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The Active Site of Arsenite Oxidase from *Alcaligenes faecalis*

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Arsenic may have a role in mammalian metabolism,[1] but it is best known for its toxicity,[2] even being exploited as a chemical warfare agent in the 20th century.[3,4] The two environmentally common water-soluble forms of arsenic are the trivalent arsenite and the pentavalent arsenate. At neutral pH arsenite is predominantly $As(OH)_3$ (often erroneously written as AsO_2^-), and arsenate is a mixture of $As(OH)_2O_2^-$ and $As(OH)O_3^{2-}$. While both arsenite and arsenate are poisonous, the former is more toxic due to its affinity for thiols, and in particular the lipoic acid of pyruvate dehydrogenase.[3] The lesser toxicity of arsenate arises primarily from its activity as a phosphate analogue. Organisms respond to arsenicals either by lowering the intracellular concentration through transport mechanisms,[5] or by chemical modification to a less toxic form.[6] The soil pseudomonad *Alcaligenes faecalis* uses this second approach, producing an arsenite-inducible periplasmic enzyme that catalyzes the two-electron oxidation of arsenite to arsenate, utilizing either azurin or a *c*-type cytochrome as an electron acceptor:[7]

$$As(OH)_3 + H_2O \rightarrow As(OH)_2O_2^- + 3\,H^+ + 2\,e^-$$

This arsenite oxidase is a member of the DMSO-reductase class of molybdenum enzymes,[8] and its crystal structure has recently been reported.[9] It consists of two subunits, the larger being similar to other members of the DMSO reductase family and containing the molybdenum site at which the oxidation of arsenite is thought to occur. Similar to other members of the DMSO reductase family the Mo coordinates two equivalents of the pterin cofactor (guanosine dinucleotide form) that is common to all molybdenum- and tungsten-containing enzymes, other than nitrogenase.[9] In the crystal structure of arsenite oxidase the molybdenum was five-coordinate, attributed to photoreduction[10] of the enzyme by the intense X-ray beam used for crystallography.[9] We present herein a study of the molybdenum site of *A. faecalis* arsenite oxidase[11] in both the oxidized and reduced forms, using X-ray absorption[12] and resonance Raman[13] spectroscopies.

The EXAFS data and curve-fitting analysis for both oxidized Mo(VI) and reduced Mo(IV) enzyme are shown in Figure 1. We find approximately four Mo−S interactions at 2.47 and 2.37 Å[14] for oxidized and reduced enzyme, respectively, confirming bis-enedithiolate coordination.[9] The arsenite-reduced enzyme possesses a single Mo═O ligand at 1.70 Å, consistent with the five-coordinate square-pyramidal coordination seen crystallographically. The oxidized enzyme additionally possesses a second Mo−O at 1.83 Å.

The difference in bond-lengths between the oxo at 1.70 Å and this second Mo−O is close to the limit of resolution of our data,[15]

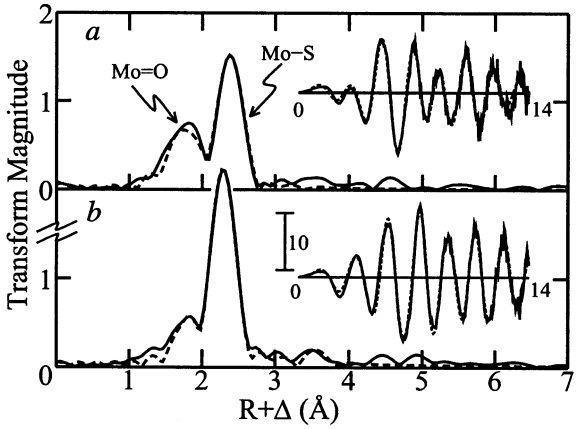

Figure 1. Fourier transforms and raw data of arsenite oxidase Mo K-edge EXAFS spectra. Both data (solid lines) and best fits (broken lines) are shown for oxidized (a) and arsenite reduced (b) enzyme. The Fourier transforms have been phase-corrected for Mo−S backscattering. The small peak at $R+\Delta \approx 1.7$ Å in the Fourier transform of the oxidized sample arises from Mo═O EXAFS, while the larger peak at $R+\Delta \approx 2.3$−2.4 Å arises from Mo−S and, to a lesser extent, Mo−O backscattering.

but if the fit is restricted to a single molybdenum−oxygen bond length, a poorer fit results with two oxygens at 1.78 Å (see Supporting Information). Since this bond length is chemically unlikely, and because the resonance Raman (see below) does not indicate a symmetric molybdenum−dioxo site, we prefer the interpretation of one Mo═O at 1.70 Å, plus one short Mo−O at 1.83 Å (a more usual Mo−O bond-length would be about 2.0 Å). The closest relevant match to the longer bond in the Cambridge Structural Data Base[16] is a Mo(V)−OH species at 1.84 Å,[17] and on this basis the 1.83 Å Mo−O interaction could be formulated as Mo−OH. However, a number of database entries with molybdenum−oxygen distances in the range 1.82−1.84 Å are formulated as dioxo Mo(VI) species, and it is possible that such an asymmetric dioxo arrangement might exist in arsenite oxidase. Were this the case, the longer 1.83 Å molybdenum−oxygen ligand would be an obvious candidate for oxygen atom transfer to arsenite, with the Mo═O acting as a spectator oxo group.[18]

Resonance Raman spectra were recorded for enzyme as isolated, redox-cycled in $H_2{}^{16}O$ and in $H_2{}^{18}O$, and arsenite-reduced. Representative spectra are shown in Figure 2.[19] Two vibrational modes dominate the high-frequency spectrum of oxidized enzyme at 1525 and 1598 cm^{-1}. By analogy to DMSO reductase,[20] where similar modes are attributed to C═C stretching in distinct P- and Q-pterins having different π-delocalization,[20] the presence of two vibrational modes provides strong evidence that molybdenum of arsenite oxidase is likewise coordinated by inequivalent pterins. The

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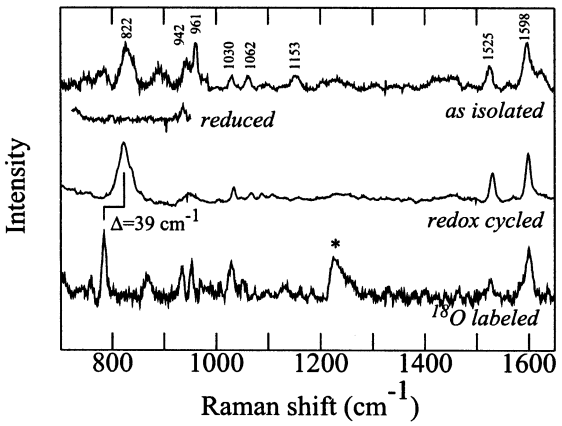

Figure 2. Resonance Raman spectra of as isolated (top spectrum), redox-cycled (middle spectrum), ^{18}O-labeled (bottom spectrum), and reduced (inset) arsenite oxidase with 647 nm excitation. The spectra were obtained with samples (~3 mM in arsenite oxidase) held at 30 K, using 80−100 mW incident laser power. The vibrational mode of residual oxidant (DCPIP) in the redox-cycled sample is marked by *.

frequency of the C═C stretch of the π-delocalized P-pterin of arsenite oxidase is very similar to that of DMSO reductase, whereas that for the Q-pterin (dithiolate-type) C═C stretch is approximately 25 cm^{-1} higher, suggesting greater double bond character in the C═C bond of the Q-pterin of arsenite oxidase.

A dominant mode in the mid-frequency region of the resonance Raman spectrum of oxidized arsenite oxidase, at 822 cm^{-1}, shifts to lower frequency by 38 cm^{-1} upon catalytic turnover of the enzyme in $H_2{}^{18}O$. This strongly suggests that the band arises from a Mo═O stretch in oxidized enzyme, comparable to that seen in DMSO reductase at 862 cm^{-1}.[20] The lower Mo═O stretch frequency in arsenite oxidase indicates that the Mo═O bond is somewhat weaker. Badger's rule indicates that this is consistent with an elongation of the Mo═O bond of ~0.02 Å, which is in reasonable accord with the EXAFS-derived bond-lengths of 1.72 and 1.68 Å for arsenite oxidase and DMSO reductase,[21] respectively. The 822 cm^{-1} band is not observed in arsenite-reduced enzyme. Since the vibrational energies of the Fe−S cluster modes overlap those arising from Mo−S vibrational modes in arsenite oxidase, it is difficult to address redox-dependent changes in the lower-frequency region of the resonance Raman spectra without more rigorous labeling studies.

The present studies indicate that arsenite oxidase from *Alcaligenes faecalis* possesses inequivalent P- and Q-pterins coordinated to the metal as is typical of this family of molybdenum enzymes. The enzyme is distinct from related enzymes, however, in lacking a metal ligand contributed by the polypeptide (a serine, cysteine, or selenocysteine). The overall coordination in fact more closely resembles tungsten-containing enzymes, which also possess two equivalents of the pterin cofactor, no polypeptide ligand, and dioxo coordination in the oxidized enzyme.[22] Our results indicate that, like the tungsten enzymes, the active site of oxidized arsenite oxidase is best formulated as $L_2MO(O)$, with L representing the bidentate enedithiolate of the pterin cofactor and the final ligand either a distended Mo═O or Mo−OH.

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Supporting Information Available: Tables of EXAFS curve-fitting results, Mo K near-edge spectra and UV-visible spectra (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(11) *Alcaligenes faecalis* strain NCIB 8687 was grown and arsenite oxidase purified as previously described.[7] Samples were prepared in 50 mM Tricine, pH 7.4. Fully oxidized enzyme was prepared by air-oxidation, and reduced enzyme, by reduction with 5 mM (final) buffered sodium arsenite solution. Exchange into $H_2{}^{18}O$ (Cambridge Isotope Laboratories, 95−97 atom % ^{18}O) was accomplished by three cycles of 10-fold dilution and reconcentration.
(12) X-ray absorption spectroscopy measurements were carried out at the Stanford Synchrotron Radiation Laboratory (SSRL) as previously described [George, G. N.; Garrett, R. M.; Prince, R. C.; Rajagopalan, K. V. *J. Am. Chem. Soc.* **1996**, *118*, 8588−8592]. The extended X-ray absorption fine structure (EXAFS) oscillations $\chi(k)$ were quantitatively analyzed using EXAFSPAK [http://ssrl.slac.stanford.edu/exafspak.html].
(13) Resonance Raman spectra were recorded as previously described [Mansy, S. S.; Xiong, Y.; Hemann, C.; Hille, R.; Sundaralingam, M.; Cowan, J. A. *Biochemistry* **2002**, *41*, 1195−1201]. Each sample was illuminated for several hours prior to data collection to reduce background fluorescence.
(14) The accuracies of EXAFS-derived bond lengths are expected to be ±0.02 Å.
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