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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Materials and Methods

Xanthine oxidase was purified from fresh unpasteurized bovine milk from an individual cow (Scott Brothers Dairy, Chino, CA) to minimize heterogeneity according to published procedures.[5a] As–islolated enzyme was typically ~50% percent functional with an AFR = 100 (activity to flavin ratio, a value of 210 for fully functional enzyme. Edmondson, D.; Massey, V.; Palmer, G.; Beacham III, L. M.; Elion, G. B. *J. Biol. Chem.* 1972, **247**, 1597–1604), reflecting the presence of inactive desulfo enzyme. An extra step using the folate column protocol[5b] developed by Nishino separated the functional enzyme from nonfunctional forms and enabling recovery of enzyme with 80% functionality (AFR = 170). PEG 200 and PEG 8000 (polyethylene glycol 200 and 8000) solutions were purchased from Hampton Research (U.S.A.). All other chemicals and reagents were obtained at the highest quality/purity available from Sigma-Aldrich (U.S.A.) or Fisher Scientific (U.S.A.), and were used without further purification.

Crystallization conditions were as previously published.[5c] Crystals were grown using microbridges to hold batch solutions in sealed wells of a 24–well tray.[5c] The batch solutions contained a 10 μl of 34.5 μM (5 mg/ml) enzyme solution mixed with 5 or 6 μl of 12% polyethylene glycol 8000 precipitant solution. The final conditions for optimal growth were a precipitant solution of 12% polyethylene glycol 8000, 0.1 M potassium phosphate at initial pH 6.5, and 0.2 mM EDTA. The enzyme solution contained 5mg/ml xanthine oxidase in 40 mM Tris–HCl, initial pH 7.8, 20 mM pyrophosphate, initial pH 8.5, 0.2 mM EDTA, and 5 mM dithiothreitol. The enzyme solution was allowed to sit on ice for 1h following the addition of dithiothreitol, prior to beginning the construction of well solutions. The final pH of the well solution was around 7.2. Crystals were grown at 25°C in the dark for 2 days.

After growth, crystals were exchanged into cryoprotectant solution containing 42% polyethylene glycol 200. In order to obtain arsenite–inhibited enzyme in the oxidized form, the crystals were then soaked with 10 mM sodium arsenite dissolved in the cryoprotectant solution for 5~8 minutes before being flash frozen in liquid nitrogen. For the arsenite–inhibited enzyme in the reduced form, the crystals were exchanged into anaerobic cryoprotectant solution containing 42% polyethylene glycol 200 which was flushed with argon gas for 20 min in advance. Then the enzyme crystals were reduced by adding enough anaerobic solution of sodium dithionite of ~ 50 mM. To ensure the reduced condition of the system, 50 μM methyl viologen was introduced as an indicator the light blue color of which indicated anaerobic condition, although anaerobic chamber was not used. The crystals under reduced conditions were then soaked with 10 mM sodium arsenite in the cryoprotectant solution for 5~8 minutes before being flash frozen in liquid nitrogen A single dataset of X–ray diffraction at 1.82 Å or 2.11 Å resolution was collected for each of the arsenite–inhibited enzyme of either the oxidized form or the reduced form using LRL–CAT beamline of synchrotron at Argonne National Laboratory.

The initial protein structure was solved by MOLREP program of the CCP4 package using the reported structure of xanthine oxidase solved by Enroth *et al.*[1b] (PDB (Protein Data Bank) code: 1FIQ with corrected assignment of equatorial sulfido and apical oxo ligands to the molybdenum coordination sphere) as the searching model and then further improved by running rigid–body and restrained refinement with REFMAC program.[6a,b] For the oxidized enzyme complex, amino acid residues 195–223 in the connecting region between the iron–sulfur domain and the FAD domain and 1316–1325 at the C–terminus were absent in the original searching model but were added to one monomer of the model during refinement based on the clear positive electron density in the $F_o - F_c$ omit map and the reported amino acid sequence of the enzyme. None of these newly introduced residues is near the molybdenum active site but rather at the surface of the protein as we observed in several previous crystal structures. In the oxidized complex, strong positive electron density for arsenite at the molybdenum center was observed in the $F_o - F_c$ omit map and arsenite (AsO_2^-) was built into the structure based on the electron density before the final round of refinement. For the reduced form of the enzyme complex, non-crystallographic symmetry constraints were set as "tight" between the corresponding peptide chains from each monomers in the asymmetric unit during refinement because this reduced the difference between R_{cryst} and R_{free} values. In the reduced complex, strong positive electron density for the arsenic atom and a non-bridging oxygen atom at one of the two molybdenum centers was observed in the $F_o - F_c$ omit map, and the arsenic and oxygen atoms

were built into the structure based on the electron density. In the other active site of the reduced complex, observed electron density was strong for the arsenic atom and much weaker for a potentially non-bridging oxygen atom both of which were built into the structure accordingly. Water molecules were subsequently added with COOT program.[6d] The refinement statistics are summarized in Table S1.

Table S1. **Statistics for Data Collection and Refinement of the Crystal Structures of Xanthine Oxidase in Complex with Arsenite in the Oxidized and Reduced Complexes**

Statistic	Oxidized XO with Arsenite	Reduced XO with Arsenite
Protein Data Bank code	3NVV	3SR6
Spacegroup	P21	P21
Cell dimensions		
a, b, c (Å)	132.8, 73.4, 138.2	133.1, 73.2, 138.0
α, β, γ (°)	90.0, 96.9, 90.0	90.0, 96.8, 90.0
Resolution (Å)	131.8 – 1.82	22.1 – 2.10
Wavelength (Å)	0.9793	0.9793
Unique Reflections (test set)	228,736 (11464)	147,820 (7451)
Completeness % (highest resolution shell, Å)	97.2 (94.6)	95.8 (88.8)
I/σ (highest resolution shell)	16.9 (2.3)	7.6 (2.4)
R_{cryst} (highest resolution shell)	19.2 (25.2)	22.5 (30.1)
R_{free} (highest resolution shell)	22.9 (29.6)	27.2 (33.8)
Ramachandran Statistics (%)	90.0, 9.6, 0.3, 0.2	88.6, 10.5, 0.4, 0.5
Mean coordinate error based on free *R* value (Å)	0.132	0.221
Mean coordinate error based on maximum likelihood (Å)	0.094	0.197
Rmsd bond length (Å)	0.012	0.014
Rmsd bond angles (°)	1.4	1.5
Average B value (Å^2)	24.6	25.5
Number of non-hydrogen atoms in refinement	20671	19834
Number of waters	1374	842

* Ramachandran statistics indicate the percentage of residues in the most favored, additionally allowed, generously allowed, and disallowed regions of the Ramachandran diagram as defined by the program PROCHECK (Laskowski, R. A.; MacArthur, M. W.; Moss, D. S.; Thornton, J. M. *J. Appl. Crystallogr.* **1993***, 26,* 283–291.). I/σ is defined as the ratio of averaged value of the intensity to its standard deviation.

Table S2. **Bond Distances of Mo Centers of Arsenite-Inhibited and Control Xanthine Oxidase and Desulfo–Aldehyde Oxidoreductase**

	Mo–As[1]	As–S/As–O[1] (/desulfo)	Mo=S/Mo–S/Mo=O (oxidized/reduced/desulfo)	Mo–S (enedithiolate)	Mo=O (apical)	Mo–O (equatorial)	As–O (bridging)	As–O (non-bridging)
Enzyme	Å	Å	Å	Å	Å	Å	Å	Å
XO +AsO_2^- (oxidized)[2]	3.18, 3.21	2.50, 2.57	2.11, 2.12	2.36, 2.41, 2.35, 2.32	1.73, 1.72	1.99, 2.00	1.94, 1.97	1.96, 1.78, 1.78, 1.96
XO +AsO_2^- (reduced)[2]	3.30, 3.07	1.77, 2.39	2.16, 2.11	2.33, 2.38, 2.36, 2.37	1.66, 1.66	2.03, 2.06	2.19, 1.93	1.78, 1.78
XO +AsO_2^- (oxidized)[3]	n.d.	n.d.	2.15	2.43	1.63	n.d.	1.78	1.78
XO +AsO_2^- + 8BrX (reduced)[3]	3.00	2.27	2.40	2.40	1.66	n.d.	1.78 or n.d.[6]	1.78 or n.d.[6]
XO (oxidized)[3]	–	–	2.15	2.44	1.67	n.d.	–	–
AOR+AsO_2^- (oxidized)[4]	3.33	3.07	1.70	2.32, 2.33	1.70	1.94	1.69	1.70, 1.70
AOR+AsO_2^- (reduced)[5]	3.25	3.24	1.80	2.37, 2.40	1.90	1.97	1.84	1.85, 1.85

[1] Distance of nonbonding Mo–As for both enzymes. Distance of either bonding As–S for the functional xanthine oxidase or nonbonding As–O for the desulfo-aldehyde oxidoreductase.

[2] From current X-ray crystal structures of oxidized and reduced forms of functional bovine xanthine oxidase in complex with arsenite. For both the oxidized form (PDB code: 3NVV. Resolution: 1.82 Å) and the reduced form (PDB code: 3RCA. Resolution 2.1 Å), parameters are shown (above and below) for both active sites in the asymmetric unit of crystal.

[3] From Ref. 3e. EXAFS data. 8-bromoxanthine (8BrX) is a nonreactive product analog of uric acid.

[4] From Ref. 4a. X-ray crystal structure of oxidized *Desulfovibrio. gigas* aldehyde oxidoreductase in complex with arsenite. PDB code: 1SIJ. Resoultion: 2.3 Å.

[5] From Ref. 4b. X-ray crystal structure of reduced *Desulfovibrio. gigas* aldehyde oxidoreductase in complex with arsenite. PDB code: 3L4P. Resoultion: 1.45 Å.

[6] One weak As–O interaction was observed, either with a bridging or non-bridging oxygen.

n.d., not determined.

–, not present.

Abbreviations being used: XO, xanthine oxidase; AOR, aldehyde oxidoreductase (desulfo–form); AST, arsenite; 8BrX, 8–bromoxanthine; EXAFS, extended x-ray absorption fine structure; PDB, Protein Data Bank.

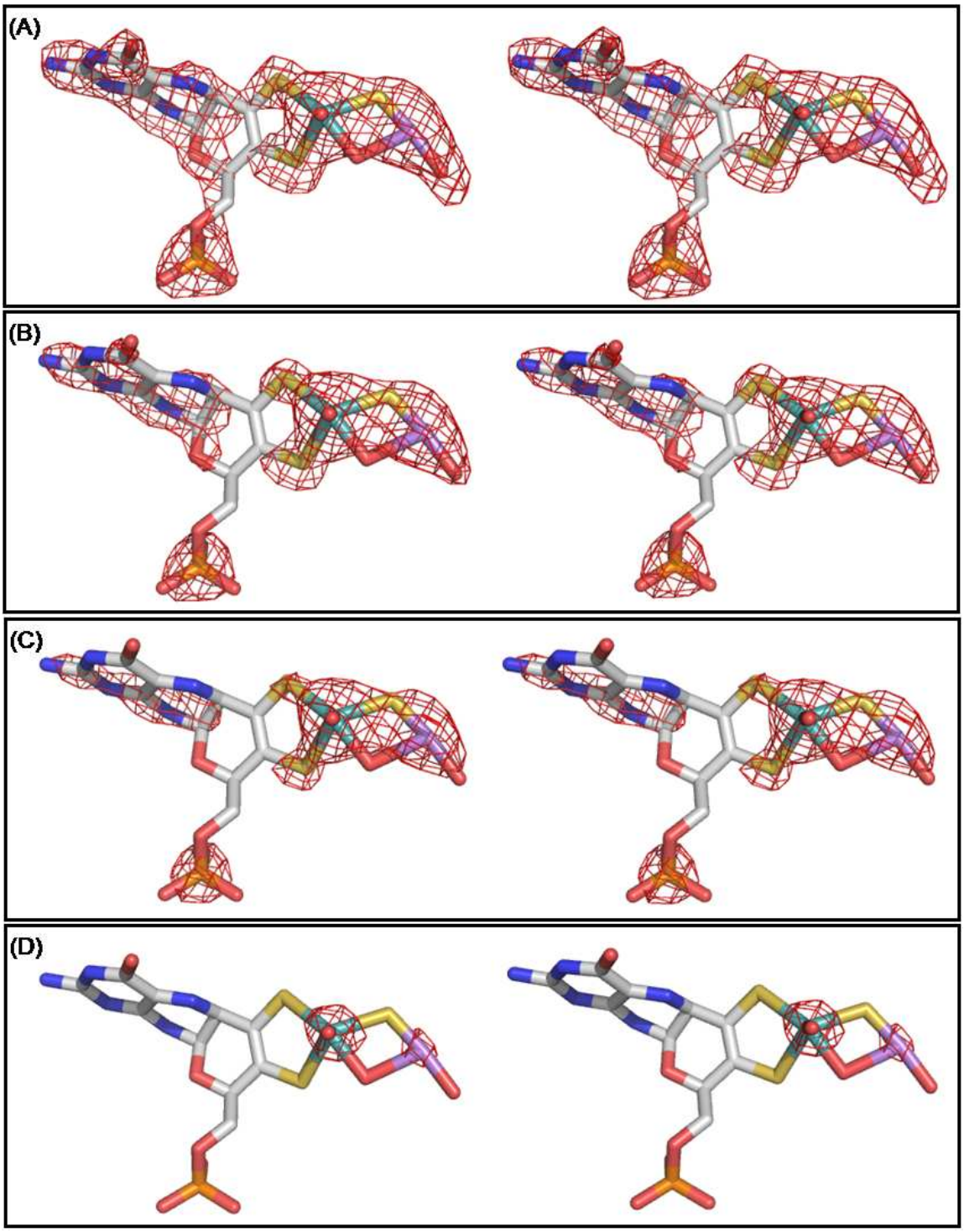

Figure S1. Stereo images of the active sites of reduced xanthine oxidase complexed with arsenite. All the electron density maps were F_o - F_c omit maps calculated before the introduction of the arsenite-inhibited molybdenum cofactor for the refinement of the model and contoured at 3.0 σ, 4.0 σ, 5.0 σ , and 10.0 σ in (A), (B), (C), (D), respectively, 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 with PyMOL (Ref. 6c).