Reconstitution of a Protein Disulfide Catalytic System*

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Disulfide bonds are important for the structure and stability of many proteins. In prokaryotes their formation is catalyzed by the Dsb proteins. The DsbA protein acts as a direct donor of disulfides to newly synthesized periplasmic proteins. Genetic evidence suggests that a second protein called DsbB acts to specifically reoxidize DsbA. Here we demonstrate the direct reoxidation of DsbA by DsbB. We have developed a fluorometric assay that allows us to directly follow the reoxidation of DsbA. We show that membranes containing catalytic amounts of DsbB can rapidly reoxidize DsbA to completion. The reaction strongly depends on the presence of oxygen, implying that electron transfer is the rate-limiting step in the overall reaction. Membranes from a dsbB null mutant display no DsbA reoxidation activity. The ability of DsbB to reoxidize DsbA fits Michaelis-Menten behavior with DsbA acting as a high-affinity substrate for DsbB with a $K_m$ of 10 $\mu$M. The in vitro reconstitution described here is the first biochemical analysis of DsbB and allows us to study the major pathway of disulfide bond formation in Escherichia coli.

Proteins start life as linear amino acid chains and rapidly fold into compact active structures. Rapid folding is a prerequisite for the survival of a protein in the cell. It is not surprising then that critical steps in the folding process are assisted in the cell. One of the rate-limiting steps in protein folding is the formation of native disulfide bonds. In the cell, they form much more rapidly than in the test tube, implying that a catalyst is present in vivo (1–3).

We and others have shown that there is a 21-kDa enzyme called DsbB that is essential for disulfide bond formation in vivo (4, 5). DsbB is a protein-folding catalyst that acts to form disulfides in newly synthesized periplasmic proteins. DsbB acts as the direct donor of disulfides to secreted proteins. The active site of DsbB consists of a pair of cysteines present in a CXXC motif that can oxidize to form a very reactive disulfide (6). This disulfide is rapidly transferred to proteins that are in the process of folding (7, 8). The ease at which DsbB can be purified and manipulated biochemically and the availability of a 1.7-A resolution crystal structure for DsbB have led to an abundance of information concerning the mechanism of DsbB action (9–16). One of the features that is thought to contribute to the extreme oxidizing power of DsbB is the very low $pK_a$ of its Cys-30 residue, which makes it a superb leaving group in disulfide exchange reactions (13–16).

In order for DsbA to act as a catalyst of disulfide bond formation, it needs to be reoxidized. Genetic studies strongly implicate an inner membrane protein called DsbB in the reoxidation of DsbA (17, 18). Evidence that DsbB is required for the reoxidation of DsbA includes the observation that DsbB accumulates in a reduced form in DsbB mutants (18). The isolation of a DsbA-DsbB dimer covalently linked by a disulfide bond implies a direct interaction between the two proteins (19, 20). We have proposed a pathway for disulfide bond formation that involves the transfer of disulfides between DsbB and DsbA (Ref. 18, Fig. 1).

DsbB contains two periplasmic domains, each of which possesses two essential cysteines (21, 22). Compared with DsbA, surprisingly little is known about DsbB, nothing is known about its redox properties, it is not known how it reoxidizes DsbA, and it is not known how DsbB in turn is reoxidized.

Our ultimate aims are 1) to understand this disulfide catalytic machine in enough detail to be able to reconstitute it in vitro, 2) to understand how disulfide bond formation is linked to the metabolism of the cell, and 3) to understand how the catalytic properties of DsbB allow it to specifically oxidize DsbA. To accomplish these aims, biochemical investigation of the properties of DsbB is clearly warranted. We report here the reconstitution of the DsbA-DsbB catalytic system in vitro and the demonstration that this system is oxygen-dependent.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Growth Conditions—We constructed an Escherichia coli strain that overproduces DsbB by cloning the protein-coding region of the dsbB gene under the isopropyl-$\beta$-D-thiogalactopyranoside-inducible T5 promoter of pQE70 (Qiagen) to generate pBJS-3. This clone was constructed by polymerase chain reaction amplification of the dsbB coding region from plasmid p24-1 (18). Primers CB8 5'GCCCCGCATCTGCGGATTTGGAACG-3' and CB9 5'-GGAGATCTCGGCAGCGAGATACGACG-3' were used to amplify DNA encoding DsbB. Primer CB8 introduces a SpI site at the initiation codon and inserts a silent mutation in the second codon. Primer CB9 generates a BglI site that replaces the termination codon. Ligating this polymerase chain reaction product cleaved with the enzymes SpI and BglI into the plasmid pQE70, which had been cleaved by SpI and BglI, fuses a six-residue histidine tag onto the C-terminus of DsbB. The sequence of DsbB was confirmed to be correct after polymerase chain reaction and cloning. The DsbB-overproducing plasmid was transformed into DHB5844 to generate JCB851. DHB5844 is MC1000 pAi250 phoR phoA34puvII p0Xgen, p0tet. It was a gift of D. Boyd (Harvard Medical School). JCB851 was grown to an A590 of 0.7, and DsbB expression was induced by the addition of isopropyl-$\beta$-D-thiogalactopyranoside to 15 $\mu$M followed by continued shaking for 4 h. Induction with higher concentrations of isopropyl-$\beta$-D-thiogalactopyranoside was found to lead to rapid growth cessation and cell lysis before detectable quantities of DsbB accumulated.

Preparation of Membranes—Two liters of cell culture were centrifuged at 5000 $\times$ g for 15 min and resuspended in 20 ml of cold buffer A (50 mm Tris-HCl, pH 8.0, 300 mm NaCl, 1 mm phenylmethylsulfonyl fluoride). All further steps were carried out at 4 $^\circ$C. Cells were broken by ultrasonication. Aggregates and cell debris were removed by two low-speed centrifugations at 5000 $\times$ g and 15,000 $\times$ g for 30 min in a Sorvall SS34 rotor. Membranes were prepared from the supernatant of the

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second low speed spin by centrifugation at 100,000 × g for 45 min in a Beckman type 50.2Ti rotor. The membrane pellet was washed with buffer B (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 1 mM phenylmethylsulfonyl fluoride) and resuspended in 10 ml of buffer B, and glycerol was added to a final concentration of 10%. Membranes containing DsbB were used directly in the DsbA reoxidation assay described below or frozen at −70 °C. Stored under these conditions, the membranes retained their DsbA oxidizing activity for at least four months.

As a negative control, we also prepared membranes from the dsbB null mutant JCB819. JCB819 is MC1000 dsbB:kan5 phoR marF lacZ 102 zih12:TrnA.

Determination of DsbB Concentration in Membranes—The DsbB protein was identified as an overproduced band that migrates on 14% SDS-polyacrylamide gels (Novex) with an apparent molecular weight of 20,000, consistent with the predicted Mr of 20,965. This band was detectable after Coomassie Blue staining of DsbB-overproducing membrane preparations but was absent from membranes prepared from WM70. WM70 carries the expression plasmid pQE70 without the n-dodecyl-β-maltoside, pH 8.0. The sample was freed of insoluble material by centrifugation at 100,000 × g for 60 min in a 50.2Ti rotor. The supernatant was then loaded on a Ni-nitrilotriacetic-agarose column (Qiagen) equilibrated with 50 mM phosphate, 300 mM NaCl, 0.1% n-dodecyl-β-maltoside, pH 8.0. After washing with the same buffer adjusted to pH 6.0, DsbB was eluted from the column by a imidazole gradient ranging from 0 to 0.5 M at pH 6.0. Peak fractions were pooled, and the concentration of DsbB was determined by UV absorption using a calculated extinction coefficient of 47,870 M−1 cm−1 at 330 nm. Measurements were made in a buffer C (10 mM citrate, 10 mM NaCl, 0.1% EDTA, pH 8.0). The extinction coefficients used for DsbA were 60,000 M−1 cm−1 at the 330-nm excitation wavelength and 491 M−1 cm−1 at the 330-nm emission wavelength. We demonstrated that after correction for the inner filter effect, the fluorescence increase upon addition of purified DsbB was linear with the increase of fluorescence over a wide range of DsbA concentrations from 0 to at least 50 μM. These corrected changes in fluorescence could be directly related to changes in the concentration of reduced DsbA. The intrinsic fluorescence of the membranes showed no change upon the addition of 2 mM reduced DTT or 2 mM oxidized GSSG. Since the membranes showed no redox-dependent changes in fluorescence, they were assumed to stay constant during the course of the reaction.

AMS Trapping—The reaction between DsbA and DsbB was performed as described above except the amount of membranes used was decreased 10-fold, and 0.2 mM EDTA was added to avoid spontaneous air oxidation of DsbA. At different times, samples were precipitated with 10% trichloroacetic acid and centrifuged at 17,000 × g for 20 min. The pellet was washed with acetone and resuspended in 50 mM Tris-HCl, pH 7.5, 20 mM AMS, 0.1% SDS, 10 mM EDTA. AMS is a reagent that covalently reacts with free thiols, adding a 490-Da group (12). The sample was then run on a 14% SDS-polyacrylamide gel electrophoresis Novex gel under nonreducing conditions. The mobility shift correspond- ing to the reaction of this reagent with reduced DsbA was detected by Coomassie staining.

Anaerobic Reaction Experiments—Rapid kinetic measurements were performed with a Hi-Tech Scientific Model SF-61 stopped flow fluorospectrophotometer that was controlled by a Macintosh Hex using KISS software (Kinetic Instruments, Inc.). The optical path length of the observation cell was 1 cm. The stopped flow apparatus was rendered anaerobic by scrubbing the flow system with anaerobic buffer solution containing 0.1 unit/ml protocatechuic acid dioxygenase (31) and 400 mM protocatechuic acid. This solution was allowed to stand in the flow system overnight and then was thoroughly rinsed out with anaerobic buffer before experiments. Reduced DsbA solution and membrane fractions containing DsbB were rendered anaerobic by 10 × gas exchange with pure argon in glass sample tubes. The excitation wavelength was set to 295 nm with a 320-nm cut-off filter. Emission was monitored over a range from 325–335 nm. K3, Determinations—DsbA can be treated as a substrate for DsbB. To obtain the K3 value of DsbB with DsbA as a substrate, we determined initial velocities of DsbA oxidation for a wide range of DsbA concentrations. The plot of substrate concentration versus initial velocity was best fitted to the Michaelis-Menten equation using Sigma Plot (Jandel Scientific).

pH Dependence—To measure the pH dependence of the reaction, we first adjusted the pH of polybuffer C by the addition of HCl or NaOH over the pH range 5.0–9.0. Buffer C contained 300 mM NaCl, and there was no significant change in salt concentration as measured by conductivity. The initial pH was measured before the pH titration. We then measured the initial rate of DsbA oxidation by DsbB-containing membranes. The concentration of DsbA used in these experiments was 20 μM, which corresponds to about 2-fold over the K3 value. The fluorescence of DsbA is slightly pH-dependent (13). To correct for this, we measured the fluorescence of DsbA in its oxidized form and after reduction using 2 mM DTT. Using the obtained correction factors, we were able to directly transform the initial vel-
eliminated by inclusion of 0.1 mM EDTA (not shown). Curve c demonstrates that DsbB-containing membranes have no effect on fully oxidized DsbA. It is obvious that the slope obtained under c reaches the same fluorescence level as under a, suggesting that DsbA gets completely reoxidized. This was further confirmed by the addition of 1 mM GSSG, which showed no additional decrease in fluorescence. Fluorescence measurements were performed at 30 °C in a total volume of 600 μl. The excitation wavelength was 295 nm, and the emission wavelength was 330 nm. All solutions were preincubated at 30 °C to avoid temperature effects on fluorescence after addition. The reaction was started by adding small volumes of highly concentrated reduced DsbA. The concentrations of DsbA_{red} or DsbA_{ox} were 10 μM, a 166-fold excess over the concentration of DsbB that was present in membranes prepared from the DsbB overproducer (60 nM).

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Isolated Membranes Containing the DsbB Protein Catalyze the Reoxidation of DsbA—The DsbA protein acts as a disulfide catalyst by directly donating its disulfide to folding periplasmic proteins (3). Genetic evidence suggests that a second protein called DsbB acts to reoxidize DsbA (18). Since it appears that the interaction between the Dsba and DsbB proteins is specific, we have started investigating this important disulfide exchange reaction in vitro. For this purpose it was necessary to develop a specific biochemical assay for DsbB activity. We have succeeded in developing a fluorescence assay for DsbB activity that allows us to directly follow the oxidation of DsbA by DsbB. Our assay is based on the observation that reduced DsbA has an about 3-fold higher tryptophan fluorescence than the oxidized form of the protein (6). This provides a very convenient way of following the oxidation or reduction of DsbA in solution (13, 16, 29).

When reduced DsbA is incubated in buffer C at 30 °C, only a very slow decline in fluorescence is observed (See Fig. 2). This effect is probably due to gradual air oxidation of DsbA. Air oxidation of thiols can be catalyzed by metal ions. It is, however, very slow for DsbA and can be eliminated by the addition of 0.2 mM EDTA (see Fig. 3). The addition of membrane fractions prepared from a dsbB null mutant strain had no effect on this gradual oxidation. The upper line in Fig. 2 shows the time course for 10 μM reduced DsbA in the presence of membranes prepared from the dsbB null mutant, JCB819.

In contrast, when a membrane fraction prepared from a DsbB-overproducing strain is added, the fluorescence decreases rapidly, indicating the rapid oxidation of DsbA. The fluorescence reaches a constant minimal value in about 30 min. suggesting that DsbA was completely oxidized (see Fig. 2). To verify that DsbA was oxidized to completion, we showed that no further decline in fluorescence accompanied the addition of oxidized glutathione to a final concentration of 2 mM, redox conditions known to completely oxidize DsbA (7).

To verify that the decline in fluorescence was due to cysteine oxidation, two experiments were performed, DTT reduction and AMS trapping. We added DTT to a final concentration of 2 mM and observed that the original fluorescence level was regained, showing that the decrease in fluorescence was due to sulfhydryl oxidation only. Evidence that DsbA is reoxidized during the assay also comes from AMS trapping experiments (Fig. 3). AMS reacts specifically and irreversibly with sulfhydryl groups, conjugating a large, 490-Dalton moiety to the free cysteines. This major change in molecular weight can be used to clearly separate oxidized from reduced DsbA on SDS-polyacrylamide gel electrophoresis gels (12). In order to minimize the amount of membrane proteins on the gel and distinguish DsbA from membrane proteins, we used 10-fold less membrane proteins than in the fluorescence assay shown in Fig. 2. The addition of DsbB-containing membranes resulted in a time-dependent change in reactivity of DsbA to AMS. The oxidation of DsbA was also followed by fluorescence. The amount of oxidized DsbA increased in a parallel fashion by both measures (see legend to Fig. 3). DsbA exposed for several hours to membranes prepared from a dsbB- strain did not show any measurable oxidation as measured by AMS reactivity (Fig. 3).

Quantification of DsbB Present in Membranes—To show that
DsbB was active in catalytic amounts, we scanned Coomassie Blue-stained gels of membrane preparations to determine the quantity of DsbB present in the membranes used in the assay. A standard curve was constructed by loading various concentrations of DsbB purified by nickel affinity chromatography as a standard (Fig. 4). The concentration of DsbB in membranes used for the measurement shown in Fig. 2 was determined to be 60 nM. Since this quantity of DsbB could oxidize to completion 10 μM DsbA, a 166-fold excess, it is clear that DsbB is acting enzymatically rather than in a stoichiometric manner. In the AMS trapping experiments shown in Fig. 3, 10-fold-less DsbB-containing membranes were used, putting DsbA (20 μM) into a 3300-fold excess.

**Oxygen Is the Final Electron Acceptor**—The finding that DsbB acts catalytically implies that it is getting reoxidized itself during the assay. In order to determine if oxygen was involved as an electron acceptor for DsbB, we ran the assay under strictly anaerobic conditions. Fig. 5 shows that DsbB is not able to oxidize DsbA in the absence of oxygen. Extensive degassing of the buffer solutions and scrubbing of the flow system with the enzymatic deoxygenation system protocatechuic acid dioxygenase was necessary to remove enough oxygen to inhibit the reaction. This implies that the DsbB-containing membranes are able to utilize even small amounts of oxygen in order to allow catalysis of disulfide bond formation. When 20% oxygen is allowed to diffuse into the anaerobic buffer, the reaction rapidly initiates, and DsbA is oxidized by DsbB. Thus, the ability of DsbB to oxidize depends strongly on the presence of oxygen. It is important to note that oxygen only very slowly oxidizes DsbA in the absence of DsbB and that this slow reaction is inhibited by EDTA, implying that it is due to metal catalyzed oxidation. The DsbB-dependent reaction, on the other hand, proceeds at the same rate in the presence of 0.2–2.5 mM EDTA (Fig. 3 and data not shown).

**Steady State Kinetic Studies**—To further investigate the catalytic role of DsbB, we measured the dependence of the reaction on DsbA concentration. The concentration of DsbA was varied, whereas the concentration of DsbB was held constant, and the initial velocities of fluorescence decrease were measured. The fluorescence decrease was linear with time for greater than 10 min. Spontaneous oxidation of DsbA by air was negligible over this time period. Fig. 6 shows the relationship between DsbB concentration and the initial velocity of DsbA oxidation. This velocity was derived from the original fluorescence data by calculating a number of 24 (= 60/2.5) for the “inner filter effect” (see “Experimental Procedures”). This effect is due to the absorption of the DsbA fluorescent signal by DsbA itself. The corrected data could be fit to the Michaelis-Menten equation, yielding a hyperbolic curve. DsbB thus shows classical Michaelis-Menten behavior. We calculate a K_m value for DsbB of 9.6 ± 0.7 μM and a k_cat = 24 ± 0.7 min⁻¹.

**pH Dependence**—Thiol disulfide interchange reactions are generally rather pH-sensitive. To determine if this is the case for the enzymatic activity of DsbB, we tested the pH dependence of its ability to reoxidize DsbA. The relationship between pH and initial velocity of DsbA oxidation is shown in Fig. 7. In sharp contrast to many disulfide exchange reactions, the reoxidation of DsbA by DsbB occurs at a similar rate over a rather broad pH range. Also, in contrast to many disulfide exchange reactions that have a pH optimum of around 9, the pH optima of DsbB is about 6.

**DISCUSSION**

We report here the first biochemical characterization of the reaction between DsbA and DsbB, two proteins that are essen-
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The concentration of DsbA in our assay is up to 3300-fold in excess over DsbB, showing that DsbB is acting as a catalyst. DsbB acts on DsbA as an enzyme with DsbB being the substrate. DsbB displays typical Michaelis-Menten behavior. The $K_m$ observed of DsbB for DsbA is 10 $\mu$M, suggesting a rather specific interaction. This specificity is strongly supported by genetic data. If DsbB was capable of directly oxidizing a wide range of substrate proteins like DsbA can, there would be no need for the DsbA protein, and mutations in dsbA would not eliminate disulfide bond formation. The $K_m$ value is remarkably close to the calculated value of the concentration of DsbA in the periplasm of about 8 $\mu$M. This value was calculated based on the number of DsbA molecules present per cell, determined to be 850, and the periplasmic volume, assumed to be 20% of the total cell volume (5, 26).

One of the unusual properties of DsbB is its ability to maintain activity over a rather wide pH range, with a pH optimum in the acidic range. Thiol groups can only serve as attacking species when they are present as the thiolate anion. This makes thiol disulfide exchange reactions very pH-sensitive. The rate of reaction generally declines 10-fold for each pH unit below the $pK_a$ of the attacking group. Disulfide exchange between small molecules containing thiol groups such as glutathione show a pH optimum of 9 and are 1000-fold slower at pH 6 (27). DsbB is able to function well over a broad pH range including mildly acidic conditions. This makes very good physiological sense. The pH of the periplasm is expected to vary widely, depending on the pH of the media. Disulfide bond formation is required to be catalyzed over this wide range of pH values, but catalysis is especially needed under acidic conditions near the minimal growth pH of E. coli, pH 4.4, where disulfide exchange with small molecules would be vanishingly slow (27, 28). DsbB shows a similarly buffered ability to oxidize substrates over a wide pH range including acidic pH (29). This is in part due to the very low $pK_a$ of the first cysteine in the active site of DsbA (16). It will be interesting to see what properties of DsbB are responsible for it still being active below pH 6.0.

Our assay opens up the door to investigate the biochemical properties of DsbB and the biochemistry of the DsbA-DsbB interaction. Our ability to test for active DsbB should allow the purification of DsbA based on activity. This should allow direct determination of which factors in addition to DsbB are required for oxidation of DsbA.

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