A Density Functional Study of Active Site Models for Xanthine Oxidase

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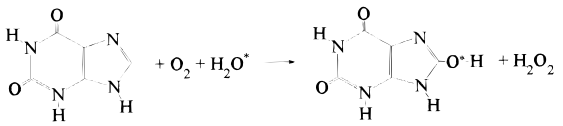
The suggestion that hydroxide is coordinated to the oxidised molybdenum site in xanthine oxidase (XnO) is tested theoretically by computing the structures of a range of four-, five-, and six-coordinate active site models. The local density approximation of density functional theory has been used with the two experimentally verified singly bonded sulfur ligands modeled by both dithiolene, $[SRCCRS]^{2-}$ (R = H and CH_3), and thiolate, $[CH_3S]^-$ groups. Both ligand types give virtually identical results for analogous species. Based on a comparison of the computed M−L distances and those reported in recent EXAFS studies, it is concluded that both four- and six-coordination are unlikely since the optimized Mo−S contacts are too short or too long respectively. Of the five-coordinate $MoOS(SR)_2X$ models, the ones with $X = [OH]^-$ give computed M−L bond lengths in excellent agreement with the reported EXAFS data while $X = H_2O$, NH_3, $[CH_3S]^-$, and O^{2-} give relatively poor agreement. The theoretical results imply that the active site represents a stable, preferred geometry rather than some imposed entatic state.

Introduction

Xanthine oxidase (XnO) is an important oxo-transfer metalloenzyme.[1−4] It catalyzes the oxidation of xanthine to uric acid using dioxygen as the electron acceptor (eq 1) and also



(1)

reacts with a range of aromatic heterocycles and simple aldehydes. XnO from cow's milk has a molecular weight of 300 kDa and is a homodimer with two 2Fe/2S iron-sulfur centers plus flavin adenine dinucleotide and a Mo center in each subunit. Oxidation occurs at Mo with concomitant reduction of the metal from the VI to the IV valence state.

Much of the interest in XnO and related enzymes concerns the unusual structure of the active site. In contrast to the $Mo^{VI}O_2$ unit normally found in model complexes and in about half of the characterized oxomolybdenum enzymes, XnO has a terminal sulfido group in place of one of the terminal oxygen atoms.

The X-ray crystal structure of a related enzyme, Aldehyde oxidase (AO), shows a five coordinate Mo^{VI} center bound to a dithiolene and three oxygens.[5] However, in the absence of definitive data for oxomolybdenum enzymes with $Mo^{VI}OS$ cores, the exact structures of the active sites of these enzymes (including XnO) are still unclear. As for AO, they appear to contain a pterin cofactor with a dithiolene side-chain[1] and magnetic circular dichroism spectra of DMSO reductase[2] and glycerol-inhibited DMSO reductase[3] suggest that the cofactor coordinates directly to the molybdenum through this side chain[4] although, in the case of DMSO reductase, this picture has recently been questioned.[6]

X-ray absorption spectroscopy and extended X-ray absorption fine structure (EXAFS) studies on the xanthine oxidase molybdenum center[7−13] suggest that the oxidized form contains a Mo^{VI} center, an oxo group, a sulfido group, and two thiolate ligands. It seems reasonable to assume that the latter arise from the dithiolene side chain although thiolate cysteine donors have also been suggested.[4] In addition to these four established ligands, most workers normally suggest a fifth donor, although its identity is often not made clear. Recent studies have indicated that this extra ligand is an O or N donor[12] possibly an hydroxide ion.

A general mechanism for oxo transfer has been proposed[4] from the early electron paramagnetic resonance (EPR) and EXAFS data. The observation[11] that the reduced enzyme with violopterin contains a regenerated Mo═O group has lead to a more specific mechanism (see Scheme 7 in ref 4). It is unclear how the Mo═O regeneration occurs though it could be from solvent present in the active site pocket or coordinated $[OH]^-$. In any event, it must be fast.

More recent EXAFS results and EPR studies on XnO model compounds[14] suggest that the oxidized form of the active site may contain a *fac*-$[Mo^{VI}(O)(S)(OH)]$ unit. This is consistent with the experimental data but leads to an alternative mechanism (see Scheme 3 in ref 14) wherein the coordinated hydroxide ligand provides the catalytically labile oxygen.

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The central question of whether $[OH]^-$ is present at the Mo^{VI} active site is still open. This problem seems to us to be ideally suited to computer modeling. The theoretical treatment of transition-metal systems has advanced significantly in recent years[15] especially with the advent of *ab initio* density functional theory (DFT). Accordingly, we have optimized the structures for a range of model active sites for XnO both with and without coordinated $[OH]^-$ in an attempt to shed further light on this problem.

Computational Procedure

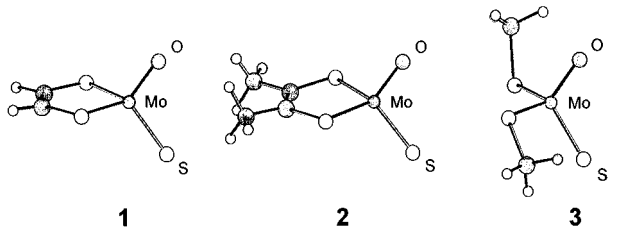
All calculations employed the local density approximation (LDA) of density functional theory as implemented in the Amsterdam Density Functional suite (ADF, Versions 1.1.2−1.1.4) due to Baerends *et al*.[16] The LDA correlation potential is based on Vosko, Wilk, and Nusair's parametrization of electron gas data.[17] STO triple-ξ-plus-polarization basis sets[18] (ADF basis sets IV) were used in conjunction with the frozen core approximation[19] (up to and including 3d for Mo, 2p for S, and 1s for C and O). Geometry optimizations were carried out using analytical energy gradients[20] and without symmetry restrictions except for the methyl groups where the C−H bond lengths were fixed at 1.09 Å and the C−C−H and S−C−H angles were fixed at 104.9°. Unless otherwise indicated, the two Mo−S distances for dithiolene models or two $Mo-SCH_3$ distances for thiolate models were constrained to adopt the same (optimized) value.

Results and Discussion

The suggestion that hydroxide is present at the active site of XnO has important implications for the source of the oxygen transferred during the enzyme's catalytic cycle. In several Mo enzymes and model MoO_2 complexes, it is one of the terminal oxo atoms which is transferred. However, there is some controversy surrounding the nature of the catalytically labile oxygen in XnO.[4] ^{18}O labeling experiments[21] showed that the primary source of transferred oxygen came directly from the enzyme itself and not from the bulk solvent.[22] At that time, the Mo═O group was the only confirmed oxygen present at the active site, so it seemed likely that this was the catalytically active oxygen. However, as mentioned above, coordinated $[OH]^-$ may provide the catalytically labile oxygen.

Hydroxide bound at the active site of XnO offers a number of attractive features.[4] It permits the coordination number at Mo to remain constant throughout the catalytic cycle and may also provide a way of rationalizing the very rapid appearance of a terminal Mo═O group in the enzyme's reduced form. However, the fit to the EXAFS data is only marginally improved by including an $[OH]^-$ group while the ^{17}O anisotropic hyperfine data are suggestive rather than definitive.[4] The question of whether $[OH]^-$ is present at the Mo^{VI} active site is still open.

Such a structural problem is amenable to computer modeling, and accordingly, we have computed the optimized geometries for a range of model active sites for XnO both with and without coordinated $[OH]^-$. Thiolate sulfurs from cysteine residues are described by $[CH_3S]^-$ ligands while the dithiolene from a supposed pterin molecule is modeled by $[SCRCRS]^{2-}$ (R = H, CH_3). We use NH_3 as a simple nitrogen donor which, in the real system, would presumably be an imine nitrogen from a histidine side chain, but as described below, this simplification does not appear to be significant.



1 **2** **3**

Figure 1. Four coordinate $[MoOS(S_2)]$ active site model complexes **1**−**3**.

There are only four firmly established Mo−L bonds for the XnO active site so the distorted tetrahedral molecules **1**, **2**, and **3** form our starting point. The computed structures are shown in Figure 1 and the LDA optimized bond lengths are given in Table 1. (A full listing of the Cartesian coordinates for the structures shown in Figures 1 and 3−7 has been deposited as Supporting Information.) All three species have O═Mo═S angles around 107° while the S−Mo−S angle is 100° for the dithiolene systems (**1**, **2**) and 110° for the thiolate molecule (**3**). There are three main points arising from these calculations. First, the computed results for the dithiolene systems do not depend on the nature of the R group, at least for R = H or methyl. We have therefore employed the simpler system in all subsequent calculations. Second, there is no significant difference between the computed metal−ligand bond distances for a dithiolene system and those for its analogue with two thiolates. This observation seems quite general. Third, the agreement between the theoretical and EXAFS data for **1**, **2** and **3** is reasonable for the Mo═O and Mo═S contacts but the Mo−S distances are calculated to be around 0.15 Å too short.

The good agreement found here and elsewhere[23] for formally multiply bonded ligands gives us confidence that, in principle, the LDA is capable of treating these systems accurately. In addition, full geometry optimizations of related MoO_2 species $MoO_2(dtc)_2$ (dtc = dithiocarbamate) and $[MoO_2(NCS)_4]^{2-}$ give M−L distances within 0.02 Å of the X-ray crystal structure data.[24] In fact, LDA results for a whole range of coordination complexes are in consistently good agreement with liquid and solid state structures.[25]

The ability to reproduce the correct bond lengths with calculations that formally refer to a vacuum implies that the potential field surrounding the molecule of interest does not greatly affect the structure at least in terms of the M−L distances. However, in XnO the S atoms are ultimately joined to the protein backbone, and perhaps this induces radial and/or angular strain which might lengthen the bonds relative to the values computed for models **1**−**3**.

In the absence of a detailed structure for XnO it is difficult to assess this possibility. In coordination chemistry, M−L distances for a particular combination of metal, ligand and coordination number can vary. For example, a search of the

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Table 1. Comparison of Calculated Bond Lengths for Models of the XnO Active Site with Experimental EXAFS Distances[a]

active site model[b]	total charge	Mo═O	Mo═S	Mo−S1	Mo−S2	Mo−S3	Mo−X	X
1	0	1.70	2.12	2.32	2.32			
2	0	1.70	2.13	2.30	2.30			
3	0	1.72	2.14	2.28	2.28			
4	−1	1.72	2.19	2.43	2.43		1.96	OH
4a	−1	1.72	2.19	2.42	2.44		1.96	OH
5	0	1.72	2.18	2.45	2.45		1.96	OH
5a	0	1.72	2.18	2.47	2.40		1.96	OH
6	0	1.71	2.16	2.38	2.38		2.15	H_2O
7	0	1.72	2.15	2.37	2.37		2.22	NH_3
7a	0	1.72	2.15	2.34	2.40		2.22	NH_3
8	0	1.72	2.16	2.35	2.35		2.26	NH_3
8a	0	1.72	2.16	2.33	2.37		2.26	NH_3
9	−1	1.72	2.19	2.42	2.42	2.36		
10	−1	1.72	2.19	2.40	2.40	2.40		
11	−2	1.75	2.26	2.54	2.54		1.77	O
12	−2	1.75	2.27	2.56	2.56		1.77	O
13	−3	1.77	2.30	2.72	2.72	2.76	1.76	O
14	−2	1.75	2.23	2.63	2.63	2.47	1.95	OH
14a	−2	1.75	2.22	2.43	2.60	2.77	1.95	OH
exptl[c]		1.73	2.17	2.46	2.46			
exptl[d]		1.74	2.18	2.47	2.47		1.98	

[a] S3 always refers to an $[SCH_3]^-$ group and is independently optimized. S1 and S2 can be either $[SCH_3]^-$ groups (thiolate) or part of an enethiolate ring {SC(R)−(R)CS}. Mo−S1 and Mo−S2 are constrained to the same (optimized) bond length except for 4a, 5a, 7a, 8a, and 14a, where both Mo−S distances are independently optimized. [b] See Figures 1 and 3−6 for structural diagrams. [c] Experimental data fitted without OH^- group. [d] Experimental data fitted with OH^- group.

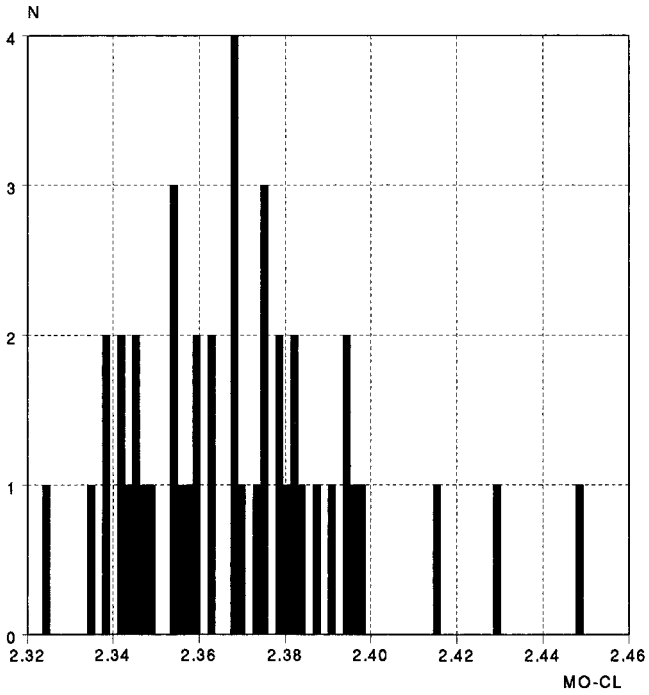
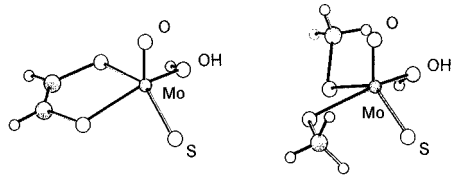


Figure 2. Histogram showing the distribution of Mo−Cl distances in molecules containing the $Mo^{IV}O_2Cl_2$ fragment. Data obtained via a connectivity search of the Cambridge Structural Database using the program VISTA.[26] (44 observations, minimum = 2.324 Å, maximum = 2.448 Å, mean = 2.369 Å, sample standard deviation = 0.025 Å).

Cambridge Structural Database[26] for complexes containing a $Mo^{VI}O_2Cl_2$ unit gives Mo−Cl distances spanning some 0.124 Å from 2.324 to 2.448 Å (Figure 2). However, such variation does not occur for a single complex. Instead, the Mo−Cl distances depend on the nature of the other ligands and the bonding in each individual case. Therefore, providing our model ligands are reasonable approximations to the real donors, the computed Mo−S distance of about 2.30 Å for molecules **1**, **2**, and **3** is a true reflection of the bond length for a notional four-coordinate complex with a $Mo^{VI}-S^-$ entity providing that the enzyme permits the Mo center to adopt a preferred geometry rather than enforcing some strained "entatic" state.[27]



4 **5**

Figure 3. Five-coordinate $[MoOS(S_2)L]^-$ (L = $[OH]^-$) active site model complexes **4** and **5**.

As described below, we believe that the DFT calculations provide strong, albeit circumstantial evidence, that the Mo center is not especially strained. The much poorer agreement for the Mo−S single bonds would then be due to inappropriate model structures. This may seem rather obvious since most workers suggest at least a five-coordinate active site. However, the theoretical results for the four-coordinate species are important since they establish the general reliability of the computational approach while specifically discounting four-coordination for the XnO active site.

The five-coordinate hydroxide species (**4** and **5**, Figure 3) have distorted trigonal bipyramidal structures. In **4**, the dithiolene occupies one axial and one equatorial site with the sulfido group in the other axial position while in **5**, the sulfido group is equatorial and the hydroxyl ligand is axial. Again, we observe good reproduction of the terminal Mo═O and Mo═S distances and virtually identical results for dithiolene and thiolate analogues. The latter applies whether the two Mo−S distances are constrained to have the same value (**4** and **5** in Table 1) or are allowed to vary independently (**4a** and **5a** in Table 1). Significantly, the computed Mo−L distances are virtually identical (i.e. within 0.02 Å) to the EXAFS bond lengths when the latter includes an $[OH]^-$ group in the fitting.[12] We believe this constitutes good evidence for the presence of

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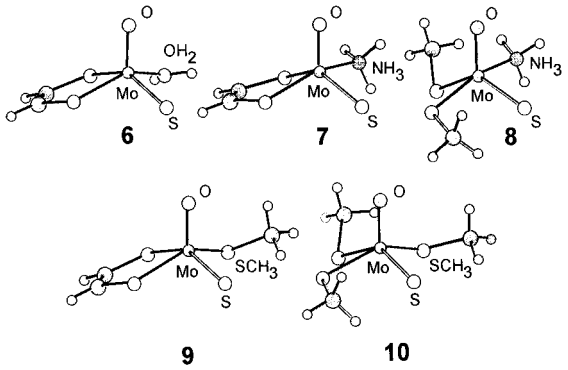

Figure 4. Five-coordinate $[MoOS(S_2)L]^{n-}$ (L = H_2O, NH_3, $n = 0$; L = $[SCH_3]^-$, $n = 1$) active site model complexes **6**−**10**.

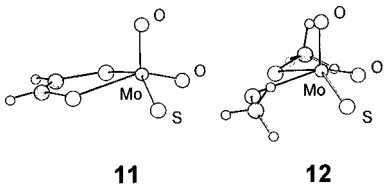

Figure 5. Five-coordinate $[MoOSO(S_2)]^{2-}$ active site model complexes **11** and **12**.

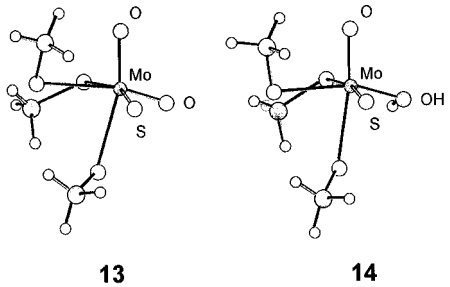

Figure 6. Six-coordinate $[MoOSL(S_3)]^{n-}$ (L = O, $n = 3$; L = $[OH]^-$, $n = 2$) active site model complexes **13** and **14**.

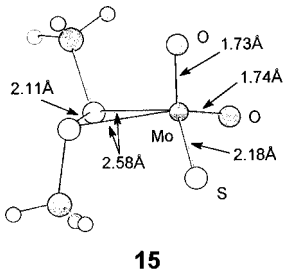

Figure 7. Structure of $[MoO_2S(CH_3S\text{-}SCH_3)]$ model complex **15** showing coordination of disulfide group.

hydroxide at the active site of XnO. In addition, these results provide the circumstantial support mentioned above for (a) the validity of the theoretical geometries and (b) the implication that the active site environment in the enzyme has little or no effect on the Mo coordination geometry. However, to test this further, we have carried out a number of other calculations.

First, the $[OH]^-$ group was replaced by water (**6**, Figure 4). The structure can best be described as a distorted square pyramid with an apical oxo ligand. However, the Mo−S distances are computed to be 0.09 Å too short while the Mo−OH_2 contact is 0.17 Å longer than the reported Mo−O/N bond length (see Table 1). Replacing the water by X = NH_3 (**7** and **8**, Figure 4) or X = $[CH_3S]^-$ (**9** and **10**, Figure 4) gave Mo−X distances respectively about 0.25 and 0.4 Å longer than the reported 1.98 Å, with Mo−S distances again too short by between 0.1 and 0.06 Å (Table 1). The computed Mo−N distance is unlikely to be shortened by the required 0.25 Å simply by replacing NH_3 with imidazole.

We also carried out some calculations with X = O, despite the lack of any experimental support for such a species, to confirm that a second oxo group is also unlikely theoretically (**11** and **12**, Figure 5). The Mo−S and Mo═S distances are about 0.08 to 0.1 Å too long (Table 1). In summary, calculations on five-coordinate models indicate the XnO active site Mo is bound to two thiolate sulfurs, a terminal oxo group, a terminal sulfido ligand, and an hydroxide ion.

Pentacoordination is also found for the active site of aldehyde oxidase (AO). This protein has been crystallographically characterized,[5] and the Mo is bound to a dithiolene and three oxygens. The binding pocket of AO has a sequence similar to XnO which provides further support for the suggestion of a five-coordinate site for XnO.

However, to complete the study, a couple of six-coordinate model species were investigated of general formula $[MoOSX(SCH_3)_3]^{n-}$, X = O and $[OH]^-$ (**13** and **14**, Figure 6). The LDA optimized Mo−S distances are up to 0.3 Å longer than experiment (Table 1) while the Mo═S distance is also overestimated by 0.05−0.13 Å. It seems that the calculations do not support a six-coordinate active site for XnO. Evidently, the Mo center becomes too crowded to accommodate the S ligands at anywhere near the bond lengths observed experimentally.

Finally, we accidentally obtained a rather unexpected and interesting result for a five-coordinate model. An incorrect overall charge of zero was initially assigned to the model species **12** such that the sulfur ligands formally referred to $CH_3S^\bullet$ radicals. During the geometry optimization, these two groups come together to form a disulfide bond (model **15**) but the sulfurs also remain bound to the metal to form a three-membered ring. (Figure 7). The S−S distance is computed to be 2.11 Å compared to the sum of the covalent radii of 2.08 Å.[28] There is no precedent for such an entity in the Cambridge Structural Database, and we are not aware of any suggestion that this moiety exists in biological molecules although Berg *et al.*[29] have synthesized various dioxo−Mo^{VI} cysteamine model complexes where the S···S distances are significantly shorter than the sum of the van der Waals radii. They suggest that the partial formation of a disulfide bond could influence the one-electron oxidations of Mo^{IV} through Mo^V to Mo^{VI} although these model complexes still have quite long S−S distances (∼2.7 Å). In contrast, the bridging S_2^{2-} units in $[Mo_3S_7]^{4+}$ clusters have S−S bond lengths of around 2.04 Å[30] which are quite similar to our theoretical results, presumably reflecting that both cases formally refer to single bonds.

Our calculations suggest that it should be possible for a range of metal−disulfide interactions depending on the stability of the metal's oxidation state—i.e. if the metal is in a state which can resist oxidation, a stable bond to disulfide is possible. This is the case for model **15** where the Mo is formally +6. However, model **12** can be notionally assigned as a Mo^{IV}−disulfide complex and the final structure is then the result of an internal redox process in which the metal is oxidized and the disulfide bond reduced, and consequently broken, giving a Mo^{VI}−dithiolate moiety. The idea that a disulfide bridge in a protein may act as a bidentate ligand is intriguing and has potentially far reaching consequences. For example, could

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transient formation of disulfide bridges be important for generating suitable protein conformations and metal binding sites during the assembly of metalloenzyme active sites? Such theoretical speculations await experimental confirmation.

Conclusions

Optimized geometries for a range of four-, five- and six-coordinate active site models for xanthine oxidase have been calculated at the local density approximation level of density functional theory. The two experimentally verified singly bonded sulfur ligands have been modeled by both dithiolene, $[SRCCRS]^{2-}$ (R = H and CH_3), and thiolate, $[CH_3S^-]$, although they give virtually identical results for analogous models. On the basis of a comparison of the computed M−L distances and those reported in recent EXAFS studies, it is concluded that both four- and six-coordination are unlikely since the optimized Mo−S contacts are too short or too long respectively. Of the five-coordinate $MoOS(SR)_2X$ models, the one with $X = [OH]^-$ gives computed M−L bond lengths in excellent agreement with the reported EXAFS data while $X = H_2O$, NH_3, $[CH_3S]^-$, or O^{2-} give relatively poorer agreement.

Although a dithiolene ligand behaves the same as two thiolates when the two Mo−S distances are constrained to the same value, some variation does occur if each bond is optimized independently. For dithiolene models (**4a** and **7a**), the two Mo−S contacts are still very similar. For thiolate systems (**5a**, **8a** and **14a**), the two bond lengths can be quite different although they average to about the constrained value. These theoretical results plus the observation of only a single Mo−S distance in the EXAFS experiment[11] favor dithiolene over thiolate at the XnO active site.

This study does not, of course, prove that the hydroxide oxygen is the one transferred during catalysis but the theoretical results do provide independent verification of the likely presence of $[OH]^-$ at the oxidized, five-coordinate active site and hence that a mechanism involving a hydroxide oxygen is at least feasible. They further suggest that the active site of the oxidized form of XnO is not unduly affected by the protein environment since the correct bond lengths are reproduced by calculations which formally refer to a vacuum. Evidently, the Mo^{VI} center in XnO is not in an entatic state. This is consistent with the proposed pentacoordinate active site and a mechanism which maintains this coordination number throughout the catalytic cycle.[4] Five-coordinate species are very flexible,[31] and hence we would expect minimal energy changes at the Mo center during turnover.

Finally, the calculations suggest that a disulfide bond, such as may cross link protein strands, can form a stable interaction with a metal providing the latter is in a state which can resist further oxidation. The disulfide acts as a bidentate ligand forming a triangular Mo−S−S unit. Such a feature has many biochemical implications, but the actual biological significance of this theoretical result, if any, awaits experimental confirmation.

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Supporting Information Available: Listings of Cartesian coordinates for models **1**−**15** (2 pages). Ordering information is given on any current masthead page.

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(31) See the discussion in, for example: Burton, V. J.; Deeth, R. J.; Kemp, C. M.; Gilbert, P. J. *J. Amer. Chem. Soc.* **1995**, *117*, 8407.