Resonance-enhanced Raman Scattering from the Molybdenum Center of Xanthine Oxidase*

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W. Anthony Oertling† and Russ Hille‡¶

From the †Department of Chemistry and the LASER Laboratory, Michigan State University, East Lansing, Michigan 48824 and the ‡Department of Physiological Chemistry, Ohio State University, Columbus, Ohio 43210

The molybdenum center of xanthine oxidase has been examined by resonance Raman spectroscopy. Making use of the long-wavelength absorption of the reduced molybdenum center in complex with violaptenin (the product of enzymic action of lumazine), resonance Raman spectra were obtained using laser excitation at 676.4 nm. Several internal vibrational modes of violaptenin were found to be resonance-enhanced, and a number of bands in the 250–1100 cm⁻¹ range, presumably arising from vibrational modes of the molybdenum coordination sphere, were also observed. Upon substitution of ¹⁸O for ¹⁶O in the molybdenum coordination sphere, bands at 1469, 853, 517, 325, and 276 cm⁻¹ exhibited shifts of 5–12 cm⁻¹ to lower energy. By analogy to previous vibrational studies of Mo–O–Mo and Mo–O–R model compounds, the 853, 517, and 276 cm⁻¹ frequencies were judged consistent with a labeled Mo–O–R linkage of the complexed violaptenin. More importantly, the relatively small frequency shifts observed in these and other vibrations upon incorporation of ¹⁸O are very similar to those observed by others for ¹⁸O-labeled phenol and metal-phenolate complexes (Pinchas, S., Sadeh, D., and Samuel, D. (1965) J. Phys. Chem. 69, 2259–2264; Pyrz, W. J., Rue, L. A., Stern, L. J., and Que, L. J., Jr. (1985) J. Am. Chem. Soc. 107, 614–620) that model iron-tyrosinate proteins. The relatively small isotope-induced frequency shifts in multiple bands are thus interpreted as resulting from vibrational mixing of internal coordinates involving the oxygen atom with internal ring motions of the aromatic species. No oxygen isotope-sensitive bands were observed in the 900–1100 cm⁻¹ region where Mo=O stretching modes typically occur. In agreement with the conclusions of previous workers (Davis, M. D., Olson, M. D., and Palmer, G. (1981); Davis et al., 1982). Kinetic studies indicate that the complex is an authentic catalytic intermediate, corresponding to E⁺⁻P in the enzymatic hydroxylation of xanthine to uric acid, a reaction that takes place at the molybdenum center of the enzyme (Hille and Massey, 1985; Bray, 1988), the latter dominating the resonance Raman spectrum in those few cases that have been examined (Willis and Loehr, 1985; Garner and Bristow, 1985). Because the molybdenum hydroxylases are unique among the monooxygenase class of enzymes by virtue of their utilization of H₂O rather than O₂ as the source of the oxygen atom incorporated into the hydroxyl group of the product, this circumstance is especially unfortunate.

Xanthine oxidase from bovine milk catalyzes the hydroxylation of xanthine to uric acid, a reaction that takes place at the molybdenum center of the enzyme (Hille and Massey, 1985; Bray, 1988). X-ray absorption spectroscopy (Tullius et al., 1979; Bordas et al., 1980; Cramer et al., 1981; Cramer and Hille, 1986; Hille et al., 1989) has shown that the oxidized enzyme contains a [Mo'O₄]²⁻ core with Mo=O and Mo=S bond lengths of 1.68 and 2.15 Å, respectively. Upon reduction, the sulfido group protonates to form a Mo=O⁻SH group with a Mo=S bond length of 1.68 and 2.15 Å, respectively. The remainder of the molybdenum coordination sphere is taken up by as many as three thiolate ligands (and perhaps a N/O ligand as well), some of which may be contributed by a pterin cofactor known to be required for activity (Cramer and Stiefel, 1985). In addition to the molybdenum center, xanthine oxidase contains flavin adenine dinucleotide and two iron-sulfur centers of the spinach ferredoxin variety. Any absorption of the molybdenum site remains ill-defined owing to the strong absorption of these other chromophores. Reduced xanthine oxidase complexed with violaptenin (2,4,7-trihydroxypteridine), however, exhibits a broad absorption centered at 650 nm (Davis et al., 1982). Kinetic studies indicate that the complex is an authentic catalytic intermediate, corresponding to E⁺⁻P in the enzymatic hydroxylation of xanthine.
Formation of this long wavelength absorbing complex requires the participation of the molybdenum center in the long wavelength charge-transfer transition. This result demonstrates that the sulfido group with a second oxygen prevents formation of the enzyme with cyanide (Massey and Edmondson, 1970) to replace the sulfido group with a second oxo prevents formation of the long wavelength absorption. This result demonstrates the participation of the molybdenum center in the long wavelength-absorbing species. The 650 nm absorption has been tentatively assigned to a molybdenum+violapterin charge-transfer transition (Davis et al., 1982). Because the extinction coefficient of the putative charge-transfer transition is substantially greater than those of the reduced iron-sulfur or flavin chromophores in the reduced enzyme, this absorption band provides an opportunity to examine vibrational modes attributable to the molybdenum center directly by means of resonance Raman spectroscopy.

MATERIALS AND METHODS

Xanthine oxidase was purified by the method of Massey et al. (1969) with the exception that unpasteurized raw milk was used rather than the buttermilk obtained from churning pasteurized cream. As a final step in the purification, ion exchange on a CM-52 column equilibrated with 0.1 M MES, pH 6.2, was used to separate lactoperoxidase from the xanthine oxidase (Morrison and Hultquist, 1963). Molybdenum enrichment was accomplished by isolating enzyme from milk obtained from a cow receiving 20 mg/day of 95Mo orally in the form of sodium molybdate over a period of 2 weeks. Milk was collected beginning on the third day of molybdenum administration and continued for the duration of the treatment. Isotopic enrichment of the enzyme isolated from this milk was estimated by EPR of the so-called Very Rapid Mo(V) signal obtained on reduction of enzyme with 2hydroxy-6-methylpurine (George and Bray, 1988) to be approximately 90%. 15O-Enriched enzyme was prepared by concentrating 95Mo-enriched enzyme to approximately 1 mM, followed by a first dilution into 18O-enriched water, recombination by centrifuge filtering and a second dilution with labeled water.

The resonance Raman samples were prepared in one of two ways. In the first, enzyme containing a slight stoichiometric excess of violapterin was made anaerobic, reduced with a small amount of solid sodium dithionite and sealed in an anaerobic resonance Raman cell. In the second, aerobic enzyme was reacted with four equivalents of lumazine to ensure labeling of the catalytically labile oxygen site of the molybdenum complex (Bray and Gutteridge, 1982; Hille and Sprücher, 1987), followed by anaerobiosis and addition of excess dithionite to ensure complete enzyme reduction. The two procedures are known to generate the same long wavelength-absorbing species (Davis et al., 1982), and this was confirmed in the present experiments. The isotopic enrichment of 15O in the sample was estimated to be approximately 88% on the basis of the volume-to-volume dilution of the concentrated enzyme stock into the labeled water. Raman spectra were measured with a LSI-11 interfaced SPEX 1401 Ramalog spectrometer equipped with photon counting detection. Each spectrum in Figs. 1 and 2 represents the sum of multiple scans (typically 6-10) using the 676.4 nm emission of a Coherent I-90 Kr+ ion laser (120-160 milli-watts incident power). Xanthine oxidase samples were maintained at 5°C in an anaerobic quartz cuvette. All experiments were carried out in 10 mM pyrophosphate buffer, pH 8.5.

RESULTS AND DISCUSSION

Fig. 1 shows the normal Raman spectrum of free violapterin (Spectrum a) and the resonance Raman spectra of reduced xanthine oxidase complexed with violapterin for enzyme containing natural abundance Mo (Spectrum b) and enzyme enriched in 95Mo (Spectrum c). The resonance enhancement effect on the spectra of the xanthine oxidase/violapterin complex is demonstrated by the 50-fold greater sensitivity of the scale in Spectrum a compared with b and c in Fig. 1. For a metal-ligand charge-transfer complex it is reasonable to expect enhancement of internal normal modes of the violapterin as well as modes of the remainder of the molybdenum coordination environment. Thus the features at 699, 1218, 1267, 1295, and possibly 1389 cm⁻¹ seen in the spectrum of the reduced xanthine oxidase/violapterin complex most likely correspond to bands at 708, 1216, 1267, 1300, and 1389 cm⁻¹ in the normal Raman spectrum of free violapterin. Complexation with the molybdenum, interactions with the pterin cofactor, interactions with the polypeptide, or any combination of these could easily account for the frequency differences observed between free and enzyme-bound violapterin. In the spectra of the enzyme complex, resonance enhancement is restricted to some but not all of the Raman-active vibrational modes of violapterin (e.g. bands at 1020, 1233, 1492, and 1524 cm⁻¹ in the normal Raman of free violapterin are not significantly resonance-enhanced). The resonance Raman spectrum of the reduced enzyme-violapterin complex obtained using enzyme enriched (~88%) in 95Mo is shown in Fig. 1 (Spectrum c). Because the natural distribution of molybdenum isotopes is sufficiently large (atomic weights of 92-100) and the abundance of the several isotopes sufficiently great, such enrichment in the 95Mo isotope should give rise to sharpening of weak low frequency vibrational modes involving molybdenum. The reduction in the fluorescence background
between Spectra b and c is due to greater contamination of the former sample with trace amounts of free flavin adenine dinucleotide (derived from the protein). Together the lower fluorescence background and molybdenum isotope enrichment improve spectral quality considerably, revealing a number of well-defined low frequency bands presumably arising not only from Mo-L vibrational modes but also internal modes of the various ligand moieties. For example, several features occur in the 450–550 cm\(^{-1}\) region where both Mo-S and Mo-O (Pence and Selbin, 1986) stretching frequencies occur. Analysis of these vibrational modes may be used to evaluate active site structure of the enzyme-product complex (see below).

To identify vibrational modes arising from exchangeable oxygen sites in the molybdenum coordination sphere experiments were undertaken with enzyme in \(^{18}\)O. Fig. 2 shows spectra obtained with samples doubly enriched in \(^{95}\)Mo and \(^{18}\)O (Fig. 2, upper) and enriched only in \(^{95}\)Mo (i.e., containing \(^{18}\)O; Fig. 2, lower). Several spectral changes are evident upon substitution of \(^{18}\)O for \(^{16}\)O. The feature at 1561 cm\(^{-1}\) in the \(^{16}\)O spectrum diminishes in intensity, while those at 1469, 853, 517, 325, and 276 cm\(^{-1}\) exhibit shifts of −12, −6, −5, −9, and −5 cm\(^{-1}\), respectively, in the \(^{18}\)O spectrum (the feature at 773 cm\(^{-1}\) also possibly exhibits a −4 cm\(^{-1}\) isotopic shift, but this is less clear). These shifts clearly indicate that at least one oxygen atom in the molybdenum center has been labeled by the \(^{18}\)O enrichment procedure used. The Mo-O stretching frequencies of various synthetic complexes occur in the 900–1050 cm\(^{-1}\) range (Nakamoto, 1986; Cotton and Wing, 1965; Buchler et al., 1978; Ledon et al., 1980; Singh et al., 1980) and, in agreement with a two-body harmonic oscillator model, exhibit 45–50 cm\(^{-1}\) shifts to lower frequency upon exchange of \(^{16}\)O for \(^{18}\)O (Miller and Wentworth, 1979; Collin et al., 1973). Because of the absence of any feature in this spectral region that displays such an isotopic frequency shift, we have no resonance Raman evidence indicative of Mo-O in the reduced enzyme/violapterin complex. It is known from x-ray spectroscopic studies of the violapterin complex, however, that the Mo-O group is present in the complex (Hille et al., 1989). Furthermore, given the manner in which the sample was prepared (multiple enzyme turnover with lumazine followed by dithionite reduction and incubation for approximately 30 min prior to the 4–6 h data acquisition period) there can be no doubt that the oxo group has been labeled (Gutteridge and Bray, 1980). The \(\nu(Mo=O)\) vibration should be both Raman- and infrared-active, and has been observed in the Raman spectra of inorganic Mo=O complexes (Collin et al., 1973) and in resonance Raman spectra of Mo=O porphyrin complexes (Ohta et al., 1979; Terner and Topich, 1984). Furthermore, it is unlikely that the \(\nu(Mo=O)\) internal coordinate is strongly coupled to other motions that could substantially alter its frequency and isotopic shift from what is observed with the model compounds. Thus, the absence of this vibrational mode in our spectra can only indicate that the \(\nu(Mo=O)\) mode is not resonance enhanced by Raman excitation at 676.4 nm.

Although the Mo=O stretching vibration is not apparent in our spectra, there clearly are oxygen isotope-sensitive vibrations in the resonance Raman spectra throughout the 200–1650 cm\(^{-1}\) region examined. In particular, the vibrations at 853 and 517 cm\(^{-1}\), which shift only modestly to 847 and 512 cm\(^{-1}\), respectively, upon substitution with \(^{18}\)O, are too low in energy to be ground-state Mo=O stretching modes, but may indicate the presence of a Mo–O bond. \(\mu\)-Oxo molybdenum dimers, for example, display symmetric and antisymmetric stretching modes at ~430 and 750 cm\(^{-1}\), respectively (a bending mode at even lower frequency is also expected; Moore and Larson, 1967; Newton et al., 1974; Melby, 1969; Matsuda et al., 1979). Furthermore, \(\text{O}=\text{MoCl}_4 (\beta,\gamma\text{-diketonate})\) complexes display IR absorptions at 500–550 cm\(^{-1}\) that likely correspond to normal modes containing significant \(\nu(\text{Mo–O})\) character (Pence and Selbin, 1969). A Mo\(^{95}\)O(purine) structure has been suggested for an EPR-detectable intermediate observed in the reaction of xanthine oxidase with xanthine and certain other substrates (Terner et al., 1989; Bray and Gutteridge, 1982) and isotope labeling of product during enzyme turnover is also consistent with such a structure (Hille and Sprecher, 1987). As the enzyme is expected to act on xanthine and lumazine by the same reaction mechanism, a plausible structure for the reduced xanthine oxidase-violapterin complex is Mo\(^{95}\)O–O(pteridine), which upon one-electron oxidation would give the EPR-detectable species. The isotopic shifts observed in our data (Fig. 2) are most likely due to the expected labeling of the 7-hydroxyl group of the violapterin formed in situ upon reduction of oxidized xanthine oxidase with lumazine (see "Materials and Methods"). In this case, in contrast to the \(\nu(Mo=O)\) stretching motion of the terminal oxo group, the internal modes of the Mo–O–C group are expected to mix extensively with the ring vibrations of the violapterin, causing a variety of normal mode frequencies to be affected by oxygen

![Fig. 2. Raman spectra of \(^{95}\)Mo-enriched xanthine oxidase obtained in unenriched (lower) and \(^{18}\)O-enriched water (upper). These spectra are the sum of seven scans measured under the same conditions as in Fig. 1c. Vertical lines indicate the proposed relationship between isotope-sensitive bands.](http://www.jbc.org/
isotope substitution, as is observed in Fig. 2. Thus a delocalization of the \( \pi \) (Mo-O) internal coordinate into several normal modes involving the entire Mo-O-pterin complex will result in \(^{18}O\) shifts much smaller than those of a vibrationally isolated Mo-O moiety: Such a situation has been illustrated by vibrational studies of Fe-\(^{18}O\)-C,H\(_4\)(CH\(_3\)) (Pyrz et al., 1985) and H-\(^{18}O\)-C,H\(_2\) (Pinchas et al., 1965). These model compounds display isotopic frequency shifts in the \(-2 \to -10\) cm\(^{-1}\) range for a variety of modes throughout the 220-1350 cm\(^{-1}\) region. In particular, the 568 cm\(^{-1}\) vibration of the Fe-phenolate complex (arising from a mode with significant Fe-O stretching character) shifts only \(-10\) cm\(^{-1}\) upon \(^{18}O\) substitution rather than the \(-25\) cm\(^{-1}\) shift expected for an isolated \( \nu \)(Fe-O) stretch. This relatively small \(^{18}O\) isotopic frequency shift, as well as the frequency shifts that occur upon deuteration of the phenolate ring and \(^{18}O\) substitution, indicate that the 568 cm\(^{-1}\) vibration is due to a delocalized normal mode with both \( \nu \)(Fe-O) and macrocyclic ring character (Pyrz et al., 1985). This vibrational mixing in metal-O-aromatic type structures has been confirmed by isotopic shifts and normal coordinate analysis (Tomimatsu et al., 1976) and can be extended to Fe-tyrosinate protein species (Que, 1983; Nagai et al., 1983; Antanaitis et al., 1982; Gaber et al., 1974). Relative to that expected for a vibrationally isolated Mo-O group, the isotopic shifts we observe for the putative Mo-O-pterin group are consistent with an even smaller metal-oxygen stretching contribution in the normal mode vibrations at 517 cm\(^{-1}\) and possibly 863 cm\(^{-1}\) than is found in the Fe-phenolate model complex. This is plausible because the violapterin macrocycle is approximately twice as large as phenolate and thus offers more extended delocalization of the Mo-O-C internal coordinates in the normal mode potential energy distributions. Thus, the presence of several \(^{18}O\)-sensitive resonance-enhanced vibrational modes, particularly attributed to the violapterin ring at 1469 cm\(^{-1}\), are consistent with direct coordination of the violapterin to molybdenum via the 7-hydroxyl group introduced by enzyme action.

The apparent absence of the \( \nu \)(Mo=O) vibration from the terminal oxo moiety in our resonance Raman spectrum despite the participation of the molybdenum center in the long wavelength-absorbing species (as evidenced by the loss of long wavelength absorption upon cyanolysis of the molybdenum center; Davis et al. (1982)) is important to the electronic assignment of the 650 nm absorption band in the reduced xanthine oxidase-violapterin complex. A variety of synthetic Mo\(^{v}\)=O and Mo\(^{v}\)=O complexes exhibit long wavelength absorption (Pence and Selbin, 1969; Gray and Hare, 1962; Ballhousen and Gray, 1962; Garner et al., 1977; Berg and Holm, 1984; Harlan et al., 1986). For the Mo\(^{v}\) complexes, these are assigned to a molybdenum \( \pi \) \( \rightarrow \) \( \delta_{\alpha} \delta_{\alpha} \) transition (Gray and Hare, 1962; Ballhousen and Gray, 1962; Garner et al., 1977; the \( \delta_{\alpha} \) highest occupied molecular orbital of these complexes is nonbonding and half-filled or filled in the Mo\(^{v}\)=O and Mo\(^{v}\)=O complexes, respectively) and we consider a similar assignment reasonable for the enzyme complex. The \( \delta_{\alpha} \) and \( \delta_{\alpha} \) orbitals, however, are mixed with the \( \pi \) and \( \delta_{\alpha} \) orbitals of the terminal oxo atom in \( \pi \) interaction that gives the \( \delta_{\alpha} \) and \( \delta_{\alpha} \) lowest occupied molecular orbitals considerable \( \pi \) * character (Gray and Hare, 1962). At 5 K this absorption is in the 600-670-nm range and exhibits fine structure with an 840 cm\(^{-1}\) spacing, attributed to vibronic coupling to the \( \nu \)(Mo-O) (Garner et al., 1977). The 840 cm\(^{-1}\) spacing presumably reflects a lowered \( \nu \)(Mo-O) frequency for the excited state and is comparable with a ground state vibrational frequency in these compounds of \( \approx 1015\) cm\(^{-1}\). Promotion of the electron from a nonbonding \( \delta_{\alpha} \) orbital to one with significant \( \pi \) antibonding character lengthens the Mo=O bond, accounting for the 175 cm\(^{-1}\) decrease in the stretching frequency in the excited state. Because of the overlap between the oxygen \( \rho \) orbitals and metal \( \delta \) orbitals, this electronic transition has considerable metal-to-oxo charge-transfer character, and the electronic transition occurs along the \( \nu \)(Mo=O) normal coordinate. Thus, resonance Raman excitation within the absorption band is expected to strongly enhance the \( \nu \)(Mo=O) vibration. Similarly, Mo=O porphyrin complexes exhibit resonance enhancement of the \( \nu \)(Mo=O) vibration owing to overlap of the metal \( \delta_{\alpha} \) and \( \delta_{\alpha} \) orbitals with the \( \pi \) systems of both the porphyrin and terminal oxo ligand (Ohta et al., 1973; Terner and Topich, 1984). For the molybdenum center chromophore that we consider here, however, the situation is quite different. Because we do not observe the \( \nu \)(Mo=O) vibration in our resonance Raman spectra we can discount the involvement of the molybdenum \( \delta_{\alpha} \alpha \) and \( \delta_{\alpha} \alpha \) orbitals in the 650 nm transition of reduced xanthine oxidase-violapterin complex, and we conclude that this absorption is not analogous to the long wavelength absorption of the small Mo-O complexes. On the other hand, the Mo \( \delta_{\alpha} \alpha \alpha \) orbital can mix with the \( \sigma \) orbitals of metal ligands other than the terminal oxo, including the violapterin (if it is indeed coordinated to the molybdenum, as suggested by the observed \(^{18}O\) isotope effects), to form a filled \( \sigma \) and empty \( \pi \) * level. If such a \( \pi \) * orbital were heavily localized on the violapterin ligand, a transition from the \( \delta_{\alpha} \) highest occupied molecular orbital to the empty \( \pi \) * orbital would have considerable charge-transfer character, consistent with the assignment of Davis et al. (1982). Such a transition would be orthogonal to the Mo=O stretching mode, and would give rise to a charge-transfer transition of a different nature from those that typically dominate the higher energy regions of the model complex absorption spectra (Gray and Hare, 1962; Ballhousen and Gray, 1962; Garner et al., 1977). The absence of obvious Mo=O stretching modes in our data is consistent with a localized molybdenum-to-violapterin charge-transfer transition, and further suggests that the violapterin is bound cis rather than trans to ther terminal oxo group.

Our results, in conjunction with the known x-ray absorption fine spectroscopy measurements suggest a structure of the following type for the complex of violapterin with reduced xanthine oxidase:

\[
\text{H} \quad \text{O} \quad \text{N} \quad \text{O} \quad \text{N} \quad \text{SH}
\]

Such a structure is consistent with the observation that the oxo group of the molybdenum coordination sphere is incorporated into product under single turnover conditions (Hille and Sprecher, 1987) only if the labeled oxygen shown is that of the oxo group present at the outset of catalysis. The oxo group shown must have been subsequently regenerated using oxygen derived from water, and under the conditions of the resonance Raman experiments both oxygens will be labeled with \(^{18}O\). We have previously proposed the corresponding structure with xanthine (but less the oxo group now known to be present) to be formed in the course of the catalytic sequence by proton abstraction from C-8 of xanthine, followed by nucleophilic attack on the oxo group to give product directly coordinated to molybdenum via the erstwhile oxo group (Hille and Sprecher, 1987). The same structure for the xanthine intermediate, specifically proposing regeneration of...
the oxo group, has been independently arrived at by Bray and coworkers (Turner et al., 1989) on the basis of evidence from X-ray absorption spectroscopy of reduced xanthine oxidase complexed with allooxanthine. Both groups propose the intermediate to undergo one-electron oxidation and deprotonation of the Mn—SH to give the catalytic intermediate giving rise to the so-called Very Rapid EPR signal that is observed transiently upon reaction of xanthine oxidase with xanthine.

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