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Crystal structure of the flavoprotein ArsH from *Sinorhizobium meliloti*

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Abstract

Purified ArsH from *Sinorhizobium meliloti* exhibits NADPH:FMN-dependent reduction of molecular O₂ to hydrogen peroxide and catalyzes reduction of azo dyes. The structure of ArsH was determined at 1.8 Å resolution. ArsH crystallizes with eight molecules in the asymmetric unit forming two tetramers. Each monomer has a core domain with a central five-stranded parallel β-sheet and two monomers interact to form a classical flavodoxin-like dimer. The N- and C-terminal extensions of ArsH are involved in interactions between subunits and tetramer formation. The structure may provide insight in how ArsH participates in arsenic detoxification.

1. Introduction

Arsenic resistance (*ars*) genes are widespread [1]. ArsH encoded by the *S. meliloti* *ars* operon has conserved domains related to the NADPH-dependent flavin mononucleotide reductases [2]. ArsH is widely distributed in bacteria (123 entries), sparsely in fungi (13 entries) and purple sea urchin, *Strongylocentrotus purpuratus* (3 entries), and, to date, none in archaea or other eukaryotes. ArsH from *Yersinia enterocolitica* confers resistance to both arsenite and arsenate [3]. Deletion of *arsH* from *Serratia marcesens* or *S. meliloti* results in loss of resistance [2,4]. In contrast, *arsH* from *Acidithiobacillus ferrooxidans* or *Synechocystis* do not appear to confer arsenic resistance [5,6]. Thus, the role of ArsH in arsenic resistance remains unclear. Here we report initial characterization and 1.8 Å resolution crystal structure of ArsH. ArsH is a H₂O₂-forming NADPH:FMN1 oxidoreductase that also reduces azo dyes, frequent industrial pollutants in waste water [7].

2. Materials and methods

2.1 Cloning, expression and purification

The *arsH* gene was amplified by polymerase chain reaction from genomic DNA of *S. meliloti* strain Rm1021, cloned into pET28a (Novagen) and expressed with a six-histidine tag in *E. coli* strain BL21 (DE3) pLysS cells (Novagen). Histagged ArsH was purified on a ProBond column (Invitrogen). ArsH concentrations were determined from A_{280 nm} (ε = 30,940 M⁻¹ cm⁻¹).

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2.2 Identification of prosthetic group

Purified ArsH was boiled for 5 min, centrifuged, and the supernatant analyzed in the dark on a cellulose thin layer plate in 1-butanol:acetic acid:acetone:water (5:1:2:3) [9]. The spots were visualized with ultraviolet light. The amount of FMN in purified ArsH was quantified by absorption at 445 nm using a molar extinction coefficient of $12,020 \text{ M}^{-1}\text{cm}^{-1}$. The concentration of FMN in a solution of $2.9 \mu\text{M}$ ArsH was determined to be $0.3 \mu\text{M}$, indicating that only 10% of the ArsH molecules contained FMN.

2.3 Enzyme Assays

ArsH was assayed for NADPH:FMN oxidoreductase activity in 1 ml of 25 mM Tris, 25 mM Bis-Tris Propane (pH 7.0), 0.1 mg/ml bovine serum albumin, 0.2 mM NADPH, 50 μM FMN, 1 mM EDTA and 10 nM ArsH at 37 °C. NADPH oxidation was followed at 340 nm ($\epsilon = 6,220 \text{ M}^{-1}\text{cm}^{-1}$). An Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen) was used to determine H_2O_2 formation, as described. [10]. Reduction of the azo dye Ponceau S was performed as described [7].

2.4 Crystallization and solution of the ArsH structure

Crystals of ArsH in 10 mM HEPES (pH 7.2) and 50 mM NaCl were grown at $23 \pm 2^\circ\text{C}$, in hanging drops, from a 1:1 mixture (1 μl of each) of protein (15 mg/ml) with 17% (w/v) PEG 6000, 0.1 M calcium acetate, and 0.1 M sodium cacodylate, pH 6.5. Micro-seeding was necessary for growing large single crystals. Crystals with a maximal size of 0.2–0.4 mm appeared within 3 days. The crystals were frozen in liquid nitrogen after soaking in cryoprotectant buffer containing 35% (w/v) PEG 6000, 0.1 M calcium acetate, 5% glycerol, and 0.1 M sodium cacodylate, pH 6.5, and a native data set was collected under cryogenic conditions at Argonne National Laboratory Advanced Photon Source (ANL-APS) beamline 32-ID-B. The data were indexed and integrated using MOSFLM [11] and scaled and merged using SCALA [12]. Heavy-atom derivatives were prepared by soaking crystals for 40 min in cryoprotectant buffer containing 5 mM ethyl mercuric phosphate. Hg-derivative crystals were flash-cooled and used for data collection with a Rigaku/MSR FR-D rotating-anode X-ray source equipped with an R-Axis HTC image-plate detector. Data were processed with MOSFLM and SCALA.

The structure was determined by single isomorphous replacement. SnB [13] was used to find the 10 Hg sites. Their positions were entered into PHENIX [14,15] for phasing, density modification. Initial model building led to tracing of about 80% of the residues. Additional residues were built manually using Xfit [16]. Refinement was carried out using Refmac5 [17]. Refinement statistics are shown in Table 1. All figures were created with PyMol [18].

3. Results

3.1 Expression and purification of ArsH

The 241-residue *S. meliloti* ArsH (NP_385180) was purified as a C-terminally six histidine tagged construct with a mass of 27,890 Da. ArsH was soluble in the cytosol and purified to >95% homogeneity by nickel-affinity chromatography. A mass of 28 kDa, that of an ArsH monomer, was observed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (data not shown). ArsH eluted as a 112 kDa tetramer by size-exclusion chromatography (data not shown).

Purified ArsH is yellow in appearance and exhibits typical flavoprotein absorption spectrum, with major peaks at 372 nm and 453 nm. On thin layer chromatography of the yellow supernatant from a boiled ArsH preparation, a single fluorescent spot was found that migrated

at the position of an FMN standard, indicating that FMN is the cofactor. However, only 10% of purified ArsH has bound FMN.

3.2 Enzymatic properties of recombinant *S. meliloti* ArsH

ArsH exhibited NADPH:FMN oxidoreductase activity with exogenously added FMN, with a $V_{\max}$ of 70 μmol of NADPH oxidized/min/mg protein. Optimal activity was observed between pH 6.5 and 7.5. The K_m for NADPH was 0.1 mM, and the concentration of FMN required for half-maximal activation was 7.5 μM . No activity was detected with NADH, and no requirement for arsenite or arsenate was observed, nor was there any conversion of arsenite into other species. It may be that the conditions for coupling the reaction to arsenicals *in vitro* have not yet been identified.

NADPH:flavin oxidoreductase from *Entamoeba histolytica* reduces O_2 to H_2O_2 [19]. Under similar conditions, ArsH produced 20 μmol of H_2O_2 $\text{min}^{-1}\text{mg}^{-1}$ during NADPH oxidation (data not shown). ArsH has weak homology with azoreductases from *Bacillus* and *Staphylococcus* species [7,20]. The ability of ArsH to reduce the azo dye Ponceau S with NADPH and FMN was assayed. Reduction of Ponceau S was assayed as the decrease in dye absorbance at 514 nm using an extinction coefficient of 33,470 $\text{M}^{-1}\text{cm}^{-1}$. The rate of reduction by purified ArsH was determined to be 363 nmol of Ponceau S reduced/min/mg protein.

3.3 Overall structure of *S. meliloti* ArsH

The *S. meliloti* ArsH structure was solved by single isomorphous replacement and refined to 1.8 Å (PDB ID code 2Q62). Most residues in the final model are well defined in the $2F_o - F_c$ electron density map. From Ramachandran plots [21], 92.9% of the residues are in the most favored regions, 7.0% in additional allowed regions, and none in disallowed regions.

ArsH crystals belong to space group $P4_2$ with eight molecules in a unit cell (molecules A, B, C, D, E, F, G, and H) in two tetramers of approximately $75 \times 60 \times 70$ Å³ each. The tetramers are identical, with RMSD of 0.43 Å between the C_α atoms and only weak interactions between tetramers. A model of the tetramer structure is shown (Fig. 1A). The final model contains residues 8–227 (monomer A), residues 8–229 (monomer B), residues 9–229 (monomer C, E, and H), residues 8–242 (monomer D), residues 9–227 (monomer F), residues 8–226 (monomer G), seven sulfate ions, and 1,682 water molecules.

Each monomer is a global α/β protein with a single core domain of flavodoxin-like architecture with a central five-stranded parallel β -sheet (strand order β_2 , β_1 , β_3 , β_4 , and β_5) flanked by helices α_1 and α_5 on one side and three helices (α_2 – α_4) on the other (Fig. 1B). There is a short 3_{10} helix (residues 178 to 180) between α_5 and β_5 . The ArsH secondary structure is consistent with that of other flavoproteins. ArsH has an N-terminal extension with two 3_{10} helices (residues 15 to 17 and residues 36 to 41) and a C-terminal extension with two helices: α_6 is almost perpendicular to α_5 , while α_7 is perpendicular to α_6 . Together these helices form a small domain involved in subunit interactions (see below). There are interactions between the N-terminal and C-terminal extensions of the same monomer. The amide nitrogens of Asn-14 and Asp-22 make hydrogen bonds with the hydroxyls of Tyr-222 and Tyr-217, respectively.

3.4 Interactions between subunits

ArsH is likely a functional tetramer: it is a tetramer in solution, and there are two tetramers in the asymmetric unit. Monomers A and B form a classical flavodoxin-like dimer, while monomers C and D form another dimer. Upon dimer formation, a surface area of 2,300 Å² is buried, comparable with other flavoproteins such as T1501, a dimeric 20-kDa FMN reductase from *Pseudomonas aeruginosa* [22]. The ArsH tetramer is a dimer of dimers. The monomers in the tetramer show non-crystallographic 222 symmetry. The buried surface area of the

tetramer is 12,000 Å², or 3,000 Å² per monomer, compared with 1,150 Å² per monomer for classical dimers, suggesting that this tetrameric structure is biologically relevant. The N-terminus helices, $\alpha 1$, $\alpha 4$, $\alpha 5$, and the C-terminal helices $\alpha 6$ and $\alpha 7$ are involved in dimer-dimer interaction. The N- and C-terminal extensions of ArsH are extensively involved in interactions between subunits and are essential for tetramer formation. If 30 residues from both the N- and C-termini of ArsH are excluded, it would resemble a classical flavodoxin, and the buried surface area would decrease to 3,600 Å² or 900 Å² per monomer. Each monomer interacts with two others to form a tetramer. For example, monomer A interacts with B and D. Molecules A and B interact with each other mainly through helices $\alpha 3$ and $\alpha 4$ to form a classical flavodoxin-like dimer (Fig. 2A). Monomer A also interacts with monomer D to form an interface that not found in the classical flavodoxin dimer. The N-terminal extensions, helices $\alpha 4$, $\alpha 5$, $\alpha 6$, and $\alpha 7$, are involved in interactions (Fig. 2B). Helix $\alpha 5$ is rich in polar residues that form salt bridges or hydrogen bonds with residues from the terminal extensions of a different subunit (Fig. 2C). For example, Glu-202 and Arg-225 from different subunits form a salt bridge.

3.5 The putative FMN binding site

T1501 from *P. aeruginosa* is an FMN-containing dimer that lacks the six-residue flavodoxin fingerprint motif (T/S)XTGXT [22]. ArsH also lacks the flavodoxin motif. The atypical T1501 FMN binding site (residues GSLRSGSYN) is superimposable on ArsH residues GSLRTVSYS, the putative FMN binding site. FMN was not observed in the ArsH structure, most likely due to the low FMN occupancy of the protein. However, in seven of the eight monomers, a sulfate was found in a deep pocket formed by Ser-43, Arg-45, Ser-48, and Tyr-49. The sulfate probably occupies a ribityl phosphate binding site, as observed in the T1501 apoprotein [22]. We predict that ArsH binds FMN similarly (Fig. 3). Most likely the FMN phosphate replaces the sulfate and participates in hydrogen bonding with the side chains of Ser-43, Arg-45, and Ser-48. The isoalloxazine ring of FMN is predicted to interact with residues 107 to 111.

4. Discussion

We have reported that disruption of *arsH* in *S. meliloti* leads to As(III) sensitivity [2], indicating that ArsH is involved in As(III) detoxification. Possibly the ability to generate H₂O₂ is indirectly involved. H₂O₂ oxidizes trivalent forms of arsenic to pentavalent forms in vitro [23], so perhaps ArsH-generated H₂O₂ oxidizes the more toxic arsenite to the less toxic arsenate. Alternately, trivalent arsenicals might serve as a terminal electron acceptor for ArsH, becoming reduced to volatile arsines such as AsH₃ or (CH₃)₃As that escape from cells. In conclusion, ArsH is an atypical flavodoxin with a noncanonical FMN binding site that catalyzes oxidation of NADPH, generating H₂O₂ and reducing azo dyes. It is a tetrameric dimer of dimers, each of which is similar to a flavodoxin dimer. The mechanism by which ArsH activity is coupled to arsenic detoxification is the subject of current study.

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References

1. Bhattacharjee, H.; Rosen, BP. Arsenic metabolism in prokaryotic and eukaryotic microbes. In: Nies, DHS.; Simon, editors. *Molecular Microbiology of Heavy Metals*. Springer-Verlag; Heidelberg/New York: 2007. p. 390

2. Yang HC, Cheng J, Finan TM, Rosen BP, Bhattacharjee H. Novel pathway for arsenic detoxification in the legume symbiont *Sinorhizobium meliloti*. *J Bacteriol* 2005;187:6991–7. [PubMed: 16199569]
3. Neyt C, Iriarte M, Thi VH, Cornelis GR. Virulence and arsenic resistance in *Yersinia*. *J Bacteriol* 1997;179:612–9. [PubMed: 9006011]
4. Ryan D, Collieran E. Arsenical resistance in the IncHI2 plasmids. *Plasmid* 2002;47:234–40. [PubMed: 12151239]
5. Butcher BG, Deane SM, Rawlings DE. The chromosomal arsenic resistance genes of *Thiobacillus ferrooxidans* have an unusual arrangement and confer increased arsenic and antimony resistance to *Escherichia coli*. *Appl Environ Microbiol* 2000;66:1826–33. [PubMed: 10788346]
6. Lopez-Maury L, Florencio FJ, Reyes JC. Arsenic sensing and resistance system in the cyanobacterium *Synechocystis* sp. strain PCC 6803. *J Bacteriol* 2003;185:5363–71. [PubMed: 12949088]
7. Suzuki Y, Yoda T, Ruhul A, Sugiura W. Molecular cloning and characterization of the gene coding for azoreductase from *Bacillus* sp. OY1-2 isolated from soil. *J Biol Chem* 2001;276:9059–65. [PubMed: 11134015]
8. Sambrook, J.; Fritsch, EF.; Maniatis, T. Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory; New York: 1989.
9. Schottel JL. The mercuric and organomercurial detoxifying enzymes from a plasmid-bearing strain of *Escherichia coli*. *J Biol Chem* 1978;253:4341–9. [PubMed: 350872]
10. Zhou M, Diwu Z, Panchuk-Voloshina N, Haugland RP. A stable nonfluorescent derivative of resorufin for the fluorometric determination of trace hydrogen peroxide: applications in detecting the activity of phagocyte NADPH oxidase and other oxidases. *Anal Biochem* 1997;253:162–8. [PubMed: 9367498]
11. Powell HR. The Rossmann Fourier autoindexing algorithm in MOSFLM. *Acta Crystallographica Section D-Biological Crystallography* 1999;55:1690–1695.
12. Pottert E, McNicholas S, Krissinel E, Cowtan K, Noble M. The CCP4 molecular-graphics project. *Acta Crystallogr D Biol Crystallogr* 2002;58:1955–7. [PubMed: 12393928]
13. Weeks CM, DeTitta GT, Hauptman HA, Thuman P, Miller R. Structure solution by minimal function phase refinement and Fourier filtering: II. implementation and applications. *Acta Cryst* 1994;A50:210–220.
14. Adams PD, et al. Recent developments in the PHENIX software for automated crystallographic structure determination. *J Synchrotron Radiat* 2004;11:53–5. [PubMed: 14646133]
15. Adams PD, et al. PHENIX: building new software for automated crystallographic structure determination. *Acta Crystallogr D Biol Crystallogr* 2002;58:1948–54. [PubMed: 12393927]
16. McRee DE. XtalView/Xfit--A versatile program for manipulating atomic coordinates and electron density. *J Struct Biol* 1999;125:156–65. [PubMed: 10222271]
17. Murshudov GN, Vagin AA, Dodson EJ. Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallographica Section D-Biological Crystallography* 1997;53:240–255.
18. DeLano, WL. The PyMOL user's manual. DeLano Scientific; San Carlos, CA: 2001.
19. Bruchhaus I, Tannich E. Purification and molecular characterization of the NAD(+)-dependent acetaldehyde/alcohol dehydrogenase from *Entamoeba histolytica*. *Biochem J* 1994;303 (Pt 3):743–8. [PubMed: 7980441]
20. Chen H, Hopper SL, Cerniglia CE. Biochemical and molecular characterization of an azoreductase from *Staphylococcus aureus*, a tetrameric NADPH-dependent flavoprotein. *Microbiology* 2005;151:1433–41. [PubMed: 15870453]
21. Laskowski RA, MacArthur MW, Moss DS, Thornton JM. Procheck - a program to check the stereochemical quality of protein structures. *Journal of Applied Crystallography* 1993;26:283–291.
22. Agarwal R, Bonanno JB, Burley SK, Swaminathan S. Structure determination of an FMN reductase from *Pseudomonas aeruginosa* PA01 using sulfur anomalous signal. *Acta Crystallogr D Biol Crystallogr* 2006;62:383–91. [PubMed: 16552139]
23. Aposhian HV, Zakharyan RA, Avram MD, Sampayo-Reyes A, Wollenberg ML. A review of the enzymology of arsenic metabolism and a new potential role of hydrogen peroxide in the detoxication of the trivalent arsenic species. *Toxicol Appl Pharmacol* 2004;198:327–35. [PubMed: 15276412]

Abbreviations

NADPH

reduced nicotinamide adenine dinucleotide phosphate

FMN

flavin mononucleotide

NADH

reduced nicotinamide adenine dinucleotide

Fig 1. X-ray crystal structure of *S. meliloti* ArsH
(A) Tetramer form of ArsH. The monomers A, B, C, and D are colored red, green, blue, and yellow, respectively. (B) Ribbon representation of the ArsH monomer with secondary structural units. Helices, strands, and loops are colored cyan, magenta, and yellow, respectively.

Fig 2. Interactions between ArsH subunits

(A) A flavodoxin-like dimer interface is formed by interactions of helices $\alpha 3$ and $\alpha 4$ from both monomer A and monomer B. Only the helices from one monomer are labeled. (B) A non-flavodoxin-like dimer interface is formed by interactions between monomers A and D through the extensions of helices $\alpha 4$, $\alpha 5$, $\alpha 6$ and $\alpha 7$ of each subunit. (c) Polar interactions (hydrogen bonds or salt bridges) between helix $\alpha 5$ of subunit A and the C-terminal extensions from subunit D. For clarity, only residues involved in polar interactions are shown as sticks. Dashed lines represent polar interactions.

Fig 3. The FMN binding site

FMN was modeled into the putative binding site of ArsH by superimposing the main chain atoms of the GSLRSGSYN loop from T1501 onto those of the GSLRTVSYS loop of ArsH (with an RMSD of 0.6 Å).

Table 1

Data collection and refinement statistics

Crystal data	Native	Hg derivative
<i>Data collection</i>		
Resolution (Å)	39.6–1.80 (1.90–1.80)	36.1–2.50 (2.64–2.50)
Space group	P4 ₂	P4 ₂
Unit cell parameters (Å)	a = b = 158.53; c = 87.95	a = b = 158.28; c = 88.21
Total number of reflections	2,104,522 (227,608)	458,434 (52,229)
Total number of unique reflections	185,319 (24,428)	75,309 (10,782)
R _{merge} (%) ^a	8.8 (53.2)	8.7 (35.3)
Completeness (%)	92.1 (83.5)	99.5 (97.9)
I/σ □(I)	18.3 (3.1)	18.0 (4.0)
<i>Phasing</i>		
Resolution (Å)		36.1–2.50
Figure of merit (after density modification)		0.25 (0.60)
<i>Refinement</i>		
Resolution (Å)	39.6–1.80 (1.85–1.80)	
I/σ cutoff	0	
Number of protein atoms	14,077	
Number of sulfate ions	7	
Number of water molecules	1,682	
R/R _{free} (%)	17.3/21.2 (25.3/32.4)	
R.m.s.d. bonds (Å)	0.016	
R.m.s.d. angle (°)	1.7	
Mean B factor (Å ²), protein/ion/water	22.2/24.0/29.4	

Values in parentheses are for highest resolution shell.

^aR_{merge} = $\sum |I - \langle I \rangle| / \sum I$, where I is the observed intensity, $\langle I \rangle$ is the statistically weighted average intensity of multiple observations of symmetry-related reflections.