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Structural and biophysical characterization of the mechanism of the inner membrane redox catalyst DsbD from *Escherichia coli*

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1. Abstract

In the periplasm of *Escherichia coli*, Dsb proteins catalyze disulfide bond formation, isomerisation, and reduction. DsbA and DsbB constitute the oxidative pathway that is responsible for the formation of disulfide bonds. DsbD has a central function in the reductive pathway: it transfers electrons from the cytosolic thioredoxin system across the inner membrane to the periplasm. These reducing equivalents are utilized for disulfide bond isomerization (involving DsbC and DsbG) and for reduction of the *c*-type apocytochromes prior to heme ligation (involving CcmG and CcmH). DsbD consists of three domains: two periplasmic domains in the N- and C-terminal region (nDsbD and cDsbD) and a central transmembrane (TM) domain. Each of these domains contains a pair of essential cysteines: C103/C109 in nDsbD, C163/C285 in the TM domain, and C461/C464 in cDsbD. It was postulated that the catalytic mechanism of DsbD is based exclusively on intra- and intermolecular disulfide exchange reactions. According to the existing model, electrons flow successively from thioredoxin to the TM domain of DsbD, then to cDsbD, nDsbD, and finally to the periplasmic substrate proteins. Available structural information on DsbD included the crystal structure of oxidized nDsbD and of the mixed disulfide between nDsbD and DsbC. In addition, a crystal structure of oxidized cDsbD had been reported.

In this thesis, the catalytic mechanism of DsbD was studied using biophysical and biochemical techniques. To study the intrinsic physical properties of the individual periplasmic domains of DsbD, isolated nDsbD and cDsbD were overexpressed and purified to homogeneity. The determined redox potentials of –232 mV and –235 mV, respectively, demonstrate that electron transfer from thioredoxin (E_0` = –270 mV) *via* DsbD to DsbC ($E_0$` = –140 mV) is thermodynamically driven. The direction of the electron flow from the periplasmic domains of DsbD to DsbC was confirmed *in vitro* by reversed phase HPLC analysis of acid-quenched reaction products. The overall electron flow from $cDsbD_{red}$ to $nDsbD_{ox}$ and finally to $DsbC_{ox}$ is rapid at 25°C and pH 7.0. Complete reduction of DsbC is achieved within about 500 s when low initial protein concentrations of 0.25 μM are used. Direct reduction of DsbC by cDsbD is extremely slow (k_{app} = 57 $M^{-1}s^{-1}$). As expected, reverse electron transfer from $DsbC_{red}$ to $nDsbD_{ox}$ or $cDsbD_{ox}$ does not occur.

We then measured the kinetics of all functional and non-functional disulfide exchange reactions between different Dsb proteins. The very fast electron transfer from $nDsbD_{red}$ to $DsbC_{ox}$ or $DsbG_{ox}$ (k_{app} = 4 x 10^6 $M^{-1}s^{-1}$ and k_{app} = 2 x 10^6 $M^{-1}s^{-1}$, respectively) ensures effective reduction of these proteins by DsbD *in vivo*. In contrast, one of the fastest nonfunctional reactions, reduction of DsbA by nDsbD, is >1000-fold slower (k_{app} = 900 $M^{-1}s^{-}$

[1]). Based on the kinetic data, we can conclude that large kinetic barriers separate the oxidative DsbA/B and the reductive DsbC/D systems and guarantee their independence.

We next showed that C109 and C461 form an inter-domain disulfide bond between nDsbD and cDsbD. This finding allowed crystallization and structure determination of the kinetically stabilized mixed disulfide complex between the C103S variant of nDsbD and the C464S variant of cDsbD (nDsbD-SS-cDsbD) that represents an essential intermediate in the catalytic cycle of DsbD. Comparison of the crystal structure of nDsbD-SS-cDsbD with the known structure of the complex between nDsbD and DsbC revealed a strong overlap between the surface areas of nDsbD that interact with DsbC and cDsbD. Consequently, electron transfer from cDsbD to DsbC via nDsbD must involve large relative domain movements. We also solved crystal structures of oxidized and reduced cDsbD and compared them with the cDsbD structure in the nDsbD-SS-cDsbD complex. cDsbD appears to be a very rigid protein that does not undergo significant conformational changes upon disulfide exchange.

Finally, we proved that DsbD rapidly and directly transfers electrons to the reductase CcmG, and showed that another Ccm protein, CcmH, is not required for this process. For kinetic and structural studies, we produced CcmG as a soluble protein that lacks the N-terminal transmembrane helix. Using stopped-flow fluorescence measurements, we showed fast electron transfer from nDsbD to soluble CcmG *in vitro* (k_{app} = 4 x 10^5 $M^{-1}s^{-1}$). We also synthesized the mixed disulfide complex between the C103S variant of nDsbD and the C83S variant of soluble CcmG (nDsbD-SS-CcmG) and determined its crystal structure. Comparison of the X-ray structures of all three mixed disulfide complexes of nDsbD (nDsbD-SS-DsbC, nDsbD-SS-cDsbD, and nDsbD-SS-CcmG) revealed the structural basis for the intriguing ability of nDsbD to interact specifically and very rapidly with different target proteins such as the membrane-anchored monomer CcmG and the periplasmic dimers DsbC and DsbG.