

Cysteine-Thiolato Dioxomolybdenum(VI) Species Supported by Water Soluble Poly(ethylene glycol) as a Molybdo-Oxidase Model. Its Enhanced Oxidative Ability in Water as Shown by the CD Spectral Investigation

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ABSTRACT: Water-soluble $\text{MoO}_2(\text{cys-O-PEG})_2$ as a model for molybdo-oxidases was prepared from *t*-butyloxycarbonyl-L-cysteine(*S*-acetamidomethyl)-O-($\text{CH}_2\text{CH}_2\text{O}$) $_n$ -OH (*t*-Boc-Cys(Acm)-O-PEG, $n=45$), and HgCl_2 (*t*-Boc-cys-O-PEG). The macromolecular Mo(VI) complex readily decomposed in water, forming a disulfide derived from the *cys*-PEG ligands. $\text{MoO}_2(\text{cys-O-PEG})_2$ in water causes the catalytic air oxidation of PPh_3 or P(OMe)_3 . The role of the hydrophobic environment surrounding the active site of molybdenum oxidase was found to protect the Mo(VI)/Mo(V) site from water in bulk. The model dioxo-Mo(VI) complexes are generally insoluble in water thus making it impossible to examine their redox behavior toward biological redox agents.

KEY WORDS Molybdo-oxidase Model / Polymer-Supported Mo(VI) Complex / Dioxo-di(cysteinylyl polyethylene glycol ester)molybdenum(VI) /

Dioxomolybdenum(VI) complexes [MoO_2L_2] ($\text{L} =$ bidentate ligand, *e.g.* chelating S-N ligands) are currently attracting interest as models for molybdo-oxidase, *e.g.* xanthine oxidase, sulfite oxidase, and aldehyde oxidase.¹ The oxidation involves O-atom transfer. We made a study of the catalytic activity of $\text{MoO}_2(\text{cys-OR})_2$, where $\text{R} =$ methyl, ethyl, isopropyl, and benzyl for the air-oxidation of PPh_3 to Ph_3PO in $\text{DMF}/\text{H}_2\text{O}$.² The EPR study of the system, $\text{MoO}_2(\text{cys-OMe})_2/\text{PPh}_3$, indicated that the reduction of the Mo(VI) complex by triphenylphosphine results in the information of a mononuclear Mo(V) complex.³ The presence of a considerable amount of water was found to be an essential factor for the catalytic activity, though the addition of excess water diminished the activity.² Thus the reactivity of dioxo-molybdenum(VI) complexes in the presence of water is important. Isolation of water-soluble *cis*-dioxoMo(VI) complexes with (S,N)ligands such as $\text{MoO}_2(\text{cys})_2$ ⁶ is considered to be quite difficult because of their high susceptibility to water. We attempted to synthesize a dioxo-

Mo(VI) complex with (S,N)ligands which is soluble in either organic solvents or water to examine the reactivity of the Mo(VI) complex in water. *Cys*-O-PEG [PEG = poly(ethylene glycol)] was prepared as a water-soluble (S,N)ligand. We previously reported the synthesis of $\text{Mo}_2\text{VO}_2(\mu\text{-O}_2(\text{cys-NH-PEG})_2)$ as a model for nitrogenase,⁴ which can be reduced with water-soluble reductants such as $\text{Na}_2\text{S}_2\text{O}_4$.

This paper describes the syntheses of water-soluble molybdenum(VI) complexes having cysteine thiolato ligands covalently bonded to poly(ethylene glycol). Further, their redox chemistry in the presence of water at various pH was examined, and the results are discussed on the basis of the active site structures of molybdo-oxidase.

EXPERIMENTAL

Materials

All the solvents were purified by appropriate procedures. *t*-Boc-L-Cys(Acm)-OH (*t*-Boc = *t*-butyloxycarbonyl, Acm = acetamidomethyl) was prepared using *t*-Boc-S (Mitsubishi Chem. Co.)

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from Cys(Acm)OH.⁵

Preparation of *t*-Boc-Cys(Acm)-O-PEG

Ten grams of PEG (M.W. 2000, Wako Chem. Co.), *t*-Boc-Cys(Acm)-OH (2.9 g), and dicyclohexyl carbodiimide (2.1 g) were added to mixed solvents of CH₂Cl₂ (100 ml) and ethyl acetate (70 ml). This mixture was stirred at 0°C for three hours and at room temperature for three days. The dicyclohexylurea formed was filtered off and the solvents were evaporated *in vacuo*. The residue was extracted with ethyl acetate to remove dicyclohexylurea. After concentrating the extract to a small volume, the addition of ether resulted in the formation of a solid which was collected, washed with ether and dried *in vacuo*. An attempt to separate the *t*-Boc-Cys(Acm)-O-PEG from the unreacted PEG was unsuccessful. The Cys content in PEG was about 50% as determined by the intensity ratio of ¹H NMR signals at 3.64 ppm due to $-(\text{CH}_2\text{CH}_2-\text{O})_n$ and at 1.46 ppm due to *t*-Boc protons.

Preparation of HCl·Cys-O-PEG

t-Boc-Cys(Acm)-O-PEG (5.0 g) was dissolved in ethyl acetate (30 ml). Hydrogen chloride gas was passed through the solution for 10 min. Ether (100 ml) was added to this solution to precipitate a solid which was collected, washed with ether, and dried *in vacuo* (4.2 g). A solution of mercuric chloride in methanol was added to the solution of HCl·Cys(Acm)-O-PEG in methanol. This mixture was stirred and after 8 h ether was added to the solution to precipitate a mercury complex which was collected, washed with ether, and dried *in vacuo*. Hydrogen sulfide was passed through a solution of the mercury complex in methanol with stirring for 30 min and then the solution was stirred for 2 h. Mercuric sulfide was filtered off and the

filtrate was concentrated cautiously *in vacuo*. The residue was dissolved in a minimum amount of methanol and ether was added to the solution to precipitate a solid which was collected, washed with ether and dried *in vacuo* (2.8 g).

Preparation of HgCl(*t*-Boc-Cys-O-PEG)

To a solution of *t*-Boc-Cys(Acm)-O-PEG in methylene chloride/methanol (1:1), HgCl₂ was added at room temperature. After the addition of a large amount of water, the methylene chloride layer was collected, washed with water, and concentrated under reduced pressure. A white solid obtained was collected and dried.

Synthesis of MoO₂(cys-O-PEG)₂

This was carried out according to Melby's method for MoO₂(cys-OEt)₂.⁷ A solution of Na₂MoO₄·2H₂O (0.22 g) in water was added to an aqueous solution of HCl·Cys-O-PEG (1. g) to give a yellow solution which was extracted with chloroform. The chloroform layer was then dried with anhydrous sodium sulfate. After filtration, ether was added to the filtrate with cooling to precipitate a yellow Mo(VI) complex. The powder was collected by filtration, washed with ether, and dried *in vacuo*. Yield 0.8 g.

Physical Measurements

¹³C NMR spectra were recorded on a JEOL FX-90Q spectrometer. CD spectra were obtained on a JASCO J-40A at room temperature. A Raman spectrum was recorded on a JASCO R800 spectrometer using an argon ion laser operated at 488 nm.

RESULTS AND DISCUSSION

Characterization of MoO₂(cys-O-PEG)₂

The ¹³C NMR spectral technique is highly useful for the analysis of oligopeptides attached to the PEG $-(\text{CH}_2\text{CH}_2-\text{O})_n$ sequence since not only methylene carbons of the PEG parts but also those of unreacted PEG give sharp signals centered at 73 ppm in Me₂SO-*d*₆ or in CDCl₃. Figure 2 shows the ¹³C NMR spectra of *t*-Boc-Cys(Acm)-O-PEG, *t*-Boc-cys(HgCl)-O-PEG, and MoO₂(cys-O-PEG)₂. The Cys C_β signal of *t*-Boc-Cys(Acm)-O-PEG at 41.9 ppm shifts to 31.5 ppm with the formation of the Hg(II) complex. Such an upfield shift can be

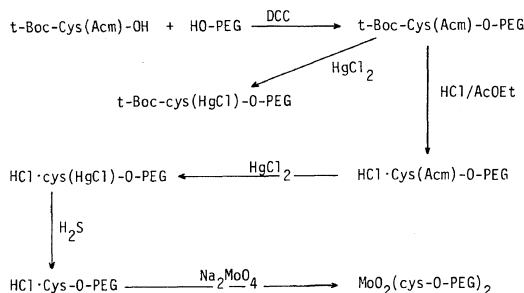


Figure 1. Synthetic scheme of MoO₂(cys-O-PEG)₂.

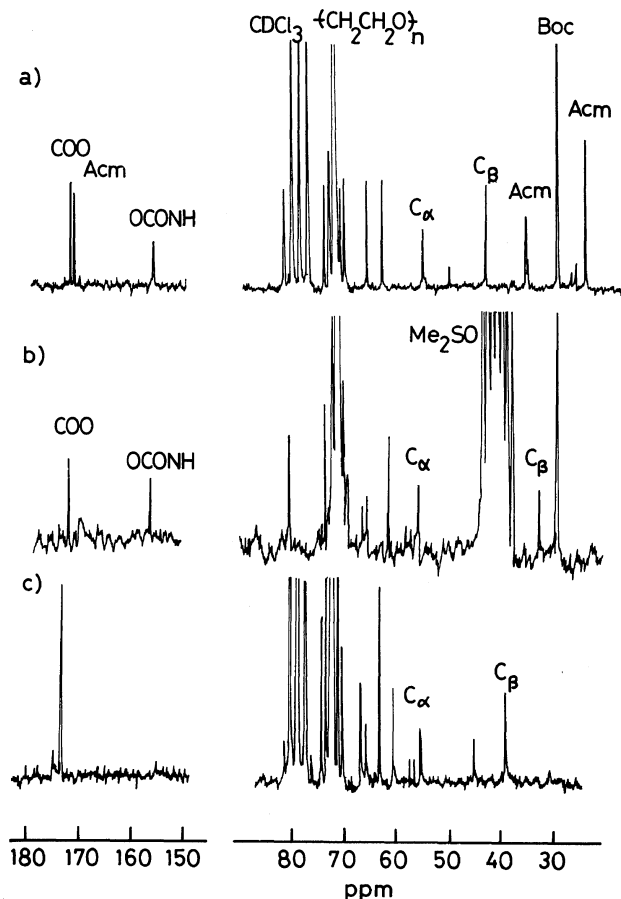
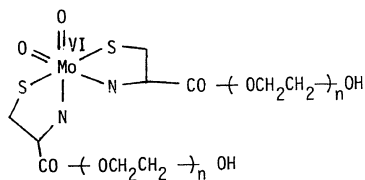


Figure 2. ^{13}C NMR spectra of a) *t*-Boc-Cys(Acm)-O-PEG in CDCl_3 , b) $\text{HgCl}(t\text{-Boc-cys-O-PEG})$ in $\text{Me}_2\text{SO}-d_6$, and c) $\text{MoO}_2(\text{cys-O-PEG})_2$ in CDCl_3 .

explained as a result of the decrease in the electronegativity of the C_β carbon since the electron cloud in the neighboring sulfur flows into the Hg(II) ion. The observation of the C_β signal for $\text{MoO}_2(\text{cys-O-PEG})_2$ at 37.7 ppm as in the case of $\text{MoO}_2(\text{cys-OMe})_2$ ⁸ indicates the coordination of the (S,N) chelate ligands to Mo(VI) ion as shown in the following structure.



Two absorption maxima were observed for $\text{MoO}_2(\text{cys-O-PEG})_2$ at 260 nm (ϵ , 3500) and 350 nm

(ϵ , 2400), corresponding to the typical absorptions of $\text{MoO}_2(\text{cys-OMe})_2$ at 262 nm (ϵ , 7300) and 354 nm (ϵ , 5400).⁷ The formation of $\text{MoO}_2(\text{cys-O-PEG})_2$ was also supported by comparing its CD spectra in Figure 3 with those of the authentic sample, $\text{MoO}_2(\text{cys-OMe})_2$. The differential dichroic absorption ($\Delta\epsilon$, -7.5) of $\text{MoO}_2(\text{cys-O-PEG})_2$ was about half the value ($\Delta\epsilon$, -16) of $\text{MoO}_2(\text{cys-OMe})_2$ at 290 nm assignable to the C-T band primarily associated with the Mo-S bond. The $\text{MoO}_2(\text{cys-O-PEG})_2$ complex showed low stability in DMF and the decrease in $\Delta\epsilon$ (-4.7) value was observed as shown in Figure 3. However, the CD shape still retained that of $\text{MoO}_2(\text{cys-O-PEG})_2$.

Reaction of $\text{MoO}_2(\text{cys-O-PEG})_2$ with Water

The CD spectra of $\text{MoO}_2(\text{cys-O-PEG})_2$ in water

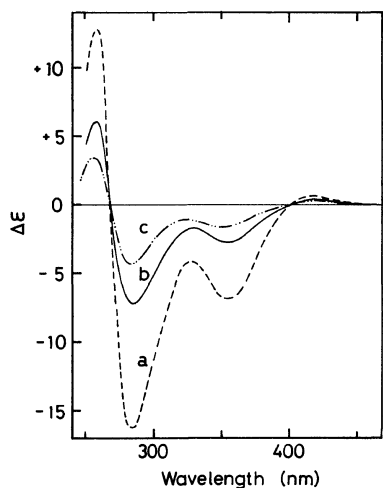


Figure 3. CD spectra of a) $\text{MoO}_2(\text{cys-OMe})_2$ in CHCl_3 , b) $\text{MoO}_2(\text{cys-O-PEG})_2$ in CHCl_3 , and c) $\text{MoO}_2(\text{cys-O-PEG})_2$ in $\text{DMF}/\text{H}_2\text{O}$ (1:0.15).

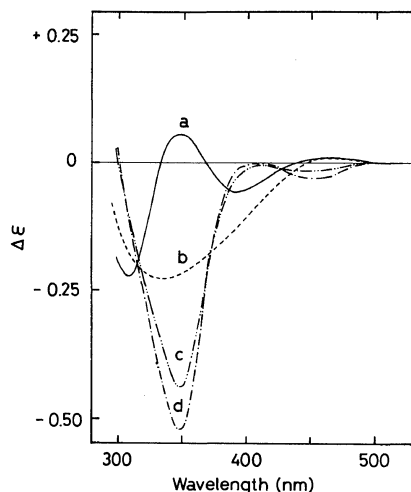
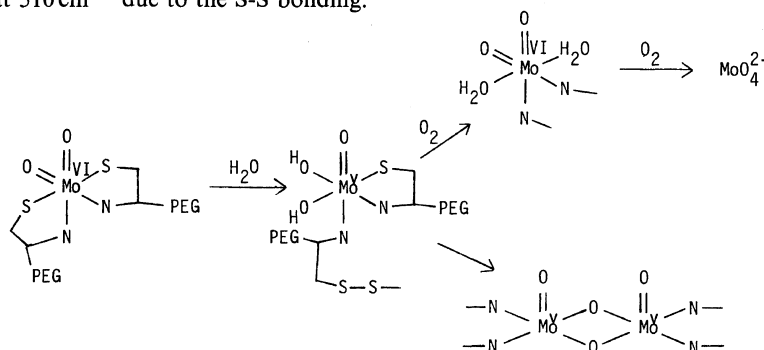


Figure 4. CD spectra of $\text{MoO}_2(\text{cys-O-PEG})_2$ in water under aerobic conditions, a) immediately, b) after 2 h, c) after 15 h, and d) after 24 h.

indicated a rapid reaction accompanied by an extreme decrease in the $\Delta\epsilon$ value as shown in Figure 4. The same CD results were obtained when a large amount of water was added to the DMF solution of $\text{MoO}_2(\text{cys-O-PEG})_2$.

The decrease in the $\Delta\epsilon$ value means that the species formed by the reaction with water probably have no chiral ligand. Since the synthesis of $\text{MoO}_2(\text{cys-O-PEG})_2$ took place in water, the small amount of water contaminated in DMF would not cause such a reaction. Therefore, the above results indicate that the $\text{Mo}=\text{O}$ moiety of $\text{MoO}_2(\text{cys-O-PEG})_2$ is activated in water and oxidizes its own thiolato ligands to form S-S bonds accompanied by a reduction of the Mo species as shown in the following scheme. The formation of a cystine derivative, $(\text{cys-O-PEG})_2$, was confirmed by a strong Raman band at 510 cm^{-1} due to the S-S bonding.

The formation of cystine by the reaction of Na_2MoO_4 with cysteine is well known.¹⁰ The absorption intensity of $\text{MoO}_2(\text{cys-O-PEG})_2$ when dissolved in water weakened immediately at 374 and 283 nm. The broad absorptions at 300 nm may indicate the formation of MoO_4^{2-} or $[\text{Mo}_2\text{O}_2(\mu\text{-O})_2]^{2+}$. The reduced Mo species gradually give a typical CD pattern of a stable binuclear Mo(V) complex showing a weak trough around 360 nm ($\Delta\epsilon$, -0.6) as observed in the CD spectrum of $\text{Mo}_2\text{O}_2(\mu\text{-O})_2(\text{histidinato})_2$ without the thiolato ligands reported by Suzuki *et al.*⁹ They also reported that the non-chelating coordination of cystine amino groups to $\text{Mo}_2\text{O}_2(\mu\text{-O})_2^{2-}$ provided a weak CD extremum. This high reactivity in water suggests that it is difficult to synthesize a dioxo Mo(VI) complex of cysteine in water. The alleged



preparation does not seem plausible, although has been proposed.⁶

The pH dependence of the CD spectra of $\text{MoO}_2(\text{cys-O-PEG})_2$ was examined. The $\text{Mo}_2\text{O}_2(\mu\text{-O})_2^{2+}$ species having bands at 290 nm ($\Delta\epsilon$, +0.2) and 370 nm ($\Delta\epsilon$, -0.23) was formed at pH 8.0. At pH 6.0 and 4.6, only a weak maximum was found over 300 nm. Of course, weak peaks due to the ligand was observed below 250 nm at low pH.

Reduction of $\text{MoO}_2(\text{cys-O-PEG})_2$ in $\text{P}(\text{OMe})_3$

A mild reduction of $\text{MoO}_2(\text{cys-O-PEG})_2$ by trimethylphosphite was studied in water and in DMF/ H_2O (1:0.15). Figure 5 shows the CD spectra of the Mo(VI) complex with various amounts of $\text{P}(\text{OMe})_3$ in DMF/ H_2O under argon. When the Mo(VI)

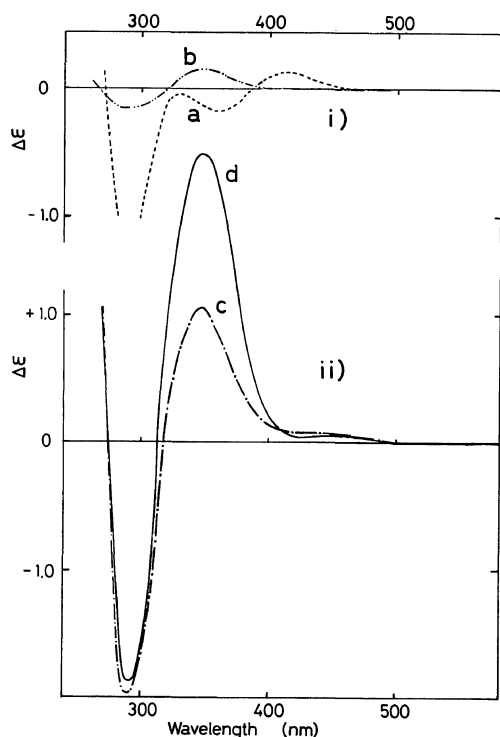


Figure 5. CD spectra of $\text{MoO}_2(\text{cys-O-PEG})_2$ in the presence of $\text{P}(\text{OMe})_3$ in DMF/ H_2O (1:0.15). a) $\text{MoO}_2(\text{cys-O-PEG})_2$ without $\text{P}(\text{OMe})_3$, b) $\text{P}(\text{OMe})_3$ was added to the solution (a) after 17 h, c) the spectrum was obtained immediately after $\text{MoO}_2(\text{cys-O-PEG})_2$ was added to the solution of $\text{P}(\text{OMe})_3$, and d) after 18 h. Conditions: $[\text{Mo}]/[\text{P}(\text{OMe})_3]=1/20$ at room temperature.

complex was added to the $\text{P}(\text{OMe})_3/\text{solvent}$ mixture, a maximum at 345 nm immediately appeared with positive sign as shown in Figure 5-i-c. The results indicate that the Mo(VI) complex was reduced smoothly by $\text{P}(\text{OMe})_3$ (Figure 5-ii-d) and gave a Mo(V) species which is probably a mononuclear Mo(V) complex. On the other hand, the addition of $\text{P}(\text{OMe})_3$ to the DMF/ H_2O solution of $\text{MoO}_2(\text{cys-PEG})_2$ results in detachment of most of the cysteinate ligands as shown by weak CD maxima in Figure 5-i-b.

In aqueous solution, the Mo(VI) complex was reduced rapidly to a Mo(V) state at pH 6. The Mo(V) species was gradually oxidized by air as shown in Figure 6. The weak maxima and shallow troughs of the CD spectrum imply that the decomposition of the complex prevails rather than the reduction to the corresponding Mo(V) complex. For example, a small maximum at 345 nm ($\Delta\epsilon$, +0.03) was observed immediately after the addition of the Mo(VI) complex to an aqueous solution of $\text{P}(\text{OMe})_3$. Thus, even with the addition of a mild reducing reagent, the decomposition of the Mo complex in aqueous solution could not be suppressed. For the air oxidation of PPh_3 with $\text{MoO}_2(\text{cys-OEt})_2$, a solvent system of DMF/ H_2O

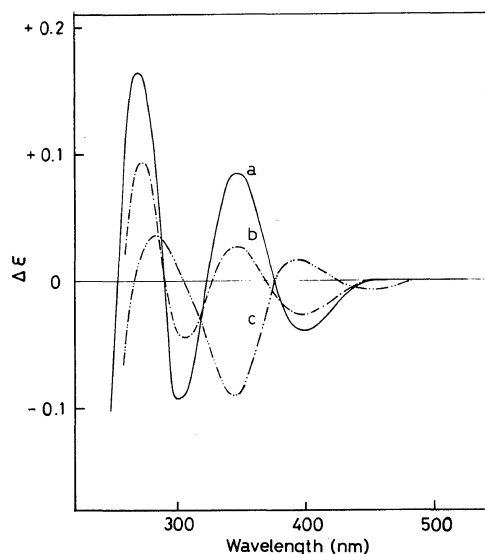


Figure 6. CD spectra of $\text{MoO}_2(\text{cys-O-PEG})_2$ which was added to the solution of $\text{P}(\text{OMe})_3$ at pH 6.0 under aerobic conditions, a) immediately, b) after 1 h, and c) after 4 h.

(1:0.15) is optimal.² In DMF/H₂O (1:0.26), the catalytic activity decreased to 1/2. This may be due to the partial inactivation of the catalyst. Inactive Mo species may be formed by decomposition of MoO₂(cys-OEt)₂. MoO₂(cys-OEt)₂ is completely insoluble in water and decomposition was not detected. MoO₂(cys-PEG)₂ rapidly decomposed when dissolved in water. Therefore, the MoO₂(S,N)₂ species will be decomposed by a homogeneous reaction with water.

The water content in an aqueous solvent alters the hydrophobic environment surrounding the molybdenum(VI) complex. More than 15% water may be effective for a hydroxylic reaction at the metal, thereby accelerating the decomposition through redox reaction resulting in S-S bond formation.

Role of the Hydrophobic Environment in the Active Site of Molybdo-enzymes

The activation of Mo=O of the Mo(VI) complex by water suggests that an active site of the native molybdo-enzyme may be associated with water to promote its oxidative ability. The participation of water in the enzymatic oxidation of xanthine oxidase has been discussed by Bray.¹¹ However, in the system of catalytic air oxidation of PPh₃ by MoO₂(S₂CNEt₂)₂, water is known to be not involved since the formation of a Mo(IV) species proceeds through an oxo-transfer reaction.^{12,13} Stiefel reported that the oxo-transfer could be postulated for the catalytic oxidation of molybdenum oxidase under hydrophobic conditions.¹⁴ Our EPR study³ indicates that a mild reduction of MoO₂(cys-OR)₂ with PPh₃ provides a mononuclear Mo(V) species similar to the Mo(V) state of xanthine oxidase¹⁵ or sulfite oxidase.¹⁶ Therefore, one electron transfer involving water seems to be important for the air oxidation catalyzed by MoO₂(cys-OR)₂.

As already mentioned, a large excess of water

greatly activates Mo=O of the Mo(VI) complexes. The hydrophobic environment surrounding the active sites of molybdenum oxidase¹⁴ controls contact with the excess water and may possibly contribute to the stability of the chelated Mo(VI) state during the redox cycle.

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