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Heterologously expressed arsenite oxidase: A system to study biogenesis and structure/function relationships of the enzyme family

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ABSTRACT

Studies of native arsenite oxidases from *Ralstonia* sp. S22 and *Rhizobium* sp. NT-26 raised two major questions. The first one concerns the mode of the enzyme's membrane-association. It has been suggested that a hypothetical not conserved protein could account for this variable association. Expression of the wild type arsenite oxidase in *Escherichia coli* allowed us to study the cellular localization of this enzyme in the absence of such a hypothetical partner. The results with the *Ralstonia* sp. S22 enzyme suggest that no additional protein is required for membrane association. The second question addresses the influence of the disulfide bridge in the small Rieske subunit, conspicuously absent in the *Rhizobium* sp. NT-26 enzyme, on the properties of the [2Fe–2S] center. The disulfide bridge is considered to be formed only after translocation of the enzyme to the periplasm. To address this question we thus first expressed the enzyme in the absence of its Twin-arginine translocation signal sequence. The spectral and redox properties of the cytoplasmic enzyme are unchanged compared to the periplasmic one. We finally studied a disulfide bridge mutant, Cys106Ala, devoid of the first Cys involved in the disulfide bridge formation. This mutation, proposed to have a strong effect on redox and catalytic properties of the Rieske protein in Rieske/cyt*b* complexes, had no significant effect on properties of the Rieske protein from arsenite oxidase. Our present results demonstrate that the effects attributed to the disulfide bridge in the Rieske/cyt*b* complexes are likely to be secondary effects due to conformational changes.

1. Introduction

The *aioA* and *aioB* genes (formerly named *aro*, *aso* or *aox* [1]) coding for the large catalytic molybdopterin and the small Rieske subunits of the enzyme arsenite (As^{III}) oxidase homologues have been identified in species from almost all phyla of the Bacteria and also in a few Archaea (for a recent inventory see [2,3]). In all characterized strains, the enzyme, AioAB, encoded by *aioBA* has been shown to be responsible for the chemolithoautotrophic (when CO_2 is the sole carbon source required) or heterotrophic (when organic matter is needed for growth) oxidation of As^{III} (for recent reviews see [4,5]). The oxidation of As^{III} is always bioenergetic but not always provides enough energy for growth. The oxidation of As^{III} therefore seems to serve bioenergetic purposes in addition to its detoxifying role. Phylogenetic analyses of both AioB and AioA even suggest that this bioenergetic process already existed in the Archaean prior to the divergence of Bacteria and Archaea [2,3,6]. Whereas the presence of *aioBA* genes was detected in a *Sulfolobus* genome and As^{III} oxidation was proposed for the Archaeon *Sulfolobus acidocaldarius* strain BC [7], only bacterial representatives of the enzyme have so far been isolated from parent species [8–12]. A three-dimensional structure of AioAB has been solved [13]. The structure of the large AioA subunit, containing a molybdopterin cofactor together with a [3Fe–4S] cluster, identifies this subunit as a member of the Complex Iron–Sulfur Molybdoenzyme superfamily of molybdopterin guanine dinucleotide containing enzymes. A total of four domains make up the large subunit of Aio (about 825 residues). Domain I binds the [3Fe–4S] cluster, whereas domains II and III, related to each other by a pseudo two-fold axis of symmetry, both possess homologous dinucleotide-binding folds. Structural similarities between the small AioB subunit (around 170 residues), harboring a [2Fe–2S] cluster, and PetA, the Rieske-subunit of cytochrome *bc* complex (or Rieske/cyt*b* complex), show that this protein is a member of the Rieske protein superfamily. AioB and PetA indeed are so closely related in terms of sequence (a common Cys-X-His-X_n-Cys-X_2-His sequence motif), structure, redox and spectral properties that the conspicuous absence of the canonical disulfide bridge in several AioB proteins was surprising [14]. This disulfide bridge has indeed long been considered to be essential for Rieske cluster catalytic, redox and spectral properties [15–17]. Native AioABs

Abbreviations: As^{III}, Arsenite; AioAB, arsenite oxidase; SDS-PAGE, Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis; FPLC, Fast protein liquid chromatography; PCR, Polymerase Chain Reaction; Tat, Twin-arginine translocation

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show similar spectral and redox properties of the [2Fe–2S] cluster regardless of the presence or absence of this bridge [14].

AioB furthermore distinguishes itself from PetA through the apparent fate of its leader sequence. Despite a similar predicted N-terminal Twin-arginine translocation (Tat) signal sequence, localization of AioB and PetA seemed to differ. Aio was either found in the periplasm [10,18] or associated with the cytoplasmic membrane, depending on the species [6,8,11,12,19] whereas PetA invariably is membrane-anchored *via* its uncleaved Tat signal peptide. Since the Tat signal sequence is predicted – and observed – to be cleaved in all isolated AioAB enzymes, the existence of a third protein responsible for membrane association of selected AioAB has been considered [10,12].

The AioAB enzymes from *Ralstonia* sp. S22 and *Rhizobium* sp. NT-26 not only differ in their observed localization but also in the presence of the disulfide bridge in the vicinity of the [2Fe–2S] cluster. The enzyme from *Ralstonia* sp. S22 has indeed been shown to be membrane-associated [12] and to contain the disulfide bridge [14] whereas the *Rhizobium* sp. NT-26 enzyme has been shown to be entirely soluble in the periplasm [9] and furthermore devoid of the disulfide bridge [14]. Heterologous expression of the AioAB from *Ralstonia* sp. S22 (this work) and *Rhizobium* sp. NT-26 (Warelow and Santini, unpublished results) in *Escherichia coli* lent itself as a promising approach to address these questions due to (a) the possibility to obtain substantial quantities of the wild type (WT) enzymes as well as mutants targeting the Tat-sequence and the disulfide bridge and (b) the absence of putative further proteins/enzymes from the parent species interacting with the enzyme. The presented work constitutes the first heterologous expression of WT AioAB.

2. Material and methods

2.1. Growth of bacteria

Ralstonia sp. S22 was grown aerobically and heterotrophically at 28 °C in the presence of 5 mM As^{III} as described previously [12]. *E. coli* was grown aerobically and heterotrophically at 37 °C (see below).

2.2. Cloning of Ralstonia sp. S22 AioAB and heterologous expression in E. coli

Based on the genomic sequence of *Ralstonia* sp. S22, PCR primers were designed that amplify DNA fragments containing both *aioB* and *aioA*, with or without the Tat leader sequence, which were subsequently cloned into pET28a (Novagen) using the *Nco*I and *Xho*I restriction sites. PCR amplification was done with *Pfu* DNA polymerase (Promega) with as template for the initial PCR genomic DNA extracted from *Ralstonia* sp. S22 using the Wizard genomic DNA purification kit (Promega). For the construct of wild type AioAB of *Ralstonia* sp. S22, AioAB–pET28a, including the Tat sequence, primers 5′-ACTAGCCATGGCCGACCCACAGATCTTCAC-3′ and 5′-CGCCGATTTGTACTCGGCTAACTCGAGACTAG-3′ were used, incorporating restriction sites (underlined) and a stop codon placed upstream of the *Xho*I site as to exclude a hexahistidine-tag (His-tag). This is rationalized by the determination that, based on the AioAB structure of *Alcaligenes faecalis* (1G8K), the placement of a His-tag should be ideally on the C-terminus of AioB. A His-tag was thus introduced there using PCR with forward primer 5′-TAAGTCGGGAGGCTCTCATG-3′ and reverse primer 5′-CGCCAGGCCAACATTCTCCACCACCACCACCACCAC-3′, using as template the AioAB–pET28a construct. The resulting DNA product was phosphorylated with T4 DNA kinase (Promega) and then ligated with T4 DNA ligase (Promega). This AioAB-His-pET28a construct was used as a template for further PCRs, described below. For an AioAB construct containing a His-tag but devoid of the Tat sequence (AioAB-HisΔTat-pET28a), destined for cytoplasmic expression in *E. coli*, the first 38 residues were excluded corresponding exactly to the mature protein purified from *Ralstonia* sp. S22 [12]. Here, PCR primer 5′-ACTAGCCATGGCTGCGCCACACACTG-3′ was employed with reverse primer 5′-CGCCGATTTGTACTCGGCTAACTCGAGACTAG-3′. For the mutation of the Cysteine (Cys) at position 106 of AioB to an Alanine (Ala), forward primer 5′-GCCCCGACAGTGTTCGACAAC-3′ and reverse primer 5′-CACGATGTGTACCCATATGGGT-3′ produced a PCR product that was subsequently circularized, as described above (AioAB-HisC106A-pET28a). All constructs were verified by DNA sequencing (GATC Biotech).

The AioAB–pET28a constructs were transformed into strain C43 (DE3) *cyo*$^-$ (obtained from R. Gennis, Department of Biochemistry, University of Illinois at Urbana–Champaign).

Expression was done aerobically in ZYM-5052 auto-induction medium [20], including 1 mM Na_2MoO_4. Cells from a petri dish were used to inoculate 0.5 L of medium in 1 L flasks and grown at 37 °C for 16–20 h at 180 rpm orbital shaking.

2.3. Cloning of Rhizobium sp. NT-26 AioAB and heterologous expression in E. coli

Cloning and expression of *Rhizobium* sp. NT-26 AioAB are performed as will be published elsewhere.

2.4. Biochemical preparations

Spheroplast and periplasm preparations were done as published previously [14]. Purification of AioAB was done from the total soluble fraction. *E. coli* cells were harvested, washed once and resuspended in purification buffer (50 mM $NaPO_4$ pH 8, 500 mM NaCl, 50 mM imidazole), and broken by passing twice through a French press cell. Unbroken cells were removed by centrifugation at 6000 *g* for 10 min, and the "membrane-fraction" was retrieved as the pellet from a subsequent ultracentrifugation for 1 h and 30 min at 250,000 *g*. The supernatant, containing both the cytoplasm and the periplasm, was used for purification at 4 °C, using first GraviTrap pre-packed Ni charged affinity chromatography column (GE Healthcare) connected to a Fast protein liquid chromatography (FPLC) system. The column was equilibrated and washed with purification buffer and the protein eluted in two different pools with 100 mM and 200 mM imidazole. The eluate was concentrated and loaded onto a Superdex S200 gel filtration column (GE Healthcare) equilibrated with Tricine 50 mM/NaCl 200 mM pH 8 and used for analysis or flash-frozen in liquid nitrogen. For activity assay controls, the enzyme was purified as from *Ralstonia* sp. S22 described previously [14].

2.5. AioAB activity assays

AioAB activity was measured optically at pH 8 using cytochromes as electron acceptors as described by Lieutaud et al. [12]. c_{555} from *Aquifex aeolicus* and c_{550} from horse heart were used with AioAB from *Ralstonia* sp. S22 and AioAB from *Rhizobium* sp. NT-26, respectively. For sulfite inhibition studies, AioAB activities were measured at pH 6 also using cytochromes. Enzyme activity was also followed in gel. The electrophoresis was in this case done on a 10% polyacrylamide Laemmli gel system [21] containing 0.1% Triton X-100 and the activity was revealed as described previously [12].

2.6. Biochemical protein analyses

Protein concentrations were determined by the BCA method using a BSA standard. The subunit composition was determined by Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE) following the procedure of Laemmli [21] on a 10%–15% gradient polyacrylamide gel.

2.7. Spectroscopic experiments

Electron Paramagnetic Resonance (EPR) spectroscopy was performed on recombinant enzymes obtained at various purification steps: after cell breaking, after HisTrap column or at the end of the