

Evidence for Conformational Changes within DsbD: Possible Role for Membrane-Embedded Proline Residues

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The mechanism by which DsbD transports electrons across the cytoplasmic membrane is unknown. Here we provide evidence that DsbD's conformation depends on its oxidation state. Our data also suggest that four highly conserved prolines surrounding DsbD's membrane-embedded catalytic cysteines may have an important functional role, possibly conferring conformational flexibility to DsbD.

Many periplasmic and secreted proteins contain disulfide bonds that are required to stabilize the protein's structure. In *Escherichia coli*, the well-studied DsbA-DsbB pathway catalyzes formation of disulfides in substrate proteins (2). A second, less studied, pathway performs disulfide bond rearrangement. In this pathway, the inner membrane protein DsbD maintains periplasmic disulfide isomerases DsbC and DsbG in the reduced and active form (8, 14). This is necessary for DsbC and DsbG to attack incorrect disulfides and catalyze disulfide rearrangements. Genetic studies have shown that DsbD receives its electrons from cytoplasmic thioredoxin and transfers these electrons across the inner membrane to DsbC/DsbG (17). DsbD therefore connects the periplasmic isomerization pathway to the reductive power of the cytoplasm by transferring electrons from the cytoplasm to the periplasm and, correspondingly, disulfide bonds from the periplasm to the cytoplasm.

DsbD is a 59-kDa protein with three domains. Two of these domains (α and γ) are periplasmic while the third, β , is located in the inner membrane. Each domain contains a conserved pair of cysteine residues, which are essential for activity (19). In vivo and in vitro experiments suggest that electrons are transferred via a succession of disulfide bond exchange reactions, from thioredoxin to β , then to γ , then to α , and finally to DsbC/DsbG (3, 11, 18). The crystal structures of both periplasmic domains α and γ have been solved, and there is a significant amount of biochemical information available for these two domains (7, 12, 18). In contrast, much less is known about the membrane domain β . In particular, the mechanism by which the β domain transports disulfides across the inner membrane remains unresolved. In fact, the question of how disulfides get across membranes has not to our knowledge been solved for any system. There are examples of systems that transport electrons across membranes, including the malate-aspartate and glycerophosphate shuttles. In these systems,

electrons are carried by metabolites that are transported from one compartment of the cell to the other. However, DsbD is thought to transport electrons without using a metabolite or a cofactor. If true, this makes DsbD unique among known electron transport proteins. We therefore wanted to examine this longstanding mystery of how reducing equivalents get across membranes.

Conformational changes. If DsbD transports disulfides without using a cofactor, major conformational changes are likely to take place to allow the membrane-embedded cysteine residues to be alternatively exposed to the cytoplasm and to the periplasm. To test this hypothesis, we compared the protease sensitivities of the oxidized and reduced protein. The protein was overexpressed and purified as described previously (3). To test the protease sensitivity of the interdomain regions, we inserted thrombin sites between the α and β domains and between the β and γ domains. DsbD is rapidly cut into two polypeptides, α and $\beta\gamma$ (2), suggesting that the α - β interdomain region is exposed. The second thrombin site, between β and γ , is less accessible to thrombin, suggesting that it is partially buried. Reduction of DsbD allows the $\beta\gamma$ polypeptide to be cleaved more rapidly (Fig. 1A). Since reduced DsbD is more protease accessible, this suggests that there is a conformational change in DsbD upon its change in redox status. In contrast, oxidized DsbD is more sensitive to proteolytic cleavage by endoGlu-C than is reduced DsbD, which is further evidence of a difference in conformation between oxidized and reduced DsbD (Fig. 1B). Altogether, these data suggest that the conformation of DsbD depends on its oxidation state. This prompted us to look for residues that may play an important role in these conformational changes.

Sequence comparisons. DsbD homologues are found in a large number of prokaryotic genomes and a few plants chloroplasts. Multiple-sequence alignments reveal that both catalytic cysteine residues (Cys163 and Cys285) of the β domain are present in two PCX₍₂₋₃₎P motifs that are highly conserved among all DsbD homologues (Fig. 2). The presence of conserved proline residues in close proximity to Cys163 and Cys285 is striking, especially considering that prolines are quite rare in α -helices. However, a number of transmembrane pro-

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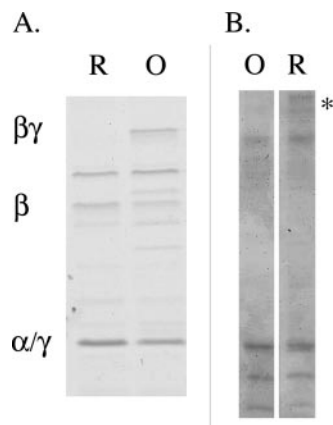


FIG. 1. Protease sensitivities of reduced (lanes R) and oxidized (lanes O) DsbD. A. Digestion pattern of a DsbD construct presenting two thrombin sites inserted between α and β and between β and γ . After reduction of the protein with dithiothreitol, followed by gel filtration to remove the reductant, DsbD is cut into three polypeptides by thrombin (α and γ , which have the same molecular mass and are therefore visible as a single band of 15 kDa [marked α/γ], and β) (3). B. A significant amount of reduced DsbD (asterisk) is still uncut after a 10-min incubation at pH 7.8 with endoGlu-C, a protease which cleaves after glutamate residues under these conditions. There are 15 glutamate residues in DsbD.

teins, including G-protein-coupled receptors and voltage-gated potassium channels, contain conserved proline residues that induce regions of helix distortion and flexibility and are essential for optimal protein function (4, 6, 13). Multiple transmembrane topology prediction programs, as well as genetic work (5, 19), localize DsbD's four conserved prolines within membrane-spanning helices 1 and 4 of the β domain, consistent with a possible conformational role in helix flexibility. We therefore tested the effect of proline-to-alanine mutations on DsbD's *in vivo* activity.

Ability to rescue the copper sensitivity of a *dsbD* mutant. Mutants containing a null mutation in the *dsbD* gene are copper sensitive (9, 15, 17). This is thought to be due to the formation of nonnative disulfide bonds by copper, causing a requirement for functional DsbC (9). To determine the importance of the four prolines to DsbD's activity, we studied the ability of single proline-to-alanine mutations to complement the copper sensitivity of a *dsbD* null strain. Plasmids pTrcD, pTrcDP162A, pTrcDP166A, pTrcDP284A, and pTrcDP289A, containing the coding sequences of His-tagged wild-type or mutant DsbD, were used to transform a *dsbD dsbA* mutant. The *dsbD dsbA* double mutant was used because the copper sensitivity of a *dsbD* mutant is augmented by the absence of *dsbA*, while the single *dsbA* mutant is not copper sensitive relative to the wild-type strain. Testing the mutant DsbD proteins in the *dsbD dsbA* mutant thus allowed us to more precisely compare the *in vivo* effects of mutant and wild-type DsbD. Strains were grown in the absence of copper to an optical density of 0.5, and dilutions were plated onto plates containing 6 μ M copper and 40 μ M IPTG (isopropyl- β -D-thiogalactopyranoside) to induce expression of the DsbD variants. As shown in Fig. 3A, in the presence of IPTG, a wild-type strain containing the empty pTrc vector formed viable single colonies at a dilution of 10^{-4} , as did the *dsbD dsbA* mutant

Transmembrane segment 1

		Cys163
<i>E. coli</i>	ALWALLI----GIGIAFTPCVLFMPYPLISGIV	
<i>S. baltica</i>	LIWTLILFFGLGVGLALTPCVFPMYPILSGII	
<i>I. loihensis</i>	LIITLLFFALGIGLAFTPCVFPMYPILSGVV	
<i>M. flagellatus</i>	FWLIVAGFFGFLLLSLTPCVLFMIPILSGII	
<i>C. tepidum</i>	LWKVFAAFLLFGLLLSFTPCVLFMPVILSSII	
<i>C. lari</i>	IFITLLTFFGYGLLLALTPCILEMIPILSSLI	
<i>P. profundum</i>	LLKIIGIALLGGLILNIMPCVLFVGLMKLSSI	
<i>R. rubrum</i>	FLAMVSVALLGGLILNLMPCVLFVLSLKILGL	
<i>C. abortus</i>	YVMIMIMAFGLGGLLNIMPCVLFVLTCLKVYGL	
<i>D. desulfuricans</i>	MGKALLFGLIAGLILNFMPCVLFVVSCLKLSTL	
	* : : : * : : : *	

Transmembrane segment 4

		Cys285
<i>E. coli</i>	GVFVMGAIAGLICSPCTTAPLSAILLYI	
<i>S. baltica</i>	GVFLMGVISGLVASPCTTAPLSGALVYV	
<i>I. loihensis</i>	GVFIIGAISGLVASPCTTAPLSGALLFI	
<i>M. flagellatus</i>	GVFAMGVSALIVSPCVAAAPLAGALLYV	
<i>C. tepidum</i>	GVFFMGAIASALIVGPCVAAPLAGLTVYI	
<i>C. lari</i>	GVAIMGFLSALIVGPCVAAPLAGALLYI	
<i>P. profundum</i>	GHFVQGMFATLLATPCS-APFLGTAVAF	
<i>R. rubrum</i>	GAFATGAFATVLTATPCS-APFLGTAVGF	
<i>C. abortus</i>	GAVFNGVLTATLVTTPT-PPFLGSILGL	
<i>D. desulfuricans</i>	QAFFTGMATLLATPCS-GPFLGGVGLW	
	* : : : * : : : *	

FIG. 2. Multiple-sequence alignments of DsbD β . The sequences of transmembrane segments 1 and 4 of *E. coli* DsbD (as predicted previously [5]) were aligned with the corresponding segments of DsbD homologues from *Shewanella baltica*, *Idiomarina loihensis*, *Methylobacillus flagellatus*, *Chlorobium tepidum*, *Campylobacter lari*, *Desulfovibrio desulfuricans*, *Chlamydomophila abortus*, *Photobacterium profundum*, and *Rhodospirillum rubrum* by using ClustalW. The conserved residues are shaded, an asterisk indicates that the residues in that column are identical in all sequences aligned, a colon indicates that conserved substitutions are present, and a period indicates that semiconserved substitutions are present.

containing wild-type DsbD expressed from pTrcD. In contrast, all of the mutants were less viable on copper than wild-type DsbD, forming from 10^1 - to 10^4 -fold fewer viable colonies on copper. This is not due to toxicity of the mutant proteins, because all strains were equally viable when protein expression was induced by IPTG in these strains in the absence of copper (not shown).

We tested the steady-state levels of the mutant and wild-type DsbD proteins by using Western blot analysis with an anti-His antibody. Western blot analysis of wild-type and mutant DsbD indicated that P162A, P166A, and P284A were present in amounts similar to that of wild-type DsbD, or even higher (Fig. 3B). P289A, however, was expressed to a lower level than wild-type DsbD. This raises the possibility that its inability to rescue copper sensitivity may be due to a lower abundance of the P289A mutant due to poor folding. We conclude that all four conserved prolines are important for DsbD's *in vivo* function or folding. The P162A, P166A, and P284A mutants do not appear to affect protein expression but rather directly affect the function of the protein.

Proline mutants are less susceptible to air oxidation. By inducing distortion in transmembrane α -helices, proline residues can act as molecular hinges (4). We hypothesized that DsbD β 's conserved prolines could be important for the correct

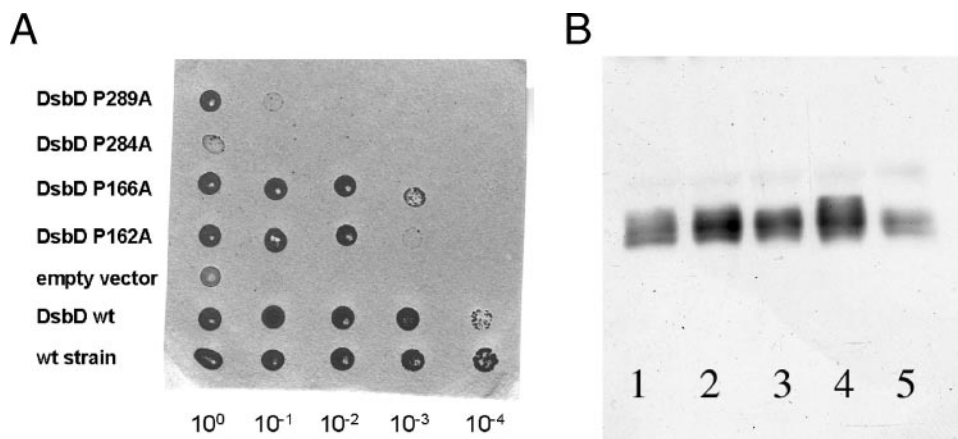


FIG. 3. A. Spot titers of wild-type (wt) and mutant DsbDs on copper plates. Strains were grown on plates containing copper (6 mM), ampicillin (200 μ g/ml), and 40 μ M IPTG to induce expression. A *dsbA dsbD* double mutant was used to express DsbD variants Pro289A, Pro284A, Pro166A, and Pro162A and wild-type DsbD from pTrc. B. Expression levels of the mutants and wild-type DsbD. BL21 cells expressing wild-type DsbD and variants were grown in LB, and protein expression was induced with IPTG. After a 4-h induction, cells were collected. Membrane pellets were prepared, and proteins were solubilized in 1% Triton. Expression levels were assessed by Western blot analysis using an anti-His tag antibody. Lanes: 1, wild type; 2, P162A, 3, P166A; 4, P284A; 5, P289A.

positioning of Cys163 and Cys285, allowing oxidation/reduction cycles to occur. We therefore postulated that replacement of these proline residues might alter the oxidation/reduction cycle of Cys163 and Cys285, possibly by inducing some rigidity in the transmembrane domain. Katzen and Beckwith have shown that Cys163 and Cys285 are found reduced *in vivo* (10). However, we have observed that they are susceptible to air oxidation, as they are found in the oxidized form after extraction and solubilization procedures. To determine whether the proline-to-alanine mutations may alter the susceptibility to air oxidation and thus possibly the oxidation/reduction cycle of Cys163 and Cys285, the individual proline mutants were overexpressed, extracted, and solubilized in 1% Triton. The free cysteine residues were modified using MalPEG, a 5,000-Da molecule that alkylates free thiols, leading to a major shift on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. In order to monitor the redox state of Cys163 and Cys285 only, we replaced all other cysteine residues present in DsbD by alanines (this protein is referred as DsbD*). Katzen and Beckwith have shown that the β domain of DsbD conserves its activity when it is expressed independently of the α and γ domains (11). It is therefore likely that the replacement of the catalytic cysteines of the periplasmic domains will have no major effect on the properties of the transmembrane domain. The proteins were detected by Western blotting using anti-His tag antibodies. After reduction of DsbD* with dithiothreitol, we observe a mobility shift of DsbD. This indicates that, as predicted, both Cys163 and Cys285 of the wild-type protein have been air oxidized upon extraction and solubilization. We noticed that His-tagged DsbD* migrates essentially as two bands (Fig. 4, bands 1 and 2), independent of the modification by MalPEG. This has been previously observed for DsbD*, and it has been proposed that these two bands may represent different SDS-denatured conformations of the protein (10). To confirm that both proteins are indeed full-length DsbD*, we performed N-terminal sequencing. Sequencing of both bands showed that their N-terminal sequences are iden-

tical. Since the His tag used to purify the protein is located at the C terminus of the protein, this result suggests that the C terminus is intact as well. After reduction, bands 1 and 2 disappear and bands 3 and 4 appear (Fig. 4). Bands 1 and 2 apparently correspond to the oxidized protein and bands 3 and 4 to the reduced one, since the protein became accessible to MalPEG only after reduction. Interestingly, whereas DsbD* migrated mainly as bands 1 and 2 (a small amount of band 3 was sometimes observed), we noted that all four bands were reproducibly present for mutant derivatives of DsbD*, i.e., P162A, P166A, and P284A (P289A expression was extremely low, and its redox state could not be characterized). After addition of dithiothreitol to the mutants, only bands 3 and 4 were observed (not shown). Our results indicate that significant amounts of P162A ($\approx 62\% \pm 6\%$), P166A ($\approx 65\% \pm 9\%$), and P284A ($\approx 66\% \pm 7\%$) are still reduced after the solubilization procedure. This shows that these mutants are less sus-

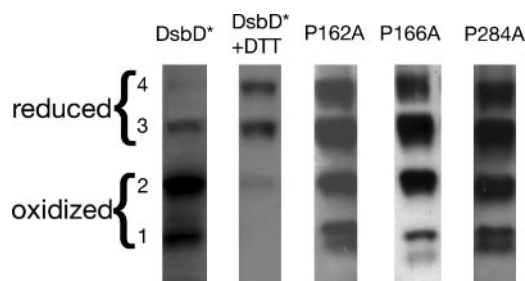


FIG. 4. Redox states of wild-type DsbD* and variants. Cells expressing wild-type DsbD* and variants were grown in LB, and protein expression was induced with IPTG. After a 4-h induction, cells were collected and disrupted and membrane pellets were prepared. Proteins were solubilized in 1% Triton and free thiols were alkylated with MalPEG as described previously (10). Proteins were separated by SDS-polyacrylamide gel electrophoresis and visualized by Western blotting using anti-His tag antibodies. Oxidized protein (bands 1 and 2) and reduced protein (bands 3 and 4) are indicated. DTT, dithiothreitol.

ceptible to air oxidation than the wild type (only $\approx 16\% \pm 3\%$ reduced). This finding suggests that P162, P166, and P284 may play an important role in the redox state of Cys163 and Cys285. When they are absent, the protein appears more likely to be able to maintain a reduced conformation. It should be noted that a band smaller than band 1 was often observed for P162, P166, and P284 (and sometimes for the wild type) and is likely to correspond to a proteolytic degradation fragment that has been modified with MalPEG.

Conclusions. In one current model of DsbD function, the membrane-spanning β domain alternates between a conformation open to the cytoplasm and a conformation open to the periplasm (16). This allows electrons to be transferred across the membrane by a succession of disulfide exchange reactions (11). Important conformational changes are likely to take place within DsbD to allow this disulfide cascade to take place. By comparing the protease sensitivities of oxidized and reduced DsbD, we provided evidence supporting such conformational changes. We then searched for residues that may play an important conformational role, and we noted the presence of four highly conserved prolines flanking the active-site cysteines in DsbD's β domain. Mutation of the four highly conserved prolines in DsbD's β domain affects DsbD's *in vivo* function or folding and stabilizes the reduced state of P162A, P166A, and P284A proteins. Our results are consistent with a conformational role for these proline residues.

While this paper was in the review process, a paper by Cho and Beckwith was published (1). Using a different approach, Cho and Beckwith studied the role of the transmembrane domain proline residues and other conserved amino acids. Their findings are in general agreement with our data.

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