Redox states of DsbA in the periplasm of *Escherichia coli*

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Abstract **DsbA is a periplasmic, disulfide bond formation factor of *E. coli*. We studied in vivo redox states of its active site cysteines. When periplasmic contents were prepared from iodoacetic acid-treated cells, according to the previously published procedures, variable but major proportions of DsbA were in the reduced form. We found that this was due to an artificial reduction that occurred after cell disruption; even purified and oxidized DsbA underwent reduction when incubated with cell extracts in the absence of any added reducing agent. Such DsbA-reducing activities were detected in both the periplasmic and the cytoplasmic fractions. To circumvent the artifact, we analyzed redox states of DsbA under denaturing conditions. Now virtually all the DsbA molecules were detected as oxidized or reduced in the *dsbB*$^+$ background or in the *dsbB*$^-$ background, respectively. Using the improved method, we also examined redox states of DsbA when it was overproduced, and followed the oxidation/reduction pathway that DsbA follows after biosynthesis. It is suggested that newly synthesized DsbA is rapidly oxidized by pre-existing DsbA, while oxidation of mature (functional) DsbA requires DsbB, whose roles might include that of antagonizing the actions of DsbA-reducing enzyme(s).**

Key words: Disulfide bond; *DsbA*; *DsbB*; Redox state; Cysteine; *Escherichia coli* periplasm

1. Introduction

Many proteins that are secreted to the cell surfaces contain disulfide bonds, which are important for their folding and stability. *Escherichia coli* and other prokaryotic cells contain a specific 'Dsb' system, which facilitates disulfide bond formation in the periplasmic space (for review see [1]). Mutational inactivation of either the *dsbA* [2,3], *dsbB* [4,5], or *dsbC* [6] gene pleiotropically impairs disulfide bond formation of envelope proteins. DsbA and DsbC are soluble proteins of the periplasm, while DsbB is a membrane protein with four transmembrane segments [7]. They all possess a Cys-X-X-Cys motif characteristic of active sites seen for the members of the thioredoxin superfamily [8]. Among these factors, the DsbA protein has been characterized in considerable detail [9–12]. It directly oxidizes secretory proteins. DsbB probably re-oxidizes the reduced form of DsbA to enable it to work again [4,7]. Little is known about DsbC, which may function in a pathway independent of the DsbA/DsbB pathway [6].

Biochemical and biophysical studies suggested that the oxidized form of DsbA is in an unstable conformation [10,11]. In apparent consistency with this notion, Bardwell et al. detected only a fraction of DsbA molecules in the wild-type cell as the oxidized form [4]. In the experiment of Bardwell et al., they fractionated the cells in the presence of iodoacetic acid and examined the periplasmic preparation by electrophoresis under non-denaturing conditions. We now show that such a method can not be used for assessing DsbA's redox states in vivo, since DsbA is subject to artificial reduction by some cellular protein(s) once cells are disrupted for fractionation. Instead, our re-examinations of the intracellular redox states of DsbA under denaturing conditions showed that DsbA molecules in wild-type cells are mostly oxidized. Examination of the redox states of overproduced DsbA revealed two modes of its oxidation; one that takes place for the newly synthesized molecules and another for the maintenance of the steady state. Possible significance of the DsbA-reducing factor(s) and the roles of DsbB in keeping DsbA oxidized are discussed.

2. Experimental

2.1. Bacterial strains and plasmids

Escherichia coli K12 strain CU141 [13] is a derivative of MC4100 and carries F′ *lacI*^{Q} *lacZ*$^+$Y$^+$A$^+$ *lacPL8*. SS141 is a CU141 derivative into which *dsbB*::kan5 [4] had been transduced. SS140 is a *dsbA33*::Tn*5* [2] derivative of CU141. pSK220, carrying a 1.2 kb chromosomal fragment of the *dsbA* region, was described previously [2]. Another *dsbA* plasmid, pSS18, was constructed by cloning the 1.0 kb *Eco*RI–*Hin*dIII fragment of the pUC19-derived *dsbA*$^+$ plasmid [14] into pMW119, a pSC101-based vector provided by Nippon Gene.

2.2. Determination of redox states of DsbA in vivo

A culture of cells growing in L medium [15] at 37°C was mixed with an equal volume of 10% trichloroacetic acid. Precipitates of denatured proteins were pelletted by centrifugation (microfuge for 2 min), washed with acetone, and dissolved in 1% SDS, 1 mM EDTA, 50 mM Tris-HCl (pH 8.1) containing 5 mM iodoacetamide. Proteins were then separated by 12.5% SDS-PAGE [16] without addition of any reducing agent. DsbA was visualized by immunoblotting as described previously [9]. In some experiments, trichloroacetic acid-denatured proteins were dissolved in 6.4 M urea, 1.5 M Tris-HCl (pH 8.7), containing 100 mM iodoacetic acid. The sample was then diluted 4-fold with 1.5 M Tris-HCl (pH 8.7) and electrophoresed under non-denaturing conditions [4,10]. Finally, the procedures described by Bardwell et al. [4] were also followed; iodoacetic acid was added to the culture, and cells were subjected to osmotic shock to obtain the periplasmic fraction, which was then analyzed by native PAGE. Anti-DsbA serum was described previously [9].

2.3. Preparation of periplasm and cytoplasm fractions and demonstration of DsbA-reducing activities

Cells were grown in L medium at 37°C, and fractionated by osmotic shock [17] or lysozyme-EDTA-sucrose treatment [18]. Shocked cells were resuspended in 10 mM Tris-HCl (pH 8.1) and then disrupted by sonication. After centrifugation at 540,000 × *g* for 30 min, the supernatant was used as the cytoplasmic extract. As an indication of separation of the periplasmic fraction from the cytoplasmic material, the distribution of GroEL was examined as described [13]. DsbA, purified from overproducing cells, was in the oxidized state [9]. It was mixed with varying amounts of each subcellular fraction, and incubated at 0°C for 60 min, followed by SDS-PAGE under non-reducing conditions.

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2.4. Pulse-chase and immunoprecipitation

Cells were grown at 37°C in synthetic medium M9 [15] supplemented with glucose (0.4%) and amino acids (20 μg/ml each, except methionine and cysteine), pulse-labeled with 3.7 MBq/ml of [^{35}S]methionine (29 TBq/mmol; obtained from American Radiolabeled Chemicals). Chase with unlabeled methionine was initiated by addition of 200 μg/ml L-methionine. Immunoprecipitation conditions were as previously described [2].

3. Results and discussion

3.1. Redox states of DsbA in vivo can only be assessed under denaturing conditions

In order to examine redox states of DsbA in vivo, Bardwell et al. [4] treated the culture with iodoacetic acid and prepared the periplasmic fraction. The redox states of DsbA were then examined by native PAGE, in which the reduced and oxidized forms can be separated by the charge difference due to the modification of the reduced cysteines by iodoacetic acid [10]. Less than 50% of DsbA molecules in wild-type cells were oxidized, while all of them were reduced in the *dsbB*::kan5 cells [4]. Using the same procedures, we obtained results that were basically consistent with the results of Bardwell et al., but somewhat variable from experiment to experiment; in many cases as much as about 90% of DsbA was detected as reduced even in the wild-type cells (Fig. 1B, lanes 1 and 2).

We employed an alternative procedure in which cultures

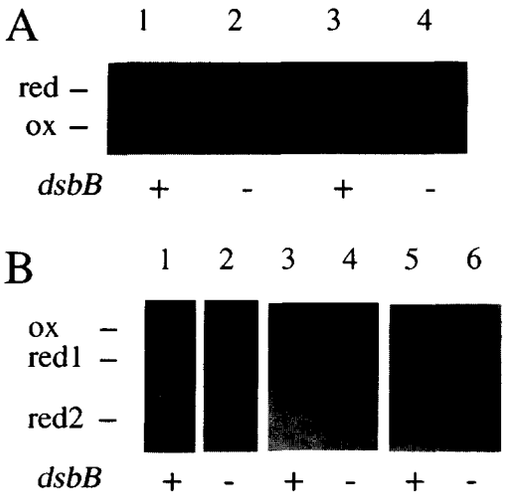

Fig. 1. Redox states of DsbA in vivo as determined by different methods. (A) Cells of CU141 (wild-type; lanes 1 and 3) and SS141 (*dsbB*::kan5; lanes 2 and 4) were treated directly with trichloroacetic acid (lanes 1 and 2), or fractionated in the presence of 10 mM iodoacetic acid [4] to obtain periplasmic fractions which were then treated with trichloroacetic acid (lanes 3 and 4). Proteins were solubilized in SDS sample buffer without reducing agent but with iodoacetamide and separated by SDS-PAGE under non-reducing conditions. DsbA was visualized by immunoblotting. red and ox represent reduced and oxidized DsbA, respectively. (B) Cells of CU141 (lanes 1, 3, and 5) or SS141 (lanes 2, 4, and 6) were subjected to the following treatments. Lanes 1 and 2: periplasmic fractions were prepared in the presence of iodoacetic acid. Lanes 3 and 4: cultures were treated with iodoacetic acid at room temperature for 10 min and then with trichloroacetic acid, followed by solubilization of proteins in urea. Lanes 5 and 6: samples were treated as described for lanes 3 and 4 except that the final urea solubilization was carried out in the presence of 100 mM iodoacetic acid. Samples were diluted and analyzed by PAGE under non-denaturing conditions [10] and immunoblotting. *red1* and *red2* represent DsbA in which only Cys^{30} was modified by iodoacetic acid and that with both Cys^{30} and Cys^{33} residues modified, respectively [10].

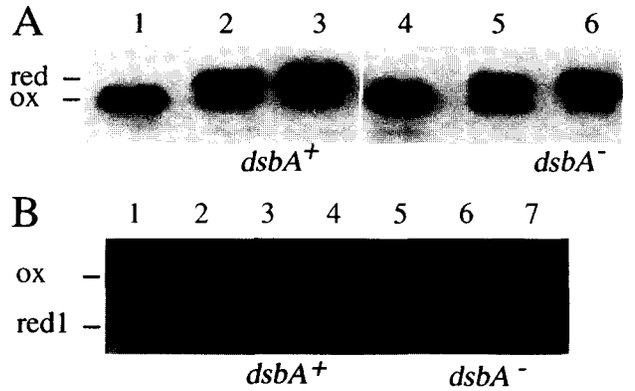

Fig. 2. Periplasmic fraction contains a factor(s) that reduces DsbA in vitro. Periplasmic fractions were prepared from CU141 (wild-type; lanes 2 and 3 of A and lanes 2–4 of B) or SS140 (*dsbA*::Tn*5*; lanes 5 and 6 of A and lanes 5–7 of B). Purified and oxidized DsbA (0.2 μg) was incubated, in a final volume of 50 μl of 3 mM Mops (pH 7), with 3 mM iodoacetic acid and the following amounts of periplasmic preparations: lanes 1 and 4 of A as well as lane 1 of B, none; lanes 2 and 5 of A, a culture-equivalent of 0.4 ml; lanes 3 and 6 of A, a culture-equivalent of 1.6 ml; lanes 2 and 5 of B, a culture-equivalent of 0.45 ml; lanes 3 and 6 of B, a culture equivalent of 1.35 ml. Redox states of DsbA were analyzed either by non-reducing SDS-PAGE (A) or native PAGE (B).

were directly treated with trichloroacetic acid to denature and precipitate all the cellular proteins, which were subsequently solubilized in SDS in the presence of iodoacetamide or iodoacetic acid. Upon SDS-PAGE under non-reducing conditions, DsbA protein from the wild-type cells migrated distinctly faster than that from the *dsbB*::kan5 cells (Fig. 1A, compare lanes 1 and 2). The former and the latter species represented oxidized and reduced forms as they migrated identically after reduction (data not shown). The successful detection of only the reduced form of DsbA in the *dsbB* mutants (Fig. 1A, lane 2) indicates that oxidation of cysteines did not occur during our in vitro manipulations of the samples.

We also used native PAGE for separation of various species of DsbA to show that our results were independent of the method of electrophoretic separation. We treated cultures first with iodoacetic acid and then with trichloroacetic acid, and protein precipitates were solubilized in urea and subjected to native PAGE. DsbA in the wild-type cell migrated mostly in the oxidized form (Fig. 1B, lane 3), while that in the *dsbB*::kan5 cells was reduced since its accessible cysteine [10] had been modified (see the *red1* band of Fig. 1B, lane 4). When iodoacetic acid was added at the point of urea solubilization, DsbA from the wild-type cells remained oxidized (Fig. 1B, lane 5), while that from the *dsbB*::kan5 cells underwent modification of both of the two cysteine residues (see the *red2* band of Fig. 1B, lane 6). Full accessibility of denatured DsbA to the iodoacetic acid modification was previously reported [10].

We found that initially oxidized DsbA was reduced during cell fractionation under non-denaturing conditions. When the same cells as used in lane 1 of Fig. 1A were fractionated to obtain the periplasmic preparation, DsbA that was otherwise identically processed was detected as the reduced form (Fig. 1A, lane 3).

These results establish that most, if not all, of DsbA molecules are oxidized in the periplasm of the wild-type cells. The

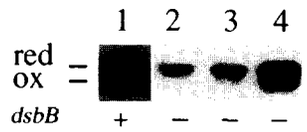

Fig. 3. Redox states of overproduced DsbA in the presence or absence of DsbB. Cells of CU141/pSK220 (lane 1), SS141 (*dsbB*::kan5) (lane 2), and SS141/pSS18 (lanes 3 and 4) were grown at 37°C in amino acid-supplemented M9 medium with 0.4% glucose (lanes 2 and 3) or 0.4% glycerol (lanes 1 and 4). Expression of *dsbA* was induced with 5 mM isopropylthio-β-D-galactoside for lanes 3 and 4. Redox states of DsbA were analyzed as described in Fig. 1A.

apparent accumulation of the reduced form in the wild-type cells [4] must have been due to artificial reduction after disruption of the cell.

3.2. E. coli cell lysates contain factors that reduce DsbA in vitro

To demonstrate DsbA reducing activities of cell extracts more directly, we incubated a purified and oxidized preparation of DsbA with periplasmic or cytoplasmic extracts. Examination by both SDS-PAGE (Fig. 2A) and native PAGE (Fig. 2B) showed that the oxidized form of DsbA was converted to the reduced form. The extent of the conversion was dependent on the amounts of added extract. It should be noted that no reducing reagent had been added to either the medium or the buffers used. Although iodoacetic acid was added in the reactions reported in Fig. 2, reduction of purified DsbA, as assessed from profiles in non-reducing SDS-PAGE, did not depend on the presence of iodoacetic acid during the incubation (data not shown). The activity found with the periplasmic preparation was partially characterized; it was non-dialyzable, largely heat stable, and adsorbed to DEAE-Sepharose, from which it could be eluted with about 0.25 M NaCl (data not shown). The active material appears to be a protein(s).

The DsbA-reducing activities did not quantitatively differ between the periplasm preparations prepared from the *dsbA*$^+$ and the *dsbA*::Tn*5* cells (compare lanes 2/3 with lanes 5/6 of Fig. 2A as well as lanes 2–4 with lanes 5–7 of Fig. 2B). This, together with the fact that pretreatment of the periplasmic material with iodoacetic acid did not abolish the activity (Fig. 1A, lane 3; Fig 1B, lane 1), indicates that reduction of DsbA was not due to fortuitous oxidation of cysteine residues that remained in some of periplasmic proteins. The same culture-equivalent amounts of periplasmic fraction and cytoplasmic fraction gave comparable activities of DsbA reduction (data not shown). The degree of contamination of the periplasmic fraction by the cytoplasmic material, as assessed by the distribution of GroEL, was less than 10%. The DsbA-reducing activity did not change when a periplasmic preparation obtained by osmotic shock and that obtained after lysozyme-sucrose treatment were compared (see [19] for the release of some cytoplasmic proteins by osmotic shock). Thus, both the periplasm and the cytoplasm of *E. coli* appear to contain material active in DsbA reduction. The cytoplasmic activity might well be ascribed to non-specific cysteine-containing proteins. However, evidence suggests that the periplasmic activity is not due to proteins with potential disulfide-bond forming cysteines. It remains to be investigated whether this activity is specific for DsbA or is due to a 'cross talk' with a system, like HelX of *Rhodobacter capsulatus* [20], which acts to keep other substrates reduced.

3.3. Redox states of overproduced DsbA

We also examined redox states of DsbA that was overproduced from a plasmid. Substantial fractions of overproduced DsbA were shown to be in the reduced state (Fig. 3, lane 1). The reduced form of DsbA appeared to represent those molecules of DsbA that escaped from oxidation by DsbB; presumably they were produced because DsbB had been overloaded by excess DsbA.

Interestingly, when DsbA was overproduced extensively in the *dsbB*::kan5 cells, a significant fraction of it was now oxidized in spite of the lack of the DsbB activity (Fig. 3, lane 4). This is striking since all DsbA molecules without hyperproduction are reduced in the absence of DsbB (Fig. 3, lanes 2 and 3; [4]). We assume that two factors may have contributed to the appearance of the oxidized DsbA in the absence of DsbB. First, as shown below, every newly synthesized DsbA molecule might be oxidized before it assumes native conformation. Although this oxidation itself will depend on the DsbA/DsbB system, overproduced DsbA is known to partially compensate for the lack of the DsbB activity [4]. Secondly, overproduced and excess DsbA may escape from the DsbA-reducing factor; thus, DsbA that is oxidized by air oxygen might be preserved.

3.4. Pathways of DsbA oxidation

We then followed the process of biosynthesis of DsbA by pulse-chase experiments. DsbA overproduced in either the *dsbB*$^+$ or *dsbB*::kan5 cells was labeled, immunoprecipitated and analyzed by non-reducing SDS-PAGE. DsbA from wild-type cells was fully oxidized at the point of the 30 s pulse label and remained so during the chase (data not shown), indicating that DsbA is rapidly oxidized in vivo like other exported proteins [2,3,21].

In the case of overproduced DsbA, it was largely oxidized just after the pulse labeling (Fig. 4A, lane 1) and then gradually

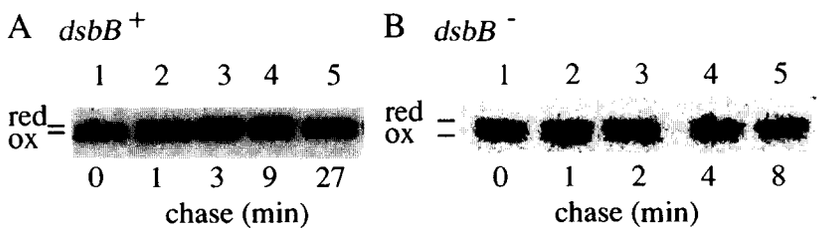

Fig. 4. Kinetics of oxidation/reduction of overproduced DsbA. Cells of CU141/pSK220 (A), or SS141/pSK220 (B) were pulse-labeled for 30 s with [^{35}S]methionine, and chased with unlabeled methionine for the indicated periods. Samples were processed for immunoprecipitation of DsbA in the presence of iodoacetamide. After SDS-PAGE under non-reducing conditions, radioactive proteins were visualized by a Bioimaging Analyzer, BAS2000 (Fuji Film).

attained the steady-state redox state (Fig. 4A, lanes 2–5), in which as much as about 60% was reduced. Thus, newly synthesized DsbA in the overproducing strain was initially oxidized and then reduced to the steady-state redox state.

As described in the preceding section, oxidized DsbA accumulates even under the *dsbB*-defective conditions, but this only happens when DsbA is extensively overproduced. Pulse-chase experiments shown in Fig. 4B indicate, however, that a significant fraction of DsbA that was moderately overproduced in the *dsbB*::kan5 mutant was oxidized just after biosynthesis (Fig. 4B, lane 1) and then reduced (Fig. 4B, lane 5).

We interpret these results as follows. During the processes of biosynthesis and export to the periplasm, DsbA molecules initially remain unfolded and behave as a substrate for the pre-existing DsbA enzyme, just as an ordinarily exported protein does. Following the oxidation, the newly exported DsbA will be folded into the functional structure. The folded DsbA molecules then gain the ability to oxidize other newly exported proteins (with concomitant reduction of themselves), to interact with DsbB, and to interact with the DsbA-reducing factor(s). Thus, DsbA is oxidized in two different modes. The one that occurs for newly synthesized molecules is probably mediated by pre-existing DsbA, since it occurs even in the *dsbB*$^-$ cells (Fig. 4B, land 1). The other that occurs for mature DsbA should be mediated by DsbB.

The present study revealed that the *E. coli* cell possesses factors that reduce DsbA. Although the activity of such factors only becomes apparent after disruption of the cell and consequent uncoupling of DsbA from DsbB, the factors (especially the one that resides in the periplasm) might have a role in regulating the redox state of DsbA. We propose that DsbB-mediated oxidation of DsbA will be important both to recycle back the reduced form of DsbA and to keep it oxidized by counteracting the reducing factor(s) in the periplasm.

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