Enzyme Catalysis

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Mechanism of the Prokaryotic Transmembrane Disulfide Reduction Pathway and Its In Vitro Reconstitution from Purified Components**

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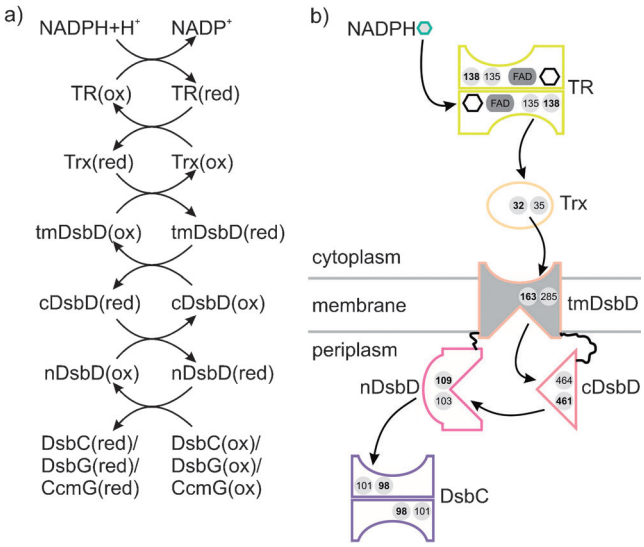
More than half of the proteins in the cell envelope of Gram-negative bacteria contain structural disulfide bonds.[1] Oxidative folding of these proteins occurs in the periplasm and requires the enzymatic introduction of disulfides. As the unfolded pre-proteins enter the periplasm, dithiol oxidase DsbA rapidly oxidizes thiol pairs to disulfides through dithiol/disulfide exchange.[2] Non-native disulfide bonds are subsequently reduced by the isomerase DsbC, allowing disulfide reshuffling to the native configuration.[1a,3]

To reduce fully oxidized, disulfide-scrambled substrates, DsbC must be kept reduced in the otherwise oxidizing environment of the periplasm. The pathway providing electrons for DsbC reduction, which we term the prokaryotic transmembrane disulfide reduction pathway, is depicted in Scheme 1a. DsbC is directly reduced by DsbD,[4] a unique inner membrane (IM) catalyst with an essential cysteine pair in each of its three redox active domains.[3a,5] Both the N-terminal, immunoglobulin-like domain (nDsbD), which directly reduces DsbC,[4a,b,6] and the C-terminal, thioredoxin-like domain (cDsbD), which reduces nDsbD,[7] are located in the periplasm. Eight transmembrane helices between nDsbD and cDsbD define the central, transmembrane domain (tmDsbD; Scheme 1b),[5a,c,8] which receives electrons from thioredoxin (Trx) on the cytoplasmic side of the membrane[5b,9] and reduces cDsbD on the periplasmic side.[5a,10] Notably, DsbD and its homologues are the only enzymes that catalyze the transfer of two reducing equivalents (corresponding to two electrons) across a biological membrane.[2c] Finally, the reduction of Trx by NADPH is catalyzed by thioredoxin reductase (TR) in the cytoplasm.[11]



Scheme 1. The prokaryotic transmembrane disulfide reduction pathway. a) The direct reaction partners and the individual redox reactions along the pathway in *Escherichia coli*. b) NADPH binds to TR in the cytoplasm to reduce its cofactor FAD, which in turn reduces the catalytic disulfide bond in TR. Active site cysteine residues are shown as circles with residue numbers, and those transiently forming covalent mixed disulfide complexes with their redox partners are indicated in bold. The reactive cysteine in tmDsbD labeled here is as identified in this work (see main text).

Besides DsbC, two additional periplasmic oxidoreductases are reduced by DsbD: DsbG and CcmG.[4c] All three DsbD substrates exhibit similar electrochemical and structural properties, but since DsbC is the best characterized, it is the primary focus of this study.

Catalytic activity of full-length DsbD has never been demonstrated directly. Initial attempts to monitor its activity by adding catalytic amounts of detergent-solubilized DsbD to reduced Trx and oxidized DsbC were unsuccessful because the rate of reduction of DsbC by Trx, catalyzed by full length DsbD in detergent micelles, was identical to the rate found when only nDsbD was present at the same concentration.[10] This fast, unnatural cross-reaction between Trx and nDsbD, which are separated by the IM in vivo, prevented the detection of electron flow through the transmembrane domain of DsbD in vitro. Hence, the true function of DsbD could not be confirmed in vitro. It remained unclear whether electrochemical membrane gradients, known to drive numerous membrane transport processes,[12] are required for catalysis, and there were no kinetic data on membrane

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transfer. Herein, we reconstituted the complete prokaryotic transmembrane disulfide reduction pathway in vitro in a three-compartment system, demonstrated the catalytic turnover, and functionally characterized full-length DsbD in its natural membrane environment. In addition, we prepared the mixed disulfides of tmDsbD with its substrates at both sides of the membrane, which represent catalytic intermediates of the membrane transfer and established how reducing equivalents are shuttled across the IM by DsbD.

To circumvent the non-natural cross-reaction between Trx and nDsbD, we used a system of two compartments separated by a third, the lipid membrane bearing DsbD. We reconstituted DsbD, purified in the presence of the mild, nondenaturing detergent decyl-β-D-maltopyranoside, into liposomes (see Experimental Section in the Supporting Information). Because of the high solubility of NADPH and Trx, and the high catalytic efficiency of TR it was feasible to incorporate these compounds (hereafter termed thioredoxin regenerating system (TRS)), into the small lumen of the DsbD-proteoliposomes. After incorporation of the TRS into the DsbD-proteoliposomes, external, unincorporated TRS was removed by ultracentrifugation/washing. Upon dilution of the proteoliposomes into a buffer containing oxidized DsbC, the entire pathway was reconstituted, and transfer of reducing equivalents across the membrane was monitored by the extent of DsbC reduction. At defined time points, the reactions were quenched with acid and remaining free thiol groups in reduced DsbC were derivatized with 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS), making the reduced DsbC about 1 kDa larger than oxidized DsbC and allowing separation of the two redox forms by SDS-PAGE band shift (Supporting Information, Figure S1).

Various gradients, present across the IM, drive membrane transport processes.[12] A pH gradient (inside basic) and membrane potential (inside negative) might be required for reductant transfer, besides the favorable differences between the redox potentials of the pathway components. Furthermore, several membrane transport processes depend on the inward-facing Na^+ gradient.[13] To test all potentially relevant membrane gradients for DsbD function, we compared the kinetics of DsbC reduction 1) in the absence of any gradient (Figure 1a), 2) with the reaction in the presence of an inward-facing pH gradient and a Na^+ gradient (Figure 1b), and 3) with an inward-facing pH gradient, a Na^+ gradient, and a difference in electrostatic potential (inside negative) across the liposomal membrane (Figure 1c). The final molar ratios of all the components in our setup were NADPH/TR/Trx/DsbD/DsbC 810:0.81:7.2:1.0:48. The presence of TR, Trx, and DsbD in catalytic amounts with respect to NADPH and DsbC enabled us to establish turnover, that is to detect multiple catalytic cycles of each enzyme along the pathway. The rate and extent of DsbC reduction was essentially identical in all these cases (Figure 1d), demonstrating that DsbD shuttled reducing equivalents across the membrane without the requirement for any gradient.

A sample of DsbD-liposomes to which the TRS was only added at the outside and then removed by ultracentrifugation/washing, thus leaving the lumen devoid of TRS, served as a control. It allowed us to determine the extent of DsbC reduction not resulting from catalyzed transmembrane reductant transfer by DsbD. This fraction amounted reproducibly to approximately 25%, and stems in part from the stoichiometric reduction of DsbC by reduced DsbD initially present (formed during its exposure to TRS during TRS internalization) and in part from reduction by traces of TRS outside the liposomes, which remained after the washing steps.

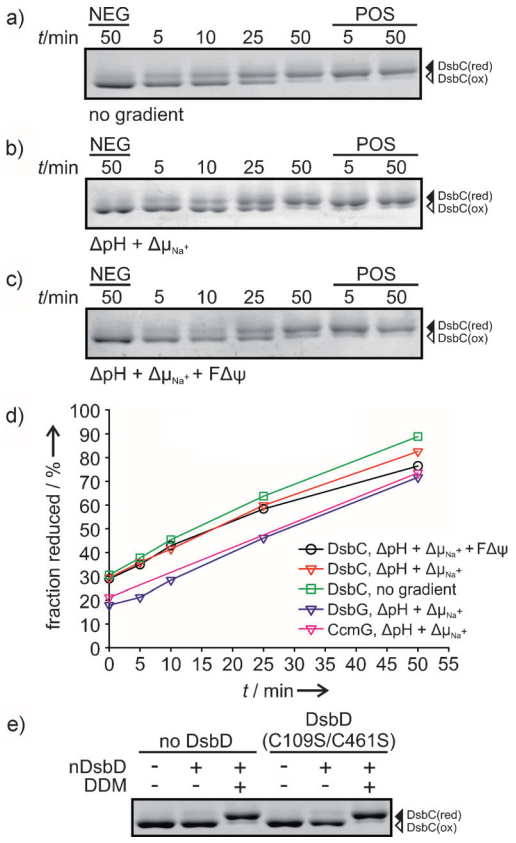

Figure 1. Kinetics of DsbC reduction by membrane-reconstituted DsbD, monitored by SDS-PAGE a) in the absence of any transmembrane gradient b) in the presence of a pH and Na^+ gradient, and c) in the presence of a pH, Na^+ gradient, and membrane potential. For a negative control (NEG), the TRS was only added to the outside of the proteoliposomes to account for the stoichiometric reduction, with no turnover, while in the positive control (POS) samples, proteoliposomes were disrupted by the addition of the nondenaturing detergent dodecyl-β-D-maltopyranoside (DDM), allowing the reduction to proceed to completion. d) Quantification of the DsbC gel bands in (a–c), and analogous experiments performed with DsbG and CcmG (Figure S2). e) SDS-PAGE analysis of the DsbC redox state in analogous reactions performed with liposomes devoid of DsbD and proteoliposomes containing the inactive variant DsbD(C109S/C461S). Samples were incubated for 50 min.

Notably, whereas DsbC reduction resulting from DsbD catalysis reached near completeness over fifty minutes, it did so within five minutes of disrupting the liposome membrane by DDM, indicating that the transmembrane steps are rate-limiting for the entire pathway under the conditions used.

In addition to the reduction of DsbC, membrane-reconstituted DsbD was capable of catalyzing the reduction of DsbG and CcmG (Figure 1d and Figure S2), in accordance with previous genetic data[14] and in vitro experiments

obtained with soluble domains of DsbD.[15] The identical rates of reduction of DsbC, DsbG, and CcmG, together with active site titration experiments (discussed in the Supporting Information) further confirmed that transmembrane steps are rate-limiting for the whole pathway.

To exclude alternative explanations for the reduction of DsbC, we performed the above experiments with TRS-containing liposomes either lacking DsbD or with the catalytically inactive variant DsbD(C109S/C461S) incorporated.[5c] In both cases, no reduction of DsbC was detected over a 50 minute period (Figure 1e). In the absence of active DsbD, DsbC was only reduced completely when nDsbD was externally added along with DDM to disrupt the liposomal membrane (Figure 1e, lanes 3 and 6). We thus conclude that the DsbC reduction in our intact, reconstituted system is a direct consequence of wild-type DsbD activity.

Based on the measured DsbD/lipid ratio in the proteoliposomes of 1:(750±80) (wt/wt; Figure S3) and assuming that all DsbD molecules were functionally reconstituted in a random orientation, the molar ratio of right-side out DsbD to external DsbC is 1:48 in our experimental setup. Determining this ratio allowed calculation of the turnover number of DsbD at 25 °C under the applied conditions to be $k_{cat} = 0.6\ min^{-1}$, or $0.3\ min^{-1}$ if all DsbD molecules had been reconstituted with their periplasmic domains outside. While this rate may be higher in vivo, for example, if not all DsbD molecules had been functionally reconstituted, it is in the same range as the k_{cat} of $0.2\ min^{-1}$ measured for the disulfide isomerization activity of DsbC.[16] The turnover numbers reported for membrane transport proteins span multiple orders of magnitude, and large conformational changes in their membrane domains are thought to result in low values of k_{cat}.[17] The moderate k_{cat} value determined for DsbD can be rationalized by the large structural rearrangements of tmDsbD required to allow alternate access of Trx and cDsbD (folded proteins larger than 11 kDa) to its single disulfide bond.[18] This value is also in agreement with proposals that conformations of tmDsbD depend on its redox state[19] and our kinetic data showing that transmembrane steps are rate-limiting.

The above experiments support the model that reducing equivalents are transferred across the bacterial inner membrane by DsbD exclusively by a series of dithiol/disulfide exchange reactions[5a] without additional cofactors. Specifically, the DsbD reaction cycle involves the transient formation of an intermolecular disulfide bond between Trx and tmDsbD, and an intramolecular disulfide between tmDsbD and cDsbD. To verify this model and identify the cysteine residues in tmDsbD forming these mixed disulfides, we isolated these intermediates from whole cells and preparations of bacterial membranes, and characterized their disulfide connectivity.

In the first set of experiments, *E. coli* membrane vesicles containing the single cysteine variants of isolated tmDsbD, tmDsbD(C285S) ($tmDsbD_{C163}$), or tmDsbD(C163S) ($tmDsbD_{C285}$) were activated with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent).[15b,20] Subsequently, the single cysteine residue variants Trx(C35A) (Trx_{C32}) or cDsbD-(C464S) ($cDsbD_{C461}$), containing only the reactive, N-terminal cysteine residue of the CXXC active site motif, were added and the complexes were solubilized and purified. The analysis of the purified complexes by reducing and non-reducing SDS-PAGE revealed that $tmDsbD_{C163}$ formed a mixed disulfide with both Trx_{C32} and $cDsbD_{C461}$. Conversely, no mixed disulfides could be detected with $tmDsbD_{C285}$ (Figure 2a).

Identical results were obtained when this experiment was repeated using whole cells depleted of outer membrane (Figure 2b). This result demonstrates that C163 is the only residue in tmDsbD capable of forming specific mixed disulfides with its thioredoxin-like substrates at both sides of the membrane, that is Trx and cDsbD.

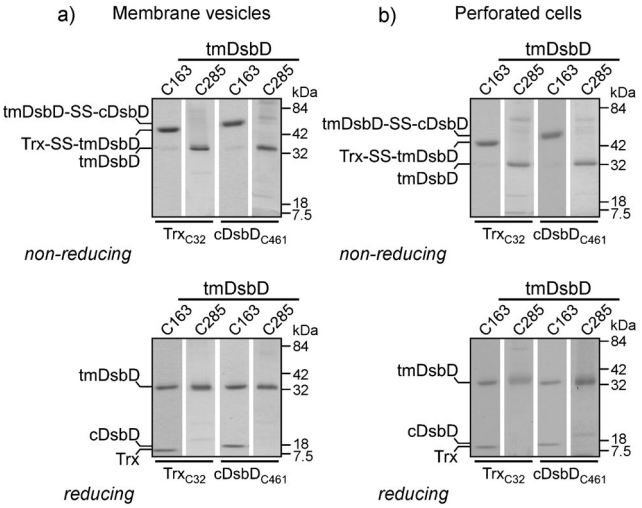

Figure 2. Catalytic intermediates of the membrane transfer process identified by non-reducing and reducing SDS-PAGE. a) Membrane vesicles containing the single cysteine residue variants $tmDsbD_{C163}$ (C163) or $tmDsbD_{C285}$ (C285) were mixed with Trx_{C32} or $cDsbD_{C461}$, solubilized, purified using a hexahistidine tag on tmDsbD, and analyzed under reducing and non-reducing conditions. b) Identical experiment as (a), but using *E. coli* cells depleted of an outer membrane, rather than membrane preparations.

Previous thiol-trapping experiments supported the existence of the same catalytic mixed disulfide intermediate with Trx in vivo, that is $tmDsbD_{C163}$–Trx_{C32},[5a] but suggested the intramolecular disulfide bond $tmDsbD_{C285}$–$cDsbD_{C461}$ instead of $tmDsbD_{C163}$–$cDsbD_{C461}$.[21] It cannot be excluded that our treatment of tmDsbD with DTNB, required to activate its cysteine residues for mixed disulfide formation, triggered this domain to assume a different conformation (for example, an oxidized-state-like conformation) with altered cysteine reactivity. On the other hand, it is plausible that the multiple amino acid replacements in regions flanking tmDsbD that had been introduced for the reported in vivo experiment with cDsbD[21] resulted in a reactivity different from that of the authentic protein.

If the preparation of the complexes of tmDsbD with Trx and cDsbD on opposite sides of the membrane were highly specific, tmDsbD would be expected to react only with cDsbD, and not with Trx added from the outside in cells depleted of an outer membrane. Similarly, preparations of IM

vesicles from cells overexpressing tmDsbD would be expected to contain an equal amount of tmDsbD in the natural and inverted orientation, and therefore react to only 50% with externally added Trx or cDsbD. Our results demonstrate that the formation of mixed disulfide complexes proceeds to completion in both cases. This can be rationalized by 1) Trx and cDsbD are very close structural and functional homologues,[7a,22] that 2) tmDsbD exhibits a pseudo-symmetrical, hour-glass-like structure,[18,21] and that 3) cDsbD likely evolved through an evolutionary fusion of a thioredoxin-like domain to a pre-existing enzymatic core of tmDsbD,[23] which would predict similar reactivity of tmDsbD with both cDsbD and Trx at both sides of the membrane.

In summary, we reconstituted the entire prokaryotic transmembrane disulfide reduction pathway from purified components and demonstrated that all components are required and sufficient for its functionality and catalytic turnover. We presented first insights into full-length DsbD activity in a membrane environment and studied its mechanism in detail. None of the gradients tested exerted any influence on DsbD catalysis, in accordance with a mechanism in which favorable differences of redox potentials provide the thermodynamic driving force for the entire pathway.[10,20,24] While other membrane electron-transfer catalysts are one-electron transporters with redox-active cofactors, such as eukaryotic cytochrome b_{561} and NADPH oxidase,[25] we demonstrated that DsbD transfers two reducing equivalents across the membrane exclusively by dithiol/disulfide redox chemistry, transiently forming a series of catalytic mixed-disulfide intermediates.

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