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Xanthine Oxidase–Product Complexes Probe the Importance of Substrate/Product Orientation Along the Reaction Coordinate

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Abstract

A combination of reaction coordinate computations, resonance Raman spectroscopy, spectroscopic computations, and hydrogen bonding investigations have been used to understand the importance of substrate orientation along the xanthine oxidase reaction coordinate. Specifically, 4-thiolumazine and 2,4-dithiolumazine have been used as reducing substrates for xanthine oxidase to form stable enzyme-product charge transfer complexes suitable for spectroscopic study. Laser excitation into the near-infrared molybdenum-to-product charge transfer band produces rR enhancement patterns in the high frequency in-plane stretching region that directly probe the nature of this MLCT transition and provide insight into the effects of electron redistribution along the reaction coordinate between the transition state and the stable enzyme-product intermediate, including the role of the covalent Mo-O-C linkage in facilitating this process. The results clearly show that specific Mo-substrate orientations allow for enhanced electronic coupling and strong hydrogen bonding interactions with amino acid residues in the substrate binding pocket.

TOC image

Resonance Raman spectroscopy has been used to probe substrate orientation and hydrogen bonding interactions in a xanthine oxidase catalytic intermediate.

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Introduction

Xanthine oxidoreductase (XO) and xanthine dehydrogenase (XDH) are prototypical members of the xanthine oxidase family of pyranopterin molybdenum enzymes.[1–4] These molybdenum hydroxylases possess highly similar active sites and catalyse the formal insertion of an oxygen atom that derives from metal-activated water into substrate C-H bonds. The coordination geometry about the oxidized active site Mo is 5-coordinate square pyramidal [5–13], being ligated by terminal oxo and sulfido ligands, the sulphur donors of a pyranopterin dithiolene chelate, and an $\text{H}_2\text{O}/\text{OH}^-$ ligand.[5, 7] The molybdenum hydroxylases catalyse the oxidation of a wide variety of substrates, which include purines and other aromatic heterocycles, formamide, and aldehydes. The reaction coordinate for XO/XDH mediated substrate oxidations has recently been evaluated by a variety of DFT[3, 14–16] and QMMM [15, 17–19] methods, and a generalized mechanistic sequence for XO and XDH mediated substrate oxidations is depicted in Figure 1 using 4-thiolumazine as the reducing substrate. Here, substrate hydroxylation is believed to be initiated by nucleophilic attack of a metal-activated water molecule (e.g. $\text{OH}^-/\text{O}^{2-}$) on a carbon atom of the substrate, which is assisted by a catalytically essential active site glutamate, leading to a tetrahedral intermediate (IM1) and transition state (TS).[1–3, 20] Breakdown of the TS occurs via a formal hydride transfer to the terminal sulfido.[1, 21] This results in protonation of the sulfido, the two-electron reduction of the Mo ion, and the oxidized heterocyclic product being covalently bound to Mo via a $\text{Mo-O-C}_{\text{product}}$ linkage as the enolate tautomer (IM2).

Figure 2 depicts a simplified two-state reaction coordinate diagram that shows how diabatic Reactant and Product potential energy surfaces (PESs) can mix via an off-diagonal electronic coupling matrix element (H_{ab}) to produce upper (ES) and lower (GS) adiabatic PESs. This mixing results in a ground state (GS) PES that possesses a point of instability commonly known as the transition state (TS). Electronic coupling between the GS and ES adiabatic surfaces results in a lowering of the transition state (TS) energy barrier that connects the reactant (R) and product (P) wells on the lower energy GS PES. The nature of the warped GS PES can also be affected by a vibronic coupling matrix element (pseudo Jahn-Teller (PJT) effect),[22–24] which directly connects the resultant adiabatic GS and ES surfaces. A key result of this two-state approximation is that the ES PES is stable at the TS geometry. Thus, the geometry at the energy minimum on the ES PES is the same as that of the TS. If one were able to directly access the minimum energy portion of the ES PES, critical information could be obtained regarding the electronic and geometric structure of the TS along the reaction coordinate. For XO and XDH, substrate oxidation is a two-electron process with the electrons being transferred from the substrate to Mo leading to its reduction. One cannot probe the ES PES directly in this depiction using electronic absorption spectroscopy since a vertical electronic transition between the electronic $|\text{GS}\rangle_{\text{P}}$ at the product minimum to the $|\text{ES}\rangle$ is forbidden due to the two-electron nature of the transition. However, one might glean a tremendous amount of information regarding the nature of the ES PES and even the TS if one could access a one-electron analog of this $|\text{GS}\rangle_{\text{P}} \rightarrow |\text{ES}\rangle$ two-electron transition. This might occur via metal-to-ligand charge transfer (MLCT) process, which formally leads to oxidation of the Mo ion and reduction of a product molecule that is covalently bound to Mo.[25] Under these conditions, resonance

Raman (rR) spectroscopy would be an ideal probe of the Mo-P interaction as it relates to reactivity, and the nature of resonantly enhanced Mo-P vibrations would probe the instantaneous distortion along an ES PES that formally reduces the Mo ion by one equivalent and oxidizes the product molecule.

The observation of such stable, reduced Mo-Product intermediates is realized with the XO/XDH catalysed oxidation of lumazine or its 2,4-dithiolumazine and 4-thiolumazine derivatives to violapterin, 2,4-dithioviolapterin, or 4-thioviolapterin, respectively.[11, 25–28] The stable catalytic intermediate (IM2) E-P complexes formed results in the observation of a low-energy CT band in the red to NIR region of the electronic absorption spectrum that can be conveniently probed by rR spectroscopy.[11, 25–27] Here, we use a combination of reaction coordinate calculations, rR spectroscopy, and an analysis of potential substrate/product hydrogen bonding interactions in the substrate binding pockets of XO/XDH to glean additional information regarding the reaction mechanism of the Mo hydroxylases, the role of the Mo-O-C covalent linkage between Mo and substrate/product in promoting the $2e^-$ oxidation of heterocyclic substrates, and the orientation of the substrate for efficient catalytic throughput.

Experimental

Enzyme Preparation

Xanthine Oxidase from bovine milk was obtained from Sigma Aldrich (Grade III, 1.0–2.0 units/mg protein. One unit will convert 1.0 μ mole of xanthine to uric acid per min at pH 7.5 at 25 °C) in the form of suspension in 2.3 M $(\text{NH}_4)_2\text{SO}_4$ and 10 mM sodium phosphate buffer (pH=7.8), containing 1 mM EDTA and 1 mM sodium salicylate. Approx. 50% of the activity is obtained with hypoxanthine as substrate. After buffer exchange with 50mM bicine/NaOH buffer (pH=8.3), the XO_{ox} sample had an A_{280}/A_{450} ratio of 5–6.[1] Enzyme concentrations were determined using the extinction coefficient at 450 nm ($\epsilon = 37,800 \text{ M}^{-1}.\text{cm}^{-1}$).[1]

Substrate Synthesis

The reducing substrate 2,4-dithiolumazine[25, 29–31] was synthesized as previously described. 4-thiolumazine[25] was synthesized using the following procedure. The starting material lumazine (Alfar Aesar) and Lawesson's reagent (Sigma Aldrich) were obtained commercially and used without further purification. Under an atmosphere of dry nitrogen gas, a slight excess of Lawesson's reagent (0.51 g, 1.25 mmol) was added into a stirred suspension of lumazine (0.61 g, 2.50 mmol) in dioxane (25 mL) contained in a three-neck flask at 100 °C. The mixture was refluxed at this temperature for 15 minutes during which time the solution color changed from pale yellow to dark red. The reaction progress was monitored by thin layer chromatography. Upon completion, the reaction mixture was cooled to room temperature and evaporated to dryness in a vacuum. The crude product was washed with acetic acid followed by water. After being dried in a vacuum, the product was collected as a dark red powder. The yield was 300 mg (75% yield) of product. Crystals suitable for x-ray diffraction were obtained by slow evaporation of a saturated 4-thiolumazine dimethylformamide solution. ESI-MS (HCOOH): $m/z_{\text{calc}} = 181.02$, $m/z_{\text{exp}} = 181.02$ (M

+H). ^1H NMR (ppm): 13.08 (s), 13.27 (s), 8.66 (d), 8.55(d). ^{13}C NMR: 191.00, 148.55, 147.67, 146.47, 141.07, 130.98.

X-ray Crystallographic Data Collection and Refinement of the Structure

X-ray Crystallographic Data were collected on a Bruker X8 Apex II CCD diffractometer equipped with a graphite monochromator and a Mo K α sealed tube ($\lambda = 0.71073 \text{ \AA}$) operated at 1500 W power (50 kV, 30 mA). An orange rod-like specimen was cut to the approximate dimensions $0.074 \text{ mm} \times 0.127 \text{ mm} \times 0.205 \text{ mm}$, coated with Paratone oil and mounted on a CryoLoop that had been previously attached to a metallic pin using epoxy for the X-ray crystallographic analysis. The X-ray intensities were measured at 173(2) K. The unit cell was determined from 5627 random reflections ($3.88^\circ < \theta < 26.41^\circ$). The structure was solved and refined using the Bruker SHELXS-97 (Sheldrick, 2008) Software Package using the space group P-1 with $Z = 2$ for the formula unit. No symmetry was crystallographically imposed on the 4-thiolumazine●DMF dimer by the P1 space group. Non-hydrogen atoms were refined anisotropically. Hydrogen atoms, except for the N-H protons that were in the diffraction map, were placed in geometrically calculated positions. The final anisotropic full-matrix least-squares refinement on F^2 with 162 variables converged at $R1 = 3.92\%$ for the observed data and $wR2 = 9.64\%$ for all data. This yields thermal parameters equal to 1.2 or 1.5 (methyl groups) times U_{iso} of that atom.

Preparation of the Enzyme-Product Complex

The XOr-4-thioviolapterin (XOr-4-TV) complex was prepared by reacting oxidized xanthine oxidase with the substrate, 4-thiolumazine followed by dithionite reduction.[25] The formation of the reduced enzyme-product complex was initiated by adding 15 μL of 7.8 mM substrate to a solution containing 70 μL 72 μM XO_{OX} and 60 μL 50mM sodium bicine-NaOH (pH=8.3) buffer. The solution mixture was incubated for 5 minutes at 22 $^\circ\text{C}$ under aerobic condition, and then anaerobically bubbled with nitrogen gas for 15 minutes. The anaerobic reaction mixture was titrated with 15 μL 0.4 M sodium dithionite in an anaerobic environment. The XOr-2,4-dithioviolapterin (XOr-2,4-TV) complex was prepared similarly. Spectrophotometric measurements established the formation of XOr-4-TV and XOr-2,4-TV complexes through the appearance of a low energy NIR charge transfer band. The same sample was then sealed in a capillary tube anaerobically and collected the resonance Raman spectrum. Additionally, the same Mo(IV)-P complexes may be formed by first reducing the enzyme with an excess of dithionite and then mixing with enzymatically generated product.

Spectroscopic Measurements

Room temperature solution electronic absorption spectra were collected using a double-beam Hitachi U-4100 UV-vis-NIR spectrophotometer (Hitachi High-Technologic Corporation) that is capable of scanning a wavelength region between 185 and 3200 nm. Absorption spectra were collected using a 1 cm path length, black-masked, quartz cuvette (Starna Cells, Inc.) equipped with a Teflon stopper. The instrument was calibrated using the 656.10 nm deuterium line.

Solution resonance Raman (rR) spectra were collected using a commercial DXR Smart Raman Instrument (Thermo Fisher Scientific Inc.). All buffered samples were sealed in 1.5–

1.8 mm diameter capillary tubes and mounted onto a capillary tube holder in the 180-degree backscattering sample access chamber. A 780 nm diode laser was used as the excitation source and the excitation power was 140 mW. Background and standard sample (NaCl/Na₂SO₄) data sets were collected before the enzyme data collection, and Na₂SO₄ was employed as an external calibrant. Spectral subtraction of the buffer spectrum from the data yields the Raman spectra of the reduced E-P complexes.

Computational Details

Spin-restricted gas phase geometry optimization, Raman frequency, excited state, and transition state computations for the XO_{red}-4-thioviolapterin and XO_{red}-2,4-dithioviolapterin complexes were performed at the density functional theory (DFT) level using the Gaussian 09W package.[32] All calculations used the B3LYP hybrid exchange-correlation functional. A 6-31g* basis set was used for all atoms except for molybdenum, where a LANL2DZ basis set that includes an effective core potential was used. Electron density difference maps (EDDMs) were generated using GaussSum (version 2.1.6).[33, 34] Time-dependent DFT (TDDFT) calculations were used to determine transition energies and assist in band assignments.

Resonance Raman frequency calculations were performed using ORCA (version 3.0.3).[35, 36] Vibrational frequencies were calculated using optimized geometries and a frequency Hessian file was generated. An excited state numerical gradient was then calculated for the metal-to-ligand charge transfer (MLCT) excited state. An Advanced Spectra Analysis (ASA) input file with necessary frequency and excitation energy information was generated to input into the ORCA_ASA program in order to simulate the resonance Raman spectrum using a given excitation energy on resonance with the MLCT transition. 2048 points in the range 0 – 2000 cm⁻¹ were used in the simulations with a 15 cm⁻¹ FWHM bandwidth for each vibrational peak. All Raman computations employed the BP86 functional with the def2-TZVPP basis set for Mo and S, and the def2-SVP for all light atoms. Computed vibrational modes descriptions were plotted using ChemCraft (version 1.7).[37]

RESULTS AND DISCUSSION

Nature of the Reaction Coordinate for the Oxidation of Lumazine-Based Substrates

Since lumazines are known to be good XO/XDH substrates[1], we have computed the energetics of a critical portion of the reaction coordinate for the hydroxylation of the substrate 4-thiolumazine, which serves as an exemplar of all XO/XDH catalyzed lumazine oxidations. The starting point of the calculation begins from a covalently bound E-S intermediate (IM1; Figure 1) that possesses a Mo(VI)-O-C_{substrate} bonding interaction. This tetrahedral intermediate that results from nucleophilic attack of metal activated water (HO⁻/O²⁻) on the C7 ring carbon atom of lumazines, which is analogous to the C8 ring carbon of xanthine. The nucleophilic attack is believed to be assisted by proton transfer to the general base E1261 in XO. A generalized path to reach the TS can be broadly described as depicted in Figure 2, where Q is the C_{substrate}-H coordinate that is lengthened as the TS is approached from IM1. We found the expected cyclic tetrahedral transition state TS, which possesses a Mo-O-C_{substrate} linkage with a C₇-H bond distance of 1.27 Å and a nascent S-H

bonding interaction. The TS was confirmed by the observation of a single imaginary vibrational frequency ($\nu_{\text{im}} = -450.54 \text{ cm}^{-1}$) that involves a dominant $\text{S}_{\text{sulfido}}\text{-H}$ stretching component, which is coupled to an $\text{O}_{\text{hydroxyl}}\text{-C}_{\text{substrate}}\text{-N}$ bending distortion. This imaginary frequency describes the motion of the substrate C-H hydrogen migrating from the C_7 substrate carbon to the terminal sulfido sulfur atom, and the hybridization of the C_7 substrate carbon changing from sp^3 to sp^2 . Since a covalent $\text{Mo-O-C}_{\text{substrate}}$ linkage is present at the TS, it is of interest to understand the nature of this bonding interaction, particularly with respect to how it may facilitate the two-electron oxidation of the substrate with the concomitant reduction of Mo(VI) to Mo(IV) and the transfer of the $\text{C}_{\text{substrate}}\text{-H}$ hydrogen to the terminal sulfido ligand.

Since the oxidation of XO/XDH substrates involves a formal hydride transfer between the substrate and Mo, with the Mo=S sulfido sulfur being ultimately protonated, the nature of the H transferred is important. Here, we compute the Mulliken charges on the transferred 4-lumazine H at the tetrahedral IM1 state, the TS, and the product bound IM2 intermediate state as +0.071, +0.071, and +0.051 respectively. This clearly points to the H being transferred to the terminal sulfido as a neutral entity. This is interesting in that XO/XDH catalyzed hydroxylations do not proceed via radical intermediates with H-atom transfer. We recently showed through an NBO analysis[3, 38] that this unique reactivity can be achieved through a mechanism that utilizes a combination of $\text{Mo=S } \pi \rightarrow \text{C-H } \sigma^*$ and $\text{C-H } \sigma \rightarrow \text{Mo=S } \pi^*$ donor-acceptor interactions. These donor-acceptor interactions represent both proton-like and hydride-like contributions to C-H bond scission that contribute to lowering the $\text{IM1} \rightarrow \text{TS}$ activation energy. Using 4-thiolumazine as the reducing substrate the $\text{IM1} \rightarrow \text{TS}$ activation energy is computed to be $\sim 16 \text{ kcal/mol}$, which is in excellent agreement with the QMMM computed free energy barriers for XO using xanthine as the reducing substrate (14 kcal/mol), [19] and with experimental results on XO and XDH ($13.9\text{--}16.3 \text{ kcal/mol}$). [19, 39–47]

A simplified description of the reaction coordinate involving frontier MOs was provided in Figure 2. On the Reactant side, the HOMO is doubly occupied and possesses dominant substrate π character, while the LUMO is dominantly Mo(xy) in nature. These orbitals effectively mix at the TS to eventually yield a doubly occupied Mo(xy) Product HOMO and a Product LUMO that is dominantly product π^* . We have shown that the degree of Mo reduction along the reaction coordinate is strongly correlated to the degree of $\text{C}_{\text{substrate}}\text{-H}$ bond cleavage for a variety of XO/XDH substrates, including lumazine.[48] A longer C-H distance observed at the TS was shown to correlate with a greater percentage of Mo reduction at the TS.[48] We define the percent Mo reduction at the TS as the $\% \text{Mo(xy)}$ character in the TS redox orbital divided by the $\% \text{Mo(xy)}$ character in the reduced IM2 E-P complex. We find that the Mo(xy) character present in the TS redox orbital is $\sim 28\%$, while that in the Mo(IV)-4-TV product HOMO is $\sim 73\%$. This allows us to determine that the percent Mo reduction at the TS is $\sim 38\%$. This correlates with a smooth transfer of electron density from the Substrate HOMO in IM1 to the Mo(IV)-Product HOMO in IM2. We note that at no point along the reaction coordinate is there a doubly occupied frontier molecular orbital that possesses dominant H 1s character, and this provides additional support that the $\text{C}_{\text{substrate}}\text{-H}$ hydrogen transferred from substrate to the terminal Mo=S sulfido does not possess hydride character. The fact that the $\text{Mo-O-C}_{\text{substrate/product}}$ covalent linkage is never

broken along the reaction coordinate from IM1 to IM2 indicates that this bonding interaction is important in TS stabilization and Mo reduction, as has been shown for aldehyde substrates.[38]

Resonance Raman Spectroscopy and Mechanistic Implications of the MLCT Excited State Distortion

The stable IM2 Mo(IV)-product complexes with 4- and 2,4-thioviolapterin that formed following the two-electron oxidation of the corresponding lumazine substrates have been shown to yield an intense MLCT transition in the red to NIR region (740 – 780 nm) of the electronic absorption spectrum allowing unprecedented access to the vibrational and electronic structure of this intermediate (IM2) by rR spectroscopy.[25, 28] This NIR MLCT transition derives almost entirely from a $\text{Mo(xy)} \rightarrow \text{P } \pi^* \text{ HOMO} \rightarrow \text{LUMO}$ one-electron promotion, which transfers electron density from the Mo ion to the product to yield a $\text{Mo(V)-P}^{\bullet-}$ description of the MLCT excited state (Figure 3). This is effectively a one-electron analog of a two-electron vertical transition between the Product minimum on the $|\text{GS}\rangle$ PES of Figure 2 and the $|\text{ES}\rangle$ configuration. We determined the concentration (20.8 μM) of oxidized XO from the known extinction coefficient of the enzyme at 450 nm ($\epsilon = 37,800 \text{ M}^{-1}\text{cm}^{-1}$). Given that the enzyme possessed ~50–60% activity, we can estimate that the extinction coefficient for the MLCT band is $\sim 18,000 \text{ M}^{-1}\text{cm}^{-1}$. The large MLCT extinction coefficient for the IM2 charge transfer intermediate indicates that the electronic coupling between the Mo(xy) redox orbital and the product π^* orbital LUMO is coupled by the presence of a $\text{Mo-O-C}_{\text{product}}$ linkage. Thus, resonantly enhanced Raman vibrations in 4- and 2,4-thiolumazine XOr-product complexes probe the nature of the product LUMO as well as the Mo-O-C linkage, providing information regarding electron density shifts between Reactant and Product PESs near the TS configuration.

The high frequency (900 – 1700 nm) rR spectra of the XOr-4-TV and XOr-2,4-TV E-P complexes are shown in Figure 4. Spectroscopic samples of oxidized XO, reduced XO, the substrates (4-thiolumazine and 2,4-dithiolumazine), and products (4-TV and 2,4-TV) show no resonance enhancement under 780 nm laser excitation. The rR spectrum of XOr-4-TV and XOr-2,4-TV display at least 10 vibrational modes in the in-plane stretching region that appear to be resonantly enhanced, and the observed differences in the XOr-4-TV and XOr-2,4-TV Raman spectra reflect the inherent molecular structure differences for these two substrates. For XOr-4-TV, we observe four vibrations that are moderately resonantly enhanced (A: 1000 cm^{-1} , B: 1214 cm^{-1} , D: 1424 cm^{-1} , and E: 1477 cm^{-1}) and three additional vibrations that are strongly enhanced (C: 1293 cm^{-1} , F: 1544 cm^{-1} , and G: 1555 cm^{-1}). Similar moderate to strongly enhanced Raman vibrations are observed for XOr-2,4-TV (A: 989 cm^{-1} , B: 1209 cm^{-1} , C: 1294 cm^{-1} , D: 1406 cm^{-1} , E1: 1468 cm^{-1} , E2: 1488 cm^{-1} , F: 1544 cm^{-1} , and G: 1555 cm^{-1}). Obtaining accurate vibrational assignments for these observed Raman bands is complicated by the fact that the data have been collected in a protein environment, where the substrate binding pocket is believed to facilitate multiple substrate/product hydrogen bonding interactions with the protein that will modify the vibrational structure of the molecule. As such, computed vs. experimental frequency differences may be used to gain insight into hydrogen bonding interactions between the enzyme binding pocket and the product molecules.

In order to make tentative assignment for these Raman bands, we have utilized a computationally assisted approach that compares both the computed frequencies and resonance enhancement patterns for XOr-4-TV and XOr-2,4-TV with the experimental data. Although all of the vibrational bands observed in the 900 – 1700 cm^{-1} region are of the correct frequency to possess dominant in-plane (ip) violapterin ring stretching character, we have chosen to make more specific assignments for bands observed in the 1250 – 1600 cm^{-1} region of the spectrum. Here, we assign Band G (Figure 5) as deriving from an ip product ring stretching vibration that possesses a considerable degree of violapterin pyrazine ring quinoidal stretching character. There is good agreement with both the computed vibrational frequency for this mode and the computed rR intensity. Band F (Figure 5) is observed at a slightly lower frequency and can be assigned as an ip violapterin stretch with O-C stretching character deriving from the Mo-O-C_{product} E-P linkage. The apparent increase in rR enhancement likely derives from some degree of Fermi resonance with Band G. Bands D and E are observed in the 1400 – 1500 cm^{-1} region and possess ip ring stretching character with additional O-C linker and C-S stretching contributions in Band E of XOr-4-TV and band E1 of XOr-2,4-TV. Observed differences in the band E region between XOr-4-TV and XOr-2,4-TV likely derive from differences in their C-S stretching components. Interestingly, our computations suggest that bands D and E (Figure S1–2) should be strongly resonantly enhanced with excitation into the MLCT band. However, the degree of resonance enhancement observed experimentally is noticeably less than the computed enhancement. Finally, we assign band C (Figure 5) as an ip ring stretching vibration with little C-S stretching character. For XOr-2,4-TV there also appears to be a low-frequency shoulder on this band of unknown origin. Although Band C is the most resonantly enhanced vibration in the experimental Raman spectra of both XOr-4-TV and XOr-2,4-TV, the computed rR intensity is markedly lower.

The observed resonance enhancement of Raman bands E (Figures S1–2) and F (Figure 5) are of mechanistic importance since they reflect excited state distortions within the Mo-O-C_{product} linkage that are correlated with a covalent pathway for electron flow between the Mo ion and the substrate. Inspection of the XOr-4-TV HOMO and LUMO wavefunctions, and the EDDM (Figure 3) indicates that the HOMO → LUMO one-electron promotion should result in a compression of the Mo-O bond (depopulation of Mo-O π^*) and an elongation of the O-C_{product} bond (population of C-O π^*), which describes the exact nature of the Mo-O-C_{product} atom motions contained in the normal mode descriptions of bands E and F. It would appear from our observed and computed rR spectra that the nature of the excited state distortion for XO/XDH bound violapterin product molecules is slightly different than what we compute in the gas phase, with the distortion being markedly more dramatic along the vibrational coordinate assigned to Band C and less so along vibrational coordinates defined by vibrations observed in the 1400 – 1500 cm^{-1} region of the Raman spectra. The observed differences in experimental vs. computed excited state distortions likely result from hydrogen bonding and other electrostatic interactions that occur between the bound product and the protein, which are not accounted for in our computations. Finally, vibrational bands in the 900 – 1250 cm^{-1} region of the Raman spectra cannot be correlated well with the computed frequencies or the resonance enhancement patterns. Again, we

attribute this to these modes being more heavily influenced by hydrogen bonding interactions with the protein.

Substrate/Product Orientation in the XO Binding Pocket

Two heterocyclic substrate orientations have been debated with respect to their mechanistic significance in XO/XDH, and these orientations are referred to as “upside” and “upside down” (Figure 6 top).[17, 19, 44] Evaluation of these orientations with respect to catalysis have focused on the examination of enzyme crystal structures[5, 7, 9–11] and mechanistic reaction coordinate QM/MM calculations.[31–33] The results of the QMMM calculations indicate that the “upside” orientation is the thermodynamically favored binding orientation[19] for the reactant species and this is in agreement with published x-ray crystal structures for good XO substrates.[1] In contrast, the QMMM calculations indicated that the “upside down” orientation led to lower TS barriers and was therefore the kinetically favored substrate orientation for catalysis.[19] A rationale for the “upside down” orientation was based on the ability of R880 to stabilize excess charge localized on a substrate ring nitrogen equivalent to N8 in the lumazines.[19] An alternative argument[1] favoring the “upside” orientation has been proposed whereby R880 can stabilize substrate charge built-up on the lumazine C4 carbonyl equivalent following nucleophilic attack (e.g. IM1).

In order to assess the hydrogen bonding capacity of the thiolumazine substrates studied here, x-ray quality single crystals of 4-thiolumazine were grown by slow evaporation of a dimethylformide (DMF) solution. The 4-thiovioloapterin structure is depicted as an ORTEP plot in Figure 7, and the molecular structure parameters are detailed in Table 1. The crystal structure of 2,4-dithiolumazine has been previously determined, and displays the same H-bonding pattern between adjacent opposed molecules.[49] Inspection of Figure 7 clearly shows the propensity for specific heterocyclic substrates to form multiple hydrogen bonding interactions using their ring N atoms. Using lumazine derivatives as an exemplar of purine substrates, it is clear that equivalents of the N1 heterocyclic ring nitrogen can serve as an effective hydrogen bond donor, while equivalents of the N8 nitrogen are available to function as a hydrogen bond acceptor.

In the “Upside” configuration, substrates are bound with nitrogen equivalents of the 4-thiolumazine N1 and N8 atoms positioned to interact with E802 (Figure 6 bottom). This orientation has been suggested to promote substrate tautomerization events following nucleophilic attack.[1, 41, 50, 51] Protonation of E802 would be expected to further stabilize “Upside” substrate/product interactions in the binding pocket, as was found in the QMMM study[19] and as depicted in Figure 6 for the interaction of E802 with 4-thiolumazine product. Additionally, in the upside orientation the xanthine keto oxygen equivalent of the 4-thiolumazine thioketone S4 is positioned to interact with R880 in order to stabilize the charge accumulation on this oxygen.[1, 10, 11] It is argued that the higher upside orientation activation barrier observed in the QMMM study may derive from the use of a protonated E802 in XO (E232 in XDH) in the QMMM calculations which increases the stabilization of IM1 relative to the TS.[1] Lumazine and its thiolumazine derivatives have been found to form very stable E-P intermediates,[25, 26, 28] this may be a direct function of strong heterocycle E802/E232 interactions involving a protonated glutamate. Thus, the hydrogen bonding

pattern observed in Figure 8 (top) may be present in IM2 and lead to its stabilization relative to product release.

In order to address the energetic stabilization due to protonation of E880 in IM2, differences between the “upside” and “upside down” orientations of the reduced enzyme-product, Mo(IV)-4-TV, with the 4-TV in both “upside” and “upside down” orientation, was modelled in the enzyme binding pocket using the crystal structure coordinates[5, 7, 9–11] for three conserved amino acid residues (E1261, E802 and R880 in XOr) as a starting point in the calculation. The results are displayed in Figure 8. As expected, the 4-TV “Upside” orientation was calculated to be 14.5 kcal/mol (0.63 eV) more stable than the “upside down” orientation when Glu 802 is protonated. Similar stabilities are computed for XOr-violapterin 13.8 kcal/mol (0.60 eV), and XOr-2,4-TV 9.2 kcal/mol (0.40 eV) in the “upside” orientation. Thus, a protonated E802 may serve to help orient the substrate such that the substrate plane is orthogonal to the square pyramidal basal plane of the ligated Mo in the enzymes. As stated earlier, this E-S orientation is important for maximal overlap between the Mo(xy) redox orbital and the substrate π^* system, allowing the covalent Mo-O-C_{substrate} linkage to effectively promote electron density shifts along the reaction coordinate for substrate oxidation. However, due to the strong stabilization incurred by protonation of E802, it may be that protonation occurs after the TS is reached. This would contribute to an enhanced stability of IM2 along the reaction coordinate allowing for the formation of stable violapterin E-P complexes that can be studied in detail spectroscopically.

Conclusions

We have evaluated key components of the XO reaction coordinate with respect to how a covalent Mo-O-C linkage and substrate/product orientation contribute to the activation barrier for substrate oxidation, the stability of E-P complexes, and hydrogen bonding interactions specific to lumazine-based substrates and products. The TS barriers and the reaction coordinate computed for 4-thiolumazine and 2,4-thiolumazine substrates are in line with those determined for other heterocyclic XO substrates. Resonance Raman spectroscopic probes of the high-frequency product in-plane stretching region has been used to confirm that the long wavelength absorption that figures prominently in the spectrum of violapterin-based E-P complexes is due to a Mo(xy) $\rightarrow$ product π^* one-electron promotion (e.g. an MLCT process). The intensity of this MLCT transition also indicates a strong orbital overlap between the Mo(IV) redox orbital and the π^* LUMO of the product. Laser excitation into the NIR MLCT absorption band produces rR enhancement patterns that directly probe the nature of the MLCT transition and provide insight into the effects of electron redistribution along the reaction coordinate between the TS and IM2, including the role of the covalent Mo-O-C linkage in facilitating this process. The combination of an intense Mo(xy) $\rightarrow$ P π^* MLCT transition and strong resonant enhancement of product vibrations in the in-plane stretching region provide compelling evidence for the importance of an E-S orientation in which the substrate plane is orthogonal to the Mo(xy) redox orbital. This geometric orientation allows for a high degree of Mo(IV)-O-C_{substrate} π orbital overlap that connects the Mo(xy) redox active orbital with the delocalized out-of-plane π system of the heterocyclic product. The large extinction coefficient observed for this MLCT band also supports strong orbital mixing between the doubly occupied Mo(xy) redox orbital and

lumazine product π^* orbitals. This is important since the Mo-O-C_{product} linkage provides a pathway for facilitating the two-electron oxidation of XO substrates, and has previously been shown to contribute to TS stabilization in XO with aldehyde substrates.[3, 38] Finally, the intrinsic hydrogen bonding capability of lumazine substrates has been analysed. Dual hydrogen bonding interactions involving the N1 and N8 violapterin ring nitrogen atoms and the carboxylate functionality of E880 may lead to proper alignment of the substrate in the binding pocket to promote electron density redistribution between Mo(xy) and substrate during the reduction half reaction of the enzyme, enhance stabilization of IM1 leading to an increase in the activation energy required to reach the TS, and an increase in E-P stabilization, with the latter leading to our ability to spectroscopically probe the rich electronic structure of this E-P intermediate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A generalized mechanism for the oxidation of heterocyclic substrates by XO/XDH. The carbon atom that is hydroxylated is the C7 carbon for lumazine and the C8 carbon for xanthine. These C atoms and the H being transferred to the sulfido ligand are highlighted red.

Figure 2.

Top: Schematic energy profile along the reaction coordinate from the IM1 Reactant state to the IM2 Product state (Mo(VI)-S to Mo(IV)-P) as a function of the C_7 -H (labelled in red in Figure 1) distance (Q) of substrate (4-thiolumazine). The red arrow depicts a hypothetical two-electron transition to the Frank-Condon ES, and the blue arrow depicts the instantaneous distorting force toward the minimum along the ES PES. Bottom: Simple diagram showing how two electrons are transferred from the Substrate HOMO to the Mo(xy) redox orbital along the reaction coordinate. In this two-state frontier orbital depiction, the Substrate HOMO smoothly transitions to the Product LUMO.

Figure 3.

Top: Computed HOMO and LUMO wavefunctions for the XOr-4-TV E-P complex. Bottom: Electron density difference map (EDDM) for the HOMO → LUMO MLCT transition. Purple represents an electron density loss in the transition and orange represents an electron density gain. The view is oriented looking down the Mo≡O bond.

Figure 4.

Experimental (red) and computed (black) resonance Raman spectra of XOr-4-TV (top) and XOr-2,4-TV (bottom) collected anaerobically in BICINE buffer (pH=8.3). The asterisk is from (NH₄)₂SO₄ in the buffer and serves as an internal standard.

Figure 5. Selected computed vibrational modes for XOR-4-TV complex, which are rendered and superimposed at the minimum and maximum values of the vibrational coordinate, Q , during the vibrational period.

Figure 6.

Top: "Upside" and "Upside down" orientations of 4-thiolumazine in IM1. Bottom: "Upside" orientation with protonated E802. (I changed to E802. E232 Should be E802 for XO if use R880; or for XDH they are E232 and R310.)

Figure 7.

ORTEP view of the hydrogen-bonded 4-thiolumazine●DMF dimer showing the thermal ellipsoids at 50% probability. Hydrogen atoms are fixed as 0.15 Å sphere. Crystal parameters: monoclinic $P1/c$. a) 5.566(3), b) 8.588(4), c) 12.217(7) Å; α) 97.751(4)°, β) 98.795(3)°, γ) 101.942(3)°. Hydrogen bonding distance: N3H3—N2: 2.086(2) Å; N4H4—O2: 1.879(2) Å.

Figure 8. Gas-phase optimized geometries for (A) the “upside” orientation and (B) the “upside down” orientation of 4-TV in the reduced Mo(IV)-P complex. Three conserved amino acids have been included in the calculation. Note that the “upside” orientation stabilizes the Mo(IV)-P complex by 14.5 kcal/mol relative to the “upside down” orientation.

Table 1

Selected bond length (Å) and bond angles (degree)

S1-C6	1.6486(19)	O1-C5	1.215(2)
N1-C1	1.341(2)	N1-C2	1.330(2)
N2-C3	1.330(2)	N2-C4	1.344(2)
N3-C4	1.367(2)	N3-C5	1.370(2)
N4-C5	1.392(2)	N4-C6	1.369(2)
N3-H3A	0.80(2)	N4-H4A	0.89(2)
C1-C4	1.393(2)	C1-C6	1.473(3)
C2-C3	1.392(3)	C2-H2	0.95
C2-N1-C1	116.37(17)	C3-N2-C4	115.80(16)
C4-N3-C5	123.65(17)	C4-N3-H3A	122.0(15)
C5-N3-H3A	114.4(15)	C6-N4-C5	127.32(16)
C6-N4-H4A	119.3(13)	C5-N4-H4A	113.4(13)
N1-C1-C4	121.36(17)	N1-C1-C6	119.79(17)
C4-C1-C6	118.85(17)	N1-C2-C3	122.03(19)
C3-C2-H2	119.0	N2-C3-C2	122.21(18)
N2-C3-H3	118.9	C2-C3-H3	118.9