The crystal structure of xanthine oxidoreductase during catalysis: Implications for reaction mechanism and enzyme inhibition

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Molybdenum is widely distributed in biology and is usually found as a mononuclear metal center in the active sites of many enzymes catalyzing oxygen atom transfer. The molybdenum hydroxylases are distinct from other biological systems catalyzing hydroxylation reactions in that the oxygen atom incorporated into the product is derived from water rather than molecular oxygen. Here, we present the crystal structure of the key intermediate in the hydroxylation reaction of xanthine oxidoreductase with a slow substrate, in which the carbon–oxygen bond of the product is formed, yet the product remains complexed to the molybdenum. This intermediate displays a stable broad charge-transfer band at ~640 nm. The crystal structure of the complex indicates that the catalytically labile Mo—OH oxygen has formed a bond with a carbon atom of the substrate. In addition, the Mo═S group of the oxidized enzyme has become protonated to afford Mo—SH on reduction of the molybdenum center. In contrast to previous assignments, we find this last ligand at an equatorial position in the square-pyramidal metal coordination sphere, not the apical position. A water molecule usually seen in the active site of the enzyme is absent in the present structure, which probably accounts for the stability of this intermediate toward ligand displacement by hydroxide.

Molybdenum hydroxylases are found in virtually all organisms, ranging from bacteria to humans, and catalyze the hydroxylation of a wide variety of heterocyclic substrates such as purines, pyrimidines, and pterins, in addition to aldehydes (1, 2). The overall reaction generates rather than consumes reducing equivalents, which sets it apart from other hydroxylation systems, such as the cytochromes P450. Xanthine oxidoreductase (XOR) is the prototypical molybdenum hydroxylase and, in humans, it catalyzes the hydroxylation of hypoxanthine to xanthine as well as xanthine to uric acid. The enzyme is a well established target of drugs against gout and hyperuricemia (3).

XOR from bovine milk, the most studied molybdenum hydroxylase, has served as a benchmark for the entire class of complex metalloflavoproteins (4). Each subunit of the homodimeric enzyme contains one molybdenum center, one flavin (FAD), and two [2Fe–2S] iron-sulfur centers (1, 2). The oxidative hydroxylation of xanthine to uric acid takes place at the molybdenum center and results in the two-electron reduction of the metal from Mo(VI) to Mo(IV). The enzyme is subsequently reoxidized by NAD⁺ or molecular oxygen (1) in a reaction that occurs at the FAD (after electron transfer from the molybdenum center via the iron-sulfur centers). The mammalian enzyme exists in two alternative forms of the same gene product. Under normal circumstances, it occurs in its dehydrogenase form [xanthine dehydrogenase (XDH)], but it can be readily converted to its oxidase form (XO) by oxidation of sulfhydryl residues or by limited proteolysis (1). XDH shows a preference for NAD⁺ as the oxidizing substrate (although it is also able to react with O₂), whereas XO is unable to react with NAD⁺ and uses dioxygen exclusively (1). X-ray crystal structures of both the XDH and XO forms of bovine XOR have recently been reported at 2.1- and 2.5-Å resolution (5, 6), respectively, and a detailed comparison of the two forms and site-directed mutagenesis studies has elucidated the structural basis for the XDH to XO switch (6). Based on the results of extended x-ray absorption fine-structure studies (2), two thiolene sulfurs (═S), one sulfo (═O), one oxo (═O), and one hydroxo (OH) group or water had previously been postulated as the ligands of the active-site molybdenum center (Fig. 1a) (2). The latter has been shown to have a distorted square-pyramidal coordination geometry, and the Mo═S group was assigned the apical position by direct analogy to the closely related enzyme, aldehyde oxidase from Desulfovibrio gigas, whose structure has been determined (7, 8). Mechanistically, the oxygen atom at the catalytically labile site on the Mo center is transferred to a substrate and is subsequently regenerated by oxygen derived from the solvent before a second round of catalysis (9). Alternative mechanisms in which either the Mo═O or Mo—OH group represents the catalytically labile group have been proposed in the past (2, 10, 11). In the present work, we have determined the crystal structure of the first and key reaction intermediate of this unique molybdenum-based hydroxylation chemistry by using a slow substrate; the results allow us to draw further conclusions about the catalytic mechanism.

Experimental Procedures

Materials and Protein Purification. Bovine milk XDH was purified by the method previously reported (12). The catalytically inactive desulfo and demolybdo forms were removed according to the method of Nishino et al. (13), with minor modifications. Bound oxipurinol was released from the complex spontaneously by incubating the enzyme solution under air-saturated conditions without additional oxidizing reagent for 48 h at 25°C. More than 95% of the purified enzyme was catalytically active (the activity to flavin ratio >200; ref. 13). Purified samples were stored on ice in a mixture of 80% 0.1 M pyrophosphate buffer (pH 8.5) and 20% 50 mM Tris-HCl buffer (pH 7.8), both containing 0.2 mM EDTA and 1 mM salicylate. The XDH concentration was determined by using an extinction coefficient of 37,800 M⁻¹ cm⁻¹ (14). 4-[5-Pyridin-4-yl-1H-[1,2,4]triazol-3-yl]pyridine-2-carbonitrile (FYX-051) and 2-hydroxy-FYX-051, shown in Fig. 1, were provided by Fuji Yakuhin, Tokyo. Titanium(III) citrate solutions were prepared in an argon glove box.
Enzyme Activity Assay and Analytical Procedures for the XDH–FYX-051 Complex.

Xanthine-O2 activity was determined in a solution containing 10% acetonitrile at 40°C by using a Hitachi (Tokyo) U3300 spectrophotometer. Absorbance spectra were recorded by using a Beckman Coulter DU-7400 spectrophotometer.

Table 1. Data collection and refinement statistics for the FYX-051 XOR complex

<table>
<thead>
<tr>
<th>Statistic</th>
<th>FYX-051–XOR</th>
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<tr>
<td>Space group</td>
<td>C2</td>
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<tr>
<td>Unit cell axes, Å; unit cell angle, degree</td>
<td>a = 168.0, b = 124.6, c = 146.9; β = 91.0</td>
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<tr>
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<tr>
<td>No. of water molecules</td>
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</table>

*aRsym = Σh[|Fobs|–|Fcalc|]/Σh|Fobs|.
†Rcryst = Σh[|Fobs|–|Fcalc|]/|Fobs|.
‡Rfree = Rsym for 3.0% of randomly selected reflections not used in refinement.

Results

Spectral Changes of XOR on Mixing with the Inhibitor FYX-051.

The chemical formula for compound FYX-051 is shown in Fig. 1b. FYX-051 is a potent inhibitor of XO with xanthine as a substrate (Fig. 1d); in the absence of xanthine, however, FYX-051 itself is very slowly hydroxylated by the enzyme. On mixing fully active

Crystallization of the XDH–Substrate Complex.

Crystals of the FYX-051 complex of bovine XDH were obtained by modifying a published method (12). Salicylate was removed from enzyme stock solutions by gel filtration, and the sample was then concentrated with a Centricon YM-100 membrane (Amersham Pharmacia) to a protein concentration of ~60 mg/mL. A 30-mg/mL enzyme solution containing 30% (wt/vol) glycerol and 5 mM DTT was made anaerobic and transferred to an argon box, where all subsequent manipulations were performed. Enzyme solutions (10 μL) were mixed with 10 μL of 50 mM potassium phosphate buffer (pH 6.5) containing 4.5–7.5% polyethylene glycol 4,000, 30% glycerol (wt/vol), 0.2 mM EDTA, 5 mM DTT, and 0.75 mM FYX-051. The solutions were mixed with a titanium(III) citrate solution to 13 mM final concentration, set up on siliconized glass plates, and kept in the dark at 20°C. Crystals grew after 5 days; they belonged to the same C2 space group as the original crystals but displayed slightly altered unit cell parameters of a = 168.0 Å, b = 124.6 Å, c = 146.9 Å, and β = 91.0°. The crystals were collected with a nylon loop, shock-frozen, and stored in liquid nitrogen.

Data Collection and Data Refinement.

A complete 1.9-Å diffraction data set was collected at Spring-8 (Harima Garden City, Hyogo, Japan), beamline BL38B1 at A = 1.000 Å by using an ADSCQ4R charge-coupled device detector (Table 1). Data were processed with the program package HKL2000. The structure was solved by molecular replacement by using the program EMPIR (17) with salicylate-bound XDH (Protein Data Bank ID 1FO4) as a search model. The molecular model was built by using the program O (18). Refinement was done following standard protocols of the program CNS, Version 1.0 (19). First, one round of rigid body refinement was followed by simulated annealing including only the protein model. Then the FAD molecule and the two [2Fe–2S] clusters were included for energy minimization and B-factor refinement, then water molecules were picked and edited, and glycerol as well as acetate molecules was added. This was followed by another round of energy minimization and B-factor refinement. Figs. 4–7 were generated with the programs MOLSCRIPT (20), RASTER3D (21), and BOBSCRIPT (22).
enzyme with a stoichiometric amount of FYX-051 under anaerobic conditions, a spectral change is seen (Fig. 2a), with the difference spectrum exhibiting a broad positive peak at 640 nm (Δε = 2.0 mM⁻¹·cm⁻¹) and a negative peak at 430 nm (Δε = 1.6 mM⁻¹·cm⁻¹) (Fig. 2a Inset). This spectral change is not caused by the reduction of FAD or the iron-sulfur centers of the enzyme (1, 23) but instead is due to formation of a charge-transfer complex between the reduced molybdenum center and the now hydroxylated inhibitor (Fig. 1c), directly analogous to what had previously been seen with violapterin (2,4,7-trihydroxypteridine, the product of enzyme action on lumazine; ref. 24). The charge-transfer complex involving the FYX-051 species forms immediately on mixing and is stable for 1 h under anaerobic conditions and for at least 30 min in air-saturated buffer (Fig. 2a). A similar spectrum is observed even after gel filtration to remove small molecules (data not shown); the complex decomposes only very slowly (t₁/₂ ≈ 22 h) even under aerobic conditions. On the other hand, no spectral change was observed when cyanide-treated XOR rather than native enzyme was used (data not shown), indicating that the cyanoxyzable and catalytically essential Mo=S ligand is required for formation of the charge-transfer complex. To investigate the nature of this complex, compounds liberated from the enzyme-inhibitor complex after protein denaturation were analyzed on a C18 reverse-phase column. The elution profile of the released compound is shown in Fig. 3. (Top) Authentic FYX-051; (Middle) 2-OH-FYX-051; (Bottom) the released compound.

Overall Structure of FYX-051-Bound XOR. The stability of the charge-transfer complex allowed us to undertake a crystallographic study of the species. Although the charge-transfer complex is quite stable, as a precautionary measure, crystals of the XDH–FYX-051 complex were grown in the presence of titanium(III) citrate under anaerobic conditions to maintain the iron-sulfur centers and FAD in their reduced states, to avoid a potential slow breakdown of the complex during crystallization. Diffraction data were collected to 1.9-Å resolution. The structure was solved by molecular replacement techniques and refined by using molecular dynamics refinement. The asymmetric unit contains one homodimer, analogous to the previously determined structures of salicylate-bound [Protein Data Bank (PDB) ID 1FO4] and TEI-6720-bound enzyme (PDB ID 1N5X). No electron density was observed for two interdomain loop regions (residues 166-191 and 532-536 in one subunit and residues 166-191 and 529-536 in the second subunit). In addition to the enzyme, 2,082 water molecules, together with eight glycero molecules and two acetate molecules from the mother liquor, are found in each asymmetric unit.

Structural Features of the Molybdenum-Substrate Complex. FYX-051 is bound in the solvent channel that provides access to the active site molybdenum (Figs. 4 and 5), with its pyridine moiety close to the molybdenum. Electron density is clearly evident between the molybdenum center and the pyridine ring of FYX-051 (Figs. 5 and 6), suggesting a covalent linkage between molybdenum and the product. This bridging electron density is bent, spanning a total of 3.3 Å and connecting to the C2 atom of FYX-051, the position that becomes hydroxylated in the course of the reaction. Our analysis suggests that this electron density represents a bridging oxygen, derived from the Mo––OH group of the oxidized enzyme. Placing the oxygen atom at the center of the bent electron density leads to Mo–O and C2–O distances of 2.0 and 1.3 Å, respectively (Fig. 6c). The latter is a typical C–O distance for a hydroxylated six-membered ring. The oxygen is displaced from the C2–Mo axis toward the backbone amide of Ala-1079 with a Mo–O–C angle of 152°. The distance between the oxygen and amido-N is 3.0 Å, consistent with hydrogen bonding between the two atoms. The structure thus shows the newly formed product still coordinated to the reduced molybdenum via the oxygen atom that is being transferred from the molybdenum center. As such, the structure presents a picture of arrested catalysis at the step of formation of the first and key intermediate through oxygen atom transfer from the molybdenum coordination sphere to the substrate carbon being hydroxylated. Several hydrogen bonds and electrostatic interactions are found between the enzyme and bound hydroxyl-FYX-051. Glu-1261 is 2.7 Å away from the N1 of FYX-051, forming a hydrogen bond in its protonated form (Fig. 4). Glu-802 is 2.8 Å from the N4 of the triazole ring of the product. In a previous study of the XDH in complex with the inhibitor TEI-6720, arguments in favor of protonation of Glu-802 have been presented (25). A similar mode of interaction might also be seen in the present complex. A 3.1-Å hydrogen bond is formed between Asn-768 and the nitrile group of FYX-051. Finally, the triazole ring of FYX-051 is sandwiched between Phe-914 and Phe-1009 (Fig. 4), reminiscent of the binding of the aromatic salicylate ring to bovine XOR (5). In addition, two water molecules (w1152 and w1810), which are shown as red spheres in Fig. 4, are found within hydrogen-bonding distance of N1 and N2 of the triazole ring (2.7 and 2.8 Å, respectively). On the other hand, water molecule w224,
interacting with the carboxylate oxygen of Glu-1261 in the salicylate complex, is not present in the FYX-051 complex structure.

**Sulfur in the Coordination Sphere of the Molybdenum.** In the present molecular model, the molybdenum center exhibits a square-pyramidal coordination geometry, as has been observed in all other structures of this class of enzymes (Fig. 6). Using an improved purification procedure resulting in almost 100% active enzyme (i.e., in enzyme possessing the full complement of the catalytically essential Mo=S group), we find the following ligands in the equatorial plane of the metal: (i) two sulfur atoms of the dithiolene group of the metal’s pterin cofactor (see Fig. 1a); (ii) the bridging oxygen atom to the pyridine ring of FYX-051 at a distance of 2.0 Å from the molybdenum; and (iii) a fourth ligand, previously assigned as the Mo=O group, but which in our structure has a quite high and clearly defined electron density (Fig. 6 a, c, and d, arrows), and which is unambiguously modeled as a Mo=SH rather than a Mo=O group. Electron density at this position can be observed even in a map contoured at a 15.0 σ cutoff (Fig. 6 b and e, arrows). The refined distance between this sulfur and the molybdenum is 2.4 Å, quite appropriate for -SH. At 15.7 and 13.6 Å, the refined B factors for the two crystallographically independent Mo=SH sulfur atoms are comparable to those calculated for dithiolene sulfurs (12.6 and 10.2 Å), sulfurs in iron-sulfur clusters (11.1–12.6 Å) and sulfurs in surrounding methionine residues (11.5–12.4 Å²), supporting the present assignment. That Mo=SH occupies the fourth equatorial position of the molybdenum coordination sphere would imply that the Mo=S group of the oxidized enzyme is also equatorial rather than apical, as previously assigned. Now, the Mo=O group occupies the apical position of the molybdenum coordination sphere, with a distance of 1.7 Å between the two atoms, typical for a Mo=O group (Fig. 6a). A new hydrogen bond was observed between Gln-767 and the molybdenum coordination sphere of XDH; the side-chain amide of this amino acid interacts with the apical Mo=O group at a distance of 3.0 Å (rather than Mo=S, which projects behind the plane of the paper; Fig. 4). The same amide is also linked to the carbonyl oxygen of Thr-1077 (2.9 Å). This interaction is likely to modulate the reactivity of the molybdenum center.
Discussion

The present crystal structure represents the structure of the first and key intermediate in the process of substrate hydroxylation by a molybdenum hydroxylase: the C2—O bond of product has formed and is clearly evident in the electron density, yet the product remains coordinated to the now reduced molybdenum center. Although xanthine oxidase displays broad substrate tolerance and catalyzes the hydroxylation of various heterocycles, we expect the underlying mechanism to be essentially the same in each case, and thus the structure reported here should be relevant to hydroxylation of xanthine and other substrates. It has long been known that the enzyme’s active site possesses a catalytically labile oxygen that represents the proximal oxygen donor to substrate in the course of the reaction (9), and both the Mo=O and Mo—OH groups have been put forward as candidates (see ref. 2 for a review) for this site. Using EPR and electron-nuclear double resonance (ENDOR) spectroscopy in conjunction with $^{17}$O labeling methods, it has been convincingly demonstrated that the Mo—OH group is the catalytically labile oxygen (10, 11). On the basis of these results, two quite different reaction mechanisms have been proposed, with the reaction proceeding either by base-catalyzed nucleophilic attack of Mo—OH on the C-8 position of the substrate (leading directly to the product coordinated to the now-reduced molybdenum via the newly introduced hydroxyl group) (11), or alternatively by addition of the C8—H bond across the Mo=S group of the molybdenum center, followed by oxygen insertion to yield an intermediate with a direct Mo—C bond (10). The work presented here provides direct information regarding the structure of the initially formed intermediate in the catalytic sequence and demonstrates that this intermediate has the product coordinated to molybdenum in a simple end-on fashion. This binding mode is clearly inappropriate for formation of a Mo—C bond, and the structure provides unambiguous support for a reaction mechanism initiated by direct nucleophilic attack of Mo—OH on the substrate.

In addition, in the present structure of the reaction intermediate of fully active sulfo-XDH, the electron density of the ligand at the fourth equatorial position of the molybdenum coordination sphere is much more intense, its bond length is quite appropriate for a Mo—SH group, and the refined B factors of the equatorial sulfur ligands are comparable, supporting the present assignment. In addition, this geometry is in agreement not only with magnetic circular dichroism studies (26) but also with a large number of structures of small inorganic compounds (27), many of which have served as structural and mechanistic models for enzyme active sites. In these complexes, the strong-field Mo=O group invariably occupies the apical position and defines the molecular z axis of the coordination sphere (27). The geometry of the molybdenum coordination sphere described here differs from that previously reported for Desulfovibrio gigas aldehyde oxidoreductase (7) and that for XOR, where, due to limited resolution of diffraction data, assignment was based on the aldehyde oxidoreductase precedent (5); the Mo=S and the Mo=O group have switched positions, making the oxygen the apical ligand. The geometry described here, however, is consistent with a recently refined 1.7-Å structure of the salicylate-bound form of XDH (B.T.E., K.O., T.N., and E.F.P., unpublished data) and is also similar to that of a recent high-resolution (1.1-Å) structure of CO dehydrogenase, although the latter contains an additional Cu ion in its active site (28).

The proposed mechanism of hydroxylation of FYX-051 and inhibition of XOR is shown in Fig. 7. A catalytically labile Mo—OH group of the oxidized (MoVI) enzyme initiates catalysis by (base-assisted) nucleophilic attack on the carbon center to be hydroxylated, with concomitant hydride transfer (Fig. 7 a and b). This yields a reduced Mo(IV)—SH that derives from the Mo(VI)—S of the oxidized enzyme (Fig. 7c), with the product
remaining coordinated to the molybdenum via the newly introduced hydroxyl group. The new geometry of the molybdenum ligands is consistent with the reaction mechanism, because an equatorial location should put the sulfide-ligand into a better position for hydride transfer from the substrate. During this reaction, Glu-1261 is well positioned to play the role of the general base abstracting the proton from Mo—OH (Fig. 7a). The now-protonated Glu-1261 is stabilized by forming a hydrogen bond to the N1-nitrogen of the substrate, and this facilitates the nucleophilic attack by the just generated Mo—O⁻ (Fig. 7b). The electron density in the crystal structure of the intermediate clearly shows product coordinated to the reduced molybdenum with a Mo—O—C angle of 152°, as predicted by earlier electron spin envelope modulation (ESEEM) (29) and ENDOR (30) studies of the so-called “very rapid” Mo(V) species (Fig. 7e) that lies immediately downstream from the Mo(IV) intermediate seen in the present work (Fig. 7c). The Mo(IV) intermediate whose existence is demonstrated here represents an obligatory intermediate in the catalytic sequence and is thought to yield the “very rapid” species by one-electron transfer to other redox-active groups in the enzyme with retention of the product at the active site. This EPR species forms to varying degrees from one substrate to the next, however, and with most substrates the Mo(IV) intermediate breaks down predominantly by direct hydroxide displacement of the product without forming the “very rapid” species. It is likely that the exclusion of water (e.g., w224 in the structure of the salicylate-bound enzyme) from the active site, due to the tight fit between the substrate FYX-051 and its binding site in the channel, is the basis for the stability of the Mo(IV) intermediate. Indeed, a steady-state kinetic study, using initial velocity measurements, indicated competitive-type inhibition with a Kᵢ value of 5.3 × 10⁻⁹ M for FYX-051 (K.M., K.O., and T.N., unpublished work).

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