The functional properties of DsbG, a thiol-disulfide oxidoreductase from the periplasm of *Escherichia coli*

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Received 14 April 1998; revised version received 29 April 1998

Abstract Genetic studies have recently identified DsbG, a new member of the *dsb* group of redox proteins, which catalyze protein disulfide bond formation in the periplasm of *Escherichia coli*. We now demonstrate that DsbG functions primarily as an oxidant during protein disulfide bond formation, which is consistent with the low stability of its active site disulfide bond. There are indications, however, that the substrate range of DsbG may be narrower than the other periplasmic oxidative enzymes, DsbA and DsbC. Our observations further elaborate the pathway of disulfide bond formation in *E. coli*.

Key words: Disulfide bond; Protein folding; Dsb protein; Thioredoxin

1. Introduction

Disulfide bond formation is an important step in the biosynthesis of many secreted proteins, which is often coupled to protein folding. Because this is a chemically slow process under physiological conditions, it is catalyzed in vivo by a number of redox enzymes that are related to thioredoxin [1,2]. In the eukaryotic endoplasmic reticulum, the principal catalyst is protein disulfide isomerase (PDI), which accelerates disulfide bond formation, breakage and rearrangement [1–4]. The situation is more complex in the bacterial periplasm, where genetic techniques have been used to identify a number of linked enzymes that participate in disulfide bond formation. These include DsbA, DsbB, DsbC, and DsbD [5–13]. DsbA functions principally to form disulfide bonds in secretory proteins, whereas DsbC is probably involved in both disulfide bond formation and rearrangement [5,12,14–16]. DsbB is required to maintain DsbA in the oxidized state [6,12], while DsbD keeps a proportion of DsbC in the reduced state and competent for disulfide bond rearrangement [9,10].

The roles of DsbA and DsbC that emerge from genetic studies are well correlated not only with their functions in vitro, but also with their biochemical properties. In common with other thioredoxin related proteins, their mechanism of action involves the cycling of the active site Cys-Xaa-Yaa-Cys sequence between the oxidized and reduced forms, via an intermediate disulfide-linked complex between the substrate and enzyme [17–19]. The active site disulfide bonds of DsbA and DsbC are very unstable and highly reactive, in line with the oxidizing properties of these proteins [16,20–24], whereas thioredoxin, which has a stable active site disulfide bond, functions as a reductant [25].

Recently, a further member of the *dsb* family of proteins, DsbG, was identified, on the basis of its ability to confer resistance to high concentrations of DTT, when present in multicopy [26]. *Escherichia coli* strains lacking the DsbG protein require low molecular weight oxidizing compounds in the medium in order to grow and exhibit a phenotype similar to *dsbA*$^-$ and *dsbB*$^-$ strains. This points to a role for DsbG in disulfide bond formation, which is supported by the observation that overexpression of DsbG can compensate for some phenotypic defects exhibited by either *dsbA* or *dsbC* mutant strains [26]. In this report, we examine the biochemical properties of the DsbG protein to further establish its function in the periplasmic redox system.

2. Materials and methods

2.1. Protein production

DsbG was expressed from plasmid pSR3801 into the periplasmic space of *E. coli* strain BL21(DE3) grown in LB medium containing 100 mg/l carbenicillin [26]. Cells from a 3 l culture volume were harvested by centrifugation and resuspended in 50 ml of 20 mM Tris-HCl (pH 8), 20% (w/v) sucrose. To release protein from the periplasmic space, 1 ml of 0.25 M EDTA and 7.5 mg of lysozyme were added and the suspension incubated for 40 min on ice. 1 ml of 1 M $MgCl_2$ was then added and the cell debris pelleted by centrifugation at 4°C for 20 min at 15 000×*g*. The supernatant was loaded onto a 30×1.6 cm S-Sepharose column equilibrated with 25 mM MES (pH 6) and eluted with a 1 l gradient from 0 to 0.4 M NaCl in the same buffer at a flow rate of 1 ml/min. The protein was located by SDS-PAGE analysis of the fractions [27]. Fractions containing pure protein were pooled, concentrated and stored frozen. Oxidized DsbG was prepared by incubation of reduced protein with 100 μM GSSG until the protein was in the oxidized form, as judged by reverse-phase HPLC, followed by desalting on a Sephadex G25 column equilibrated with 10 mM Tris-HCl (pH 7.4). The protein was quantified using the calculated extinction coefficient of 42 650 M^{-1} cm^{-1} [28].

DsbA and DsbC were purified and quantified as previously described [16,20].

2.2. Separation of the different redox forms of DsbG

The oxidized and reduced forms of DsbG and the mixed disulfides with glutathione were separated by reverse phase HPLC. Separations were carried out on a Vydac C-18 column at 25°C in 0.1% (v/v) TFA using the following gradients: 0–4 min, 15–30% (v/v) acetonitrile; 4–16 min, 30–38% (v/v) acetonitrile; 16–66 min, 38–43% (v/v) acetonitrile.

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Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; $DsbG^{S}_{S}$ and $DsbG^{SH}_{SH}$, the disulfide and dithiol forms of protein DsbG, respectively; DTT, dithiothreitol; P^{SH}_{SH}, peptide substrate containing cysteine residues at positions 2 and 27; GSSG and GSH, the oxidized and reduced forms of glutathione, respectively; HPLC, high pressure liquid chromatography; PDI, protein disulfide isomerase; TFA, trifluoroacetic acid

PII S0014-5793(98)00539-0

2.3. Redox titration

The equilibrium constants for disulfide bond formation were determined in both folded and unfolded DsbG, with glutathione as thiol-disulfide reagent. DsbG was incubated in different redox mixtures of excess GSH and GSSG in 0.1 M Tris-HCl (pH 7.4), 0.2 M KCl, and 1 mM EDTA at 25°C, in the presence or absence of 8 M urea. Reactions were quenched after 30–60 min by the addition of HCl to 0.1 M (reactions in the presence of 8 M urea) or 0.3 M (reactions in the absence of 8 M urea), and analyzed by HPLC. Higher concentrations of acid are required to trap reactions involving the folded form of the protein, as previously described [16,29]. Multiple time points were taken to ensure that the reaction had reached equilibrium.

2.4. Rearrangement of BPTI folding intermediate (30–51, 14–38)

The intermediate (30–51, 14–38) was isolated from a BPTI refolding mixture as previously described [3], lyophilized and re-dissolved in 10 mM HCl. Assays of (30–51, 14–38) rearrangement were carried out in 0.1 M Tris-HCl (pH 7.4), 0.2 M KCl, 1 mM EDTA at 25°C with 10 μM substrate and 1 μM catalyst, as previously described [3,15].

2.5. Thiol-disulfide exchange activities

The ability of DsbG to oxidize or reduce various substrates, such as DTT^{SH}_{SH}, a model peptide (P^{SH}_{SH}) and GSSG was examined. Assays were carried out in 0.1 M Tris-HCl (pH 7.4), 0.2 M KCl, 1 mM EDTA at 25°C with varying concentrations of substrate, which was always in excess, and the oxidized or reduced forms of DsbG. Reactions were initiated by the addition of the appropriate amount of substrate to a buffer solution containing DsbG. At various time intervals the reactions were quenched by the addition of HCl to a final concentration of 0.1 M, and the mixture analyzed by HPLC to separate the different forms of DsbG present. The rate of reaction between DsbG and either DsbA or DsbC was determined in a similar way, but in this case an excess of DsbG was used in the reactions and the products quantified by HPLC analysis were the oxidized and reduced forms of DsbA or DsbC.

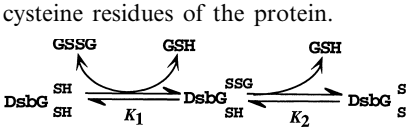
3. Results

3.1. DsbG purification

DsbG was purified by a single ion exchange step to give a protein that was essentially pure by SDS-PAGE. The N-terminal sequence of the protein was determined to be EELPA, as expected, and the molecular mass was 25 669.4 (expected, 25 671.4, oxidized form). This corresponds to cleavage after residue 17 of the precursor form, as previously reported [26]. One contaminant, about 10% of the total protein, was visible by reverse phase HPLC analysis and was probably a form of DsbG, as it behaved similarly during oxidation-reduction reactions. It eluted well away from the major DsbG peak, however, and did not interfere with any of the assays performed. The contaminant had a mass of 26 239.2, which might correspond to processing after residue 11 of the precursor (mass difference expected, 570.8; mass difference observed, 569.8) leaving an N-terminal extension of PAIAFA.

3.2. Thiol-disulfide equilibrium with glutathione

The equilibrium for thiol-disulfide exchange between the thiol and disulfide forms of glutathione and DsbG was examined by HPLC separation of acid trapped species in the presence and absence of 8 M urea. These reactions occur via mixed disulfides between glutathione and one or both of the cysteine residues of the protein.



(1)

The overall equilibrium constant for the reaction is defined as follows:

$$K_{ss} = \frac{[DsbG^{S}_{S}][GSH]^2}{[DsbG^{SH}_{SH}][GSSG]} \quad (2)$$

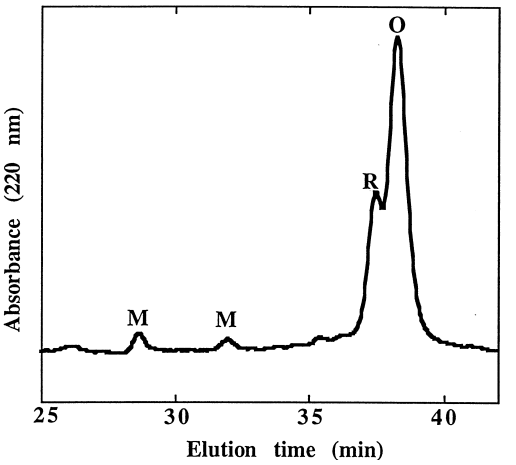


Fig. 1. HPLC reverse-phase elution profile of acid trapped DsbG that had been incubated at 25°C with 10 mM GSH and 1 mM GSSG in 0.1 M Tris-HCl (pH 7.4), 0.2 M KCl, 1 mM EDTA and 8 M urea. O stands for oxidized DsbG, R for reduced and M for the two species with a single mixed disulfide with glutathione.

All the possible species of Eq. 1 could be resolved by HPLC after acid trapping (Fig. 1), but the mixed disulfide forms only accumulated to a significant extent in the presence of 8 M urea. By determining the ratio of oxidized to reduced DsbG as a function of redox conditions (Fig. 2), the value of K_{ss} in each case could be determined. In the absence of urea, DsbG forms an unstable disulfide bond at its active site, with an overall equilibrium constant for the reaction of 0.18 mM, whereas in the presence of urea this value is 180 mM. The 1000-fold difference in the values of these two equilibrium constants indicates that the active site disulfide bond destabilizes the folded conformation of the protein.

The glutathione mixed disulfide forms of DsbG only accumulate when DsbG is unfolded, not when it is in the native state. The values of K_1 and K_2 (Eq. 1) were determined in the presence of 8 M urea to be 0.97 and 150 mM respectively.

3.3. The reactivity of the DsbG active site

The reactivity of the active site thiol group and disulfide bond towards a number of different compounds was examined. Two substrates were used to assess the ability of DsbG to function as an oxidant, DTT^{SH}_{SH} and P^{SH}_{SH}. P^{SH}_{SH} is a 28 residue peptide based upon the sequence of BPTI, which contains two cysteine residues; assay of disulfide bond formation in this peptide has previously been used in mechanistic studies on PDI, DsbA and DsbC [4,18,30]. Both DTT^{SH}_{SH} and P^{SH}_{SH} were readily oxidized by $DsbG^{S}_{S}$, with rate constants of 2200 s^{-1} M^{-1} and 12 000 s^{-1} M^{-1}, respectively (Fig. 3).

The reaction of $DsbG^{SH}_{SH}$ with GSSG to form $DsbG^{S}_{S}$ and GSH was also examined. The rate constant for this process was 1.1 s^{-1} M^{-1} (data not shown).

3.4. The reaction of DsbG with DsbA and DsbC

In the periplasm it is possible that DsbG may be affecting the redox state of both DsbA and DsbC. The kinetics of the

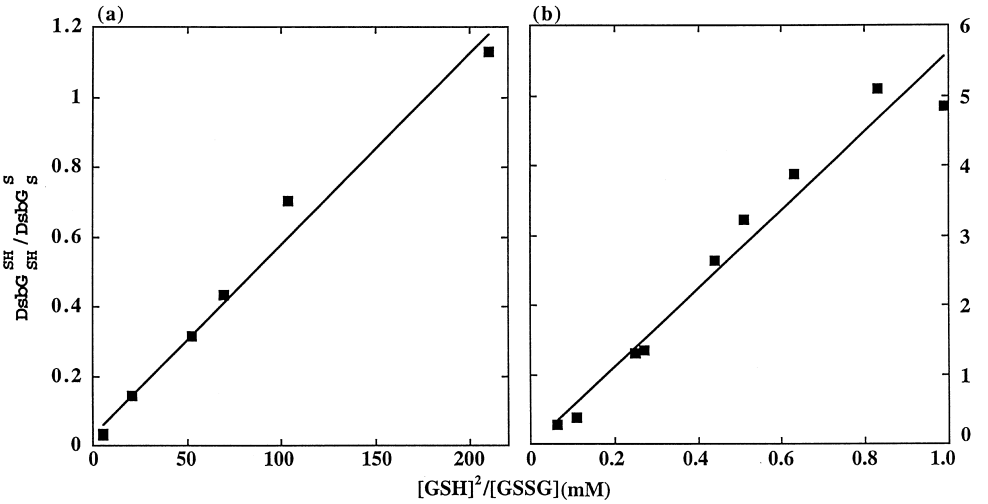


Fig. 2. Measurement of the thiol-disulfide equilibrium of DsbG with glutathione. DsbG was incubated in various redox buffers in the presence (a) or absence (b) of 8 M urea, as described in Section 2. Reactions were acid quenched and the relative levels of oxidized and reduced DsbG determined by HPLC. The plot of $[DsbG^{SH}_{SH}]/[DsbG^{S}_{S}]$ against $[GSH]^2/[GSSG]$ should be linear and gives the value of $1/K_{ss}$ (Eq. 2).

reaction between DsbA and DsbG were examined by mixing an excess of $DsbG^{S}_{S}$ with $DsbA^{SH}_{SH}$ and analyzing the products by reverse phase HPLC, as described in Section 2. The data were consistent with a pseudo-first order reaction, giving a rate constant of 36 s^{-1} M^{-1} (data not shown). From the equilibrium constants of DsbA and DsbG with glutathione, the value of the equilibrium for their direct thiol-disulfide exchange can be calculated to be 0.46, allowing the reverse rate constant to be calculated:

$$DsbG^{S}_{S} + DsbA^{SH}_{SH} \underset{78\ s^{-1}M^{-1}}{\overset{36\ s^{-1}M^{-1}}{\rightleftharpoons}} DsbG^{SH}_{SH} + DsbA^{S}_{S} \quad (3)$$

A similar approach was adopted to determine the kinetics of reaction between DsbC and DsbG. Partial overlap of the $DsbC^{SH}_{SH}$ and DsbG peaks, however, complicated the quantitation and only allowed a very approximate rate constant (61 s^{-1} M^{-1}) to be determined. Combining these data with the equilibrium constant for this reaction, which was calculated from the equilibrium constants of DsbC and DsbG with glutathione, gave:

$$DsbG^{S}_{S} + DsbC^{SH}_{SH} \underset{56\ s^{-1}M^{-1}}{\overset{61\ s^{-1}M^{-1}}{\rightleftharpoons}} DsbG^{SH}_{SH} + DsbC^{S}_{S} \quad (4)$$

3.5. The activity of DsbG in disulfide bond rearrangement

The activity of DsbG as a catalyst of disulfide bond rearrangement was determined using the BPTI folding intermediate (30–51, 14–38) as substrate; the numbering refers to the cysteine residues of BPTI that are paired in disulfide bonds. Rearrangements of (30–51, 14–38) to two other intermediates, (5–55, 14–38) and (30–51, 5–55), are significant steps on the BPTI folding pathway, which are catalyzed by PDI [3] and at high catalyst concentrations by DsbC [16]. In contrast, DsbA does not catalyze this rearrangement process [15].

In the absence of any catalyst, (30–51, 14–38) rearrangement occurs with a half-life of about 100 min. Inclusion of 1 μM DsbC in the reaction mixture accelerates this by about a factor of 5, reducing the half-life for reaction to 17 min. In contrast, DsbG at this concentration had no discernible effect on (30–51, 14–38) rearrangement (data not shown).

4. Discussion

Genetic studies have indicated that DsbG functions as a catalyst of disulfide bond formation and might be responsible for the residual oxidative activity that exists in the periplasm of *dsbA*$^-$ *E. coli* [6,31]. This conclusion is supported by the present biochemical studies, which show that DsbG forms an unstable active site disulfide bond, which can be transferred rapidly to a substrate molecule. The active site disulfide bonds of DsbC and DsbG have very similar stabilities and are only a little more stable than the disulfide bond of DsbA. The values of K_{ss} for these proteins are in the range of 80–200 μM [16,20,22,29], whereas disulfide bonds in folded proteins are

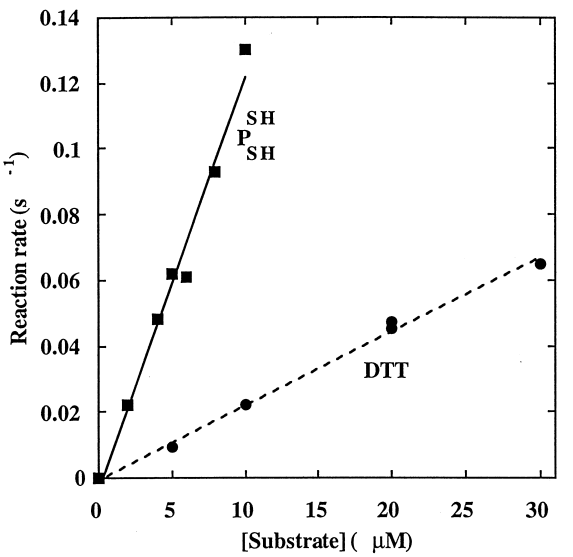


Fig. 3. The rate of reduction of $DsbG^{S}_{S}$ by two substrates, DTT^{SH}_{SH} and P^{SH}_{SH}, as a function of their concentrations. Reactions and analysis were carried out as described in Section 2.

very much more stable, with K_{ss} values in the range of 10^2–10^5 M [32]. Consequently, transfer of the active site disulfide bond from each of the catalysts to a substrate protein can be a very favorable process.

Catalysis of disulfide bond formation by DsbA and DsbC is a very rapid process, with rate constants of 10^5 s^{-1} M^{-1} to 10^7 s^{-1} M^{-1} for reactions of the oxidized forms of the catalysts with the synthetic peptide substrate, P^{SH}_{SH} [18,30]. These high rates are attributed to the very reactive disulfide bond at the active site, and to the ability of DsbA and DsbC to bind substrate proteins [18,19,30]. The reaction rate of $DsbG^{S}_{S}$ with the same substrate was somewhat slower, about 10^4 s^{-1} M^{-1}. Such differences may be related to a number of factors, such as differences in substrate binding or the reactivity of the active site. There was some indication that thiol-disulfide exchange at the DsbG active site was generally slower than in DsbA and DsbC; the rate of reaction of reduced DsbG with GSSG was about 30-fold lower than the corresponding reactions with DsbA and DsbC. More detailed studies are required to investigate the role of substrate binding in the catalytic mechanism of DsbG, but it seems likely that such interactions also occur. The reaction of oxidized DsbG with a reduced peptide was much more rapid than its reaction with DTT, which may be related to better substrate binding interactions between the larger peptide molecule and catalyst than are possible with a low molecular weight compound.

In vivo experiments have shown that in a *dsbG* null mutant, both DsbA and DsbC accumulate in the reduced state, suggesting that the redox states of DsbA, DsbC and DsbG may be directly linked [26]. Although it was demonstrated that $DsbG^{S}_{S}$ can oxidize $DsbA^{SH}_{SH}$ and $DsbC^{SH}_{SH}$, it does so at a relatively low rate, compared to the rate at which it oxidizes DTT^{SH}_{SH} or P^{SH}_{SH}. In the periplasm, oxidation of a newly translocated protein by $DsbG^{S}_{S}$ would probably occur in preference to the oxidation of either DsbA or DsbC. DsbG is thus unlikely to directly regulate the redox state of DsbA or DsbC, raising the question of why the latter two proteins accumulate in the reduced state in a *dsbG* null mutant. One possibility is that in the absence of DsbG, turnover of the oxidized forms of DsbA and DsbC increases because they oxidize substrates that are normally oxidized selectively by DsbG. This suggests that DsbA and DsbC can to some extent catalyze disulfide bond formation for some substrates of DsbG. This is supported by the fact that multicopy *dsbA* or *dsbC* compensate only partly for the loss of *dsbG* [26]. It is possible that such substrate specificity may confer the stronger phenotype observed with *dsbG* null mutants if DsbG is essential for the folding/oxidation of a particular subset of proteins that are essential for the normal growth of *E. coli*.

Acknowledgements: We thank the EMBL Peptide and Protein Group for mass spectrometry and N-terminal sequence analysis and Bayer AG for the gift of BPTI.

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