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**Toshihiro Kuroita · Takuya Kanno · Atsushi Kawai
Bunsei Kawakami · Masanori Oka · Yaeta Endo
Yuzuru Tozawa**

Functional similarities of a thermostable protein-disulfide oxidoreductase identified in the archaeon *Pyrococcus horikoshii* to bacterial DsbA enzymes

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Abstract We have isolated and characterized a gene for a putative protein-disulfide oxidoreductase (*phdsb*) in the archaeon *Pyrococcus horikoshii*. The open reading frame of *phdsb* encodes a protein of 170 amino acids with an NH_2-terminal extension similar to the bacterial signal peptides. The putative mature region of PhDsb includes a sequence motif, Cys-Pro-His-Cys (CPHC), that is conserved in members of the bacterial DsbA family, but otherwise the archaeal and bacterial sequences do not show substantial similarity. A recombinant protein corresponding to the predicted mature form of PhDsb behaved as a monomer and manifested oxidoreductase activities in vitro similar to those of DsbA of *Escherichia coli*. The catalytic activity of PhDsb was thermostable and was shown by mutation analysis to depend on the NH_2-terminal cysteine residue of the CPHC motif. Thus, in spite of their low overall sequence similarities, DsbA-like proteins of archaea and bacteria appear to be highly similar in terms of function.

Keywords Protein-disulfide oxidoreductase · DsbA · Archaea · *Pyrococcus horikoshii*

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T. Kuroita · Y. Endo · Y. Tozawa
Graduate School of Science and Engineering, Ehime University, Bunkyo-cho, Matsuyama, Ehime 790-8577, Japan

T. Kuroita · A. Kawai · B. Kawakami · M. Oka
Tsuruga Institute of Biotechnology, Toyobo Co. Ltd., Tsuruga, Fukui 914-0047, Japan

T. Kanno · Y. Endo · Y. Tozawa (✉)
Cell-Free Science and Technology Research Center, Ehime University, Bunkyo-cho, Matsuyama
Ehime 790-8577, Japan
E-mail: tozaway@ccr.ehime-u.ac.jp
Tel.: +81-89-9278274
Fax: +81-89-9278276

Introduction

The correct folding of many secreted proteins requires the formation of intramolecular covalent links between pairs of cysteine residues. Members of the DsbA family of proteins present in the periplasm of Gram-negative bacteria (Raina and Missiakas 1997) and protein-disulfide isomerases (PDIs) present in the endoplasmic reticulum of eukaryotic cells (Frand and Kaiser 1998) catalyze oxidation of such pairs of cysteines within substrate proteins to yield disulfide moieties. Bacterial DsbA enzymes possess a thioredoxin-like sequence motif, Cys-X-X-Cys (CXXC), that participates in direct thiol-disulfide exchange reactions with protein substrates. Mature DsbA of *Escherichia coli* is a 21.1 kDa monomeric protein that contains two active cysteine residues, Cys^{30} and Cys^{33} (corresponding to Cys^{49} and Cys^{52} in the premature polypeptide sequence, respectively), in the consensus motif CPHC and promotes disulfide formation in nascent polypeptides in the periplasm (Raina and Missiakas 1997; Martin et al. 1993). The reduced form of DsbA is reoxidized by DsbB, which is localized in the inner membrane, and the reduced form of DsbB then releases electrons to quinones and thereby returns to the oxidized state (Bader et al. 1999; Kadokura and Beckwith 2002). The endoplasmic reticulum-resident PDIs of eukaryotes possess a CG(H/P)C sequence motif as the active center for thiol-disulfide exchange, and the reduced form of these proteins is reoxidized by Ero1(Frand and Kaiser 1999; Freedman et al. 1994; Pollard et al. 1998).

Recent analyses of the genomes of several hyperthermophiles have revealed similarities and differences among the three kingdoms of life—bacteria, eukaryotes, and archaea (Adams 1993; Bult et al. 1996; Kawarabayasi et al. 1998, 1999, 2001; Klenk et al. 1997). Protein-disulfide oxidoreductases have been purified and characterized from hyperthermophiles such as *Sulfolobus solfataricus* (Guagliardi et al. 1994), *Methanococcus*

jannaschii (*Mj*Trx) (McFarlan et al. 1992), and *Pyrococcus furiosus* (*Pf*PDO) (Guagliardi et al. 1995), and a gene encoding a protein homologous to *Pf*PDO has been identified in *Pyrococcus horikoshii* (Kashima and Ishikawa 2003). Structural and biochemical analyses of *Pf*PDO have revealed two thioredoxin fold units and PDI-like functional features (Pedon et al. 2004; Ren et al. 1998). A thioredoxin reductase has also been identified in *P. horikoshii* (Kashima and Ishikawa 2003). These findings indicate that archaea possess cytosolic redox homeostasis systems as do bacteria and eukaryotes.

We now describe the cloning of a gene (*phdsb*) for a Dsb-like protein from the hyperthermophilic archaeon *P. horikoshii*. Predicted amino acid sequence from the gene contains a putative signal peptide in the amino terminus, and synthesis of recombinant wild-type and mutant forms of this protein revealed that it exhibits protein-disulfide oxidoreductase activities similar to those of *E. coli* DsbA. Furthermore, a database search identified genes homologous to *phdsb* in other archaea. Our study thus suggests that the PhDsb is a Dsb-like protein in archaea.

Materials and methods

Gene isolation and vector construction

Genomic DNA of *P. horikoshii* OT3 was obtained from American Type Culture Collection, and that of *E. coli* was prepared from the JM109 strain. Fragments of genomic DNA containing the coding sequences of PH1130 (*P. horikoshii*), DsbA (*E. coli*), or DsbC (*E. coli*) were amplified by PCR. The sequences of the primers used in this study are listed in Table 1. The primers PH1130-F (or PH1130LL-F) and PH1130-R were used for PhDsb (or PhDsbΔL, which lacks a corresponding leader sequence), DsbA-F and DsbA-R for DsbA, and DsbC-F and DsbC-R for DsbC. A DNA fragment encoding a form of PhDsb in which the native leader sequence was replaced with the corresponding sequence of *E. coli* DsbA was constructed by a two-step PCR with the primers DsbA-F and DsbA-linker-R for the leader sequence of DsbA and the primers PH1130-linker-F and PH1130-R for PhDsb without the presumed native leader sequence. The products of the first reactions were purified, mixed together, and subjected to PCR with the primers DsbA-F and PH1130-R. All PCR products were digested with *Bam*HI and inserted into the *Eco*RV–*Bam*HI site of pBluescript SK$^-$ (Stratagene, La Jolla, CA, USA) for sequence confirmation, after which the inserts were transferred to the *Nde*I–*Bam*HI site of pET11c (Stratagene). The open reading frame for the mature portion of human salivary amylase (S-amylase) was amplified from human cDNA (Clontech, Palo Alto, CA, USA) by PCR with the primers S-AMY-F and S-AMY-R (Table 1). The amplicon was digested with *Bam*HI and inserted into the *Eco*RV–*Bam*HI site of pEU3-NII (Toyobo). Transcripts were derived from pEU-S-AMY for cell-free protein synthesis with the use

Table 1 Oligonucleotide primers used in the present study

Primer	Sequence	Description
PH1130-F	5′-ATCCATATGAAGAGGGATTTGGCCCTACTC-3′	Forward primer for PhDsb, *Nde*I
PH1130LL-F	5′-ATCCATATGCAAACTAGTCAAGAAAAGTGGTTAGAG-3′	Forward primer for PhDsbΔL, *Nde*I
PH1130-R	5′-CCGGATCCCTAGTGTCCATTGAGCTTAAAC-3′	Reverse primer for PhDsb and PhDsbΔL, *Bam*HI
DsbA-F	5′-CCATATGAAGAAGATTTGGCTGGCGCTGGC-3′	Forward primer for DsbA, *Nde*I
DsbA-R	5′-CCGGATCCTTACTTCTTCTCGGACAGATATTTCAC-3′	Reverse primer for DsbA, *Bam*HI
DsbC-F	5′-ATCCATATGAAGAAAGGTTTCATGTTGTTTACTTTG-3′	Forward primer for DsbC, *Nde*I
DsbC-R	5′-CCGGATCCTTATTTACCGCTGGTCATCTTTTGGTG-3′	Reverse primer for DsbC, *Bam*HI
DsbA-linker-R	5′-GACTAGTTTGCGCCGATGCGCTAAACGCTA-3′	Reverse primer for leader of DsbA, linker sequence
PH1130-linker-F	5′-CGCATCGGCGCAAACTAGTCAAGAAAAGTG-3′	Forward primer for PhDsbΔL, linker sequence
S-AMY-F	5′-ATGCAGTATTCCTCAAATACACAAC-3′	Forward primer for human S-amylase
S-AMY-R	5′-CGGGATCCTATTACAATTTAGATTC-3′	Reverse primer for human S-amylase, *Bam*HI
Dsb-C53S	5′-TTTGGCTTAAACACGtccCCTCACTGTCAAAGA-3′	Middle-forward primer for PhDsbΔL(C53S) mutant
Dsb-C53S-R	5′-TCTTTGACAGTGAGGggaCGTGTTTAAGCCAAA-3′	Middle-reverse primer for PhDsbΔL(C53S) mutant
Dsb-C56S	5′-AACACGTGCCCTCACtctCAAAGAATGAAGAAA-3′	Middle-forward primer for PhDsbΔL(C56S) mutant
Dsb-C56S-R	5′-TTTCTTCATTCTTTGagaGTGAGGGCACGTGTT-3′	Middle-reverse primer for PhDsbΔL(C56S) mutant
Dsb-P100A	5′-GGAATTACTGGGgttGCCCTTATAGGAATATTC-3′	Middle-forward primer for PhDsbΔL(P100A) mutant
Dsb-P100A-R	5′-GAATATTCCTATAAGGGCaacCCCAGTAATTCC-3′	Middle-reverse primer for PhDsbΔL(P100A) mutant
T7-pro	5′-TAATACGACTCACTATAGGG-3′	For site-directed mutagenesis
T7-ter	5′-CTAGTTATTGCTCAGCG-3′	For site-directed mutagenesis

Single underlines indicate introduced restriction sites. Double underlines indicate linker sequences. Lowercase letters indicate a mutagenized codon

of a ScriptMAX Thermo T7 transcription kit (Toyobo) and were purified by phenol–chloroform extraction.

Site-directed mutagenesis

Site-directed mutagenesis of the PhDsbΔL gene was performed by in vitro overlap-extension PCR (Higuchi et al. 1988). Two separate PCRs were performed with a total of four primers to amplify two halves of the PhDsbΔL gene (Table 1). An outside-forward primer (T7-pro) was paired with a middle-reverse mutation primer (Dsb-C53S-R, Dsb-C56S-R, and Dsb-P100A) to generate the first half of the gene, and an outside-reverse primer (T7-ter) was paired with a middle-forward mutation primer (Dsb-C53S, Dsb-C56S, and Dsb-P100A-R) to synthesize the second half. The resulting two half-fragments of the PhDsbΔL gene, which bore overlapping sequences introduced by the two middle primers, were then mixed together and subjected to three PCR cycles. The resulting mutated DNA fragment was amplified by a further PCR with the T7 promoter (T7-pro) and T7 terminator (T7-ter) primers. The final PCR product was digested with *Nde*I and *Bam*HI and cloned into the corresponding sites of pET11c. The nucleotide sequences of the constructs were confirmed by DNA sequencing.

Protein expression and purification

Escherichia coli BL21 Codon Plus (DE3) RIL (Stratagene) cells harboring pET expression plasmids were grown in 100 ml of LB broth at 37°C until the optical density (OD) at 600 nm reached 0.6, after which the expression of recombinant proteins was induced by the addition of isopropyl-β-D-thiogalactopyranoside to a concentration of 1 mM and cultured for an additional 4 h. The cells were then harvested by centrifugation at $6{,}800 \times g$ for 15 min.

The preparation of periplasmic proteins was performed essentially as described (Thorstenson et al. 1997). The cells were resuspended in 10 ml of a solution containing 0.5 M sucrose, 0.2 M Tris–HCl (pH 8.0), and 0.5 mM EDTA and incubated on ice for 15 min. After the addition of 40 ml of TE buffer [10 mM Tris–HCl (pH 8.0), 1 mM EDTA], the cells were incubated on ice for an additional 30 min and then centrifuged at $6{,}800 \times g$ for 15 min. The recombinant proteins were purified from the resulting supernatant as described below. For preparation of cytosolic proteins, cells were resuspended in 10 ml of 50 mM Tris–HCl (pH 7.0) and then disrupted by ultrasonic treatment. The cell lysates were centrifuged at $17{,}700 \times g$ for 20 min, and the recombinant proteins in the supernatant were purified as described below.

Cells transformed with the expression plasmids for DsbA and DsbC were used for the preparation of DsbAΔL and DsbCΔL, which lack the corresponding leader sequences of the full-length proteins. Each protein was purified from the periplasmic fraction by ion-exchange column chromatography with DEAE-Sephadex (Amersham Biosciences, Piscataway, NJ, USA). The cytosolic fraction of cells transformed with the expression plasmid for PhDsbΔL and its mutants were fractionated by ion-exchange column chromatography with S-Sepharose (Amersham Biosciences). The fractions containing PhDsbΔL were heated at 85°C for 20 min and then centrifuged at $13{,}500 \times g$ for 10 min. The resulting supernatant was then subjected to the same chromatography once again. All fractions containing recombinant proteins were dialyzed against 20 mM HEPES–KOH (pH 7.6).

Gel filtration analysis

Pooled and concentrated fractions (40 μg of protein) of the S-Sepharose column containing PhDsbΔL were applied to a Superdex-75 10/300GL gel filtration column (Amersham Biosciences). Elution was performed with 50 mM Tris–HCl (pH 8.0) containing 150 mM NaCl at a flow rate of 0.5 ml min^{-1}. The column was calibrated with a Gel Filtration LMW Calibration kit (Amersham Biosciences) containing blue dextran (2,000 kDa, void volume), ovalbumin (43 kDa), and chymotrypsinogen A (25 kDa).

Assay of insulin disulfide reduction

The assay was performed essentially as described (Holmgren 1979). Solutions of bovine insulin (Roche Diagnostics, Tokyo, Japan) at a concentration of 1 mg ml^{-1} in 0.1 M potassium phosphate (pH 7.0) and 2 mM EDTA and of 10 mM dithiothreitol (DTT) were prepared freshly and maintained on ice. The insulin solution (150 μl) and enzyme solution were added to each well of a 96-well microtiter plate and the final volume was adjusted to 180 μl. The reaction was started by the addition of 7.5 μl of 10 mM DTT to each well, thorough mixing, and incubation at 25°C. The OD at 650 nm was measured every 5 min, and the nonenzymatic reduction of insulin by DTT was measured as a negative control.

Assay of oxidative folding of RNase A

The oxidative renaturation of RNase A was measured by monitoring the hydrolysis of 2′,3′-cytidine monophosphate (cCMP) as described (Lyles and Gilbert 1991). Reduced and denatured RNase A was generated by incubation of 5 mg of the native enzyme (Sigma-Aldrich, Tokyo, Japan) overnight in 1 ml of a solution containing 0.1 M Tris-acetate (pH 8.0), 2 mM EDTA, 6 M guanidine hydrochloride, and 0.14 M DTT. Immediately before use, the enzyme was separated from

the DTT and guanidine hydrochloride by centrifugal gel filtration with a G-25 spin column (Amersham Bioscience) that had been equilibrated with 0.1% acetic acid. A reaction mixture (100 µl) containing 50 mM Tris-acetate (pH 8.0), 4.5 mM cCMP, 1 mM glutathione (GSH), 0.2 mM glutathione disulfide (GSSG), and 5 µM enzyme was equilibrated at 25°C, and the assay was initiated by the addition of 2.2 µl of the reduced and denatured RNase A. The hydrolysis of cCMP resulting from the restoration of RNase A activity was recorded continuously as an increase in absorbance at 296 nm.

Cell-free protein synthesis and assay of human S-amylase activity

Cell-free protein synthesis with a wheat embryo extract was performed using PROTEIOS™ Wheat germ cell-free protein synthesis kit (Toyobo) according to the bilayer method as previously described but with slight modifications (Madin et al. 2000; Sawasaki et al. 2002). The reaction buffer of the PROTEIOS™ kit contains a relatively high concentration of DTT (2.5 mM), so we exchanged the DTT-based buffer for a glutathione-based one as described (Ryabova et al. 1997). The reaction mixture (50 µl) thus contained 10 µl of wheat embryo extract, 30 mM HEPES–KOH (pH 7.8), 95 mM potassium acetate, 2.65 mM magnesium acetate, 1.2 mM ATP, 0.25 mM GTP, 16 mM creatine phosphate, 0.38 mM spermidine, 0.3 mM of each of the 20 amino acids, 0.4 mM GSSG, 1 mM GSH, creatine kinase (0.5 mg ml^{-1}), RNase inhibitor (800 U ml^{-1}), mRNA (0.25 mg ml^{-1}) derived from pEU-S-AMY, and 3 µM of test enzyme or bovine serum albumin. As a control, 20 mM HEPES–KOH (pH 7.6) was added instead of the test protein. The substrate mixture (250 µl), containing the same components as the reaction mixture with the exception of mRNA, creatine kinase, RNase inhibitor, and test protein, was carefully overlaid on the reaction mixture (50 µl) in each well of a 96-well microtiter plate, which was then incubated at 26°C for 16 h. The contents of each well (300 µl) were then collected as crude amylase solutions.

The activity of the synthesized human S-amylase (5 µl) was measured with the use of a Diacolor Liquid AMY kit (Toyobo) and monitoring of the increase in absorbance at 415 nm. The amount of human S-amylase in each reaction was estimated by immunoblot analysis with specific antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Results

Isolation of a protein-disulfide oxidoreductase gene from *P. horikoshii*

Protein-disulfide oxidoreductases such as bacterial DsbA and eukaryotic PDIs share the active site sequence motif CXXC. To identify such a protein in archaea, we searched a database of the complete *P. horikoshii* genome sequence (Kawarabayasi et al. 1998) with nucleotide sequences corresponding to the CXXC motif as the query. The PH1130 gene (DDBJ accession no. D71054) was found to encode one CXXC motif, specifically Cys-Pro-His-Cys. The predicted PH1130 protein comprises 170 amino acids, and we designated the PH1130 gene and product as *phdsb* and PhDsb, respectively. A search of the DDBJ database with the sequence of *phdsb* as the query yielded sequences encoding a similar protein in *Pyrococcus abyssi* (accession no. F75086), *Thermococcus kodakaraensis* (Q5JGG9), and *P. furiosus* (Q8U2V4) (Fig. 1). The predicted mature region (see below) of PhDsb (residues 28–170) exhibits sequence similarities and identities of 96.5 and 71.3, 84.7 and 39.5, and 82.1 and 37.5% to the putative mature regions of these Dsb-like proteins of *P. abyssi* (residues 30–183), *T. kodakaraensis* (residues 20–194), and *P. furiosus* (residues 23–194), respectively. The proteins of *P. horikoshii* and *P. abyssi* thus appear highly related evolutionarily. All of the archaeal Dsb-like proteins possess the CPHC sequence, which is also conserved in many bacterial DsbA proteins (Fig. 1). The deduced protein sequence of each archaeal Dsb homolog also includes an NH_2-terminal extension similar to the signal sequences of bacterial DsbA enzymes. The cleavage sites for a signal peptidase were predicted with the use of the Signal P version 1.1 program (Nielsen et al. 1997). The charge distribution in the putative archaeal signal sequences was similar to that in bacterial DsbA signal sequences (Fig. 1). A positively charged n-region (containing lysine or arginine residues) is followed by an h-region (containing hydrophobic residues such as leucine or isoleucine) in the archaeal NH_2-terminal sequences, reflecting features found in the predicted signal sequences of *M. jannaschii* (Nielsen et al. 1999). A valid sequence alignment of the archaeal Dsb-like proteins with bacterial DsbA family members was not possible (data not shown), with obvious similarity being limited to the signal sequences and CPHC motif regions. The COOH-terminal regions of the archaeal Dsb homologs are shorter than those of bacterial DsbA proteins. The calculated molecular masses of the putative mature forms of PhDsb (16.5 kDa) and *P. abyssi* Dsb (17.6 kDa) are smaller than those of the mature forms of bacterial DsbA proteins such as that of *E. coli* (21.1 kDa). The isoelectric points (p*I*) of the putative mature forms of PhDsb and the Dsb proteins of *P. abyssi*, *T. kodakaraensis*, and *P. furiosus* were calculated to be 9.23, 8.12, 4.32, and 5.22, respectively.

Biochemical study on *E. coli* DsbA protein also indicates that *cis*-Pro^{151} (corresponding to proline-170 in the premature polypeptide sequence) has significant roles for stabilizing DsbA structure (Charbonnier et al. 1999). Results from structural analysis suggest interaction between *cis*-Pro^{151} and His^{32} (corresponding to histidine-51 in the premature polypeptide sequence in Fig. 1) residue in the CPHC catalytic motif (Charbonnier et al. 1999). This proline is conserved within a

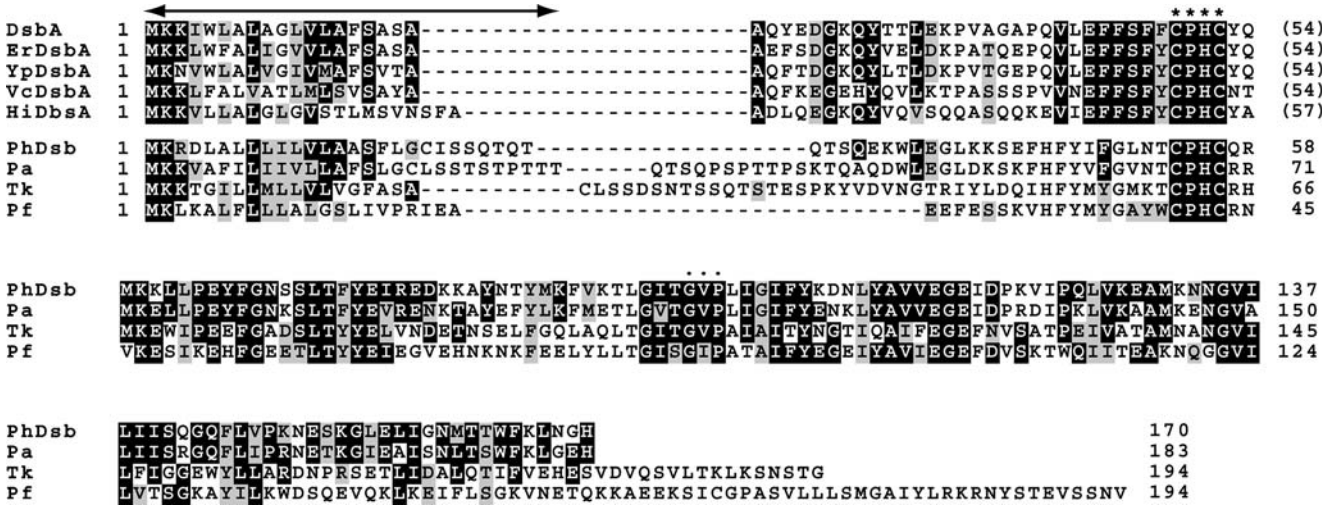

Fig. 1 Alignment of the predicted amino acid sequence of PhDsb with those of bacterial DsbA proteins and archaeal homologs. The sequences shown include *E. coli* DsbA (DsbA, accession no. P24991), *Erwina carotovora* DsbA (ErDsbA, Q9RB10), *Yersinia pestis* DsbA (YpDsbA, Q9XBV2), *Vibrio cholerae* DsbA (VcDsbA, P32557), *Haemophilus influenzae* DsbA (HiDsbA, P31810), *P. abyssi* homolog (Pa, F75086), *T. kodakaraensis* homolog (Tk, Q5JGG9), and *P. furiosus* homolog (Pf, Q8U2V4). Sequence similarity between bacterial DsbA proteins and archaeal Dsb homologs is restricted to the NH_2-terminal regions, so only these regions are shown for the bacterial sequences. *Hyphens* indicate gaps introduced to optimize alignment. Identical and similar residues among the various proteins are indicated by *dark and light shadings*, respectively, and residue numbers are shown on the *right*. *Asterisks* and *dots* indicate the redox active site and the Gly-(Val/Ile)-Pro consensus sequence conserved among DsbA family members, respectively. The *arrow* delineates the bacterial signal sequences and predicted archaeal signal sequences

consensus sequence of Gly-Val-Pro among bacterial DsbA proteins. The alignment of the archaeal Dsb proteins also shows conservation of the similar motif sequence, Gly-Val/Ile-Pro among four protein sequences (Fig. 1), suggesting functional similarity of the proline residue in these proteins to the *cis*-Pro^{151} residue in bacterial DsbA.

Expression of PhDsb in *E. coli*

To evaluate the activity of the *phdsb* product, we expressed the recombinant protein in *E. coli*. The signal sequences of various archaeal proteins expressed in *E. coli* mediate their localization to the periplasm (Horlacher et al. 1998; Jørgensen et al. 1997). We compared the function of the signal sequences of PhDsb and *E. coli* DsbA by constructing expression vectors for DsbA, the full-length PhDsb polypeptide, a chimeric protein (EcDL + PhDsbΔL) consisting of the NH_2-terminal signal sequence of *E. coli* DsbA, and the putative mature region of PhDsb, and an NH_2-terminally truncated form of PhDsb (PhDsbΔL) corresponding to the putative mature protein. We also constructed a vector for full-length *E. coli* DsbC. Bacterial cells expressing the various recombinant proteins were subjected to fractionation to isolate periplasmic and cytosolic components, and the fractions were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 2a). Whereas recombinant *E. coli* DsbA was detected in the periplasmic fraction, PhDsbΔL was present in the cytosolic fraction, and both full-length PhDsb and the EcDL + PhDsbΔL chimera were expressed but not present in either fraction. Recombinant *E. coli* DsbC was also detected in the periplasmic fraction (data not shown). We further purified PhDsbΔL, DsbA, and DsbC by ion-exchange chromatography. Analysis of the purified PhDsbΔL protein by gel filtration chromatography revealed it to behave as a monomer (Fig. 2b), similar to *E. coli* DsbA, which exists as a monomer in the periplasm (Raina and Missiakas 1997). The molecular mass of PhDsbΔL calculated on the basis of its elution from the gel filtration column was 16 kDa, consistent with the theoretical value of 16.5 kDa. The various purified recombinant protein preparations were estimated to be > 95% homogeneous by SDS-polyacrylamide gel analysis and Coomassie blue staining (data not shown).

Protein-disulfide oxidoreductase activity of PhDsb

For precise functional study of the archaeal protein, it is important to adjust all experimental conditions to physiologically optimum ones for *P. horikoshii*. On the other hand, it is difficult to estimate enzymatic functions of the novel protein without comparable data obtained from standard enzyme. Practically, it is very hard to compare functions of thermophilic PhDsb with other mesophilic Dsb enzymes in the range of high temperature (> 50°C). To examine basic function of PhDsb, we performed conventional oxidoreductase assay originally established for analyzing mesophilic Dsb enzymes and compared the activity with *E. coli* enzymes, DsbA and DsbC. The protein-disulfide reductase activity of

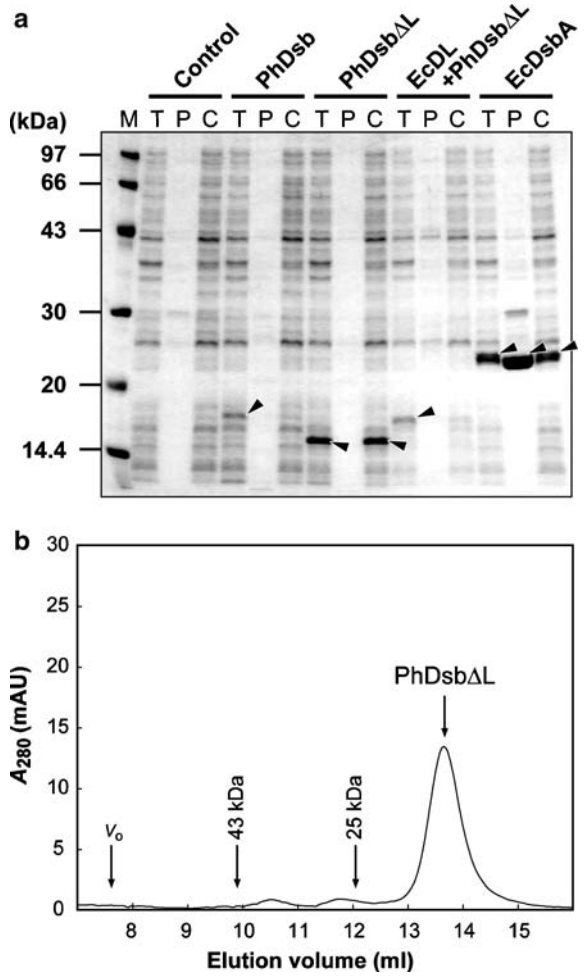

Fig. 2 Expression of PhDsb variants in *E. coli*. **a** SDS-polyacrylamide gel electrophoresis of recombinant PhDsb, PhDsbΔL, EcDL + PhDsbΔL, and *E. coli* DsbA (EcDsbA) in total cell lysates (*T*), periplasmic fractions (*P*), and the supernatants of cell lysates prepared by centrifugation at 17,700 × *g* for 20 min (*C*). Fractions prepared from *E. coli* transformed with an empty vector (pET11c) were used as controls. Equal volumes of protein solution (5 μl) were applied to each lane, and the gel was stained with Coomassie blue. *Lane M* molecular size markers. *Arrowheads* indicate recombinant proteins. **b** Gel filtration analysis of purified PhDsbΔL. Recombinant PhDsbΔL was purified from the cytosolic fraction of transformed *E. coli* cells and subjected to gel filtration on a Superdex-75 10/300GL column. Elution was monitored by measurement of absorbance at 280 nm (mAU, 10^{-3} absorbance unit). The void volume (V_o) and the elution positions of molecular mass markers are indicated

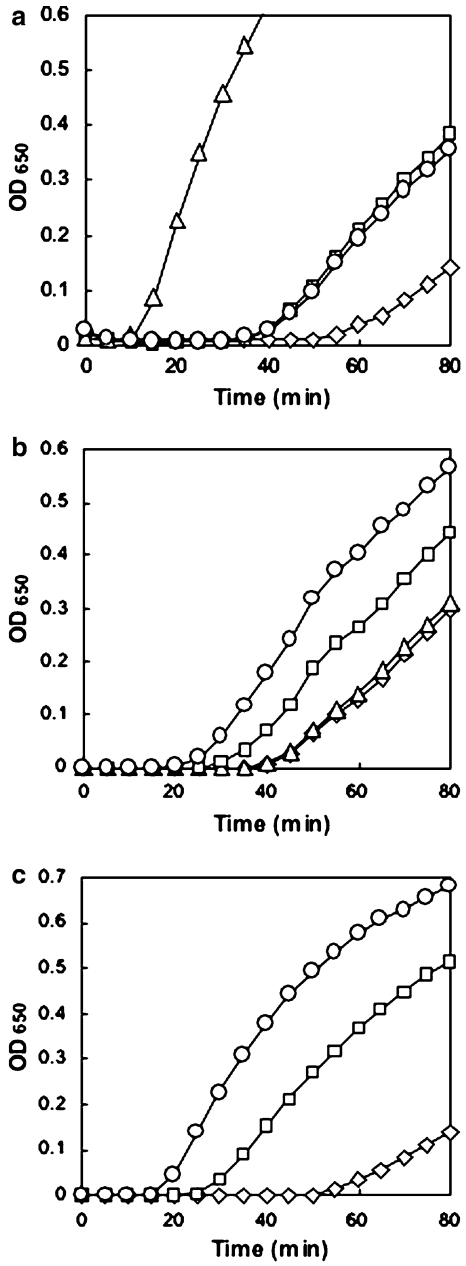

Fig. 3 DTT-dependent insulin reduction activity of PhDsb. **a** The DTT-dependent reduction of bovine insulin was assayed in the absence (*diamonds*) or presence (final concentration, 2 μM) of purified recombinant PhDsbΔL (*circles*), *E. coli* DsbA (*squares*), or *E. coli* DsbC (*triangles*). **b** The assay was performed in the absence (*diamonds*) or presence (final concentration, 3 μM) of wild-type PhDsbΔL (*circles*), PhDsbΔL(C56S) (*squares*), or PhDsbΔL(C53S) (*triangles*). **c** The assay was also performed in the absence (*diamonds*) or presence (final concentration, 3 μM) of wild-type PhDsbΔL (*circles*), or PhDsbΔL(P100A) (*squares*)

purified PhDsbΔL was examined in the presence of DTT with the oxidized form of bovine insulin as a substrate at 25°C (Holmgren 1979; Urban et al. 2001). The enzymatic activities of PhDsbΔL were compared with those of purified *E. coli* DsbA and DsbC. On the basis of the difference in the initial slope of the reaction rate obtained with PhDsbΔL and that apparent in the absence of enzyme, PhDsbΔL manifested a DTT-dependent insulin reduction activity (at 25°C) of 19.2 OD units min^{-1} μmol^{-1} (Fig. 3a). Although the temperature was lower than the physiological condition for the archaeon, PhDsbΔL showed similar activity to *E. coli* DsbA (21.4 OD units min^{-1} μmol^{-1}) in this experimental condition, but was lower than *E. coli* DsbC (58.1 OD units min^{-1} μmol^{-1}).

To investigate the contribution of the cysteine residues in the CPHC active site motif to the enzymatic activity of the protein, we prepared recombinant mutants

of PhDsbΔL in which either of these two cysteines was changed to serine. The PhDsbΔL(C53S) mutant, in which the NH_2-terminal cysteine of the CPHC motif was replaced by serine, showed no activity in the insulin reduction assay, whereas PhDsbΔL(C56S), in which the COOH-terminal cysteine was similarly replaced, exhibited partial activity (Fig. 3b). The NH_2-terminal cysteine of the CPHC motif thus appears to make a major contribution to the reductive activity of PhDsb.

Next, to examine the functional contribution of the proline-100 residue to the enzymatic activity, we prepared a mutant PhDsbΔL protein, in which the proline-100 was substituted by alanine, and performed oxidoreductase assay. The PhDsbΔL (P100A) mutant showed reduced reductase activity, which was about 60% of the wild-type PhDsbΔL protein (Fig. 3c), suggesting functional significance of the proline-100 residue in the PhDsb protein.

We also measured the ability of PhDsbΔL to catalyze the oxidative renaturation of RNase A (Lyles and Gilbert 1991) and compared the enzymatic activity of PhDsbΔL with that of purified *E. coli* DsbA. The oxidation activity of PhDsbΔL measured with the RNase A folding assay at 25°C was slightly greater than that of *E. coli* DsbA (30.5 vs 20.8 absorbance units min^{-1} μmol^{-1}) (Fig. 4). In addition, we evaluated the protein-disulfide oxidase activity of PhDsb in a cell-free protein synthesis system (Madin et al. 2000; Sawasaki et al. 2002) with GSH–GSSG instead of DTT and with human S-amylase, which contains five essential disulfide bridges (Nishide et al. 1986), as the substrate. The activity of S-amylase synthesized by the wheat embryo extract in the presence of PhDsbΔL was 1.7 times that of S-amylase synthesized in its absence (Fig. 5). The effect of PhDsbΔL was greater than that of DsbA. The wheat germ extract did not itself manifest amylase activity and did not interfere with amylase activity (data not shown). The specific activity of the S-amylase synthesized in the absence of test protein was estimated to be 10% of the enzyme isolated from human saliva, and the amounts of the synthesized protein were virtually identical, as revealed by semiquantitative immunoblot analysis, among the various reaction conditions (data not shown).

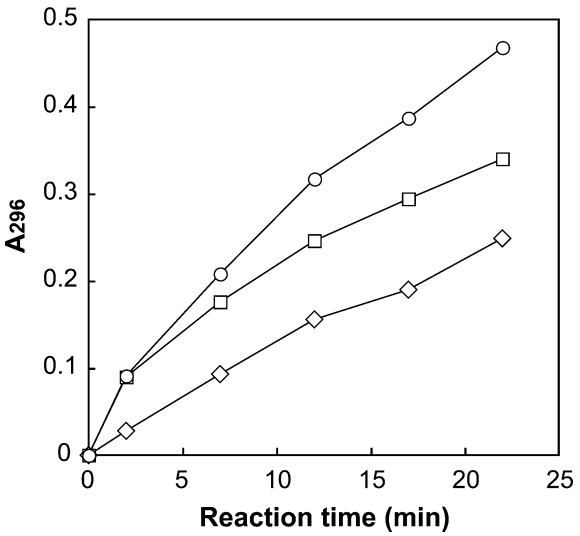

Fig. 4 Oxidative renaturation of RNase A by PhDsb. Restoration of the activity of reduced and denatured RNase A was assayed in the absence (*diamonds*) or presence (final concentration, 5 μM) of purified recombinant PhDsbΔL (*circles*), *E. coli* DsbA (*squares*)

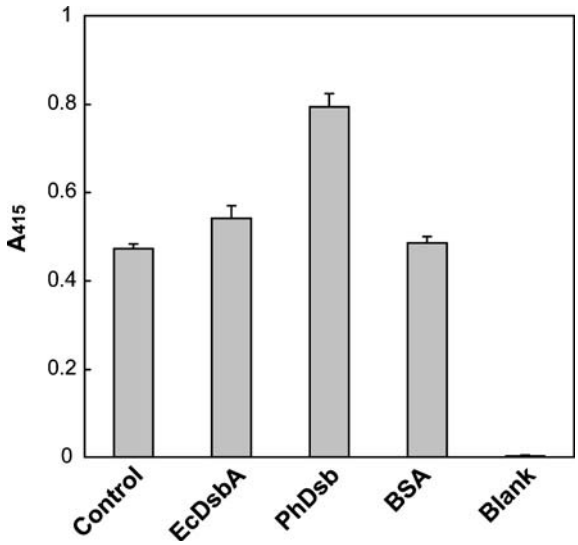

Fig. 5 Effect of PhDsb on the activity of human S-amylase synthesized in a cell-free protein synthesis system. Human S-amylase was synthesized for 16 h at 26°C in the absence (*Control*) or presence of the indicated proteins (final concentration, 3 μM). *BSA* bovine serum albumin. *Blank* refers to a reaction mixture without template mRNA. Data are means ± SE of values from four experiments

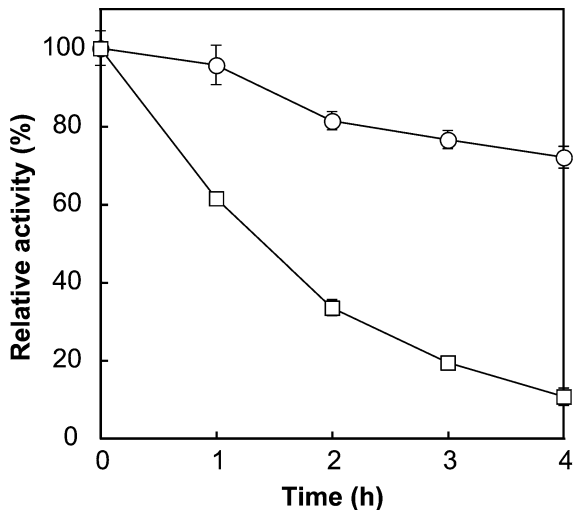

Fig. 6 Thermostability of the protein-disulfide oxidoreductase activity of PhDsb. Purified recombinant PhDsbΔL (*circles*) or *E. coli* DsbA (*squares*) was heated at 100°C for the indicated times in 50 mM potassium phosphate buffer (pH 7.0), after which each protein (final concentration, 5 μM) was assayed for DTT-dependent insulin reduction activity. Data are expressed as a percentage of the activity observed for the corresponding enzyme not subjected to heat treatment and are means ± SE of values from three experiments

No amylase activity was detected when the protein was synthesized in a reaction buffer containing 2 mM DTT (data not shown).

Thermostability of PhDsb

To examine the thermostability of PhDsb, we measured the reductase activity of recombinant PhDsbΔL after treatment at 100°C for various times. More than 70% of the activity of PhDsbΔL remained after treatment of the enzyme at 100°C for 4 h (Fig. 6). In contrast, the activity of DsbA was reduced by 90% after the same treatment.

Discussion

We have described the isolation of a gene for a novel archaeal protein-disulfide oxidoreductase as well as the characterization of the encoded protein. We also identified genes similar to that for the *P. horikoshii* enzyme (PhDsb) in other archaea, indicating that PhDsb-like proteins are conserved among members of the archaeal kingdom. All the predicted archaeal Dsb proteins possess a putative signal sequence at their NH_2-termini and a CPHC sequence, a conserved active site motif of bacterial DsbA proteins, in their mature regions. At present, we have no direct evidence that the PhDsb localizes into periplasm. However, the putative NH_2-terminal signal sequences of the archaeal proteins suggest an extracytoplasmic localization for the mature forms of the archaeal proteins.

Although the function of archaeal signal sequences remains to be characterized, a gene for a type-I signal peptidase has been identified in archaeal genomes (Tjalsma et al. 1998). It is therefore possible that Gram-negative bacteria and archaea share a common mechanism of signal sequence cleavage. However, expression of the PhDsb preprotein in *E. coli* did not result in its translocation into the periplasm but rather led to its aggregation in the cytoplasm. Moreover, replacement of the PhDsb signal peptide with that of *E. coli* DsbA did not result in translocation of the chimeric protein into the periplasm. We assume that the signal peptide region of the exogenous PhDsb disturbed *E. coli* translocation system and eventually affected translation efficiency. In the case of chimeric protein, EcDL + PhDsbΔL, high folding efficiency of the mature PhDsb region might cause inhibitory effects on the translocation process. The putative mature form of PhDsb was expressed in and isolated from the cytoplasm of transformed *E. coli* cells. The recombinant mature form of PhDsb behaved as a monomer (Fig. 2b), another feature of bacterial DsbA proteins (Raina and Missiakas 1997). Although the status of the native protein in *P. horikoshii* remains to be characterized, this observation suggests that functionally mature PhDsb is a monomeric enzyme.

Under the conditions established to characterize enzymes from mesophilic bacteria, the functional properties of PhDsb were similar to those of *E. coli* DsbA in protein-disulfide reductase. Despite the low sequence similarity of the mature proteins, the insulin reduction activities of PhDsb and DsbA appeared virtually identical (Fig. 3a). In the case of oxidative activity, the ability of PhDsb to mediate the oxidative renaturation of RNase A was slightly greater than that of *E. coli* DsbA even under this limited temperature condition (Fig. 4). Similar tendency was also observed in the cell-free protein synthesis of S-amylase (Fig. 5). Cell-free protein synthesis in the presence of protein-disulfide oxidoreductases or molecular chaperones has been pursued as a tool for protein engineering. A modified cell-free system that relies on GSH–GSSH instead of DTT has been shown to be effective for the synthesis of active periplasmic proteins (Ryabova et al. 1997). We have now shown that PhDsb promoted the folding of human S-amylase synthesized in such a system. In spite of the relatively low temperature (26°C) at which the assay was performed, PhDsb was more effective than *E. coli* DsbA in this regard. PhDsb or *E. coli* DsbA did not increase the specific activity of S-amylase after its synthesis (data not shown). These results indicate that PhDsb and DsbA catalyze co-translational formation of disulfide bonds in substrate proteins in a cell-free system. Given that the reduced form of PhDsb is reoxidized by GSSG, this archaeal protein might prove suitable for a cell-free protein expression system in which continuous supplementation or exchange of constituents is regulatable (Madin et al. 2000).

The calculated p*I* values of the mature forms of PhDsb and *P. abyssi* Dsb were markedly higher than that of *E. coli* DsbA (5.3), whereas those of the *T. kodakaraensis* and *P. furiosus* Dsb proteins were similar to that of *E. coli* DsbA. Other bacterial DsbA homologs have a high p*I*, with that of *Burkholderia cepacia* DsbA (accession no. Q9RHV8), for example, being 8.93. The broad substrate specificity of *E. coli* DsbA has been attributed to the uncharged surface features surrounding the active site (Guddat et al. 1997). The large differences in p*I* among DsbA-like proteins from both bacteria and archaea may thus not affect their common enzymatic functions. On the other hand, the characteristic p*I* of the PhDsb protein might positively affect interaction with substrate proteins. This may be one of the possible explanations for the observed functions of the recombinant PhDsb protein in the oxidoreductase assay and the cell-free protein expression analysis under the condition of low temperature (at 25–26°C).

In general, archaeal proteins are thermostable and function well at temperatures of > 70°C. We have now shown that PhDsb is markedly more thermostable than *E. coli* DsbA (Fig. 6). In addition, we have found that the purified mature form of PhDsb is stable during storage in aqueous buffer for > 6 months (data not shown).

The CPHC motif present in PhDsb is highly conserved in the bacterial DsbA family of proteins, and the functional roles of each cysteine residue and of the internal dipeptide sequence, Pro-His, of *E. coli* DsbA

have been well characterized (Bessette et al. 2001; Gane et al. 1995; Ondo-Mbele et al. 2005; Wunderlich et al. 1995). Site-directed mutagenesis of the cysteine residues in the CPHC sequence of PhDsb revealed that the PhDsbΔL(C53S) mutant was devoid of reductase activity, whereas the activity of PhDsbΔL(C56S) was decreased compared with that of the wild-type protein. The pK_a (where K_a is the acid constant) of the NH_2-terminal cysteine in the CPHC sequence of *E. coli* DsbA is markedly lower than that of the COOH-terminal cysteine (Grauschopf et al. 1995), and the oxidized form of DsbA, in which the two cysteines of the CPHC sequence form an internal disulfide bond, is more stable than is the reduced form (Raina and Missiakas 1997; Martin et al. 1993). Our results now indicate that the oxidative potential of the NH_2-terminal cysteine of the CPHC motif in PhDsb is also greater than that of the COOH-terminal cysteine.

It has been suggested that the *cis*-Pro^{151} residue of the *E. coli* DsbA plays an important role in exhibiting enzyme activity (Charbonnier et al. 1999). The DsbA (P151A) mutant was reported to exhibit an activity less than half that of the wild-type enzyme with respect to the folding of alkaline phosphatase in vivo (Charbonnier et al. 1999). They indicated that the substitution destabilized DsbA structure and changed the conformation of the active site, especially His^{32} in the CPHC motif, significantly. Our results using PhDsbΔL (P100A) mutant suggests that proline-100 residue in the PhDsb also plays a significant role in the catalytic function (Fig. 3c). Although it is necessary to perform further structural analysis to clarify precise function of the proline-100 residue in PhDsb, conservation of the CPHC motif and G(V/I)P motif among Dsb family suggests functional similarity between the PhDsb proline-100 and the bacterial DsbA Pro^{151}.

Together, the enzymatic function, active site motif consensus sequence, putative *cis*-Pro including consensus sequence, monomeric status of recombinant enzyme, and presumptive periplasmic localization of PhDsb suggest that this archaeal protein is a homolog of bacterial DsbA-type enzymes.

Although we have not ruled out the possible existence of a eukaryotic PDI-type enzyme in archaea, it is likely that the PhDsb and related proteins described in the present study play a major role in promoting the folding of secretory proteins in archaea. Our findings thus provide a basis for further elucidation of the mechanisms of protein folding in the third kingdom of life.

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