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X-ray Crystal Structure of Arsenite-Inhibited Xanthine Oxidase: μ-Sulfido,μ-Oxo Double Bridge between Molybdenum and Arsenic in the Active Site

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Supporting Information

ABSTRACT: Xanthine oxidoreductase is a molybdenum-containing enzyme that catalyzes the hydroxylation reaction of sp^2-hybridized carbon centers of a variety of substrates, including purines, aldehydes, and other heterocyclic compounds. The complex of arsenite-inhibited xanthine oxidase has been characterized previously by UV−vis, electron paramagnetic resonance, and X-ray absorption spectroscopy (XAS), and the catalytically essential sulfido ligand of the square-pyrimidal molybdenum center has been suggested to be involved in arsenite binding through either a μ-sulfido,μ-oxo double bridge or a single μ-sulfido bridge. However, this is contrary to the crystallographically observed single μ-oxo bridge between molybdenum and arsenic in the desulfo form of aldehyde oxidoreductase from *Desulfovibrio gigas* (an enzyme closely related to xanthine oxidase), whose molybdenum center has an oxo ligand replacing the catalytically essential sulfur, as seen in the functional form of xanthine oxidase. Here we use X-ray crystallography to characterize the molybdenum center of arsenite-inhibited xanthine oxidase and solve the structures of the oxidized and reduced inhibition complexes at 1.82 and 2.11 Å resolution, respectively. We observe μ-sulfido,μ-oxo double bridges between molybdenum and arsenic in the active sites of both complexes. Arsenic is four-coordinate with a distorted trigonal-pyramidal geometry in the oxidized complex and three-coordinate with a distorted trigonal-planar geometry in the reduced complex. The doubly bridged binding mode is in agreement with previous XAS data indicating that the catalytically essential sulfur is also essential for the high affinity of reduced xanthine oxidoreductase for arsenite.

Xanthine oxidoreductase catalyzes the final two steps in purine metabolism in mammals, the oxidation of hypoxanthine to xanthine and the oxidation of xanthine to uric acid. The the enzyme's active site is a square-pyramidal LMo(VI)OS(OH) center, where L represents a pyranopterin cofactor coordinated to the metal via a bidentate enedithiolate side chain (see Chart 1, which depicts the molybdenum coordination sphere and the structure of the pyranopterin moiety).[1] The sulfido ligand is essential for enzyme activity toward both xanthine and aldehyde substrates, and its replacement by a second oxo ligand via cyanolysis yields an inactive desulfo form.[2]

Chart 1. Structure of the Molybdenum Center in Xanthine Oxidase

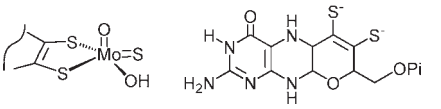
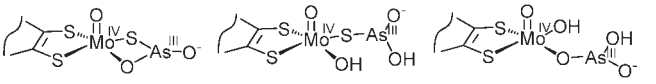


Chart 2. Previously Proposed Structures for (left, center) Arsenite-Inhibited Forms of Xanthine Oxidase Based on EXAFS Data and (right) Aldehyde Oxidoreductase Based on Crystallographica Data



Arsenite is known to inhibit xanthine oxidase, binding particularly tightly to the reduced form of the enzyme.[3a,b] Electron paramagnetic resonance studies of the arsenite-complexed enzyme have demonstrated strong anisotropic hyperfine and quadrupole coupling of arsenic ($I = {}^3/_2$) to the electronic spin of Mo(V) ($S = {}^1/_2$, d^1 system) and also that the inactive desulfo enzyme, as well as the native form, can bind arsenite.[3a−d] X-ray absorption spectroscopy (XAS) studies have indicated a Mo−S−As core with a bond angle of 80 ± 4° and the following distances in the reduced enzyme: Mo−S, 2.39 Å; Mo−As, 3.00 Å; and As−S, 2.27 Å.[3e] Two alternate binding modes for arsenite have been proposed, with either a single μ-sulfido bridge or a μ-sulfido,μ-oxo double bridge (Chart 2).[3]

Recent X-ray crystallographic studies of the arsenite-inhibited desulfo form of aldehyde oxidoreductase (AOR) from *Desulfovibrio gigas* (which is closely related to xanthine oxidase but has been reported to be active in a MoO_2 form lacking the Mo=S moiety required by xanthine oxidase for activity[4c]) showed a single μ-oxo bridge between As and Mo in the arsenite-inhibited complex (Chart 2).[4a,b] The observed binding mode for arsenite was distinctly different from that proposed for the functional form of xanthine oxidase,[3] and a crystal structure of arsenite-inhibited xanthine oxidase is thus highly desirable to elucidate the mechanism of arsenite inhibition.

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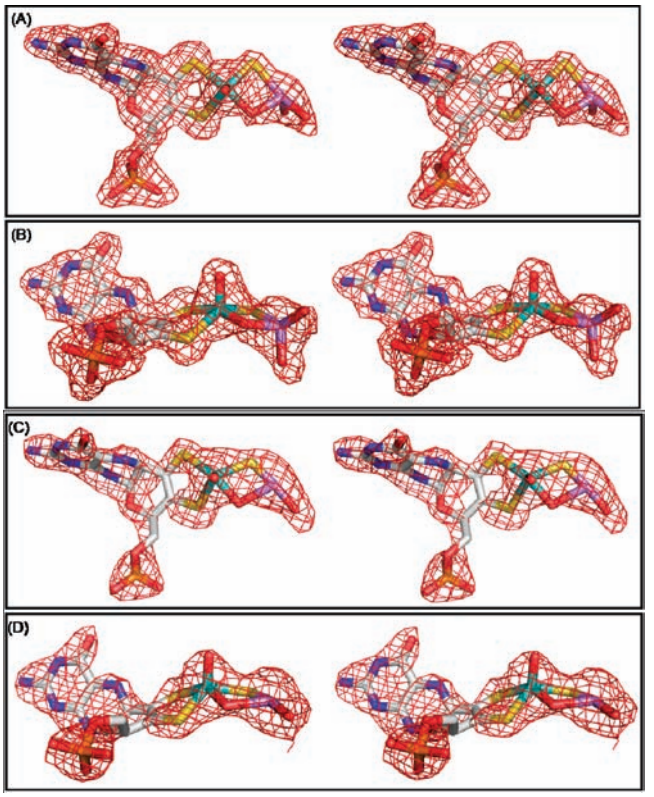

Figure 1. Stereo images of the active sites of xanthine oxidase complexed with arsenite: (A, B) oxidized form; (C, D) reduced form. (B) and (D) are rotated ~90° about the horizontal axis from the perspectives shown in (A) and (C), respectively. All of the electron density maps were $F_o - F_c$ omit maps calculated before the introduction of the arsenite-inhibited molybdenum cofactor for the refinement of the model and contoured at 3.0σ within 2 Å of all atoms shown. Molybdenum is in teal, carbon in white, nitrogen in blue, oxygen in red, sulfur in yellow, phosphorus in orange, and arsenic in purple. Figures were rendered using PyMOL.[6c] Only one of the two active sites of xanthine oxidase in each complex is shown, although both sites in the asymmetric unit showed similar structures.

Xanthine oxidase was purified and crystals were grown as described in the Supporting Information. Arsenite was introduced to enzyme crystals through soaking at a concentration of 10 mM. For the arsenite-inhibited enzyme in the reduced form, dithionite was used to reduce the crystals after addition of arsenite. X-ray data sets were collected at the LRL-CAT beamline of Argonne National Laboratory with 1.82 Å resolution for the complex of arsenite with oxidized enzyme and 2.11 Å resolution for the complex with reduced enzyme. The protein structures were solved using the MOLREP program of the CCP4 package,[6a] with the reported structure of xanthine oxidase solved by Enroth et al.[1b] (PDB entry 1FIQ) as the search model and then further improved by running rigid-body and restrained refinement using REFMAC.[6b] For the reduced form of the enzyme complex, noncrystallographic symmetry constraints between the corresponding peptide chains were set as "tight" during refinement. In the somewhat lower-resolution data for the reduced complex, bond distance restraints based on EXAFS-derived values[3e] were incorporated during refinement. In this way, uncertainties in the metal−ligand distances due to Fourier truncation artifacts in the immediate vicinity of the metal were minimized. Refinement statistics are summarized in Table S1 in the Supporting Information (SI).

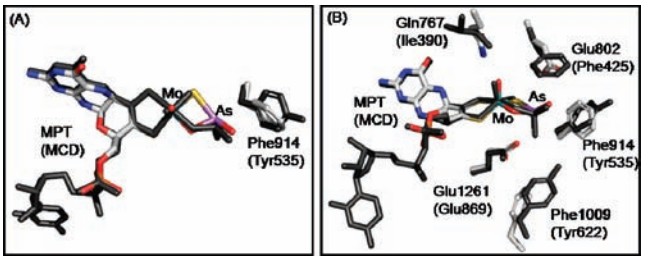

Figure 2. Superposition of the active sites of arsenite-inhibited xanthine oxidase and AOR. (B) is rotated ~90° about the horizontal axis from the perspective shown in (A). The desulfo-AOR complex (PDB entry 1SIJ, ref 4a) is shown in dark-gray. The current xanthine oxidase complex is color-coded as in Figure 1. Amino acids for both structures are labeled, with those for AOR in parentheses. The superposition was performed using COOT[6d] to maximize the overlap between the molybdenum atoms and the enedithiolate ligands of the two structures. One of the two active sites of xanthine oxidase was used for the superposition, although both yielded similar results. Figures were rendered using PyMOL.[6c]

The overall protein structure of arsenite-inhibited xanthine oxidase in both the oxidized and reduced forms as determined here is very similar to that previously reported for the oxidized enzyme, with all differences limited to the active site. Figure 1 shows an overlay of the molybdenum centers with the $F_o - F_c$ omit map (the map omitted the molybdenum cofactor and arsenite). In both the oxidized and reduced structures, molybdenum has a square-pyrimidal coordination geometry with the same ligand set and similar metal−ligand distances as seen in the free enzyme (Table S2). As shown in Figure 2, the oxidized structure has a bridging oxygen between molybdenum and arsenic similar to that in the arsenite-inhibited (desulfo) AOR from *D. gigas*.[4a] The data for the reduced structure also suggest a similar bridging oxygen, although its electron density is not as strong as in the oxidized structure. In contrast to the AOR structure, however, in both the oxidized and reduced xanthine oxidoreductase we see clear and strong electron density connecting the equatorial sulfido ligand to the arsenic atom, with As−S bond lengths of 2.53 Å in the oxidized structure and 2.08 Å in the reduced structure (Figure 1 and Table S2); the latter distance is generally consistent with the As−S distance determined for the reduced structure by XAS (2.27 ± 0.03 Å).[3e] These distances are significantly shorter than the corresponding As−O distances for the nonbridging equatorial oxo ligand of Mo as observed in the X-ray crystal structures of the oxidized (3.07 Å) and reduced (3.24 Å) desulfo *D. gigas* enzyme (PDB entries 1SIJ and 3L4P, respectively).[4a,b] In addition, the Mo−As distance of 3.19 Å seen here for reduced xanthine oxidoreductase is similar to that seen previously by XAS (3.00 Å)[3e] and too long for a direct Mo−As bond (Table S2). The Mo−S−As angle of 100° in reduced xanthine oxidoreductase is somewhat larger than that seen by XAS (80°).[3e] However, the corresponding Mo−S−As angle with oxidized xanthine oxidoreductase is 86°.

The current structures of arsenite-complexed xanthine oxidoreductase (with functional sulfurated enzyme) contrast with that seen previously for the desulfo form of the *D. gigas* enzyme in clearly demonstrating a double bridge between molybdenum and arsenic that involves μ-sulfido bonding in addition to μ-oxo bonding (Figure 1). In the structure of the complex with oxidized xanthine oxidoreductase, arsenic is four-coordinate with a distorted trigonal-pyramidal geometry in which the bridging S and two nonbridging O atoms form the base plane and the bridging O

to molybdenum occupies the apical position. Its bonding suggests that arsenite was oxidized to arsenate in the synchrotron beam. (Xanthine oxidase is not known to possess arsenite oxidase activity, and extensive efforts on our part were unable to identify any here.) Chamberlain[7] has previously reported that certain elements of inorganic compounds are subject to reduction or oxidation by X-ray radiation on the basis of different stabilities of alternative electron configurations where, for exmple, the reduction of iodic acid and potassium permanganate and the oxidation of sulfide and sulfite to sulfurous acid were found. The bridging oxygen occupies a position in the molybdenum coordination sphere equivalent to that of the catalytically labile oxygen of the native enzyme.[1] In the complex with reduced enzyme, arsenic is three-coordinate with a distorted trigonal-planar geometry consisting of the bridging S and O atoms and one nonbridging O (although the nonbridging O shows strong electron density in only one active site of the dimer, with considerably weaker density in the other). In the strongly reducing conditions under which the sample was prepared, the arsenic appears to have remained in the As(III) state. In the AOR structure, arsenic is bound only to three oxygens (one bridging and two nonbridging) in a distorted trigonal-planar geometry (Figure 2). In the oxidized xanthine oxidase structure, the Mo−O−S−As unit is orthogonal to the trigonal base plane of the arsenic coordination sphere and essentially planar, with a butterfly angle of ∼44° relative to the dithiolene−Mo plane; similar butterfly angles are seen in the complex with reduced enzyme.

Figure 2 shows a superposition of the active site of the current structure for the arsenite complex of oxidized xanthine oxidase and that of *D. gigas* AOR.[4a] The alignment was performed using COOT, maximizing the overlap of the corresponding enedithiolate ligands and the molybdenum atoms. The arsenic atom occupies similar positions in the two structures, with a root-mean-square deviation of ∼0.6 Å. In the following discussion, the amino acid residue numbers for the bovine enzyme are given first, followed by those for *D. gigas* enzyme. Glu1261 and Glu869, which are universally conserved in the xanthine oxidase family, occupy almost precisely the same position in the two structures and are within hydrogen-bonding distance of one of the two nonbridging oxygen atoms of the bound arsenite (2.52 and 2.71 Å for xanthine oxidase and AOR, respectively). Glu802 (which corresponds to the nonconserved Phe425 in the *D. gigas* protein) is within hydrogen-bonding distance (2.52 Å) of the other nonbridging oxygen of arsenite; Phe425 itself lies significantly further away at 3.76 Å.

Phe1009 and Tyr622 occupy similar but not identical positions. The phenolic oxygen of Tyr622 is 3.52 Å from one of the nonbridging oxygens of arsenite, but Phe1009 is 5.37 Å away from the corresponding oxygen of arsenite. Still, the key difference is the bond between arsenic and the cyanolyzable sulfur in the molybdenum coordination sphere in the xanthine oxidoreductase structure, which is absent in the structure of the *D. gigas* enzyme because of the desulfo nature of the latter. The sulfur content of the bridging sulfido position of the molybdenum center of xanthine oxidase used for the current crystal structures was in excess of 80% based on the percentage functionality determined by standard enzymatic assays of the activity to flavin ratio for xanthine oxidase (as described in the SI). An examination of the electron densities around the bridging sulfide and oxo groups at different σ levels in the reduced complex clearly indicated that the bridging sulfido group shows an electron density comparable to those of the two sulfur ligands from the enediolate moiety of the pterin cofactor whose identities were known, while the density for the bridging oxo group was much weaker, indicating a less electron-rich ligand (Figure S1 in the SI).

In summary, the current crystal structures of arsenite-inhibited xanthine oxidase in the oxidized and reduced states clearly demonstrate a double bridge between Mo and As that involves the catalytically essential sulfur of this enzyme, consistent with earlier XAS work.[3e] This bidentate binding appears to be essential for the high affinity of reduced xanthine oxidoreductase for arsenite.[3a,b] Our structure provides a structural basis for understanding enzyme inhibition by arsenite, which simultaneously blocks the equatorial Mo−OH ligand that initiates nucleophilic attack on the substrate at the outset of catalysis and the Mo=S moiety that serves as a hydride acceptor during the course of the reaction.[1,5c]

■ ASSOCIATED CONTENT

Supporting Information. Details of crystallization and structure refinement. This material is available free of charge via the Internet at http://pubs.acs.org.

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