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Electron transfer through arsenite oxidase: Insights into Rieske interaction with cytochrome *c*

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ABSTRACT

Arsenic is a widely distributed environmental toxin whose presence in drinking water poses a threat to > 140 million people worldwide. The respiratory enzyme arsenite oxidase from various bacteria catalyses the oxidation of arsenite to arsenate and is being developed as a biosensor for arsenite. The arsenite oxidase from *Rhizobium* sp. str. NT-26 (a member of the Alphaproteobacteria) is a heterotetramer consisting of a large catalytic subunit (AioA), which contains a molybdenum centre and a 3Fe-4S cluster, and a small subunit (AioB) containing a Rieske 2Fe-2S cluster. Stopped-flow spectroscopy and isothermal titration calorimetry (ITC) have been used to better understand electron transfer through the redox-active centres of the enzyme, which is essential for biosensor development. Results show that oxidation of arsenite at the active site is extremely fast with a rate of > 4000 s^{-1} and reduction of the electron acceptor is rate-limiting. An AioB-F108A mutation results in increased activity with the artificial electron acceptor DCPIP and decreased activity with cytochrome *c*, which in the latter as demonstrated by ITC is not due to an effect on the protein-protein interaction but instead to an effect on electron transfer. These results provide further support that the AioB F108 is important in electron transfer between the Rieske subunit and cytochrome *c* and its absence in the arsenite oxidases from the Betaproteobacteria may explain the inability of these enzymes to use this electron acceptor.

1. Introduction

Arsenic, in the inorganic forms arsenite (+ III) and arsenate (+ V), is toxic to most organisms [1]. The reduction potential of the arsenite/arsenate couple (+ 60 mV) [1] is such that certain phylogenetically diverse bacteria can either use arsenite as an electron donor or arsenate as a terminal electron acceptor for growth [2]. Aerobic arsenite oxidation is catalysed by arsenite oxidase, Aio, which couples the oxidation of arsenite to the reduction of oxygen to water generating ATP and in some cases NADH for carbon dioxide fixation [3]. The physiological electron acceptor for Aio has been shown to be *c*-type cytochromes or azurin [4–7].

Aio is a member of the dimethyl sulfoxide reductase (DMSOR) superfamily of molybdoenzymes, which all contain two equivalents of an organic pyranopterin cofactor coordinated to the molybdenum, usually present as the dinucleotide of guanine and termed MGD (for molybdopterin guanine dinucleotide). Aio is unique among members of the DMSOR superfamily, however, in that the molybdenum is not coordinated to the protein by an amino acid side chain [8]. The molybdenum centre of Aio also exhibits highly cooperative two electron transfer, with the intermediate Mo(V) oxidation state not typically observed upon reduction of Mo(VI) to Mo(IV) [9] until recently in a mutant with altered hydrogen bonding to the MGD [10]. Aio also contains a high potential 3Fe-4S cluster in the large catalytic subunit, AioA, rather than the more common 4Fe-4S cluster, and a Rieske centre in the small subunit, AioB, homologous to the Rieske protein in the bc_1 and b_6f complexes [11,12]. Aio is the only molybdoenzyme to contain a Rieske 2Fe-2S cluster (in which one of the Fe atoms is complexed by two histidine residues instead of two cysteines) [13]. An overview of the redox-active centres in Aio catalysis is shown in Fig. 1. It has been suggested that the electrons from arsenite oxidation pass to the molybdenum centre, to the 3Fe-4S cluster, the Rieske cluster and finally to an electron acceptor [8,13].

Heterologous expression of the NT-26 Aio in *Escherichia coli* has facilitated a more detailed study of the mechanisms of electron transfer [7,8,10,14] and its development as a biosensor for arsenite [15]. In this

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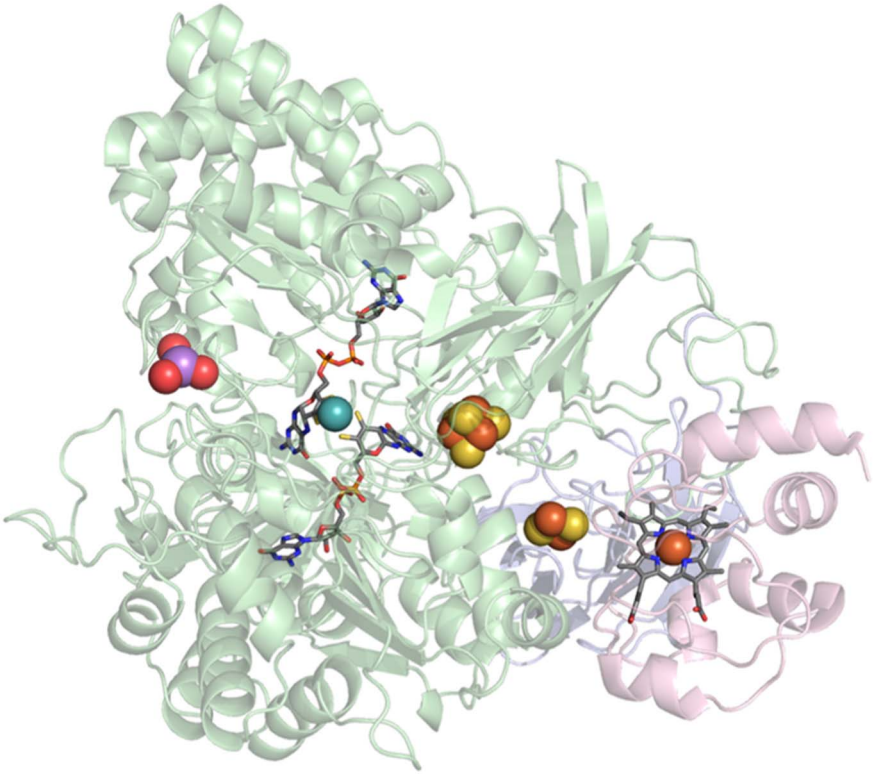

Fig. 1. Structure of Aio (AioA, green and AioB, blue) with arsenite (purple) and cytochrome *c* (pink). The molybdenum centre is shown as a blue ball (Mo) and sticks (MGD). The [Fe-S] clusters are shown in yellow and orange. PDB ID: 4AAY (NT-26 Aio) and 1HRC (horse-heart cytochrome *c*).

study, we use a combination of steady-state and stopped-flow kinetics as well as isothermal titration calorimetry (ITC) to determine the rate-limiting step of catalysis. Crystallization and structure determination of the AioB F108A mutant provides evidence of protein integrity and details of the atomic model of the enzyme. Understanding electron transfer through the enzyme is critical to further biosensor development.

2. Experimental procedures

2.1. Heterologous Aio expression, purification and site-directed mutagenesis

The NT-26 *aioBA*-pPROEX-HTb + construct and *E. coli* strain DH5α were used for the aerobic expression of Aio where the final culture volume was 2 L in a 5 L flask, induction with isopropyl β-D-1-thiogalactopyranoside was at 21 °C for 24 h and the enzyme purified as described previously [8]. Site-directed mutagenesis to obtain the AioB-F108A mutant was done as described previously [8] with the following primers, AioBF108A forward 5′ GTCCTCACAAGGGTGCTCCTCTGAGCTACTCCGC 3′ and AioBF108A reverse 5′ GCGGAGTAGCTCAGAGGAGCACCCTTGTGAGGAC 3′.

2.2. Enzyme assays

Aio assays measuring steady-state kinetic parameters for arsenite were performed as described previously using dichlorophenolindophenol (DCPIP) [6] (the ε for DCPIP at pH 5.5 used was 8.2 mM^{-1} cm^{-1} [16] instead of 23 mM^{-1} cm^{-1} [6]) and horse heart cytochrome *c* (Sigma-Aldrich) [14] as electron acceptors. The steady-state kinetic parameters of cytochrome *c* were determined using an excess of arsenite (2.5 mM) and followed the reduction of cytochrome *c* at 416 nm ($\Delta\varepsilon$ = 57.5 mM^{-1} cm^{-1}; based on UV–visible spectra in 50 mM Tris-HCl (pH 8) using ε_{550} = 8.4 and 29.5 mM^{-1} cm^{-1} for oxidised and reduced cytochrome *c* to determine the concentration [17]). Kinetics experiments were performed on three separate occasions with three separate enzyme preparations. NaCl was not added to any buffers as 100 mM reduced the activity of Aio with cytochrome *c* to 35% (data not shown). Using salt is also not physiologically relevant as the Aio is a periplasmic enzyme which means that the pH and salt concentration would be in equilibrium with the environment and NT-26 was isolated from a low-salt environment [18].

2.3. Stopped-flow UV–visible spectroscopy

All experiments were conducted in 50 mM Tris-HCl (pH 8) at 5 °C using a SX-20 stopped-flow spectrophotometer (Applied Photophysics, Inc.) equipped with photodiode array and photomultiplier tube detection and running ProData SX 2.2.5.6 acquisition software. A volume of 1.2 mL of 30 μM Aio was placed in a glass tonometer and made anaerobic by stirring in an O_2 scrubbed Argon rich environment for 1 h. Aio was reacted with 500 μM arsenite in a stopped-flow apparatus to follow the reductive half-reaction. Reduction of the molybdenum centre and [Fe-S] reduction was followed at 680 nm and 450 nm, respectively. To observe the interaction with cytochrome *c*, 30 μM Aio was mixed with 60 μM cytochrome *c* and 30 μM arsenite to catalyse a single turnover. The rate of cytochrome *c* reduction was followed at 551 nm. Four to six replicates were performed with one enzyme preparation. Rate constants thus determined are presented at 25 °C based on the Arrhenius equation [19].

2.4. Isothermal titration calorimetry

ITC was carried out using a MicroCal200 ITC and analysed with the accompanying software (GE Healthcare). A volume of 350 μL of 75 μM reduced Aio was loaded into the cell in 50 mM Tris-HCl (pH 8) and 40 μL of 750 μM reduced cytochrome *c* in 50 mM Tris-HCl (pH 8) was loaded into the injection syringe. The cytochrome *c* was injected into the enzyme solution in 1.5 or 2 μL volumes with 1000 rpm stirring at 25 °C. All proteins were reduced prior to the experiment by the addition of excess dithionite which was removed by gel filtration chromatography using either a Superdex 200 (for Aio) or 75 (for cytochrome *c*) gel filtration column (GE Healthcare). ITC experiments were performed