invariably find a match among filled and vacant $d\pi$ orbitals, respectively, in compounds **1–6**. The experimental and theoretical results to date lead us to hypothesize that numerous new compounds containing both π-acid and π-base ligands in the coordination sphere should be accessible. Synthetic efforts to realize this chemistry where segregation of empty $d\pi$ orbitals overlapping with π-donor ligands will complement retrodative metal-to-ligand bonding to π-acid ligands are continuing in our laboratory.

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Molybdenum Sites of Sulfite Oxidase and Xanthine Dehydrogenase. A Comparison by EXAFS

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Abstract: The molybdenum enzymes sulfite oxidase and xanthine dehydrogenase have been investigated by fluorescence-detected EXAFS using synchrotron radiation, and the results have been interpreted with improved EXAFS analysis procedures. A new treatment of EXAFS amplitudes has been developed which allows the extraction of meaningful Debye–Waller factors using experimentally derived functions. A search profile procedure has also been developed to aid in the treatment of minor EXAFS components. These methods have been used for a more detailed analysis of sulfite oxidase molybdenum EXAFS. They have also been used for analysis of the EXAFS of intact and cyanolyzed xanthine dehydrogenase, in both oxidized and reduced forms. Although the results are in qualitative agreement with recent work by Bordas et al., some significant quantitative differences are found. For oxidized sulfite oxidase, the analysis revealed two oxygens at 1.68 Å and two or three sulfurs at 2.41 Å, changing to one oxygen at 1.69 Å and three sulfurs at 2.38 Å upon reduction. For oxidized, intact xanthine dehydrogenase the prediction was one oxygen at 1.70 Å, one sulfur at 2.15 Å, and two sulfurs at 2.47 Å, changing to one oxygen at 1.68 Å and three sulfurs at 2.38 Å upon reduction. Finally, in cyanolyzed xanthine dehydrogenase, two oxygens at 1.67 Å and two sulfurs at 2.46 Å were found, which upon reduction changed to one oxygen at 1.66 Å and two or three sulfurs at 2.33 Å. In all cases there may be extra ligands which complete the molybdenum coordination sphere but contribute only weakly to the EXAFS.

Molybdenum enzymes exhibit a broad range of chemical and spectroscopic behavior,[1] and comprehending the structural basis for this diversity is a prerequisite for understanding and synthetically modeling their catalytic mechanisms. Previous results from X-ray absorption spectroscopy have suggested that the Mo sites of these enzymes can be classified as either "cluster" type such as in nitrogenase[2-4] or "oxo" type such as contained in xanthine oxidase,[5-7] sulfite oxidase,[8] and nitrate reductase.[9] Specifically, the EXAFS studies of nitrogenase have shown that the molybdenum is present in an Fe,Mo,S cluster, whereas the other Mo enzymes appear to have a Mo site with both sulfur donor and terminal oxo ligands. The structural differences between these two types of sites may be related to the fact that nitrogenase possesses an iron–molybdenum cofactor ("FeMo-co"),[10] whereas the other enzymes cited contain a molybdenum cofactor ("Mo-co") which is free of iron but contains a novel pterin component.[11]

Even within the class of oxo-type Mo proteins there are substantial differences in properties. Xanthine oxidase, xanthine dehydrogenase, and aldehyde oxidase all have relatively low Mo redox potentials[12] and a unique "cyanolyzable" sulfur.[1] Upon treatment of these enzymes with cyanide, SCN^- is released and "desulfo" Mo proteins are formed which have no catalytic activity and even lower redox potentials. A molybdenum protein from *Desulfovibrio gigas* has properties similar to these desulfo proteins.[13] In contrast, sulfite oxidase[14] and nitrate reductase[15] have substantially higher Mo redox potentials, and although they are inhibited by cyanide in their reduced states, this process is reversible by oxidation and no SCN^- is released.[16] Thus, a distinction can be made between low potential and high potential

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oxo-type Mo sites, and it would be useful to have a structural explanation for this division.

In this paper, the results of an X-ray absorption study of sulfite oxidase and xanthine dehydrogenase are presented. Differences were found both in the nature of the terminal chalcogenides and in the Mo–S bond lengths. Refinements in EXAFS data analysis, with regard to the treatment of disorder and the interpretation of minor components, are also discussed. Finally, comparisons are drawn with previous xanthine oxidase EXAFS studies.[5-7]

Experimental Section

Sample Preparation. Sulfite oxidase was prepared from chicken liver[17] and concentrated in 50 mM potassium phosphate buffer (pH 7.8) to greater than 100 mg/mL. Reduced samples were prepared by addition of excess solid sodium dithionite. Xanthine dehydrogenase was also prepared from chicken liver,[18] and its activity was measured by the procedure of Coughlan et al.[16] Two different samples of xanthine dehydrogenase were used. The first had an activity/flavin ratio (AFR) of 325 before data collection and 203 afterward, while the second had a ratio of 240 before and after the experiment. With the assumption that fully active enzyme with a 1:1 Mo:flavin ratio has an AFR of 400,[16] it is estimated that the averaged spectrum represents roughly 65% ± 10% active molybdenum. Cyanolyzed xanthine dehydrogenase was prepared by the method of Coughlan[19] and later reduced by addition of 0.1 M sodium dithionite. The buffer used for all xanthine dehydrogenase experiments was 5 mM potassium phosphate (pH 7.8) with 0.1 mM EDTA.

A sample which had been anaerobically reduced with dithionite was also prepared. This sample had an AFR of 200 before data collection and 190 afterward. However, its Mo:flavin ratio was 0.5. Accordingly, this was deemed to represent better than 95% active molybdenum.

Data Collection. The spectra were recorded at the Stanford Synchrotron Radiation Laboratory on a bending magnet line using a channel cut Si[2,2,0] crystal monochromator. The electron storage ring SPEAR was running in dedicated mode at an energy of 3.0–3.4 GeV with a current of 30–70 mA. The spectrometer was calibrated by using the Mo foil first inflection point as 20003.9 eV. The foil spectrum was obtained simultaneously with the protein data by using a three ion chamber geometry.

Data was collected on protein samples in 2.0 cm × 2.0 cm × 0.3 cm lucite cuvettes with 0.1 cm thick windows. The sulfite oxidase spectra were recorded at ambient temperatures by fluorescence or in the transmission mode, using argon-filled ionization chambers to measure the incident and transmitted intensity. The reduced, intact xanthine dehydrogenase spectrum was recorded at −8 °C, while the remainder of the xanthine dehydrogenase spectra were recorded at 5 °C. For these measurements a 24 detector fluorescence system with NaI detectors and zirconium filters was used.[20] Typically, about 16 20-min scans were averaged to generate a useful spectrum.

Data Preparation. A weighted average of all the fluorescence detector signals and the incident ion chamber signal were made into a ratio to generate the fluorescence excitation spectrum. A base line spectrum corresponding to buffer only was first subtracted, followed by a linear preedge subtraction. A three-region cubic spline was used to extract the EXAFS signal. An E_0 of 20025 eV was used to define the wavenumber k. Finally, the data were smoothed by convolution with a Gaussian (0.25 Å^{-1}, full width 1/e).

Data Analysis

An equation commonly used to describe EXAFS is

$$\chi(k) = \sum_b (N_b/kR_{ab}^2)|f_b(\pi,k)|e^{-2\sigma_{ab}^2k^2} \sin(2kR_{ab} + \alpha_{ab}(k)) \quad (1)$$

where N_b is the number of atoms with backscattering amplitudes f_b at distance R_{ab} from the X-ray absorbing species, α_{ab} is a total phase shift, and k is the photoelectron wavenumber. In this expression σ^2 represents the mean square deviation of an assumed Gaussian distribution of absorber–backscatter distances.

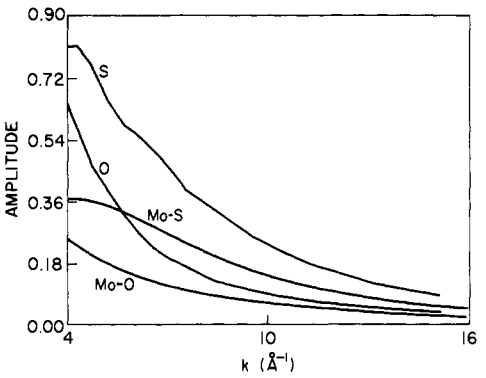

Figure 1. A comparison of experimentally derived Mo—O and Mo—S EXAFS amplitudes with the theoretical electron-atom backscattering amplitudes calculated by Teo and Lee.[22] For this comparison an E_0 of 20016 eV was used.

In previous work,[21] it was shown that by choosing model compounds with bond lengths and strengths similar to those in the sample to be analyzed, the effects of thermal motion could be combined with other amplitude terms to yield a roughly transferable total amplitude function. Keeping the shape of this amplitude envelope fixed, the overall amplitude could then be varied to obtain an estimate of the number of scatterers. Although the average accuracy of this procedure was ±20%,[21] this approximation breaks down at high k values or when model and unknown have significantly different bonding. Therefore, a more general procedure for treatment of EXAFS amplitudes was needed.

One alternative approach to the treatment of EXAFS amplitudes is the use of theoretical electron-atom backscattering amplitudes.[22] However, it is now recognized that determination of coordination numbers requires recourse to the spectra of standard materials to correct for various loss effects[23,24] which reduce the EXAFS amplitude. One source of these diminished EXAFS amplitudes is multielectron excitation (shake-up or shake-off), and another source is inelastic scattering of the excited-state photoelectron wave. These effects are difficult to calculate, and an alternative is to define a totally experimental EXAFS amplitude which combines all first-shell amplitude effects *except* thermal and static disorder. The latter would be accounted for by the normal Debye–Waller factor, thus

$$\chi(k) = \sum_b (N_b/kR_{ab}^2)A_{ab}(k)e^{-2\sigma_{ab}^2k^2} \sin(2kR_{ab} + \alpha_{ab}(k)) \quad (2)$$

where

$$A_{ab}(k) \simeq Y_a(k)|f_b(\pi,k)|T_{ab}(k) \quad (3)$$

In this expression Y_a is the single electron excitation yield factor for a particular X-ray absorbing atom, and $T_{ab}(k)$ is an elastic transmission function. Typical values for Y_a are reported to be on the order of 0.7.[25]

Clearly, the determination of an experimental EXAFS amplitude function $A(k)$ requires knowledge of the appropriate Debye–Waller factor σ. Fortunately, a large body of literature exists on the determination of mean square amplitudes from vibrational spectra,[26] and for two critical models, MoO_4^{2-} and MoS_4^{2-}, the appropriate values for σ have already been calculated.[27] It was thus possible to derive new Mo–O and Mo–S

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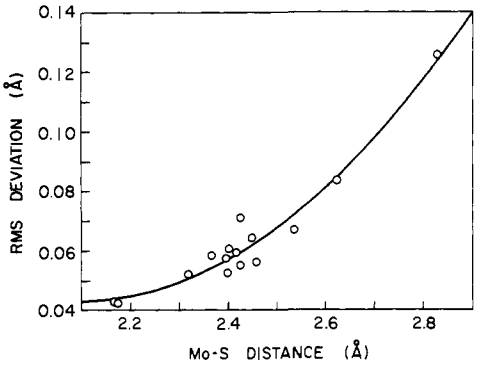


Figure 2. The calculated σ vs. bond length for a variety of compounds with Mo—S bonds. The solid line represents the best quadratic fit of σ vs. R.

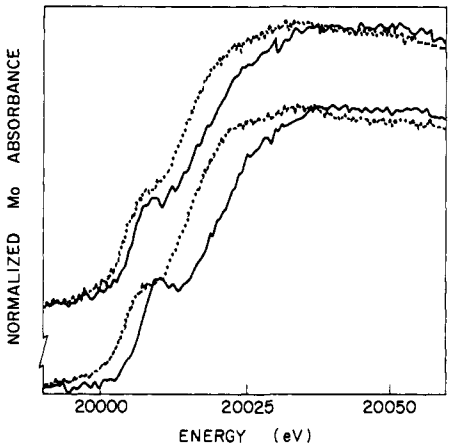


Figure 3. The molybdenum K absorption edges of xanthine dehydrogenase: top, oxidized, intact XDH (—) vs. reduced, intact XDH (---); bottom, oxidized, cyanolyzed XDH (—) vs. reduced, cyanolyzed XDH (---).

amplitude functions (Figure 1) using previously described curve-fitting procedures[21] and, in subsequent fits, to use integral numbers of scatterers and variable Debye–Waller factors. The new fits, which have the same number of variables as before, actually have slightly smaller residuals than those obtained by the earlier procedure.

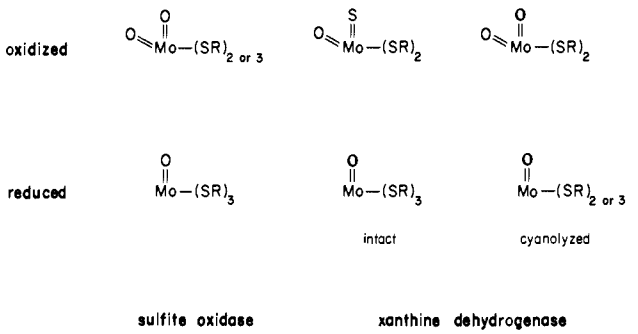
As a test of the precision and utility of this new procedure, the correlation between the bond length R and the calculated root-mean-square deviation σ in a set of molybdenum and sulfur-containing compounds was investigated. The results for 15 Mo–S distances are illustrated in Figure 2, and a clear trend for σ to increase with distance can be seen. This is as expected, because the vibrational modes involving Mo–S stretching fall to lower frequency with increasing bond length. This results both in more zero-point motion along the Mo–S bond as well as greater thermal population of excited vibrational states.

A final refinement of the analysis procedure pertains to the treatment of minor EXAFS components. Although the major components of the EXAFS can be visualized by means of a Fourier transform, inclusion of minor components has in the past been somewhat arbitrary. Therefore, a protocol was developed which involves fixing the major components at their optimized values and then calculating the quality of fit as a function of the type, number, and distance of the extra component. A search profile for a given atom type is a plot of the fit quality vs. distance for a fixed number of atoms being added. Such a plot permits visualization of the importance of a new component and helps guarantee finding an absolute rather than local minimum. Several illustrations of these search profiles are provided later in this text.

Results and Discussion

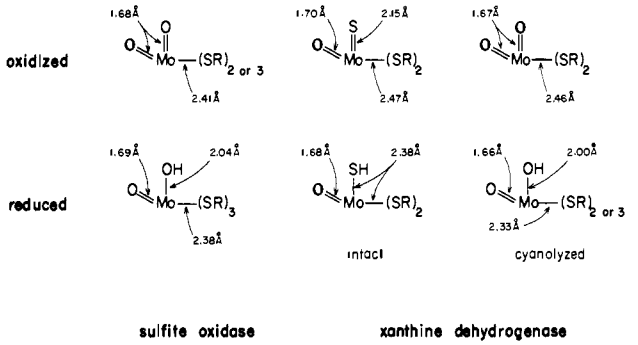
Edges. The Mo X-ray absorption edge of xanthine dehydrogenase (Figure 3) exhibited a characteristic low-energy

Chart I. The Primary Structural Differences between Sulfite Oxidase and Xanthine Dehydrogenase, as Deduced from EXAS Alone[a]



[a] Additional ligands are probably present.

Chart II. An Interpretation of the EXAFS Results in light of Known EPR hyperfine Splittings[a]



[a] Other ligands may be present.

shoulder under all conditions examined. Similar results have already been reported for sulfite oxidase.[8] For the oxidized proteins, the intensity of the bound-state transition increases progressing from intact xanthine dehydrogenase to sulfite oxidase to cyanolyzed xanthine dehydrogenase. In all cases, this feature is weaker for the reduced proteins. In line with previous results,[2] the reduced xanthine dehydrogenase edges are shifted to lower energy than their oxidized counterparts.

It is now established that most of the intensity in these features results from unresolved, bound-state transitions from the molybdenum 1s orbital to vacant molecular orbitals oriented along the Mo–O bond(s), and the detailed electronic aspects of these features have been discussed.[28,29] For current purposes the bound-state transition is used as a necessary characteristic of terminal oxo group coordination. However, because terminal sulfido groups can also produce such a feature, although of weaker intensity, the shape of these edges is not a sufficient condition for proof of oxo coordination.

EXAFS. Figure 4 shows the Fourier-transformed EXAFS data for oxidized and reduced samples of sulfite oxidase, intact xanthine dehydrogenase, and cyanolyzed xanthine dehydrogenase. In all six cases peaks assignable to Mo=O and Mo—S bond lengths are observed. For sulfite oxidase and cyanolyzed xanthine dehydrogenase, reduction of the enzyme causes a lowering of the Mo=O peak, consistent with the hypothesis that an oxo group is lost in this process. However, for intact xanthine dehydrogenase, the peak in the oxo region actually gets larger upon reduction of the enzyme.

Curve-fitting analysis of the Mo protein EXAFS was done initially with two-component (Mo=O and Mo—S) fits. Then, amplitude and distance space was scanned with either an additional oxygen or sulfur component. The results of these fits, as sum-

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Table I. Molybdenum Enzyme Curve-Fitting Results

sample	Mo–O_t no.[a]	Mo–O_t R, Å	Mo–O_t σ, Å	Mo–S no.[a]	Mo–S R, Å	Mo–S σ, Å	Mo–X no.[a]	Mo–X type	Mo–X R, Å	Mo–X σ, Å	residual $(1/N)\Sigma_i(x_{obsd} - x_{calcd})^2 k^6$
sulfite oxidase	2	1.68	0.053	2	2.41	0.071					0.46
(as isolated)	2	1.68	0.054	3	2.41	0.086					0.27
(4–13 $Å^{-1}$)	2	1.69	0.052	2	2.42	0.067	1	O	2.17	0.043	0.26
	2	1.69	0.054	3	2.41	0.089	1	O	2.19	0.086	0.24
	2	1.68	0.054	3	2.41	0.086	1	S	2.86	0.095	0.25
sulfite oxidase	1	1.69	0.040	3	2.38	0.056					0.91
(dithionite reduced)	1	1.69	0.041	3	2.38	0.055	1	O	2.04	0.092	0.83
(4–14 $Å^{-1}$)	1	1.69	0.040	3	2.38	0.055	1	S	2.82	0.049	0.65
xanthine dehydrogenase	1	1.70	0.026	2	2.47	0.060					1.31
(as isolated)	1	1.70	0.028	2	2.46	0.070	1	S	2.15	0.054	0.42
(4–14 Å)	1.3	1.70	0.044	2	2.47	0.071	0.7	S	2.15	0.32	0.32
xanthine dehydrogenase	1	1.68	0.007	3	2.38	0.061					0.74
(dithionite reduced)	1	1.68	0.008	3	2.38	0.061	1	S	2.97	0.14	0.72
(4–13 $Å^{-1}$)	1	1.68	0.005	3	2.38	0.062	1	O	2.16	0.11	0.69
xanthine dehydrogenase	2	1.67	0.040	2	2.46	0.069					0.70
(cyanolyzed, oxidized)	2	1.66	0.040	2	2.46	0.088	1	O	2.31	0.042	0.62
(4–13 Å)	2	1.67	0.037	2	2.46	0.068	1	O	1.89	0.081	0.55
	2	1.67	0.039	2	2.46	0.072	1	S	2.09	0.11	0.55
	1.7	1.67	0.030	2	2.46	0.070	0.3	S	2.08	0.072	0.55
xanthine dehydrogenase	1	1.66	0.049	3	2.33	0.069					1.67
(cyanolyzed, dithionite reduced)	1	1.65	0.060	3	2.33	0.062	1	O	2.00	0.036	1.31
(4–13 $Å^{-1}$)	1	1.66	0.052	3	2.33	0.070	1	S	2.84	0.037	1.21

[a] Constrained to value listed.

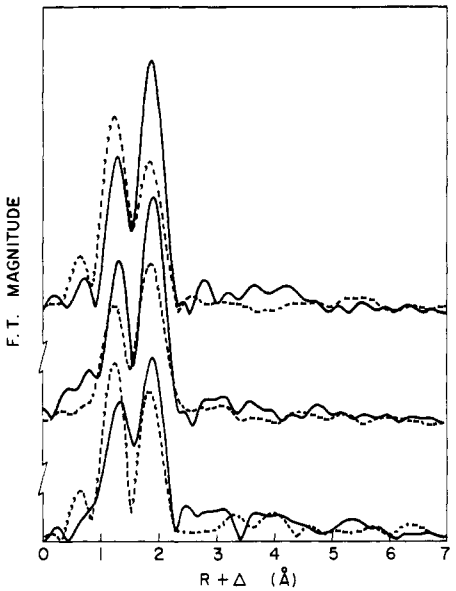

Figure 4. EXAFS Fourier transforms of sulfite oxidase and xanthine dehydrogenase. Top to bottom: reduced SO (—), oxidized SO (---), reduced, intact XDH (—), oxidized intact XDH (---), reduced, cyanolyzed XDH (—), oxidized, cyanolyzed XDH (---). Transform range: 4–12 $Å^{-1}$, k^3 weighting.

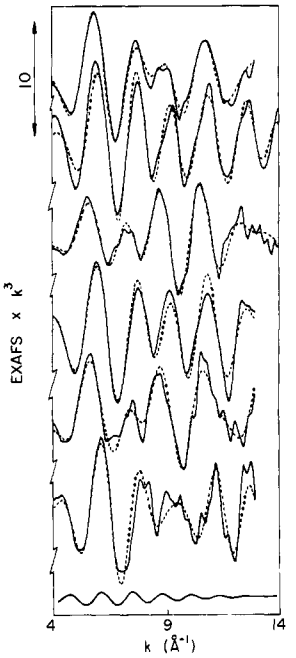

Figure 5. Measured EXAFS (—) and best fits (---) for sulfite oxidase and xanthine dehydrogenase: top to bottom, oxidized SO; reduced SO; oxidized, intact XDH; reduced, intact XDH; oxidized, cyanolyzed XDH; reduced, cyanolyzed XDH; bottom, the expected contribution of a single sulfur at 2.85 Å with a σ of 0.1 Å.

marized in Table I, Figure 5, and Charts I and II, indicate considerable variability in the Mo-site structure. Although sulfur donor ligation was a common feature in all cases, there is evidence of a short Mo–S distance in intact xanthine dehydrogenase which was not present in either sulfite oxidase or cyanolyzed xanthine dehydrogenase. The results for the six different samples are discussed in detail below.

Sulfite Oxidase (Oxidized). The new data permitted a slightly wider fitting range, but the calculated distances were essentially the same as previously reported.[8] The best two-component fit to the EXAFS is obtained by assuming two terminal oxygens at 1.68 Å and three sulfurs at 2.41 Å. However, the resultant σ (0.086 Å) for the Mo—S distance is larger than expected for vibrational disorder alone, and the presence of only two sulfurs with a smaller σ (0.071 Å) cannot be ruled out. Although a long sulfur at 2.86 Å improves the fit somewhat, the most valuable third component is a single nitrogen or oxygen at 2.19 Å. Neither component improves the fit enough to be considered definite.

As an illustration of the poor sensitivity of EXAFS to long distance Mo—S interactions, Figure 6 presents search profiles for addition of a single sulfur to the fits for oxidized sulfite oxidase, $MoO_2((SCH_2CH_2)_2N(CH_2CH_2SCH_3))$,[30] and $MoO_2(etcys)_2$.[31] As expected, a minimum at the appropriate distance is seen in

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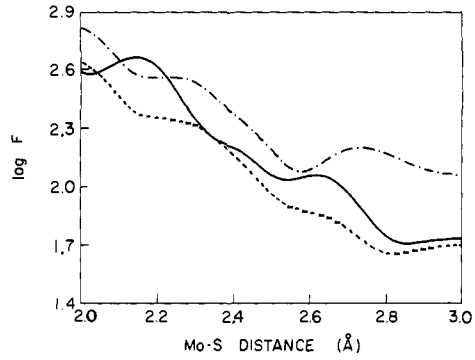

Figure 6. Search profiles for oxidized sulfite oxidase (—), $MoO_2((SCH_2CH_2)_2NCH_2CH_2SCH_3)$ (---), and $MoO_2(cys\text{-}et)_2$ (---).

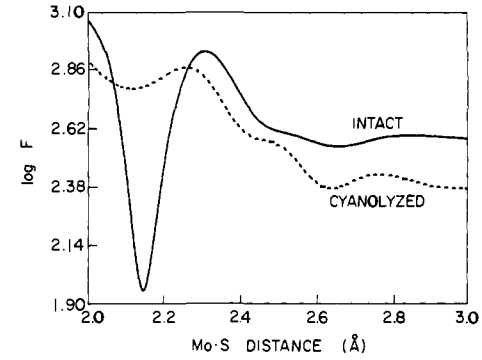

Figure 7. Search profiles for oxidized intact (—) and cyanolyzed (---) xanthine dehydrogenase.

the model data for the compound with a Mo–thioether linkage, and not for the cysteine ester complex. However, it is also clear that these minima are quite shallow. Stronger evidence seems necessary before a long methionine-like Mo—S interaction in sulfite oxidase can be unambiguously demonstrated. Future low-temperature studies are planned to clarify this point.

Given the large body of model compound data available for dioxomolybdenum(VI) thiolate complexes, it is tempting to draw further conclusions about the geometry of the sulfite oxidase Mo site. The overwhelming majority of Mo(VI) dioxo complexes have *cis*-oxo groups,[32] and it is likely that a similar geometry exists for the sulfite oxidase oxo groups. The sulfurs at 2.41 Å fall in a region typical for thiolates, and it was originally proposed that these thiolates were cis to the oxo's in an octahedral geometry.[8] However, recent model chemistry has shown that partially interacting thiolates can assume unusual geometries.[33] The question of the orientation of the sulfurs is further complicated by the ambiguity about whether there are two or three medium distance sulfurs present in the oxidized state. Thus, nothing can be said about the geometry of these thiolate-like sulfurs with respect to the oxo's from the bond lengths alone.

Sulfite Oxidase (Reduced). A single terminal oxygen at 1.69 Å, and three sulfurs at 2.38 Å gave a good fit to the data. A long Mo—S at 2.82 Å gave the most improvement to this two-wave fit, and a less substantial improvement was gained by introducing a nitrogen or oxygen at 2.04 Å. However, neither of the latter components made a strong enough contribution to the unambiguously identified by EXAFS alone.

A number of monooxomolybdenum(IV) complexes have now been synthesized,[32] and the loss of one oxo upon reduction of the original dioxomolybdenum(VI) species is not surprising. Similarly, complexes with a single oxo group generally have shorter Mo—S bonds than comparable dioxomolybdenum compounds. Since there are no known Mo(III) species with terminal oxo groups, the EXAFS can be taken as an indication that dithionite reduction stops at Mo(IV). A schematic representation of the minimum requirements for both oxidized and reduced sulfite oxidase is given in Chart I.

Intact Xanthine Dehydrogenase (Oxidized). A minimal picture for the xanthine dehydrogenase Mo site is also presented in Chart I. In contrast with sulfite oxidase, both the Fourier transform and a two-wave fit for this sample indicate only about one terminal oxygen at 1.70 Å, as well as two sulfurs at a longer distance of 2.47 Å. Using this two-wave fit as a starting point, a search profile was generated to investigate what other components might be present. As shown in Figure 7, a dramatic improvement to the fit was obtained by addition of a sulfur at 2.15 Å, a reasonable terminal Mo—S bond length. Enemark et al. found an average Mo=S distance of 2.10 Å in a series of Mo complexes,[34] and a value of 2.19 Å was recently found in $[MoO_2S_2]^{2-}$.[29] No evidence was found for a long (2.7–3.0 Å) Mo—S interaction.

Keeping in mind that the enzyme was not completely active, a series of fits with x short sulfurs and $2 - x$ short oxygens was run. The most reasonable fits were had with 0.7 sulfurs and 1.3 oxygens, and as shown in Table I, the σ values obtained in this case were more reasonable as well. In light of the conclusion (see below) that desulfo xanthine dehydrogenase has two terminal oxygens and no terminal sulfur, these results are roughly in line with the original estimation of 65 ± 10% active enzyme.

Cyanolyzed Xanthine Dehydrogenase (Oxidized). Upon cyanide treatment, the Mo—O component of the xanthine dehydrogenase EXAFS becomes larger, consistent with an increase in the number of terminal oxygens. The enhancement of the bound-state feature in the absorption edge (Figure 3) is also consistent with such a change. The best two wave fit required two oxygens at 1.67 Å, as well as two sulfurs at 2.46 Å. In sharp contrast with the intact enzyme, little improvement was had by addition of a short Mo—S component (Figure 7). Although the fits could accommodate a Mo—S distance of about 2.08 Å, the resultant σ was physically unrealistic. Assuming partial occupation produced a better σ but not a better fit. An equivalent fit was had by addition of a nitrogen or oxygen component at 1.89 Å, and a second minimum for a nitrogen or oxygen was found at 2.31 Å. Neither component improved the fit sufficiently to be definitively assigned.

The resulting picture of the molybdenum site for cyanolyzed xanthine dehydrogenase is quite complementary with the structure predicted for the intact enzyme, in that the major effect of cyanide treatment can be viewed as the replacement of a terminal sulfido ligand with a terminal oxo group. It is interesting that virtually no change was seen in the bond lengths of the remaining sulfur ligands and that they therefore remained about 0.05 Å longer than the corresponding sulfite oxidase values. The source of this difference between the enzymes is thus not in the nature of the terminal chalcogenides but may rest in the geometry of the molybdenum binding site.

Intact Xanthine Dehydrogenase (Dithionite Reduced). In this case, a single terminal oxygen was indicated, at a distance of 1.68 Å. As with sulfite oxidase, dithionite reduction resulted in a shortening of the thiolate-like Mo—S distance to 2.38 Å. The amplitude of this component also increased, and the best fit resulted from the use of three sulfurs. There was no longer any sign of a Mo—S interaction in the 2.1–2.2-Å region.

In view of the evidence for a terminal sulfide for xanthine dehydrogenase in the active, oxidized form, an obvious question is what becomes of this sulfur upon reduction. The EXAFS results clearly show that a short Mo—S bond no longer exists upon dithionite reduction. They are consistent with the hypothesis that protonation of the sulfur results in a bond lengthening which makes this sulfur unresolvable from the thiolate-like sulfurs. A Mo(IV)—SH bond length of 2.49 Å was found in $[MoO(SH)(16\text{-ane}[S_4])](CF_3SO_3)]$,[35] and in the absence of a Mo=O trans effect, one would expect a Mo—SH bond length of about 2.4 Å.

Cyanolyzed Xanthine Dehydrogenase (Dithionite Reduced). Unfortunately, only a single and relatively noisy spectrum was

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Table II. Comparisons of EXAFS Conclusions

	Mo-O_t			Mo-S			Mo-X			Mo-Y			
sample	no.	R, Å	σ, Å	no.	R, Å	σ, Å	no.	R, Å	σ, Å	no.	R, Å	σ, Å	ref
MoS_4^{2-}				4	2.17	0.0427[a]							this work
MoS_4^{2-}				4	2.18	0.075							7
$MoO_2(et\text{-}cys)_2$	2	1.70	0.039	2	2.42	0.054							this work
$MoO_2(et\text{-}cys)_2$	2	1.74	0.018	2	2.48	0.030	2 N	2.38	0.025				7
intact XDH[b]	1	1.70	0.044	2	2.47	0.071	1 S	2.15	0.032				this work
intact XO[c]	1	1.75	0.010	2	2.46	0.030	1 S	2.25	0.005	1 S	2.89	0.040	7
mixed XO	1.5	1.71	...	2.1	2.54	...				1.1 S	2.84	...	5
cyanolyzed XDH	2	1.67	0.040	2	2.46	0.069							this work
cyanolyzed XO	2	1.74	0.010	2	2.49	0.045	1 S	2.91	0.080				7

[a] Calculated from vibrational spectrum.[27] [b] XDH: chicken liver xanthine dehydrogenase. [c] XO: milk xanthine oxidase.

obtained for this sample. However, the loss of an oxo group upon dithionite reduction can still be deduced from the Fourier transform, and the fit indicated a 1.66-Å bond length for the remaining Mo=O bond. The two-wave fit also predicted three sulfurs at the much shorter distance of 2.33 Å. Some improvement in the two-wave fit was achieved by addition of either an oxygen at 2.00 Å or a long sulfur at 2.84 Å, or both. Neither of the latter components can be considered significant without additional confirming evidence.

Discussion of General Results

Although the current results are in agreement with Bordas et al.[7] with regard to the presence of a short Mo—S bond in xanthine dehydrogenase, there are several quantitative and qualitative discrepancies. Most importantly, a distance of 2.15 ± 0.03 Å is calculated, which is in a chemically reasonable range for a terminal sulfide. In contrast, the value of 2.25 Å is quite long for a Mo=S bond. The Mo—O bond lengths of Bordas et al. also tend to be consistently longer than the values obtained in this work. A third point is that their calculated Debye–Waller factors are substantially different. For example, their value for σ of 0.075 Å in MoS_4^{2-} is 70% higher than the 0.043-Å value deduced from vibrational spectroscopy,[27] and there is little correlation between their mean square amplitudes and bond lengths. Finally, in view of the search profiles of Figures 6 and 7, one should be cautious about the case for a long methionine-like Mo—S bond in the 2.8–2.9-Å vicinity, since no minimum is found in this region by the search procedure. An illustration of the expected size of a single Mo—$S_{methionine}$ interaction at 2.85 Å is given at the bottom of Figure 5. The paper of Bordas et al. makes three bond length predictions for the structure of MoO_2(ethyl-L-cysteinate)$_2$. Independently, the EXAFS of this compound was measured and analyzed by using experimental phase shifts and amplitudes. A comparison of the results with the previous predictions is made in Table II. Solution of the crystal structure of this material should provide an excellent test of the accuracy of the two methods.

Up to this point, the EXAFS results have been discussed independently of any other spectroscopic data for the molybdenum sites of these enzymes. However, there is an abundant amount of information available on the Mo(V) states of sulfite oxidase and xanthine dehydrogenase or oxidase based on EPR spectroscopy.[36] Hyperfine splittings due to ^1H and ^{17}O have been deserved for both types of enzymes[37,38] and ^{33}S splittings have been seen for the "very rapid" signal of xanthine oxidase.[39] All of these data can be explained by invoking the protonation of a terminal oxygen or sulfur upon reduction of the molybdenum.[36] Since the EPR data refer to the Mo(V) state and the dithionite-reduced samples are presumably Mo(IV), it is conceivable that further protonation of the molybdenum centers has occurred. Still, it is reasonable to search for evidence of –OH, $-OH_2$, or –SH coordination in the reduced forms of the enzymes.

With the EXAFS of reduced sulfite oxidase and reduced, cyanolyzed xanthine dehydrogenase, some improvement was had by including an oxygen at 2.00–2.04 Å. This is a reasonable value for a Mo—OH bond length. It was not possible to resolve a separate Mo—SH distance for reduced, intact xanthine dehydrogenase. Such a bond would probably be unresolvable from a Mo—SR at 2.38 Å. Although this could explain the increased Mo—S amplitude in reduced, intact xanthine dehydrogenase, one would then expect a smaller Mo—S component in reduced, cyanolyzed xanthine dehydrogenase, and this was not the case. Further work to clarify the number of thiolate-like sulfurs in all cases is clearly needed. Still, when the EXAFS results are combined with the EPR information, a more detailed model for the molybdenum sites of sulfite oxidase and xanthine dehydrogenase can be formulated, and this expanded model is presented in Chart II.

It should be emphasized that EXAFS analysis alone has not unambiguously defined the entire coordination sphere for any of the molybdenum sites discussed. Despite the incompleteness of the picture provided by EXAFS, the current results can explain some of the differences between xanthine dehydrogenase and sulfite oxidase. Two major differences between xanthine dehydrogenase and sulfite oxidase will be discussed—the behavior upon cyanide treatment and the redox potentials.

The fact that cyanide treatment of xanthine dehydrogenase (and related enzymes) results in inactivation and release of SCN^- has been known for years,[40] and the nature of the cyanolyzable sulfur has been the subject of prolonged speculation. Williams and Wentworth first proposed in 1973[41] that this sulfur was linked to molybdenum as a terminal sulfide group. The first direct evidence for this terminal sulfur was the EXAFS work of Bordas et al., but their 2.25-Å Mo—S distance was disturbing. The 2.15-Å Mo—S distance currently found removed any problems with the Mo=S hypothesis. One clear effect of CN^- is therefore reaction with this terminal sulfide and eventual replacement with a second oxo group. Such a reaction has already been observed on small molecules.[42] This kind of reactivity is impossible for sulfite oxidase, which has a dioxomolybdenum site in the oxidized form.

A second important difference between sulfite oxidase and xanthine dehydrogenase is their Mo redox potentials. The reduction potentials for sulfite oxidase are in the range of –150 to +50 mV (vs. SHE),[14] whereas intact xanthine dehydrogenase has potentials near –300 mV, and cyanolyzed xanthine dehydrogenase potentials are near –400 mV.[12] It is well established that sulfur ligands raise the redox potentials of Mo compounds with respect to oxygen-ligated analogues. Therefore, the difference between intact and cyanolyzed xanthine dehydrogenase potentials is readily explained by the replacement of a terminal sulfur with a terminal

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oxygen. However, there remains a large difference between cyanolyzed xanthine dehydrogenase and sulfite oxidase. One possible source of this difference is a diminished molybdenum thiolate interaction in xanthine dehydrogenase. Whereas the Mo—S bonds in sulfite oxidase fall at 2.42 Å, a value typical for dioxo dithiolate complexes, the xanthine dehydrogenase Mo—S bond lengths are 0.05 Å longer. Perhaps this decreases the Mo—S interaction and thereby lowers the reduction potential. It is also possible that sulfite oxidase has an extra thiolate ligand in comparison with xanthine dehydrogenase. In any case, it would be useful to have molybdenum thiolate complexes which exhibit these longer Mo—S bonds.

Summary

This work has shown unambiguously that the primary difference between the molybdenum sites of sulfite oxidase and xanthine dehydrogenase is in the nature of the terminal chalcogenides. Some differences in Mo—SR bond lengths were also found. Major changes were always observed upon reduction of the enzymes.

Previous EXAFS results on the molybdenum in nitrogenase[2,3] prompted a wealth of model-building activity, resulting in the synthesis of Mo,Fe,S clusters with one, two, or three irons bridged by sulfur to molybdenum.[43] This has permitted comparison of the properties of model compounds with those of the isolated Fe—Mo cofactor and the intact proteins. An analogous development in mononuclear molybdenum chemistry would certainly be informative. As yet, no mononuclear oxosulfidomolybdenum complexes have been synthesized, apart from the tetrahedral $(MoO_xS_{4-x})^{2-}$ series. Furthermore, there are no molybdenum thiolate complexes which exhibit the somewhat longer Mo—O bonds (ca. 2.47 ± 0.03 Å) found in xanthine dehydrogenase, although dithiocarbamate complexes with trans effects are known.[44] Finally, it would be extremely useful to have $Mo^{VI}(O)_2$ or $Mo^{VI}(O)(S)$ complexes which are stable upon reduction to Mo(V) or Mo(IV) and which exhibit EPR hyperfine splittings similar to those found in the enzymes. The lack of models for the reduced forms in Scheme II may reflect a tendency for such species to dimerize. The synthetic challenges of modeling the active sites of the nonnitrogenase molybdenum enzymes are probably of similar difficulty to those in modeling nitrogenase itself.

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^{1}H NMR Rate Constants and Mercury-199 FT NMR Equilibrium Constants Involved in Disulfide Cleavage by Methylmercury

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Abstract: A ^{1}H NMR kinetic investigation of the cleavage of dimethyl disulfide with methylmercury acetate and triethyl phosphite is described. The metal-assisted –SS– bond rupture is first order in both CH_3Hg^{II} and CH_3SSCH_3. Mercury-199 FT NMR has been employed to measure the equilibrium constants for complexation of CH_3HgOAc with CH_3SSCH_3 and $P(OEt)_3$. A concomitant electrophilic and nucleophilic mechanism for –SS– bond cleavage is suggested that involves attack by $P(OEt)_3$ on the –SS– σ^* orbital of the CH_3Hg^{II} complex of CH_3SSCH_3.

Few compounds have received the instant notoriety earned by methylmercury after the large scale outbreak of CH_3Hg^{II} poisoning in Minimata Bay, Japan.[1] The realization that alkylmercury derivatives are highly toxic and cause irreversible damage to the central nervous system has stimulated considerable research on the biochemistry and dynamics of methylmercury derivatives.

Our own research efforts have been aimed at elucidating the mechanism of CH_3Hg^{II} migration in living systems by attempting to pinpoint specific targets for CH_3Hg^{II} complexation. After initial ingestion, CH_3Hg^{II} is bound almost exclusively to the sulfhydryl functional group in the cysteine residue of peptides and proteins. The formation constants of a series of methylmercury mercaptides (CH_3HgSR) range from 10^{14} to 10^{18}, reflecting the thermodynamic stability of sulfur-bound mercury.[2] Indeed the generic term mercaptan is derived from the Latin *Mercurinium captans* (lit., seizing mercury). The unusually strong covalent bond between mercury and sulfur provides a favored binding site in biological systems. Despite such high formation constants, we have found that mercaptide anion exchange[2] in $RHgSR'$–$RHgSR''$ systems is remarkably fast and provides a potential pathway for migration of organomercurials in nature. The rapid exchange of RS^- has been suggested by Rabenstein[1] to be of paramount importance in the biological pathway for alkylmercury migration. Such rapid ligand exchange reactions play a key role in the

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