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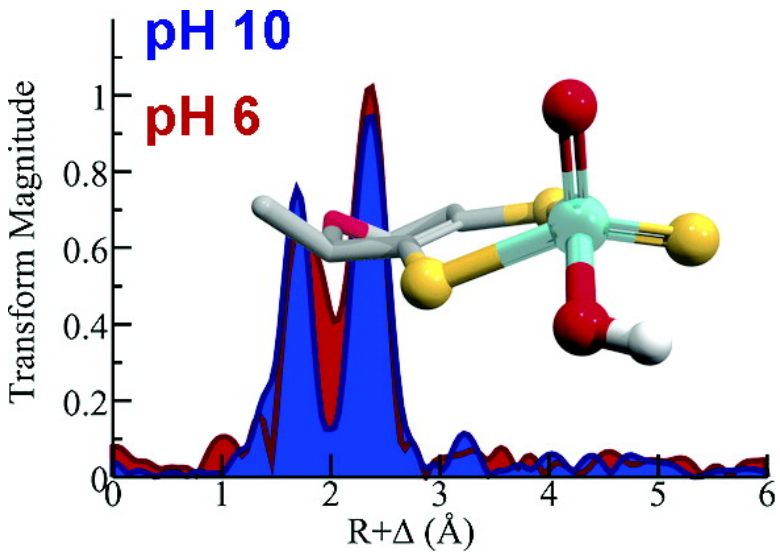
J. Am. Chem. Soc., **2005**, 127 (12), 4518-4522• DOI: 10.1021/ja042500o • Publication Date (Web): 08 March 2005



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Nature of the Catalytically Labile Oxygen at the Active Site of Xanthine Oxidase

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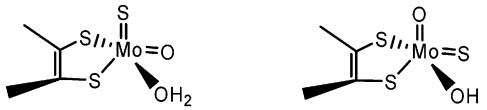
Abstract: In this paper we report the results of molybdenum K-edge X-ray absorption studies performed on the oxidized active site of xanthine oxidase at pH 6 and 10. These results indicate that the active site possesses one terminal oxygen ligand (Mo═O), two thiolate ligands (Mo−S), one terminal sulfido ligand (Mo═S), and one Mo−OH moiety. EXAFS analysis demonstrates that the Mo−OH bond shortens from 1.97 Å at pH 6 to 1.75 Å at pH 10, which is consistent with the generation of a Mo−O^- moiety. This study provides convincing structural evidence that the catalytic oxygen donor at the oxidized active site of xanthine oxidase is Mo−OH rather than the Mo−OH_2 ligation previously suggested by X-ray crystallography. These results support a mechanism initiated by base-assisted nucleophilic attack of the substrate by Mo−OH.

Introduction

Xanthine oxidase (XO) is the prototypical member of the molybdenum hydroxylase family of enzymes. It can catalyze the hydroxylation of purines in the 2, 6, and 8 ring positions (e.g. purine to hypoxanthine to xanthine to uric acid), as well as the oxidation of a wide range of aldehydes and other substrates, although its physiological role is thought to be in purine oxidation.[1] Mammalian XO is a 290 kDa homodimer containing one molybdenum, one flavin, and two different [Fe_2S_2] centers per subunit.[1−3] In contrast to monoxygenase systems, XO generates rather than consumes reducing equivalents during catalysis and utilizes water as opposed to O_2 as the source of oxygen for substrate hydroxylation.[1] Substrate oxidation occurs at the molybdenum site, which becomes reduced from Mo^{VI} to Mo^{IV} in the process. The catalytic cycle is completed by electron transfer from molybdenum to the [Fe_2S_2] clusters and then the flavin, where the electrons are donated to an acceptor such as O_2. The molybdenum is anchored to its active site by an organic pyranopterin dithiolene cofactor (often called molybdopterin) with the molybdenum covalently bound to the cofactor via the dithiolene moiety.

Detailed structural information on the active site of XO is available from both protein crystallography and spectroscopy, and this has provided valuable insights into the catalytic mechanism. It is generally agreed that the active site molybdenum atom is ligated by one short terminal sulfido unit (Mo═S), two long thiolate ligands (Mo−S) (from the dithiolene), and one short terminal oxygen (Mo═O). Two major controversies about the active site structure remain outstanding: the position of the Mo═S group in the molybdenum coordination sphere and the nature of the catalytically labile oxygen. With regard to the position of the Mo═S group, the initial crystallographic studies of xanthine oxidase and related enzymes placed this ligand in the apical position of a distorted square-pyramidal coordination geometry, with the Mo═O ligand occupying one of the equatorial positions (Figure 1).[3−6] By contrast, a detailed magnetic circular dichroism analysis of the so-called very rapid Mo^V form of the enzyme[7] concluded that the Mo═O group must be in the apical position in this form of the enzyme.[8] The issue has recently been further addressed by a high-resolution X-ray crystallographic study of the reduced enzyme in complex with a mechanism-based inhibitor. In reduced enzyme the Mo═S group of oxidized enzyme is replaced by Mo−SH by protonation, which accompanies reduction.[1] This study clearly identified the Mo═O in the apical



A B

Figure 1. Crystallographic (A) and spectroscopic (B) pictures of the active site structure of xanthine oxidase.

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(1) Hille, R. *Chem. Rev.* **1996**, *96*, 2757−2816.
(2) Bray, R. C. Molybdenum iron−sulfur flavin hydroxylases and related enzymes. In *The Enzymes*; Boyer, P. D., Ed.; Academic Press: New York, 1975; pp 299−419.
(3) Enroth, C.; Eger, B. T.; Okamoto, K.; Nishino, T.; Nishino, T.; Pai, E. F. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 10723−10728.
(4) Huber, R.; Hof, P.; Duarte, R. O.; Moura, J. J.; Moura, I.; Liu, M. Y.; Legall, J.; Hille, R.; Archer, M.; Romao, M. J. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 8846−8851.
(5) Romao, M. J.; Archer, M.; Moura, I.; Moura, J. J.; LeGall, J.; Engh, R.; Scnieder, M.; Hof, P.; Huber, R. *Science* **1995**, *270*, 1170−1176.
(6) Truglio, J. J.; Theis, K.; Leimkühler, S.; Rappa, R.; Rajagopalan, K. V.; Kisker, C. *Structure* **2002**, 115−125.
(7) The very rapid intermediate is the Mo^V form of an intermediate of catalytic turnover that forms at high pH in which the substrate is covalently bound to the Mo site.
(8) Jones, R. M.; Inscore, F. E.; Hille, R.; Kirk, M. L. *Inorg. Chem.* **1999**, *38*, 4963−4970.

10.1021/ja042500o CCC: $30.25

position, with the Mo−SH of the reduced enzyme (derived from the Mo═S) in the equatorial plane.[9] Thus, either a large conformational change occurs on association with substrate[8] or the crystal structures of fully oxidized enzyme are incorrect in their assignment of the Mo═S ligation.[9] Since this conformational change would include an inversion of symmetry at the molybdenum center, it seems more likely that the initial assignments were incorrect.[9] The nature of the catalytically labile oxygen, however, remains totally unresolved. X-ray crystallographic analysis indicates that this is an Mo−OH_2 ligand (based upon a Mo−O distance of 2.2 Å),[4,5] but mechanistic considerations favor a Mo−OH ligation instead, which would yield a significantly better nucleophile, Mo−O^-, upon base-assisted deprotonation (Figure 1).[10] In the present work, we use X-ray absorption spectroscopy (XAS) as a structural probe of the active site of bovine xanthine oxidase to address this issue and to provide definitive evidence that at moderate pH this group is a Mo−OH, which deprotonates to Mo−O^- at pH 10.

Experimental Section

Samples. Xanthine oxidase was purified from unpasteurized cow's milk (obtained from the experimental dairy herd at The Ohio State University) as previously described.[11] Fully active xanthine oxidase samples were prepared by submitting as-isolated 70% active enzyme to two rounds of reactivation using a modification of the procedure of Wahl and Rajagopalan.[12] Three milliliters of 0.2 mM enzyme (in 0.1 M sodium pyrophosphate, pH 8.5, 0.1 M KCl, 0.3 mM EDTA) plus 25 μM in methyl viologen was made anaerobic by flushing with argon for 1 h. The enzyme solution was then reduced by adding just sufficient sodium dithionite solution to maintain the blue color of reduced methyl viologen during the reaction, and 15 μL of freshly prepared 2 M sodium sulfide was added. The reaction was incubated on ice for 45 min and then gel-filtered into either 100 mM CAPS, pH 10.0, or 100 mM MES, pH 6.0. Enzyme activity was assayed by monitoring uric acid production at 295 nm and estimated at 95−97% activity on the basis of its activity-to-flavin ratio. Samples were concentrated to 2.0 mM Mo using 30 000 MW cutoff Microcon filters, transferred to (2 × 10 × 10 mm) Lucite sample cuvettes, and frozen in liquid nitrogen.

XAS Data Collection. XAS measurements were conducted at the Stanford Synchrotron Radiation Laboratory with the SPEAR 3 storage ring containing between 75 and 100 mA at 3.0 GeV. Molybdenum K-edge data were collected on the structural molecular biology XAS beamline 9-3 with a wiggler field of 2 T and employing a Si(220) double-crystal monochromator. Beamline 9-3 is equipped with a rhodium-coated vertical collimating mirror upstream of the monochromator and a downstream bent-cylindrical focusing mirror (also rhodium-coated). Harmonic rejection was accomplished by setting the cutoff angle of the mirrors to 23 keV. Incident and transmitted X-ray intensities were monitored using argon-filled ionization chambers, and X-ray absorption was measured as the Mo Kα fluorescence excitation spectrum using an array of 30 germanium detectors.[13] During data collection, samples were maintained at a temperature of approximately 10 K using an Oxford instruments liquid helium flow cryostat. For each sample, eight to 12 scans were accumulated, and the energy was calibrated by reference to the absorption of a molybdenum metal foil measured simultaneously with each scan, assuming a lowest energy inflection point of 20 003.9 eV. The energy threshold of the extended X-ray absorption fine structure (EXAFS) oscillations was assumed to be 20 025.0 eV.

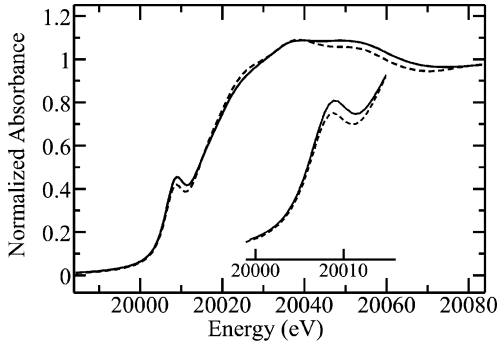

Figure 2. Molybdenum K-edge X-ray absorption near-edge spectra of oxidized xanthine oxidase at pH 6 (broken lines) and pH10 (solid lines). The inset shows the 1s → 4d region, showing the subtle increase in intensity between pH 6 and 10. This transition gains dipole-allowed intensity from admixture of orbitals with significant p-character.

XAS Data Analysis. The EXAFS oscillations $\chi(k)$ were quantitatively analyzed by curve-fitting using the EXAFSPAK suite of computer programs[14] as described by George et al.,[15] using ab initio theoretical phase and amplitude functions calculated using the program FEFF version 8.2.[16,17] No smoothing, filtering, or related operations were performed on the data.

Results

Mo K Near-Edge X-ray Absorption Spectra. Figure 2 displays the molybdenum K near-edge spectra of oxidized xanthine oxidase at pH 6 and 10. The spectra shown in Figure 2 are similar to, but sharper than, those previously reported for xanthine oxidase.[18−20] In particular, the lowest energy feature at ca. 20 009 eV is a resolved peak in our data, rather than just the shoulder observed previously.[18−20] This can be attributed to the better instrumental energy resolution afforded by the use of a vertical collimating mirror in our experiments and does not indicate any gross differences in active site structure. The individual spectra of low and high pH samples are subtly but significantly different than each other. Near-edge spectra are comprised of transitions of the core electron (Mo 1s in our case) to bound states involving the frontier molecular orbitals of the system and are sensitive to electronic structure. The presence of subtle differences between the two spectra in Figure 2 clearly indicates a pH-dependent structural change at the active site. The lowest energy peak, at ca. 20 009 eV, is observed in both spectra and is attributed to a Mo 1s → Mo═O π^* transition.[21] This pre-edge feature is characteristic of the presence of Mo═O ligation,[15,18,21,22] and its intensity is related to the number of Mo═O ligands.[23] The ∼20 009 eV peak is more intense in

(9) Okamato, K.; Matsamuto, K.; Hille, R.; Eger, B. T.; Pai, E. F.; Nishino, T. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 7931−7936.
(10) Choi, E.-Y.; Stockert, A. L.; Leimkühler, S.; Hille, R. *J. Inorg. Biochem.* **2004**, *98*, 841−848.
(11) Kim, J. H.; Ryan, M. G.; Knaut, H.; Hille, R. *J. Biol. Chem.* **1996**, *271*, 6771−6780.
(12) Wahl, R.; Rajagopalan K. V. *J. Biol. Chem.* **1982**, *257*, 1354−1359.
(13) Cramer, S. P.; Tench, O.; Yocum, M.; George, G. N. *Nucl. Instr. Methods* **1988**, *A266*, 586−591.
(14) http://ssrl.slac.stanford.edu/exafspak.html
(15) George, G. N.; Garrett, R. M.; Prince, R. C.; Rajagopalan, K. V. *J. Am. Chem. Soc.* **1996**, *118*, 8588−8592.
(16) Rehr, J. J.; Mustre de Leon, J.; Zabinsky, S. I.; Albers, R. C. *J. Am. Chem. Soc.* **1991**, *113*, 5135−5140.
(17) Mustre de Leon, J.; Rehr, J. J.; Zabinsky, S. I.; Albers, R. C. *Phys. Rev. B* **1991**, *44*, 4146−4156.
(18) Cramer, S. P.; Wahl, R.; Rajagopolan, K. V. *J. Am. Chem. Soc*. **1981**, *103*, 7721−7727.
(19) Turner, N. A.; Bray, R. C.; Diakun, G. P. *Biochem. J.* **1989**, *260*, 563−571.
(20) Cramer, S. P.; Hille. R. *J. Am. Chem. Soc.* **1985**, *107*, 8164−8169.
(21) Kutzler, F. W.; Natoli, C. R.; Misemer, D. K.; Donaich, S.; Hodgson. K. O. *J. Chem. Phys.* **1980**, *73*, 3274−3288.
(22) George, G. N.; Hilton, J.; Temple, C.; Prince, R. C.; Rajagopolan, K. V.; *J. Am. Chem. Soc.* **1999**, *121*, 1256−1266.
(23) Kutzler, F. W.; Scott, R. A.; Berg, J. M.; Hodgson. K. O.; Donaich, S.; Cramer, S. P.; Chang, C. H. *J. Am. Chem. Soc.* **1981**, *103*, 6083−6088.

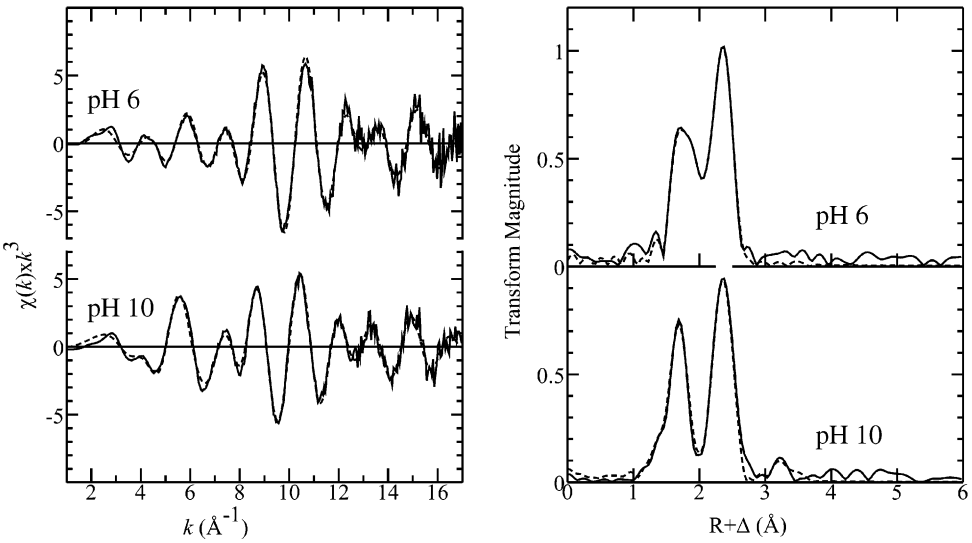

Figure 3. Comparison of the Mo K-edge EXAFS data (solid lines) and best fits (broken lines) of xanthine oxidase at pH 6 and 10, together with the corresponding Fourier transforms. The Fourier transforms have been phase-corrected for Mo−S backscattering.

Table 1. EXAFS Curve-Fitting Parameters[a]

	Mo═O			Mo═S			Mo−S			Mo−O			
sample	N	R	σ^2	N	R	σ^2	N	R	σ^2	N	R	σ^2	F
pH 6	1	1.694(1)	0.0014(1)	1	2.154(2)	0.0061(4)	2	2.432(2)	0.0028(1)	1	1.978(4)	0.0027(1)	0.251
	1	1.702(2)	0.0006(1)[b]	1	2.159(2)	0.0032(2)	2	2.445(3)	0.0035(1)				0.313
	1	1.707(2)	0.0008(1)[b]	1	2.171(2)	0.0024(1)	2	2.452(3)	0.0035(1)	1	2.2[c]	0.0035[c]	0.363
pH 10 [d]	2	1.721(1)	0.0034(1)	1	2.186(2)	0.0051(2)	2	2.467(2)	0.0029(1)				0.216
	1	1.719(1)	0.0008(1)[b]	1	2.150(3)	0.0094(2)	2	2.471(2)	0.0028(2)	1	2.067(5)	0.0018(3)	0.264
	1	1.719(1)	0.0010(1)[b]	1	2.213(4)	0.0054(3)	2	2.464(1)	0.0031(1)				0.273

[a] Coordination numbers, N; interatomic distances R (Å); Debye−Waller factors σ^2 (Å^2). Values in parentheses are the estimated standard deviations obtained from the diagonal elements of the covariance matrix. The fit-error function F is defined as $\{\sum k^6(\chi_{calc} - \chi_{expt})^2/\sum\chi_{expt}^2\}^{1/2}$, where the summations are over all data points included in the refinement. A selection of alternative fits is presented. [b] This value is in the physically impossible range (smaller than σ_{vib}^2 [27]), and this fit can therefore be discounted. [c] Interatomic distance and Debye−Waller factor was fixed for this refinement, and the Mo−OH_2 distance was estimated from crystallographic studies. [d] Two carbon atoms (presumably belonging to the dithiolene moiety) at a distance of 3.45 Å from the molybdenum were modeled in each fit to account for a small peak in the Fourier transform.

the spectrum of the pH 10 sample than in the spectrum of the pH 6 sample (Figure 2), suggesting increased oxygen π-orbital character in the molecular orbital manifold of the high pH molybdenum site. This enhancement could be due to either a change in coordination geometry or the nature of metal−ligand bonding, or a combination of the two. The spectrum of the high pH sample is also shifted to slightly higher energy. In general, shifts of the absorption edge energy are due to changes in the electronic environment at the metal center, with oxidation state and degree of metal−ligand covalency both contributing to the edge position.

Molybdenum K-Edge EXAFS. Figure 3 shows the high and low pH xanthine oxidase Mo K-edge EXAFS spectra plus best fits, together with the corresponding Fourier transforms. The parameters obtained from curve-fitting analysis are given in Table 1. The data presented in the present work are of substantially better signal-to-noise than those previously reported,[18−20] and this can be predominantly attributed to recent improvements in synchrotron radiation and detector technology. Significant differences in the data from samples at two different pH values can be seen (Figure 3). The low pH sample shows two peaks in its EXAFS Fourier transform: a broad flat-topped peak at ∼1.8 Å and a more intense peak at ∼2.3 Å. The EXAFS Fourier transform of the data of the high-pH sample, on the other hand, shows a well-resolved sharp peak at ∼1.7 Å plus a 2.3 Å peak similar to that observed for the pH 6 sample. This observation is in agreement with the near-edge data and indicates significant pH-dependent structural changes at the molybdenum site of oxidized xanthine oxidase.

The curve fitting analysis of the pH 6 EXAFS data set indicates that the active site possess a terminal Mo═O bond at 1.69 Å, a short Mo═S bond at 2.15 Å, two Mo−thiolate bonds at 2.43 Å, and an Mo−O interaction at 1.98 Å (Table 1). This analysis agrees qualitatively with previous xanthine oxidase EXAFS,[18−20] and the Mo═O and Mo═S bond lengths are consistent with bond lengths derived from EXAFS[24] and from X-ray crystallography[25] of model compounds with a *cis*-[Mo^{VI}OS] moiety.[25] The Mo−O bond length at 1.98 Å is significantly shorter than that determined from crystallography (2.2 Å). Attempts to coerce the EXAFS to fit a long Mo−O interaction resulted in this parameter converging to 1.97 Å, if the initial bond length was less than 2.3 Å, or a large fit-error and theoretically impossible Debye−Waller factors for both Mo═O and Mo−O (Table 1), if the initial Mo−O bond length was greater than 2.3 Å. Curve-fitting analysis of the pH 10 EXAFS data set indicated two short molybdenum−oxygen interactions at 1.72 Å, two Mo−S bonds at 2.47 Å and a short Mo═S bond at 2.19 Å (Table 1). The active site at high pH is thus quite different than that observed at low pH, showing a

(24) Smith, P. D.; Slizys, D. A.; George, G. N.; Young, C. G. *J. Am. Chem. Soc.* **2000**, *122*, 2946−2947.
(25) Doonan, C. J. Ph.D. Thesis, University of Melbourne, 2004.

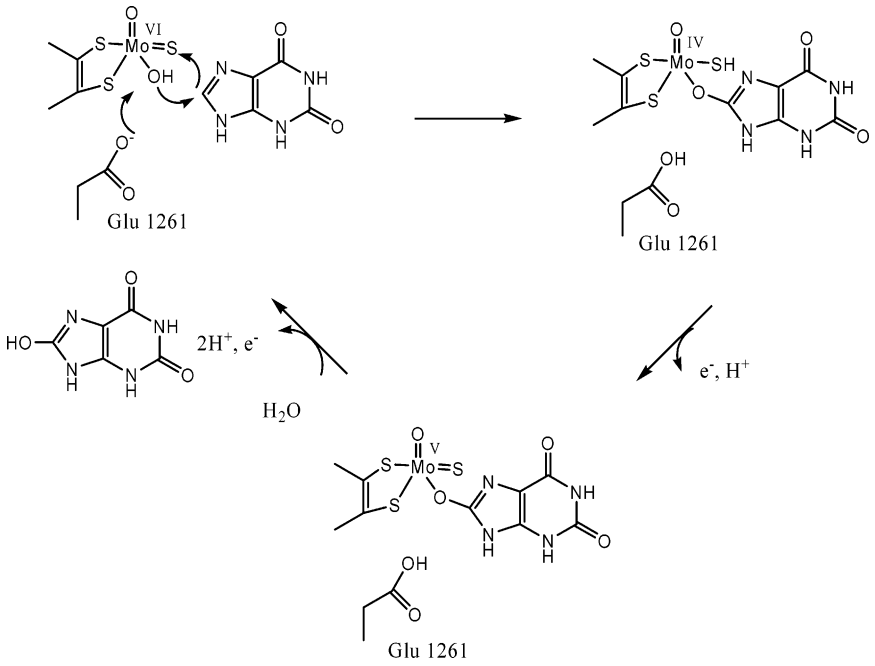

Figure 4. Postulated catalytic mechanism for xanthine oxidase.

slight elongation of the Mo═S and Mo−S bond lengths (0.03 and 0.04 Å, respectively). The most significant change is that two short molybdenum−oxygen interactions are present at pH 10, rather than one long and one short at pH 6. Efforts to resolve these two interactions into a short Mo═O bond (∼1.70 Å) and a longer Mo−O group resulted in physically unreasonable Debye−Waller factors,[22] and we conclude that the two short molybdenum−oxygen interactions differ in bond length by less than the EXAFS resolution ($\Delta R \sim \pi/2k$). We can compute an approximate value for the bond length of the longer oxygen of 1.78 Å if we assume that the shorter one has similar bond length and Debye−Waller factor to that observed at pH 6.[26,27] Such a contraction in bond length is expected if the Mo−OH were deprotonated at high pH to form a Mo−O$^-$ ligand:

$$\mathrm{Mo{-}OH} \leftrightarrow \mathrm{Mo{-}O^- + H^+}$$

A search of the Cambridge Structural Database[28] indicates an average bond length of 1.77 Å for Mo−O$^-$ ligation. Assuming only a single ligand ionization in the pH range 6−10, our results strongly suggest that the changes in the EXAFS in going from low pH to high are due to deprotonation of an Mo−OH group to form a Mo−O$^-$ ligand. Thus, our data are consistent only with the presence of Mo−OH, and not with Mo−OH$_2$.

Previous work on X-ray absorption spectroscopy of oxidized xanthine oxidase was conducted using samples prepared in phosphate buffer at pH 7.8[18] or in bicine buffer at pH 8.2[19] or 8.5.[20] Experiments at pH 8.5 (not illustrated) suggest the presence of a mixture of the pH 6 and 10 species, and because of this, previous work might have suffered from ambiguities involving mixed species. Additionally, phosphate buffer can lower its pH by as much as two units on freezing,[30] and the data of Cramer et al.[18] might therefore predominantly correspond to the low pH structure, although contamination with the desulfo form of the enzyme complicated their analysis.[18] Finally, our data have considerably better signal-to-noise and higher *k*-ranges than previously reported. Thus, while previous EXAFS studies have provided vital structural insights, they were unable to accurately determine the catalytically important Mo−O bond length. Our observation of a Mo−O bond length of 1.98 Å at low pH shortening to 1.78 Å at high pH, in conjunction with the observed changes in near-edge structure, combine to provide compelling evidence for Mo−OH coordination, rather than Mo−OH$_2$ at low pH, with ionization to Mo−O$^-$ at high pH.

Discussion

A detailed understanding of the catalytic mechanism of XO is the ultimate goal of all spectroscopic and crystallographic studies of the molybdenum site of this enzyme. Insights into mechanism have been provided by combination of rapid reaction, spectroscopic, and structural studies. During catalysis the active site of XO is reduced from Mo^{VI} to Mo^{IV} upon

(26) The Debye−Waller factor can be represented as the sum of vibrational and static components, $\sigma^2 = \sigma_{vib}^2 + \sigma_{stat}^2$. The static component is given by $\sigma_{stat}^2 = (1/N)\sum_i (R_i - R)^2$, where N is the coordination number, R the mean bond length, and R_i are the individual bond lengths. The vibrational component[27] can be assumed to be the value obtained at pH 6 (as we have only a single Mo═O bond in this case, so that $\sigma_{stat}^2 = 0$). Assuming that the shorter of the two bond lengths at pH 10 is the same as the Mo═O bond length at pH 6 (1.694 Å), we compute a bond length for the longer of the two oxygen interactions of 1.78 Å.

(27) The vibrational component of the Debye−Waller factor can be approximately calculated by using the equation for a diatomic harmonic oscillator, $\sigma_{vib}^2 = (h/8\pi^2\mu\nu)\coth(h\nu/2kT)$, where ν is the bond-stretch vibrational frequency, μ is the reduced mass, k is Boltzmann's constant, and T is temperature. Using a value of 984 cm^{-1} obtained for model compounds, we obtain $\sigma_{stat}^2 = 0.0015$ Å^2, which is very close to that determined from curve-fitting (0.0014 Å^2).

(28) Allen F. H.; Kennard, O. *Chem. Des. Autom. News* **1993**, *1*, 31−37.

(29) The search of the Cambridge Structural Database yielded 133 individual hits, and these were examined manually to eliminate hits that were in fact reduced molybdenum species and hits with obvious problems (such as a high *R* factor). We note that assignment of Mo−O$^-$ is obviously problematic in some cases, as this often represents a longer than normal terminal oxygen bond in crystal structures, where charge considerations mandate the formal presence of Mo−O$^-$ or the equivalent. We also note that charge localization upon the oxygen may not be an accurate picture of the site, and the structure of the pH 10 species might be alternatively formulated as containing two short bonds to oxygen (with one slightly longer than the other) and a decreased overall charge for the whole site.

(30) van den Berg L.; Rose D. *Arch. Biochem. Biophys.* **1959**, *81*, 319−329.

substrate oxidation with subsequent reoxidation achieved by coupled electron and proton-transfer reactions, involving a transient Mo^{V} intermediate (Figure 4).[1] From the pH-dependence of the reaction, the initial reductive event is thought to be base-catalyzed,[10] with Glu1261 acting as the active site base.[9,10] The catalytic mechanism of XO shown in Figure 4 depicts the catalytically labile oxygen site as Mo−OH (as found in the present work), rather than $Mo-OH_2$. From a chemical standpoint, the base-catalyzed deprotonation of Mo−OH to $Mo-O^-$, rather than deprotonation of $Mo-OH_2$ to Mo−OH, is expected to yield a significantly better nucleophile that can initiate the catalytic sequence by attacking the C-8 position of substrate.[31,32]

Our EXAFS and near-edge analyses indicate that the fully oxidized active site of XO possesses an Mo−OH unit at pH 6 that is deprotonated at pH 10. This is consistent with the changes in the near-edge spectrum between pH 6 and 10. Here, a rise in the intensity of the dipole-allowed Mo 1s → Mo−O π^* transition at pH 10 is associated with increased O2p−Mo4d character in the excited state due to the formation of $Mo-O^-$. In addition, EXAFS analysis provides unambiguous evidence that the long Mo−OH of 1.98 Å at pH 6 contracts to a characteristic $Mo-O^-$ distance of ca. 1.78 Å at pH 10. The results presented herein allow us to unambiguously assign the catalytically labile oxygen ligand at the active site of XO. The assignment of a hydroxide ligand is consistent with a reaction mechanism involving substrate hydroxylation initiated by a base-assisted deprotonation of Mo−OH and nucleophilic attack of the substrate. It has been suggested that this is facilitated by a nearby glutamate residue (Glu1261). Our work supports this hypothesis, providing compelling evidence that the labile active site oxygen is Mo−OH and not $Mo-OH_2$.

Acknowledgment. Portions of this work were carried out at the Stanford Synchrotron Radiation Laboratory, which is funded by the U.S. Department of Energy, Office of Basic Energy Sciences and Office of Biological and Environmental Sciences, and the National Institutes of Health, National Center for Research Resources. Research at the University of Saskatchewan (G.N.G.) was supported in part by Canada Research Chair award, the University of Saskatchewan, the Province of Saskatchewan, the National Institutes of Health (GM5735), the Natural Sciences and Engineering Research Council (Canada) (283315), and the Canadian Institute of Health Research. Research at The Ohio State University (R.H.) is supported by the National Institutes of Health (GM59953).

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(31) Toba, S.; Colombo, G.; Merz, K. M., Jr. *J. Am. Chem. Soc*. **1999**, *121*, 2290−2302.
(32) Benkovic, S. J.; Hammes-Sciffer, S. *Science* **2003**, *301*, 1196−1202.