Ferric Reductase Activity of the ArsH Protein from Acidithiobacillus ferrooxidans

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The arsH gene is one of the arsenic resistance system in bacteria and eukaryotes. The ArsH protein was annotated as a NADPH-dependent flavin mononucleotide (FMN) reductase with unknown biological function. Here we report for the first time that the ArsH protein showed high ferric reductase activity. Glu104 was an essential residue for maintaining the stability of the FMN cofactor. The ArsH protein may perform an important role for cytosolic ferric iron assimilation in vivo.

Keywords: Acidithiobacillus ferrooxidans, ArsH, flavoprotein, ferric reductase

Arsenic resistance genes are widespread in nature. The arsenic resistance operon in the genome of Acidithiobacillus ferrooxidans consists of four genes, arsR, arsB, arsC, and arsH [2, 3, 11]. Three of these genes, arsB, arsC, and arsR, have been studied and their arsenic resistance mechanisms have been elucidated [13, 14]. The function of ArsH of the arsenic resistance pathway is not clear so far. ArsH from Yersinia enterocolitica confers resistance to both arsenite and arsenate [12]. Deletion of arsH from Serratia marcesens results in loss of resistance [18]. In contrast, ArsH from A. ferrooxidans or Synechocystis showed no arsenic resistance phenotype [2, 8]. Thus it is reasonable that the the ArsH from A. ferrooxidans may perform another specific function. Bioinformatic classification of the ArsH protein based on sequence homology showed that the ArsH protein belongs to the NADPH-dependent flavin mononucleotide (FMN) reductases family, and it was later purified to be a flavoprotein containing a FMN cofactor, and showed NADPH-dependent FMN reductase activity [16, 19].

Iron is an essential element for virtually all living organisms. In aerobic environments the stable form is Fe3+, which has little solubility in neutral aqueous solutions (10−17 M at physiological pH). The cell has to convert cytosolic ferric iron (free or chelated) into ferrous iron before its incorporation into heme and nonheme iron-containing proteins. Ferric reductase catalyzes the reduction of complexed Fe3+ to complexed Fe2+ using NAD(P)H as the electron donor. The resulting Fe2+ is subsequently released and incorporated into iron-containing proteins [17].

Here we report for the first time that the ArsH protein showed high ferric reduction activity. The ArsH from A. ferrooxidans may perform an important role as a NADPH-dependent ferric reductase for cytosolic ferric iron assimilation in vivo.

Materials and Methods

Materials
Acidithiobacillus ferrooxidans ATCC 23270 was obtained from the American Type Culture Collection. A Hi-Trap chelating metal affinity column was purchased from GE Healthcare Ltd. Top10 competent cells and E. coli strain BL21(DE3) competent cells came from Invitrogen Life Technologies. The Plasmid Mini kit, a gel extraction kit, and synthesized oligonucleotides were obtained from Sangon Company of Shanghai. Taq DNA polymerase and restriction enzymes came from MBI Fermentas. All other reagents were of research grade and obtained from commercial sources.

Cloning of the arsH Gene from A. ferrooxidans
Genomic DNA from A. ferrooxidans ATCC 23270 was prepared using an EZ-10 spin column genomic DNA isolation kit from Bio Basic Inc., according to the manufacturer’s instructions for bacterial DNA extraction. This genomic DNA was used as a template for PCR reaction. The sequence of the forward primer was 5'-CGCGCGAATTCAGGAGGAATTTAAAATGAGAGGATCGCATCACCATCACCATCAC-3', containing an EcoRI site (GAATTC), a ribosome binding site (AGGAGGA), codons for the last 10 amino acids, and codons for amino acids 2–11 of ArsH protein. The sequence of the reverse primer was 5’-CTGCAGGGATCCCTTTAAGGTTCTGTAAGATTGACGCGCCGGGAAAG-3’, containing a BamHI site (GGATCC), a stop codon, and the samples were subjected to 25 cycles of 45 s of
denaturation at 95°C, 1 min of annealing at 61°C, and 2 min of
elongation at 72°C in a Mastercycler Personal of Eppendorf Model
made in Germany. The amplification products were analyzed by
electrophoresis on a 1% agarose gel and stained with ethidium bromide.
The resulting PCR product was gel purified, double digested, and
ligated into a p.M1 expresion vector, resulting in the p.M1::ARSH
plasmid. The constructed p.M1::ARSH plasmid was then transferred
into TOP10 competent cells for screening purposes. A positive colony
with a gene insert in the plasmid was identified by single restriction
digestion of the plasmid with EcoRI and double restriction digestion
of the plasmid with EcoRI and BamHI, followed by an agarose gel
analysis. The identified positive colony was grown in an LB medium
containing ampicillin (50 mg/l), and the plasmid p.M1::ARSH was
isolated from harvested bacteria cells using a plasmid extraction kit.
The isolated p.M1::ARSH plasmid was then transformed into E.
coli strain BL21(DE3) competent cells for expression purposes. DNA
sequencing of the cloned ArsH protein gene was performed and the
inserted gene sequence identified as previously reported without any
mutation.

**Construction of A. ferrooxidans ArsH Mutant Plasmids**
A QuickChange mutagenesis kit (Stratagene) was applied for
constructing the pArsH(E104A), pArsH(E104D), and pArsH(E104Q)
mutation expression plasmids. The plasmid p.M1::ARSH was used
as a template for constructing mutant expression plasmids through
PCR reaction. The following primers and their antisense primers were
synthesized to introduce the mutated sequences:

1. E104A, 5'-CTGTTTCTCGCGGCACATGGGAC-3',
   condon GAG for glutamic acid (E) was changed to condon GCG
   for alanine (A)
2. E104D, 5'-TGGTTTCTCGCGGACATGCGAC-3',
   condon GAG for glutamic acid (E) was changed to condon GAT
   for aspartic acid (D)
3. E104Q, 5'-CTGTTTCTCGCGGCACATGGGAC-3',
   condon GAG for glutamic acid (E) was changed to condon GCA
   for glutamine (Q)

PCR amplification was performed using Pfu DNA polymerase
and samples were subjected to 13 cycles of 0.5 min of denaturation
at 95°C, 1 min of annealing at 61°C, and 12 min of elongation at 72°C
in a Mastercycler Personal of Eppendorf Model made in Germany.
DpnI restriction enzyme was used to digest the parental supercoiled
double-stranded DNA. The constructed mutant plasmids were transferred
into TOP10 competent cells for screening purposes. The positive colonies with
the mutant plasmids were identified by sequencing. The isolated mutant plasmids were then used to transform E.
coli strain BL21(DE3) competent cells for expression.

**Expression of Recombinant ArsH Wild-Type and Mutant Proteins**
The E. coli strain BL21(DE3) cells with p.M1::ARSH plasmid or
its mutants were grown at 37°C in 500 ml of LB medium containing
ampicillin (100 mg/l) to an OD600 of 0.6. At this point, the cells were
incubated at room temperature with the addition of 0.5 mM IPTG
overnight with shaking at 180 rpm. Thereafter, the cells were harvested
by centrifugation and the cell pellet was washed with an equal volume
of sterile water. The cells were then reharvested by centrifugation,
suspended in a start buffer (20 mM potassium phosphate, pH 7.4,
0.5 M NaCl), incubated with 5 mg of lysozyme at 37°C for 0.5 h,
and stored at −80°C for purification.

**Purification of the Recombinant ArsH Wild-Type and Mutant Proteins**
The cells were then harvested and lysed by sonication using a 150-
Watt Autotune Series High Intensity Ultrasonic sonicator. The insoluble
debris was removed by centrifugation and the clear supernatant was
used for protein purification with a Hi-Trap affinity column by a
standard procedure. The Hi-Trap column was first equilibrated with
0.1 M nickel sulfate to charge the column with nickel ions, followed
by 5 column volumes of MilliQ water to remove any unbound nickel
ions from the column, and then 5 column volumes of a start buffer
(20 mM potassium phosphate, pH 7.4, 0.5 M NaCl) to equilibrate the
column. The clarified sample was applied to the Hi-Trap column
after being filtering through a 0.45 µm filter. Next, the column was
washed with 5 column volumes of the start buffer, followed by 5
column volumes of a wash buffer (20 mM potassium phosphate, pH 7.4,
0.5 M NaCl, 50 mM imidazole), and then the protein was eluted
with an elution buffer (20 mM potassium phosphate, pH 7.4, 0.5 M
NaCl, 500 mM imidazole). The eluted fractions were analyzed by
SDS polyacrylamide gel electrophoresis (SDS-PAGE) with 15%
of acrylamide according to Laemmli [9]. The purified enzyme fractions
were dialyzed against a 20 mM potassium phosphate buffer, pH 7.4,
5% glycerol and then stored in a freezer at −80°C.

The method of Bradford was used to determine the protein
content with bovine serum albumin as the standard [4].

**Identification of the Prosthetic Group**
Purified ArsH was visualized with ultraviolet light in a Shimadzu
UV-1800 spectrophotometer. The ratio of absorbance of the ArsH
solution at 267 nm and 373 nm was calculated [16]. The amount
of flavin mononucleotide in purified ArsH was quantified by absorption
at 445 nm using a molar extinction coefficient of 12,020 M
−1 

**Enzyme Assays**
The ferric reductase assay was performed as described previously
[17]. Electron acceptors other than Fe
3+
-EDTA were used at a
concentration of 0.25 mM. NADPH:flavin oxidoreductase activity
was measured with 0.1 mM NADPH, and Fe
3+
-EDTA was not
included. One unit of activity is defined as 1 µmol of NADPH oxidized
per minute.

**Molecular Structure Modeling of the ArsH from A. ferrooxidans**
All simulations were performed with Discovery Studio 2.1 software
developed by Accelrys Software Inc., running on the Dell Precision
470 workstation with Windows system. The first step was to search
for a number of related sequences to find a related protein as a
template by the FASTA program. Then, the templates were aligned to
find the conserved core and build the model. After a further improved
and optimization procedure of the initial model, the simulation model
was accomplished.

**RESULTS AND DISCUSSION**

**Cloning of the arsH Gene from A. ferrooxidans**
A PCR technique was used to successfully add six continuous histidine residues to the N-terminal of the ArsH from A.
ferrooxidans, which greatly accelerated the protein purification
process. Mutant expression plasmids of pArsH(E104A), pArsH(E104D) and pArsH(E104Q) were constructed, and their sequences were verified for the presence of the directed mutation and the absence of PCR-generated random mutations by DNA sequencing. The expression plasmid for the ArsH was transformed into *E. coli* BL21(DE3) for expression.

**Expression and Purification of the ArsH**

Nickel metal-affinity resin column was used for single-step purification of His-tagged ArsH wild-type and their mutant proteins. The purity of the ArsH wild-type and its mutant proteins were further examined by SDS–PAGE and a single band corresponding to the 28 kDa protein was observed with >95% purity (Fig. 1). The protein fractions were dialyzed against a 20 mM potassium phosphate buffer, pH 7.5, 5% (v/v) glycerol as soon as possible after the purification.

The purified ArsH wild type was a yellow color protein and exhibited a typical flavoprotein absorption spectrum, with major peaks at 373 nm and 455 nm, indicating the presence of the FMN cofactor. The concentration of FMN in the supernatant solution was 198 µM, and ArsH concentration was determined to be 206 µM, indicating that more than 95% of the ArsH molecules contained the FMN cofactor. It was reported that the enzyme–prosthetic group ratio was 10:1 for ArsH from *Sinorhizobium meliloti* [19], and 12:1 for ArsH from *Shigella flexneri* [16]. Our result clearly indicated that the ArsH from *A. ferrooxidans* fully binds FMN cofactor after affinity chromatography.

To our knowledge, this is the first report of a ferric reductase with a fully bound FMN cofactor and that shows high enzyme activity without addition of FMN cofactor, which will greatly facilitate our further research on resolving the molecular structure of the ArsH with bound FMN cofactor. After mutation, the E104D and E104Q mutants of ArsH showed a yellow color, which means the FMN cofactor was still bound to the protein, whereas E104A had no color.

**Enzymatic Properties of Recombinant *A. ferrooxidans* ArsH**

The ArsH was first assayed for NADPH-dependent FMN reductase activity. The protein showed activity towards NADPH, as shown in Fig. 2, where the enzyme-bound FMN was rapidly reduced in the presence of NADPH. The ArsH had FMN reductase activity, and the specific activity was determined to be 3.2 µmol min^{-1} mg^{-1}. To examine whether Fe³⁺-EDTA could serve as the electron acceptor for the ArsH, Fe³⁺-EDTA was incubated with the above ArsH and NADPH reaction mixture. FMN was rapidly oxidized by Fe³⁺-EDTA, as shown in Fig. 2. FMN remained oxidized in the presence of Fe³⁺-EDTA, consistent with the notion that the cofactor serves as an electron mediator. Additions of Fe³⁺-EDTA resulted in an immediate oxidation of the FMN, as shown by the increase of the absorption at 457 nm (Fig. 3). Complete oxidation was achieved at two molar equivalents of Fe³⁺-EDTA per mol of reduced FMN in the active site of ArsH. The results indicated that the reduced FMN bound to the ArsH transferring electrons to the Fe³⁺-EDTA may follow the ping-pong mechanism [7]. Therefore, the ArsH functions as a NADPH-dependent ferric reductase. Kinetic constants were then measured, where rates were calculated at five substrate concentrations and averages of two assays were used for each point. The $K_m$ value for NADPH was 76 µM, and the $K_m$ value for

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**Fig. 1.** Coomassie blue-stained SDS–PAGE of the purified ArsH. Lane 1, molecular mass standards; lane 2, ArsH wild-type protein, lane 3, E104A mutant protein; lane 4, E104D mutant protein; lane 5, E104Q mutant protein.

**Fig. 2.** Absorption spectrum changes of the ArsH in the absence and presence of Fe³⁺-EDTA. Line 1, purified ArsH (80 µM) in 50 mM sodium phosphate buffer (pH 6.5); Line 2, ArsH incubate with 150 µM NADPH in 50 mM sodium phosphate buffer (pH 6.5); Line 3, purified ArsH incubate with 150 µM NADPH, then 100 µM Fe³⁺-EDTA was added to the reaction mixture.
Fe\textsuperscript{3+}-EDTA was 30 µM, and V\textsubscript{max} was 21.6 µmol min\textsuperscript{-1} mg\textsuperscript{-1}. The ferric reductase activity of the ArsH from \textit{A. ferrooxidans} was at least 50-fold higher than bacterial ferric reductase activities that have been measured so far, but its activity was lower than that from \textit{Archaeoglobus fulgidus} [17].

NADH was used as the electron donor to test the FMN reductase activity. No catalytic activity was detected, which was in agreement with previous report for ArsH from \textit{Sinorhizobium meliloti} and \textit{Shigella flexneri} [16, 19]. Ferric reductase activity was also not found with NADH as electron donor. It was reported that both NADH and NADPH were substrates for ferric reductase from \textit{E. coli}, \textit{Saccharomyces cerevisiae}, and \textit{Archaeoglobus fulgidus} [5, 6, 15, 17, 19].

The ArsH exhibited a pH optimum of 6.5 for its ferric reductase activity, as shown in Fig. 4, and optimum reaction temperature was observed to be 35°C (Fig. 5).

**Electron Acceptors of the ArsH**

Ferric reductase also exhibited activity with other complexes that contained Fe\textsuperscript{3+} as the electron acceptor, as shown in Table 1. The highest activity was obtained with Fe\textsuperscript{3+}-EDTA, where it was 1.5 times higher than the activity measured with potassium ferricyanide. The activity of 17% was obtained with Fe\textsuperscript{3+}-NTA as electron acceptor and only 10% for Fe\textsuperscript{3+}-citrate. No activity was obtained with FeCl\textsubscript{3}, so the results indicated that the ArsH showed activity only towards complexed Fe\textsuperscript{3+}. The ferric reductase from \textit{Saccharomyces cerevisiae} and \textit{Archaeoglobus fulgidus} all showed highest activity with ferricyanide as the electron acceptor [9, 17]. The ArsH showed no reductase activity for EDTA-chelated Ag\textsuperscript{+} and Cu\textsuperscript{2+}.

![Fig. 3. Oxidation of reduced ArsH Fe\textsuperscript{3+}-EDTA under anaerobic conditions.
ArsH (50 µM) was incubated with 50 µM NADPH, and then Fe\textsuperscript{3+}-EDTA was added to the reaction mixture at a final concentration of 0 µM, 20 µM, 40 µM, 60 µM, 80 µM, and 100 µM respectively (from below to above).](image)

![Fig. 4. pH dependence of the ferric reductase activity of the ArsH from \textit{A. ferrooxidans}.
The pH optimum of the ferric reductase activity of the ArsH was determined using 50 mM sodium phosphate buffer at different pHs. The reaction mixture contained 50 mM buffer, 100 µM NADPH, 250 µM Fe\textsuperscript{3+}-EDTA, and 15 nM of protein.](image)

![Fig. 5. Temperature dependence of the ferric reductase activity of the ArsH from \textit{A. ferrooxidans}.
The reaction mixture contained 50 mM buffer phosphate buffer at pH 6.5, 100 µM NADPH, 250 µM Fe\textsuperscript{3+}-EDTA, and 15 nM of protein.](image)

**Table 1. Substrate specificity for various electron acceptors of the ArsH from \textit{A. ferrooxidans}.
Electron acceptor | Specific activity (µmol min\textsuperscript{-1} mg\textsuperscript{-1}) | Relative activity (%)
--- | --- | ---
Fe\textsuperscript{3+}-EDTA | 13.1 | 100
Fe\textsuperscript{3+}-citrate | 1.3 | 10
Fe\textsuperscript{3+}-NTA | 2.2 | 17
Ferricyanide | 8.6 | 66
FeCl\textsubscript{3} | 0 | 0
Cu\textsuperscript{2+}-EDTA | 0 | 0
Ag\textsuperscript{+}-EDTA | 0 | 0
Role of the Conserved Glu104 of the ArsH in Catalytic Reaction

Sequence alignment of ArsH from various sources showed that the Glu104 is a highly conserved residue (Fig. 6). This conserved glutamic acid of the ArsH might be important in ligation with the FMN cofactor. To confirm this, we mutated Glu104 into A, D, and Q mutant plasmids, and then the mutant plasmids were expressed in *E. coli* and purified by affinity chromatography to accomplish homogeneity. After purification, E104D and E104Q mutant proteins also had absorption between 373 and 455 nm, and showed the FMN cofactor still bound to the enzyme, whereas E104A mutant proteins had no color and no typical absorption for FMN molecule (data not shown).

Compared with the ArsH wild-type enzyme, only E104D and E104Q mutant proteins showed ferric reductase activity, where their specific activities were lower than ArsH wild type, as shown in Table 2. No activity was detected for E104A mutant protein. The results indicated that the carbonyl group of the glutamic acid was crucial for maintaining the stability of the FMN cofactor for electron transfer, where removal of the carbonyl group resulted in loss of FMN cofactor and inactivity of the enzyme.

Molecular Structure Modeling of the ArsH from *A. ferrooxidans*

In the result of FASTA search, the ArsH protein from *Sinorhizobium meliloti* (PDB code 2Q62) had a high level of sequence identity of 69% with the ArsH protein from *A. ferrooxidans*, which should guarantee a relative reliable homology structure building. Based on the structure of 2Q62, through the procedures of homology modeling and molecular simulations, the final structure of the ArsH protein expressed from *E. coli* BL21(DE3) was obtained.

The modeled structure of ArsH from *A. ferrooxidans* is shown in Fig. 7. The FMN cofactor is shown binding in the protein. Sequence analysis indicated that the putative FMN binding site in ArsH (GSNRECSYS) from *A. ferrooxidans* is similar to that of the FMN binding site (GSLRTVSYS) from *Sinorhizobium meliloti*.

As shown in the modeled structure, Ser39, Arg41, Ser44, and Tyr45 comprise a sulfate interaction site that probably occupies a ribitol phosphate binding site, whereas residues 103–107 interact with the isoalloxazine ring of FMN directly through oxygen and nitrogen atoms of the main chain or mediated by water molecules.

Moreover, Glu104 is the only acidic residue binding the FMN cofactor from the modeled structure, which is also a highly conserved residue (Fig. 6). The negative charge of Glu104 is shown in light gray, the conserved glutamic acid 104 was colored in black.

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**Table 2.** Specific activities of the ArsH and mutant Glu104 proteins with NADPH as electron donor and Fe$^{3+}$-EDTA as electron acceptor.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Specific activity (µmol min$^{-1}$ mg$^{-1}$)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>13.1</td>
<td>100</td>
</tr>
<tr>
<td>E104A</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>E104D</td>
<td>1.05</td>
<td>0</td>
</tr>
<tr>
<td>E104Q</td>
<td>2.62</td>
<td>20</td>
</tr>
</tbody>
</table>

**Fig. 6.** The sequences alignment of ArsH from *A. ferrooxidans* and other sources.

*A. f:* Acidithiobacillus ferrooxidans ATCC 23270; *B. a:* Burkholderia ambifaria MC40-6; *Fm:* Pseudomonas mendocina ympr; *Sm:* Sinorhizobium meliloti; *Sf:* Shigella flexneri. Residues conserved in all sequences are marked with *.* Residues not conserved in all sequences but conserved in some sequences are marked with : or based on the degree of conservation. The conserved glutamic acid residue was marked with black color.

**Fig. 7.** The modeled molecular structure showing the FMN binding pocket of the ArsH protein from *A. ferrooxidans*. The FMN cofactor was colored in light gray, the conserved glutamic acid 104 was colored in black.
the residue might be important for the stability of the FMN cofactor and also plays a critical role in mediating an electron-transfer pathway between the cofactor and protein surface. In our experimental results, the carbonyl group of Glu104 is essential for maintaining the stability of the cofactor. Removal of the carbonyl group resulted in loss of activity, which is in good agreement with the modeling results.

From the enzymatic data, it is still difficult to conclude on the exact biochemical function of the ArsH, although several specific reductase activities have been characterized using NADPH as an electron donor and FMN as a cofactor. We propose that ferric iron in chelated form may be the natural substrate for the reduction catalyzed by this protein. Ferric iron reduction is the central reaction in cellular iron metabolism. The cell has to convert cytosolic ferric iron (free or chelated) into ferrous iron before its incorporation into heme and nonheme iron-containing proteins. The cytosolic protein responsible for ferric iron reduction is still not clear so far. From our data, the ArsH protein is a good candidate for cytosolic ferric iron reductase activity in vivo.

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References