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Reconstitution of a Disulfide Isomerization System*

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Jean-François Collet‡§¶, Jan Riemer‡, Martin W. Bader‡‖, and James C. A. Bardwell‡**

From the ‡Department of Molecular, Cellular and Developmental Biology, University of Michigan, Ann Arbor, Michigan 48109-1048, the §Laboratoire de Chimie Physiologique, Université Catholique de Louvain, Brussels B-1200, Belgium.

Isomerization of disulfide bonds is vital for the proper folding of proteins that possess multiple disulfides. In prokaryotes, the catalytic pathway responsible for disulfide isomerization involves thioredoxin, thioredoxin reductase, and the DsbC, DsbG, and DsbD proteins. To be active as isomerases, DsbC and DsbG must be kept reduced. This task is performed by the cytoplasmic membrane protein DsbD. DsbD in turn is reduced by the cytoplasmic thioredoxin and is composed of three domains. The β domain is membrane-embedded, whereas the α and γ domains are localized to the periplasm. It had been proposed that electrons are transferred within DsbD by a succession of disulfide exchange reactions between the three domains. To test this model using biochemical methods, we purified to homogeneity different polypeptides corresponding to the α, β, γ, and βγ domains. Using these domains, we could reconstitute a DsbD activity and, for the first time, reconstitute *in vitro* the electron transport pathway from NADPH and thioredoxin to DsbC and DsbG. We showed that electrons are transferred from thioredoxin to the β domain then successively to the γ domain, the α domain, and finally on to DsbC or DsbG. We also determined the redox potential of the γ domain to be −241 mV, and that of the α domain was found to be −229 mV. This shows that the direction of electron flow within DsbD is thermodynamically driven.

A critical step in the folding of newly synthesized proteins is the formation of native disulfide bonds between the thiol groups of two cysteine residues (for a review, see Ref. 1). In prokaryotes, this disulfide formation occurs in the periplasm and is catalyzed by a protein called DsbA[1] (DsbA stands for disulfide bond) (2). DsbA is a powerful (3) and rather nonspecific oxidant that has the dangerous potential of introducing non-native disulfides into proteins with multiple cysteines, an event that, if uncorrected, can lead to protein inactivation. To correct for these mistakes, the cell needs a disulfide isomerization system. The isomerization system in *Escherichia coli* involves three additional Dsb proteins: DsbC, DsbG, and DsbD.

DsbC and DsbG are periplasmically localized proteins that have disulfide isomerase activity *in vitro*. The three-dimensional structure of DsbC solved recently by McCarthy *et al.* (4) showed that DsbC is a V-shaped dimer of two thioredoxin-like folds. The active site C*XX*C motifs, one from each monomer, face into the interior of the V. It appears that dimerization of DsbC is required for its isomerase activity (5).

To be able to correct non-native disulfides, the cysteines of the C*XX*C motif within DsbC and DsbG need to be kept reduced in the oxidizing environment of the periplasm. In the isomerization reaction, the first cysteine of the C*XX*C motif, present in the reduced form, is thought to attack a non-native disulfide in a misfolded protein. This results in the formation of a mixed-disulfide between the isomerase and the target protein. This mixed disulfide is resolved either by the attack of the second cysteine of the C*XX*C motif or by another cysteine contained in the misfolded protein. If the isomerase becomes oxidized, it needs to be re-reduced before it can carry out a second catalytic cycle. The protein responsible for the reduction of DsbC and DsbG is the inner-membrane protein DsbD (6).

DsbD is a 59-kDa protein that is synthesized as a precursor with a cleavable signal peptide (7, 8). DsbD has three different domains. Two domains (α and γ) are located in the periplasm. They are connected by the membranous β domain. Sequence analysis suggests that the γ domain has a thioredoxin-like fold, like DsbA, DsbC, and DsbG. Each domain of DsbD possesses a conserved pair of cysteine residues, which have been shown by site-directed mutagenesis experiments to be required for activity (7–9).

Genetic studies have shown that DsbD receives its electrons from thioredoxin, a cytoplasmic protein that keeps ribonucleotide reductase and other cytoplasmic proteins in the reduced state (10). Thioredoxin itself is reduced by thioredoxin reductase and NADPH.

A model has been proposed for the direction of electron flow within DsbD. This model relies on two types of *in vivo* experiments: first, the identification of mixed disulfide bonds between thioredoxin and the β domain and between the α domain and DsbC (11, 12) and second, the demonstration that, when the γ domain is removed from the system, the β domain is found reduced and the α domain is found oxidized (12). This suggests that electrons are successively transferred from thioredoxin to the β domain, to the γ and α domains, and finally on to DsbC or DsbG. In addition, *in vitro* 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonate trapping experiments also suggested that the α domain and DsbC interact (13).

There are a number of reasons to suspect that this pathway may be incorrect or incomplete. One significant problem with the model is that the evidence for it is based largely on two difficult techniques: *in vivo* thiol trapping and isolation of reaction intermediates from whole cells. Quantitative thiol trapping is difficult to achieve even *in vitro*, where the availability

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‖ Current address: Department of Microbiology, Medical School, University of Washington, Seattle, Washington 98195-7720.

** A Pew Scholar. To whom correspondence should be addressed: Department of Molecular, Cellular and Developmental Biology, University of Michigan, 830 N. University, Ann Arbor, MI 48109-1048. Tel.: 734-764-8028; Fax: 734-647-0884; E-mail: jbardwel@umich.edu.

[1] The abbreviations used are: Dsb, disulfide bond; MES, 4-morpholineethanesulfonic acid; HPLC, high performance liquid chromatography.

This paper is available on line at http://www.jbc.org

of extraneous thiols can be rigorously controlled. Effective quenching is expected to be an especially severe problem when dealing with proteins such as DsbD that are designed to undergo very rapid thiol exchange reactions as part of their enzymatic mechanism. Removal of cysteines within DsbD by mutagenesis results in the accumulation of stable mixed disulfides between proteins and between domains for some of these mutants. These cross-linking experiments have interpreted the presence of these stable disulfide bonds as an indication that these bonds are a normal intermediate in the reaction mechanism. Unfortunately, cross-linking experiments are especially difficult to interpret when studying disulfide exchange reactions within a multicysteine protein. Here, removal of one cysteine may not stop the reaction in its tracks leading to accumulation of the intermediate normally present prior to the block. Rather, the unstable reaction intermediates may rapidly rearrange to a more stable configuration. The cross-linking of DsbC with cysteine mutants of DsbD's α domain was taken as an indication that the two interact in DsbD's catalytic cycle. It may, however, have another explanation. It has been reported that mutations that alter the cysteines 103 or 109 of the α subunit destabilize this subunit (12). The role of DsbC *in vivo* is to form mixed disulfides with partially unfolded miss-oxidized proteins. DsbC will be expected to interact with any cysteine-containing misfolded protein, including destabilized mutants of DsbD. Thus DsbC may be treating DsbD mutants that lack these cysteines as isomerization targets.

For these reasons we decided that it would be appropriate to test the current model of DsbD function using a biochemical approach.

To test this model, we produced and purified separately the three domains of DsbD. Using the purified domains, we could reconstitute a DsbD activity *in vitro*. We showed that thioredoxin is able to reduce the β domain and that electrons are then successively transferred to the γ domain, the α domain, and DsbC or DsbG. These results are consistent with the direction that electron flow has been previously proposed. We also determined the kinetic parameters of the reaction between the α domain and DsbC and DsbG and the redox potentials of the α and γ domains. We also showed that DsbA cannot effectively oxidize the α domain of DsbD. This result helps explain how the DsbA-dependent disulfide oxidation system fails to oxidize and thus inactivate the DsbD-dependent isomerization system.

EXPERIMENTAL PROCEDURES

Materials—Glucose and MES were from Acros. *n*-dodecyl-β-D-maltoside was from Antrace. All other chemicals were from Sigma Chemical Co., Fisher, or ICN. PD-10 gel filtration columns, HiTrap affinity columns, and Q-Sepharose were from Amersham Biosciences. *Pfu* polymerase was from Stratagene, and the restriction enzymes were from Promega. SDS-PAGE gels were from NOVEX, and thrombin was from Novagen. Plasmid pFK053 was provided by F. Katzen and J. Beckwith (Harvard), and purified thioredoxin and thioredoxin reductase were provided by Charles Williams, Jr.

Plasmids Construction—The coding DNA sequence of DsbD was amplified by PCR using primers DsbD1 and DsbD2 (Table I). The amplified DNA was first inserted into a pCR2.1 vector, cut with *Nco*I and *Bam*HI, and introduced into a pQE60 plasmid to generate pMB87. Primer pairs DsbD3/DsbD4 and DsbD5/DsbD6 were amplified to generate deletion derivatives of pMB87 that only contained the portion of DsbD that corresponded to the α or γ domains of DsbD. The amplification products were re-circularized to generate pJFC1 (DsbD α) and pJFC2 (DsbD γ), respectively. Primers DsbD7/DsbD8 were used to amplify pJFC1 to generate a derivative of the α domain that lacks the sequence of DsbD's signal peptide, for cytoplasmic expression. Re-ligation of the amplification product generated pJFC3.

TABLE I
Primers used in this study

DsbD1	5′-CAGATTACCTCATATGGCTCAACG
DsbD2	5′-CCACTGCAAGGATCCTTCACGGTTG
DsbD3	5′-GGATCCAGATCTCATCACCATCAC
DsbD4	5′-GTTGTTGGCGACCACTTCGCTTAA
DsbD5	5′-CATACCGCGCAAACTCAGACG
DsbD6	5′-CATCCATGGTTAATTTCTCCTCTTTAATGA
DsbD7	5′-GGATTATTCGACGCGCCGGGAC
DsbD8	5′-CATCCATGGTTAATTTCTCCTCTTTAATGA
DsbD9	5′-CCATGGCAGCGCCACAGCCT
DsbD10	5′-GGATCCCGTCGCACCAAATGC
DsbD11	5′-CGCGGCAGCGCAGCGCCACAGCCTGTG
DsbD12	5′-CGGCACCAGGTTGTTGGCGACCACTTC
DsbD13	5′-CGCGGCAGCCATCTCAACTTTACACAAATC
DsbD14	5′-CGGCACCAGCGTCTGAGTTTGCGCGGTATG
DsbD15	5′-AACCCCATGGATGTCATT
DsbD16	5′-CGGATCCTGCAAGTGGGCGCACGCTAAC
DsbD17	5′-CTTTATGCCGACTGGGCCGTCGCGGCTAAAGAGTTTGAGAAATAC
DsbD18	5′-GTATTTCTCAAACTCTTTAGCCGCGACGGCCCAGTCGGCATAAAG

The coding sequence for the β domain of DsbD was amplified with primers DsbD9 and DsbD10. The PCR product was cut with *Nco*I and *Bam*HI and inserted into a pQE60 plasmid to generate pJFC4 (DsbD β).

A thrombin site was introduced between the α and β domains using "back to back" primers DsbD11/DsbD12 to PCR amplify pMB87. The PCR product was re-ligated to generate pJFC5. pJFC5 was used in a PCR reaction with primers DsbD13 and DsbD14 to introduce a thrombin site between the β and γ domains. Re-ligation of the PCR product generated pJFC6. The two thrombin sites were inserted after residues 145 and 452, respectively. Plasmid pJFC6 and primers DsbD17 and DsbD18 were used to replace the two cysteine residues present in the γ domain by alanine residues generating pJFC7.

The β-MalF sequence was inserted in front of the β domain by PCR amplification of pFK053 with primers DsbD15 and DsbD16. The PCR product was digested with *Nde*I and *Bam*HI and inserted into pET11 to generate pJFC7.

PCR reactions were performed using *Pfu* DNA polymerase. That the desired sequence was obtained was verified by Sanger sequencing performed at the University of Michigan core facilities.

Expression and Purification of the Proteins—BL21(DE3) harboring the various DsbD domain expression plasmids were grown aerobically in 1 liter of LB medium at 37 °C until an A_{600} of 0.8–1.0 was reached. Isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 0.5 mM, and shaking was continued for 5 h to allow protein expression. Cultures were then centrifuged at 5000 rpm and 4 °C for 15 min. From this point on, all the steps were performed at 4 °C. Bacteria were resuspended in 25 ml of buffer A (50 mM sodium phosphate, pH 8.0, 300 mM NaCl) containing 1 mM phenylmethylsulfonyl fluoride and 1 mM EDTA. For the preparation of the more protease-sensitive species, DsbD with thrombin sites, the β domain, and MalF-β, a tablet of Complete protease inhibitor mixture (Roche Molecular Biochemicals) was also added. Cells were disrupted by two passes through a French Press at 1200 p.s.i.

Two different purification protocols were followed, one for soluble domains and the other for membrane-associated domains. Bacterial extracts containing the soluble α or γ domains were centrifuged for 40 min at 20,000 × *g* and 4 °C in a Sorvall SS34 rotor. The resulting supernatant (~20 ml) was diluted 2-fold with buffer A and applied onto a 5-ml Hi-Trap nickel-affinity column. The column was washed with 3 volumes of buffer A. The His-tagged DsbD derivatives were eluted with buffer A containing 60 and 300 mM imidazole, successively.

Both the α and γ domains were found to elute at 300 mM imidazole.

Bacterial extracts containing the membrane-associated proteins, DsbD with thrombin sites, the β domain, and MalF-β, were centrifuged at 100,000 $\times$ g for 90 min in a Beckman type 50.2Ti rotor. The membrane pellet was resuspended in 10 ml of buffer A, and membranes were solubilized in 1.5% n-dodecyl-β-D-maltoside. The His-tagged proteins were then bound to a 5-ml Hi-Trap nickel-affinity column that had been equilibrated with 3 volumes of buffer A, containing 0.02% n-dodecyl-β-D-maltoside. The column was washed with the same buffer, and proteins were eluted with various concentrations of imidazole (15, 30, 45, 60, and 300 mM). DsbD with thrombin sites eluted with 300 mM imidazole.

All purified proteins were concentrated by ultrafiltration and desalted by gel filtration on PD-10 columns in 20 mM Hepes, pH 7.5. For membrane proteins, 0.02% n-dodecyl-β-D-maltoside was added to the buffer.

After gel filtration, 3 ml of DsbD with thrombin sites containing 15 mg of protein was incubated with 6 thrombin units in 20 mM Tris-HCl, pH 8.4, 150 mM NaCl, 2.5 mM $CaCl_2$, and 0.02% n-dodecyl-β-D-maltoside. A thrombin unit is the amount of enzyme required to cleave 1 mg of a test protein when incubated in the above conditions at 20 °C for 16 h. After an overnight incubation at 4 °C, DsbD with thrombin sites was completely cut between the α domain and $\beta\gamma$. The digestion mixture was then loaded onto a 5-ml Hi-Trap nickel-affinity column, and proteins were eluted in two steps with buffer A containing 60 and 300 mM imidazole, respectively. The fraction that eluted with 60 mM imidazole contained the α domain; those that eluted with 300 mM imidazole contained the $\beta\gamma$ polypeptide. This polypeptide was concentrated to 3 mg/ml and desalted by gel filtration on PD-10 columns. 400 μg of the $\beta\gamma$ polypeptide was incubated overnight at 37 °C with 20 thrombin units to cleave at the thrombin site that had been engineered between the β and γ domains. The digestion mixture was then loaded onto a column containing 200 μl of nickel-nitrilotriacetic acid (Qiagen). The column was washed with 3 volumes of buffer A. The β domain was found in the flow-through fraction, while the γ domain was eluted with 3 volumes of buffer A containing 300 mM imidazole. The domains were desalted by PD-10 chromatography.

DsbC, DsbG, and DsbA were expressed and purified as previously described (14, 15).

Reduction and Oxidation of Recombinant Proteins—DsbA, DsbC, DsbG, thioredoxin, and both the α and γ domains were oxidized by incubation in 20 mM oxidized glutathione for 30 min at 4 °C. Oxidized glutathione was then removed by gel filtration on PD-10 Sephadex columns, and the proteins were concentrated using an Amicon 50-ml ultrafiltration unit (YM10 filters). The thiol content was measured using the Ellman assay (16), and the proteins were shown to be >95% oxidized. Proteins were stored in 25 mM Hepes, pH 7.5, whereas proteins used in redox equilibria were in 100 mM potassium phosphate, pH 7.0, 1 mM EDTA.

Thioredoxin and the α and γ domains were reduced by incubation in 10 mM dithiothreitol for 1 h at 4 °C. Dithiothreitol was then removed by gel filtration on PD-10 Sephadex columns. Gel filtration was performed using a solution purged of N_2 of 100 mM potassium phosphate, pH 7.0, 1 mM EDTA. For the determination of redox potential, reduced proteins were prepared immediately before use. The thiol content was measured using the Ellman assay (16), and the proteins were shown to be >95% reduced.

Redox Potentials Determination—Redox potentials of the γ and α domains of DsbD were determined by redox equilibria with thioredoxin ($E^{0\prime} = -270$ mV) as described in a previous study (17). 50 μM of either the γ or the α domain was incubated separately with 50 μM thioredoxin in 200 μl of an argon-purged solution of 100 mM potassium phosphate, pH 7.0, 1 mM EDTA. One protein of the pair was added in the oxidized state, and the other was added in the reduced state. After 4 and 14 h, 50-μl aliquots were quenched by addition of 50 μl of 1% H_3PO_4 and analyzed by reverse-phase HPLC (Waters, 2695 Separations Module). The oxidized and reduced forms of each protein were separated on a C18 column (Phenomenex Primesphere, 250 $\times$ 4.60 mm). The oxidized and reduced forms of both thioredoxin and the γ domain were separated using a linear aqueous 39–51% acetonitrile gradient in 0.1% trifluoroacetic acid over 25 min at a flow rate of 1 ml/min. The oxidized and reduced forms of the α domain were separated using an isocratic elution with 15% methanol and 28% acetonitrile in 0.1% trifluoroacetic acid over 20 min at a flow rate of 0.5 ml/min. Absorbance was recorded at 214 and 280 nM. Integration of the chromatograms allowed us to calculate the amounts of the oxidized and reduced forms of the different proteins. Similar results were obtained from integration of chromatograms recorded at 214 and 280 nM. The equilibrium constant of the reactions and the redox potentials were calculated as in a previous study (17).

Enzyme and Proteins Assays—The fluorescence of DsbA and DsbG was monitored in a Hitachi F-4800 spectrofluorometer. The wavelength used for tryptophan excitation was 295 nm, and the emission was monitored at 330 nm.

The α domain activity was assayed spectrophotometrically at 340 nm by coupling its reduction to NADPH consumption via thioredoxin and thioredoxin reductase. The assay was performed at 30 °C in 1 ml of a mixture containing 25 mM Hepes, pH 7.5, 30 μM NADPH, 40 nM α domain, 2 μM thioredoxin, 20 nM thioredoxin reductase, and various amounts of the substrate protein (DsbC or DsbG).

The β domain activity was assayed spectrophotometrically at 340 nm. The assay was performed at 30 °C in 0.4 ml of a mixture containing 25 mM Hepes, pH 7.5, 0.02% n-dodecyl-β-D-maltoside, 20 μM NADPH, ~200 nM β domain, 10 μM oxidized γ domain, 2 μM thioredoxin, and 20 nM thioredoxin reductase.

Protein concentrations were calculated using the following extinction coefficient (ϵ_{280}, in M^{-1} cm^{-1}): 20,340 for the α domain, 8,370 for the γ domain, 62,730 for the $\beta\gamma$ domain, and 83,470 for DsbD with thrombin sites. These extinction coefficients were calculated using the ProtParam program of the ExPASy website (us.expasy.org/tools/protparam.htm).

RESULTS AND DISCUSSION

It has previously been shown that DsbD consists of three domains, the α, the β, and the γ domains. These domains of DsbD, when expressed separately, can assemble *in vivo* into a functional enzyme that is capable of transferring electrons from thioredoxin to the disulfide isomerases DsbC and DsbG (12). It has been postulated that electron flow within DsbD occurs via a succession of disulfide exchange reactions, where disulfides are transferred from DsbC to the α domain, then on to the γ domain, and finally on to the β domain. The β domain is then reduced by thioredoxin and thioredoxin reductase in an NADPH-dependent reaction (Fig. 1). To test this model of electron flow within DsbD, our experimental strategy was to first purify all the components of the reaction, including the individual domains of DsbD. Then we determined which of the individual components when mixed together are capable of rapid electron transfer reactions. By this means we postulated that we should be able to determine the direction of electron flow within DsbD.

Production of the Two Periplasmic Domains of DsbD—The α domain of DsbD (residues 1–145) was first overexpressed with

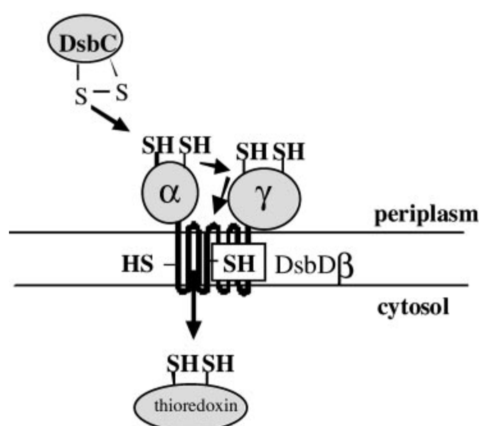

FIG. 1. **Proposed model for the disulfide bonds isomerization pathway in the *E. coli* periplasm.** The *arrows* show disulfide flow.

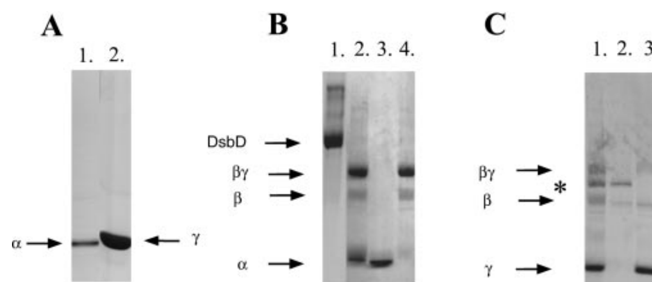

FIG. 2. **Purification of the three domains of DsbD.** *A*, purified α (*lane 1*) and γ (*lane 2*) domains. B, purified DsbD with thrombin sites (*lane 1*) was digested with thrombin. Two polypeptides (*lane 2*) corresponding to the βγ polypeptide and the α domain are clearly visible. A small amount of the β domain could also been observed. The digestion mixture was loaded onto a nickel-affinity column. The α domain was eluted with 60 mM imidazole (*lane 3*), whereas the βγ and β domains were eluted with 300 mM imidazole (*lane 4*). *C*, incubation of the βγ polypeptide isolated from the DsbD that contained the thrombin site, with thrombin partially cutting the protein into two polypeptides (*lane 1*) corresponding to the β and γ domains. This preparation was purified by nickel-affinity chromatography. The flow-through (*lane 2*) contained some β domain and thrombin (*asterisk*), whereas the γ domain, uncleaved βγ, and some β domains were eluted at 300 mM imidazole (*lane 3*).

the signal peptide targeting the domain into the periplasm and flanked by a C-terminal His tag to allow rapid purification. Unfortunately, SDS-PAGE analysis of periplasmic extracts revealed that only minor amounts of the α domain were produced (data not shown). We then attempted cytoplasmic expression by expressing a version of the α domain from which the signal sequence (residues 1–19) had been removed. In this case, much higher amounts of the α domain were produced. 30 mg of the α domain could be purified from 1 liter of culture. The γ domain (residues 440–565) flanked by a C-terminal His tag was also highly overexpressed in the cytoplasm. 100 mg of this domain could be purified from 1 liter of culture. The two proteins were >95% pure (Fig. 2*A*).

Production and Purification of the β Domain—There are at least two reasons to suspect that the β domain is key to transport of disulfides across the membrane. First, the β domain is the only membrane-embedded domain within DsbD, and it contains two strictly conserved cysteines. Second, many organisms do not have DsbD but only the β domain. For these reasons, we decided that it was worth some considerable effort to purify the β domain in an active form. Efforts to express the isolated β domain of DsbD on its own failed (data not shown). This was not surprising because the β domain is entirely membrane-embedded, and membrane proteins are notoriously hard to purify. We also attempted, but failed, to express a variant of the β domain that had its N-terminal end fused to the first transmembrane segment of the membrane protein MalF. We picked this construct because it had been shown to be expressed *in vivo* in at least sufficient quantities to complement a DsbD deletion when coexpressed with the α domain and the γ domain (12). This MalF-β fusion, however, did not express in sufficient quantities to allow purification.

We knew that the intact DsbD protein could be expressed, solubilized, and purified in high quantities.[2] Thus, we decided to construct a variant of DsbD that contained two thrombin cleavage sites (LVPRGS) introduced at the N- and C-terminal ends of the β domain. We called this variant "DsbD with thrombin sites." We then purified this variant by using a His tag and then cleaved out and purified the β domain. A membrane fraction was prepared from cells overexpressing this variant and solubilized, and the DsbD thrombin construct was purified by nickel-agarose chromatography. About 16 mg of ~92% pure DsbD with thrombin sites could be obtained from 6 liters of culture (Fig. 2*B*).

The incubation of 15 mg of DsbD with thrombin sites with 6 units of thrombin cleaved the protein mainly into two polypeptides of 15 and 45 kDa, respectively (Fig. 2*B*). This indicated that only one thrombin site had been completely cleaved. A band of ~30 kDa (the molecular mass of the β domain) could also be seen but was very faint. The digestion mixture was then loaded onto a nickel-agarose column, and proteins were eluted with imidazole. The 15-kDa polypeptide was found in the fraction that eluted with 60 mM imidazole, and the 45-kDa polypeptide in the fraction eluted with 300 mM imidazole (Fig. 2*B*). The presence of the His tag at the C terminus of DsbD with thrombin sites (at the C terminus of the γ domain) indicated that the 15-kDa protein corresponded to the α domain, and the 45-kDa fragment corresponded to a protein that contained the β and γ domains still linked together (βγ). The polypeptide corresponding to the β domain was also found in the fraction containing 300 mM imidazole. The absence of a His tag in the β domain suggested that the β domain has a high affinity for the γ and/or βγ domains. About 7 mg of the βγ polypeptide was then concentrated and desalted by gel filtration.

A small amount of the βγ polypeptide (~0.4 mg) was then incubated with 20 units of thrombin. After 16 h of incubation, the βγ polypeptide was ~60% cleaved into two polypeptides of 30 and 15 kDa, respectively (Fig. 2*C*), corresponding to the molecular masses of the β and γ domains. The digestion products were loaded onto a 0.2-ml nickel-agarose column. The 30-kDa β domain was found in the flow-through and in the fraction eluted with 300 mM imidazole. This fraction also contained the 15-kDa polypeptide (the γ domain). Using this approach, about 0.1 mg of the β domain could be obtained from 0.4 mg of βγ polypeptide.

In another approach to obtain a domain that had similar redox properties to the isolated β domain but that might be easier to purify, we prepared a βγ polypeptide in which the cysteines of the C*XX*C motif in the γ domain were replaced by alanine residues (βγC-A). Because this protein only contains the two cysteines from the β domain, it should have similar thiol disulfide exchange properties to that of the isolated β domain. Using the approach described above for the βγ polypeptide, 6 mg of βγC-A could be purified from 6 liters of culture.

Reconstitution of DsbD Activity—The reaction that we chose to follow is the reduction of the disulfide isomerases DsbC and DsbG by NADPH in a DsbD-catalyzed reaction (Fig. 1). Ideally, our goal was to monitor both products of the reaction, the

[2] J.-F. Collet, J. Riemer, M. W. Bader, and J. C. A. Bardwell, unpublished data.

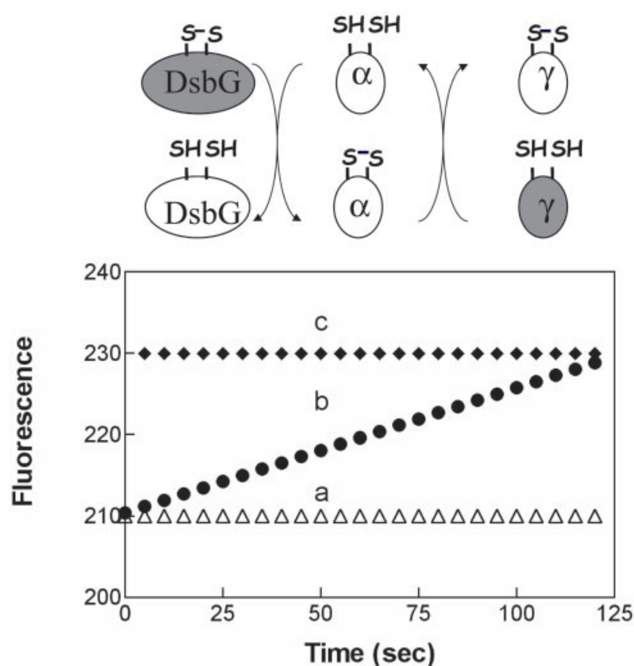

FIG. 3. **Electron flow between the periplasmic domains of DsbD.** *Curve a* shows that the fluorescence of DsbG does not change in the presence of a stoichiometric amount (1 μM) of the γ domain. *Curve b* shows the increase of fluorescence of DsbG when a catalytic amount of the α domain (10 nM) is added to this system. Fluorescence was measured as described under "Experimental Procedures." *Curve c* shows the immediate increase in fluorescence of DsbG, corresponding to the reduction of DsbG that occurs when 1 μM of reduced α is mixed with 1 μM of oxidized DsbG. *Above the graph* is a diagram of the reaction.

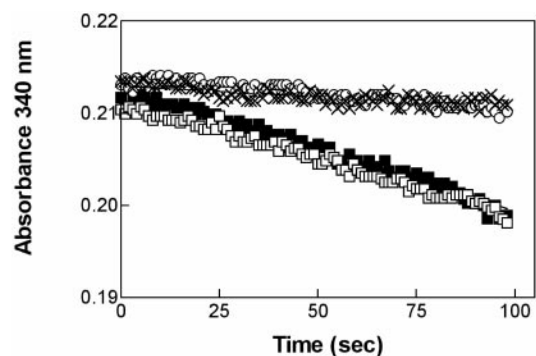

FIG. 4. **Reconstitution of a DsbD activity.** When the thioredoxin system (20 μM NADPH, 2 μM thioredoxin, 20 nM thioredoxin reductase) was incubated with DsbC (20 μM), a small consumption of NADPH was observed (○). When the three domains were added together (40 nM each), the NADPH consumption increased (■). The same increased was observed when 40 nM of the α domain was added alone (□), whereas the addition of 40 nM $\beta\gamma$ (×) had no effect.

oxidation of NADPH and the reduction of the isomerases. The oxidation of NADPH to $NADP^+$ can be monitored at 340 nm. The reduction of DsbG can be monitored by the 1.3-fold increase in tryptophan fluorescence that accompanies its reduction. This provides a convenient way to measure the oxidation state of DsbG and measure how it changes in real-time.

To be able to determine which of the domains of DsbD can directly reduce DsbG, we decided to mix stoichiometric quantities of oxidized DsbG with reduced versions of the periplasmic domains of DsbD. For this experiment to be unambiguous, it was first important to determine how the tryptophan fluorescence of the isolated α and γ domains of DsbD changed with their oxidation status. The α domain does not exhibit a change in fluorescence upon oxidation or reduction, and the isolated γ domain exhibits almost undetectable levels of fluorescence at 340 nm in either the oxidized or reduced forms (not shown). This lack of fluorescence change in the domains of DsbD is fortunate, because it allows us to directly monitor the oxidation status of DsbG in the presence of various mixtures of the DsbD periplasmic domains, without interference from simultaneous changes occurring in the oxidation status of these domains.

When stoichiometric quantities of reduced γ domain were mixed with oxidized DsbG, no reaction was observed, indicating that the γ domain is unable to interact directly with DsbG. However, when stoichiometric amounts of reduced α domain and oxidized DsbG were mixed, a rapid reaction took place and DsbG was immediately reduced (<3 s) (Fig. 3 and data not shown). This indicates that electrons can be directly donated from the α domain to DsbG, as it has been previously reported for DsbC (13).

We then asked if the α domain is reduced by the γ domain as proposed by the model. As shown above, when stoichiometric amounts (1 μM each) of reduced γ domain and oxidized DsbG were incubated, the DsbG fluorescence was not modified (Fig. 3). This indicated that electrons cannot be efficiently transferred directly from the γ domain to DsbG. However, when a catalytic amount (10 nM) of the α domain was added to that system, the fluorescence increased (Fig. 3). This indicated that the α domain could act in a catalytic manner to transfer electrons from the γ domain to DsbG. We calculated that the rate of the reaction was about 5 nM/s. Our results indicated that electrons flow from the γ domain to the α domain of DsbD and then on to DsbG.

Our fluorescence method has a significant advantage over the previously reported 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonate trapping methodology because it allows us to determine rates of reaction rather than just end points. This is important in measuring thiol disulfide exchange reactions, because when one mixes any disulfide-containing molecule with a dithiol-containing molecule the two will eventually reach an equilibrium that is dependent on their oxidation potential. This allows one to determine whether one protein is more oxidizing than the other but does not show that they react at a physiologically relevant rate. DsbA is, for instance, more oxidizing than DsbC but is kinetically isolated from DsbC both *in vivo* and *in vitro* (14).

There is good genetic evidence that DsbD uses the reducing power of the cytosolic thioredoxin reductase/thioredoxin/NADPH system to effect the reduction of the periplasmic disulfide isomerases DsbC and DsbG (10). We next attempted to reconstitute the entire *in vitro* electron pathway from NADPH to DsbC by incubating together the different components. The consumption of NADPH can be easily followed by a decrease in absorption at 340 nm. When stoichiometric amounts of NADPH and oxidized DsbC (20 μM) were incubated with catalytic amounts of thioredoxin and thioredoxin reductase but without the three domains of DsbD, the consumption of NADPH was observed to occur at (5 nM/s) (Fig. 4). When catalytic amounts of the three domains from DsbD were added together, the rate of NADPH consumption was increased almost 10-fold to 42 nM/s. This indicated that at least one of these domains was capable of transferring electrons between thioredoxin and DsbC. The rate of NADPH consumption was very similar when a catalytic amount of intact DsbD was added instead of the separate α and $\beta\gamma$ domains of DsbD (not shown). This initially led us to suspect that we had succeeded in reconstituting the entire isomerization system *in vitro*.

As a control, we then added separately the α and $\beta\gamma$ domains. The addition of β did not increase the NADPH consumption significantly (Fig. 4). Surprisingly, in the presence of a catalytic amount of the α domain alone, we observed a reduction of DsbC

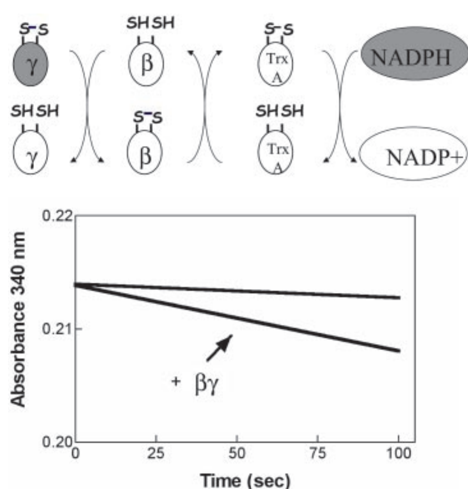

FIG. 5. **Electrons are transferred from thioredoxin to the γ domain by the β domain.** When the thioredoxin system is incubated with oxidized γ domain, a very small NADPH consumption was observed (*upper line*). After the addition of the βγ polypeptide (900 nM), the consumption increased (*lower line*). The activity was assayed spectrophotometrically at 340 nm. The assay was performed at 30 °C in 0.4 ml of a mixture containing 25 mM Hepes, pH 7.5, 0.02% *n*-dodecyl-β-D-maltoside, 20 μM NADPH, 10 μM oxidized γ domain, 2 μM thioredoxin, and 20 nM thioredoxin reductase. *Above the graph* is a diagram of the reaction.

at a rate comparable to that measured with the three domains together (Fig. 4). This indicated that the activity observed in the presence of the three domains could be explained by the contribution of the α domain alone. Additional control experiments, where we mixed stoichiometric quantities of the α domain with thioredoxin, showed that thioredoxin can efficiently reduce α directly *in vitro* (not shown). This reaction presumably does not occur *in vivo*, because the α domain of DsbD is periplasmically localized and thioredoxin is in the cytoplasm. Fortunately, our separation of the domains of DsbD allowed us the possibility of avoiding this non-physiological reaction by separately analyzing the interdomain interactions. We decided to investigate how well the individual domains interacted with each other. Is the γ domain of DsbD reduced by the β domain? To answer this question we incubated the oxidized γ domain (10 μM) with the thioredoxin system (thioredoxin, thioredoxin reductase, and NADPH). In the absence of the β domain, a very low amount of NADPH consumption (~1.4 nM/s) was assayed (Fig. 5). When a catalytic amount (~200 nM) of the β domain was added, the rate of NADPH consumption was increased to ~2.7 nM/s (not shown). Because only a minor amount of the β domain was available, we confirmed this result with the purified βγ polypeptide that was available in much higher amounts. We assumed that in this case the β domain of the βγ construct would be able to function catalytically to reduce large quantities of the γ domain free in solution. When 0.9 μM βγ polypeptide was added to the system, the NADPH consumption increased by a factor of 5 to ~8 nM/s (Fig. 5). The same result was obtained using the purified βγC-A construct. These results revealed that the β domain is able to reduce the γ domain and that thioredoxin can reduced the β domain. We note that the apparent rate at which the β domain can catalyze the reduction of γ is significantly slower than the rate at which the γ domain reacts with the α domain or the rate with which the α domain reacts with DsbC or DsbG. We have considered two possible explanations for the low reactivity. First, the transfer of electrons from the β domain to the γ domain could be a non-physiological reaction. However, the membrane-embedded β domain is the only domain that is in an appropriate topological position to transfer electrons from the cytoplasmic thioredoxin

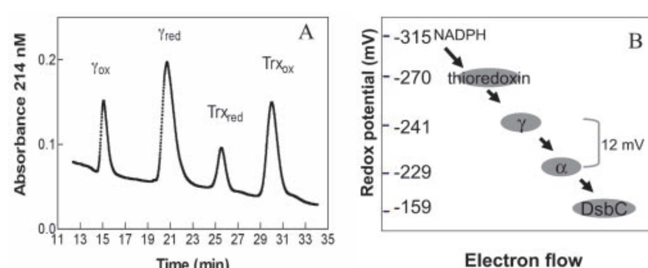

FIG. 6. *A*, analysis of the equilibrium between thioredoxin and the γ domain of DsbD by reverse-phase HPLC. The proteins (50 μM each) were incubated at room temperature in 100 mM potassium phosphate, 1 mM EDTA. Aliquots (50 μl) were taken after 14 h and quenched by the addition of 50 μl of H_3PO_4. HPLC was performed as described under "Experimental Procedures." From the amount of oxidized and reduced proteins present at equilibrium, the redox potential could be calculated as described previously (17). *B*, electron flow in the isomerization pathway. The redox potential increases as electrons flow down the isomerization pathway from NADPH to DsbC.

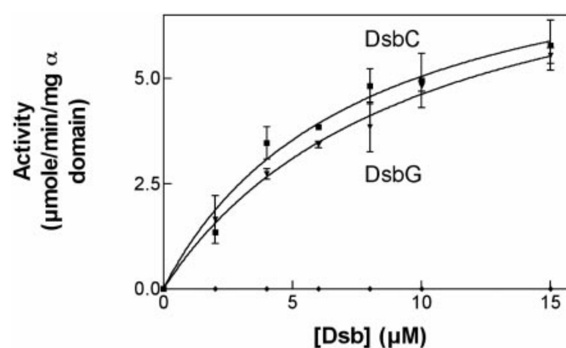

FIG. 7. **Michaelis-Menten kinetics of the reaction between the α domain and DsbC or DsbG.** Initial velocities were obtained from the decrease of the absorbance of NADPH measured at 340 nm. Data were corrected to take into account the NADPH consumption contributed by the direct reduction of DsbC and DsbG by thioredoxin. Reactions were performed in the conditions described under "Experimental Procedures."

to the periplasmic isomerases. Second, the β domain could be partially inactivated by the purification procedure either by partial denaturation by the detergent or by the partial loss of a cofactor.

It appears that that electrons are transferred from thioredoxin to the β domain and then to the γ domain. The low activity observed when thioredoxin is incubated with oxidized γ domain only (Fig. 5) was also observed when thioredoxin was incubated with DsbC (Fig. 4) and DsbG (not shown). This points out the importance of measuring rates of reaction not just the end point. We presume that these reactions represent non-physiological thiol-disulfide exchange reactions expected to occur between any two cysteine pairs. However, even with this reaction in the background, the addition of the β domain clearly increased the rate of the reduction of the γ domain. It was even more striking with the βγ construct, because we could use more of this protein.

The second part of the pathway was reconstituted by following the increase of fluorescence of DsbG upon reduction, as shown above. We showed that electrons can be transferred from the γ domain to DsbG only if a catalytic amount of the α domain is present.

Taken together, these results indicate that electrons flow from NADPH to thioredoxin, then successively to the β, γ, and α domains and to DsbC or DsbG. Our data agree with the previously proposed model, and we have shown that the electron flow is in the direction previously proposed. Our current investigative goals are to determine 1) whether this flow re-

sults from direct disulfide interchange between the domains and 2) if additional electron-transporting cofactors are also involved.

The fact that DsbD activity can be reconstituted *in vitro* by incubating the three independent domains together is reinforced by the fact that in certain organisms such as *Streptomyces*, *Corynebacterium*, and *Mycobacterium* a β-like protein (CcdA) and a γ-like protein are adjacent. It is tempting to consider that evolution has linked CcdA to other proteins to form DsbD. However, DsbD is more than a CcdA domain fused to a thioredoxin-like fold and has certainly some very specific characteristics. This is strongly suggested by our results. We show that the γ domain can only react with the α domain and not with DsbC and DsbG and that the α domain is absolutely required to reduce DsbC and DsbG. This is difficult to reconcile with the fact that any of the genomes containing a CcdA protein present an α-like protein. Moreover, our results show that the β and γ domains stick to each other so well that they are rather resistant to cleavage, even with other proteases.[2] Our purifications also showed that the β domain has some affinity for the γ domain. These data suggest that the thioredoxin-like fold of the γ domain is not just fused to the β domain but deeply interacts with it, probably more so than two separate proteins.

Determination of the Redox Potential of α and γ—We have shown that the electron flow in the isomerization pathway is kinetically driven. To determine if the flow is also thermodynamically driven, we measured the redox potentials of the α and γ domains. The redox potentials of thioredoxin and of DsbC have previously been determined to be -270 and -159 mV, respectively (17). The redox potentials of the γ and α domains were determined by direct protein-protein redox equilibria (17). In this method the protein of interest (Prot A) is incubated with a protein whose redox potential is well characterized (Prot B). At the equilibrium, the amounts of each protein in the reduced and the oxidized state are determined and the equilibrium constant of the reaction is calculated (K_{AB}). The difference between the redox potentials of the two proteins is then given by the equation, $\Delta E_{AB} = RT/nF \ln K_{AB}$.

The α and γ domains were incubated with thioredoxin. At equilibrium, the reactions were quenched and aliquots were analyzed by HPLC. The oxidized and reduced forms of each protein could be separated as shown in Fig. 6 for thioredoxin and the γ domain. Integration of the chromatograms allowed us to calculate the equilibrium constants. For each protein pair, we verified that similar equilibrium constants were obtained irrespective of the redox state of the initial mixture. We determined a value of 9.4 for the equilibrium constant of the reaction between γ and thioredoxin and a value of 23.7 for the equilibrium constant of the reaction between α and thioredoxin. According to the equation above, thioredoxin is 29 mV more reducing than γ and 41 mV more reducing than α. Thus the redox potentials of the γ domain is -241 mV and that of the α domain is -229 mV.

These two values were verified by incubating α and γ together. An equilibrium constant of 0.38 was determined corresponding to a difference of redox potential of 12 mV. This indicates that the electron flow between thioredoxin, γ, α, and DsbC is thermodynamically driven (Fig. 6).

Characterization of the DsbC and DsbG Reduction by the α Domain—We showed that thioredoxin is able to efficiently reduce the α domain. We took advantage of this reaction to characterize the reduction of DsbC and DsbG by the α domain. Catalytic amounts of the α domain were incubated with an excess of the thioredoxin system and with concentrations of DsbC or DsbG that varied from limiting to saturating. By following the decrease of the absorbance at 340 nm, we determined the saturation curve of the α domain for these two isomerases (Fig. 7). We showed that the α domain reduces DsbC and DsbG with comparable V_{max} (~6 μmol/min/mg of α domain) and K_m (~5 μM) values.

There are two disulfide exchange systems present in the periplasm of bacteria: the isomerization system described here and the DsbA-dependent oxidation system. In the oxidation system, DsbA needs to be oxidized to be active whereas the isomerases DsbC and DsbG need to be reduced to be active. What prevents the powerful oxidase DsbA from oxidizing and thus inactivating the isomerization system? We previously showed that DsbB can distinguish between DsbA, which it is able to oxidize, and the isomerases DsbC and DsbG, which it cannot oxidize (14). It is known that DsbC is kinetically isolated from the oxidation system. Is DsbD similarly isolated? The fluorescence of DsbA increases upon reduction (3.5 times) (18). However, when we incubated a stoichiometric amount of reduced γ domain with oxidized DsbA in the presence of a catalytic amount of the α domain, no reaction was observed (not shown). This indicates that DsbA is kinetically isolated from the periplasmic domains of DsbD and helps explain how a futile cycle is avoided. We now report that the α domain cannot reduce oxidized DsbA. Our results indicate that the two systems are kept separated from each other both at the DsbB and the DsbD level.

By separating DsbD into three domains we could reconstitute a DsbD activity and, for the first time, reconstitute *in vitro* the electron transport pathway from NADPH and thioredoxin to DsbC and DsbG. We provide evidence that electrons are transferred from thioredoxin to the β domain then successively to the γ domain, to the α domain, and finally on to DsbC or DsbG. This reconstitution makes possible extensive biochemical and mechanistic analyses of the process of disulfide isomerization. Our results provide biochemical vindication for the conclusions about the direction of electron flow within DsbD that were based on genetic techniques.

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