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Resonance Raman Studies of the Flavin and Iron-Sulfur Centers of Milk Xanthine Oxidase†

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ABSTRACT: Resonance Raman spectroscopy has been used to study milk xanthine oxidase, an enzyme containing molybdenum, binuclear iron-sulfur clusters, and FAD as cofactors. The contribution of FAD dominates the resonance Raman spectrum at frequencies above 500 cm^{-1}. As expected, no bands assignable to FAD are observed in deflavo xanthine oxidase. The resonance Raman spectrum below 500 cm^{-1} reveals the contribution of the $Fe_2S_2(Cys)_4$ groups with frequencies similar to those of adrenodoxin and putidaredoxin. Resonance enhancement profiles of the $Fe_2S_2(Cys)_4$ clusters indicate intensity variations among the $Fe_2S_2(Cys)_4$ peaks that are attributed to different excitation wavelength maxima of their bridging and terminal iron-sulfur vibrations. No evidence for Mo-ligand vibrations could be obtained by using excitation wavelengths between 363.8 and 514.5 nm.

Milk xanthine oxidase (EC 1.2.3.2) is a multicomponent molybdenum enzyme that catalyzes the two-electron oxidation of xanthine to uric acid (Spence, 1983). Important cofactors are two Mo(VI) centers, four $Fe_2S_2(Cys)_4$ groups, and two flavin adenine dinucleotide (FAD)[1] moieties per molecule of enzyme. Information about these cofactors has been obtained through the use of EPR, EXAFS, electrochemistry, and electronic absorption spectroscopy (Spence, 1983; Bray, 1980).

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[1] Abbreviations: FAD, flavin adenine dinucleotide; EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption fine structure; CT, charge transfer; S_b and S_t, bridging and terminal sulfur, respectively; Cys, cysteine; XO, xanthine oxidase.

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The visible absorption spectrum of xanthine oxidase primarily shows the electronic transitions of the flavins (Bray, 1975). When the FAD cofactors are removed (Komai et al., 1969), the remaining absorption is characteristic of binuclear iron–sulfur proteins. No charge-transfer transitions of the Mo(VI) are discernible on account of the much stronger absorptions of FAD and $Fe_2S_2(Cys)_4$ groups. Even though the FAD-free enzyme can be prepared, attempts to remove the Fe_2S_2 groups result in denaturation of the enzyme. Weak absorptions due to Mo(IV) in reduced, inhibited xanthine oxidase have been reported in the ultraviolet (Massey et al., 1970).

In resonance Raman spectroscopy, vibrational modes associated with the electronic absorptions of a chromophore may be selectively enhanced over the many protein vibrational modes. Many resonance Raman spectroscopic studies of flavins and flavoproteins have been reported (Benecky et al., 1979; Kitagawa et al., 1979; Nishina et al., 1980; Irwin et al., 1980; Schopfer & Morris, 1980; Schmidt et al., 1983). The fluorescence of flavins, which usually interferes with resonance Raman spectra, can be partially overcome by the natural quenching of the protein, by quenching with potassium iodide, or by the use of coherent anti-Stokes' Raman scattering experiments (Dutta et al., 1977; Dutta & Spiro, 1980; Visser et al., (1983).

More recently, high-quality resonance Raman spectra of $Fe_2S_2(Cys)_4$ proteins are being reported (Johnson et al., 1982; Ozaki et al., 1983; Yachandra et al., 1983). The increased resolution of these spectra show the presence of three bands where previously only a broad, weak band at ~330–340 cm^{-1} had been reported. These bands are all assigned to various stretching modes of the $Fe_2S_2(Cys)_4$ group.

The strong visible absorptions of the flavin moiety and the iron–sulfur centers of xanthine oxidase make this enzyme ideally suited for study by resonance Raman spectroscopy. Here we report the results of our resonance Raman characterization of these chromophores in xanthine oxidase.

Experimental Procedures

Xanthine oxidase was prepared by a modification of the method of Massey et al. (1969). After elution from the cellulose–calcium phosphate column, the enzyme fractions were concentrated by using an Amicon TCM 10 ultrafiltration apparatus prior to gel filtration with Sephacryl S-300. After elution from the Sephacryl, the enzyme was concentrated by ultrafiltration and stored at 5 °C until use.

Deflavo xanthine oxidase was prepared by the method of Kanda et al. (1972). Desulfo xanthine oxidase was prepared by the method of Gutteridge et al. (1978). Desulfo–deflavo xanthine oxidase was prepared by the above two procedures, one after the other. The activity of xanthine oxidase was measured by the method of Avis et al. (1955), and typical values ranged from 1.36 to 6.3 units/mg. Concentrations of enzyme samples used for resonance Raman spectral analysis were 20–70 mg/mL.

Digitized Raman spectra were obtained on a modified Jarrell-Ash 25-300 Raman spectrophotometer and analyzed according to procedures previously described (Loehr et al., 1979). Samples were contained in glass melting point capillaries and cooled to ~77 or ~275 K in a copper rod cold finger immersed in a Dewar flask (Sjöberg et al., 1982). Spectra-Physics 164-01 and 164-05 Kr and Ar ion lasers were used in these experiments, and the scattered Stokes light was collected in a backscattering geometry. The spectra shown in the figures have been subjected to background subtraction and smoothing (25 point).

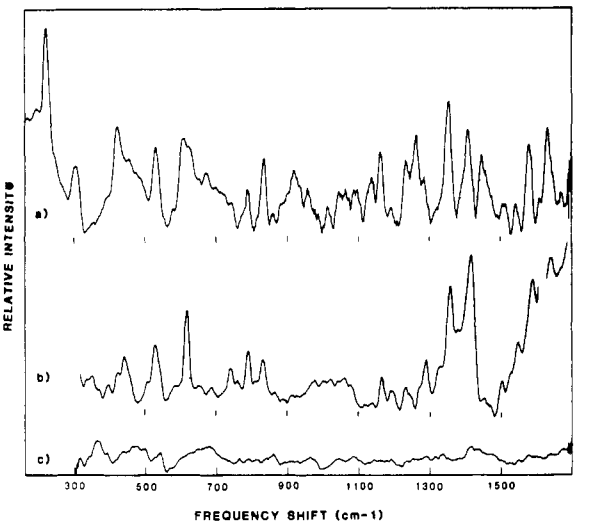

FIGURE 1: Resonance Raman spectra of (a) FAD (1.2×10^{-4} M plus KI added to quench fluorescence), (b) native xanthine oxidase, and (c) deflavo xanthine oxidase. Scan parameters: laser excitation 488.0 nm, 25–40 mW; slits 10 cm^{-1}; scan rate 2 cm^{-1}/s; number of scans (a) 56, (b) 90, and (c) 137; sample temperature ~77 K. Raman scattering at ~230 and 310 cm^{-1} is from ice.

Table I: Resonance Raman Peaks of Xanthine Oxidase and FAD[a]

XO	FAD	XO	FAD
351		977	
396		1164	1161
423	427	1233	1230
439		1269	1261
506		1287	1283
527	523	1352	1351
585	582	1408	1403
617	613	1470	1460
687	673	1501	1507
740		1546	1541
760		1586	1579
790	791	1636	1631
832	833	1679	1672

[a] Frequencies in cm^{-1}.

Results

Xanthine oxidase produces a large fluorescence background when illuminated by laser radiation, making the resonance Raman signal extremely small by comparison and impossible to see based upon a single scan. However, multiple scans allow the resonance Raman signal to emerge from the noise, and computer-assisted subtraction of the fluorescence background allows the resonance Raman signal to be observed on an expanded intensity scale (Loehr et al., 1979).

The resonance Raman spectrum of xanthine oxidase obtained with 488.0-nm excitation is shown in Figure 1b and compared with the resonance Raman spectrum of FAD (Figure 1a) and the Raman spectrum of deflavo xanthine oxidase (Figure 1c). The resonance Raman spectrum of FAD was measured over a wider frequency range than is normally reported since it was necessary to distinguish between peaks arising from FAD and possible metal–ligand vibrations which occur at low frequencies. Table I lists the observed frequencies for the xanthine oxidase and FAD spectra and shows a near one-to-one correlation between the two spectra.

The low-frequency resonance Raman spectrum of deflavo xanthine oxidase is shown in Figure 2. Three major peaks occur at 287, 344, and 389 cm^{-1}, and three peaks of lower intensity at 312, 335, and 364 cm^{-1} were reproducibly observed in all samples of xanthine oxidase. These include native xanthine oxidase, deflavo xanthine oxidase, desulfo xanthine

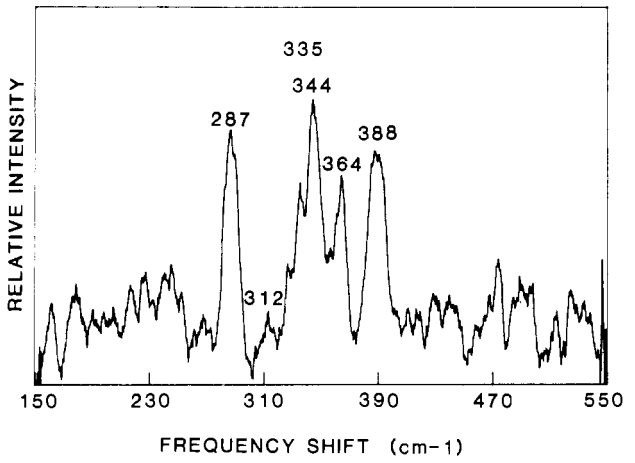

FIGURE 2: Resonance Raman spectrum of deflavo xanthine oxidase. Scan parameters: laser excitation 476.5 nm, 45 mW; slits 10 cm^{-1}; scan rate, 2 cm^{-1}/s; sample temperature ~275 K; number of scans 280.

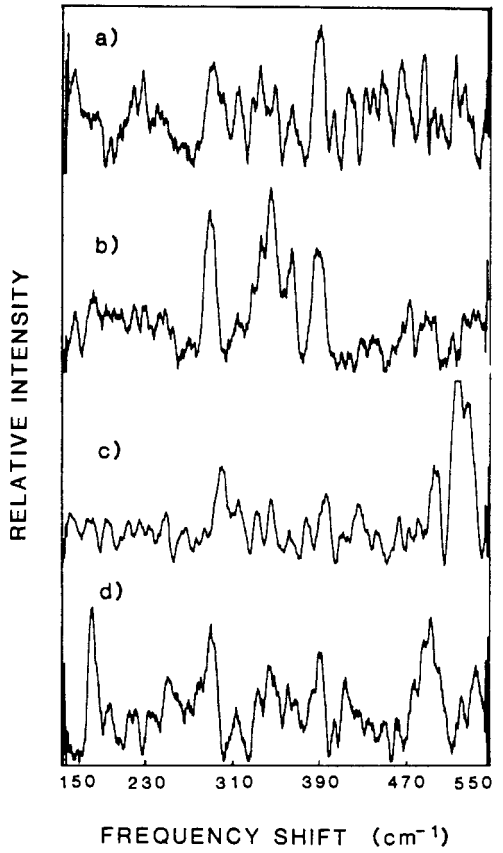

FIGURE 3: Resonance Raman spectra of the low-frequency region of (a) xanthine oxidase, (b) deflavo xanthine oxidase, (c) desulfo xanthine oxidase, and (d) desulfo–deflavo xanthine oxidase. Scan parameters as in Figure 2. Number of scans (a) 317, (b) 280, (c) 328, and (d) 615.

oxidase, and desulfo–deflavo xanthine oxidase (Figure 3). A summary of peaks observed for these different forms of the enzyme is presented in Table II.

The excitation wavelength dependence of the resonance Raman spectrum of deflavo xanthine oxidase in the region between 150 and 550 cm^{-1} is shown in Figure 4. The three major peaks at 287, 344, and 389 cm^{-1} show resonance enhancement from 406.7-nm excitation through 488.0-nm excitation. Resonance enhancement for all peaks is nearly lost with 514.5-nm excitation. The other three peaks at 312, 335, and 364 cm^{-1} show maximal enhancement with 488.0- and

Table II: Resonance Raman Peaks of Xanthine Oxidase, Deflavo Xanthine Oxidase, and Desulfo Xanthine Oxidase[a]

XO[b]	deflavo XO[b]	desulfo XO[b]	desulfo–deflavo XO[b]
288	288	sh	290
299		298	
313	313	315	312
334	333	331	333
348	344	344	344
363	364	362	361
390	389	395	391

[a] Frequencies in cm^{-1}. [b] 476.5-nm excitation frequency.

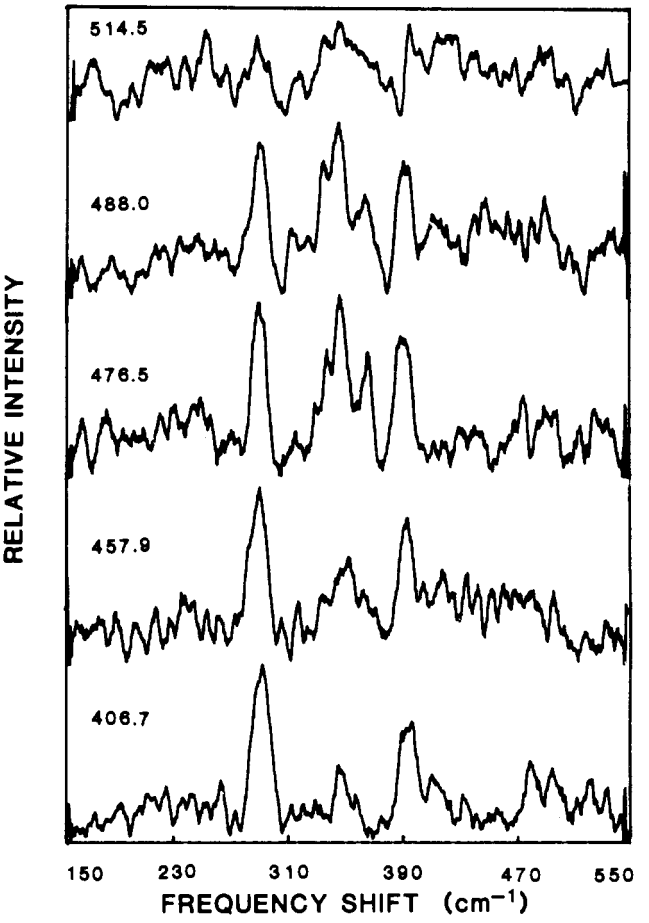

FIGURE 4: Excitation wavelength dependence of the low-frequency peaks of deflavo xanthine oxidase. The same sample was used for all of the spectra; the 476.5-nm spectrum is identical with that shown in Figure 2.

476.5-nm excitation; they start to lose intensity with 457.9-nm excitation and go out of resonance with 406.7-nm excitation. It was not possible to plot an excitation profile for the low-frequency vibrations due to lack of an internal intensity standard. Solutions of xanthine oxidase to which sulfate was added as a standard failed to give resonance Raman spectra.

Discussion

The resonance Raman spectra of FAD and xanthine oxidase are observed to be very similar (Figure 1). A correlation of the peaks in these two spectra shows that, for every FAD peak, there is a corresponding peak in the xanthine oxidase spectrum (Table I). The Raman spectrum of deflavo xanthine oxidase (Figure 1c) shows none of these FAD peaks, thus confirming that the resonance Raman spectrum of xanthine oxidase as seen in Figure 1b arises from the flavin cofactor.

The low-frequency spectrum of deflavo xanthine oxidase (Figure 2) shows three major peaks at 287, 344, and 389 cm^{-1} which correspond to peaks observed in the resonance Raman

spectra of other Fe_2S_2 proteins, putidaredoxin, spinach ferredoxin, and adrenodoxin (Tang et al., 1973; Yamamoto, 1974; Adar et al., 1977; Blum et al., 1977; Champion et al., 1978; Yachandra et al., 1983). The frequencies of these three peaks most closely match those of the $Fe_2S_2(Cys)_4$ group from adrenodoxin. Assignment of these peaks has been achieved by substitution of selenium for labile sulfur in adrenodoxin (Tang et al., 1973). The peaks at ~290 and ~395 cm^{-1} in adrenodoxin are associated with labile sulfur atoms whereas the 350-cm^{-1} peak is assigned to iron–sulfur (cysteine) stretching vibrations.

The ~290- and ~395-cm^{-1} peaks of various Fe_2S_2 proteins appear to be constant in frequency, indicating that the bridging structure of the binuclear center is consistent among these proteins (Ozaki et al., 1983). However, the 350-cm^{-1} band shows variation in frequency among Fe_2S_2 proteins. In spinach ferredoxin, the Fe–S(Cys) vibration occurs at ~330 cm^{-1} as a doublet peak. In adrenodoxin and putidaredoxin, this feature is observed as a single strong band at ~345 cm^{-1}. These frequency variations have been suggested to indicate differences in bonding between iron and the cysteine sulfur ligands (Ozaki et al., 1983). In addition, Schopfer has proposed that the position of this band correlates with protein functions, i.e., whether the protein is involved in electron transfer in photosynthetic pathways or in hydroxylation reactions. The plant ferredoxins fall into the former category, whereas adrenodoxin and putidaredoxin fit into the latter category (L. Schopfer, 1982, personal communication). In xanthine oxidase, the corresponding vibrational mode is seen at 344 cm^{-1}, thus substantiating Schopfer's hypothesis since its function is purine hydroxylation.

Three other bands at 312, 335, and 364 cm^{-1} are identified in addition to the three major $Fe_2S_2(Cys)_4$ peaks of xanthine oxidase (Figure 2). Although these spectral features are weak, they have been reproducibly observed in other samples and in different forms of the enzyme. To determine whether any of the peaks in the $Fe_2S_2(Cys)_4$ region could be attributed to molybdenum–ligand or flavin vibrations, resonance Raman spectra were measured on several different forms of the enzyme (Figure 3). The Raman frequencies summarized in Table II indicate that, with exception of a peak at 299 cm^{-1}, all of the peaks are present in all four forms of the enzyme. The peak at 299 cm^{-1} is present only in the native and desulfo forms of xanthine oxidase, both of which contain FAD, and is therefore likely to be a low-frequency deformation mode of the flavin moiety. It was not observed in the FAD or xanthine oxidase spectra measured at 77 K (Figure 1) because of interference from an ice band at ~300 cm^{-1}. However, a band of moderate intensity at ~300 cm^{-1} has been recorded in resonance Raman spectra of riboflavins (Kitagawa et al., 1979).

The desulfo form of the enzyme is inactive toward xanthine oxidation following removal of a sulfur ligand from the molybdenum moiety (Bordas et al., 1980). This modification is expected to produce changes in the molybdenum–ligand vibrational spectrum. From the results in Table II, however, no evidence is seen for any molybdenum–ligand stretching vibrations in the region of the $Fe_2S_2(Cys)_4$ vibrations.

Further information on the $Fe_2S_2(Cys)_4$ vibrations can be obtained from a study of their intensity behavior with laser excitation from violet (406.7 nm) to green (514.5 nm) (Figure 4). The three major peaks at 287, 344, and 389 cm^{-1} all show resonance enhancement with violet through blue (488.0 nm), but lose intensity with green excitation. This behavior corresponds with the electronic absorption spectrum of deflavo xanthine oxidase and indicates that the electronic absorption is due to the $Fe_2S_2(Cys)_4$ centers. The three peaks at 312, 335, and 364 cm^{-1} show the most marked enhancement with blue excitation at 476.5 and 488.0 nm but loss of intensity at both longer and shorter excitation wavelengths. The visible absorption spectrum of the $Fe_2S_2(Cys)_4$ centers displays maxima at 465 and 417 nm before rising into the UV. The three Raman peaks at 312, 335, and 364 cm^{-1} show maximal enhancement within the 465-nm absorption band whereas the three major peaks at 289, 344, and 389 cm^{-1} are resonance enhanced throughout the $Fe_2S_2(Cys)_4$ absorption envelope. The differential resonance enhancement of Fe–S vibrations in the $Fe_2S_2(Cys)_4$ clusters has been noted previously by Spiro and co-workers, who have assigned the vibrational modes of adrenodoxin and ferredoxin on the basis of a normal coordinate analysis of a structural model possessing D_{2h} symmetry (Yachandra et al., 1983). Our results for the iron centers in xanthine oxidase, assigned by analogy to their study, are listed in Table III.

Table III: Vibrational Frequencies (cm^{-1}) and Assignments for Fe–S Modes in $Fe_2S_2(SR)_4$ Species[a]

assignment (D_{2h} symmetry)	calcd frequency	xanthine oxidase[b]	adrenodoxin	spinach ferredoxin
B_{3u}, b	411	416[c]	419	424
A_g, b	398	389	391	393
B_{2g}, b	345	344	372	369
B_{1u}, t	360	364	346	336
B_{2u}, t	338			
B_{3g}, t	338	335	312	326
A_g, t	310	312	326	310
B_{1u}, b	286	287	289	284

[a] Assignments, calculated frequencies, and spectral data for adrenodoxin and ferredoxin are from Yachandra et al. (1983). [b] This work. [c] Uncertain; see text.

Yachandra et al. (1983) noted that the bridging and terminal sulfur modes showed different resonance enhancement profiles owing to the slightly different energies of the S → Fe charge-transfer bands. In xanthine oxidase, the 287- and 389-cm^{-1} peaks are assigned to bridging sulfur vibrational modes, which are enhanced throughout the blue and violet region, whereas the terminal sulfur (cysteine) modes at 312 and 335 cm^{-1} appear to be enhanced only in the blue region. These data suggest that the absorption bands at 465 and 417 nm are due principally to S_t → Fe CT and S_b → Fe CT, respectively.

The peak at 344 cm^{-1} is assigned as a bridging sulfur vibrational mode because it follows the 287- and 389-cm^{-1} peaks in its excitation wavelength dependence (Figure 4; Table III). We assign the peak at 364 cm^{-1} as a terminal sulfur vibrational mode because it follows the 312- and 335-cm^{-1} bands in its intensity behavior. Furthermore, these values are in excellent agreement with the calculated frequencies of their assigned modes (Table III). Although the low-frequency resonance Raman spectrum of xanthine oxidase is generally similar to that of adrenodoxin, our proposed assignments for the 344- and 364-cm^{-1} bands are opposite to those given by Yachandra et al. (1983). Their results were based upon frequency shifts in ^{34}S-reconstituted enzyme preparations. Such an experiment is not possible with xanthine oxidase because of denaturation of the enzyme with attempts to remove the acid-labile sulfurs or to extrude its iron cores (Bray, 1975). Thus, our proposed assignments of the 344- and 364-cm^{-1} bands to B_{2g} and B_{1u} modes, respectively, rest on their correlation with the observed intensity variations of the other bridging and terminal Fe–S modes and are strengthened by the agreement with the cal-

culated frequencies. A resolution of the differences in the proposed assignments for the B_{2g}(b) and B_{1u}(t) modes must await further experimental work.

In the normal coordinate analysis, the fourth bridging sulfur mode of B_{3u} symmetry was calculated to be at 411 cm^{-1}. Corresponding peaks are observed in adrenodoxin and spinach ferredoxin (Yachandra et al., 1983; Table III). Some of our resonance Raman spectra of xanthine oxidase do show a weak feature at 416 cm^{-1} that may correspond with this B_{3_u} mode. However, because it is within the noise level, its presence must be suspect. In adrenodoxin and spinach ferredoxin, this peak is of weak to medium intensity; therefore, its low intensity in the xanthine oxidase spectrum is not unexpected.

An alternate interpretation for the excitation wavelength behavior of the two sets of Fe–S vibrations (287, 344, and 389 cm^{-1} vs. 312, 335, and 364 cm^{-1}) would be to suggest that the two $Fe_2S_2(Cys)_4$ sites in xanthine oxidase exhibit different resonance Raman signatures. However, site-specific structural differences are not evident from EPR data at temperatures >40 K (Bray, 1975), and it would be difficult to rationalize any resonance Raman spectral differences of samples at ~275 K. Furthermore, the optical absorption spectrum of deflavo xanthine oxidase (Komai et al., 1969) is typical of the iron–sulfur proteins containing but a single $Fe_2S_2(Cys)_4$ cluster. Moreover, resonance Raman excitation profiles of bridging and terminal Fe–S vibrations in spinach ferredoxin have been shown to maximize at different wavelengths (Yachandra et al., 1983). We, therefore, rule out separate contributions to the resonance Raman spectrum from the $Fe_2S_2(Cys)_4$ sites in xanthine oxidase.

No evidence for a Mo(VI)–ligand vibration has been obtained for any of the several xanthine oxidase samples studied. Since electronic absorptions involving Mo(VI) are expected in the UV portion of the spectrum, visible excitation would not be expected to produce a resonance Raman effect with this chromophore. Although no distinct Mo(VI) absorptions are known for xanthine oxidase, a difference spectrum between native [Mo(VI)] and allopurinol-inhibited [Mo(IV)] xanthine oxidase reveals a Mo(IV) absorption at ~380 nm (Massey et al., 1970). Attempts to obtain a resonance Raman spectrum of the Mo(IV) active site by exciting into this band with 363.8-nm radiation have been unsuccessful. In future experiments, it may be of interest to work with the isolated Mo cofactor to reduce interferences from protein vibrational modes and fluorescence.

Registry No. XO, 9002-17-9; FAD, 146-14-5.

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