterin, the Ser-147 O_γ and the Mo define an equatorial plane, with the oxo group and $S^{1'}$ of the Q pterin positioned as apical ligands on either side of this plane. Consequently, the dithiolene sulfurs of the Q pterin are positioned approximately *trans* to the oxo group. This suggests that the asymmetric co-ordination of the Mo by the P and Q pterins may reflect the influence of the 'trans' effect,[29,30] in which an oxo group weakens the co-ordination of a ligand on the opposite side of the metal.

Significant changes are observed at the active site of dmso reductase upon reduction (Fig. 7).[5] These changes include the expected loss of the oxo ligand and a different co-ordination of the Mo atom by the pterin sulfur atoms. Only three sulfur ligands remain co-ordinated to the Mo; the two sulfurs of the P pterin at 2.5–2.6 Å from the Mo atom and one sulfur ($S^{1'}$) of the Q pterin at 3.0 Å, in addition to O_γ of Ser-147 at 1.7 Å. $S^{2'}$ in the Q pterin has shifted to a position 3.8 Å from the Mo atom. Reduction of dmso reductase is accompanied by an increase in the distance between $S^{1'}$ and $S^{2'}$ of the Q pterin from 2.6 to 2.9 Å, which suggests that the disulfide bond character of this interaction is diminished. For comparison, the sulfur–sulfur distance in the P pterin remains essentially unchanged (3.2 Å in the oxidized and 3.1 Å in the reduced form).

In two independent crystal structures of the oxidized form of *R. capsulatus* dmso reductase, different co-ordination environments of the Mo have been observed; in one structure,[6] two sulfur atoms from the P pterin, two oxo groups and the Ser side chain were found co-ordinated to the Mo, while in the other structure,[7] all four dithiolene sulfurs co-ordinate to Mo equivalently, in addition to two oxo groups and the Ser side chain. The 1.35 Å resolution structure of the oxidized form of the *R. sphaeroides* dmso reductase, currently under refinement,[28] suggests yet another Mo co-ordination environment that includes two sulfur atoms from the P pterin, two oxygen ligands [an oxo group and a second oxygen at a somewhat longer distance (1.9 Å)] and the Ser side chain. In addition, the Mo appears to be co-ordinated by a possibly oxygenated dithiolene sulfur from the Q pterin, along with the possible oxidation to a sulfenic acid of the Cys-219 side chain, some 21 Å from the Mo. The latter observations of sulfur oxidation are particularly intriguing given the ability of the enzyme to oxidize dimethyl sulfide to dmso. Electron paramagnetic resonance spectroscopy has suggested the existence of multiple states of the molybdenum center of *R. capsulatus* dmso reductase.[31] An important objective for future research is to establish the relationships between these multiple forms of dmso reductase that have been observed crystallographically and spectroscopically, and to determine the mechanistic significance, if any, of these states.

Conclusion

Although Moco containing enzymes share common features of a mononuclear Mo/W ion co-ordinated by the dithiolene sulfurs of a non-planar, tricyclic molybdopterin ligand, it is important to recognize that Moco containing enzymes can also exhibit significant differences. In addition to the diversity of polypeptide folds (four, at present) that have been observed to associate with Moco, differences are also evident between enzymes in the number of molybdopterin ligands, the presence of nucleotides covalently linked to the molybdopterin, the presence and nature of co-ordinating protein ligands, the presence and nature of non-protein oxygen and sulfur ligands, in addition to the overall polypeptide fold. This variability emphasizes that the molybdenum cofactor is not a single, well defined entity, but rather the term represents a broad class of cofactors that share common elements combined in diverse ways to achieve a variety of enzymatic properties. Our challenge now is to mesh the increasingly detailed understanding of the structural properties of Moco containing enzymes with the elegant and extensive spectroscopic and biochemical studies to establish the molecular mechanism of these fascinating enzymes.

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