Tungstoenzymes

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Contents

I. Introduction

Tungsten (W, atomic number 74) and molybdenum (Mo, 42) are chemically analogous elements, and both are relatively scarce in natural environments.[1−3] The notion that either of them might have a significant biological role is perhaps surprising when one considers that, with the exception of iodine (53), life forms on this planet typically thrive by utilizing elements with atomic numbers below 35.[4,5] Yet, from a biological perspective, W and Mo provide a fascinating study in contrasts. The essential role of Mo in various fundamental biological conversions carried out by both microorganisms and higher (larger) life forms has been known for many decades.[6−8] Molybdoenzymes are ubiquitous in nature and play intimate roles in the global cycles of nitrogen, carbon, and sulfur, with nitrogenase, nitrate reductase, formate dehydrogenase, and xanthine oxidase being prime and extensively studied examples.[9−17] On the other hand, W has traditionally been regarded as an antagonist of the biological functions of Mo. Because of the great similarities in the properties of the two elements, it was reasoned that insight into the catalytic role of Mo in various enzymes might be provided by replacing Mo with W.[18−20] Various organisms, including plants and rats, were therefore grown with or exposed to W, but they produced either inactive metal-free molybdoenzymes or W-substituted enzymes with little or no activity.[21−26] Clearly, the chemical properties of W and Mo are sufficiently different that biology can distinguish between them, either at the levels of their uptake and/or incorporation into enzymes or in the properties of the enzymes themselves, which function with Mo but not with W.

That W might have a positive biological role has a very short history compared to that of Mo. The first indications were in the 1970s when it was reported that tungstate stimulated the growth of certain acetate- and methane-producing microorganisms,[27−34] but it was not until 1983 that the first naturally occurring tungstoenzyme was purified, in this case from one of the acetogens.[35] However, these seminal studies did not generate a great deal of interest. By 1990, the stimulatory growth effect of tungstate had been reported with only one other group of microorganisms, the hyperthermophilic archaea, which thrive near 100 °C,[36] and only two more tungstoenzymes had been purified, a second from an acetogen and one from a hyperthermophile.[37,38] Since then, and particularly in just the last year, rather dramatic progress has been made in the study of tungstoenzymes. At present, more than a dozen tungstoenzymes have been purified (Table 1), the genes for three of them have been cloned and sequenced,[39,40] and the crystal structure of one of them has been determined to 2.3-Å resolution.[41] Notably, the structures of two molybdoenzymes have subsequently been determined.[42,43] Thus, for the first time, direct comparisons at the atomic level between these two classes of enzyme are now possible. Moreover, in addition to acetogens, methanogens, and hyperthermophiles, tungstoenzymes have been purified from acetylene-utilizing[44] and sulfate-reducing[45] anaerobes, and although not purified, a tungstoenzyme appears to be present in some aerobic, methylotrophic organisms.[46,47]

A role for tungsten in at least some biological systems has, therefore, been firmly established. Yet, although the complete spectrum of organisms that might utilize this element has yet to be fully explored, the fact remains that for almost all of the known tungstoenzymes there is an analogous molybdoenzyme that is present within the same organism or in

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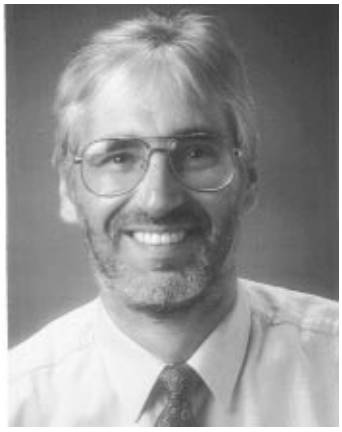

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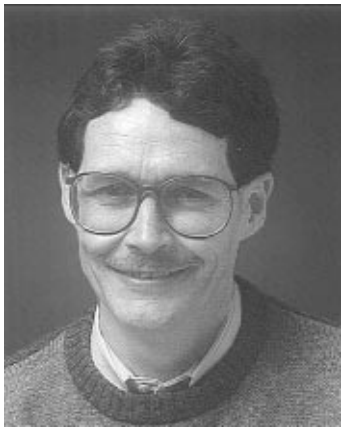
Douglas C. Rees was born in New Haven, CT, and returned there for college, graduating from Yale with a B.S. in Molecular Biophysics and Biochemistry. In between, he grew up in Lexington, KY, where was independently introduced to both biochemistry and nitrogen fixation. For his Ph.D. work in Biophysics at Harvard University under "the Colonel" (W. N. Lipscomb), he worked on the crystallographic analysis of complexes of the protease carboxypeptidase A. As a postdoc with J. B. Howard at the University of Minnesota, he began working on the nitrogenase system, which has been a continuing research interest. He was appointed to the faculty at UCLA from 1982 to 1989, and is currently a Professor of Chemistry at the California Institute of Technology. His research interests continue to focus primarily on structure determinations and functional analyses of metalloproteins and membrane proteins (Photo: Bob Paz, Caltech.)

a very closely related species.[48] In other words, of the vast array of life forms that utilize Mo, a very small subset are also able to use W. In fact, the only exceptions known at present are the hyperthermophilic archaea. These are the only organisms whose growth appears to be obligately dependent upon W and are incapable of utilizing Mo.[48,49]

The aim of this review is, therefore, not only to summarize what is known about tungstoenzymes but also to try and rationalize the dichotomy between the



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biological relevance of W and Mo. We begin with a description of the biochemical properties of tungstoenzymes and then consider their spectroscopic and structural properties. These aspects are discussed in light of the recent advances on the structure of molybdoenzymes. The abundance and chemical forms of W and Mo in natural environments, and the effects of W on microbial growth, were the subject of a recent review.[48] Only the salient features are summarized here, and the reader is referred to this and earlier, albeit brief, reviews of tungstoenzymes[10,31,50,51] for additional information and relevant references.

II. Biochemical Properties of Tungstoenzymes

A. AOR Family

The molecular properties of tungstoenzymes are summarized in Table 1. They are divided into three categories. The majority fall within the so-called AOR-type, with aldehyde ferredoxin oxidoreductase (AOR) from the hyperthermophile, *Pyrococcus furiosus* (Pf; maximum growth temperature, T_{max}, 105 °C) being the best studied example.[39,41,52] Its gene sequence has been determined,[39] and crystallographic analysis[41] revealed that it is a homodimeric enzyme wherein each subunit (67 kDa) contains a [4Fe-4S] cluster and a single W atom. The two subunits are bridged by a monomeric Fe site, coordinated by the side chains of a histidine and a glutamate residue from each subunit. Hence Pf AOR holoenzyme contains two W and nine Fe atoms. The crystallographic study,[41] which is discussed in more detail in section III, also provided the first structure for the pterin cofactor, the organic entity that binds each W atom. A prior study[53] had shown that Pf AOR contains the so-called mononucleotide from of molydopterin, where the latter is the pterin cofactor that

Table 1. Molecular Properties of Tungstoenzymes

organism and enzyme[a]	holoenzyme mass (kDa)	subunits	subunit mass (kDa)	W content[b]	pterin cofactor[c]	FeS or cluster content[d]
			I. AOR Type			
P. furiosus AOR[39,41,52]	136	α_2	67	2 W	non-nuc	2 [Fe_4S_4] + 1 Fe
Thermococcus sp. ES-1 AOR[55]	135	α_2	67	2 W	non-nuc	2 [Fe_4S_4] + 1 Fe?
Pyrococcus sp. ES-4 AOR[56]	135	α_2	67	2 W	non-nuc	2 [Fe_4S_4] + 1 Fe?
P. furiosus FOR[56]	280	α_4	69	4 W	non-nuc	4 [Fe_4S_4]
T. litoralis FOR[56,57]	280	α_4	69	4 W	non-nuc	4 [Fe_4S_4]
P furiosus GAPOR[58]	63	α	63	1 W	non-nuc	~6 Fe
C. thermoaceticum CAR form I[37,67,68,70]	86	$\alpha\beta$	64, 14	1 W	non-nuc	~29 Fe, ~25 S
C. thermoaceticum CAR form II[37,67,68,70]	300	$\alpha_3\beta_3\gamma$	64, 14, 43	3 W	non-nuc	~82 Fe, ~54 S (2 FAD)
C. formicoaceticum CAR[66,69]	134	α_2	67	2 W	non-nuc	~11 Fe, ~16 S
D. gigas ADH[45]	132	α_2	65	2 W	yes	~10 Fe
(*P. vulgaris*[76]	800	α_8	80	1 Mo	yes	~4 Fe)
			II. F(M)DH Type			
C. themoaceticum FDH[35,117]	340	$\alpha_2\beta_2$	96, 76	2 W	yes	2 Se, 20−40 Fe
M. wolfei FMDH II[91−94]	130	$\alpha\beta\gamma$	64, 51, 35	1 W	nuc[e]	2−5 Fe
M. thermoautotrophicum FMDH II[40,86,90]	160	$\alpha\beta\gamma\delta$	65, 53, 31, 15	1 W	nuc[e]	~8 Fe
			III. AH Type			
Pr. acetylenicus AH[44]	73	α	73	1 W	yes	4−5 Fe

[a] The sources of the data are indicated. [b] Expressed as an integer value per mole of holoenzyme. [c] Indicates whether the enzyme contains pterin (yes) with (nuc) or without (non-nuc) an appended nucleotide. [d] Cluster contents are expressed per mole of holoenzyme and are based on EPR spectroscopy or crystallography. See text for details. [e] The nucleotide is GMP.

coordinates the Mo atom in all molybdoenzymes, with the notable exception of nitrogenase.[54] The mononucleotide form has a pteridine nucleus with a four-carbon side chain containing a dithiolene group and a terminal phosphate, whereas the dinucleotide form has a mononucleotide (AMP, GMP, CMP, or IMP) attached to the phosphate. To avoid any confusion that might arise, for example, in referring to molybdopterin in a tungstoenzyme that lacks Mo, herein we will use the terms non-nucleotide and nucleotide forms of the pterin cofactor, rather than the mono- and dinucleotide forms of molybdopterin. Thus, Pf AOR contains the non-nucleotide form of the pterin cofactor. Surprisingly, the structural study[41] of Pf AOR revealed that the W atoms were coordinated, not to one, but to two pterin molecules (see section III). On the basis of the similarities in spectroscopic properties (see section IV), this is likely to be true for the other members of the AOR family (Table 1). AOR has also been purified from the hyperthermophilic archaea *Thermococcus* strain ES-1[55] and *Pyrococcus* strain ES-4.[56] The molecular properties of both enzymes appear to be virtually identical to those of Pf AOR.

The hyperthermophilic archaea contain two other types of tungstoenzyme besides AOR. Formaldehyde ferredoxin oxidoreductase (FOR) has been purified from Pf and *Thermococcus litoralis* (Tl; T_{max}, 98 °C).[56,57] Both are homotetrameric enzymes with each subunit containing one W and four Fe atoms. Chemical analyses have shown that both FORs contain the non-nucleotide form of the pterin cofactor.[53] The gene encoding Tl FOR has been sequenced (621 residues),[39] and this revealed its close structural similarity with Pf AOR (605 residues). The two enzymes are 59% similar at the amino acid level, and the four cysteinyl residues that coordinate the [4Fe-4S] cluster in Pf AOR are conserved in Tl FOR, but the latter lacks the equivalent of the His and Glu residues which coordinate the monomeric Fe site in Pf AOR (see section III). Thus, although these two enzymes differ in quaternary structure and apparently in the nature of the subunit interactions, the size, cofactor content, and tertiary structures of Pf AOR and Tl FOR are highly similar (Table 1).

The third tungstoenzyme from the hyperthermophiles is glyceraldehyde-3-phosphate ferredoxin oxidoreductase (GAPOR), which so far has been purified only from Pf.[58] This monomeric enzyme is the least characterized of the three and unlike AOR and FOR contains two Zn atoms per subunit. However, its N-terminal amino acid sequence (Figure 1) and its size and W and Fe contents (Table 1) strongly suggest that GAPOR is very closely related in structure. In fact, we have suggested that all three enzymes arose from an ancestral AOR-type subunit containing the tungstodipterin site and a single [4Fe-4S] cluster.[48,59] Moreover, since the hyperthermophilic archaea such as species of *Pyrococcus* and *Thermococcus* are regarded as the most slowly evolving of all known organisms,[60] our hypothesis is that this AOR subunit was also the evolutionary precursor to all of the tungstoenzymes in the AOR family (Table 1).

In addition to their structural similarities, a unifying feature among the three types of tungstoenzyme in the hyperthermophiles is that they catalyze the oxidation of aldehydes and use the redox protein ferredoxin (Fd) as the physiological electron acceptor (Table 1). Aldehyde oxidation is a two-electron process (eq 1) but the ferredoxins of both Pf[61,62] and

$$CH_3CHO + H_2O \leftrightarrow CH_3COO^- + 3H^+ + 2e^- \quad (1)$$

Tl[63] contain a single [4Fe-4S] cluster and undergo only a one-electron redox reaction. Hence, assuming each W-containing subunit of these enzymes functions independently, one catalytic turnover per subunit requires the reduction of two molecules of Fd.

AOR-type

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Pf   AOR[a]   MYGNWGRFI RVNLSTGDIK VEEYDEELAK KWLGSRGLAIY LLLKEMDPTV DPL
ES4  AOR      MYGYWGMIL RVNLSDGTIK GXTADXK
ES1  AOR      MFGYHGKIL RVNLTT
Pf   FOR      MYGWWGRIL RVNLTTGXVK VQEYPEXVA
Tl   FOR[a]   MKGWWGKIL RVDLTNNKVW VQEYSPEVAK NFIGGRGLAAW ILWNE.AKNV DPL
Pf GAPOR        MKFSVL KLDVGKREVE AQEIERE... DIFGVVDYGI. MRHNE.XRTY EVN
Ct   CAR64   MYGWTGQLL RVNLSN
Cf   CAR64     MNKFI RVDMTTLKVT XTEX.EVPAK .YAG
Dg  ALDH       MDKIL RIDVGAEGGP KLTTLPVG.. EYA
(Pv HVOR_Mo MINGWTGNIL RINLTTGAIS)
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F(M)DH-type

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Mt  FMDH65[a] MEYIIKNGFV Y.PLNNVDGE  MDI
Mw  FMDH64   MEYIIKNGFV YXPLNGVDGE XMD
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AH-type

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Pa    AH    ASKKHVVCQ CCDINCVVEA
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Figure 1. N-terminal amino acid sequences of tungstoenzymes. Abbreviations for the organisms: Pf, *Pyrococcus furiosus*; ES-4, *Pyrococcus sp.* ES-4; ES-1, *Thermococcus* sp. ES-1; Tl, *Thermococcus litoralis*, Ct, *Clostridium thermoautotrophicum*, Cf, *Clostridium formicicum*, Dg, *Desulfovibrio gigas*, Pv, *Proteus vulgaris*, Mt, *Methanobacterium thermoautotrophicum*, Mw, *Methanobacterium wolfei;* Pa, *Pelobacter acetylenicus.* Sequence references: Pf AOR,[39] ES4 AOR and Pf FOR,[56] ES1 AOR,[55] Tl FOR,[39] Pf GAPOR,[58] Ct CAR and Cf CAR,[68] Dg ALDH,[45] Pv HVOR,[76] Mt and Mw FMDH,[91] and Pa AH.[44] For proteins with more than one subunit, the molecular mass (in kDa) of the relevant subunit is indicated (see Table 1). Identical positions and conservative exchanges are shown in boldface. Modified from ref 48. [a]Complete amino acid sequences are available for Pf AOR, Tl FOR, and Mt FMDH.[39,40]

Tetrameric FOR must therefore interact with eight Fd molecules per holoenzyme, while AOR and GAPOR interact with four and two, respectively. The [4Fe-4S] cluster within each of the subunits of these enzymes is thought to reduce Fd, but whether each subunit has one or two Fd binding sites is unknown.

The primary factor that distinguishes the three hyperthermophilic tungstoenzymes is their substrate specificity. AOR oxidizes a broad range of both aliphatic and aromatic aldehydes and shows the highest catalytic efficiency with the aldehyde derivatives of the common amino acids, such as acetaldehyde (from alanine), isovalerylaldehyde (from valine), and phenylacetaldehyde (from phenylalanine).[52,55] These compounds are generated by the transamination of amino acids and their subsequent decarboxylation by 2-keto acid oxidoreductase, which also produce the corresponding coenzyme A derivatives.[55,64] Thus, AOR is thought to play a key role in peptide fermentation.[59] On the other hand, FOR has a very limited substrate specificity and only oxidizes small (C1−C3) aliphatic aldehydes.[57] In fact, even with these substrates the catalytic efficiencies are very low, and the physiological reaction of FOR is not known. In further contrast, GAPOR is absolutely specific for glyceraldehyde 3-phosphate (GAP), a substrate for which it has high affinity.[58] GAPOR is thought to play a key role in the unusual glycolytic pathway in the fermentative hyperthermophiles,[58,65] in which it functions in place of NAD(P)-dependent glyceraldehyde-3-phosphate dehydrogenase, the enzyme found in more conventional organisms.[58] Neither GAPOR, AOR, nor FOR is able to utilize nicotinamide nucleotides as electron carriers. In addition, AOR and FOR do not oxidize aldehyde phosphates. The presence of zinc in GAPOR but not in AOR or FOR may therefore not be coincidental. For example, we have suggested[48,59] that during the evolution of these enzymes from an ancestral AOR-type subunit exhibiting broad substrate specificity, (a) Pf AOR formed a dimer and maintained the substrate range, (b) FOR acquired a much more limited specificity perhaps in part by forming a tetrameric structure, while (c) GAPOR remained monomeric and gained its absolute specificity for GAP in part by the incorporation of zinc, which specifically orients the negatively charged phosphate at the catalytic site. At present, however, this remains pure speculation.

AOR, FOR, and GAPOR from Pf do not incorporate Mo when cells are grown in the presence of this element, nor does the organism produce active Mo-containing, aldehyde-oxidizing isoenzymes.[49] For example, AOR and FOR purified from cells growing with Mo (100 μM) lacked detectable Mo and their W contents and specific activities were greater than 70% of the values for the enzymes obtained from W-sufficient grown cells. Remarkably, the organism is able to scavenge the W that contaminates the medium (~15 nM) even in the presence of a 6500-fold excess of Mo. The specific activity of GAPOR in the Mo-grown cells was only 1% of that in cells grown in the presence of sufficient W, so this enzyme is also not replaced by an active Mo-containing isoenzyme form.[49]

In addition to the three hyperthermophilic tungstoenzymes, the AOR family includes carboxylic acid reductase (CAR) found in certain acetogenic clostridia.[37,66−70] As its name suggests, CAR was first identified by its ability to catalyze the reduction of nonactivated carboxylic acids. CAR also catalyzes the reverse reaction, aldehyde oxidation. In fact, because the acid/aldehyde redox couple has such a low potential (acetaldehyde/acetate, $E_o' = -580$ mV),[71] aldehyde oxidation is much more thermodynamically favorable. For example, the calculated equilibrium concentrations of acetate and acetaldehyde are 100 mM and 1.5 μM, respectively, when the electrons are

used to form H_2 (25 °C, 1 atm H_2).[71] Hence, both CAR[66] and AOR[55] typically catalyze the oxidation reaction at more than 10 times the rate of the reduction reaction under the usual assay conditions.

The molecular properties of CAR from *Clostridium formicoaceticum* (Cf) are very similar to those of Pf AOR, including subunit size, pterin type, metal content (Table 1), and N-terminal sequence (Figure 1). Moreover, like AOR, Cf CAR exhibits a broad substrate specificity. On the other hand, the physiological electron carrier for CAR is not known, nor is its physiological role, in spite of the fact that it represents ~4% of the total cytoplasmic protein in Cf. As shown in Table 1, two forms of CAR have been purified from the related species, *Clostridium thermoaceticum* (Ct). Compared to the Cf enzyme (α_2-subunit structure), both Ct CAR I ($\alpha\beta$) and Ct CAR II ($\alpha_3\beta_3\gamma$) contain additional subunits, a much higher Fe content and, in the case of CAR II, a flavin group (FAD in the γ-subunit). Nevertheless, the large α-subunit in both forms of the Ct enzyme are similar in size to, and show N-terminal sequence similarity with, Cf CAR and with the hyperthermophilic tungstoenzymes (Figure 1). Ct CAR was recently shown to contain the non-nucleotide form of the pterin cofactor.[70] When grown in the presence of Mo rather than W, Cf produces a Mo-containing equivalent of CAR termed Mo-aldehyde oxidoreductase (Mo-AOR).[72] The enzyme has a single subunit type but its molecular mass (100 kDa) is higher than that of CAR's (67 kDa) and the two do not share any N-terminal sequence similarity, suggesting they are unrelated structurally. Cf Mo-AOR is multimeric (α_{2-3}) wherein each subunit contains one Mo atom and approximately seven Fe atoms, apparently in the form of [2Fe-2S] clusters.[69,72] Growth studies[67] suggest that Mo-AOR may also be present in Ct, but such an enzyme has not been purified.

Considering the molecular properties of the clostridial CARs and their similarity to those of the hyperthermophilic tungstoenzymes and to Pf AOR in particular (Table 1), it is clear that all of these enzymes belong to the same family. Moreover, it is tempting to speculate that the α-subunit of the CARs directly evolved from the ancestral AOR subunit now represented in Pf AOR and that the dimeric form is retained by Cf CAR. In Ct, CAR acquired additional unrelated subunits (β and γ) that presumably do not contain W. On the other hand, the so-called Mo-AOR that replaces CAR in the acetogens when they are grown with Mo appears not to be related to the W-containing AOR family. Rather, it is part of the much larger group of molybdoenzymes, as further discussed below.

The other tungstoenzyme that is included in the AOR family is the aldehyde dehydrogenase (ADH) from the sulfate-reducing bacterium, *Desulfovibrio gigas* (Dg).[45] As shown in Table 1, its quaternary structure, subunit size, and W and Fe contents match those of Pf AOR. ADH also has a broad substrate specificity although its physiological electron carrier is not known. That Dg ADH and Pf AOR are related enzymes is also demonstrated by the similarity in their N-terminal sequences (Figure 1). As might be expected, ADH contains the pterin cofactor although the type is not known.[45] In any event, Dg ADH is also thought to be an evolutionary derivative of the hyperthermophilic AOR subunit. A Mo-containing aldehyde-oxidizing enzyme has also been purified from Dg, and this was originally termed aldehyde oxidase (AOX).[73] Although more recent references[42,74,75] refer to it as aldehyde dehydrogenase, aldehyde oxidoreductase, and mop (for molybdenum protein), we will use the AOX designation herein to distinguish it from other aldehyde-oxidizing enzymes under discussion. AOX uses flavodoxin as a physiological electron carrier, but its function is not known. It is a homodimeric enzyme and each subunit (97 kDa) contains one Mo atom and four Fe atoms in the form of two [2Fe-2S] clusters. In contrast to Pf AOR, AOX contains a monopterin which is present as the GMP form. Dg AOX shows no N-terminal sequence similarity to Dg ADH or to any other member of the AOR family (Figure 1). Accordingly, the complete sequence of AOX[42,75] shows no similarity to the complete sequences of either Pf AOR or Tl FOR.[39] Instead, AOX is related to other molybdoenzymes, showing 52% sequence identity with the prototypical xanthine oxidase.[42] Very recently, the crystal structure of Dg AOX has been determined,[42] the first for a pterin-containing molybdoenzyme (see section III).

Surprisingly, the only other member of the W-containing AOR family known at present is not a tungstoenzyme. It is a molybdoenzyme known as hydroxycarboxylate viologen oxidoreductase (HVOR) and is purified from the mesophilic bacterium *Proteus vulgaris* (Pv).[76] As shown in Table 1, its subunit size and metal content (exchanging W for Mo) are comparable to those of Pf AOR, and HVOR also has a broad substrate specificity and contains the non-nucleotide form of pterin. Moreover, of all the available amino acid sequences in the databases, the N-terminal sequence of HVOR shows similarity only to the N-termini of members of the AOR family (Figure 1). Interestingly, W inhibits the growth of Pv,[76] so HVOR appears to be a "true" Mo-containing enzyme and not a Mo-substituted form of a tungstoenzyme. It therefore seems likely that HVOR also evolved from the ancestral AOR subunit, although in this case, W has been replaced by Mo and the enzyme formed a multimeric structure.

Two distinct families of aldehyde-oxidizing enzymes are therefore apparent. One is the W-based AOR family which includes Mo-containing HVOR, while the other is Mo-based and includes Cf Mo-AOR and Dg AOX. The latter group are part of the broad class of molybdoenzymes, whereas the AOR family shows no sequence similarity with any Mo-containing enzyme (except HVOR) or with the other two types of tungstoenzyme discussed below.

B. F(M)DH Family

The second family of tungstoenzymes involves two types, both of which utilize CO_2 as the substrate. One is formate dehydrogenase (FDH), which was the first W-containing enzyme to be purified.[35] So far this is the only W-containing FDH that has been well characterized both biochemically and spectroscopically and it was obtained from Ct, the same organism that contains CAR. Although FDHs from anaerobic

organisms are typically Mo-containing enzymes,[77−79] growth studies have indicated that certain species of both Gram-positive bacteria[77,80] and methanogens[33,34] have W-containing FDHs, and several of them have been partially purified.[28,80−82] In the acetogenic Ct, FDH catalyzes the first step in CO_2 conversion to acetate, the production of formate (eq 2). It uses NADPH as the physiological electron

$$CO_2 + H^+ + 2e^- \leftrightarrow HCOO^- \qquad (2)$$

donor. As shown in Table 1, Ct FDH is a complex tetrameric enzyme of two different subunits and it contains selenium (Se) as well as W and Fe. Se is present as selenocysteine, which is located in the α-subunit.[35] The finding of two W atoms per holoenzyme and the presence of a pterin cofactor[35] suggests that each α-subunit contains one W atom coordinated to both Se and a pterin moiety of unknown type. The genes for the two subunits of Ct FDH were recently cloned.[83] The preliminary sequence data show no similarity to the sequences of Pf AOR and Tl FOR, rather, they are similar to those of Mo-containing FDHs, such as that represented by FDH from the methanogen *Methanobacterium formicicum* (Mf).[84] As discussed below, the latter is part of a much larger class of molybdoenzymes that contain the nucleotide form of pterin, suggesting this is also the case with Ct FDH. Ct also produces a Mo-containing FDH but this has not been well studied due mainly to its extreme O_2 sensitivity.[77] FDH has been purified and characterized from the related species, *Clostridium pasteurianum*.[85] The subunit size and cofactor contents of this Mo-containing FDH are very similar to those of both Mf FDH and Ct FDH. It therefore seems likely that Ct produces two very closely related FDHs, one containing W and one containing Mo, both of which are members of the general family of molybdoenzymes.

The other member of the second class of tungstoenzyme is *N*-formylmethanofuran dehydrogenase (FMDH). This enzyme catalyzes the first step in the conversion of CO_2 to methane in methanogens, where the other substrate is methanofuran (MFR; eq 3). The

$$CO_2 + MFR^+ + H^+ + 2e^- \leftrightarrow CHO\text{-}MFR + H_2O \qquad (3)$$

physiological electron donor is not known.[86] FMDH has been purified from several methanogens and is typically a molybdoenzyme.[87−89] However, two examples of W-containing FMDHs are known, from *Methanobacterium thermoautotrophicum* (Mt) and *M. wolfei* (Mw). In fact, both of these organisms each produce two FMDHs, one containing W (FMDH II)[90−93] and one Mo (FMDH I),[86,90,91,93] but they differ in their responses to W and Mo in the growth media. Thus, Mw preferentially expresses Mo-FMDH I in the presence of Mo, but both isoenzymes are produced if W is present in the medium. Mo does not substitute for W in FMDH II, but W does substitute for Mo in FMDH I.[94] Remarkably, the opposite is true for Mt.[90] Mo-grown cells contain both isoenzymes, whereas W-grown cells contain W-FMDH II, and Mo can replace W in FMDH II but W does not replace Mo in FMDH I. Hence, the Mo-substituted form of FMDH II of Mt and the W-substituted form of FMDH I of Mw have been separately purified and characterized.[91,94] So far, these are the only examples of where Mo and W can be interchanged to yield enzymes with comparable activities.

As shown in Table 1, the W-containing FMDH IIs of both Mw and Mt contain either three or four subunits. The N-terminal sequences of the two largest subunits show a high degree of similarity between the two enzymes, and each is virtually identical to the Mo-containing FMDH I isoenzyme from the same organism, showing that all four FMDHs are closely related. Indeed, it has been suggested that the largest (α) subunit may be shared between FMDH I and FMDH II.[40] However, one of the major problems in characterizing the cofactor contents of these enzymes is their intrinsic lability coupled with their O_2 sensitivity. They lose substantial amounts of activity upon purification, and typically contain less than 0.5 atom of W (or Mo) per mole of holoenzyme.[90] The Fe contents of these enzymes are also likely to be underestimates (Table 1). All of the FMDHs from Mw and Mt contain the GMP derivative of the pterin cofactor.[89] The complete amino acid sequences of the four subunits of Mt FMDH II were recently reported, and these showed no relationship to those of Pf AOR or Tl FOR.[40] In fact, the α-, γ-, and δ-subunits showed no similarity to any other protein in the databases, but the β-subunit contained sequence motifs characteristic of molybdoenzymes that contain the nucleotide form of the pterin cofactor, such as the large (α) subunits of FDH of Mf and of FDH, nitrate reductase, and biotin sulfoxide of *Escherichia coli* (Ec). Note that this group now includes the tungstoenzyme Ct FDH.[83] Clearly, the β-subunit of FMDH II can be assumed to contain W, and the two tungstoenzymes FMDH II and FDH are closely related.

The genes for Mt FMDH II were part of an operon that contained three other genes, all of which encoded proteins that would be expected to contain two or more [4Fe-4S] clusters, although their function and relationship to FMDH is not known.[40] In any event, W-containing FMDH II of the methanogens, and the W-containing FDH of the acetogens, are part of molybdoenzyme family and appear to be part of the subclass that contains the nucleotide form of the pterin cofactor. Obviously they are not part of the AOR family of tungstoenzymes, which probably all contain the non-nucleotide form. Moreover, the complexity of both FMDH and FDH, particularly in their subunit composition, suggests that they appeared more recently on the evolutionary time scale than the AOR-type enzymes, although whether the original pterin-containing moiety that ultimately gave rise to both enzyme families contained the non- or the nucleotide version is an interesting question that cannot be answered at present.

C. Acetylene Hydratase

The third class of tungstoenzyme has just one member, and it is the most recently discovered and the least characterized. It is termed acetylene hydratase (AH) and was purified from the acetylene-utilizing anaerobe *Pelobacter acetylenicus* (Pa).[44] The

enzyme catalyzes the hydration of acetylene to acetaldehyde, according to eq 4. As shown in Table 1,

$$HC{\equiv}CH + H_2O \leftrightarrow CH_3CHO \qquad (4)$$

the molecular properties of AH (subunit size, cofactor content) closely resemble those of Pf AOR but the N-terminal amino acid sequence of AH shows no similarity to the sequences of any of the AOR family or to FDH or FMDH, see Figure 1. Another reason to suggest that AH represents a new class of tungstoenzyme is the reaction that it catalyzes, namely, a hydration. This is in contrast to the oxidoreductase-type reaction catalyzed by all other tungstoenzymes and, indeed, by all molybdoenzymes. However, AH is catalytically active only in the presence of strong reducing agents (sodium dithionite or titanium citrate), so the reaction that it catalyzes might involve the initial reduction of acetylene, followed by hydration and subsequent oxidation.[44] Such a reaction sequence would presumably involve both W and the FeS cluster(s) in the enzyme. Unfortunately, the physiological electron carrier for AH is not known (assuming it does need one) and the enzyme has not been characterized spectroscopically. Understanding the role of tungsten in AH will obviously require further study, and determination of its relationship to the other tungstoenzymes must await complete amino acid sequence comparisons.

III. Structural Properties of Tungstoenzymes

The only tungstoprotein presently characterized at atomic resolution is Pf AOR.[41] As discussed in section II.A, this enzyme is a dimer of two identical 605 residue (67 kDa) subunits of known sequence. Three different types of metal sites are found in the AOR protein dimer: a mononuclear tungsten center and a [4Fe-4S] cluster that are contained within each subunit and a single tetrahedral metal atom located at the dimer interface. The structure of AOR described below was determined by X-ray crystallography at 2.3-Å resolution.[41] In addition to AOR, the structures of two molybdoenzymes have recently been determined: for Dg AOX[42] and for DMSO reductase (DMSOR)[43] from *Rhodobacter sphaeroides* (Rs). These will be briefly discussed in relation to the structure of Pf AOR.

A. Tungstopterin Cofactor of Pf AOR

Pf AOR had been earlier shown to contain the non-nucleotide form of the pterin cofactor.[53] The pterin cofactor was first identified by Johnson and Rajagopalan,[96] who isolated and characterized its bis-(carboxamidomethyl) derivative from various molybdoenzymes by fluorescence and mass spectroscopy. From these studies, Rajagopalan proposed a structure that was based on a pterin ring system, with a side chain extending from C6 that contained dithiolene, hydroxyl, and phosphate groups (Figure 2a). The metal was proposed to be coordinated to the cofactor through the dithiolene sulfurs. In general, molybdoenzymes from eukaryotes and archaea contain this unmodified (non-nucleotide) form of the cofactor, while the bacterial enzymes usually contain

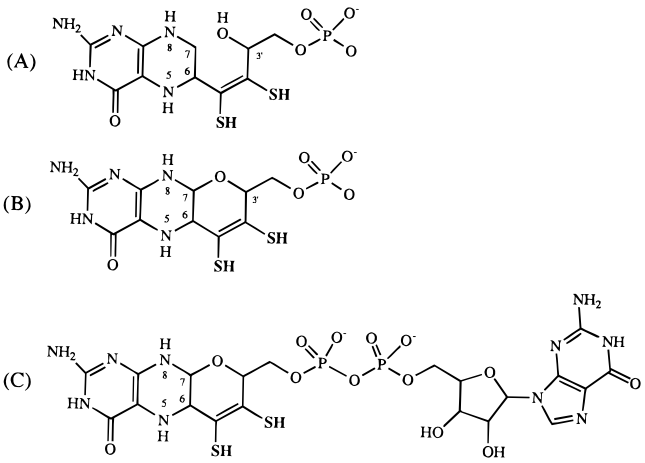

Figure 2. Structural models for the pterin cofactors: (A) model deduced by Johnson and Rajagopalan[96] for the pterin cofactor; (B) structure of the tungstopterin center observed in AOR;[41] (C) model for the nucleotide (GMP) form of the pterin cofactor.

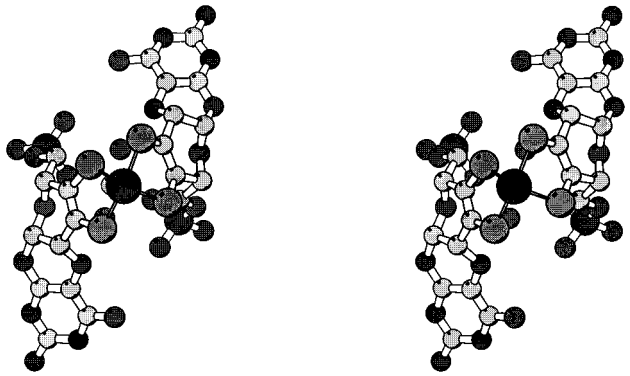

Figure 3. Stereoview of the tungstopterin center of AOR.[41] Structural figures for this paper were prepared with the program MOLSCRIPT.[140]

the alternate nucleotide version with a GMP, CMP, AMP, or IMP nucleotide appended via a pyrophosphate linkage.[15,54] The role of the nucleotide in the chemistry of this system is unknown. As with molybdoenzymes, all tungstoenzymes that have been examined contain the same pterin cofactor in one or other form (Table 1). Moreover, all members of the AOR family contain the non-nucleotide form, whereas the F(M)DH type appears to contain the nucleotide form. Note that each of these families contains both archaeal (Pf, Tl, Mw, Mt) and bacterial (Ct, Dg, Cf) members. Therefore, the general phylogenetic conclusion on the distribution of the non-nucleotide and nucleotide forms of the pterin cofactor in molybdoenzymes[54] does not appear to be applicable to tungstoenzymes.

The structure of Pf AOR established the essential correctness of the model for the pterin cofactor, with one modification (Figures 2 and 3). Specifically, the cofactor in AOR contains a third ring formed by closure of the side-chain hydroxyl with the pterin ring at C7. The three chiral centers, at carbons 6, 7, and 3′, exhibit the *R* configuration. Such a cyclization would not be unprecedented, in that related compounds have been synthesized.[97] A plausible mechanism for formation of the third ring would be by attack of the side-chain hydroxyl on the C7 carbon of a 5,6 dihydropterin (Figure 2). Provided that this

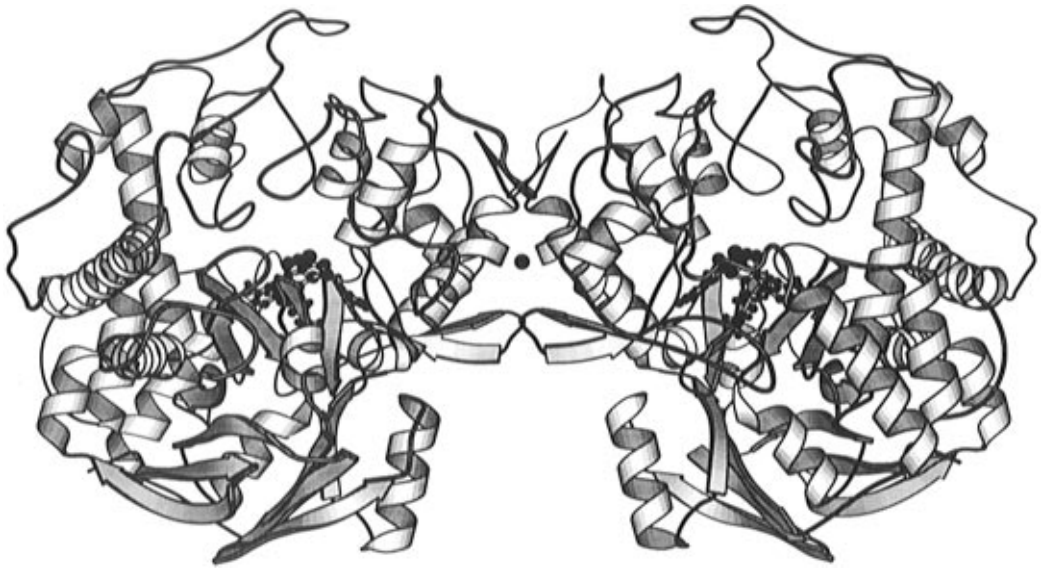

Figure 4. Ribbons diagram of the AOR dimer viewed perpendicular to the dimer 2-fold axis.

ring closure is reversible, there is no inconsistency between the three-ringed structure seen in the protein and the two-ringed form observed for the isolated pterin. The nonplanar structure of the pterin ring system seen in AOR is consistent with a reduced form for the protein-bound pterin, which would likely be equivalent to a dihydropterin (possibly the 5,6-dihydropterin) in the ring-opened form. This revised structure of the pterin cofactor is likely a common feature of all pterin-containing enzymes and has been observed subsequently in the AOX and DMSOR structures.[42,43]

As proposed by Rajagopalan and Johnson,[15,54,96] the pterin cofactor does bind to the tungsten via the dithiolene sulfurs in Pf AOR. Two pterin cofactors, however, instead of the expected one molecule, are coordinated to the tungsten. The two cofactors are approximately related by a 2-fold rotation axis that passes through the tungsten. The arrangement of the tungsten and the two pairs of dithiolene sulfurs may be described as a distorted square pyramid, with an angle between the planes of the dithiolene ligands of ~81°. No protein ligands are coordinated to the tungsten atom, although the present analysis of the electron density around the tungsten suggests that two additional coordination sites may be occupied by glycerol or oxo ligands (or both) to yield an overall distorted trigonal prismatic arrangement at the tungsten site. The glycerol presumably comes from the protein storage buffer and may represent a substrate analog. Spectroscopic studies (see section IV) also provide evidence that diols, such as glycerol, inhibit AORs by direct binding to tungsten. Unless substantial rearrangements in the tungsten coordination sphere occur during substrate oxidation, the presence of two pterin ligands in AOR enforces cis coordination for the substrate and oxo groups, which would have stereochemical consequences for the mechanism of the aldehyde oxidation reaction.

In addition to interactions between the dithiolene sulfurs and tungsten, the two pterin ligands are also linked through their phosphate groups, which coordinate axial sites of the same magnesium ion (Figure 3). The two pterin ligands are approximately related by a 2-fold rotation about an axis that passes through both the tungsten and magnesium sites. The magnesium ion exhibits octahedral geometry. In addition to the two coordination sites filled by phosphate oxygens, two waters bind at cis sites on the side facing the tungsten atom, and the two backbone carbonyl oxygens from residues Asn93 and Ala183 bind at cis sites on the Mg directed away from the tungsten. The two waters form part of a hydrogen-bonding network within the tungsten cofactor. Each water is hydrogen bonded to at least one phosphate oxygen and an N5 nitrogen of the pterin ring, with one of the two waters also within hydrogen bond distance of the pyran ring oxygen of one of the pterin ligands.

B. Protein Structure of Pf AOR

AOR exists as a dimer (Figure 4) with a tungstopterin cofactor and a [4Fe-4S] cluster contained within each subunit in close proximity. The shortest W−Fe distance is ~8 Å. A mononuclear center, most likely Fe, is positioned on the dimer 2-fold axis ~25 Å from the other metal centers. The [4Fe-4S] clusters and tungsten sites in different subunits of the dimer are separated by ~50 Å. Each subunit of AOR folds into three domains (Figure 5) with the binding sites for the tungstopterin cofactor and [4Fe-4S] cluster located at the interfaces of these domains. A comparison of the AOR structure against the all structures in the Protein Data Bank indicates that AOR exhibits a unique fold.[98] Domain 1 (residues 1−210) forms a base on which the saddlelike tungsten cofactor sits, while domains 2 (residues 211−417) and 3 (residues 418−605) enclose the opposite surface of the tungsten cofactor and provide residues that form specific polar and ionic interactions with the different metal centers. Each subunit exhibits a pseudo 2-fold axis that coincides approximately with the 2-fold axis of the tungsten cofactor. This rotation axis passes through the center of domain 1 and approximately relates domains 2 and 3. The 2-fold symmetric arrangement of the polypeptide chain in domain 1 is particularly striking (Figure 5c). The similarity of domains 2 and 3 is less apparent, which

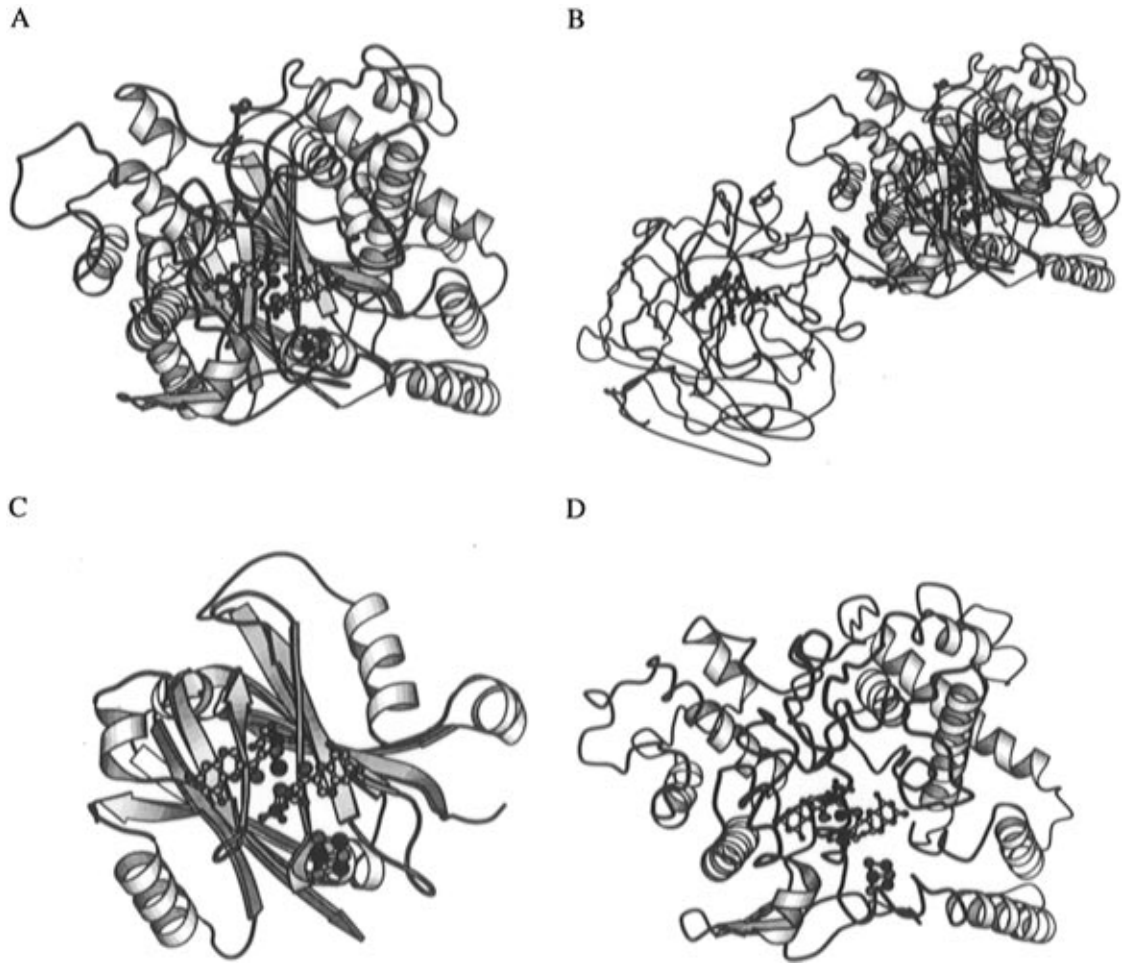

Figure 5. (A) Ribbons diagram of a single subunit of AOR, viewed roughly down the 2-fold axis of the pterin cofactor. (B) Ribbons diagram of the AOR dimer viewed from the same orientation as (a), with the second subunit indicated by a coiled chain. (C) Ribbons diagram of domain 1 of the AOR subunit, viewed in the same orientation as (a). (D) Ribbons diagram of domain 2 and 3 of the AOR subunit, viewed in the same orientation as (a).

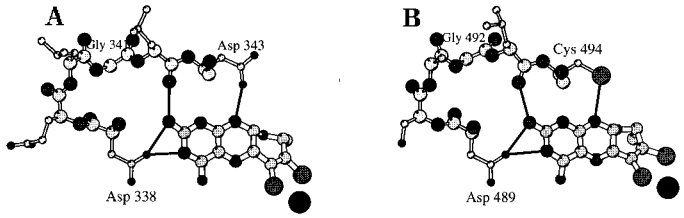

Figure 6. Interactions between the pterin and the DXXGL(C/D) loop in AOR: (A) structure of residues Asp338−Asp343, with hydrogen bonds to the pterin indicated; (b) structure of residues Asp489−Cys494, with hydrogen bonds to the pterin indicated.

may result from the involvement of domain 2 in both generating the dimer interface and providing most of the [4Fe-4S] cluster binding site (Figure 5d).

Domain 1 consists primarily of 12 β-strands arranged in two six-stranded, predominantly antiparallel β-sheets that are related approximately by the 2-fold rotation axis described above (Figure 5c). Residues Asn93 and Ala183, which coordinate the magnesium ion through their carbonyl oxygens, are located within adjacent edge strands of both sheets. Within domain 1 is residue Arg182, which forms a salt bridge to the phosphate group of the pterin nearer the [4Fe-4S] cluster. Additional hydrogen-bonding interactions to the phosphates are provided by the main-chain amide nitrogens of residues 93, 95, 183, 185, and 186. Residue Arg76, which hydrogen bonds both a pyran ring oxygen and a [4Fe-4S] inorganic sulfur, is also located within this domain.

The regular secondary structure of domains 2 and 3 consists primarily of α-helices, with few β-sheet regions. Although these two domains are related approximately by the 2-fold axis described above, this structural relationship is less apparent than for domain 1 and the tungsten cofactor. Consistent with an underlying structural similarity between the two domains, residues 338−344 and 489−495 in domains 2 and 3, respectively, contain Asp-X-X-Gly-Leu-(Cys or Asp)-X sequences, where the Asp carboxylate group and Leu main-chain carbonyl oxygen are arranged to bind the primary amine group of their respective pterin ligand (Figure 6). Asp343 and Cys494 interact with the secondary ring nitrogen, N8, near the pyran ring linkage, while the amide nitrogens from the carboxy-terminal end of each motif,

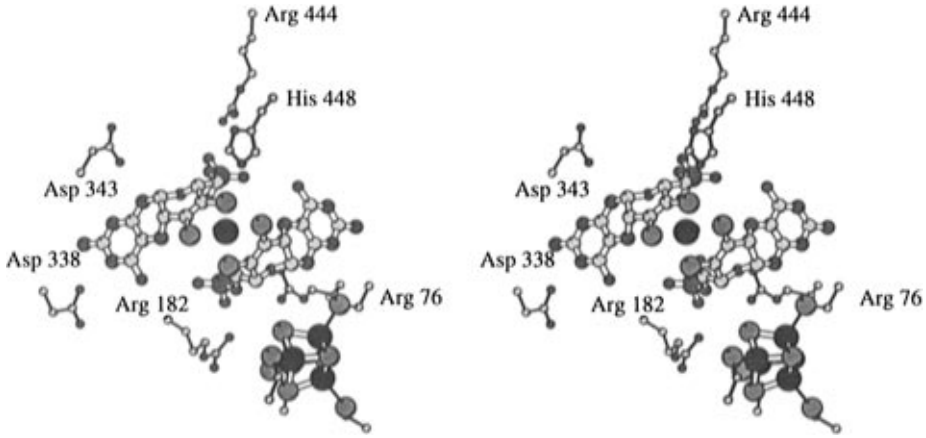

Figure 7. Stereoview of the active center of AOR, with the tungsten, pterin cofactor, [4Fe-4S] cluster, and surrounding residues indicated.

Thr344 and Leu495, form a hydrogen bond with a secondary ring nitrogen on the pterin. This sequence motif occurs in both domains at the end of α-helices, with the Gly residue adopting the left-handed helical (Lα) conformation characteristic of a helical C-cap interaction.[99] An additional polar interaction to one of the two pterin rings is generated by a hydrogen bond between the side chain of Lys450 and a pterin carbonyl oxygen. The side chains of residues Glu313 and His448 in these two domains are in the vicinity of the substrate-binding site on the tungsten and could participate in proton transfers associated with oxidation−reduction reactions.

As discussed in section II, Pf AOR has many properties in common with the tungstoenzyme FOR, which is also found in hyperthermophiles such as Pf and Tl (Table 1). The sequences of the Pf AOR and Tl FOR exhibit 38% sequence identity, and residue conservation is especially striking for domain 1, where the two proteins share 48% identity.[39] In general, residues that interact with the tungstopterin cofactor through their side chains are identical in both AOR and FOR, including Arg182, Glu313, Asp338, Asp343, His448, Cys497, and Lys450 (AOR numbering). In a few cases, conservative substitutions are observed, such as the replacement of Arg76 and Asp489 in AOR by Lys and Glu, respectively, in FOR. However, there are some distinct sequence differences between the two enzymes. For example, I449 near the tungstopterin cofactor is replaced by His in FOR, and the second DXXGL motif, which contains Asp489 in AOR, is not present in FOR, although it is possible that the Glu can serve as a replacement for this residue.

In addition to maintaining specific binding interactions with the pterin, another role for domains 2 and 3 may be to regulate substrate access to AOR. A hydrophobic channel of ~15-Å length that leads from the tungsten site to the surface of the protein may be defined[100] between these two domains. The non-pterin coordination sites on the tungsten atom occupy one end of this cavity, consistent with the role of these coordination sites in substrate binding and catalysis. The channel seems large enough to accommodate a variety of substrates, which may help to explain the ability of AOR to oxidize both aliphatic and aromatic aldehydes. In general, the channel is lined with apolar residues, except in the vicinity of the tungsten site. The channel opening from the protein surface contains residues V233, L238, V245, I249, L459, Y461, and F499, while the middle region of the channel is lined by the side chains of T243, L246, I250, Y312, Y427, and Y452. The channel leads into the tungsten center from the side toward the pterin that interacts with the [4Fe-4S] cluster, with the side chains of Glu311, Glu313, His448, and Ile449 nearby. Sequence comparisons between AOR and FOR suggest that, with the exception of T243, Y312, E313, Y427, and H448, residues lining the channel in AOR are substituted in Tl FOR, although the polar character of each residue is generally conserved. Variation in the channel size of these two enzymes might contribute to the differences in the substrate specificities of AOR and FOR.

The [4Fe-4S] cluster in AOR is positioned ~10 Å from the tungsten atom and is buried ~6 Å below the van der Waals surface of the protein. This arrangement is consistent with the postulated role of the cluster as an intermediary for electron transfer between the tungsten cofactor and Fd, the physiological electron acceptor of AOR. Four cysteine ligands, provided by the S^{γ} of Cys288, Cys291, Cys295, and Cys494, coordinate the [4Fe-4S] cluster. While the first three Cys residues are part of a characteristic iron−sulfur cluster binding sequence, the polypeptide conformation of this region is distinct from that adopted by the [4Fe-4S] binding site found in ferredoxins. The [4Fe-4S] cluster is linked to one of the two pterin cofactors of the tungsten site by two distinct sets of interactions (Figure 7). The side chain of Arg76 bridges these two groups by forming hydrogen bonds to an inorganic sulfur of the [4Fe-4S] cluster and to two sites on the pterin ring, the pyran ring oxygen and a phosphate oxygen. In addition, the S^{γ} of Cys494, a [4Fe-4S] cluster ligand, is positioned to accept a hydrogen bond from the pterin ring nitrogen N8, which is nearest the pyran ring linkage of the pterin. These interactions could provide electron transfer pathways between the two metal centers. This arrangement suggests that the pterin ligand does not merely play a passive structural role but may be an active participant in the redox chemistry of AOR.

C. Comparisons with the Structures of Molybdoenzymes

The structures of two enzymes containing the Mo-coordinated form of the pterin cofactor, Dg AOX and Rs DMSOR, have been recently determined.[42,43] Although these enzymes contain this cofactor, they are unrelated to AOR in terms of both amino acid sequence and in their polypeptide folds. AOX is a member of the protein family that includes xanthine oxidase, and it contains two different [2Fe-2S] clusters, in addition to the pterin cofactor. DMSOR belongs to another family of pterin-containing enzymes, which includes the molybdoenzymes, FDH, nitrate reductase, and biotin sulfoxide, as well as the tungstoenzymes FMDH II from methanogens and Ct FDH (see section II). Thus, DMSOR is distinct from both AOX and AOR, and is unusual in that, except for the pterin cofactor, it contains no other redox-active groups.

The structures of both AOX and DMSOR reveal that their pterin cofactors also form a tricyclic system, with the same stereochemistry as observed in Pf AOR. Likewise, the molybdenum ion is coordinated by the dithiolene sulfurs of the pterin. However, unlike AOR, these enzymes contain the nucleotide form of the cofactor. The phosphate group is modified by CMP and GMP in AOX and DMSOR, respectively. Moreover, there is one pterin cofactor molecule per Mo atom in AOX, while, as in AOR, two pterin cofactors are coordinated to the Mo in DMSOR. Although there are extensive interactions between the pterin ring system and the protein in both AOX and DMSOR, the detailed interactions are distinct from each other and from those seen in the AOR structure.

The details of the metal coordination are also distinct for AOR, AOX, and DMSOR. In the AOX structure, the Mo coordination sphere in the oxidized (VI) state has two dithiolene sulfurs from a single pterin cofactor and three non-protein oxygen ligands. In DMSOR, the Mo coordination sphere in the oxidized (VI) state includes four dithiolene sulfurs from the two pterin cofactors (one of which has a long Mo−S distance of 3.1 Å), an oxo group, and the O^{γ} atom from the side chain of Ser147. Upon reduction of DMSOR to the reduced Mo(IV) form, the coordination sphere changes to four coordinate, with only three dithiolene sulfurs (again, with one long Mo−S bond distance at 2.9 Å) and the serine side chain still bound to the Mo. The fourth dithiolene sulfur shifts to a position too remote to directly bind Mo, at a distance of 3.7 Å. This behavior is suggestive that the substrate DMSO can bind to an open coordination site on the Mo, perhaps generated by the change in dithiolene geometry. There is also evidence that the coordination environment of Mo in DMSOR is sensitive to environmental conditions, such as the presence of glycerol, which perhaps can act as a substrate analog. More extensive analysis will be required to distinguish the consequences of metal coordination on the oxidation state and solution conditions for DMSOR. Note, however, that many of the conclusions already drawn about the structure of the pterin site in DMSOR are likely to be relevant to those of the tungstoenzymes, FMDH II and Ct FDH.

In the three pterin cofactor-containing enzymes that have been structurally characterized to date, the W-containing AOR and the Mo-containing AOX and DMSOR, the cofactor adopts a tricyclic system with a pyran ring fused to the pterin system. Metal coordination by the pterin cofactor in all cases utilizes the dithiolene sulfur groups. The remaining coordination sites are occupied by non-protein ligands such as oxo groups, water, or sulfur-containing groups, while in at least DMSOR, amino acid side chains may also bind to the metal. It seems likely that these pterin cofactors can participate in the redox mechanism of the enzymes, although direct demonstration of this has not been achieved. The functional significance of a nucleotide form of the cofactor, when present, is unclear. Likewise, the factors influencing whether a given protein contains one or two pterin ring systems are also unknown, although possible reasons are discussed in section VI.

It is clear from the AOR, AOX, and DMSOR structures that multiple protein folds can accommodate this cofactor, rather than having a unique protein structural motif associated with pterin binding. An important role for the protein appears to be to provide a predominantly buried environment for the metal site with regulated access for ligand binding. In this regard, pterins are rather like hemes or flavins in that these cofactors are found bound to a diverse set of proteins. There is no apparent reason from a protein structure perspective that would prevent the same polypeptide fold from being utilized to bind both W- and Mo-containing forms of the pterin cofactor. While no examples of this behavior have been structurally studied, it most likely reflects the relatively few numbers of pterin-containing enzymes that have been crystallographically characterized. For tungstoenzymes, on the other hand, the situation, at least at present, is more well defined. That is, except for AH, all can be placed into two families—AOR and F(M)DH—and within each there appears to be a high degree of structural similarity, with the AOR type reflecting variations on the structure of Pf AOR, while F(M)DH groups are closely related to the DMSOR structure.

IV. Spectroscopic Properties of Tungstoenzymes

Compared to the extensive electron paramagnetic resonance (EPR), X-ray absorption (XAS), electron−nuclear double resonance (ENDOR), resonance Raman, and variable-temperature magnetic circular dichroism (VTMCD) studies that have provided a wealth of mechanistic, structural and electronic information for molybdenum oxotransferases,[13−15] spectroscopic characterization of tungstoenzymes is limited to a handful of preliminary studies. Indeed, Pf AOR provides one of the few examples in metalloenzymes where the X-ray crystal structure has preceded detailed spectroscopic studies of the active site. Very recent results with AORs and FORs in particular demonstrate that spectroscopic studies have tremendous potential to provide insight into the structural diversity and catalytic mechanisms of tungstoenzymes. However, tungstoenzyme spectroscopy is still in its infancy, and the following should be viewed in the spirit of a progress report on what

is certain to be a rapidly evolving area of future research.

A. X-ray Absorption

So far, biological W XAS analyses have been limited to Ct FDH and Pf AOR. Preliminary W XAS studies of dithionite-reduced samples of Ct FDH were interpreted in terms of multiple W−O/N and W−S interactions at 2.13 and 2.39 Å, respectively, but surprisingly gave no evidence for W=O bonds.[101] However, the quality of the data prevented detailed assessment of the W coordination sphere and, in particular, left unresolved the question of W coordination by Se, which is known to be present as selenocysteine.[35] W XAS studies have been reported[102] for a dithionite-reduced form of Pf AOR known as the red tungsten protein (RTP). This species has since been found to be catalytically inactive (<3% of the activity of subsequent samples prepared by rapid, anaerobic purification in the presence of dithiothreitol and glycerol[52]). The results indicated a first coordination sphere involving two W=O at 1.74 Å, approximately three W−S at 2.41 Å, and possibly an additional W−O/N at 2.10 Å.[102] The presence of two oxo ligands strongly suggested a formal W(VI) oxidation state. Subsequent studies of dithionite-reduced active samples of AOR revealed only a single W=O at 1.75 Å, four or five W−S at 2.40 Å, and possibly an additional W−O/N at 1.97 Å.[103] This coordination sphere is in good agreement with the X-ray structure[41] that was obtained with crystals exhibiting good catalytic activity on dissolution. Moreover, it leaves open the question of the oxidation state of W in active enzyme and suggests that the dioxo W(VI) form corresponds to an inactive species.

B. EPR and VTMCD

Mo(V) and W(V) have d^1-configurations and generally exhibit EPR resonances with $g_{av} < 2$ that are slow relaxing and observable at temperatures above 100 K. Larger deviations from the free-electron g value are expected and observed for W(V) compared to Mo(V) due to much larger spin−orbit coupling (spin−orbit coupling constants are typically 3−5 times larger for W than Mo).[104,105] This usually translates to greater g value anisotropy and lower g_{av}-values for W(V) compared to equivalent Mo(V) species.[104−106] With the exception of recently discovered AH class of tungstoenzymes (Table 1), resonances attributed to W(V) species have been observed for each type of tungstoenzyme, i.e., AOR, FOR, GAPOR, CAR, and ADH from the AOR class and FDH and FMDH from the F(M)DH class (Table 2). In some cases, the assignment has been confirmed by observation of ^{183}W hyperfine ($I = {}^1/_2$, 14.4% natural abundance) in natural abundance or ^{183}W-enriched samples. In others, the assignment is based on relaxation properties and/or g values. While it is not obvious on initial inspection of the published data, we show here that the available EPR data can be interpreted in terms of two distinct classes of tungstoenzyme with different active site structures.

1. W-Substituted Sulfite Oxidase

The inactive W-substituted sulfite oxidase (SO) from livers of W-treated rats provides a useful starting point for the discussion of W(V) EPR signals in naturally occurring tungstoenzymes, since EPR studies strongly suggest identical coordination for Mo and W.[106] Recent mutagenesis results,[107] coupled with the EPR and Mo XAS data,[108] provide strong evidence for a Mo(V) coordination sphere in SO involving one oxo, one OH, one Cl (present only in the low pH form), one Cys S, and two S from the dithiolene side chain of a single pterin cofactor. The g value anisotropy of the Mo(V) resonance in sulfite-reduced native SO is dependent on pH and the presence of anions such as phosphate and fluoride, and this behavior is mirrored by the sulfite-induced W(V) signals of W-substituted SO.[106] For example, the low-pH Mo(V) species has $g_{1,2,3} = 2.007$, 1.974, 1.968 and $A_{1,2,3} = 56.7 \times 10^{-4}$, 25.0×10^{-4}, and 16.7×10^{-4} cm^{-1},[109] compared to $g_{1,2,3} = 1.98$, 1.89, 1.87 and $A_{1,2,3} = 81 \times 10^{-4}$, $\sim 41 \times 10^{-4}$, and $\sim 41 \times 10^{-4}$ cm^{-1} for the low-pH W(V) species,[106] and the high-pH Mo(V) species has $g_{1,2,3} = 1.990$, 1.966, 1.954 and $A_{1,2,3} = 54.4 \times 10^{-4}$, 21.0×10^{-4}, and 11.3×10^{-4} cm^{-1},[109] compared to $g_{1,2,3} = 1.93$, 1.87, and 1.84 (A-values not determined) for the high-pH W(V) species.[106] These results illustrate that the overall line shape is preserved, albeit with greatly amplified g value anisotropy, on substitution of Mo(V) by W(V). Hence, they provide a structural benchmark for these two types of biological W(V) resonance. In addition, they show that the line widths are substantially larger (approximately doubled), the metal hyperfine is larger and simpler to interpret (^{183}W ($I = {}^1/_2$) vs ^{95,97}Mo ($I = {}^5/_2$)), and the resonances are less readily saturated for W(V) compared to the equivalent Mo(V) species. The increased line width of the W resonance is particularly significant since it obscures proton hyperfine splitting. For example, the proton splitting (~0.7 mT) on the low-field component of the low-pH resonance is clearly resolved for the Mo(V) resonance but is only apparent when H_2O and D_2O samples are compared for the equivalent W(V) resonance. To our knowledge, this is the only proton hyperfine that has been observed thus far for any biological W(V) resonances. Detailed EPR and/or ^{1}H ENDOR studies of all W(V) species in H_2O and D_2O will be required to assess the possibility of directly coordinated OH^-, SH^-, or H_2O.

2. AOR and FOR

In principle, Pf AOR should provide another structurally characterized benchmark for interpreting biological W(V) EPR signals. However, recent EPR studies of this enzyme,[110] and the closely related ES-4 AOR[110] and Tl FOR,[111] have revealed that the W centers in the active forms of these enzymes are not homogeneous. Two, three, or four distinct W species, each with distinctive W(V) EPR signals and redox properties, are present in each enzyme. However, the close similarity in the nature of the W(V) EPR signals, at least two of which are common to all three enzymes, attests to similar active site structures involving coordination by two pterin cofactors for both AORs and FORs.[110,111] The W(V) resonances in these

Table 2. EPR *g*-Values, ^{183}W *A*-values ($cm^{-1} \times 10^4$), and Midpoint Potentials (mV vs NHE) for Biological W(V) Centers and Model Complexes

sample	g_{av}	A_{av}	g_1	g_2	g_3	A_1	A_2	A_3	E_m (pH 7.8)[a] "W^{IV}/W^{V}"	E_m (pH 7.8)[a] "W^{V}/W^{VI}"	ref
Models											
$[WO(bdt)_2]^-$ [b]	1.962	52	2.044	1.931	1.911	78	40	37			131
$[WO(edt)_2]^-$ [b]	1.973		2.105	1.919	1.894						132
$[WO(mnt)_2]^-$ [b,c]	1.959		2.104	1.913	1.860						133
$[WO(SPh)_4]^-$	1.936	55	2.018	1.903	1.903	78	44	44			105
$[WO(S\text{-}p\text{-}tolyl)_4]^-$	1.935	55	2.014	1.903	1.903	78	44	44			105
$[WO(SePh)_4]^-$	1.971	51	2.086	1.923	1.923	74	43	43			105
Enzymes											
P. furiosus AOR											110
spin coupled									<−550	−443	
low potential	1.918	42	1.989	1.901	1.863	52	27	46	−436	−365	
glycerol inhibited	1.930	61	1.965	1.941	1.884	67	37	79	−355	>+100	
glycol inhibited	1.928	60	1.958	1.938	1.887	66	35	79	nd[j]	nd	
midpotential	1.963		1.988	1.961	1.940				−345	>+100	
high potential	1.949	51	1.992	1.962	1.892	48	43	62	+157	>+300	
re-reduced 1	1.989		2.021	1.988	1.957				optimal at ∼−190 mV		
re-reduced 2	1.905		1.949	1.916	1.850				optimal at ∼−190 mV		
Pyrococcus sp. ES-4 AOR											110
low potential	nd		nd	1.900	1.860				−385	−310	
glycerol inhibited	1.927		1.961	1.940	1.881				−315	>+300	
high potential	1.945		1.986	1.960	1.890				+50	>+300	
T. litoralis FOR											
low potential	1.905		1.976	1.898	1.842				−335	−280	
midpotential	1.954		1.983	1.950	1.930				−34	>+110	
high potential	1.940	52	1.981	1.954	1.884	45	52	58	+184	>+320	
P. furiosus GAPOR[d]											116
spin coupled	1.891		1.954	1.890	1.830						
D. gigas ADH[e]											45
spin-coupled 1	1.898		1.943	1.902	1.850						
spin-coupled 2	1.894		1.949	1.889	1.844						
glycerol-inhibited 1	1.925		1.974	1.932	1.869						
glycerol-inhibited 2	1.919		1.962	1.921	1.874						
C. formicoaceticum CAR[f]	1.964		2.035	1.959	1.899						
	2.016		2.028	2.017	2.002						
C. thermoaceticum FDH[g]	2.010		2.101	1.980	1.950				≤−450		117
W-sub. *M. wolfei* FMDH[h]	2.008	40	2.049	2.012	1.964	48	43	28			94
W-sub. rat liver SO[i]											106
low pH	1.91	∼54	1.98	1.89	1.87	81	∼41	∼41			
high pH	1.88		1.93	1.87	1.84						

[a] Midpoint potentials at pH 7.8 for the appearance and disappearance of W(V) EPR signals in oxidative or reductive redox titrations. [b] Abbreviations used: bdt, 1,2-benzenedithiolate, $(S_2C_6H_4)^{2-}$; edt, 1,2-ethanedithiolate, $(S_2C_2H_4)^{2-}$; mnt, 1,2-dicyanoethylenedithiolate, $(S_2C_2(CN)_2)^{2-}$. [c] W^V species formed *in situ* by oxidation of W(IV) complex with I_2. [d] Dithionite reduced. [e] As purified in the presence of dithionite. [f] As purified in the absence of dithionite. The assignment of these sets of *g* values to W(V) species may not be correct, and a possible reassignable is given in the text. [g] Poised at −450 mV vs NHE, pH not specified. [h] Air-oxidized, W-substituted Mo−FMDH. [i] Generated by reduction with sulfite. [j] nd, not determined.

enzymes are by far the best characterized in terms of redox properties and ^{183}W coupling constants, and the picture that has emerged for the origin, activity, and possible structures of each W species is outlined below.

The EPR spectra of AORs and FORs at 4 K are dominated by resonances from $S = {}^3/_2$ $[4Fe\text{-}4S]^+$ clusters, see Figure 8. A rhombic resonance, $g = 4.7$, 3.4, and 1.9, that originates from the lower doublet of a zero-field split $S = {}^3/_2$ ground state ($E/D = 0.12$, $D = +4.0 \pm 0.5$ cm^{-1}) is common to all three AORs investigated thus far, i.e., from Pf, ES-4 and ES-1.[38,55,110] While mixed spin, $S = {}^3/_2$ and $S = {}^1/_2$, $[4Fe\text{-}4S]^+$ clusters have been observed in many iron−sulfur proteins, clusters that have exclusively $S = {}^3/_2$ ground states with predominantly axial zero-field splitting are unique to AORs. FORs can be distinguished from AORs on the basis of the EPR properties of their $[4Fe\text{-}4S]^+$ clusters. Both Pf and Tl FORs have mixed-spin $[4Fe\text{-}4S]^+$ clusters with a dominant $S = {}^3/_2$ component that is evident by a broad low-field resonance at $g = 5.4$ ($E/D = 0.33$ predicts effective *g* values of 5.4, 2.0, and 1.4 for both doublets) and a minor $S = {}^1/_2$ component, $g = 2.03$, 1.93, and 1.86.[56,57]

Inactive samples of dithionite-reduced Pf AOR (RTP) also exhibit a broad, complex resonance spanning almost 600 mT, with inflections at $g = 10.0$, 6.5, 5.4, 3.6, 2.9, 2.3, 1.8, 1.6, and 1.3, that is fast relaxing and only observable below 20 K.[38] EPR redox titrations indicated that this complex resonance arises from weak spin−spin interaction between the $S = {}^3/_2$ $[4Fe\text{-}4S]^+$ cluster and a lower potential center that becomes paramagnetic only on one-electron reduction and is present in a 1:1 stoichiometry with the cluster.[38] Subsequent VTMCD studies and broadening of the complex resonance in ^{183}W-enriched samples have shown that this lower potential center is a $S = {}^1/_2$ W(V) species.[110] In hindsight, it is now apparent that our inability to observe magnetically isolated W(V) in Pf RTP is a consequence of almost all the W being present in the inactive W(VI)/W(V) species that

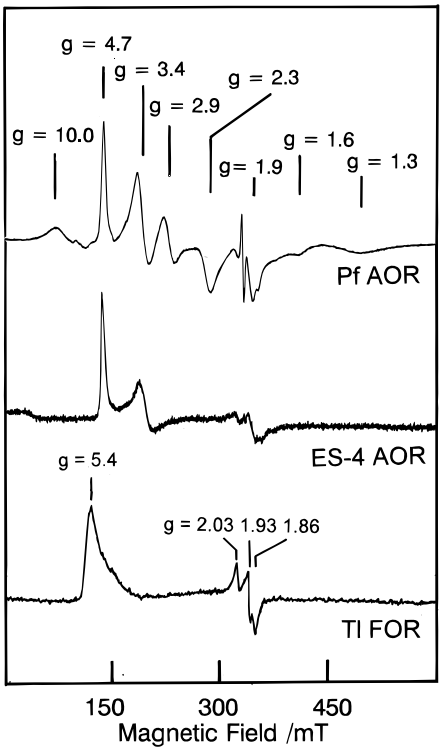

Figure 8. EPR of active, as-prepared samples of dithionite-reduced Pf AOR, ES-4 AOR, and Tl FOR. Conditions of measurement: temperature, 7 K; microwave power, 10 mW; modulation amplitude, 1.02 mT; microwave frequency, 9.60 GHz.

results in the complex spin-coupled resonance on reduction. Active samples of Pf AOR from this laboratory invariably exhibit the same complex resonance (Figure 8), but it is present at levels ~20% of those found in RTP. In contrast, this complex resonance is much weaker or not observed in active samples of ES-4 and ES-1 AORs.[55,110] While the W EXAFS results for Pf RTP indicate that the W(VI) species (and possibly the W(V) species) responsible for this inactive form is likely to have dioxo coordination, in the absence of EPR g values it is not possible to evaluate more precisely the nature of this W(V) species (the possibility that this W(V) species corresponds to those observed in Pf GAPOR and Dg ADH is discussed in sections IV.B.3 and IV.B.4). Moreover, it is not to be equated with the product of O_2 inactivation of active AOR. The spin-coupled W(V) species was not enhanced in samples of Pf and ES-4 AOR that were inactivated by prolonged exposure to O_2 followed by dithionite reduction.[110]

A "low-potential" magnetically isolated W(V) species, $g = 1.98-1.99$, 1.90, and 1.85−1.86 (Figure 9a) is common to all active AORs and FORs investigated thus far.[110,111] This resonance increases and subsequently decreases in intensity in oxidative redox titrations indicative of closely spaced W^{IV}/W^{V} and W^{V}/W^{VI} couples and is maximal at −400 mV in Pf AOR, −350 mV in ES-4 AOR, and −310 mV in Tl FOR. Maximally it accounts for approximately 0.15−0.20 spins/W, which translates to a W species corresponding to ~30% of the W in each enzyme, given the separation of the midpoint potentials (Table 2). Moreover, all the available evidence indicates that this pool of W is catalytically competent.[110] First, this resonance is observed in samples treated anaerobically with the substrate under physiological conditions, 85 °C, and then frozen rapidly for EPR. Second, it is the only resonance that is lost, together with enzymatic activity, when AORs are treated anaerobically at 85 °C with high concentrations (60−70% (v/v)) of diols such as glycerol or ethylene glycol. Third, while redox cycling of this species is reversible for potentials in the range −500 to 0 mV, this W(V) resonance is not observed and enzymatic activity is irreversibly lost in Pf AOR samples that have been exposed to O_2 or anaerobically oxidized to +300 mV. In its place, re−reduced samples of Pf AOR have been found to exhibit two new W(V) resonances that are maximal at ~−190 mV, $g = 2.021$, 1.988, and 1.957 (re-reduced 1) and $g = 1.949$, 1.916, and 1.850 (re-reduced 2).

Our current working model for the structure of the low-potential W(V) species is shown in Figure 10. It involves W coordination by three dithiolene sulfurs with the fourth only weakly attached, in addition to a single oxo and hydroxy ligand. In large part, this model is based on the close similarity in the g values and ^{183}W A values with those of the low-pH W(V) species in W-substituted SO and the available crystallographic data for Pf AOR; see above. The suggestion that one of the dithiolene sulfurs is only weakly coordinated originates from the recent crystal structure of Rs DMSOR[43] and is tentatively supported by the correspondence with W-substituted SO and the g_{av}-value, which is significantly lower than that of crystallographically W(V) model complexes with bis(dithiolene) or four thiolate ligands (Table 2). Studies of oxo−Mo model complexes suggest a direct correlation between the number of thiolate ligands and the g_{av} value.[109] Further work involving the effect pH, D_2O/H_2O exchange, and the presence of anions such as fluoride, chloride, or phosphate, is clearly required to test the current model.

As indicated above, the active W species responsible for the low-potential W(V) resonance in AORs is irreversibly and quantitatively converted into a diol-inhibited form by the addition of high concentrations of glycerol or ethylene glycol at physiological temperatures. This form exhibits a characteristic W(V) resonance ($E_m(W^{IV}/W^{V}) \sim -350$ mV) that is dependent on the specific diol, i.e., $g = 1.965$, 1.941, and 1.884 for the glycerol-inhibited W(V) species and $g = 1.958$, 1.938, and 1.887 for the glycol-inhibited W(V) species in Pf AOR, and persists at potentials up to at least +100 mV (Table 2 and Figure 9).[110] Partial (10−20%) conversion to the glycerol-inhibited form also occurs in AORs purified in the standard purification and storage buffer, which contains 10% (v/v) glycerol. In contrast, FORs are not affected by the addition 50% (v/v) glycerol at room temperature; this may be a consequence of the differences in the active site accessibility[111] (see section IV). A very similar glycerol-inhibited Mo(V) species with equivalent g value anisotropy has also been observed in Rs DMSOR, $g = 1.990$, 1.984, and 1.962.[112,113]

The similarity between the glycerol-inhibited Mo(V) center in Rs DMSOR and the glycerol- or glycol-inhibited W(V) species in Pf AOR extends to the excited state electronic structure as revealed by VTMCD studies (Figure 11).[110,112] Both exhibit an

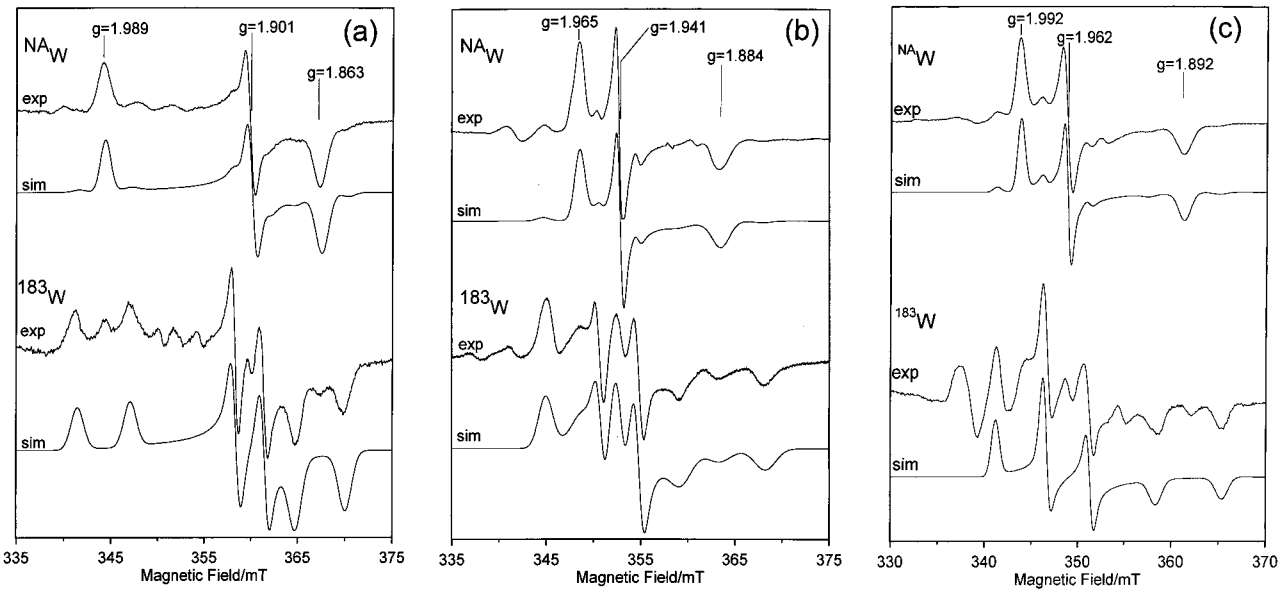

Figure 9. ^{NA}W- and ^{183}W-enriched EPR spectra and simulations for W(V) species in Pf AOR: (a) ^{NA}W- and ^{183}W-enriched low-potential W(V) species, prepared by anaerobic incubation of dithionite-reduced enzyme at 85 °C with 2 mM formaldehyde followed by and rapid freezing in liquid N_2; (b) ^{NA}W- and ^{183}W-enriched glycerol-inhibited W(V) species, prepared as (a) except for the addition of 60% (v/v) glycerol after the addition of formaldehyde; (c) ^{NA}W- and ^{183}W-enriched high-potential W(V) species, prepared by poising a sample at +250 mV (vs NHE) via ferricyanide oxidation in the presence of mediator dyes. Only the ^{183}W component is simulated in the ^{183}W-enriched samples, and the resulting parameters were used to simulate the ^{NA}W spectra (14.4% $I = {}^1/_2$ ^{183}W; 85.6% $I = 0$ W isotopes). The simulation parameters are given in Table 2. Conditions of measurement for experimental spectra: temperature; 40−50 K; microwave power, 1 mW; modulation amplitude, 1.02 mT; microwave frequency, 9.60 GHz. Taken from ref 110.

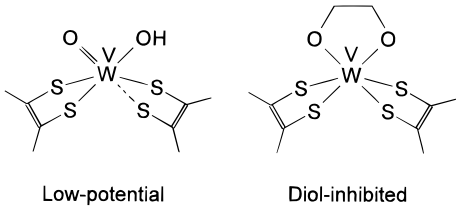

Figure 10. Proposed structures for the low-potential and diol-inhibited W(V) species in AOR-type tungstoenzymes.

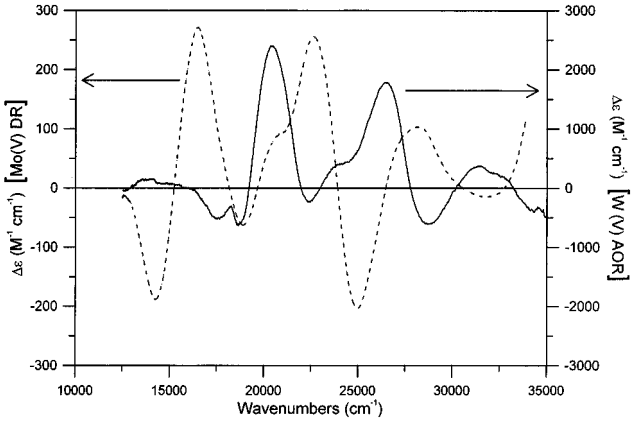

Figure 11. Comparison of the VTMCD spectra for the glycol-inhibited W(V) species in Pf AOR (solid line) and the glycerol-inhibited Mo(V) species in Rs DMSOR (broken line). The spectra were recorded at 4.2 K with a magnetic field of 4.5 T, and the spin values are based on the W^V/Mo^V concentrations as assessed by quantitation of parallel EPR samples. Taken from ref 110.

analogous pattern of temperature-dependent MCD bands with corresponding transitions shifted up in energy by ~4000 cm^{-1} for W relative to Mo. The transitions in the 12 000−35 000 cm^{-1} region can be rationally assigned to S → Mo/W charge transfers from four thiolate ligands,[110] and the increase in energy for W is consistent with the higher energy of the W 6d orbitals. The intensities of the corresponding transitions (normalized to the Mo(V) and W(V) concentrations as assessed by EPR spin quantitations) are 1 order of magnitude larger for W. This is in accord with the larger spin−orbit coupling for W and illustrates the potential of VTMCD to provide an extremely sensitive optical probe for W(V) centers, even in the presence of paramagnetic Fe-S clusters. The close similarity in the ground and excited state electronic properties of the diol-inhibited Mo^V/W^V forms of Rs DMSOR and Pf AOR indicates very similar coordination environments. Hence the proposed structure for the diol-inhibited W(V) species in AORs, shown in Figure 10, is based largely on the recent Mo XAS data for the glycerol-inhibited Mo(V) form of DMSOR[113] and the available crystallographic data for DMSOR[43] and AOR,[41] as discussed in section III.

EPR redox titrations of AORs and FORs revealed two additional W(V) species that appeared at higher potentials. A minor "midpotential" W(V) species, g ~ 1.99, 1.96, and 1.94, accounting for <0.05 spin/W, appears with midpoint potential of −351 mV in Pf AOR and −34 mV in Tl FOR and persisted up to at least +100 mV. At still higher potentials, a major "high-potential" W(V) species, g = 1.98−1.99, 1.96, and 1.89, accounting for ~0.3 spin/W, appears with a midpoint potential of +157 mV in Pf AOR, +50 mV in ES-4 AOR, and +184 mV in Tl FOR and persists up to at least 300 mV (Figure 9). Both W(V) resonances are the product of reversible redox processes. While the high-potential W(V) resonance results from a form of W that constitutes ~30% of the W in the enzyme, it does not appear to arise from

a functional form of W. The main evidence for this is that it is also observed without change in intensity or redox properties in samples that have been irreversibly inactivated by prolonged exposure to O_2. Moreover, such a high-potential redox process is unlikely to be physiologically relevant in light of the extremely low potentials of acid/aldehyde couples, <-500 mV.[71] Clearly the obvious interpretation is to assign this species to a catalytically inactive W^{IV}/W^{V} couple. However, in light of the high midpoint potential, it is possible that this net one-electron oxidation primarily involves ligand oxidation. For example, two-electron oxidation of one of the coordinated dithiolene ligands coupled with one-electron reduction of a W(VI) center could equally well account for a net one-electron oxidation. The possibility of ligand-based oxidation is supported by VTMCD studies which show major differences in the electronic transitions for the high-potential W(V) species compared to the diol-inhibited W(V) species in Pf AOR.[110] For example, S → W(V) charge transfer transitions occur to much lower energies (down to 10 000 cm^{-1}) for the high-potential W(V) species.[110] Such ligand-based redox chemistry is not without precedent in the chemistry of thio−molybdenum centers.[114,115] While it is premature at this juncture to suggest structures or assign the VTMCD spectrum of the high-potential W(V) species, it is important to recognize that it could originate from a catalytically inactive W(IV) or W(VI) species in the as-purified samples of FORs and AORs.

The overall picture that emerges is that the W sites in active AORs and FORs are not homogeneous in the samples as prepared. There at least two major components with each corresponding to ~30% of the W in the enzyme. One of these cycles between the $W^{IV}/W^{V}/W^{VI}$ states at physiological relevant potentials (−450 to −250 mV at 25 °C) and is responsible for catalytic activity and the low-potential W(V) resonance. The other is an inactive W(VI) or W(IV) species that gives rise to the high-potential W(V) species at nonphysiologically relevant potentials (>0 mV). In addition, purified samples of Pf AOR, in particular, can have up to 20% of the W as an additional inactive species that is responsible for the spin-coupled W(V) species and cycles between the W^{VI}/W^{V} states with a midpoint potential close to −450 mV. W XAS studies indicate that this species is likely to be dioxo−W species in one or both of the available oxidation states. The available X-ray crystallographic and W XAS data for Pf AOR are also likely to correspond to average structures with W(IV), W(V), and W(VI) species all contributing.

3. GAPOR

GAPOR has only recently been purified from Pf, and spectroscopic studies have been limited to preliminary absorption, EPR, and VTMCD.[58,116] The enzyme is purified under anaerobic conditions, but in the absence of dithionite, which is an inhibitor producing a reversible loss in activity.[58] However, reduction with dithionite does result in the appearance of a slow-relaxing rhombic $S = {}^1/_2$ resonance, $g = 1.954$, 1.890, and 1.830, accounting for ~0.5 spin/W, that is observable without significant broadening up to at least 100 K.[116] ^{183}W satellites were not clearly resolved, but the relaxation properties, coupled with all three g values of <2, point to a W(V) species. However, this resonance is quite distinct from those observed in FORs and AORs, with the possible exception of the re-reduced 2 signal in Pf AOR. On the basis of the very low g_{av} value ($g_{av} = 1.891$; see Table 2), it seems likely that this W center is coordinated by no more than three sulfur ligands. This suggests that the W center in GAPOR may be coordinated by the dithiolene side chain of a single pterin cofactor or that two pterin cofactors are attached with one being weakly coordinated through only one dithiolene sulfur in reduced forms, as is the case for the Mo center in Rs DMSOR.[43]

The VTMCD spectra of dithionite-reduced Pf GAPOR are dominated by transitions that are readily identified with a $[4Fe\text{-}4S]^{+}$ cluster and magnetization studies indicate a $S = {}^1/_2$ ground state.[116] However, the characteristic EPR signal of a $S = {}^1/_2$ $[4Fe\text{-}4S]^{+}$ cluster was not observed. Instead, EPR studies at temperatures of <20 K revealed a broad fast-relaxing resonance centered around $g \sim 1.9$ that is attributed to spin−spin interaction between the W(V) species and a *lower* potential $[4Fe\text{-}4S]^{+}$ cluster. In accord with this interpretation, this resonance increases in intensity, while the magnetically isolated W(V) species decreases in intensity, in samples treated with a larger excess of dithionite at higher pH. Since this W(V) species can interact magnetically with the $[4Fe\text{-}4S]^{+}$ and appears to correspond to an inactive form of W, it is tempting to correlate this with the spin−coupled W(V) species in Pf AOR (see section IV.B.2). In the case of Pf AOR, the magnetic interaction is between a W^{VI}/W^{V} species and a higher potential $[4Fe\text{-}4S]^{2+/+}$ cluster ($S = {}^3/_2$ ground state for the reduced cluster) and this prevents direct observation of the g values for the spin−coupled W(V) species. More detailed spectroscopic and redox studies of Pf GAPOR are currently in progress.

4. ADH

Preliminary EPR and UV−visible absorption data have been reported for the W-containing ADH from Dg.[45] The anaerobically purified enzyme (in 10 mM phosphate buffer with 1 mM dithionite, 2 mM dithiotheitol, and 9% glycerol) exhibits a complex W(V) resonance which has been resolved by simulations as the sum of four separate resonances.[45] Two of the major components, $g = 1.943$, 1.902, and 1.850 (40%) and $g = 1.949$, 1.889, and 1.844 (24%), have g values most similar to those of the spin-coupled W(V) species in Pf GAPOR. The other two components, $g = 1.974$, 1.932, and 1.869 (26%) and $g = 1.962$, 1.921, and 1.874 (10%) have g values close to those of the glycerol-inhibited W(V) species in Pf AOR. This observation is important in two respects. First, it suggests that the presence of glycerol in the purification buffers is responsible in large part for the heterogeneity at the W site. Second, it suggests a similar active site structure to Pf AOR with bis(dithiolene) coordination from two pterin cofactors. Loss of the W(V) resonances was reported on reduction with larger excesses of dithionite at higher pH, and this occurred with concomitant increase of a

poorly resolved, fast-relaxing resonance with apparent g values of 2.04 and 1.92, indicative of a $S = {}^1/_2$ $[4Fe\text{-}4S]^+$ cluster magnetically interacting with a nearby paramagnet.[45] The results were tentatively interpreted in terms of all four W species having W^V/W^{IV} midpoint potentials of <-400 mV with $S = 1$ W(IV) responsible for the magnetic interaction. However, paramagnetic W(IV) is *extremely* unlikely in a low-symmetry, biological environment. A more plausible interpretation in light of the results for Pf AOR and GAPOR (see above) is that the glycerol-inhibited W(V) species are lost due to reduction to a W(IV) state, but that the GAPOR-type W(V) species are lost because of spin−spin interaction with a $[4Fe\text{-}4S]^{2+/+}$ cluster ($S = {}^1/_2$ ground state for the reduced cluster) that has a midpoint potential less than that of the W^{VI}/W^V couple. Our current working hypothesis is that the unique W(V) species with similar g values seen in Dg ADH and Pf GAPOR, $g = 1.94-1.96$, 1.89−1.90, and 1.83−1.85, correspond to the inactive spin−coupled W(V) species in Pf AOR (see section IV.B.2).

5. CAR

The W-containing Ct CAR is purified anaerobically but in the absence of dithionite and has been investigated by UV−visible absorption and EPR both as-prepared and after dithionite reduction.[69] In common with the other oxidized tungstoenzymes in the AOR class, the UV−visible absorption spectrum of the as-prepared enzymes comprises a broad band at 400 nm indicative of the presence of $[4Fe\text{-}4S]^{2+}$ cluster. Weak EPR resonances, accounting in total for 0.1 spin/molecule, were observed at 30 K for the same enzyme preparation. On the basis of power- and temperature-dependence studies that were not shown, the spectrum was deconvoluted into two resonances, $g = 2.035$, 1.959, and 1.899 and $g = 2.028$, 2.017, and 2.002, and both were attributed to W(V) species. However, this analysis and interpretation seems unlikely since W(V) resonances with all g values greater than 2 are experimentally unprecedented. In light of the EPR data discussed above for AORs and FORs,[110,111] this spectrum can be reinterpreted in terms of a high-potential W(V) resonance of the type seen in FORs and AORs, $g = 1.98-1.99$, 1.96, and 1.89, with the low-field component obscured by the intense derivative centered around 2.01 that most likely originates from impurities or $[3Fe\text{-}4S]^+$ clusters produced via partial oxidative degradation of $[4Fe\text{-}4S]^{2+}$ clusters. The origin of the feature at $g = 2.035$ is unclear at present, but weak derivative-shaped features in this region have been observed in some oxidized samples of FORs and AORs.[110,111] The observation of a W(V) EPR signal from Ct CAR that is also observed in the structurally characterized Pf AOR suggests a similar active site structure for both enzymes. Dithionite-reduced samples of Ct CAR exhibited a spectrum at 16 K that was simulated with $g = 2.047$, 1.926, and 1.914.[69] In the absence of temperature-dependence studies, this can be attributed to either a magnetically isolated $S = {}^1/_2$ $[2Fe\text{-}2S]^+$ or $[4Fe\text{-}4S]^+$ cluster, with the latter assignment more probable in light of the absorption spectra.

6. FDH

Ct FDH was not only the first tungstoenzyme to be purified to homogeneity, it was also the first enzyme in which a biological W(V) resonance was observed.[117] In addition to EPR resonances attributed to two $[2Fe\text{-}2S]^+$ and two $[4Fe\text{-}4S]^+$ clusters, a novel slow-relaxing resonance, $g = 2.101$, 1.980, and 1.950, accounting for 0.08 spin/W and observable up to 200 K, was reported in samples poised at −450 mV.[117] On the basis of relaxation properties, Deaton et al.[117] argued that this slow-relaxing resonance was due to a W(V) species, despite the anomalous g values, i.e., $g_{av} > 2$ and one g value of $\gg 2$. The essence of the argument was ligand field calculations that readily rationalize one g value of >2 in W(V) or Mo(V) complexes having low-energy charge transfer excited states.[117] Moreover, this effect should be enhanced for Se as opposed to S coordination due to lower energy charge transfer and larger ligand spin−orbit coupling. Although direct Se coordination in Ct FDH has yet to be proven by W XAS, EPR studies of the homologous Mo-containing FDH_H from Ec[118] lend support to the view that the anomalous EPR properties of the W(V) center in Ct FDH are at least in part a consequence of W coordination by selenocysteine. Selenocysteine coordination of the Mo in Ec FDH_H has been elegantly demonstrated by EPR studies of ^{77}Se-enriched samples ($I = {}^1/_2$) and mutants in which selenocysteine was substituted with cysteine.[118] Moreover, the presence of selenocysteine appears to be responsible for the anomalous g values of the formate-induced Mo(V) species, $g = 2.094$, 2.001, and 1.989, since a more normal Mo(V) resonance, $g = 1.995$, 1.984, and 1.973 (estimated from the published spectrum) is observed in the SeCys → Cys mutant. This mutant is functional, albeit with greatly reduced activity.

As discussed above, the genes for the two subunits of Ct FDH have recently been cloned and sequencing is in progress.[83] On the basis of partial sequence alignments it seems likely that this enzyme is a member of the monooxo Mo(VI) containing family of enzymes, typified by the crystallographically defined Rs DMSOR.[83] This family of enzymes has conserved residues interacting with the two guanine-dinucleotide pterin cofactors and a single conserved protein ligand (Ser, Cys or SeCys). By analogy with Rs DMSOR,[43] a structure for the W(V) form of Ct FDH is proposed in Figure 12. The anomalous EPR properties are therefore attributed to the S-rich environment provided by the two dithiolene side chains, the coordinated selenocysteine and the lack of an oxo ligand in the W(V) state. Once again this proposal should be viewed in the spirit of a working model and additional spectroscopic and redox studies are clearly required.

7. FMDH

No W(V) EPR signals have been reported for the naturally occurring W-containing FMDHs (FMDH II) from Mt and Mw. However, the W-substituted form of Mw FMDH I, which appears to have activity comparable to that of the nonsubstituted Mo enzyme, exhibited a W(V) resonance, $g = 2.049$, 2.012, and 1.964 (0.63 spin/W), in air-oxidized samples at 55 K.[94]

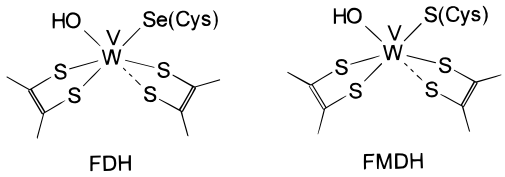

Figure 12. Proposed structures for active W(V) species in Ct FDH and Mw FMDH.

The observation of well-resolved ^{183}W satellites unambiguously identified this resonance as a W(V) species and facilitated estimation of ^{183}W hyperfine coupling constants (Table 2). While the g_{av}-value ($g_{av} = 2.008$) is significantly higher than that of any of the W(V) resonances from the AOR class, the A_{av}-value ($A_{av} = 40 \times 10^{-4}$ cm^{-1}) is significantly smaller. Such behavior is indicative of an increase in the number of S donors and/or the absence of an oxo ligand for the W(V) species in air-oxidized Mw FMDH I. It is tempting to interpret this result in terms of a W(IV) oxidation state in the as-isolated enzyme. However, the fact that this resonance is observed as a stable W(V) species in air-oxidized samples raises the possibility that it is primarily the result of ligand oxidation at nonphysiological potentials. Such a possibility was discussed above for the stable high-potential W(V) species in AORs and FORs, and clearly similar chemistry originating from a W(VI) species could be occurring here in FMDH.

Air-oxidized, inactive samples of Mo-FMDH I from Mw, Mt, and Mb all exhibit a similar EPR resonance, $g = 2.00-2.01$, 1.98−1.99, and 1.94−1.95.[91,93] Active samples of substrate-reduced or as-prepared samples of Mo−FMDH I have an additional albeit very similar Mo(V) resonance, $g = 2.00-2.01$, 1.98−1.99, and 1.94−1.95, that disappears on oxidation, in addition to more rapidly relaxing resonances from $S = {}^1/_2$ [2Fe-2S]$^+$ and [4Fe-4S]$^+$ clusters.[91,93] However, it is not appropriate to compare the W(V) and Mo(V) EPR signals in Mw FMDH I given the widely different conditions required to elicit these resonances, and the relationship, if any, between these resonances is unclear at present.

The complete amino acid sequence of the four subunits of W−FMDH II from Mt has been recently determined.[40] On the basis of the recent structure of Rs DMSOR,[43] the β-subunit clearly has the conserved residues required for interacting with two guanine dinucleotide pterin cofactors, with cysteine as the protein ligand to W in this case. Hence, even in the absence of EPR data for an active W(V) species in a W−FMDH, it is possible to propose a structure by analogy with Ct FDH (Figure 12). Further EPR experiments on this enzyme are awaited with great interest.

The preliminary picture that emerges, therefore, from the available spectroscopic and sequence data for tungstoenzymes is of two distinct classes, both with W coordinated by the dithiolene side chains from two pterin cofactors. In the AOR class, the W is coordinated by non-nucleotide forms of the pterin cofactors but it has no protein ligands. In the F(M)-DH class, the W is coordinated by the GMP form of the pterin cofactor with one cysteine or selenocysteine ligand. While there is considerable heterogeneity at the W center in the AOR type, one or more resonances analogous to those in Pf AOR are also found in other AORs, as well as FOR, GAPOR, ADH, and CAR. EPR studies suggest that the active W(V) center has an oxo and possibly an hydroxyl ligand which would imply a dioxo−W(VI) form. However, by analogy with xanthine oxidase, the possibility that this represents a slow form of the enzyme with the rapid form corresponding to mixed oxo−thio−W^{VI} species that is formed under physiological sulfiding conditions cannot be discounted at this stage. In contrast, the W(V) forms of the F(M)DH-type probably have a single hydroxyl as the sixth ligand, which would imply a monooxo−W^{VI} form.

V. Synthetic Analogs of the Tungsten Site

Structurally characterized synthetic analogs of the active sites of W- and Mo-containing enzymes are essential for the interpretation of spectroscopic results and for addressing the fundamental differences in the chemistry of equivalent Mo and W complexes. However, the chemistry of oxo−W complexes with S donor ligands has been slow to develop compared to the equivalent Mo chemistry. This is primarily a consequence of the difficulty in reducing W(VI) species to corresponding W(IV) species.[119] Indeed the thermodynamic instability of oxo−W^{IV} complexes compared to the equivalent oxo−Mo^{IV} complexes raised questions concerning the catalytic competence of such centers in oxo transfer reactions.[119] However, appropriate structural and functional models have recently become available involving sulfur-rich ligation with bis(dithiolate) and bis(dithiolene) ligands.[120,121] This leads to the hypothesis that bis-(dithiolene) coordination via two pterin cofactors may be required for the W sites of all tungstoenzymes in order to tune the W^{VI}/W^{V}/W^{IV} redox potentials to the range required for catalyzing low-potential biological oxotransferase reactions such as acid/aldehyde transformations.

Numerous hexacoordinated dioxo−W^{VI} complexes relevant to tungstoenzyme systems have been reported. These include W^{VI}O$_2$(R$_2$dtc)$_2$ (R = Et, Me; dtc = dithiocarbamate),[119,122] [NH$_4$]$_2$[W^{VI}O$_2$(O$_2$CC(S)-Ph$_2$)$_2$],[123] and Schiff base complexes of the type W^{VI}O$_2$-(ssp) (ssp = 2- (salicylideneamino)benzenethiolate).[119] However, none could be reduced to stable W(IV) and oxygen atom transfer reactions resulted in the formation of a W(V) dimer.[119] In addition W(VI) disulfur complexes of the type W^{VI}X(S$_2$)(S$_2$CNR$_2$)$_2$ (X = O, S; R = Me, Et) with an η^2-S$_2$ ligand have been synthesized.[124−126] Recently the synthesis was reported of a hexacoordinate W(IV) complex with no oxo ligands that is clearly relevant to the active site of Ct FDH.[127] This complex, LW^{IV}(SePh){S$_2$C$_2$(Ph)(2-quinoxalinyl)} (L = hydrotris(3,5-dimethyl-1-pyrazolyl) borate), involves a coordination by a selenate and a dithiolene in addition to the three nitrogens from L.

As indicated above, stable monooxo−W^{IV}/W^{V} and dioxo−W^{VI} complexes have recently been synthesized with bis(benzene-1,2-dithiolate) (bdt) and bis(1,2-dicyanoethylene-1,2-dithiolate) (mnt) ligation,[120,121] and the structures are shown in Figure 13. The

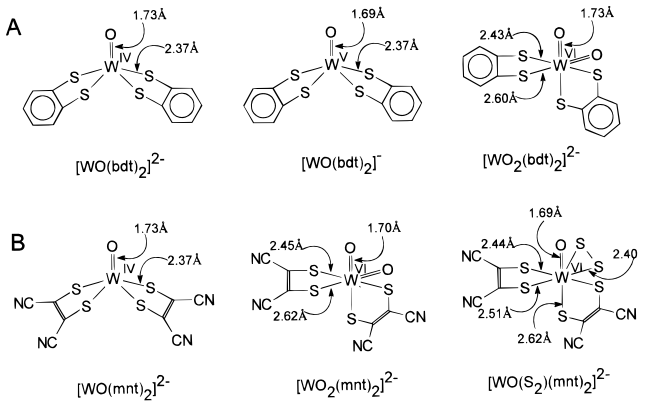

Figure 13. Structures for W(IV), W(V), and W(VI) bis-(dithiolate) or bis(dithiolene) model complexes. The structures shown in (A) with the benzene-1,2-dithiolate (bdt) ligand are taken from ref 120. The structures shown in (B) with the 1,2-dicyanoethylene-1,2-dithiolate (mnt) ligand are taken from ref 121. Average bond lengths for each distinct type of W=O or W−S bond are indicated.

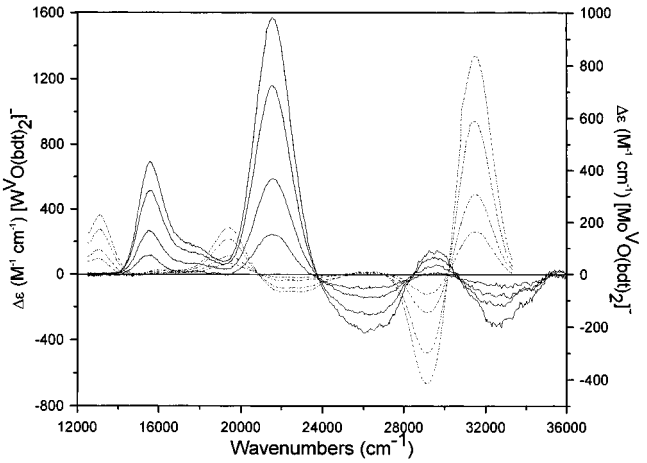

Figure 14. Comparison of VTMCD spectra for $[W^{V}O(bdt)_2]$ (solid lines) and $[Mo^{V}O(bdt)_2]$ (dot−dash lines) in 60:40 toluene−DMF. The spectra were recorded at 6 T at temperatures of 1.7, 4.2, 10, and 50 K, and all transitions increase in intensity with decreasing temperature.[133]

monooxo−W^{IV} and −W^{V} complexes have square-pyramidal coordination whereas the dioxo−W^{VI} complexes have distorted octahedral coordination geometry with the W−S bonds trans to the oxo groups longer by ∼0.17 Å than those cis to the oxo groups. A stable $[W^{V}O(mnt)_2]$ complex has yet to be crystallized, but refluxing the $[W^{IV}O(mnt)_2]^{2-}$ complex in acetone the presence of elemental S resulted in the formation of a W(VI) complex with a terminal oxo and a η^2-S_2 ligand, $[W^{VI}O(S_2)(mnt)_2]^{2-}$ (Figure 13), similar to those reported with the $S_2CNR_2^{2-}$ ligand.[124−126] Such complexes or related species may be present in AORs from hyperthermophilic archaea, which grow at high temperatures in sulfur-rich environments. The ability of the $[W^{IV}O(bdt)_2]^{2-}$ and $[W^{VI}O_2(bdt)_2]^{2-}$ to participate in oxygen atom transfer reactions was demonstrated using trimethylamine *N*-oxide and benzoin as model substrates.[120] By comparing the structures and resonance Raman spectra with those of the equivalent Mo complexes, it is apparent that both the W^{VI}−S and W^{VI}=O bonds are significantly stronger than the equivalent Mo^{VI}−S and Mo^{VI}=O bonds in the oxidized complexes due to greater π-interactions.[120,128] This is also the case for the W^{IV}−S bonds compared to the Mo^{IV}−S bonds, but the W^{IV}=O and Mo^{IV}=O bonds strengths are comparable in the reduced complexes.[120,129] Sarkar and Das have shown that $[W^{IV}O(mnt)_2]^{2-}$ is able to reduce CO_2/HCO_3^- to $HCOO^-$ in aqueous media in the presence of dithionite, in a reaction that mimics the W−FDH reaction.[129] In addition they have demonstrated the sulfur reductase activity of $[W^{IV}O(mnt)_2]^{2-}$ by showing that it catalyzes the reactions $Ph_3P + S \rightarrow Ph_3PS$ and $H_2 + S \rightarrow H_2S$, and the AOR activity of $[W^{VI}O(S_2)(mnt)_2]^{2-}$ by showing that it reacts with crotonaldehyde to form crotonic acid.[121]

The most detailed comparison of the EPR properties of oxo−W^{V} and oxo−Mo^{V} complexes with sulfur- or selenium-rich environments comes from studies of complexes of the type $[M^{V}O(XPh)_4]$ (M = Mo, W; X = S, Se).[105,130] These complexes have effective C_{4v} symmetry and give rise to axial EPR signals with ^{183}W or ^{98}Mo hyperfine interactions; $g_{\|} = 2.02$, $g_{\perp} = 1.90$, and $A_{\|} = 78 \times 10^{-4}$ cm^{-1}, $A_{\perp} = 44 \times 10^{-4}$ cm^{-1} for $[W^{V}O(SPh)_4]^-$, compared to $g_{\|} = 2.02$, $g_{\perp} = 1.98$, and $A_{\|} = 52 \times 10^{-4}$ cm^{-1}, $A_{\perp} = 22 \times 10^{-4}$ cm^{-1} for $[Mo^{V}O(SPh)_4]^-$; $g_{\|} = 2.09$, $g_{\perp} = 1.92$, and $A_{\|} = 74 \times 10^{-4}$ cm^{-1}, $A_{\perp} = 43 \times 10^{-4}$ cm^{-1} for $[W^{V}O(SePh)_4]^-$, compared to $g_{\|} = 2.07$, $g_{\perp} = 2.00$, and $A_{\|} = 48 \times 10^{-4}$ cm^{-1}, $A_{\perp} = 21 \times 10^{-4}$ cm^{-1} for $[Mo^{V}O(SePh)_4]^-$. Such studies provide the groundwork for correlating *g* values and *A* values between equivalent oxo−W^{V} and −Mo^{V} complexes in sulfur-rich environments and show that Se coordination produces an increase in g_{av} and a decrease in A_{av}, relative to S coordination.

Model complexes with dithiolate chelates are more relevant to biological W centers, and as expected, this removes the axial symmetry, resulting in rhombic EPR signals, e.g., $g = 2.044$, 1.931, and 1.911 for $[W^{V}O(bdt)_2]^-$, compared to $g = 2.023$, 1.986, and 1.977 for $[Mo^{V}O(bdt)_2]^-$;[131] $g = 2.105$, 1.919, and 1.894 for $[W^{V}O(edt)_2]^-$ (where edt = ethylene-1,2-dithiolate) compared to $g = 2.012$, 1.997, and 1.975 for $[Mo^{V}O(edt)_2]^-$;[132] $g = 2.104$, 1.913, and 1.860 for $[W^{V}O(mnt)_2]^-$,[133] compared to $g = 2.015$, 1.982, and 1.960 for $[Mo^{V}O(mnt)^2]^-$.[134] Compared to W(V) resonances in AOR-type tungstoenzymes (Table 2), the high g_{av}-values and the presence of one *g* value of >2 indicates that such symmetrical oxo−W(V) complexes are unlikely to be good structural models for any W(V) species identified thus far. This is supported by recent VTMCD studies of $[M^{V}O(bdt)_2]^-$ complexes (M = W, Mo) Figure 14.[135] The VTMCD spectrum of $[W^{V}O(bdt)_2]^-$ is quite distinct from that observed thus far for any W(V) species in AORs. As expected, the pattern of bands is the same for both the Mo and W complexes with equivalent transitions shifted to higher energy by ∼3000 cm^{-1} and exhibiting greater VTMCD intensity for the W complex. VTMCD studies of such structurally characterized model complexes are crucial for the emerging understanding of the excited state electronic structure of Mo(V) and W(V) centers in sulfur-rich environments, and the spectra shown in Figure 14 have been assigned exclusively to S → Mo^{V}/W^{V} charge transfer transitions from a square-pyramidal arrangement of thiolate ligands.[135]

VI. Why Tungsten and Not Molybdenum?

Finally, we come to the questions of why some microorganisms have chosen to use W rather than Mo at the active site of key enzymes, why some can utilize both elements, and why the majority of life forms on this planet use exclusively Mo. As a preface to considering these issues, it is useful to summarize some of the differences between relevant oxo−W and oxo−Mo complexes with sulfur donor ligands[120,121] and relate them to differences in the physiological functions of biological W and Mo centers. In other words, under what conditions are such complexes stable and maintain the IV, V, and VI oxidation states within the biological range of temperature (0−110 °C) and reduction potential (approximately −500 to +800 mV, vs NHE)? For example, the $[W^{IV}O(mnt)_2]^{2-}$ and $[W^{IV}O(bdt)_2]^{2-}$ complexes are *very* oxygen sensitive compared to the equivalent Mo complexes, indicating that the W complex would need to be rigorously protected from O_2 if used in aerobic organisms. Similarly, the strongly enhanced thermal stability of W(VI) complexes compared to Mo(VI) complexes, due to stronger π-interactions, suggests that only the former would be stable enough to be utilized at temperatures near the normal boiling point. This enhanced bond strength of W(VI) complexes may also account for the observation that such complexes are generally kinetically slower than the equivalent Mo(VI) complexes in oxygen atom transfer reactions, as in acid/aldehyde interconversion. Thus, one would expect that higher temperatures might be a requirement for W centers to catalyze reactions at high rates, reactions that could be performed at similar rates by Mo centers at much lower temperatures. In addition, the much lower redox potentials for the W complexes compared to the Mo complexes (typically 300−400 mV more negative) indicate they are better suited to catalyze lower potential redox processes. The problem, however, is stabilizing the W(IV) state within the biological range, and from the discussion in section V, it seems that bis(dithiolene) coordination is likely to be an obligate requirement for tuning the W redox potentials to the appropriate level. Even then, one would expect reactions to be limited to the lower potentials, such that W sites could be involved in catalyzing the interconversion of formate/CO_2 ($E_o' = -432$ mV),[71] but not nitrate/nitrite ($E_o' = +420$ mV).[71] On the other hand, Mo complexes can be stabilized throughout this potential range.

Hence, *a priori*, one would predict that W could only be utilized by biological systems to catalyze low-potential reactions under anaerobic conditions and then significant catalytic rates would be observed only at high temperatures. Conversely, the instability of relevant Mo complexes at high temperatures might preclude their utilization, otherwise such complexes should be catalytically competent over the whole biological range of potentials under both aerobic and anaerobic conditions. Remarkably, our current knowledge of the properties of tungstoenzymes fits this scenario quite well. With the exception of AH, where the catalytic function of W is unknown, all the other tungstoenzymes listed in Table 1 catalyze reactions of extremely low potential (≤ -420 mV vs NHE). The values for the carboxylic acid/aldehyde, CO_2/formylmethanofuran, and CO_2/formate couples are −580, −497 and −432 mV, respectively.[71,136] Similarly, all of the organisms that contain these enzymes are obligate anaerobes, so the W sites of their enzymes are not normally exposed to O_2. Interestingly, there is evidence that a tungstoenzyme is present in some aerobic bacteria,[46,47] so in this case there would appear to be some protective mechanism. One would also expect that molybdoenzymes could substitute for tungstoenzymes in organisms that grow at low or moderate temperatures, and this also is the case. With the notable exception of the hyperthermophiles, the organisms that produce the tungstoenzymes listed in Table 1 also synthesize Mo-containing isoenzymes. These include Mo-containing forms of FMDH (FMDH I) in the thermophilic methanogens (optimal growth, T_{opt} 50−60 °C),[87−89] CAR (Mo-AOR)[69,72] and FDH[35,137] in mesophilic (T_{opt} ~30 °C) and thermophilic acetogens, ADH in mesophilic sulfate reducers (AOX),[73] and AH in the mesophilic acetylene utilizer.[44] Unfortunately, there are no data to test the prediction that a given tungstoenzyme would be less active at a given temperature than its Mo counterpart. The FMDH system is the only one for which virtually identical Mo- and W-containing isoenzymes are available, but these enzymes are extremely unstable with dramatic losses of activity during purification, which precludes meaningful comparisons.

On the other hand, AOR, FOR, and GAPOR of hyperthermophiles such as Pf (T_{opt} 100 °C) are not replaced by Mo-containing isoenzymes during cell growth in the presence of Mo with no added W, and all three enzymes appear to be obligately dependent upon W for catalytic activity. All three enzymes also catalyze the oxidation of aldehydes of one type or another, reactions that have one of the lowest reduction potentials in biochemistry. Thus, we previously suggested[59] that these hyperthermophilic organisms might be carrying out some chemical conversions near the limits of biological systems—very low potential reactions at extreme temperatures. Moreover, it seems that it is only under such conditions that the chemical properties of W, but not Mo, are compatible with enzyme catalysis. Indeed, hyperthermophiles frequently inhabit and thrive not only in hot but also in sulfiding conditions, such as found near deep sea hydrothermal vents. It seems likely that W, but not Mo, would be capable of remaining in the VI oxidation state under these conditions, a state that is essential for catalytic activity. Of course, we should also consider the opposite situation: can molybdoenzymes function at 100 °C? Thus, it will be intriguing to determine whether the FMDH of the hyperthermophilic methanogen *Methanopyrus*, which grows up to 110 °C, exists as both Mo- and W-containing forms. Similarly, is the nitrate reductase (nitrate/nitrite, $E_o' = +420$ mV)[71] of *Pyrobaculum*, a nitrate-reducing microaerophilic archaeon (T_{opt} 100 °C),[139] a Mo or a W-containing enzyme? From the above discussion we would predict that only W-containing FMDHs can function near 100 °C, and since it would seem unlikely that the W(IV) oxidation state could be stabilized near +400 mV, even at high

temperatures, the hyperthermophilic nitrate reductase is likely to be a molybdoenzyme. It will be interesting to determine whether such predictions are correct.

VII. Conclusions

Even just five years ago a review of this type would not have been possible. At that time, only a couple of tungstoenzymes were known and a role for tungsten in biology was largely unappreciated. Now more than a dozen tungstoenzymes have been purified, and for some of them, gene sequences and even one crystal structure are available. We have demonstrated herein that the known tungstoenzymes fall into two major classes. The AOR type, which are found in hyperthermophilic archaea and in acetogenic and sulfate-reducing bacteria, are "true" tungstoenzymes in that they are phylogenetically distinct from the major classes of molybdoenzyme. We suggest that they all evolved from an ancestral aldehyde-oxidizing enzyme of the type now represented by AOR in the hyperthermophiles. Based on Pf AOR, which is the only tungstoenzyme whose structure is known, it seems likely that all of the tungstoenzymes in the AOR class contain a W atom that is coordinated in part by four S atoms from two molecules of the non-nucleotide form of the pterin cofactor but not by protein ligands. In contrast, the other class of tungstoenzyme, the F(M)DH type, appears from sequence comparisons to be closely related to a major class of molybdoenzyme, which includes FDH, biotin sulfoxide, and DMSOR, the structure of which was recently determined. This showed that the Mo atom of DMSOR is coordinated by a side chain of a serine residue together with four S atoms from two molecules of the GMP form of the pterin cofactor. We suggest that the F(M)DH type of tungstoenzyme is structurally very similar to DMSOR, but with cysteine or selenocysteine replacing the active site serine. However, although the two classes of tungstoenzyme must have diverged very early on the evolutionary time scale, a common feature of their W sites is coordination by a bis(dithiolene) group from two pterin cofactors. Such coordination may well be a requirement for tuning the $W^{VI}/W^{V}/W^{IV}$ potentials to the appropriate range for low-potential oxotransferase reactions. From a comparison of the chemical properties of analogous W and Mo complexes it is apparent that W should only be utilized by biological systems to catalyze low-potential reactions (more negative than NHE) under anaerobic conditions, with high catalytic rates only at high temperature, and to a large extent these conclusions appear valid. Moreover, only the hyperthermophilic archaea, which contain three distinct types of aldehyde-oxidizing tungstoenzyme, are obligately W-dependent, as tungstoenzyme-producing organisms that grow at lower temperatures also synthesize Mo-containing isoenzymes to catalyze the same reaction. It appears that the hyperthermophiles require W to catalyze certain conversions that are near the limits of biological systems—very low potential reactions at extreme temperatures—a requirement fulfilled by W but not by Mo.

VIII. Abbreviations

Abbreviations used: AOR, aldehyde ferredoxin oxidoreductase; FOR, formaldehyde ferredoxin oxidoreductase; GAPOR, glyceraldehyde-3-phosphate ferredoxin oxidoreductase; Fd, ferredoxin; CAR, carboxylic acid reductase; ADH, aldehyde dehydrogenase; AOX, aldehyde oxidase; HVOR, hydroxycarboxylate viologen oxidoreductase; FDH, formate dehydrogenase; FMDH, *N*-formylmethanofuran dehydrogenase; AH, acetylene hydratase; DMSOR, DMSO reductase; RTP, red tungsten protein; SO, sulfite oxidase; GAP, glyceraldehyde 3-phosphate; Pf, *Pyrococcus furiosus*; Tl, *Thermococcus litoralis*; Ct, *Clostridium thermoaceticum*; Cf, *Clostridium formicoaceticum*; Dg, *Desulfovibrio gigas*; Pv, *Proteus vulgaris*; Mf, *Methanobacterium formicicum*; Mt, *Methanobacterium thermoautotrophicum*; Mw, *Methanobacterium wolfei*; Pa, *Pelobacter acetylenicus*; Rs, *Rhodobacter sphaeroides*; Ec, *Escherichia coli*; EPR, electron paramagnetic resonance; VTMCD, variable-temperature magnetic circular dichroism; XAS, X-ray absorption; EXAFS, extended X-ray absorption fine structure; ENDOR, electron−nuclear double resonance; dtc, dithiocarbamate; ssp, 2-(salicylideneamino)benzenethiolate; bdt, bis(benzene-1,2-dithiolate); mnt, bis(1,2-dicyanoethylene-1,2-dithiolate); edt, ethylene-1,2-dithiolate.

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