In Vitro Catalysis of Oxidative Folding of Disulfide-bonded Proteins by the Escherichia coli dsbA (ppfA) Gene Product*

Yoshinori Akiyama, Shigeki Kamitani†, Noriko Kusukawa§, and Koreaki Ito

From the Institute for Virus Research, Kyoto University, Kyoto 606-01, Japan and the §Takara Shuzo Biotechnology Research Laboratories, Settsu 520-21, Japan

It was shown previously that the Escherichia coli gene ppfA (dsbA) encodes a periplasmic protein, and its inactivation leads to a deficiency in disulfide bond formation of envelope proteins (Kamitani, S., Akiyama, Y., and Ito, K. (1992) EMBO J. 11, 57–62; Bardwell, J. C. A., McGovern, K., and Beckwith, J. (1991) Cell 67, 581–589). The DsbA/PpfA protein was overproduced, purified, and examined for its activities in vitro. Its abundance in a wild-type cell was estimated to be about 850 molecules which probably exist as homodimers as suggested by size exclusion chromatography. Purified DsbA markedly stimulated disulfide bond formation of E. coli alkaline phosphatase, either in vitro synthesized or purified and denatured, as well as of reduced bovine ribonuclease A. The DsbA-catalyzed rapid disulfide bond formation occurred after a lag period which appeared to be determined by the redox state of the reaction mixture and concentration of DsbA. Inclusion of higher concentrations of oxidized glutathione or DsbA shortened the lag period. We propose that DsbA, which proved to directly catalyze disulfide bond formation, may also have a role in maintaining the bacterial periplasm oxidative.

Until recently, protein folding was considered to occur spontaneously, being dictated by primary sequences and the laws of thermodynamics (Anfinsen, 1973). Indeed, in vitro refolding of purified and denatured proteins can in many cases be achieved without the aid of any other protein factors. However, such in vitro folding does not necessarily proceed at the rate and efficiency that could account for in vivo biosynthesis. At least two classes of cellular factors are currently known to assist in protein folding in vivo or in vitro. The first class includes a number of proteins collectively known as “molecular chaperones” represented by the HSP70 and the chaperonin (HSP60) families. Being located ubiquitously among a variety of subcellular compartments, molecular chaperones are believed to prevent undesirable folding pathways such as premature folding, aggregation, and misfolding, and thereby facilitate formation of biologically active protein structures (for reviews, Rothman, 1988; Ellis and Hemmingsen, 1989; Deshaies et al., 1988; Wienhues and Neupert, 1992; Gething and Sambrook, 1992). The second class includes enzymes that catalyze in vitro protein folding by stimulating rate-limiting reactions such as the formation and interchange of disulfide bonds and isomerization of peptidyl-prolyl bonds (for reviews, Gething and Sambrook, 1992; Fischer and Schmid, 1990). The respective enzymes are protein disulfide isomerase and peptidylprolyl cis-trans isomerase.

Protein disulfide isomerase, also known as the glycosylation site binding protein or β-subunit of prolyl hydroxylase, resides in the lumen of the endoplasmic reticulum (for reviews, Freedman, 1989; Noiva and Lennarz, 1992), but its physiological role has not been fully established. The gene disruption study with Saccharomyces cerevisiae indicates that protein disulfide isomerase is essential for viability of this species (LaMantia et al., 1991; Tachikawa et al., 1991).

Escherichia coli possesses cytoplasmic chaperones such as DnaK (HSP70 family), GroEL-GroES (chaperonin family), and SecB (for reviews, Kumamoto, 1991; Landry and Gierasch, 1991), as well as cytoplasmic and periplasmic peptidylprolyl cis-trans isomerases (Hayano et al., 1991; Liu and Walsh, 1990). The counterpart of protein disulfide isomerase had not been found in the periplasmic space of E. coli, which is topologically equivalent to the endoplasmic reticulum. Although the E. coli thioredoxin (for review, Holmgren, 1989) could exhibit protein disulfide isomerase-like activity under certain conditions in vitro (Pigiet and Schuster, 1986), its localization in the cytoplasm does not justify the physiological relevance of this activity. Recently, we and others isolated E. coli mutants defective in disulfide bond formation of cell envelope proteins (Kamitani et al., 1992; Bardwell et al., 1991). The gene affected (ppfA or dsbA)1 encodes a periplasmic protein of 21 kDa with a possible redox active site sequence, Cys-Pro-His-Cys. The purified DsbA protein was shown to be able to reduce oxidized insulin (Bardwell et al., 1991). However, there has been no direct evidence that it catalyzes the oxidation of thiols, the reaction that we believe is of physiological relevance.

In the work reported here, we estimated the cellular abundance of the DsbA protein, purified the protein from an overproducing strain, and examined its activity to oxidize reduced and denatured protein substrates. The results indicate that DsbA can catalyze directly and efficiently the formation of disulfide bonds of in vitro translated bacterial alkaline phosphatase (PhoA),2 as well as denatured re-

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1 In our previous study (Kamitani et al., 1992), ppfA mutants were screened based on defective folding of PhoA, and ppf stood for periplasmic phosphatase folding. However, since we have now shown that this gene product directly acts at a more specified step, the disulfide bond formation, the alternative nomenclature dsbA given by Bardwell et al. (1991) should be more appropriate. We hence adopt the latter nomenclature hereafter.

2 The abbreviations used are: PhoA, alkaline phosphatase; DTT, dithiothreitol; MOPS, 3-(N-morpholino)propanesulfonic acid.
duced purified PhoA or similar preparations of bovine pancreatic RNase A. Its possible role in maintaining oxidative states of the periplasm is also discussed in light of the experimental observations made in this study.

**EXPERIMENTAL PROCEDURES**

**Plasmids—**Plasmid pSK220, in which the dsbA gene is placed under the lac promoter-operator, was described previously (Kamitani et al., 1992) and used for overproducing DsbA. pBA74 carried a bla (β-lactamase)-phoA gene fusion in which the transcription-translation initiation region of bla is directly followed by an initiation codon and the mature sequence of phoA. This was constructed from pBA71 (Kamitani et al., 1992) by site-directed deletion using a synthetic oligonucleotide designed to remove the 5′ proximal coding region of bla and the linker sequence derived from TuphA as well as to reconstruct the 6 amino acids of the phoA sequence missing in TuphA (Manoil and Beckwith, 1985). Thus, pBA74 directs the synthesis of PhoA without its signal sequence under the control of the bla promoter.

**Purification of DsbA—**Cells of *E. coli* strain HB101 or MC4100 harboring pSK220 were grown in LB broth at 37°C to a late log phase, harvested by centrifugation, and treated with cold osmotic shock procedure (Neu and Heppel, 1965) to obtain the periplasmic fraction. This fraction was dialyzed against 10 mM Tris-HCl, pH 8.1, and applied to a DEAE-Sepharose CL-6B anion exchange column equilibrated with the same buffer. Under these conditions, most of the DsbA protein in the crude cell sample passed through the column. The flow-through fraction was dialyzed against the same buffer and rechromatographed. In this time, DsbA was retained in the column due presumably to the removal of the more polar other proteins. The column was then developed with a linear gradient (0-0.3 M KCl) elution. DsbA eluted at about 0.15 M KCl. The pooled peak fractions were dialyzed, concentrated, and applied to Sephacryl S-200 column equilibrated with 10 mM Tris-HCl, pH 8.1, which was developed with the same buffer. The column had been calibrated with the following molecular mass standards: γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and cyanocobalamin (1.35 kDa). The molecular mass of monomeric DsbA was determined by liquid chromatographic mass spectrometry (API II, Perkin-Elmer Sciex, Ontario, Canada).

**Determination of Protein Concentration and Immunoblotting of DsbA—**Protein concentration was determined using a Bio-Rad protein assay kit and bovine serum albumin as standard. For quantitation of DsbA in the cell, total cell proteins (from 4-40 mg/ml) were separated by SDS-polyacrylamide gel electrophoresis, electroblotted onto an Immobilon P (Millipore) and immunologically stained with rabbit antiserum against the purified DsbA protein. Cross-reacting materials were visualized using an ECL Western blotting detection system (Amersham International, Great Britain). Intensities of the DsbA bands were quantitated by a Biomed laser scanning densitometer (Biomed Instruments).

**Assay of Dsba Bond Formation of in Vitro-translated PhoA—**PhoA was synthesized and labeled with [35S]methionine using the coupled transcription-translation system of Zubay (1973) with pBA74 DNA as template, as described previously (Baba et al., 1990). Translation was terminated by adding 100 μg/ml chloramphenicol. Typically, 5 μl of the translation mixture was diluted to a final volume of 16.1 μl with ZnCl₂ (final concentration, 0.16 mM), Tris acetate, pH 8.0 (47 mM), and either DsbA protein and/or GSSG (final concentration as indicated). The mixture was incubated at 37 °C, and samples were withdrawn and mixed with equal volumes of 10% trichloroacetic acid. Protein precipitates were collected by centrifugation, washed with 10% TCA, and then dissolved in SDS sample buffer (Laemmli, 1970) containing 0.1 M iodoacetamide (to block free sulfhydryl groups) but no 2-mercaptoethanol (Kamitani et al., 1992).

Proteins were separated by SDS-polyacrylamide gel electrophoresis (15% acrylamide system described in Akiyama and Ito, 1983, or the 10% polyacrylamide slab gel used in this laboratory) and immunologically stained with rabbit antiserum. Electrophoresis, autoradiography, and visualized densitometric scanning. Quantitation of the oxidized and the reduced PhoA species that had been labeled with [35S]methionine was done by a Fujix bioimage analyzer BAS2000 (Fuji Film, Tokyo, Japan).

**Assay of Dsba Bond Formation of Purified and Denatured PhoA—**PhoA purified from E. coli (Takara Shuzo, Kyoto, Japan) at the concentration of 0.5 mg/ml was denatured and reduced by incubation at 37°C for 30 h in the presence of 6 M urea, 0.12 mM DTT, 1 mM MgCl₂, 50 mM Tris-HCl, pH 8.1, 25% glycerol, 50 mM KCl, 0.5 mM MgSO₄, and 2 mM EDTA.

For renaturation, the mixture was diluted 200-fold into solution containing 10 mM Tris-HCl, 0.16 mM ZnCl₂, 47 mM Tris acetate, pH 8.0, 1 mM MgCl₂, 0.55 mg/ml DsbA, and 0.9 mM GSSG. Samples were treated as described above except that silver staining was used for visualization of protein bands.

**Assay of Oxidative Folding of Reduced RNase A—**Reduced RNase A was prepared as follows: 20 mg of bovine pancreatic RNase A (Sigma, Type I-A) was dissolved in 2 ml of 6 M guanidine HCl, 0.2 M DTT, 2 mM EDTA, 50 mM Tris-HCl and was incubated at 37°C for 2 h in the dark. The solution was adjusted to pH 3.0 with 1 N HCl and applied to a Sephacryl G-25 column equilibrated with 0.2 M NaCl and 10 mM HCl. Reduced RNase A was recovered as a 1 mg/ml solution, sealed in nitrogen gas, and stored in the dark at −80°C. Measurement of RNase A activity was performed as follows: reduced RNase A (final concentration 0.2 mg/ml) was incubated in 0.6 mM DTT, 0.9 mM GSSG, 1 mM MgCl₂, 40 mM Tris acetate, pH 8.0, in the presence or absence of 0.55 mg/ml purified DsbA. Eighty-microliter portions were withdrawn from the reaction solution at appropriate intervals and were mixed with 1.12 ml of cyclic 2',3'-CMP (0.1 mg/ml), 0.1 M MOPS, pH 7.0, and incubated at 25°C for 3 min. Hydrolysis of cyclic 2',3'-CMP was measured by the increase of absorption at 284 nm (Blackburn, 1979).

**RESULTS**

**Purification of the Dsba Protein—**Periplasmic fraction prepared from a strain harboring the DsbA-overproducing plasmid, pSK220, was fractionated through two rounds of DEAE Sepharose CL-6B anion exchange chromatography as described under "Experimental Procedures," yielding a preparation that contained more than 90% pure DsbA protein (electrophoretogram not shown; also see Fig. 1A, lane 1). About 22 mg of DsbA was obtained from each liter of culture. The amino-terminal amino acid sequence was determined to be Ala-Gln-Tyr-Glu-Asp-Gly-Lys−, and the mass spectrometry measurement gave a covalent molecular mass of 21,132 ± 3 daltons. These results indicate that the 19-amino acid signal sequence is cleaved and there is no further processing. This preparation was used for the experiments described in this study.

The DsbA protein eluted slightly behind ovalbumin in Sephacryl S-200 size exclusion chromatography (Fig. 1B). The estimated molecular mass was about 40-50 kDa, raising the possibility that DsbA (subunit molecular mass, 21 kDa) is dimeric. Although results were unchanged when the Superose H12 column was used, independent measurements are required to establish the quaternary structure of DsbA. Mobility of DsbA in SDS-polyacrylamide gel electrophoresis was examined in the presence or absence of 2-mercaptoethanol. DsbA treated with the reducing agent was electrophoresed slightly but reproducibly slower than the untreated one (Fig. 1A). As such, differential mobilities should reflect the presence and absence of intramolecular disulfide bonds, the results indicate that the pair of cysteine residues at the proposed active site of DsbA (Kamitani et al., 1992; Bardwell et al., 1991) are mostly oxidized at least after purification.

**Quantification of Dsba in the Cell—**Anti-Dsba rabbit serum was raised against the purified preparation. Various amounts of total cell extracts from strain MC4100 were electrophoresed, and the Dsba protein was immunologically stained...
after blotting onto a membrane filter. Comparison with the intensities obtained with known amounts of the purified DsbA led to an estimation that a single cell contains about 850 molecules of DsbA subunit. Based on this result, the subunit concentration of DsbA in the periplasm can be calculated as about 4 to 8 μM, assuming that the periplasm occupies 20 to 40% of a typical cell volume (Stock et al., 1977) of 0.88 μm³ (Goodsell, 1991).

Promotion of Disulfide Bond Formation of in Vitro-synthesized PhoA by Purified DsbA—We used PhoA, the model protein in our previous in vivo screening of the ppfA (dsbA) mutants (Kamitani et al., 1992), as a substrate to study in vitro activities of the DsbA protein. PhoA is a homodimeric, Zn²⁺- and Mg²⁺-containing protein having two intrasubunit disulfide bonds. It can assume protease-resistant and enzymatically active structure only after translocation into the periplasmic space in vivo; the disulfide bonds of PhoA are not formed in the cytoplasm (Derman and Beckwith, 1991). We constructed a plasmid, pBA74, carrying a phoA derivative devoid of its signal sequence-coding region and controlled by the transcription and translation initiation regions of bla. This plasmid directed the synthesis of the “mature”-sized PhoA protein in the coupled transcription-translation system in vitro (Fig. 2A). However, the in vitro-synthesized PhoA molecules were enzymatically inactive and completely trypsin-sensitive (data not shown) when left in the milieu of in vitro translation.

The presence and absence of intrachain disulfide bonds of PhoA can be distinguished by a mobility difference in SDS-polyacrylamide gel electrophoresis under nonreducing conditions (Kamitani et al., 1992; Bardwell et al., 1991). The in vitro-synthesized PhoA molecules were exclusively in the reduced form and remained so for at least 4 h.³

When the translation products were supplemented with Zn²⁺ (0.16 mM) and GSSG and incubated at 37 °C, the oxidized form of PhoA was generated gradually with time (Fig. 2C). The ratio of reduced to total PhoA molecules decreased with the first order kinetics that depended on the concentration of GSSG (Fig. 2D).

We then incubated the translation products in the presence of purified DsbA. In the absence of GSSG and in the presence of 0.55 mg/ml DsbA, PhoA remained mostly reduced until about 90 min, after which abrupt oxidation took place within 5–10 min (Fig. 2, B and D). Similar experiments with varied concentrations of DsbA showed that the oxidation of PhoA always occurs abruptly within a short time window, and higher concentrations of DsbA shorten the lag period preceding this conversion. The DsbA concentrations and the lag lengths were inversely proportional (Fig. 3), suggesting that a DsbA-

³ DTT was carried over and present during this reaction, but it was not known what proportion of it had remained reduced after the transcription-translation.
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DsbA-dependent linear reaction proceeded during the lag period. In addition to higher concentration of DsbA, the addition of GSSG shortened or eliminated the lag period. For instance, inclusion of 0.075 mM GSSG shortened the lag period from 90 min to 30 min in the presence of 0.55 mg/ml DsbA (Fig. 4B), and from 70 min to almost zero in the presence of 1.1 mg/ml of DsbA (Fig. 4C). GSSG alone at this concentration was virtually ineffective (Fig. 4D). In the presence of 0.1 mM GSSG, 0.55 mg/ml DsbA was able to catalyze the oxidation without a lag period (Fig. 4A).

These results indicate that although the disulfide bond formation of PhoA can take place slowly in the presence of nonspecific oxidant (GSSG), it can be remarkably stimulated by DsbA under certain conditions, namely in the presence of a low concentration of GSSG or after preincubation with DsbA itself.

DsβA-mediated Oxidation of Purified PhoA—The reaction mixtures used in the above experiments contained cytoplasmic proteins and ribosomes as well as free cysteine and DTT carried over from the S30 transcription-translation mixture. The catalytic activity of DsbA on purified PhoA was examined to exclude possible effects from other unrelated proteins and to verify redox requirements for the reaction. Reduced and denatured PhoA was diluted into renaturation buffer and incubated in the presence of 0.55 mg/ml DsbA plus either 0.1 mM or 0.9 mM GSSG. The carry-over of DTT from the reduced-denatured substrate amounted to 0.6 mM. In a control experiment without DsbA (Fig. 5A), or even in the presence of DsbA and 0.1 mM GSSG (data not shown), PhoA remained reduced for 60 min or longer. In contrast, a majority of the PhoA molecules were oxidized in the presence of DsbA upon 20 min of incubation with 0.9 mM GSSG (Fig. 5B). As was the case with the in vitro-translated samples, the DsbA-mediated oxidation was not linear and was accompanied by a lag period. Thus, DsbA acts directly to stimulate disulfide bond formation of PhoA without involving any other protein mediators. The presence of oxidized sulfhydryls, in this case GSSG, in stoichiometric excess against reduced thiols (DTT) favors the reaction.

DsβA-stimulated Oxidation and Disulfide Bond Isomerization of RNase A—The ability of DsbA to stimulate oxidative refolding of reduced RNase A was examined. Oxidation was initiated by addition of DsbA and/or GSSG as described under “Experimental Procedures.” When incubated in the presence of DsbA, GSSG (0.9 mM), and DTT (0.6 mM) for 60 min, about 40% of RNase activity was recovered, whereas the recovery was only 15% in the absence of DsbA (Fig. 6).

To examine whether DsbA can catalyze exchange of preformed disulfide bonds, an RNase A preparation with scrambled disulfide bridges was incubated with DsbA in the presence of excess GSH over GSSG (Fig. 7). The recovery of RNase activity was significantly higher in the presence of DsbA than in its absence, and the DsbA effect was concentration-dependent (Fig. 7, squares and triangles). This indicates that DsbA can stimulate disulfide bond interchange reactions. The redox conditions we tuned for the oxidative folding (0.6 mM DTT and 0.9 mM GSSG) did not allow either spontaneous or DsbA-stimulated activation of the scrambled RNase A (Fig. 7, circles).

**DISCUSSION**

Taking advantage of the simple electrophoretic assay, we focused our analyses of in vitro activity of DsbA on the disulfide bond formation step of the PhoA folding. This approach is much simpler than examining the total process of PhoA folding that should include, in addition to general folding reactions, some PhoA-specific events such as dimerization and incorporation of metal ions. We have evidence...
under the buffer condition used for the oxidative folding of proteins. PhoA and RNase A (Fig. 7), suggesting that interchange bonded species. Thus, the physiological relevance of the weak partial trypsin resistance and then by acquisition of complete serine proteinase. This enzyme may exist in excess in the cell and may function to maintain the periplasmic oxidative. Lyles and Gilbert (1991) proposed that the highly concentrated protein disulfide isomerase in the endoplasmic reticulum may contribute to the redox buffering of the compartment. DsbA itself contributes to the oxidative state of the periplasm. We have indeed shown that DsbA is able to change redox state of the "solvent" in vitro. Although fairly high concentrations of DsbA were used in our in vitro reactions, they were comparable to the estimated concentration of DsbA in the periplasm. This enzyme may exist in excess in the cell and may function to maintain the periplasmic oxidative. DsbA can directly oxidize protein thiols, extending the observation of Bardwell et al. (1991) that the periplasmic space is in a redox state that favors the DsbA action. On the other hand, the fact that the disulfide bond formation of PhoA is negligible in the mutants defective in dsbA (Kamitani et al., 1992; Bardwell et al., 1991) indicates that the periplasmic space without DsbA is not as oxidative as allowing significant levels of spontaneous thiol oxidation. Possibly, DsbA also possesses a weak disulfide interchange activity. However, neither DsbA-mediated nor spontaneous disulfide interchange was detected under the buffer condition used for the oxidative folding of PhoA and RNase A (Fig. 7), suggesting that interchange reactions could be disregarded in these reactions; DsbA-aided disulfide bond formation appears to occur correctly without going through any isomerization steps. Recently, oxidative folding of pancreatic trypsin inhibitor polypeptide was re-examined in detail (Weisman and Kim, 1991). The results seriously questioned the validity of the notion that intermediate steps of protein folding involves incorrectly disulfide-bonded species. Thus, the physiological relevance of the weak isomerase activity of DsbA remains obscure. We have shown that DsbA is able to stimulate the refolding of reduced bovine RNase A, which contains 8 cysteines, and therefore conclude that DsbA can assist in the formation of correctly positioned multiple disulfide bonds of both prokaryotic and eukaryotic proteins.

The DsbA-catalyzed oxidation of PhoA occurred abruptly after a lag the length of which was dependent on the concentration of DsbA (Figs. 2–4). More detailed kinetic study showed that the conversion completes within 5 min. The oxidation kinetics are in sharp contrast to the gradual oxidation induced by a low molecular weight oxidant, GSSG. Increased DsbA concentrations as well as the simultaneous presence of GSSG markedly shortened or abolished the lag time. We interpret these observations as follows. The rapid oxidation that occurs within the short time frame represents the intrinsic catalytic ability of DsbA. This activity of DsbA requires certain levels of oxidative environment that can be fulfilled by the inclusion of GSSG. In the absence of GSSG, DTT in the translation mixture initially inhibits the DsbA action. Subsequently, DsbA overcomes the inhibition by catalyzing the oxidation of DTT, and the time required for this oxidation will appear as a lag time before DsbA can act on reduced PhoA. Perhaps, DsbA acts catalytically, with the air oxygen as an electron acceptor, and the time required for quenching DTT should be inversely correlated to the concentration of DsbA, in accordance with this hypothesis.

The rapid formation of disulfide bonds in vitro (Kamitani et al., 1992; Bardwell et al., 1991) suggests that the periplasmic space is in a redox state that favors the DsbA action. On the other hand, the fact that the disulfide bond formation of PhoA is negligible in the mutants defective in dsbA (Kamitani et al., 1992; Bardwell et al., 1991) indicates that the periplasmic space without DsbA is not as oxidative as allowing significant levels of spontaneous thiol oxidation. Possibly, DsbA itself contributes to the oxidative state of the periplasm. We have indeed shown that DsbA is able to change redox state of the "solvent" in vitro. Although fairly high concentrations of DsbA were used in our in vitro reactions, they were comparable to the estimated concentration of DsbA in the periplasm. This enzyme may exist in excess in the cell and may function to maintain the periplasmic oxidative. Lyles and Gilbert (1991) proposed that the highly concentrated protein disulfide isomerase in the endoplasmic reticulum may contribute to the redox buffering of the compartment. DsbA protein was suggested to be dimeric from the gel filtration profiles. Protein disulfide isomerase is a homodimer each having two "active sites," whereas another thiol-related enzyme thioredoxin is a monomer having only one active site. Our results using the purified system clearly showed that DsbA can directly oxidize protein thiols, extending the observation of Bardwell et al. (1991) who demonstrated the activity of this enzyme in the opposite direction in the presence of DTT. It remains possible, however, that some other cellular factors, either proteinaceous and/or of low molecular weight, participate in the recycling (reoxidation) of DsbA. The immediate and eventual electron acceptors in the cell remain to be identified.

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