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MODELING THE MOLYBDENUM CENTERS OF THE MOLYBDENUM HYDROXYLASES

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CONTENTS

ABBREVIATIONS

AO	aldehyde oxidase
EPR	electron paramagnetic resonance
EXAFS	extended edge X-ray absorption fine structure
hae	*N*,*N*′-bis(2-hydroxyphenyl)-1,2-diaminoethane
hbpd	*N*,*N*′-bis(2-hydroxybenzyl)-*o*-phenylenediamine
hfs	hyperfine splitting
mab	*N*,*N*′-bis(2-mercaptophenyl)-2,3-diaminobutane
mae	*N*,*N*′-bis(2-mercaptophenyl)-1,2-diaminoethane
mee	*N*,*N*′-bis(2-mercaptoethyl)ethylenediamine
mpe	*N*,*N*′-bis(2-mercapto-2-methylpropyl)ethylenediamine
nit-1	*N. crassa* mutant lacking nitrate reducing ability
SDS	sodium dodecyl sulfate
shfs	superhyperfine splitting

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SO	sulfite oxidase
tox	8-mercaptoquinoline
XAS	X-ray absorption spectroscopy
XDH	xanthine dehydrogenase
XO	xanthine oxidase

A. INTRODUCTION

The molybdenum hydroxylases comprise a group of multicomponent enzymes which catalyze two electron oxidations of purines, aldehydes and sulfite in animals and microorganisms [1,2]. This group includes xanthine oxidase, xanthine dehydrogenase, aldehyde oxidase, sulfite oxidase and the as yet poorly characterized formate dehydrogenases [3]. In addition, a molybdenum nonheme iron protein of unknown function isolated from *Desulfovibrio gigas* [4] and the recently reported carbon monoxide:methylene blue oxidoreductase [5], isolated from bacteria which utilize carbon monoxide as a carbon source, appear to have properties similar to the hydroxylases (Table 1).

TABLE 1

Molybdenum hydroxylases

Enzyme and source	*g*-atoms/Mo molecule enzyme	Other cofactors	Substrate
Xanthine oxidase (bovine milk)	2	4 Fe_2S_2 2 FAD	Purines
Xanthine dehydrogenase (avians, insects, microorganisms)	2	4 Fe_2S_2 2 FAD	Purines
Aldehyde oxidase (mammals)	2	4 Fe_2S_2 2 FAD	Aldehydes
Sulfite oxidase (mammals, fish microorganisms)	2	2 cytochrome *b*	SO_3^{2-}
Formate dehydrogenase (microorganisms)	4	4 cytochrome *b* 4 Se, 56 Fe_nS_n W(?)	CO_2 (HCOOH)
Mo protein from *Desulfovibrio gigas*	2?	3 Fe_2S_2	?
Carbon monoxide: methylene blue oxidoreductase (microorganism)	2?	Fe_nS_n FAD	CO

While the function of xanthine oxidase in mammals has not been firmly established, evidence indicates a role in xanthinuria, gout and uricemia [1,2], and a recent controversial hypothesis proposes it to be a causative factor in atherosclerosis and coronary heart disease in humans [6a]. Recent work also suggests it may be involved in iron absorption in mammals [6b]. Sulfite oxidase appears to be responsible for sulfite detoxification in humans and its absence has been linked to neurological abnormalities, mental retardation and early death in children [7]. As a result of these findings, and of the great interest in metallobiomolecules, there is currently considerable research concerning the structure and function of the molybdenum centers of these enzymes.

The literature concerning molybdenum chemistry of biological interest and the biochemistry of the molybdenum hydroxylases has been comprehensively reviewed in a number of articles [1,2,8–10]. The present review will first consider the most recent results of EPR (electron paramagnetic resonance), XAS and EXAFS (X-ray absorption spectroscopy and extended X-ray absorption fine structure), and electrochemical investigations in the context of structural possibilities and limitations for the enzyme molybdenum centers. Next, the problems these results pose for model studies will be discussed. Finally, research directions which might be explored in seeking solutions to these problems will be suggested.

B. BIOCHEMICAL RESULTS

(i) EPR

Early EPR investigations established Mo(V) (a $4d^1$ ion) as an intermediate during reduction of the enzymes by substrate or dithionite [1,2]. A number of different Mo(V) EPR signals were observed, depending on conditions and substrate. With xanthine oxidase (XO) for example, four major types of signals have been described: very rapid, a reduced form with bound substrate; rapid, reduced forms without bound substrate; slow, arising from the denatured (cyanolized, desulfo) enzyme; and inhibited, resulting from complexes of the Mo(V) center with certain inhibitors [1,2]. While initial two electron reduction of the enzyme by substrate gives the EPR-silent Mo(IV) state, rapid equilibration of electrons between the Mo, Fe_2S_2 and flavin centers, governed by the reduction potentials of the various couples, results in the appearance of the Mo(V) signal. Only upon complete reduction (six electrons) of all centers does the Mo(V) signal disappear; all states may be detected during turnover experiments. A mathematical model, based on the relative reduction potentials of the redox centers, gives reasonable agreement with the experimental results [11].

EPR experimental results are generally expressed in g values (determined

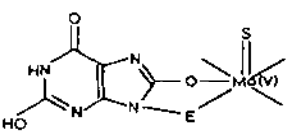
by the ratio of the magnetic field (H) to the microwave frequency at which resonance occurs and A values which indicate the strength of coupling of magnetic nuclei ($I \neq 0$) to the Mo(V) signal. Coupling of the $I = 5/2$ ^{95,97}Mo nuclei, which make up approximately 25% of naturally occurring Mo, results in hyperfine splitting (hfs) of the Mo(V) signal; coupling of other magnetic nuclei results in superhyperfine splitting (shfs). Both parameters give information concerning the coordination environment of Mo. EPR g values for the enzymes have been measured with considerable precision, using computer simulation techniques. Hfs of ^{95,97}Mo has not been resolved in most cases because of line width and sensitivity problems. Substitution of ^{95}Mo in the enzymes would permit the determination of the ^{95}Mo coupling constants; this can only be achieved, however, by ^{95}Mo feeding experiments, and has been reported only for the very rapid signal of XO [2,12,13].

Superhyperfine splitting (shfs) of the Mo(V) EPR signal by exchangeable protons has been observed for XO, XDH and SO during substrate turnover [1,2]. In the case of the rapid signal for XO, it has been shown the proton responsible originates from the C-8 position of xanthine and is bound to the enzyme during catalysis [1,2]. More recently, splitting by a second proton has been reported [14]. The location of these protons with respect to Mo has been the subject of some controversy. Additional shfs by ^{17}O incorporated by exchange in $H_2{}^{17}O$, has been observed, and the coupling constant for this splitting of 1.6 mT has been taken as evidence for the presence of ligated oxygen on Mo [15]. Similar coupling of ^{17}O to Mo(V) has also been found with sulfite oxidase (SO) [16].

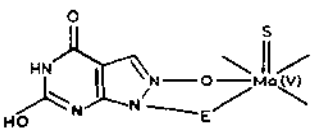
The presence of a labile sulfide, necessary for catalytic activity has been demonstrated for XO and XDH [1,2,17]. Treatment of these enzymes with CN^- liberates the sulfur as SCN^- with consequent loss of enzymatic activity. Activity of the desulfo form can be restored by addition of sulfide to the reduced enzymes, and this was made use of to label XO with ^{33}S [18]. Coupling of this ^{33}S to the Mo(V) signal was observed, providing evidence for a sulfur ligand on Mo. Most recently coupling of ^{33}S to the various Mo(V) signals seen with XO has been studied in detail, and the ^{33}S coupling constants determined [19]. Strong, highly anisotropic coupling of 1.27 mT to the very rapid signal and weaker coupling (0.3–0.4 mT) to the rapid signal was found. It was suggested the large coupling of the very rapid signal arises from a sulfur in an equatorial position (in the plane of the d_{xy} orbital), while the weaker coupling seen for the rapid signal comes from a sulfur in an axial position (orthogonal to the plane of the d_{xy} orbital) [17].

Treatment of XO with NO_3^-, which binds to reduced enzyme, affects the Mo(V) signal; from this it was concluded the enzyme has an anion binding site which binds the substrate xanthine at the N-9 position in a Michaelis complex [20]. It is not clear whether the anionic binding site is on Mo or

another site of the enzyme close to Mo. Further evidence concerning substrate binding comes from experiments with the XO inhibitor, allopurinol. Inhibition results from complex formation of the reduced (Mo(IV)) enzyme by the product of oxidation of allopurinol, alloxanthine. By briefly exposing the inhibited enzyme to air, a new, broader Mo(V) EPR signal was generated, which was attributed to a one electron oxidized product, a Mo(V) complex with alloxanthine [21]. This signal shows no ^1H coupling; upon treatment with a computer line sharpening technique, however, this signal gave evidence of resolution into three lines arising from isotropic coupling of 0.36 mT of a single ^{14}N. This is the first observation of ^{14}N coupling to Mo(V) found in an enzyme and suggests binding of the inhibitor to Mo at the N-8 position of alloxanthine [21]. This EPR signal is very similar, except for line broadening, to the very rapid signal seen with xanthine, which is thought to be bound to Mo at C-8 via a Mo–O bond [2].

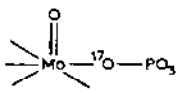


xanthine enzyme complex

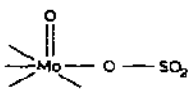


alloxanthine enzyme complex

Treatment of partially reduced SO with ^{17}O labeled PO_3^{3-} produces an Mo(V) signal coupled to the ^{17}O; this coupling remains after equilibration of the enzyme with H_2O [22]. This was attributed to a Mo(V)PO_4^{3-} complex bound by an ^{17}O of the phosphate group. Similarly, a "dead end" complex of SO_3^{2-} with reduced SO has been detected at low pH in which SO_3^{2-} is proposed to be bound to Mo(V) by a sulfite oxygen [23]; neither signal shows evidence of ^1H coupling.

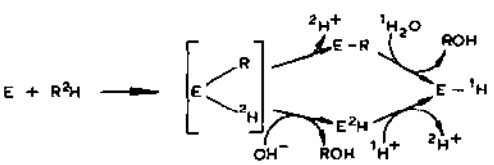


$P^{17}O_4^{3-}$ complex



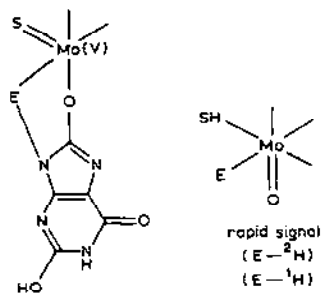
SO_3^{2-} complex

The EPR signals observed for active XO in the presence of substrate have been rationalized by the following scheme [2]



In this scheme, xanthine labeled with ^2H in C-8 (R^2H) is bound at the anionic site via N-9. This Michaelis complex then breaks down by two pathways. In the upper pathway, $^2H^+$ is lost, xanthine is bound to Mo by an

Mo–O–R bond at C-8 (E–R) and two electrons are transferred from xanthine to Mo, reducing it to Mo(IV). One electron oxidation of this complex by flavin or Fe_2S_2 gives the Mo(V) complex responsible for the very rapid signal (no H coupling). In the lower pathway, nucleophilic attack by OH^- (or H_2O) and simultaneous transfer of two electrons to Mo gives uric acid and ^2H is transferred to the Mo sulfido group (E–^{2}H). Rapid one electron oxidation by flavin or Fe_2S_2 gives the Mo(V) rapid (H coupled) signal



Hydrolysis of E–R and H^+ exchange of E–^{2}H give the same product (E^{1}H), which also gives the rapid (H coupled) signal.

A rearrangement must take place in the transition from the very rapid signal species to the rapid signal species, since very rapid has large (1.27 mT) ^{33}S coupling, while rapid signal ^{33}S coupling is much smaller (0.3–0.4 mT) [19]. For the rapid signal which is strongly coupled to ^1H (or ^2H), it might be expected that the ^{33}S coupling would also be large, since the proton is presumably an SH proton. The small ^{33}S coupling observed in the rapid signal, however, might indicate the proton is, in fact, located on another ligand (the strongly coupled ^{17}O, for example). Alternatively, it was suggested the ^{33}S might lie on a nodal plane of the d_{xy} orbital, giving minimum overlap, while the nonlinear Mo–SH bond could result in the proton projecting from the nodal plane into a region of greater overlap, accounting for the larger ^1H coupling [19]. Such disparities of coupling constants have been observed with Mo(V) complexes with N–H ligands, in which the ^1H coupling is much larger than the ^{14}N coupling [23].

There is no direct evidence that the proton responsible for the shfs of the Mo(V) EPR signal in XO and XDH is bound to sulfur, although the hypothesis seems reasonable. Furthermore, the location of the second proton which is more weakly coupled is unknown. It may possibly be located on the nitrogen of an amino or imidazole group (vide infra). While the EPR parameters for the Mo(V) signals suggest additional sulfur ligands (high g,

low *A*), no further conclusions concerning ligand donors may be drawn from the EPR results.

For SO and the desulfo forms of XO and XDH, the sulfido is replaced by a second oxo group, and, in the reduced forms, the proton responsible for the ^1H coupling is presumed to be an OH proton [2]. For SO this proton appears to originate from the HSO_3^- substrate since it is not seen at high pH, where the substrate is present as SO_3^{2-} [25].

The EPR spectra for aldehyde oxidase (AO) have recently been reported [26]. The Mo(V) signals were found to be quite similar to those from XO and it was concluded the ligands of Mo and the overall coordination geometries must be essentially the same for the two enzymes.

The EPR data is found in Table 2.

(ii) XAS, EXAFS

Much data concerning the Mo centers of the hydroxylases has recently been obtained from absorption edge (XAS) and extended fine structure (EXAFS) spectroscopy. The absorption edge is characteristic of an element and, to some extent, of its environment. The EXAFS technique has been particularly useful, since it provides much more information about the identity and number of donor atoms and Mo–donor bond lengths. The EXAFS spectrum arises from back scattering of the electron wave of the ejected K shell electron by atoms in the coordination sphere of Mo. While bond length measurements are relatively precise (± 0.01 Å) the number of donors of a given kind can only be determined to ca. $\pm 20\%$, and differences between atoms of similar mass (e.g. O vs. N) are difficult to detect; no information concerning bond angles can be obtained by solution EXAFS. Nevertheless, EXAFS is a powerful technique for probing the metal centers of enzymes and proteins. In a field in which X-ray crystal structures are non-existent, it has provided, in combination with EPR, the most important data yet available. For a discussion of the EXAFS technique, see ref. 27.

The most recent EXAFS data for XDH indicates a terminal oxo and a terminal sulfido are coordinated to Mo in the oxidized (Mo(VI)) state of the native enzyme [28]. In addition, two thiolate sulfurs are present. Inasmuch as Mo(VI) is almost always 6 coordinate, 2 other donors may be present (in the case of the Mo(VI) complex, $MoO_2(SCH_2CH_2)_2NCH_2CH_2SCH_3$, the Mo–N bond, known to be present from the X-ray structure, was not detected by EXAFS) [29]. Upon reduction, the short (2.15 Å) Mo=S bond disappears and an additional thiolate sulfur at 2.38 Å is found. In the desulfo (cyanolized) enzyme, the Mo=S has been replaced with a terminal oxo (Mo=O), in agreement with the previous results obtained by CN^- treatment of the enzyme [2,17]. These results are in general agreement with previous EXAFS

TABLE 2

Mo hydroxylase Mo(V) EPR parameters

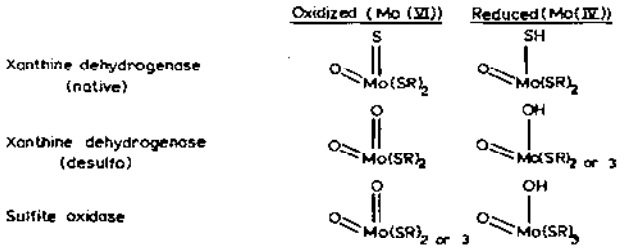
Enzyme	Signal	Parameter [a]	1 [b]	2 [b]	3 [b]	Average	Ref.
Xanthine oxidase	Very rapid	g	2.025	1.955	1.949	1.976	2, 64
		A (^{95}Mo)	4.1	2.4	3.7	3.4	
		A (^{17}O)	1.34	1.40	1.36	1.37	13
		A (^{33}S)	0.30	2.80	0.70	1.27	17
	Rapid type 1	g	1.990	1.970	1.965	1.975	2, 65
	(xanthine)	A (^{1}H)	1.20	1.30	1.30	1.27	
		A (^{1}H)	1.47	0	0	0.16	
	Rapid type 2	g	1.995	1.971	1.961	1.976	2, 66
	(xanthine)	A (^{1}H)	1.07	0.98	0.93	1.01	
		A (^{1}H)	1.07	0.98	0.93	1.01	
	Inhibited	g	2.0269	1.9593	1.9444	1.9769	19
	(alloxanthine)	A (^{14}N)	0.33	0.36	0.39		
Xanthine dehydrogenase	Very rapid	g	2.023	1.954	1.949	1.975	2, 67
Xanthine oxidase	Slow	g	1.971	1.967	1.955	1.964	2, 65
(desulfo)		A (^{1}H)	1.66	1.56	1.56	1.63	
		A (^{1}H)	0.16	0.16	0.16	0.16	

Xanthine dehydrogenase (desulfo)	Slow	g	1.971	1.968	1.957	1.965	2, 67
		A (^{1}H)				1.6	
Sulfite oxidase	Low pH	g	2.003	1.972	1.965	1.980	2, 25
		A (^{95}Mo)	6.3				
		A (^{1}H)	0.85	0.80	1.20	0.95	
		A (^{17}O)		0.80	0.80		16
	High pH	g	1.987	1.964	1.953	1.968	2, 25
		A (^{95}Mo)			5.5		
		A (^{17}O)	1.1				16
	Sulfite complex	g	2.000	1.972	1.963	1.978	21
	Phosphate complex	g	1.9917	1.9692	1.9614	1.9741	20
		A (^{17}O)	0.10	1.30	1.25		
Aldehyde oxidase	Inhibited (HCHO)	g	1.9926	1.9764	1.9501	1.9730	63
		A (^{1}H)	0.65	0.52	0.70	0.62	

[a] A values in mT.

[b] Numbers 1, 2, 3 indicate anisotropic g and A values as they appear in the spectra.

investigations of XO [30] and the two enzymes undoubtedly have essentially identical Mo centers. With SO, two oxo groups and 2–3 thiolates sulfurs are present in the oxidized (Mo(VI)) state, and one oxo and three thiolate sulfurs in the reduced (Mo(IV)) state. The data do not unambiguously indicate the presence of other donors in either state; an oxygen with a Mo—O bond length corresponding to Mo—OH (2.04 Å) in the reduced state is compatible with the data, and in view of the EPR evidence for ^1H coupling, seems probable. The results, incorporating conclusions from EPR studies as well, are summarized below [28].



In all cases, it is assumed an oxo or sulfido group of the oxidized enzyme is converted to an OH or SH group upon reduction, although the data cannot distinguish between SH and SR, and may not detect other ligands such as NH. Furthermore, the EPR data requires the proton coupled to the Mo(V) EPR center to be present in the Mo(V) state, for which no EXAFS data is available [30].

Additional evidence for the presence of a sulfido group on Mo in oxidized XO, XDH and AO and its conversion to SH on reduction has been obtained from studies of the reactivation by sulfide of the desulfo forms of these enzymes [17]. It was demonstrated that the oxidized forms (Mo(VI)) of the enzymes are labile towards CN^-, producing SCN, while the reduced forms (Mo(IV)) are inert. Reactivation by sulfide, on the contrary, occurs readily only from the reduced (Mo(IV)) state, accounting for the synergistic effect of dithionite on the reactivation process. As was observed earlier, removal of labile sulfur leaves the enzyme in the reduced state. These results were explained on the basis that sulfide on Mo(VI), due to the high charge on Mo, may be considered to be in a resonance form in which two electrons are transferred to Mo(VI), leaving sulfide as S^0.

$$Mo(VI)—S^{2-} \leftrightarrow Mo(IV)—S^0$$

The S^0, in analogy with the known inorganic reaction of S^0 with CN^-, reacts to form SCN^- (reaction 1) [31], leaving the enzyme in the reduced form (reaction 2)

$$S^0 + CN^- \rightarrow SCN^- \qquad (1)$$

$$Mo(IV)\text{-}S^0 + CN^- \rightarrow SCN^- + Mo(IV) \quad (2)$$

The reduced form of the native enzyme, in which sulfur is present as SH, is inert to CN^-, again in analogy with inorganic results. On the other hand, OH in the reduced desulfo enzyme is labile and therefore easily replaced by sulfide (SH^-) to reactivate the enzyme

$$Mo(IV)OH + SH^- \rightarrow Mo(IV)SH + OH^- \quad (3)$$

The rate data for ^{17}O exchange reactions for oxidized and reduced enzymes support this interpretation [15].

(iii) Electrochemistry

The reduction potentials for Mo in XO, XDH (both native and desulfo forms) and SO have been determined by a combination of EPR and potentiometric measurements (Table 3) [32–34]. This method assumes potentials calculated from EPR data obtained at the low temperatures required (77–110 K) do not differ significantly from room temperature values. Recently reduction potentials for XO have been obtained at room temperature by a microcoulometric method [35]. Results indicate this assumption is generally valid, in zwitterionic buffers, for the Mo(VI)/(V) couple; the value for the Mo(V/IV) couple, however, is shifted approximately 30 mV more positive in samples analyzed by EPR after freezing than the potentials obtained at room temperature. Since the Mo(VI)/(V) values are not altered, this effect was not attributed to change in pH upon freezing, but rather to a difference in the entropy of reduction with temperature of the Mo(V)/(IV) couple ($dE'm/dt = \Delta S/nF$). This result indicates calculations of internal electron distribution in XO based on EPR and spectrophotometric measurements may be suspect, and the generally accepted model based on these measurements may need modification [11]. The microcoulometric method

TABLE 3

Reduction potentials [a]

Enzyme	Mo(VI)/(V)	Mo(V)/(IV)	Ref.
Xanthine oxidase [b]	−355	−355	32
Xanthine oxidase [b] (desulfo)	−440	−480	
Xanthine dehydrogenase [b]	−357	−337	33
Xanthine dehydrogenase [b] (desulfo)	−397	−433	
Sulfite oxidase [c]	+38	−163	34
Mo protein from *Desulfovibro gigas* [d]	−415	−530	4

[a] mV vs. NHE. [b] Pyrophosphate, pH 8.2. [c] Tris buffer, pH 7.0. [d] Tris buffer, pH 8.2.

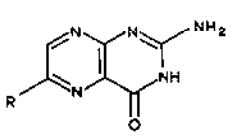
also confirmed changes in reduction potentials with change of pH, predicted from the EPR results, and found a specific effect of pyrophosphate on the Mo(V)/(IV) couple.

The data (Table 3) indicate both Mo(VI)/(V) and Mo(V)/(IV) potentials are lower in desulfo forms than native forms of the enzymes, an effect observed in model complexes when sulfur is replaced by oxygen [36].

The large difference (ca. 400 mV for the Mo(VI)/(V) couple) in Mo reduction potentials between XO (or XDH) and SO is striking. It might have been expected the potentials for SO would be lower, since the sulfido group in XO is replaced by an oxo in SO; this is, however, not the case. Furthermore, while the two couples Mo(VI)/(V) and Mo(V)/(IV) have the same potential in XO, there is a difference of approx. 200 mV between them in SO. While the protein undoubtedly has considerable influence on the potentials, differences of this magnitude argue for major differences in geometry and bonding at the sites, even though the donor sets may be similar (for an estimate of protein vs. axial ligand effects on the reduction potential of cytochrome *c*, see ref. 37). Finally, since the enzymes have similar EPR parameters, the EPR technique appears to be relatively insensitive to the factors determining reduction potentials.

(iv) The molybdenum cofactor

In 1970, a mutant (*nit-1*) of *N. crassa*, which lacked nitrate reductase activity was reported [38]. Acidified samples of XO, XDH, AO and SO, when added to an extract of *nit-1* produced an active nitrate reductase. It was proposed all the Mo enzymes contain a common cofactor which is released upon acid treatment which, when added to Mo deficient *nit-1*, forms an active nitrate reductase. This appears to be the case for all Mo enzymes except nitrogenase (which has a different cofactor). The cofactor appears to be a small peptide (ca. 2000 mol. wt.), the structure of which is not known. Amino acid analysis of the cofactor obtained from XO and SO reveals approximately the same kind and number of amino acids [39]. Recently, using an SDS treatment method, good yields of cofactor were isolated from the Mo hydroxylases [40]. Under aerobic conditions, a strong fluorescence was found associated with cofactor. Although the fluorescent fraction was not active in the *nit-1* assay, it was identified as originating from the active fraction by oxidation. Analysis of the fluorescence spectrum led to its identification as a 6-alkyl substituted pterin:



Since the fluorescence appeared only on air oxidation, with concomitant loss of *nit-1* activity, it was proposed the cofactor contains the pterin in a reduced form (dihydro or tetrahydro) which does not fluoresce. It was suggested the function of the pterin may be binding of the cofactor to apoprotein, Mo coordination, or in redox reactions of the enzymes. In view of the EXAFS and EPR evidence, coordination to Mo is unlikely, but not impossible, since the complete donor set of Mo has not yet been identified (vide infra). A redox function is also improbable since microcoulometric titration of XO indicates a maximum of 6 electrons are taken up on reduction and these are accounted for by the Mo, Fe_2S_2 and flavin centers.

C. MODEL STUDIES

It is clear that the most recent biochemical EXAFS and EPR results, while greatly improving our understanding, have not yet established the complete donor set nor defined the geometry at the Mo center for any of the hydroxylases. Model studies, therefore, are not nearly as advanced for these enzymes as for cytochromes or nonheme iron sulfur proteins [41], and it can be unequivocally stated no model complex that satisfactorily mimics the EXAFS, EPR and electrochemical properties of the hydroxylases, or their reactivities with substrates, has been described. Nevertheless, a considerable number of Mo complexes which mimic to a degree some of the properties of the biological sites have been synthesized and characterized [8–10]. Much valuable data concerning structures, Mo—ligand bond lengths, EPR and electrochemical parameters, and reactivity have been obtained [8–10]. This data has been useful in interpreting corresponding properties of the Mo hydroxylase centers and points the way towards the design and synthesis of more satisfactory models.

The recent biochemical results present a number of difficult and important problems in modeling the Mo centers of the hydroxylases. Current model studies will be discussed in relationship to these problems.

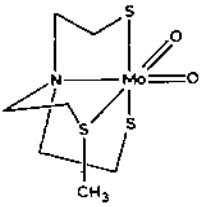
(i) Ligand donor sets

Oxidized enzymes

For SO, the EXAFS results indicate, as a minimum, Mo is coordinated to two oxos and 2–3 thiolate sulfurs in the oxidized state [28]. It may be safely assumed on the basis of model studies and Mo=O bond lengths in the enzyme, the oxos are in a *cis* relationship [8]. The identity of the other donor(s) is unknown but it could be nitrogen, oxygen or thioether sulfur.

The complex that comes closest to this set of donors is

$MoO_2N(CH_2CH_2S)_2(CH_2CH_2SCH_3)$, reported by Stiefel and co-workers [29]



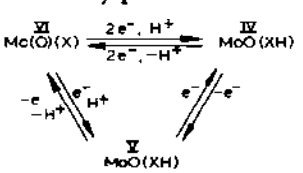
This complex is described as having distorted octahedral geometry, with N and thioether S of the tripodal ligand *trans* to oxo. The Mo=O and Mo—S (thiolate) bond lengths are quite similar to those found by EXAFS for SO and the EXAFS spectrum is close to that for SO [29]. It would be most interesting to know the redox behavior and the reactivity with biological substrates of this complex; unfortunately no data concerning these properties have appeared.

A number of dioxo Mo(VI) complexes containing N_2S_2 and NSO donor sets have been reported. While they are somewhat less satisfactory models (with respect to ligand donors), the redox behavior and reactivity of some are quite interesting and will be described below.

No Mo(VI) complex having both oxo and sulfido ligands, as required by the EXAFS results for XO and XDH is known. In fact, only a few monomeric Mo complexes with sulfido ligands in a relevant oxidation state have been reported, and these have not been well characterized [42]. A number of dimeric Mo(V) complexes with both sulfido and bridging sulfide are known [43,44]. Data on Mo=S bond lengths obtained from such dimers have proven helpful in calibrating EXAFS measurements for the enzymes; otherwise, they do not appear to be biologically relevant.

Reduced enzymes (Mo(V), Mo(IV))

According to the interpretation of recent EXAFS and EPR investigations described above, an Mo oxo (SO) or sulfido (XO, XDH) is reduced during turnover to an OH or SH and is present as such in both the Mo(IV) and EPR-active Mo(V) states [28]. The OH or SH proton is proposed to be coupled to Mo(V), giving rise to the ^1H shfs seen in the Mo(V) EPR signal. The electrochemical and EPR data for the enzymes indicate rapid, reversible electron/proton transfer processes occur between the three states



X = S ; XO, XDH
X = O ; SO

With one exception [45], oxo Mo(IV) and (V) complexes with OH or SH ligands are unknown; one or two electron reduction of dioxo Mo(VI) complexes results in oxo bridged Mo(V) dimers in the majority of cases [36,46]:

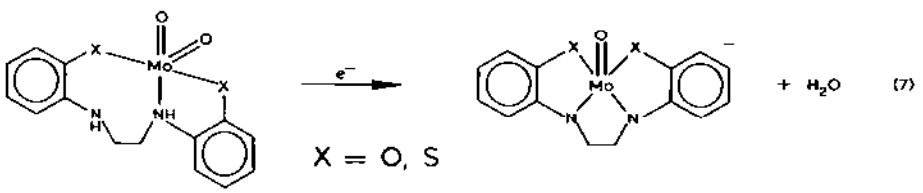
$$2\ \overset{VI}{MoO_2}L \xrightarrow[H^+]{2\,e^-} \overset{V}{L}MoO\text{—}O\text{—}\overset{V}{MoO}L + OH^- \tag{4}$$

$$\overset{VI}{MoO_2}L \xrightarrow[H^+]{2\,e^-} \overset{IV}{MoO}L + OH^- \tag{5}$$

$$\overset{VI}{MoO_2}L + \overset{IV}{MoO}L \rightleftarrows \overset{V}{L}MoO\text{—}O\text{—}\overset{V}{MoO}L \tag{6}$$

The comproportionation reaction (6) is an equilibrium which lies in most cases completely to the right [47]. The driving force for these reactions appears to be the great thermodynamic stability of the oxo bridged dimers. Sulfide bridged dimers formed by reduction of dioxo Mo(VI) complexes with H_2S, are equally stable [43].

Tetradentate aromatic dioxo Mo(VI) complexes have recently been prepared which, upon one electron reduction, give stable oxo Mo(V) monomers [48]. These are unusual, however, inasmuch as the stability of the Mo(V) complex results from ligand amino group deprotonation, imposing a planar ligand geometry and blocking dimerization (which occurs only through a *cis* position)



The EPR spectra of the Mo hydroxylases are characterized by highly anisotropic Mo(V) signals with high g values (1.96–2.03). A number of oxo Mo(V) complexes with N, O and S ligands have been prepared, and their EPR parameters determined (Table 4) [50]. High g values and low ^{95,97}Mo A values are observed with sulfur ligands. For the majority of such complexes, the largest ^{95,97}Mo A value (A_z) is associated with the highest g value (g_z) and is considerably larger than A_x and A_y [50]. In the one case where the anisotropic ^{95}Mo A values have been reported (XO, very rapid signal, Table 2), this is not the case, suggesting the ground state orbital of the Mo(V) center of the enzyme is different than for the model complexes. Until more ^{95,97}Mo coupling constants for the enzymes are known, however, such a conclusion is highly tentative. While no ^{33}S coupling constants for Mo(V) complexes are known, a small ^{17}O coupling (ca. 0.23 mT) has been observed

TABLE 4

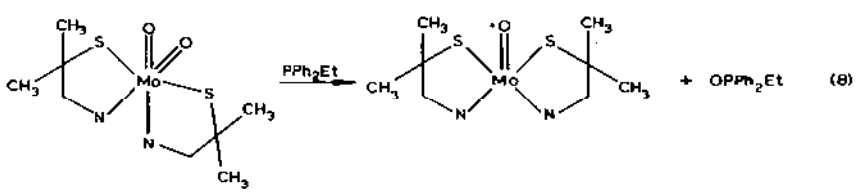
EPR parameters of selected complexes

Complex [a]	Equatorial ligands	(^{95,97}Mo) [b]		
		g_x	g_y	g_z
$MoOCl(tox_2)$	NS_2Cl	1.948	1.952	2.003
MoOCl(mae)	NS_2Cl	1.940	1.951	2.006
MoOCl(mpe)	NS_2Cl	1.943	1.958	2.001
$[Et_4N][MoO(SPh)_4]$	S_4	1.979	1.979	2.017
$[Et_4N][MoO(mae)]$	N_2S_2	1.9740	1.9820	2.0025
$[Et_4N][MoO(mab)]$	N_2S_2	1.9730	1.9790	2.0040
$[Et_4N][MoO(hae)]$	N_2O_2	1.9550	1.9645	1.9875
$[Et_4N][MoO(hbpd)]$	N_2O_2	1.9685	1.9645	1.9505
$[Mo(SC_6H_4NH)_3]^-$	N_2S_2			
$Mo(S_2CNEt_2)(SC_6H_4NH)_2$	[c]			

[a] For ligand abbreviations, see beginning of this paper. [b] All A values in $cm^{-1} \times 10^4$. $A_{(cm^{-1})} = 10$ A (mT) $g/2.142$. [c] Trigonal prismatic.

for $[Et_4N][Mo^{17}O(SPh)_4]$ [16,51]. In this case, the ^{17}O is in the axial position; the larger values (ca. 1.6 mT) seen for XO and SO (Table 2) suggest either the ^{17}O is in an equatorial position, or the geometry is greatly distorted from octahedral. Both ^1H and ^1N coupling have been reported for the non-oxo Mo(V) complexes, $Mo(SC_6H_4NH)^-$ [24] and $Mo(S_2CNEt_2)(SC_6H_4NH)_2$ [52], while coupling of two equivalent ^{14}N nuclei has been observed for oxo Mo(V) complexes with N_2S_2 [48] or N_2O_2 [53] ligands (Table 4). It is interesting to note ^{14}N and ^1H shfs have only been found for complexes with deprotonated aromatic amino ligands, in which the nitrogen is trigonal.

A small number of oxo Mo(IV) complexes have been prepared by oxo abstraction by PPh_2Et from the dioxo Mo(VI) complexes [47,49]



(8)

For these complexes, equilibrium (6) is not completely to the right, allowing isolation of the oxo Mo(IV) complex by the use of excess phosphine.

In both these cases (oxo Mo(V) and (IV)), an oxo group is lost upon reduction of the dioxo Mo(VI) complex, instead of protonating to form OH, as occurs with SO. Similar results are most likely if Mo(VI)(O)(S) complexes

					A			Ref.
A_x	A_y	A_z	$\langle g \rangle_0$	$\langle A \rangle_0$	(^{1}H) [b]	(^{14}N) [b]	(^{17}O) [b]	
19.5	35.5	59.2	1.967	37.9				50
30	23	61	1.966	37.8				50
22.5	36.0	57.5	1.969	38.0				50
22.3	22.3	52.3	1.990	32.3			2.2	51
		54.5	1.986	33.0		2.14		48
		50.0	1.986	33.0		2.14		48
67.3	29.5	20.0	1.975	38.0		2.15		53
29.0	33.0	73.0	1.959	46.0		2.23		53
			1.988	35	5.8	1.9		24
			1.990	35	6.9	2.2		52

are synthesized, with loss of the sulfido group. While the addition of OH^- or SH^- to these oxo Mo(V) and (IV) monomers might be possible, the OH^- or SH^- would be in a *trans* position (orthogonal to the d_{xy} orbital). The large ^{1}H coupling to Mo(V) seen with the enzymes, however, indicates a *cis* OH or SH, where overlap with the Mo(V) d_{xy} orbital is maximum.

Clearly, the synthesis of oxo Mo(V) and (IV) complexes with *cis* OH or SH ligands will be a most difficult synthetic task.

(ii) Substrate binding

The biochemical results for XO indicate the substrate and some inhibitors are bound to Mo in a Michaelis complex [20]. The nature of this binding is uncertain; binding via an Mo—O is suggested for xanthine, while the EPR data for the inhibitor alloxanthine (^{14}N coupling) are also compatible with direct Mo inhibitor (N) binding [21]. It is important to know how substrate is bound, since direct substrate—Mo binding, in contrast to Mo—O— or Mo—S— binding, requires an open coordination site. EXAFS results appear to be compatible with either mode. The EPR data for SO indicate PO_4^{3-} binds to reduced enzyme (Mo(IV), (V)) through a phosphate oxygen by replacement of a labile OH on Mo [22]. The binding mode of the substrate (SO_3^{2-}), however, is uncertain [23].

The reduction of NO_3^- (which also binds to XO) by oxo Mo(V) complexes

involves an obligatory binding of NO_3^- through a nitrate oxygen in a *cis* (equatorial) position [54,55]. The observation of sulfite oxidation by dioxo Mo(VI) complexes in nonaqueous solvents suggests Mo—O—SO_3 binding but details of the reaction have not been obtained [48,56]. Complexing of xanthine or other purines by dioxo Mo(VI) complexes has not been reported.

A number of dioxo Mo(VI) and oxo Mo(V) and (IV) complexes with an open coordination site or a site occupied by a weakly coordinated ligand, are known, but no studies of substrate binding by these complexes have been made [36,49,50].

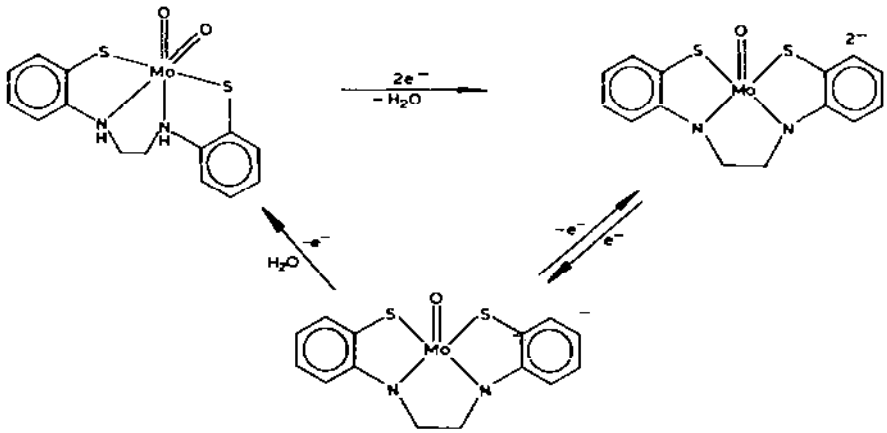
(iii) Electrochemistry

Reduction potential measurements for the Mo(VI)/(V) and Mo(V)/(IV) couples of the enzymes indicate reversible pH dependent behavior, suggesting coupled electron/proton processes [32–34]. Such reversible behavior for dioxo Mo(VI) complexes has not been observed. Upon reduction, these complexes lose an oxo instead of protonating it [36,46,48]. This loss and subsequent reorganization of the ligand undoubtedly accounts for the irreversible behavior. In fact, until quite recently, only two electron reductions of dioxo Mo(VI) complexes to oxo bridged Mo(V) dimers or oxo Mo(IV) complexes were known. One electron irreversible reductions of dioxo Mo(VI) complexes with aromatic N_2O_2 ligands involving deprotonation of ligand amino groups have now been observed (see reaction (7) above) [53].

The one electron reduction of many oxo Mo(V) complexes to oxo Mo(IV) complexes, on the other hand, are reversible, since this involves only electron transfer [36,49].

The oxidation of substrate by XO and XDH involves an initial two electron reduction of the Mo(VI) center, followed by two one electron oxidations of Mo(IV) to Mo(V) and Mo(VI) by the flavin and/or Fe_2S_2 centers. Dioxo Mo(VI) complexes with aromatic N_2S_2 ligands exhibit similar behavior at a Pt electrode [49]. The two electron reduction of the dioxo Mo(VI) complex probably involves a one electron irreversible step with loss of an oxo, and a one electron reversible step, with almost identical reduction potentials (if the reduction is stopped at one equivalent of electrons, the oxo Mo(IV) complex reacts with the remaining dioxo Mo(VI) complex to give the oxo Mo(V) complex (reaction (7) above)). Identical reduction potentials for the Mo(VI)/(V) and Mo(V)/(IV) couples are observed for XO and XDH (Table 3) [32,33].

A major problem in modeling the Mo centers of the hydroxylases is the great difference in Mo reduction potentials between XO (or XDH) and SO. As the EXAFS and EPR results indicate, the two sites do not appear to differ greatly. The major difference, the presence of a sulfido ligand for XO,



should result in a higher potential for XO, rather than the reverse (Table 3).

While large differences in reduction potentials for Mo(VI) and Mo(V) complexes are found, they result from different ligand donor sets; in cases with similar ligands or with geometrical isomers, the potentials do not differ greatly [36]. The large differences seen for the enzymes might reflect the constraints of the protein, perhaps forcing the ligands to adopt unusual or unfavorable geometries (e.g. S or O *trans* to oxo) [57]. If so, ligand design will be crucial in the development of satisfactory models.

(iv) Reactions

Oxidation of substrates

No reports of the oxidation of xanthine or other purines by Mo(VI) complexes have appeared. Preliminary studies of the oxidation of SO_3^{2-} by dioxo Mo(VI) complexes with aromatic N_2S_2 ligands have been made [55], and the oxidation of aldehydes by O_2, catalyzed by MoO_2 (ethyl-L-cysteine)$_2$, has been investigated [58].

It is not clear whether substrate oxidations by the enzymes proceed via atom transfer or coupled electron/proton mechanisms. Sulfite oxidation seems more likely to involve oxygen transfer, while purine oxidation may go by the electron/proton pathway. If so, the oxidation of phosphines to phosphine oxides by dioxo Mo(VI) complexes (reaction (8)) which has been studied in some detail [47], might be considered a model for sulfite oxidation; similar reactions with sulfite, which do not appear to have received much attention, would be more interesting and might provide valuable data. A major difficulty in selecting useful complexes for such studies is a lack of thermodynamic data. Most dioxo Mo(VI) complexes undergo irreversible electrochemical reductions, making electrochemical studies of little use in

this regard, and few thermochemical measurements have been made [59]. Similar considerations, as well as the poor solubility of xanthine and other purines in aprotic solvents, are perhaps responsible for the lack of studies of purine oxidations by dioxo Mo(VI) complexes.

Oxidation of oxo Mo(V) and (IV) complexes by flavins, hemes, and nonheme iron complexes

After reduction by substrate, the other redox centers of the enzymes (flavin and Fe_2S_2 for XO and XDH; heme for SO) reoxidize the Mo centers and subsequently transfer electrons to the biochemical acceptors.

No reduction of model Fe_2S_2 clusters or flavins by monomeric Mo(V) or (IV) complexes has been reported. The reduction potentials of the Fe_2S_2 clusters are quite negative (ca. -1.00 V) [60] and, in most cases where comparisons in similar solvents can be made, the Mo potentials are not sufficiently negative to effect such reductions [36,49]. Furthermore, since the Mo and Fe_2S_2 centers are ca. 11 Å apart in XO [61], and the mechanism by which electron transfer over such distances is unknown, the relevance of studies involving relatively small and simple molecules is questionable.

It has been proposed the Mo and heme centers of SO approach each other closely during electron transfer [62]; this process may possibly involve the heme edge, as has been shown to be the case with the reactions between cytochrome *c* and a number of small redox molecules [63], or between SO and cytochrome *c* [64]. If so, an investigation of the reduction of cytochrome *c* by monomeric oxo Mo(V) or (IV) complexes could prove valuable. The requirement for monomeric oxo Mo(V) and (IV) complexes that are stable in aqueous solution, however, imposes severe limitations on such studies.

(v) Molybdenum cofactor

Little is known of the composition or nature of the Mo cofactor. It appears to be a small polypeptide which is associated with (at least when isolated from SO), a pterin [39]. At this point, no evidence exists for a structural or redox role for a pterin in the cofactor. It is tempting to suggest a ligand of Mo, not identified by EXAFS, might be the 2-amino group of a pterin; if so, one of the protons coupled to the Mo(V) EPR signal in XO and XDH could be the amino proton of the pterin. No evidence for coordination of Mo to a pterin in the cofactor, however, exists and furthermore, no studies of Mo coordination by pterins have been reported.

D. CONCLUSIONS

The most recent results of the investigation of the Mo hydroxylases by the methods discussed above have not unambiguously defined the complete set

of ligand donors for the Mo center in any of these enzymes [28]. Furthermore, the results offer few clues as to the origins of the large differences in Mo reduction potentials between XO (or XDH) and SO [28]. While the high g values for the enzyme Mo(V) EPR signals are satisfactorily explained by the presence of sulfur ligands, the somewhat unusual ^{95}Mo hyperfine coupling observed for the XO very rapid signal suggests a geometry substantially different from that of model Mo(V) complexes so far examined. Finally, the results provide little insight into the enzymatic mechanisms of biological substrate oxidations.

The solution of these problems depends to a great extent on the development of well characterized molybdenum complexes having ligand donors quite similar to those found by EXAFS and EPR to be present in the enzymes. In addition to this general requirement, models which fulfill the following specific demands are greatly needed at this time:

(1) Monomeric Mo(VI) complexes having the oxo, sulfido core ($Mo(O)(S)^{2+}$). Replacement of an oxo of dioxo Mo(VI) complexes with S, oxidative addition of S to Mo(IV) sulfido complexes (themselves essentially unknown) or synthetic routes starting with tetrathio (MoS_4^{2-}) or oxotrithio ($MoOS_3^{2-}$) molybdates should be explored in this respect.

(2) Mo(VI) complexes that undergo reversible reduction to stable MoO(OH) and MoO(SH) complexes in both Mo(V) and Mo(IV) oxidation states. Ligands that prevent dimerization or comproportionation of such species by steric, electronic or geometrical factors suggest themselves.

(3) Monomeric Mo(V) complexes that exhibit ^{1}H, ^{17}O and ^{33}S coupling constants comparable to the values found with the enzymes. Complexes with the donor of interest in both axial and equatorial (octahedral) positions as well as complexes with distorted geometries need to be synthesized.

(4) Dioxo Mo(VI), oxo sulfido Mo(VI) and oxo Mo(V) and (IV) complexes having a range of Mo—donor bond lengths and reduction potentials. The relationship between these parameters and structure, particularly for complexes with unusual and presently not reported geometries and donor arrangements (e.g. *cis* thiolate ligands) needs careful investigation.

(5) Mo(VI) complexes that effect oxidation of biological substrates (purines, SO_3^{2-}, aldehydes). The factors, both thermodynamic and kinetic, governing the reactivity of such complexes in these oxidations need elucidation.

The syntheses of such complexes present many problems, the difficulty of which cannot be over emphasized. The solution of these problems depends on the imaginative design of ligands and the development of new synthetic methods. Continued investigation of the Mo hydroxylases by the powerful techniques now available should further define these problems and provide more precise guidelines to the coordination chemist for their solution.

NOTE ADDED IN PROOF

The molybdenum cofactor has been shown to contain a novel pterin which has sulfur bound to the pterin ring at position 7 (K.V. Rajagopalan, J.L. Johnson and B.E. Hainline, Fedration Proc., 41 (1982) 2608). This raises the possibility that one (or two) of the thiolate sulfur ligands of Mo in the hydroxylases are part of the cofactor.

Room temperature potentiometric and EPR measurements of reduction potentials of XO have demonstrated both a pH and temperature dependence (A.G. Porras and G. Palmer, J. Biol. Chem., 257 (1982) 11617). Due to differences in buffers, it is difficult to compare the results with those obtained by microcoulometric titration [35].

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