RESEARCH ARTICLE
doi: 10.2306/scienceasia1513-1874.2010.36.100

ScienceAsia 36 (2010): **100–104**

The putative thiol-disulphide interchange protein DsbG from *Acidithiobacillus ferrooxidans* has disulphide isomerase activity

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Received 18 Aug 2009
Accepted 26 Mar 2010

ABSTRACT: The putative thiol-disulphide interchange protein, DsbG, is involved in the formation and rearrangement of disulphide bonds in *Acidithiobacillus ferrooxidans* but its exact role is so far unclear. The gene encoding DsbG from *A. ferrooxidans* ATCC 23270 was cloned and expressed in *Escherichia coli* BL21 (DE3). The protein was purified by one-step affinity chromatography. The scRNaseA could be rescued to 89% of the native RNaseA activity by using the purified DsbG of *A. ferrooxidans*. This characteristic is unique and different from that of DsbG from *E. coli*, which does not catalyse the oxidative refolding of RNaseA in vitro. Site-directed mutagenesis of the DsbG protein revealed that Cys119A and Cys122A do not possess the disulphide isomerization activity, indicating Cys119 and Cys122 are catalytic residues and play a crucial role in disulphide isomerization.

KEYWORDS: thiol-disulphide interchange protein, disulphide isomerization activity, site-directed mutagenesis

INTRODUCTION

The formation and rearrangement of disulphide bonds is an important step in the biosynthesis of many secreted proteins. Such processes provide correct protein folding and stability. In Gram-negative bacteria, this step normally occurs during exporting the proteins into the periplasmic space. Genetic and biochemical studies on disulphide bond formation in *Escherichia coli* have uncovered five different disulphide bond formation-related proteins, namely, DsbA, DsbB, DsbC, DsbD, and DsbG[1]. It is known that DsbA randomly oxidizes cysteines to forms non-native disulphide bonds in the proteins, DsbB restores the reduced form of DsbA, and the other three Dsb proteins are involved in both disulphide bond formation and rearrangement by isomerization[2,3]. It has been found that the inner-membrane protein DsbD keeps DsbC and DsbG reduced in the oxidizing environment of the periplasma, and DsbC functions as isomerase and has chaperone activity. DsbG, which shares 24% of amino acid identity with DsbC when expressed at approximately 25% of the level of DsbC, can compensate for some phenotypic defects exhibited by either *dsbA* or *dsbC* mutant strains. However, the *dsbG* null mutants display no defect in folding of multiple disulphide bond-containing heterologous proteins[4–6]. In contrast to DsbC, DsbG is narrower in substrate range as it does not catalyse the oxidative refolding of RNaseA in vitro[6] and is not critical for the RNaseA activity in vivo[7]. All these inconsistencies justify a further study of the DsbG function.

In the present study, the gene encoding the putative thiol:disulphide interchange protein DsbG (AEF0162) from *Acidithiobacillus ferrooxidans* ATCC 23270 was cloned and expressed in *E. coli*. The expressed DsbG was used to examine the disulphide-bond isomerase activity and the key amino acid residues of active sites involved in DsbG disulphide-bond isomerization.

MATERIALS AND METHODS

A. ferrooxidans ATCC 23270 was obtained from the American Type Culture Collection. *E. coli* BL21 (DE3) competent cells and the pET28a(+) expression vector were from Invitrogen (Life Technologies). A HiTrap chelating metal-affinity column was purchased from GE healthcare LTD. The Plasmid Mini kit, a gel extraction kit, and synthesized oligonucleotides were from Sangon Company of Shanghai. *Taq* and *Pfu* DNA polymerase, T4 DNA ligase, and restriction enzymes were from MBI Fermentas.

Cloning of the *dsbG* gene from *A. ferrooxidans*

Genomic DNA of *A. ferrooxidans* ATCC 23270 was prepared according to the manufacturer's instructions by using the EZ-10 spin column genomic DNA isolation kit (Bio Basic Inc.). Oligonucleotide primers were designed based on the possible open reading frames (ORFs) in the genomic sequence of *A. ferrooxidans* ATCC 23270 (www.tigr.org), and were synthesized by Bio Basic Inc. The *dsbG* gene was cloned from the total DNA of *A. ferrooxidans* by PCR with the following primers: DsbG-sin-NH_2-*Nco*I, 5′-GGCATAGAAT*CCATGG*AAACGGAAG-ATCGGAGC-3′; DsbG-NH_2-*Nco*I, 5′-GGCATAGA-AT*CCATGG*GGTGCGCCACCACCC-3′; and DsbG-CO_2-*Xho*I, 5′-TCCAA*CTCGAG*GGGGGTGCAGC-CTTCCGCG-3′. The *dsbG* gene with signal peptide was obtained by PCR with DsbG-sin-NH_2-*Nco*I and DsbG-CO_2-*Xho*I primers, and the *dsbG* gene without signal peptide was cloned with the DsbG-NH_2-*Nco*I and DsbG-CO_2-*Xho*I primers.

The DNA amplifications were carried out in a Perkin-Elmer DNA Thermal Cycler under heating at 93 °C for 3 min followed by 29 cycles (30 s denaturation at 95 °C, 30 s annealing at 58 °C, and 30 s elongation at 72 °C) and by a final DNA extension step at 72 °C for 7 min. The amplified product was maintained at 4 °C after cycling.

The resulting PCR product was gel purified, double digested, and ligated into a pET28a (+) expression vector, resulting in the pET28a-dsbG-S and pET28a-dsbG plasmid. The isolated pET28a-dsbG-S and pET28a-dsbG plasmid were then transformed into *E. coli* BL21 (DE3) competent cells for expression purposes.

Construction of *A. ferrooxidans* DsbG mutants

Site-directed mutagenesis by overlap extension was applied to obtain the DsbG(C119A) and DsbG(C122A) mutant expression plasmids. The plasmid pET28a-dsbG was used as a template for constructing mutant expression plasmids through a PCR reaction. The overlapping primers (with the mutated bases underlined) were: DsbG-C119A-5, 5′-GACCCCAAC<u>GCC</u>ATCTACTGCC-3′, codon TGC for cysteine was changed to codon GCC for alanine; DsbG-C119A-3, 5′-GGCAGTAGAT<u>GGC</u>GTTGGGGTC-3′, codon GCA for cysteine was changed to codon <u>GGC</u> for alanine; DsbG-C122A-5, 5′-CTGCATCTAC<u>GCC</u>CACCTGCTC-3′, codon TGC for cysteine was changed to codon GCC for alanine; DsbG-C122A-3, 5′-GAGCAGGTG<u>GGC</u>GTAGATGCAG-3′, codon TGC for cysteine was changed to codon <u>GGC</u> for alanine. Two separate PCR reactions were performed, each with one of the overlapping primers cited above and one flanking primer (DsbG-NH_2-*Nco*I or DsbG-CO_2-*Xho*I). The PCR amplification was performed by using *Pfu* DNA polymerase and the samples were subjected to 29 cycles of 30 s denaturation at 95 °C, 30 s annealing at 60 °C, and 30 s elongation at 72 °C. The resulting whole DNA fragment was obtained by further PCR amplification with two flanking primers. The products of the two PCR amplifications were purified and used as the templates. The isolated mutant plasmids pET28a-dsbG(C119A) and pET28a-dsbG(C122A) were constructed and transformed into the competent cells of *E. coli* BL21 (DE3) for expression.

Expression and purification of recombinant DsbG proteins

E. coli BL21 (DE3) cells with pET28a-dsbG plasmid and the mutant plasmids were grown at 37 °C in LB medium containing 40 μg/ml of kanamycin. Once the cultures reached an optical density of 0.6 at 600 nm, 1 mM IPTG was added to induce expression. Cells were then incubated at 30 °C for 4 h with shaking at 180 rpm. Expression of the recombinant DsbG with or without signal peptide was analysed by 15% (v/v) SDS-PAGE of total cell extracts. The *E. coli* BL21 (DE3) cells with pET28a-dsbG plasmid or mutant plasmid vectors were grown in 500 ml LB medium. The cells were harvested by centrifugation, and then suspended in start buffer (20 mM phosphate buffer, pH 7.4, 0.5 M NaCl). The cells were lysed by sonication 20 times for 30 s each time by using a 150-W Autotune Series High Intensity Ultrasonic sonicator equipped with an 8 mm-diameter tip.

The insoluble debris was removed by centrifugation, and a clear supernatant was further purified through a Hi-Trap column (GE Healthcare Ltd) according to the instructions from the manufacturer. The contents of the proteins were determined by the Bradford method with BSA as a standard. The purified DsbG was dialysed against a 20 mM potassium phosphate buffer, pH 6.0, 5% (v/v) glycerol, and then stored at −4 °C.

Preparation of scrambled RNase and isomerase assay

Scrambled RNaseA (scRNaseA) was prepared from wild-type RNaseA from bovine pancreas (Sigma) as described in Ref. 8. Disulphide-bond isomerase

activity was assayed by measuring the recovery of misfolded scRNaseA.

The reactivation of scRNaseA was monitored by DsbG. The reaction mixtures consisted of 100 mM Tris/AcOH acetate buffer (pH 8.0), 4.5 mM cCMP, 100 μM oxidized glutathione (GSSG), and 1.0 mM GSH. The purification of *A. ferrooxidans* recombinant DsbG wild-type protein and its mutant protein were performed at concentrations of 30 μM. After equilibrating the reaction at 25 °C, the reaction was initiated by the addition of scRNaseA to a final concentration of 8.5 μM. cCMP hydrolysis by the refolded RNaseA was monitored by the UV-visible spectrophotometer at 296 nm[9].

RESULTS AND DISCUSSION

Expression and purification of DsbG wild type and mutant proteins

The expression vectors pET28a-dsbG-S and pET28a-dsbG containing the ORF corresponding to the putative DsbG (AEF0162) sequence, with or without leader peptide, respectively, were used to transform *E. coli* BL21 (DE3). The expressed peptide derived from a plasmid containing the putative DsbG gene without signal peptide is shown in Fig. 1 (lane b), and the corresponding form with the signal peptide in Fig. 1 (lane c). The migrations of these bands were in agreement with the predicted sizes. This suggests that the Sec machinery of *E. coli* might recognize the secretion signals of *A. ferrooxidans*. The presence of another *A. ferrooxidans* periplasmic protein, P21, was confirmed and its signal peptide sequence could be recognized and incised during the cloning procedure in *E. coli*[10]. Most of the *A. ferrooxidans* periplasmic proteins (62.3%) possessed export signals of secretory proteins according to their sequences[11].

A Ni metal-affinity resin column was used to purify His-tagged putative DsbG and its mutant proteins in a single step. After dialysing the sample against 20 mM potassium phosphate buffer, pH 7.5, with 5% (v/v) glycerol, the purity of the putative DsbG and its mutant proteins was examined by SDS-PAGE (Fig. 2). The dominating bands correspond to a molecular mass of about 30 kDa, which is in agreement with the deduced molecular mass of DsbG.

Isomerase activity and the key active sites of DsbG

Isomerase activity of DsbG was monitored by DsbG-catalysed reactivation of scRNaseA to native RNaseA (Fig. 3). The DsbG-catalysed scRNaseA isomerization led to a time-dependent increase in cCMP cleavage activity of RNaseA. The addition of DsbG to

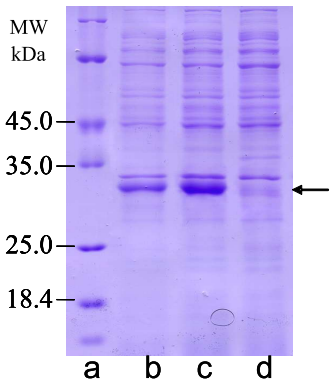

Fig. 1 The total cell proteins were separated by SDS-PAGE and stained with Coomassie blue. In vivo expression of the putative thiol:disulphide interchange protein DsbG (AEF0162) gene sequence in *E. coli*. The pET28a (+) plasmid containing the putative *dsbG* gene insert with or without leader peptide (lanes b and c) or the vector without the insert (lane d) was used to transform *E. coli* BL21(DE3).

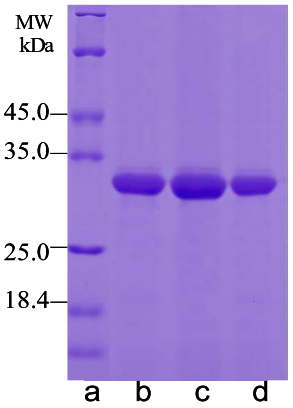

Fig. 2 Coomassie blue-stained SDS-PAGE of the purified DsbG from *A. ferrooxidans* ATCC 23270 and its mutant proteins. Lane a: molecular mass standards; lane b: purified DsbG wild type; lane c: purified DsbG C119A mutant; lane d: purified DsbG C122A mutant.

scRNaseA can significantly reactivate scRNaseA to native RNaseA activity, e.g., in 81% and 89% after about 40 min and 60 min of reactivation, respectively (Fig. 3 A and B). The mutants DsbG(C1119A) and DsbG(C122A) basically led to loss of that reactivation (Fig. 3 A(c, d)) of the native RNaseA activity (positive control) and about 10% of the native RNaseA activity from the scRNaseA (negative control). It suggests the two conserved cysteines Cys119 and Cys122 were crucial to DsbG disulphide isomerization activity. In contrast, the DsbG of *E. coli* is unable to catalyse the oxidative refolding of RNaseA in vitro[6,12].

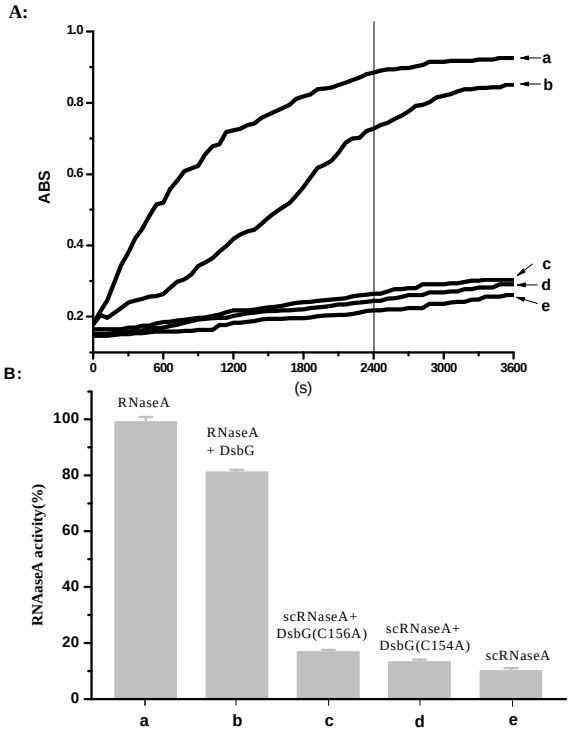

Fig. 3 DsbG-catalysed scRNaseA reactivation: (A) Continuous spectroscopic monitoring at 296 nm of the cleavage of 4.5 mM cCMP by (a) 10.0 μM wild type RNaseA (b) 10.0 μM scRNaseA and 30.0 μM DsbG, (c) 10.0 μM scRNaseA and 30.0 μM DsbG(C119A), (d) 10.0 μM scRNaseA and 30.0 μM DsbG(C122A), (e) 10.0 μM scRNaseA. (B) The (a) was set to 100% RNaseA activity, and all other reaction rates (b, c, d and e) were expressed as a percentage of this activity at 40 min.

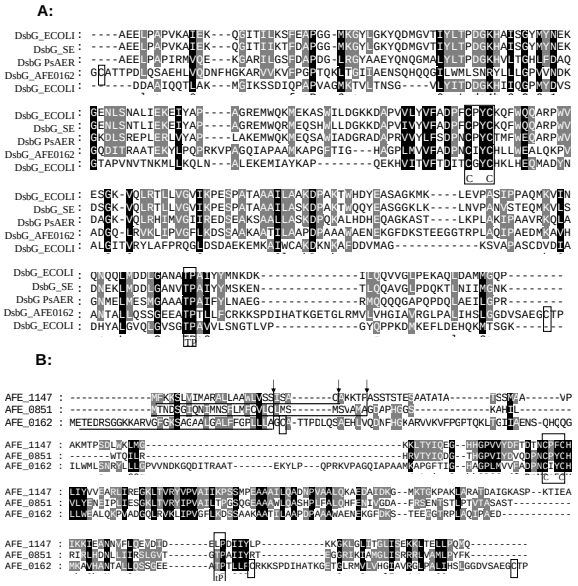

Fig. 4 (A) The sequence alignment of the putative thiol:disulphide interchange protein DsbG (AEF0162) of *A. ferrooxidans* with those of representative thiol-disulphide interchange proteins. DsbG_ECOLI (DsbG from *E. coli*); DsbG_SE (DsbG from *Salmonella enterica*); DsbG_PsAer (DsbG from *Pseudomonas aeruginosa*); DsbC_ECOLI (DsbC from *E. coli*); and DsbG_AEF0162 (DsbG from *A. ferrooxidans*). (B) Sequence alignment of *A. ferrooxidans* proteins with similarity to DsbG. Three protein sequences all contain leader peptides as indicated by the numbered lines below. The arrow indicates the site where the cleavage of the leader peptide would occur. AFE_0162, the putative thiol:disulphide interchange protein DsbG, contains a 302-amino-acid peptide from *A. ferrooxidans*. AFE_1147, a DsbG domain protein, contains a 225-amino-acid peptide from *A. ferrooxidans*. AFE_0851, a dsbG domain protein, contains a 201-amino-acid peptide from *A. ferrooxidans*.

A. ferrooxidans, one of the most studied molithoautotrophic bacteria in acidic mine drainage, obtains its energy from the oxidation of ferrous iron, elemental sulphur, or partially oxidized sulphur compounds [10,13]. The thiol groups, the breaking of disulphide bonds, and the rearrangement of proteins have been considered to play a key role in the sulphur oxidation [14]. Dsb proteins and closely related proteins contain thiol groups and disulphide bonds. The genome sequence of *A. ferrooxidans* ATCC 23270 has been shown to annotate the putative DsbG and DsbD, but does not give any functional annotation of DsbC protein (www.tigr.org). The present result proposes that the *A. ferrooxidans* DsbG protein may have an extended function, unlike that of *E. coli*, compensating for the absence of DsbC protein in *A. ferrooxidans* to enlarge the survival ability in extreme environments.

The BLASTP analysis of the amino acid sequence of the putative thiol:disulphide interchange protein DsbG (AEF0162) of *A. ferrooxidans* indicates that this protein has 23% identity and 37% similarity to the DsbG from *E. coli* and comparable values with those of many other DsbGs. The amino acid sequences of the putative DsbG (AEF0162) from *A. ferrooxidans* and DsbC from *E. coli* only has 13% sequence identity and 25% sequence similarity. The DsbG and DsbC from *E.coli* have 24% sequence identity. Two characteristically highly conserved motifs (CXXC and TP) were also present in all of the proteins compared (Fig. 4 A). The thioredoxin-like proteins share no overall sequence homology, yet they all contain a CXXC motif and a *cis*-Pro loop. The Thr in the *cis*-Pro loop may favour the reduced form of the

protein by interaction with the sulphur of the first Cys in the active site[12]. The active site of the putative DsbG (AEF0162) (Cys-Ile-Tyr-Cys) has an Ile residue. However, the active site of the other DsbG (Cys-Pro-His-Cys) has a Pro residue. There are also another three separate cysteine residues in the putative DsbG (AEF0162) sequence which are absent in other sequences (Fig. 4). Such differences may be related to the kind of substrate binding or affect the functions of the putative DsbG (AEF0162).

Upon analysing the genome sequence annotation of *A. ferrooxidans*, we found that not only AEF0162 was annotated as the putative DsbG, but also AFE_1147 and AFE_0851 were annotated as DsbG domain proteins. AEF0162 differs from AFE_1147 and AFE_0851 in the highly conserved motifs (CXXC and TP); the active site of the putative DsbG (AEF0162: Cys-Ile-Tyr-Cys) has an Ile residue but the active site of the DsbG domain proteins (AFE_1147 and AFE_0851: Cys-Pro-His-Cys) have a Pro residue, and the *cis*-Pro loop of AEF0162 and AFE_0851 (Thr-Pro) have Thr but the AFE_1147 has a Leu residue. It is also interesting that only the AEF0162 contains a cysteine at its N-terminal and two cysteines at its C-terminal, which are absent in AFE_1147 and AFE_0851, suggesting different functions. Whether the putative dsbG domain proteins (AFE_1147 and AFE_0851) catalyse oxidative refolding of RNase or have isomerase and chaperone activities needs further research.

Acknowledgements: This work was supported by the National Basic Research Programme of China (No. 2010CB630902) and National Natural Science Foundation of China (No. 50674101, No. 50974140). We thank Integrated Genomics Inc for making the *A. ferrooxidans* genome data available.

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