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# DsbG, a Protein Disulfide Isomerase with Chaperone Activity*

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**Feng Shao‡, Martin W. Bader§, Ursula Jakob§, and James C. A. Bardwell§¶**

*From the §Department of Biology and the ‡Program in Biomedical Sciences, University of Michigan, Ann Arbor, Michigan 48109-1048*

**DsbG, a protein disulfide isomerase present in the periplasm of *Escherichia coli*, is shown to function as a molecular chaperone. Stoichiometric amounts of DsbG are sufficient to prevent the thermal aggregation of two classical chaperone substrate proteins, citrate synthase and luciferase. DsbG was also shown to interact with refolding intermediates of chemically denatured citrate synthase and prevents their aggregation *in vitro*. Citrate synthase reactivation experiments in the presence of DsbG suggest that DsbG binds with high affinity to early unstructured protein folding intermediates. DsbG is one of the first periplasmic proteins shown to have general chaperone activity. This ability to chaperone protein folding is likely to increase the effectiveness of DsbG as a protein disulfide isomerase.**

In the cell, protein folding is assisted by two classes of proteins, the folding catalysts and the molecular chaperones (1). Folding catalysts accelerate rate-limiting steps in the folding pathway. The formation and isomerization of disulfide bonds is one rate-limiting step in protein folding (2). Consequently, both prokaryotic and eukaryotic cells have developed systems that catalyze disulfide bond formation (DsbA) and isomerization (protein disulfide isomerase PDI) *in vivo* (2–4). Molecular chaperones, the second class of folding helpers, act by preventing off pathway reactions such as misfolding and aggregation. They recognize and bind to aggregation-sensitive folding intermediates, thereby keeping their free concentration low and inhibiting aggregation (5). It has been found that eukaryotic PDI, a folding catalyst in the endoplasmic reticulum, also exerts chaperone activity *in vitro* (4, 6–8). This chaperone activity likely supports the isomerase activity of PDI.

Disulfide bond formation and isomerization is catalyzed in the periplasm of Gram-negative bacteria by a battery of Dsb enzymes. All the known Dsb proteins contain the Cys-Xaa-Xaa-Cys sequence motif, and nearly all are likely to incorporate the thioredoxin fold (9). Biochemical and genetic studies demonstrated that DsbA, a soluble periplasmic protein, donates its disulfide bond directly to folding proteins. DsbA is reoxidized by the inner membrane protein DsbB (10–13). Recently, we have reported that DsbB is, in turn, reoxidized by the electron transport system using quinones as electron acceptors (14). Because DsbA has the most oxidizing disulfide known, the oxidation of protein thiols by DsbA is very rapid, and the formation of some incorrect disulfide bonds in periplasmic proteins seems unavoidable. DsbC, a periplasmic protein that has both disulfide isomerase and chaperone activity (15–18), is most likely involved in unscrambling these misformed disulfide bonds.

Recently, a new member of the Dsb family, DsbG, has been identified (19–21). Although the exact *in vivo* role of DsbG is controversial, recent experiments indicated that DsbG contributes to the isomerization activity in the periplasm of *Escherichia coli* (21). DsbG shows disulfide isomerase activity *in vitro* (19, 21). In this report we demonstrate that DsbG functions also as a molecular chaperone. DsbG is capable of interacting with both highly structured, late folding intermediates and unstructured, early folding intermediates of citrate synthase and luciferase. The folding helper activity of DsbG is not dependent on its active site cysteines.

## EXPERIMENTAL PROCEDURES

*Expression and Purification of DsbG*—The expression strain BL21/pETdsbG was very generously provided by Paul Bessette and George Georgiou (University of Texas, Austin) (21). The expression strain was grown at 30 °C in Luria broth medium supplemented with 100 mg/liter ampicillin to midlog phase ($A_{600}$ = ~0.5), and DsbG expression was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside. After 12 h of growth at 30 °C, the cells were harvested by centrifugation (6,000 × *g*, 15 min, 4 °C), and the cell pellet was resuspended in 40 mM HEPES, and 5mM EDTA, pH 7.5. Polymyxin B sulfate was added to a final concentration of 1 mg/ml to permeabilize the outer membrane. After 30 min of incubation on ice, the periplasmic proteins were separated from the protoplasts by centrifugation (16,000 × *g*, 30 min, 4 °C). The supernatant was dialyzed against 20 mM MES,[1] pH 6.0, 1 mM EDTA overnight, and DsbG was purified according to Straaten *et al.* (20). The purified protein was then dialyzed against 20 mM HEPES, pH 7.5, and stored at −70 °C.

Protein concentration of DsbG was determined spectrophotometrically at 280 nm using a calculated extinction coefficient of 16,570 $M^{-1}$ $cm^{-1}$ (22). Purified DsbG was in its oxidized state as judged by fluorescence measurements (21). Reduced DsbG was generated by incubating purified DsbG in 10 mM DTT (in 20 mM HEPES-KOH, pH 7.5) for 20 min on ice. DTT was purified away from reduced DsbG by PD10 gel filtration (Amersham Pharmacia Biotech). The extent of reduction was >95% as monitored by fluorescence (21). The reduced DsbG was stored at −70 °C and stable for more than 2 months.

*Construction of a Cysteine-less DsbG Mutant*—The two cysteines (Cys-126 and Cys-129) of DsbG were both mutagenized to serine by oligonucleotide-directed mutagenesis, following the protocol of QuikChange ™ site-directed mutagenesis kit (Stratagene). This was achieved by polymerase chain reaction amplification using the primer 5′-cgccgatccgttctCcccatatAgtaaacagttctggc-3′ and its complementary primer. The resulting plasmid carrying the mutated gene encoding a cysteine free DsbG variant was verified by DNA sequencing using T7 promoter sequencing primer.

*Chaperone Activity of DsbG*—The chaperone activity of DsbG was determined using citrate synthase (CS) or luciferase as chaperone substrate proteins. To monitor the influence of DsbG on the thermal aggregation of CS or luciferase (Roche Molecular Biochemicals), the proteins were diluted to final concentrations of 0.15 or 0.10 μM, respectively, into prewarmed 40 mM HEPES, pH 7.5, at 43 °C under

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¶ To whom correspondence should be addressed: University of Michigan, Dept. of Biology, Ann Arbor, MI 48109-1048. Tel.: 734-764-8028; Fax: 734-647-0884; E-mail: jbardwel@umich.edu.

[1] The abbreviations used are: MES, 4-morpholinoethanesulfonic acid; DTT, dithiothreitol; CS, citrate synthase.

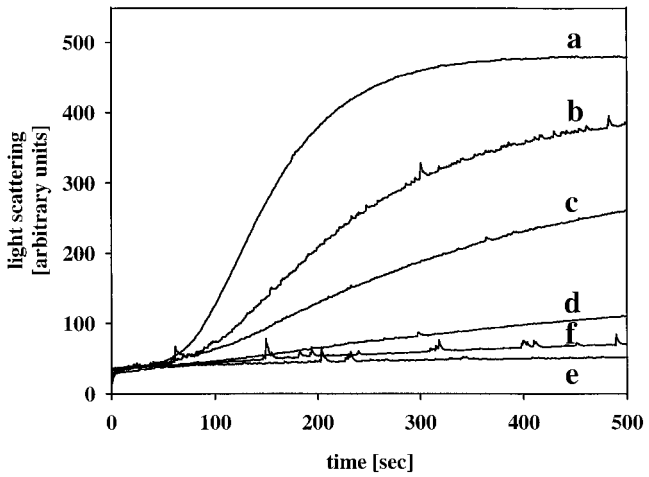


FIG. 1. **DsbG suppresses the thermal aggregation of luciferase at 43 °C.** Luciferase was diluted to a final concentration of 0.10 $\mu$M into 40 mM HEPES-KOH buffer, pH 7.5, equilibrated at 43 °C in the absence (*a*) or in the presence of 0.05 $\mu$M (*b*), 0.1 $\mu$M (*c*), 0.25 $\mu$M DsbG (*d*), or 0.5 $\mu$M DsbG (*e*), respectively. *line f* shows the influence of 0.5 $\mu$M reduced DsbG on the thermal aggregation of luciferase, monitored in the presence of 2 mM DTT. Light scattering measurements were performed.

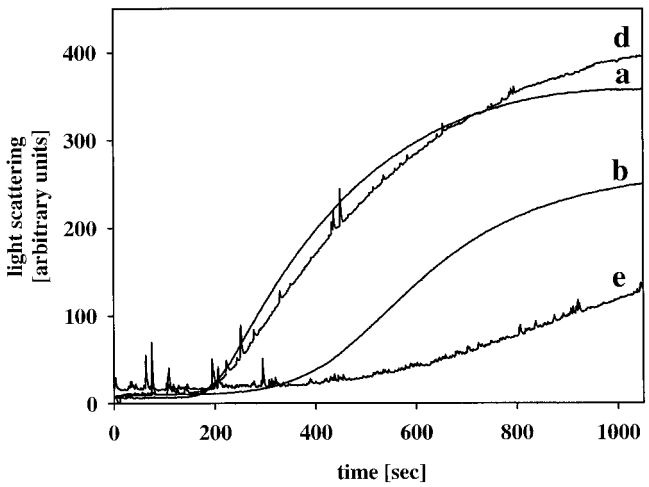


FIG. 2. **Influence of DsbG on the thermal aggregation of citrate synthase.** 30 $\mu$M CS was diluted 200-fold into prewarmed 40 mM HEPES-KOH, pH 7.5, at 43 °C in the absence (*a*) or presence of 1.5 $\mu$M (*b*) or 3.0 $\mu$M DsbG (*c*), respectively. To exclude unspecific protein effects, control experiments in the presence of 2.2 $\mu$M bovine serum albumin (*d*) were conducted. Light scattering measurements were performed.

constant stirring (23, 24). Protein aggregation was monitored with light scattering measurements using a Hitachi Spectrofluorometer F4500 equipped with thermostated cell holder and stirrer. The excitation and emission wavelengths were set to 350 nm for measurements when luciferase served as a substrate protein and to 500 nm when citrate synthase served as a substrate protein. The excitation and emission slit widths were set to 2.5 nm. To test the influence of the redox state of DsbG on its chaperone activity, we monitored the thermal aggregation of luciferase also in the presence of reduced DsbG and DTT.

To analyze whether DsbG influences the aggregation of refolding intermediates of chemically denatured substrate protein, CS was denatured in 6 M guanidine hydrochloride, 2 mM DTT for 2 h (CS concentration, 30 $\mu$M) and diluted 1:200 into 40 mM HEPES-KOH, pH 7.5, to initiate renaturation and aggregation. Light scattering measurements at 25 °C were performed as described above. To test the influence of DsbG on the reactivation kinetics of chemically denatured CS, CS was denatured as described (CS concentration, 15 $\mu$M) and was then diluted 100-fold into 40 mM HEPES-KOH, pH 7.5, at room temperature to initiate the refolding reaction. Enzyme activity was determined according to Srere *et al.* (25) using a Beckman DU640 spectrophotometer. The influence of DsbG on the reactivation of CS was performed in the absence and presence of 0.75 mM DTT.

## RESULTS

*DsbG Prevents the Thermal Aggregation of Luciferase and CS*—DsbG is a soluble periplasmic protein. When overexpressed under the T7 promoter, DsbG comprises >80% of the periplasmic protein content. This good overexpression allows its straightforward purification from periplasmic extracts to >99% purity as judged by SDS-polyacrylamide gel electrophoresis (19–21). According to its fluorescence properties, purified DsbG was in its oxidized form (data not shown). To analyze the potential chaperone activity of DsbG, its influence on the thermal aggregation of two commonly used chaperone substrate proteins was tested. In the absence of molecular chaperones, both luciferase and citrate synthase rapidly and irreversibly aggregate at 43 °C, temperatures that resemble heat shock *in vivo* (23, 24). In the absence of DsbG, luciferase is completely aggregated within 7 min of incubation at 43 °C as monitored by light scattering (Fig. 1). The presence of DsbG in quantities as small as 0.5:1 molar ratio decreased significantly the extent of the aggregation. A 5:1 molar ratio of DsbG to luciferase was sufficient to completely suppress the thermal aggregation of luciferase. Similar results were obtained using citrate synthase as a substrate protein except that higher concentrations of DsbG were needed to suppress the aggregation of this dimeric enzyme (Fig. 2). To verify that the chaperone activity of DsbG is a specific effect, a 15-fold molar excess of bovine serum albumin was used as a control in a parallel experiment but failed to inhibit the aggregation of CS (Fig. 2).

These results suggest that DsbG fulfills the main criteria that defines molecular chaperones (26); it is capable of recognizing and binding to folding intermediates of two different thermolabile proteins, luciferase and citrate synthase, and effectively prevents their nonspecific aggregation *in vitro*.

*DsbG Prevents Refolding Citrate Synthase from Aggregating*—To further characterize the chaperone activity of DsbG, we studied its effect on the aggregation of refolding CS following complete denaturation in guanidine hydrochloride. In the absence of chaperones, CS immediately aggregates upon its dilution from the denaturant into refolding buffer (27). This aggregation is nucleated by high local concentration of aggregation-sensitive folding intermediates (27). The aggregation reaction goes to completion within the first 3 min after initiating the refolding of CS, as monitored by light scattering (Fig. 3). A 20-fold molar excess of DsbG was sufficient to significantly suppress the formation of light scattering CS aggregates. This indicated that DsbG is able to recognize and bind very early, presumably unstructured folding intermediates with the goal to keep them from aggregating. To analyze how this suppression of aggregation affects the refolding yield of CS, reactivation studies using CS were performed in the absence and presence of DsbG. Surprisingly, a 5-fold molar excess of DsbG reduced the refolding yield from about 20% in the absence of DsbG to about 5% in its presence of DsbG (Fig. 4*A*). To rule out the possibility that this phenomena was simply due to disulfide cross-linking between DsbG and citrate synthase molecules, which would prevent the refolding citrate synthase from assuming the native conformation, the reactivation assays were performed with reduced DsbG in the presence of 0.75 mM DTT. Reduced DsbG showed a similar ability to prevent the CS reactivation, indicating that this effect is not due to the formation of mixed disulfides between DsbG and CS folding intermediate.

*Role of the Disulfide Bonds of DsbG in Chaperone Activity*—For DsbG to act as a disulfide isomerase, the two cysteines, Cys-126 and Cys-129, are essential and must remain in the

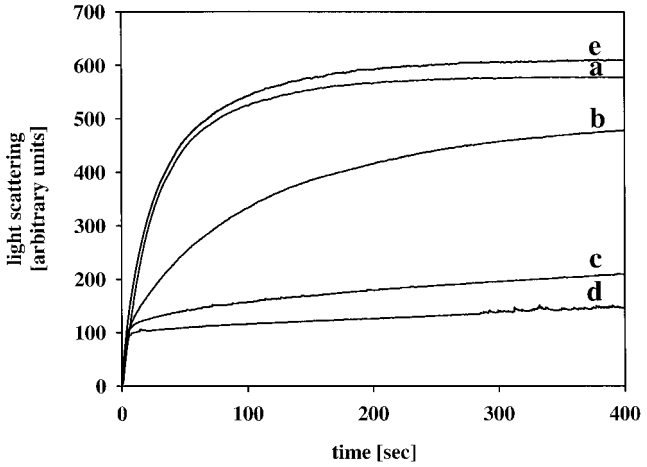


FIG. 3. **Influence of DsbG on the aggregation of refolding intermediates from chemically denatured citrate synthase.** Chemically denatured citrate synthase was diluted to a final concentration of 0.15 $\mu$M at room temperature into 40 mM HEPES, pH 7.5. Light scattering was monitored in the absence (*a*) and presence of 1.5 $\mu$M (*b*) and 3.0 $\mu$M (*c*) oxidized DsbG; *line d* shows the influence of 4.5 $\mu$M DsbG on the aggregation of CS in the presence of 2 mM DTT. To exclude unspecific protein effects, control experiments in the presence of 0.116 mg/ml bovine serum albumin (*e*) were carried out. Light scattering measurements were performed.

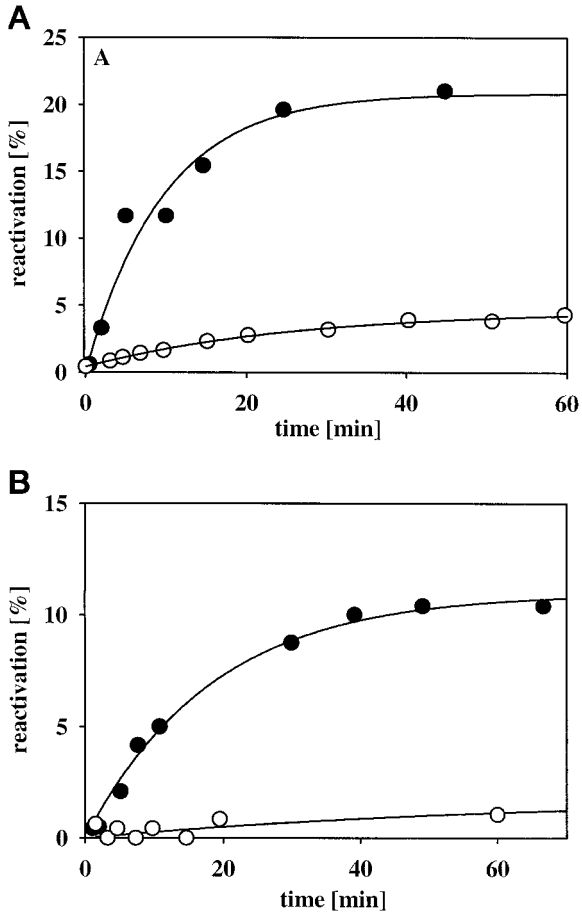


FIG. 4. **Oxidized and reduced DsbG prevents the reactivation of chemically denatured citrate synthase.** Denatured enzyme was diluted to a final concentration of 0.15 $\mu$M into 40 mM HEPES, pH 7.5. *A*, reactivation of CS under nonreducing conditions in the (●) absence or (○) presence of 0.75 $\mu$M oxidized DsbG. *B*, reactivation of CS under reducing conditions (0.75 mM DTT) in the (●) absence or (○) presence of 0.75 $\mu$M reduced DsbG.

reduced form. DsbG, used in all but the last experiments, was entirely in the oxidized form, as judged by fluorescence (21). These results demonstrated that DsbG displays molecular chaperone activity in its disulfide isomerase inactive, oxidized form. To confirm that the chaperone activity of DsbG is indeed not dependent on the redox status of its cysteines and, therefore, not connected to the isomerase activity of the enzyme, chaperone activity assays were performed with reduced DsbG in the presence of 2 mM DTT. The presence of DTT should exclude the possibility of DsbG reoxidizing during the test assay. The equilibrium constants of DTT and DsbG with glutathione are known to be −320 and −125 mV, respectively (21). From these redox potentials one can calculate the equilibrium constant between DTT and DsbG to be $0.3 \times 10^{-6}$ M. This implies that under assay conditions DsbG will be completely reduced. Reduced DsbG displayed the same chaperone activity as oxidized DsbG as judged by its ability to prevent the aggregation of thermally unfolding luciferase (Fig. 1) and refolding CS (Fig. 3). We therefore, conclude that the chaperone activity of DsbG is not affected by its redox state.

To completely eliminate the possibility that mixed disulfide complexes between DsbG and the substrate are the source of the observed chaperone activity, we constructed a mutant of DsbG that removes both active site cysteines. This leaves DsbG entirely cysteine-free and thus unable to form mixed disulfide complexes with any substrate. The ability of this cysteine free mutant of DsbG to influence the aggregation kinetics of luciferase was compared side by side with the wild type DsbG protein in aggregation assays equivalent to those reported in Fig. 1. The curves generated by the cysteine free mutant protein were virtually superimposable with those from wild type DsbG protein. Quantification of the difference between wild type and mutant showed that the mutant had 99.8% of the activity of the wild type (data not shown). This demonstrates unequivocally that the chaperone activity we are measuring is not due to the formation of mixed disulfides between the enzyme and the substrate.

## DISCUSSION

Disulfide bond formation and protein folding are known to be error-prone processes. They are assisted in the cell by folding catalyst and molecular chaperones (1). In the endoplasmic reticulum, a number of different protein folding catalysts and molecular chaperones are present that assist in the folding of proteins (3). The periplasm, on the other hand, appears rather devoid of general molecular chaperones. Although several substrate specific periplasmic chaperones such as Skp and PapD have been identified (28–30), general chaperone activity has only been observed for one protein to date: the disulfide isomerase, DsbC. We decided to attempt to identify additional molecular chaperones in the periplasm. We describe here that DsbG, a new member of the Dsb family, functions as a molecular chaperone. In this report, we show that DsbG is able to significantly suppress the aggregation of two classical chaperone substrate proteins, citrate synthase and luciferase. This strongly suggests that it has the ability to recognize and bind exposed, aggregation-prone surfaces of folding intermediates to prevent their nonspecific aggregation. Eukaryotic PDI has been reported to have similar, isomerase-independent chaperone activity (31). This strongly suggests that DsbG serves as a prokaryotic counterpart of PDI. Both proteins assist in protein folding processes in oxidizing cell compartments by at least two different means: one is to catalyze disulfide exchange allowing formation of the proper disulfides, and the other is to protect the proteins in nonnative conformation from aggregation, by acting as a chaperone.

We also conclude that the chaperone activity of DsbG, al-

though general, differs in its effectiveness depending on the substrate proteins used. Comparison of the influence of DsbG on the thermal aggregation of cellular proteins revealed that the chaperone activity of DsbG is at least 10-fold more efficient with the monomeric luciferase then with dimeric CS. The substrate binding affinity of DsbG is also higher for early, presumably unstructured and monomeric folding intermediates that are generated from chemically denatured CS than for the dimeric CS unfolding intermediates that are known to be generated by thermal unfolding processes (32). This suggests that DsbG may have a higher affinity for monomeric, poorly structured folding intermediates. Because protein translocation requires at least partial unfolding of the polypeptide, the implied preference of DsbG for unstructured polypeptides might reflect its affinity for extending polypeptide chains that are newly translocated into the periplasm. DsbG prevented CS molecules from reactivation, suggesting that it forms a rather stable complex with the refolding intermediates of CS. Addition of DTT or removal of all cysteine residues in DsbG by mutagenesis did not influence this interaction, indicating that intermolecular disulfide bond formation is not involved in the chaperone activity of DsbG. The mechanism by which DsbG releases its substrate proteins is still under investigation. It is unlikely to involve ATP because ATP has not been detected in the periplasm and DsbG does not show any consensus motif indicative of an ATP binding motif. The chaperone activity of DsbG is reminiscent of the chaperone activity recently reported for DsbC (18). DsbC and DsbG are the most homologous proteins among the Dsb family with 28% sequence identity and 56% sequence similarity (19). Both proteins are dimeric, form an unstable disulfide bond, and are maintained in the reduced state via the action of DsbD (21). They are both predicted to exhibit the thioredoxin fold and have disulfide isomerase activity (19, 21). Our finding that DsbG also works as a general folding helper protein *in vitro* doubles the number of known chaperones with wide substrate specificity that are known to exist in the periplasm of *E. coli*.

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