Pyranopterin Dithiolene Distortions Relevant to Electron Transfer in Xanthine Oxidase/Dehydrogenase

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Supporting Information

ABSTRACT: The reducing substrates 4-thiolumazine and 2,4-dithiolumazine have been used to form MoIV-product complexes with xanthine oxidase (XO) and xanthine dehydrogenase. These MoIV-product complexes display an intense metal-to-ligand charge-transfer (MLCT) band in the near-infrared region of the spectrum. Optical pumping into this MLCT band yields resonance Raman spectra of the Mo site that are devoid of contributions from the highly absorbing FAD and 2Fe2S clusters in the protein. The resonance Raman spectra reveal in-plane bending modes of the bound product and low-frequency molybdenum dithiolene and pyranopterin dithiolene vibrational modes. This work provides keen insight into the role of the pyranopterin dithiolene in electron-transfer reactivity.

Mammalian xanthine oxidoreductase (XOR) and R. capsulatus xanthine dehydrogenase (XDH) are molybdenum hydroxylases with broad substrate specificities.†‡ These enzymes possess a high degree of sequence homology and virtually identical coordination geometries.¶ Unlike monoxygenases, the oxygen atom incorporated into substrate C−H bonds derives from metal-activated water, and the enzymes generate rather than consume reducing equivalents.‡ These reducing equivalents are transferred sequentially from the reduced MoIV center via an apparent electron-transfer (ET) chain consisting of the pyranopterin dithiolene (Figure 1), two 2Fe2S clusters, and FAD.¶¶ The ultimate electron acceptor for the oxidase form of XOR (xanthine oxidase, XO) is O2, and this results in the formation of reactive oxygen species that have been implicated in reperfusion injury following ischemia.¶ The ultimate electron acceptor for XDH and the dehydrogenase form of XOR is NAD.¶ Integral to the ET regeneration of the catalytically competent MoVI site is the pyranopterin dithiolene chelate,§¶ which has been hypothesized to facilitate ET and modulate the molybdenum reduction potential.¶¶ The pyranopterin dithiolene is one of the most electronically complex ligands in biology,¶¶−¶‡ containing a redox noninnocent dithiolene,¶¶,¶‡ a pyran ring that can exist in both ring-opened¶¶,¶§ and ring-closed forms, and a redox-active pterin ring system. The Mo ion is not covalently linked to the protein but is anchored via the pyranopterin dithiolene through an extensive hydrogen-bonding network with the protein. Recently, we showed that pyranopterin dithiolene distortions can be correlated with enzyme function.¶ As a result of this analysis, XO family enzymes are proposed to possess a tetrahydropyranopterin dithiolene (Figure 1) that is intimately involved in the transfer of redox equivalents from Mo to the proximal 2Fe2S center. In spite of the intense interest in metallopterins,¶ and, more specifically, in the complexity of the pyranopterin dithiolene,¶¶,¶‡ there is a dearth of spectroscopic studies that have been directed toward understanding how the pyranopterin dithiolene facilitates ET in XO/XDH. Although XO has been studied by resonance Raman (rR) spectroscopy,¶¶,¶‡ modes attributed to the pyranopterin dithiolene have not been assigned. In order to address this issue, we have synthesized new XO/XDH reducing substrates that, when oxidized, bind tightly to the MoIV form of the enzyme. The MoIV−product bonding interaction results in the appearance of an intense near-infrared (NIR) metal-to-ligand (product) charge-transfer (MLCT) band in the electronic absorption spectrum.¶¶ Specifically, we generated MoIV−product charge-transfer complexes for bovine XO and R. capsulatus XDH by the enzyme-catalyzed oxidation of 4-thiolumazine and 2,4-dithiolumazine to 4-thioviolapterin (4-TV) and 2,4-dithioviolapterin (2,4-TV), respectively, in a manner similar to that used for the seminal studies on violapterin.¶¶,¶‡ Alternatively, enzymatically generated product collected and concentrated by centrifugation/filtration, then incubated with reduced XO/XDH, generates the same MoIV−product MLCT complex, as evidenced by electronic absorption spectroscopy.

We anticipated that heavy-atom congeners of lumazine would result in bathochromic shifts of the MLCT absorption maximum relative to the analogous complex formed with lumazine.¶¶,¶‡ The

Figure 1. Left: oxidized and reduced XO/XDH. Right: Reduced "tetrahydro" structure proposed for the pyranopterin dithiolene ligand in the XO family of enzymes. The metalated form of pyranopterin dithiolene is often referred to as the molybdenum cofactor, or Moco.
bathochromic MLCT shift would result in high-quality rR data because it effectively eliminates the dominant higher-energy absorption contributions from the 2Fe2S and FAD centers and deleterious contributions from free FAD fluorescence. The electronic absorption spectra for Mo-product complexes with 4-TV (758 nm) and 2,4-TV (778 nm) possess NIR absorbance maxima that are red-shifted by ∼3000 and ∼4000 cm\(^{-1}\), respectively, relative to the lumazine MLCT complex (Figure 2).\(^{14,16}\) Because the MLCT transition derives from a Mo(xy)→product(π*) (HOMO→LUMO) one-electron promotion (Figure S3 in the Supporting Information, SI), optical pumping of this transition creates an excited state with appreciable MoV−P− character, and interrogation of this MLCT state by rR spectroscopy (Figure 3 and Table 1) provides important information regarding the nature of low-frequency Mo-(pyranopterin dithiolene) distortions that are coupled to a one-electron oxidation of the MoIV site. These are the same distortions anticipated for the MoIV→MoV ET event in the oxidative half-reaction of the enzyme, providing new insight into the extent to which the pyranopterin dithiolene is coupled into ET regeneration processes in XO/XDH.

**Table 1. Selected Vibrational Frequencies**

| Mode                                      | Mo\(^{IV}\)-4-TV | Mo\(^{IV}\)-2,4-TV |
|-------------------------------------------|------------------|-------------------|
| dithiolene fold + MoIVO                   | \(\nu_{26}\) 234 | \(\nu_{28}\) 234  |
| rocking + pyranopterin dithiolene         | \(\nu_{26}\) 328 | \(\nu_{26}\) 326  |
| S−Mo−S symmetric core stretch             | \(\nu_{28}\) 333 | \(\nu_{28}\) 331  |
| product in-plane ring stretching           | \(\nu_{50}\) 493 | \(\nu_{45}\) 411   |
|                                          | \(\nu_{51}\) 513 | \(\nu_{51}\) 501  |

“Vibrational frequencies in wavenumbers (cm\(^{-1}\)).”

Low-frequency rR spectra for reduced XOR and XDH product-bound species, collected on resonance with the MoIV-product MLCT band (780 nm excitation), are essentially identical (Figure 3). However, the spectra are dependent on the nature of the product molecule bound to molybdenum and display multiple resonantly enhanced vibrations in the 200–600 cm\(^{-1}\) region. Because the sulfur heteroatoms are part of the heterocyclic \(\pi\) system of the product and the mass of sulfur is approximately twice that of oxygen, vibrations with appreciable product character will display frequency shifts relative to other resonantly enhanced modes due to force constant and reduced mass changes. This heavy-atom-congener approach, coupled with vibrational-frequency calculations, represents a powerful method for the assignment of vibrational modes that are localized on either the product half or the pyranopterin dithiolene half of the MoIV-product complex. Product-dependent spectral differences are clearly apparent at Raman shifts greater than ∼375 cm\(^{-1}\). The experimental dependence of these vibrational bands on the nature of the product allows us to assign the MoIV-4-TV resonantly enhanced vibration at 493 cm\(^{-1}\) (Figure 4A) and the MoIV-2,4-TV enhanced vibrations at 411 and 513 cm\(^{-1}\) (Figure S2 in the SI) as in-plane product bending modes localized on the product.

The rR spectra at frequencies lower than ∼375 cm\(^{-1}\) are virtually identical. Thus, these vibrational bands derive from...
modes localized primarily on the Mo-(pyranopterin dithiolene) half of the MoIV-product complex because they are not dependent on the nature of the product. We assign the resonantly enhanced bands at 328 and 326 cm$^{-1}$ in Mo$_{IV}$-4-TV and Mo$_{IV}$-2,4-TV, respectively, as the Mo-dithiolene core vibration that possesses S$\cdots$Mo$\cdots$S symmetric stretching and bending character (Figure 4B). To our knowledge, these are the first definitive assignments of a core Mo-dithiolene vibrational mode in a Mo-hydroxylase enzyme. Additional support for this assignment is based on polarized rR spectra of the benchmark Mo-dithiolene complex Tp*$\text{Mo(bdt)}$ (bdt = benzene-1,2-dithiolate), where the S$\cdots$Mo$\cdots$S symmetric stretch and bend are observed at 393 and 362 cm$^{-1}$, respectively.\textsuperscript{17,18} Although low-frequency rR data for pyranopterin-molybdenum enzymes are sparse, vibrational data for dimethyl sulfoxide reductase (DMSOR) have been collected and analyzed.\textsuperscript{19–22} In contrast to XO/XDH, DMSOR family enzymes possess two pyranopterin dithiolenes bound to the Mo ion.\textsuperscript{23} In the reduced form, DMSOR$_{\text{red}}$ displays S$\cdots$Mo$\cdots$S symmetric character in this mode indicates that the electron-hole generation on Mo, induced by photo-excitation into the MLCT band. The use of S/O substitution for the pyranopterin dithiolene) character (Figures S1 and S2 in the SI) and further evidence for the pyranopterin dithiolene being coupled to redox pathways that involves the coordinated cysteine. Similarly, the four stretching coordinate, underscoring the importance of an ET reaction in the oxidative half-reaction of XO/XDH.

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