Basis of the disulfide isomerase activity of DsbC

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Diss. ETH NO. 16485
Basis of the disulfide isomerase activity of DsbC

A dissertation submitted to the

SWISS FEDERAL INSTITUT OF TECHNOLOGY ZURICH

for the degree of

Doctor of Natural Sciences

presented by

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2006
Parts of this thesis have appeared in the following publication:


**Frei P**, Grauschopf U, Glockshuber R. 2006. The disulfide isomerase activity of DsbC does not require the presence of two active sites (in preparation)
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<tr>
<td>Amp</td>
<td>ampicillin</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
</tr>
<tr>
<td>3’CMP</td>
<td>3’-cytidinemonophosphate</td>
</tr>
<tr>
<td>cCMP</td>
<td>cyclic-2’,3’-cytidinemonophosphate</td>
</tr>
<tr>
<td>Dsb</td>
<td>disulfide bond protein</td>
</tr>
<tr>
<td>D-DsbA</td>
<td>dimerized DsbA</td>
</tr>
<tr>
<td>DsbC&lt;sub&gt;CCAA&lt;/sub&gt;</td>
<td>DsbC with active site cysteines exchanged to alanines</td>
</tr>
<tr>
<td>DsbC-C</td>
<td>catalytic domain of DsbC</td>
</tr>
<tr>
<td>DsbC-S</td>
<td>DsbC heterodimer with one active site</td>
</tr>
<tr>
<td>DsbC H45D</td>
<td>monomerized DsbC with His54 to Asp exchange to prevent dimerization</td>
</tr>
<tr>
<td>DsbG-C</td>
<td>catalytic domain of DsbG</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5’-dithio-bis-(2-nitrobenzoic acid)</td>
</tr>
<tr>
<td>DTT</td>
<td>1,4-dithiothreitol</td>
</tr>
<tr>
<td>GdmCl</td>
<td>Guanidinium chloride</td>
</tr>
<tr>
<td>GroEL-AD</td>
<td>apical domain of GroEL</td>
</tr>
<tr>
<td>G7D</td>
<td>fusion protein between GroEL-AD and DsbA via a 7 amino acid residue linker</td>
</tr>
<tr>
<td>G12D</td>
<td>fusion protein between GroEL-AD and DsbA via a 12 amino acid residue linker</td>
</tr>
<tr>
<td>G17D</td>
<td>fusion protein between GroEL-AD and DsbA via a 17 amino acid residue linker</td>
</tr>
<tr>
<td>GSH</td>
<td>glutathione, reduced form</td>
</tr>
<tr>
<td>GSSG</td>
<td>glutathione, oxidized form</td>
</tr>
<tr>
<td>HPLC</td>
<td>high pressure liquid chromatography</td>
</tr>
<tr>
<td>NADPH</td>
<td>$\beta$-nicotinamide adenine dinucleotide phosphate (reduced form)</td>
</tr>
<tr>
<td>nDsbD</td>
<td>NH$_2$-terminal domain of DsbD</td>
</tr>
<tr>
<td>Q1</td>
<td>ubiquinone 1</td>
</tr>
<tr>
<td>RBI</td>
<td><em>ragi</em> bifunctional inhibitor</td>
</tr>
<tr>
<td>RNaseA</td>
<td>ribonuclease A</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------------------------------------</td>
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<tr>
<td>scRNaseA</td>
<td>scrambled ribonuclease A</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>UV</td>
<td>ultra violet</td>
</tr>
<tr>
<td>v/v</td>
<td>volume per volume</td>
</tr>
<tr>
<td>w/v</td>
<td>weight per volume</td>
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<tr>
<td>Xaa</td>
<td>any amino acid residue except Cys</td>
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Abstract

Oxidative protein folding in the periplasm of *E. coli* is mediated by two independent pathways. The introduction of new disulfide bonds in proteins is carried out by the DsbA-DsbB pathway, where two electrons from the new disulfide bond are transferred to quinones. In proteins with more than two cysteines, DsbA can introduce non-native disulfide bonds. To reshuffle these disulfide bonds, an additional, reductive pathway exists, in which two electrons, originating from cytoplasmic NADPH, are transported across the inner membrane through DsbD. DsbD provides these reduction equivalents either to the disulfide isomerases DsbC and DsbG, which are responsible for the correction of non-native disulfide bonds in proteins, or to CcmG, which is required for the maturation of cytochrome c proteins. The soluble, periplasmic disulfide oxidoreductases DsbA, DsbC and DsbG share two similarities: the thioredoxin-like fold that forms the catalytically active domain and the Cys-Xaa-Xaa-Cys active site motif. In contrast to the monomeric disulfide oxidase DsbA, the disulfide isomerases DsbC and DsbG are homodimers.

In this PhD thesis, the function of DsbC as a disulfide isomerase was studied using different variants of the proteins DsbC and DsbA. To investigate the structural basis for disulfide isomerase function, the catalytic domain of DsbC alone, an inactivated variant of DsbC and a dimerized DsbA variant were expressed in *E. coli* and purified. Additionally, wild type DsbG and its catalytic domain were expressed and purified separately. It was shown that a dynamic exchange of subunit occurs in DsbC, whereas no subunit exchange was detected in DsbG. The dynamic subunit exchange of DsbC allows the formation of a heterodimeric form of DsbC, where only one active subunit is present in each dimer. Disulfide isomerase or reductase activities of the different proteins were tested for the catalysis of reshuffling of disulfide bonds in scrambled RNaseA or catalyzed reduction of insulin disulfide bonds. The disulfide isomerase or reductase activity of DsbC was independent of the number of active sites present in the DsbC dimer. However, the monomerization of DsbC led to the loss of both disulfide isomerization and reduction activity, whereas the dimerization of DsbA increased both the disulfide isomerization and reduction activity, albeit only slightly. No disulfide isomerization and reduction activities were found for DsbG or its catalytic domain. DsbG prevented aggregation of reduced insulin by binding the insulin β-chain and thus possesses a chaperone-like activity.
To test if the presence of two active sites has an influence on the function of disulfide oxidoreductases, dimeric DsbA and the monomeric catalytic domain of DsbC were expressed in a \textit{dsbA} deficient \textit{E. coli} strain. Notably, dimeric DsbA fully complemented the \textit{dsbA} deficiency and the catalytic domain of DsbC partially complemented the deficiency, while wild type DsbC could not restore disulfide bond formation. Thus, both the dimerized DsbA and the catalytic domain of DsbC interact with DsbB. This disulfide exchange with DsbB was confirmed \textit{in vitro}. Monomeric DsbC reacts with DsbB five times faster than wild type DsbC, although the rate is still 700 times slower than that with DsbA. The Michaelis-Menten parameters for both the DsbB-catalyzed oxidation of dimeric DsbA and wild type DsbA by ubiquinone 1 are in the same range. Similarly, the reaction of the different variants with the NH\textsubscript{2}-terminal domain of DsbD, which directly reduces DsbC by disulfide exchange, was tested \textit{in vitro}. Disulfide exchange between nDsbD and DsbA is $10^5$ times slower than between nDsbD and DsbC, whereas monomeric DsbC reacts $10^3$ fold slower than wild type DsbC. Dimeric DsbA reacts by a factor of 10 faster with nDsbD than wild type DsbA. No differences between DsbG and its catalytic domain were observed for disulfide exchange rates with either DsbB or DsbD. Overall, the ability of the catalytic DsbC to partially replace DsbA in \textit{dsbA} deficient strains results from a dramatically decreased reduction rate by DsbD, rather than from faster oxidation by DsbB.

In conclusion, it was shown that, in addition to the oligomeric state of the proteins, other structural factors are involved in the separation of the two independent pathways in oxidative protein folding. Furthermore, DsbC needs only one active site per dimer for its disulfide isomerase activity, but its dimerization is necessary to form a large contiguous peptide binding site, which is required for disulfide bond isomerization.
**Zusammenfassung**


In dieser Doktorarbeit wurde die Funktion von DsbC im Hinblick auf seine Rolle als Disulfidisomerase mit Hilfe verschiedener Varianten der Proteine DsbA und DsbC untersucht. Um mögliche Modelle für die Funktion zu testen wurden die katalytische Domäne von DsbC, eine inaktivierte Variante von DsbC sowie eine dimerisierte Variante von DsbA in *E. coli* hergestellt und gereinigt. Zusätzlich wurden ebenfalls DsbG und die katalytische Domäne von DsbG überexprimiert und gereinigt. Im Rahmen der Arbeit konnte gezeigt werden, dass DsbC einen dynamischen Austausch seiner Untereinheiten aufweist, welcher in DsbG nicht stattfindet. Mittels dieses dynamischen Untereinheitenaustausches von DsbC konnte eine heterodimere Form von DsbC, bestehend aus nur einem aktiven Zentrum pro Dimer, hergestellt werden. Um die Disulfidisomerasefunktion der verschiedenen Varianten zu testen, wurden die Disulfidisomerasefunktion anhand der Rückfaltung falschverbrückter RNaseA und die Disulfidreduktaseaktivität über die Fähigkeit zur Reduktion von Insulin untersucht. Die Aktivität in beiden Tests erwies sich als unabhängig von der Anzahl aktiver Zentren pro DsbC-Dimer. Hingegen verliert DsbC bei der Monomerisierung seine Disulfidisomerase- und Disulfidreduktaseaktivität, während beide Aktivitäten in DsbA durch Dimerisierung von DsbA teilweise gesteigert werden konnten, aber nie dieselben Aktivitäten wie in DsbC erreicht. Es konnte keine Disulfidisomerase- und Disulfidreduktaseaktivität von
DsbG und seiner katalytischen Domäne festgestellt werden. Hingegen konnte gezeigt werden, dass DsbG die Aggregation der β-Kette von Insulin im Insulinreduktions-Test wie ein Chaperon verhindern kann.


Zusammenfassend konnte in dieser Arbeit gezeigt werden, dass neben dem oligomeren Zustand von DsbC und DsbG für die Reaktivität mit DsbB und DsbD weitere sterische Faktoren eine Rolle spielen. Ebenso konnte gezeigt werden, dass DsbC nur ein aktives Zentrum pro Dimer für die Disulfidisomerasefunktion benötigt, aber die Dimerisierung für eine effiziente Isomeraseaktivität unerlässlich ist.
Introduction

1. Protein folding

How does a newly synthesized polypeptide fold to acquire its defined active structure? The three-dimensional structure and thereby the function of a protein is exclusively determined by its amino acid sequence, and the native structure in general represents the lowest free energy state of a polypeptide chain. This was first shown in 1961 in the classical experiment of Christian Anfinsen, where the refolding of denatured, reduced RNaseA was investigated \textit{in vitro}, without the addition of helper enzymes or co-factors (Anfinsen \textit{et al.}, 1961). From this experiment it was concluded that “the information for the correct pairing of the half-cystine residues in disulfide linkage, and for the assumption of the native secondary and tertiary structures, is contained in the amino acid sequence itself”. However, protein folding is still not fully understood. It can be shown that an identical sequence of up to eleven amino acid residues may be part of different secondary structure elements in the same protein depending on its environment (Minor and Kim, 1996). Conversely in other proteins, 25% amino acid sequence identity is sufficient to obtain protein folds that are virtually identical.

Although protein folding \textit{in vitro} is often slower than \textit{in vivo}, it is clearly too fast for sampling every possible conformation on the way to the protein native structure. This was first discussed in 1968: Protein folding by random search would take longer than the age of the universe (Levinthal, 1968), an observation now known as the Levinthal paradox.

In the middle of the 1990s, a new view on protein folding began to emerge. In that view the search for the native conformation can be seen as the search for the energetically most favorable structure through multiple pathways. The protein folding process can be depicted by a so-called “folding funnel” (Onuchic \textit{et al.}, 1995), which representing the free energies of a polypeptide chain during the folding process. Computational simulations and experiments with model proteins are pointing to the existence of intermediate states that are stabilized by the intrinsic tendency of the polypeptide chain to form secondary structure elements and subdomains before tertiary interactions are formed. However, small one-domain proteins may not fold through such folding intermediates (Radford, 2000). The solving of the protein folding mechanism would give us tools to predict not only the structure of a protein, but also its function. Proteins with pre-defined structures, functions and stabilities could be designed (Daggett and Fersht, 2003; Dinner \textit{et al.}, 2000; Radford, 2000).
Protein folding \textit{in vivo}

\textit{In vivo}, proteins have to fold under cellular conditions, which are very different from \textit{in vitro} conditions. Most remarkably, the environment is very crowded with about 200-300 g/l total protein, representing about 20-30\% of the cell volume, at temperatures that are usually higher than those used for \textit{in vitro} folding (Ellis, 2001). This increases the cellular concentrations of non-native proteins with exposed hydrophobic patches that are aggregation prone. To alleviate the effects of this situation, cells have evolved a set of helper proteins (Bukau \textit{et al.}, 2000). These so-called molecular chaperones bind to exposed, hydrophobic patches in unfolded or partially folded polypeptides, thereby preventing them from aggregation. Due to the low specificity and the flexibility of the folding intermediates, chaperones can interact with a large number of target proteins. Several classes of molecular chaperones are known; although no sequence homology between chaperones from different classes exists, chaperones often share similar functional features. Some molecular chaperone classes are ATP-independent, in others substrate binding and release are regulated by ATP-hydrolysis (Bukau and Horwich, 1998). Many molecular chaperones are heat shock proteins (Hsp), others are involved in cellular processes such as protein translocation, signal transduction and protein degradation (Martin and Hartl, 1997).

In addition to the molecular chaperones, the cell contains enzymes that are essential for efficient protein folding and assembly. In protein folding two rate-limiting steps can be observed; the \textit{cis-trans}-isomerisation of Xaa-Pro peptide bonds and the formation of disulfide bonds in proteins. Cells evolved enzymes to catalyze these reactions.

Normally the peptide bonds of amino acid residues are in the \textit{trans}-conformation. In peptidyl-prolyl-peptide bonds, the proline ring causes the \textit{trans}-conformation to be energetically almost as unfavorable as the \textit{cis}-conformation. In unfolded proteins, 10 to 30\% of the prolyl-peptide bonds are found in the \textit{cis}-conformation (Schmid, 1993) and their isomerization occurs in the time scale of around 100 s at room temperature. The \textit{cis-trans}-isomerisation contributes to the conformational heterogeneity of the unfolded state. So far four classes of different peptidyl-prolyl isomerases (PPI) are known: cyclophilins (Fischer \textit{et al.}, 1989), FK500 binding proteins (FKBP’s) (Harding \textit{et al.}, 1989), parvulins (Rahfeld \textit{et al.}, 1994) and the trigger factor of \textit{E. coli} (Stoller \textit{et al.}, 1996).

The spontaneous formation of disulfide bonds through air oxidation is very inefficient and is catalyzed by the ubiquitous protein disulfide oxidoreductases.
2. Disulfide bond formation in proteins

Disulfide bond formation results from the oxidation of pairs of cysteine side chains. Disulfide bond-containing proteins can be divided into two classes. In proteins of the first class the disulfide bond contributes to their folding pathway and to the stability of their native state, whereas in proteins of the second class the disulfide bond either is central for their activity (e.g. DsbA, PDI, ribonucleotide reductases) or is involved in protein activation and deactivation (e.g. OxyR) (Kadokura et al., 2003).

Disulfide bonds are normally formed in oxidative compartments, which is either the endoplasmic reticulum (ER) of eukaryotes or the periplasm of prokaryotes. Recently a large numbers of disulfide bonded proteins were found in the cytoplasm of certain archaea (Mallick et al., 2002). Thiol-disulfide oxidoreductases, capable of catalyzing protein disulfide bond formation, are found in all living cells. Many of these proteins belong to the thioredoxin-like family, which is defined by an active site containing a Cys-Xaa-Xaa-Cys motif (where Xaa can be any amino acid except cysteine) and by a thioredoxin-like fold as seen in the prototypical thioredoxin 1 of E. coli.

Enzymes are continuously being identified that use redox-active cysteines in transferring electrons in oxidative or reductive pathways, without being a member of the thioredoxin-like family. In addition, these proteins may use electron donor and receptor cofactor, such as FAD, NADPH, NADH, quinones and lipoic acid. Many of these proteins exchange electrons with members of the thioredoxin-like family (Kadokura et al., 2003).

2.1 The thioredoxin-like protein family

Thioredoxin-like oxidoreductases share the thioredoxin-like fold, a central four-stranded β-sheet with three flanking α-helices (see Figure 1), and the Cys-Xaa-Xaa-Cys active site motif.
Figure 1: The thioredoxin-like fold. (A) Schematic drawing of the thioredoxin-like fold, where the placement of the α-helices with respect to the central β-sheet is shown. The shaded helices, α1 and α3, are behind the β-sheet in this orientation, whereas helix α2 is in front of the β-sheet. Positions, where sequence insertions are normally found, are indicated by asterisks. (B) Architecture of the thioredoxin-like fold with the β-sheets drawn as arrows and α-helices as rectangles. The NH₂-terminal (βαβ) and COOH-terminal (ββα) motif is connected by α-helix 2 (Martin, 1995).

The name of this protein family is derived from its first member. Thioredoxin was initially identified as the hydrogen donor of ribonucleotide reductase (Laurent et al., 1964; Reichard, 1962). Later, further functions of thioredoxin were found, such as hydrogen donation to sulfate reductase (Russel and Holmgren, 1988), participation in the light and dark cycle of chloroplasts (Wolosiuk and Buchanan, 1978) and the redox control of transcription factors (Zheng et al., 1998). In 1975, the x-ray structure of E. coli thioredoxin 1 was solved (Holmgren et al., 1975). Most of the soluble disulfide oxidoreductases belong to the thioredoxin-like family. Their structures contain different amino acid sequence insertions in the thioredoxin-like fold (see Figure 1). This fold consists of 80 core amino acid residues, which are only partially conserved in the family. Thioredoxin itself consists of 108 amino acid residues; the additional 28 residues are inserted between β-sheet 2 and α-helix 2 (Katti et al., 1990). The Cys-Xaa-Xaa-Cys active site motif of all thioredoxin-like disulfide oxidoreductases is located at the NH₂-terminal end of α–helix 1 of the thioredoxin-like fold. The thiolate anion of the NH₂-terminal active site cysteine is stabilized by the helix dipole, causing the pKₐ of the cysteine to be considerably lower (pKa 7.4 in the case of thioredoxin) (Mossner et al., 1998) than normally observed pKₐ of a cysteine (9.5). The NH₂-terminal
cysteine is, in contrast to the COOH-terminal cysteine of the active site, solvent exposed and can thus interact with other disulfide bonds. The COOH-terminal cysteine only interacts with the NH2-terminal cysteine of the active site (Freedman et al., 1994; Zapun et al., 1994). The different behavior of the two active site cysteines is important for the function as a disulfide oxidoreductase. The redox potential of the active site, i.e. the equilibrium between the dithiol and disulfide state, depends on the stabilization of the thiolate. As the redox equilibrium of the active site in cellular conditions is important for the function of the enzyme, the amino acid residues that influence the pKₐ will directly influence the function of the protein. In the case of DsbA, the bacterial dithiol oxidase, there has been a considerable interest in the amino acid residues which are situated between the active site cysteines. These amino acid residues determine the redox potential (Grauschopf et al., 1995; Huber-Wunderlich and Glockshuber, 1998; Mossner et al., 1999). Amino acid residues that are close to the active site in the tertiary structure also have a direct influence on the pKa of the active site cysteines (Gane et al., 1995).

2.2 Mechanism of disulfide exchange reactions

Disulfide bond formation is too slow to occur spontaneously through air oxidation during protein folding, even when the cysteine residues come close to each other. The formation of disulfide bonds involves the transfer of two electrons to an oxidant, such as GSSG, oxygen or a protein.
In thiol-disulfide exchange, a thiolate anion \( (\text{R}1\text{S}^-) \) displaces one sulfur of a disulfide bond \( (\text{R}2\text{SSR}3) \). In the transition state, the negative charge of the thiolate appears to be delocalized among the three sulfur atoms (Narayan et al., 2000).

Disulfide exchange is a chemical reaction of the \( S_N2 \) type, where a thiolate anion nucleophilically attacks an existing disulfide bond.

\textit{In vitro}, electrons from two cysteine thiols can be transferred directly to an adequate electron acceptor, like molecular oxygen, resulting in the generation of a new disulfide bond.

\textit{In vivo}, thiol-disulfide exchange reaction occurs, where a disulfide-bonded molecule is attacked by a molecule carrying free thiols via transient formation of a mixed disulfide bond. Another thiolate anion then attacks the mixed disulfide bond, either resulting in the transfer of a disulfide bond or a restoring the original situation (see Figure 3).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{disulfide_exchange_vivo.png}
\caption{Disulfide exchange \textit{in vivo}. In an oxidation reaction, the disulfide bond of a disulfide oxidase (grey) is transferred to a substrate protein. This involves the formation of a mixed disulfide between the oxidase and the substrate protein. If this species is resolved through the attack of a further cysteine in the substrate protein, the result is an oxidized substrate protein and a reduced enzyme (Sevier and Kaiser, 2002).}
\end{figure}

\section{2.3 Oxidative refolding of proteins \textit{in vitro}}

For over 40 years, the study of disulfide bond formation during protein refolding \textit{in vitro} has provided key insights into the process of oxidative protein folding. A few proteins have dominated the studies on disulfide-linked protein refolding \textit{in vitro}: bovine pancreatic trypsin inhibitor (BPTI) (Darby and Creighton, 1993; Weissman and Kim, 1995), bovine pancreatic ribonuclease A (RNaseA) (Talluri et al., 1994), bovine \( \alpha \)-lactalbumin and Ribonuclease T1 (Freedman, 1995).
Oxidative refolding of reduced, unfolded protein \textit{in vitro} without the addition of helper enzymes or co-factors is normally slow and, if more than one pair of disulfide bonds can be formed, can lead to the formation of incorrect disulfide bonds (2 disulfide bonds, 3 possibilities; 3 disulfide bonds, 15 possibilities; 4 disulfide bonds, 105 possibilities, etc). To prevent this from happening, low molecular weight thiols in their reduced and oxidized form (e.g. GSH/GSSG, cysteine/cystine or DTT\textsubscript{red}/DTT\textsubscript{ox}) have often been added as “Redox-shuffling” reagents. “Redox-shuffling” reagents allow thiol-disulfide exchange reactions in both directions and increase yield of proteins with correct disulfide bonds through reshuffling of improper disulfide bonds (Rudolph and Lilie, 1996).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{disulfide_bonds.png}
\caption{Disulfide bond formation and isomerization in glutathione redox buffers. Disulfide bond formation (A) and isomerization (B) \textit{in vitro} using low molecular weight thiols, indicated with G, in their reduced or oxidized state.}
\end{figure}

Oxidative refolding of proteins \textit{in vitro} can also be performed in the presence of disulfide oxidoreductases as catalysts. The disulfide exchange rate of a disulfide oxidoreductase with a peptide substrate can vary between $10^3$-$10^7$ M\textsuperscript{-1}s\textsuperscript{-1} (Darby \textit{et al.}, 1998b), which is several orders of magnitude faster than disulfide exchange between alkyl thiols (Shaked \textit{et al.}, 1980).
3. Catalyzed disulfide bond formation \textit{in vivo}

Cells have evolved specialized compartments, like the ER in eukaryotes or the periplasm of Gram-negative bacteria, where disulfide bond formation and isomerization occur. These specialized compartments are, in contrast to the reductive cytoplasm, of oxidative nature and are separated by biological membranes. Most of the proteins from these compartments, and most of the secretory proteins, which also fold in the oxidative compartment, contain disulfide bonds (Hiniker and Bardwell, 2004b).

Eukaryotes and prokaryotes both use separate systems for disulfide bond formation and isomerization. Nevertheless, Disulfide bond formation in the ER and in bacteria have a lot in common. Both pathways use a pair of enzymes, which transfer electrons from the substrate protein to electron acceptors and ultimately to molecular oxygen (Tu and Weissman, 2004). One of these enzymes is membrane associated (Ero1p, DsbB) and the other is soluble and belongs to the thioredoxin-like family (PDI/DsbA) (Hiniker and Bardwell, 2004a).

3.1 Disulfide bond formation and isomerisation in the ER

In eukaryotes, oxidative protein folding occurs in the ER. Early studies on RNaseA folding led to the identification of protein disulfide isomerase (PDI) as the first protein folding catalyst, which either forms, cleaves or rearranges disulfide bonds \textit{in vitro} (Goldberger \textit{et al}., 1963). Despite of numerous evidence that PDI increases the rate of oxidative protein folding \textit{in vivo} and \textit{in vitro}, the source of the oxidation equivalents remained unknown (Tu and Weissman, 2004).

A combination of genetic and biochemical studies using yeast \textit{Saccharomyces cerevisiae}, and more recently mammalian and plant systems, have recently revealed the proteins and mechanisms behind this fundamental protein folding process. Most importantly, the conserved, ER-resident protein ER oxidoreductin 1 (Ero1p) was found to reoxidize PDI after oxidation of soluble proteins (Frand and Kaiser, 1999). Ero1p transfers the electrons from PDI to molecular oxygen in a FAD-dependent manner (Tu and Weissman, 2002), rendering PDI able to form new disulfide bonds in a substrate protein. Ero1p is specific for PDI, so that the numerous PDI homologs in the PDI remain in the reduced state and can carry out separate redox functions aside from protein oxidation. In yeast, four PDI homologs (EUG1, MPD1,
MPD2 and EPS1) and dozens more in higher eukaryotes have been found (Ellgaard and Ruddock, 2005).

Initial results suggest that in yeast Ero1p cannot interact with several of these PDI homologs, and this specificity for PDI may also be present in the human system (Mezghrani et al., 2001). But where do the reduction equivalents come from to keep the PDI homologs in their reduced state? Glutathione is the major redox buffer in eukaryotes (Hwang et al., 1992). The ratio of reduced (GSH) to oxidized (GSSG) glutathione is ~100:1 in the cytosol, whereas in the ER the ratio is ~3:1. This abundance of GSSG in the secretory pathway was long thought to be the source of oxidizing equivalents for disulfide formation (Hwang et al., 1992). But now it is clear that the Ero1p-catalyzed disulfide formation proceeds independent of glutathione \textit{in vivo} and \textit{in vitro}. Furthermore, glutathione functions as a net reductant in the ER (Cuozzo and Kaiser, 1999). Ero1p is not able to oxidize GSH to GSSG (Tu et al., 2000). The presence of a GSH/GSSG redox buffer in the ER is the result of Ero1p-mediated oxidative and glutathione-mediated reductive processes. The kinetic shuttling of oxidizing equivalents by Ero1p and PDI could explain how the ER supports rapid disulfide formation while maintaining the ability to reduce or rearrange incorrect disulfide bonds through glutathione or PDI and its homologs (Ellgaard, 2004; Mezghrani et al., 2001).
**Figure 5: Scheme of oxidative protein folding in yeast ER.** The formation of disulfide bonds is catalyzed by PDI and Ero1p. Oxygen oxidizes Ero1p with bound FAD. Oxidized Ero1p then recovers oxidized PDI which directly oxidizes substrate proteins by disulfide exchange. FAD can readily enter the ER lumen and stimulate Ero1p. Reduced glutathione (GSH) is also able to enter the ER lumen and represents reducing equivalents, which are oxidized by PDI and its homologs. GSH provides PDI and its homologs, Eug1p, Mdp1, Mdp2p and Eps1p with reducing equivalents so that they can act as disulfide isomerases and probably as disulfide reductases (Tu and Weissman, 2004).

**Protein Disulfide Isomerase**

Protein disulfide isomerase (PDI) was identified in 1963, when RNaseA was refolded in presence of microsomal extracts (Goldberger et al., 1963). This archetypical disulfide oxidoreductase from ER consists of five domains, two thioredoxin-like domains with active sites (a, a’), two thioredoxin-like domain without active sites (b, b’) and one acidic domain (c) (Freedman et al., 1998; Kemmink et al., 1997).

![Figure 6: Domain structure of PDI.](image)

PDI is a 55 kDa protein and has a COOH-terminal KDEL-ER retrieval sequence. PDI has been shown to act as a disulfide oxidase, isomerase and reductase in vitro (Creighton et al., 1980; Darby and Creighton, 1995a).

Both catalytically active thioredoxin-like domains contain the Cys-Gly-His-Cys active site motif and share significant sequence identity with thioredoxin. As in all thioredoxin-like disulfide oxidoreductases, the active site is located at the NH$_2$-terminus of $\alpha$-helix 1 in the thioredoxin-like fold. The NH$_2$-terminal cysteine is solvent exposed, in contrast to the buried COOH-terminal cysteine of the active site. The determination of the pK$_a$ for the cysteine thiol
Introduction

gave two different values, 4.5 (Kortemme et al., 1996) and 6.7 (Hawkins and Freedman, 1991). The PDI a and a’ domains are thus destabilized in their oxidized form (Alanen et al., 2003; Darby and Creighton, 1995b), which reinforce their ability to act as oxidases. Using glutathione as reference, the redox potential of full length PDI has been determined to be -175 mV (Lundstrom and Holmgren, 1993).

The redox-inactive thioredoxin-like domains b and b’ share significant sequence similarity, but no obvious sequence similarity between these domains and thioredoxin or the a or a’ domain of PDI can be observed.

The individual redox-active domains catalyze substrate oxidation, but display weak isomerase activity (Darby and Creighton, 1995b; Darby et al., 1998a). Thus, the peptide-binding region, which is mostly formed by the b’ domain (Klappa et al., 1998), and with it, the multidomain structure of PDI have probably evolved in order to expand the catalytic repertoire of this disulfide oxidoreductase. In S. cerevisiae, PDI is an essential protein, but its predominant function is still debated (Farquhar et al., 1991; LaMantia et al., 1991; Scherens et al., 1991).

In mammalian cells, PDI is found predominantly in the reduced state (Mezghrani et al., 2001), whereas in yeast PDI is mostly oxidized (Frand and Kaiser, 1999; Tu et al., 2000). This difference might be explained by different cellular conditions, as the redox state of PDI depends on the load of folding proteins and the activity of its own oxidase, Ero1p. In vitro data so far do not clearly reveal different functions of the a and a’ domain of PDI (Darby and Creighton, 1995b) and their common mechanism of isomerization is the formation of transient mixed disulfide bonds between folding proteins and PDI (Molinari and Helenius, 1999).

In mammalian cells, PDI is also a subunit of prolyl 4-hydroxylase (Pihlajaniemi et al., 1987) and of the microsomal triacylglycerol transfer protein (Wetterau et al., 1990).

Ero1p

Genetic studies in yeast identified the conserved, ER membrane-associated protein, Ero1p, as an essential component of the oxidative folding machinery (Frand and Kaiser, 1998; Pollard et al., 1998). Mutations in this gene lead to the accumulation of reduced proteins, that normally contain disulfide bonds in the ER, a phenotype resembling that of bacteria lacking DsbB function (Bardwell et al., 1993; Missiakas et al., 1993). A single isoform of Ero1p is found in yeast cells; human cells have two isoform, hERO1Lα and hERO1Lβ (Cabibbo et al., 199
Ero1p is a 65 kDa protein, which possesses seven conserved cysteins and two active site disulfide bonds (see Figure 7) (Tu and Weissman, 2004).

![Figure 7: Ribbon diagram of the X-ray structure of Ero1p from S. cerevisiae. The α-helices are shown in red and β-strands in blue. The cysteins and the cofactor FAD are shown as ball and stick representation. (A) and (B) indicate the active site cysteins (Gross et al., 2004).](image)

Ero1p only accepts electrons from PDI and possesses a FAD binding site (Tu et al., 2000). It transfers the electrons from the substrate protein via PDI and its active site disulfide bonds to FAD, and finally to molecular oxygen. Under anaerobic conditions, Ero1p probably uses alternate terminal electron acceptors, similar to DsbB in bacteria, though the identity of these acceptors remains unknown (Tu and Weissman, 2004).
Other proteins involved in oxidative protein folding in eukaryotes

In addition to PDI and Ero1p, several other proteins are known to be involved in the oxidative protein folding in eukaryotes. In yeast, Erv2p is found to be a supplementary membrane associated protein that is likely to be also involved in de-novo disulfide bond formation. Erv2p restores Ero1p deficiency and interacts with PDI when overexpressed. The function of Erv2p is not entirely clear (Sevier et al., 2001).

Furthermore, Eug1p (Tachibana and Stevens, 1992), Mpd1 (Tachikawa et al., 1995), Mpd2 (Tachikawa et al., 1997) and Eps1 (Wang and Chang, 1999) have been found in the yeast ER and are structurally related to PDI. Their function is not clear. In higher eukaryotes over 14 PDI homologs are found (Ellgaard and Ruddock, 2005).

3.2 Disulfide bond formation and isomerisation in bacteria

In Gram-negative bacteria, disulfide bond formation and rearrangement occurs in the periplasm. At least 300 proteins in E. coli contain two or more cysteine residues and are predicted to be secreted to the periplasm. These proteins are therefore candidates for undergoing oxidative protein folding (McCarthy et al., 2000). The proteins that support the oxidative protein folding process in the periplasm are named Dsb proteins (Disulfide Bond). DsbA was discovered in 1991 by mutational screening for lacking disulfide bond formation (Bardwell et al., 1991). Since then, disulfide bond formation and isomerization in the E. coli periplasm has been studied in great detail (Nakamoto and Bardwell, 2004). The periplasm forms a compartment for disulfide bond formation, which has similarities to the ER of eukaryotes. Most notable are the phenotypic parallels between mutants in these compartments. Disruption of DsbA interferes with the oxidation of secretory proteins (Bardwell et al., 1991; Frand and Kaiser, 1999), whereas the loss of DsbB function causes defects in the reoxidation of DsbA and this gives the same phenotype as a dsbA deletion mutant (Bardwell et al., 1993). Given the functional similarity between Ero1p and DsbB, it is surprising that these proteins share no amino acid sequence homology; but they share a four helix bundle motif as a common structural feature (Sevier et al., 2005).

A striking difference between the prokaryotic and eukaryotic systems for disulfide bond formation is the apparent absence of a specialized isomerization pathway in eukaryotes analogous to the prokaryotic DsbC-DsbD system (Sevier and Kaiser, 2002).
Figure 8: Schematic model of oxidative protein folding in the *E. coli* periplasm.

Disulfide bonds are introduced into newly secreted polypeptides by DsbA. DsbA is then reoxidized by the inner membrane protein DsbB. Under aerobic conditions, DsbB passes electrons to ubiquinone. Then electrons flow to molecular oxygen via the electron transport chain and the respiratory chain, whereas under anaerobic conditions, DsbB passes electrons to menaquinone and final electron acceptors. DsbA can also form non-native disulfide bonds in proteins with more than two cysteine residues. The non-native disulfide bonds are corrected by DsbC via disulfide reshuffling. DsbC is kept in its active reduced state by the inner-membrane protein DsbD. DsbD receives the electrons from cytoplasmic thioredoxin. In addition, the reductive pathway via DsbD also keeps DsbG in the reduced state (adapted from A. Frand and coworkers (Frand et al., 2000)).

In addition to the reductive pathway for DsbC and DsbG, the transfer of electrons from the cytoplasmic thioredoxin via DsbD is involved in cytochrome *c* maturation. Cytochrome *c* proteins are either soluble or membrane anchored proteins involved in electron transfer to nitrate, nitrite, trimethylamine *N*-oxide and biotin sulfoxide. *E. coli* synthesizes cytochrome *c* only under anaerobic condition. A gene cluster that is essential for cytochrome *c* maturation has been identified. The cluster includes essential genes for proteins that are involved in the maturation of all *c*-type cytochromes of *E. coli* (Thöny-Meyer, 2002). Most of the proteins
encoded by the *ccmABCDEFGH* gene cluster form a complex in which the different steps of the pathway are coordinated. Three different major functions are found in the protein complex: an ABC transporter, a heme delivery system and a redox-control system (Thöny-Meyer, 2002). The general secretory pathway (Sec-system) and the cellular redox control system (thioredoxin and Dsb proteins) are also involved in the cytochrome *c* maturation (Crooke and Cole, 1995; Grove *et al*., 1996; Metheringham *et al*., 1995; Sambongi and Ferguson, 1994; Sambongi and Ferguson, 1996). Apocytochrome *c* has one or more Cys-Xaa-Xaa-Cys-His heme binding motifs, where the heme is covalently bound via two thioethers (Sambongi *et al*., 1996). The formation of the thioether occurs in the oxidizing environment of the periplasm; initially the heme binding cysteines of apocytochrome *c* have to be in the reduced state. *E. coli* uses the reduction pathway, whereas the electrons are transported from the thioredoxin via DsbD to the periplasm and to the cytochrome *c* maturation complex. CcmG (DsbE) and CcmH transfer the electrons from DsbD to apocytochrome *c*. CcmG, a periplasmic, monomeric 20 kDa protein, which is membrane anchored, belongs to the thioredoxin-like family. CcmG is the third natural partner of DsbD besides DsbC and DsbG and transfers the electron from DsbD via CcmH to apocytochrome *c* (Stirmann *et al*., 2005).

**DsbA**

DsbA was found in 1991, when *E. coli* was screened for proteins lacking proper disulfide bonds (Bardwell *et al*., 1991). The disulfide bond formation in of β–lactamase, alkaline phosphatase and OmpA was disrupted, when the *dsbA* gene was mutated. *dsbA* deficient strains showed pleiotropic phenotypes like hypersensitivity to benzylpenicillin, DTT and metal ions (Missiakas *et al*., 1993; Rensing *et al*., 1997; Stafford *et al*., 1999) or showed impaired folding of the flagellar protein FlgI (Dailey and Berg, 1993). Many virulence factors contain disulfide bonds; DsbA is important for disease causing properties of enteropathogenic and uropathogenic *E. coli*, *Vibrio cholera* and *Shigella flexneri* (Donnenberg *et al*., 1997; Jacob-Dubuisson *et al*., 1994; Peek and Taylor, 1992; Watarai *et al*., 1995; Yu, 1998).
DsbA is a 21 kDa soluble, monomeric protein in the periplasm of *E. coli*. The DsbA structure shows the typical thioredoxin-like fold and the Cys-Xaa-Xaa-Cys active site motif of soluble disulfide oxidoreductases (Martin *et al.*, 1993). Compared to the thioredoxin-like fold (see Figure 1), DsbA contains a 75 amino acid residue insertion, so called $\alpha$–helical domain, between $\beta$-strand 2 and $\alpha$–helix 2. Despite their structural similarities, DsbA and thioredoxin fulfill different functions and exist in different cellular compartments. While thioredoxin acts as a reductant of disulfide bonds in the cytosol (Rietsch and Beckwith, 1998), DsbA introduces disulfide bonds consecutively (Berkmen *et al.*, 2005) in newly synthesized proteins.

**Figure 9: Ribbon diagram of the X-ray structure of oxidized DsbA from *E. coli*.** The thioredoxin-like domain (A) and $\alpha$–helical domain (B) are indicated. $\alpha$–helices are shown in red and $\beta$-strands in blue. The active-site cysteines are shown as ball and stick representation (Guddat *et al.*, 1998).
What are the features that make DsbA a disulfide oxidase? The active site disulfide bond of DsbA is highly reactive; oxidized DsbA reacts rapidly with dithiols, resulting in disulfide bond formation. DsbA reacts with glutathione about $10^3$ fold faster than a normal protein disulfide bond does (Zapun et al., 1993). The highly oxidizing nature of DsbA is evident from its redox potential of $-120$ mV (Wunderlich and Glockshuber, 1993; Zapun et al., 1993). The active site disulfide bond in DsbA is very unstable. Normally a disulfide bond contributes to the stability of a protein, but in DsbA the disulfide bond destabilizes the protein (Zapun et al., 1993).

What is the reason for this destabilization of the protein by its disulfide bond? In vivo, DsbA donates disulfide bonds to substrate proteins; the reduced form is the preferred form of DsbA. In the active site Cys-Pro-His-Cys of DsbA, the NH$_2$-terminal Cys30 is solvent exposed and has a pK$_a$ of 3.5 (Nelson and Creighton, 1994). The Cys 30 is in the fully deprotonated state at physiological pH. It is the stabilization of this negative charge that accounts for the difference in stability of the two different redox states of DsbA (Grauschopf et al., 1995; Nelson and Creighton, 1994).

Figure 10: The DsbA active site in its reduced state. The hydrogen bonds (dashed lines) that stabilize the thiolate anion of Cys 30 are indicated. The interaction between the Cys 30 thiolate and the Cys 33 thiol is shown as a dotted line. Distances are in angstrom (Guddat et al., 1998).

Several studies have shown that the Cys 30 thiolate anion is stabilized by hydrogen bonds (see Figure 10), electrostatic interactions and the helix dipole (Guddat et al., 1998; Martin et al., 1993). These stabilizations of the negative charge make the DsbA reduction favorable and this drives the formation of new disulfide bonds by DsbA.
The protein folding in newly synthesized proteins competes with DsbA-mediated formation of disulfide bonds. Therefore DsbA has to introduce the disulfide bonds before the cysteines are masked by the folded protein. DsbA interacts non-covalently with unfolded substrate proteins (Couprie et al., 2000; Darby and Creighton, 1995b; Frech et al., 1996; Wunderlich and Glockshuber, 1993); non-covalent binding of substrate protein to DsbA could prevent the target protein from folding prior to disulfide bond formation (Nakamoto and Bardwell, 2004). A potential peptide binding site has been identified in the crystal structure (Guddat et al., 1997), but no structure of a DsbA-substrate complex has been solved so far. The helical domain that covers the active site of DsbA contains several amino acid residues forming a hydrophobic patch. This patch, together with a groove running in the thioredoxin domain below, is likely to interact with substrate proteins (see Figure 9) (Guddat et al., 1997). The non-covalent binding of a substrate protein to DsbA should be terminated immediately after the intermolecular disulfide exchange, so that protein folding can proceed. After the release of the substrate protein, DsbA is in the reduced state and therefore inactive as a disulfide oxidase. To reactivate DsbA, it has to be reoxidized by the inner membrane protein DsbB.

**DsbB**

The dsbB gene was originally identified in a similar way as the dsbA gene (Bardwell et al., 1993). In an independent approach, the dsbB gene was isolated by a genetic screen for multicopy suppressor of DTT sensitivity (Missiakas et al., 1993). dsbB null mutations accumulate reduced DsbA in the periplasm, whereas DsbA from a wild type background is found mostly oxidized. This implied that DsbA and DsbB are part of the same pathway; DsbB is upstream relative to DsbA. Furthermore, a mixed disulfide complex of DsbA and DsbB was isolated, strongly indicating that DsbB directly reoxidizes reduced DsbA (Guilhot et al., 1995; Kishigami et al., 1995b). The direct oxidation of DsbA by DsbB was also shown *in vitro* (Bader et al., 1998).
Figure 11: Predicted structure of DsbB. Predicted membrane topology of DsbB is shown with the one amino acid residue code. Cysteine are shown in bold sphere (Jander et al., 1994).

DsbB is a 20 kDa protein and located in the inner membrane. Topology predictions indicate four transmembrane helices and two periplasmic loops. Each periplasmic loop contains a pair of conserved cysteines. Cys 41 and Cys 44 are located in the NH₂-terminal loop and Cys 104 and Cys 130 in the COOH-terminal loop. These two pairs of cysteines can undergo oxidation-reduction cycles and removal of any of these cysteine residues causes loss of DsbB activity (Guilhot et al., 1995; Jander et al., 1994; Kishigami et al., 1995b). A Cys-Val-Leu-Cys active site motif is present in the NH₂-terminal periplasmic loop of DsbB. However, both periplasmic loops are too short to form a thioredoxin-like fold. Heme- or quinone-depleted E. coli strains accumulate reduced DsbA and DsbB. This fact suggested that the oxidation power for disulfide bond formation is provided by the respiratory chain (Kobayashi et al., 1997). It was shown that DsbB catalyzes the electron transfer from DsbA to ubiquinone in vitro (Nakamoto and Bardwell, 2004). Under anaerobic conditions, DsbB switches to menaquinone as the immediate electron acceptor.
A model for the mechanism of DsbB function was developed on the basis of in vivo (Kishigami and Ito, 1996) and in vitro (Grauschopf et al., 2003) experiments. According to this model, the solvent exposed Cys30 of DsbA attacks the DsbB disulfide bond of Cys104-Cys130 and a mixed disulfide bond is formed. The Cys33 of DsbA attacks this mixed disulfide bond and the disulfide bond of DsbB Cys104-Cys130 is transferred to DsbA. The reduced DsbB cysteine pair 104-130 is then reoxidized by the second DsbB cystein pair 41-44 and these reduced cysteine residues eventually transfer the two electrons to ubiquinone or menaquinone. The Cys41-Cys44 disulfide bond has a redox potential of – 69 mV, compared to – 186 mV of Cys104-Cys130 (Grauschopf et al., 2003). The Cys41-Cys44 disulfide bond is the most oxidizing disulfide bond known in a protein. In contrast, the redox potential of the other disulfide bond is even more reducing than the redox potential of DsbA (-120 mV). This very strong oxidizing redox potential of Cys41-Cys44 in DsbB drives the reaction.

DsbC

A protein with four disulfide bonds, for example, can form 105 different disulfide bond patterns in its fully oxidized state. DsbA is therefore likely to introduce non-native disulfide bonds in proteins with multiple disulfide bonds (Rietsch et al., 1996) and trap the protein in non-native conformations. In vivo, DsbA is found in its oxidized state (Kishigami et al., 1995a) and has a low ability to reduce incorrect disulfide bonds. But incorrect disulfide bonds need to be reshuffled and corrected before the protein can attain its proper folding. By further screening of E. coli mutants that display DTT hypersensitive phenotypes, an additional Dsb protein was found, DsbC (Missiakas et al., 1994; Shevchik et al., 1994). DsbC was isolated from a multicopy plasmid library by its ability to confer resistance to DTT. dbsC null mutants have no strong effect on the growth of E. coli (Rietsch et al., 1996). The yield of eukaryotic proteins containing multiple disulfide bonds, targeted to the E. coli periplasm, can be increased in the presence of DsbC (Maskos et al., 2003; Rietsch et al., 1996).
DsbC is a 23.5 kDa soluble, homodimeric protein in the periplasm of *E. coli*. The DsbC structure shows an overall V-shape. Each arm of the V is made by a monomer consisting of two separated domains, a NH2-terminal domain and a COOH-terminal domain. The NH2-terminal domain or dimerization domain exclusively contributes to the dimer interface, which consists mostly on a symmetric β-sheet. The COOH-terminal domain with thioredoxin fold is the catalytic active domain and has a Cys-Xaa-Xaa-Cys active site motif (McCarthy *et al.*, 2000). The two redox-active Cys-Gly-Tyr-Cys motifs are facing each other and facing inside the V-shape. This interior between the two subunits of DsbC is covered with uncharged amino acid residues, forming a large hydrophobic cleft (McCarthy *et al.*, 2000). As in DsbA, the NH2-terminal Cys98 of the active site is solvent exposed and therefore the nucleophilic species. DsbC displays an equilibrium constant with glutathione of 0.12 mM and has a redox potential of -140 mV. This makes DsbC highly reactive and only slightly less oxidizing than DsbA (Sone *et al.*, 1997).
Proteins with non-native disulfide bonds display probably hydrophobic patches which can bind non-covalently to the inner cleft of DsbC and present their disulfide bonds to the reduced Cys98. The Cys98 thiolate anion attacks the non-native disulfide bond in the substrate protein and opens the disulfide bond by forming a mixed disulfide with a cysteine of the substrate. Mixed disulfide bonds between DsbC and model proteins are 40-100 fold more stable than between DsbA and model proteins (Darby et al., 1998b). The second cysteine from the reopened disulfide bond in the substrate can then probably attack another disulfide bond and so on, until the right disulfide bond pattern is formed. The higher stability of the mixed disulfide bond between DsbC and the substrate protein could result from the enhanced peptide binding site of DsbC’s large cleft. This allows probably the substrate to search various conformations for the correct disulfide bond pattern. Burial of hydrophobic amino acid residues upon protein folding, that accompanies correct disulfide bond formation, may destabilize the DsbC-substrate-complex, allowing the correctly folded protein to dissociate from DsbC (Nakamoto and Bardwell, 2004). Further support for the hypothesis that the interior of DsbC is responsible for the peptide binding by DsbC, comes from the observation that the dimeric nature of DsbC is necessary for its function in vivo (Sun and Wang, 2000).

On the other hand another model could explain the function of DsbC. The disulfide bond of a substrate protein is reduced by DsbC, whereby DsbC is oxidized. The reduced disulfide bond in the substrate protein starts an internal reshuffling until the most stable conformation is formed. During the internal reshuffling the substrate is non-covalently bound in the hydrophobic cleft of DsbC. In the end the last disulfide bond has to be oxidized by DsbC and then DsbC is present in its reduced state. This model is supported by the fact that no DsbC-substrate complexes were found.

The second Cys101 of DsbC is not necessary for the first model. Cys101 might only be important for the resolution of kinetically trapped complexes between DsbC and substrate proteins. The attack of Cys101 on such a trapped mixed disulfide leads to the oxidation of DsbC. In any case, only reduced DsbC is found in vivo (Joly and Swartz, 1997) and able to isomerise incorrect disulfide bond. Therefore, there is a need to keep DsbC in the reduced state in vivo.

DsbD

DsbD was found by further screening for E. coli mutants that display DTT hypersensitive phenotypes (Missiakas et al., 1995). dsbD null mutant strains accumulate DsbC and DsbG in
their oxidized form (Rietsch et al., 1997). Additionally, it was shown that dsbD null mutants abolish the formation of holocytochromes.

**Figure 13: Predicted structure of DsbD.** The predicted topology of DsbD consists of an NH$_2$-terminal periplasmic domain (nDsbD), a transmembrane domain (TM DsbD) and a COOH-terminal periplasmic domain (cDsbD). The six essential cysteine of DsbD are indicated as bold sphere (Chung et al., 2000; Gordon et al., 2000; Stewart et al., 1999).

DsbD is 59 kDa protein, located in the inner membrane of *E. coli*. DsbD full length structure was predicted by alkaline phosphatase fusion and computational algorithms. DsbD consist of three domains: a periplasmic NH$_2$-terminal domain, nDsbD or $\alpha$-domain, a central transmembrane domain, TM DsbD or $\beta$-domain, and a COOH-terminal domain, cDsbD or $\gamma$-domain. Each of the domains contains a conserved pair of cysteines and mutation of any of these conserved cysteines leads to an inactive DsbD *in vivo* (Stewart et al., 1999). The single domains can be coexpressed and show DsbD activity (Katzen and Beckwith, 2000), but the removal of one of these domains from DsbD leads to the interruption of the transmembrane electron flow and to oxidized DsbC. Additionally, it was shown that the remove of the transmembrane domain leads to oxidized periplasmic domains, whereas the removal of the NH$_2$-terminal domain has no influence on the redox state of the other two domains in DsbD. In addition, mixed disulfide bond reaction intermediates between either thioredoxin Cys32 and the transmembrane Cys163 of DsbD and between DsbC Cys98 and the NH$_2$-terminal Cys109 have been found (Katzen and Beckwith, 2000). It was proposed that the catalytic
mechanism of DsbD is exclusively based on disulfide exchange reactions. According to this model, the transmembrane domain of DsbD accepts electrons from thioredoxin and transfers the electrons across the inner membrane to cDsbD, followed by successive reduction of the substrate proteins, DsbC, DsbG and CcmG via the nDsbD (Chung et al., 2000; Katzen and Beckwith, 2000; Katzen and Beckwith, 2002; Krupp et al., 2001).

The structures of both soluble periplasmic domains, nDsbD and cDsbD, have been solved.

Figure 14: Ribbon diagram of the X-ray structure of oxidized nDsbD from *E. coli*. β-strands are shown in blue. The active-site cysteins are shown in ball and sticks representation (Goulding et al., 2002).

Oxidized nDsbD has an immunoglobulin-like fold. In contrast to the immunoglobulin-like fold, nDsbD has a catalytic subdomain insertion, consisting of additional four β-sheets and the active site at one end of the immunoglobulin-like fold. The active site cysteines are located in two different β-strands and is protected from the environment by the active site cap-loop region formed by two β-strands (Goulding et al., 2002). nDsbD has a reducing redox potential of -232 mV (Rozhkova et al., 2004).

Complexes of mixed disulfide between nDsbD and either DsbC or CcmG have been solved by X-ray structure determination (Haebel et al., 2002; Stirnimann et al., 2005).
The structures of the complexes between the two substrates and nDsbD show a movement of the active site cap-loop region, compared to the nDsbD structure, which e.g. allows Cys109 of nDsbD to form a mixed disulfide with the substrate protein. The mechanism of this movement and how nDsbD distinguishes between oxidized and reduced substrates is still unclear.
Figure 16: Ribbon diagram of the X-ray structure of oxidized cDsbD from *E. coli*. α–helices are shown in red and β-strands in blue. The active-site cysteins are shown as ball and sticks representation (Kim *et al*., 2003).

The structure of the COOH-terminal domain of DsbD contains a thioredoxin-like fold with a Cys-Val-Ala-Cys active site motif. cDsbD shares 28 % sequence identity with thioredoxin and has an additional NH₂-terminal extension (Kim *et al*., 2003). The redox potential of cDsbD (-235 mV) is slightly more reducing compared to the redox potential of nDsbD (Rozhkova *et al*., 2004). It is shown that no electron transfer between cDsbD and DsbC or DsbA is possible and all electrons need to be transferred via nDsbD (Rozhkova *et al*., 2004). In the same publication the structure of the mixed disulfide complex between the NH₂-terminal domain and the COOH-terminal domain of DsbD was described.

Figure 17: Ribbon diagram of the X-ray structure of cDsbD and nDsbD complex from *E. coli*. α–helices are shown in red and β-strand in blue. The active-site cysteins are shown as ball and sticks representation (Rozhkova *et al*., 2004).

Comparison of the nDsbD orientation in the complex with DsbC and cDsbD that large domain movements in DsbD seems to be necessary to allow the electron transfer to its substrates (Rozhkova *et al*., 2004).
**DsbG**

By further screening for _E. coli_ mutants that display DTT hypersensitive phenotypes, an additional Dsb protein was found, DsbG (Andersen _et al._, 1997). Initially, it was reported that _dsbG_ deficiency is lethal and can only be substituted by small oxidation agents in the medium. However, these results were not confirmed and in contrast, it was shown that a _dsbG_ null mutation is not lethal and no effects on oxidative protein folding were found (Bessette _et al._, 1999).

**Figure 18: Ribbon diagram of the X-ray structure of reduced DsbG from _E. coli_.** The dimerization domain (A) and thioredoxin-like domain (B) are indicated. α–helices are shown in red and β-strands in blue. The active-site cysteines are shown in ball and sticks representation (Heras _et al._, 2004).

DsbG is a 25.7 kDa homodimeric, soluble protein in the periplasm of _E. coli_. The DsbG structure reminds of DsbC and shows the typical V-shaped homodimer. DsbG shares 26% sequence identity and 40% sequence homology with DsbC. As in DsbC, each arm of the V is made by a monomer consisting of a NH2-terminal dimerization domain and a COOH-terminal catalytic domain. Both domains are joined by a linker α-helix, which is 2.5 turns longer than the linker α-helix in DsbC (Heras _et al._, 2004). The two redox active Cys-Pro-Tyr-Cys motifs
are not facing inside the V-shape, in contrast to DsbC. The inside of the V-cleft of dimeric DsbG is covered with many acidic residues (Heras et al., 2004). As in the other thioredoxin-like folded proteins, the first cysteine of the active site in the catalytic domain of DsbG, Cys 109, is solvent exposed and therefore the reactive species. DsbG shows an equilibrium constant with glutathione (0.14 mM) between that of DsbA and DsbC and has a redox potential of -126 mV (Bessette et al., 1999). Overexpression of DsbG in a dsbC null mutant strain can partially restore DsbC activity, but no activity was found in disulfide isomerase and reductase activity assay in vitro (van Straaten et al., 1998). Additionally, DsbG shows chaperone-like activity and prevents aggregation of citrate synthase, but does not, in contrast to real chaperones, increase the yield of citrate synthase folding (Shao et al., 2000). Until now, the function of DsbG reminds unclear.

Homologs of E. coli DsbA and DsbB are found in many Gram-negative bacteria, but homologs of DsbC and DsbD are mostly restricted to the β- and γ-subdivision of eubacteria (Katzen et al., 2002). The DsbA-DsbB pathway plays an important role in the virulence of many bacteria (Yu and Kroll, 1999). In Gram-positive bacteria, only a few extracytoplasmic proteins with disulfide bonds have been identified. It is not clear if this class of bacteria has a catalytic system to introduce disulfide bonds. Recently, such a system was found in Bacillus subtilis (Kadokura et al., 2003). An unusual, high number of cytoplasmic proteins with disulfide bonds are found in some archaea and therefore the question arises, how these disulfide bond are formed in the cytoplasm (Mallick et al., 2002).

4. Aim of the thesis

The first part of this work is focused on the understanding of the DsbC disulfide isomerase function. Two models for the structural basis of the DsbC isomerase activity were assumed. Either the dimerization of DsbC leads to the formation a large, contiguous peptide binding site or the formation of a mixed disulfide bond between one active-site of DsbC and a substrate increases the effective concentration of the second active site in the DsbC dimer. To test these models, disulfide isomerase and reductase activity assays were developed or adapted. Different DsbA and DsbC variants were purified and tested for their function as disulfide isomerases and reductases. These variants included dimeric DsbA, the isolated catalytic domain of DsbC and a DsbC heterodimer with only one active site.
In the second part, the putative disulfide isomerase DsbG was tested for disulfide isomerase and reductase activities and compared to those of its homolog DsbC. Additionally, the structural basis for the independence of the disulfide bond formation and isomerization pathways was investigated by different variants of DsbA, DsbC and DsbG. These kinetic measurements allowed a better understanding of the coexistence of the two pathways without cross reactions in the same compartment.

The structure of DsbC led to the question of whether a fusion of a good peptide binding site to a thioredoxin-like disulfide oxidoreductase may result in disulfide isomerase activity. Therefore the apical domain of GroEL, which is a good peptide binding site, was fused to DsbA via different linkers. The fusion proteins were tested for their disulfide isomerase and reductase activities.
Materials and Methods

1. Materials

1.1 Chemicals

Acetic acid
Acrylamid / bisacrylamid (37.5 : 1 v/v)
Agarose (ultra pure)
Ammonium sulfate
Ammonium peroxodisulfate (APS)
Ampicillin (Amp)
Bacto Agar
Bacto Trypton
Bacto yeast extract
Bis-Tris
Boric acid
5-Bromo-4-chloro-3-indolyl phosphate, p-toluidine salt (BCIP)
Bromphenol blue sodium salt
Liquid Wax Chill-out 14TM
Coomassie brilliant blue G-250
3’-Cytidine nucleotide
Cyclic-2’-3’-cytidine nucleotide
5,5’-Dithio-bis-(2-nitrobenzoic acid)
1,4-Dithiothreitol (DTT)
Dodecyl-β-D-maltoside (DDM)
Ethidium bromide
Ethylenediamintetraacetate Na salt (EDTA)

Fluka (Buchs, Switzerland)
Serva (Heidelberg, Germany)
Bethesda Research Laboratories (Neu Isenburg, Germany)
Fluka (Buchs, Switzerland)
Serva (Heidelberg, Germany)
Sigma (Buchs, Switzerland)
Difco Laboratories (Detroit, United States)
Difco Laboratories (Detroit, United States)
Difco Laboratories (Detroit, United States)
Applichem (Darmstadt, Germany)
Fluka (Buchs, Switzerland)
Sigma (Buchs, Switzerland)
Fluka (Buchs, Switzerland)
MJ Research (Bio-Rad Laboratories, Las Vegas, United States)
Sigma (Buchs, Switzerland)
Sigma (Buchs, Switzerland)
Sigma (Buchs, Switzerland)
Sigma (Buchs, Switzerland)
Applichem (Darmstadt, Germany)
Anatrace (Maumee, United States)
Applichem (Darmstadt, Germany)
Applichem (Darmstadt, Germany)
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<td>Fluka (Buchs, Switzerland)</td>
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<tr>
<td>Glucose</td>
<td>Fluka (Buchs, Switzerland)</td>
</tr>
<tr>
<td>Glutathione, oxidized form (GSSG)</td>
<td>Sigma (Buchs, Switzerland)</td>
</tr>
<tr>
<td>Glutathione, reduced form (GSH)</td>
<td>Sigma (Buchs, Switzerland)</td>
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<tr>
<td>Guanidinium chloride (GdmCl)</td>
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<tr>
<td>Glycerol</td>
<td>Schweizerhall (Basel, Switzerland)</td>
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<tr>
<td>Glycine</td>
<td>Fluka (Buchs, Switzerland)</td>
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<tr>
<td>HEPES</td>
<td>Sigma (Buchs, Switzerland)</td>
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<tr>
<td>Imidazole</td>
<td>Fluka (Buchs, Switzerland)</td>
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<tr>
<td>Isopropyl-β-thiogalactopyranoside(IPTG)</td>
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<tr>
<td>Laurylsarcosine (LS)</td>
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<td>MOPS</td>
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<td>Phenylmethylsulfonyl fluoride (PMSF)</td>
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<tr>
<td>Tris base Trizma</td>
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</tr>
<tr>
<td>Urea</td>
<td>USB (Cleveland, United States)</td>
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</table>

All other chemicals were from Merk (Darmstadt, Germany) and of the highest purity available.
1.2 Standards and Kits

DNA molecular weight standard 100 bp ladder New England Biolabs (Schalbach, Germany)
DNA molecular weight standard 1 kbp ladder New England Biolabs (Schalbach, Germany)
Protein molecular weight standard low range Bio-Rad Laboratories (Richmond, United States)
DNA Gel Extraction Kit QIAquick Qiagen (Hombrechtikon, Switzerland)
PCR Purification Kit QIAquick Qiagen (Hombrechtikon, Switzerland)
Site–directed mutagenesis Kit QuikChangeII Stratagene (La Jolla, United States)
DNA Purification System Kit Wizard Plus SV Promega (Catalysis, Wallisellen, Switzerland)
DNA Sequencing Kit Vistra DNA Labstation 625 GE Healthcare (Otelfingen, Switzerland)

1.3 Proteins and Enzymes

BamHI (20 U/μl) New England Biolabs (Schalbach, Germany)
NarI (4 U/μl) New England Biolabs (Schalbach, Germany)
NdeI (20 U/μl) New England Biolabs (Schalbach, Germany)
NheI (10 U/μl) New England Biolabs (Schalbach, Germany)
XhoI (20 U/μl) New England Biolabs (Schalbach, Germany)
Taq DNA polymerase (5 U/μl) New England Biolabs (Schalbach, Germany)
Pwo DNA polymerase (5 U/μl) Roche Molecular Biochemicals (Mannheim, Germany)
T4 DNA ligase (400 U/μl)  
benzonase (25 U/μl)  
glutathione reductase (100-300 U/mg)  
insulin (27 U/mg)  
lysozyme (150'000 U/mg)  
ribonuclease A (RNaseA) (90 U/mg)  
trypsin  

New England Biolabs (Schalbach, Germany)  
Merck (Darmstadt, Germany)  
Sigma (Buchs, Switzerland)  
Fluka (Buchs, Switzerland)  
Serva (Heidelberg, Germany)  
Fluka (Buchs, Switzerland)  
Roche Molecular Biochemicals (Mannheim, Germany)  

1.4 Media and antibiotics  

LB  
see Appendix  

DYT  
see Appendix  

M63 minimal medium  
see Appendix  

Ampicillin stock solution  
100 mg/ml ampicillin in H2O (stored at -20 °C)  

1.5 Materials for chromatography  

anion exchange column Resource Q  
anion exchange resin DE 52 cellulose  
anion exchange resin QA 52 cellulose  
cation exchange resin CM 52 cellulose  
cation exchange resin SE 52 cellulose  
high performance column Phenyl Sepharose Hydroxy apatite  

GE Healthcare (Otelfingen, Switzerland)  
Whatman (Maidstone, United Kingdom)  
Whatman (Maidstone, United Kingdom)  
Whatman (Maidstone, United Kingdom)  
Whatman (Maidstone, United Kingdom)  
GE Healthcare (Otelfingen, Switzerland)  
BDH (Chemie Brunschwig, Basel, Switzerland)
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<th>Supplier/Location</th>
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</tr>
<tr>
<td>PD 10 prepacked cartridge</td>
<td>GE Healthcare (Otelfingen, Switzerland)</td>
</tr>
<tr>
<td>Reversed-phase HPLC column <em>ZORBAX Poroshell</em></td>
<td>Agilent Technologies (Basel, Switzerland)</td>
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<tr>
<td>SB-C18</td>
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<td>Size exclusion column <em>Superdex 75 HiLoad</em> 16/60</td>
<td>GE Healthcare (Otelfingen, Switzerland)</td>
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<td>Size exclusion column <em>Superdex 75 HiLoad</em> 26/60</td>
<td>GE Healthcare (Otelfingen, Switzerland)</td>
</tr>
<tr>
<td>Size exclusion column <em>Superdex 75 HR 10/30</em></td>
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</tr>
</tbody>
</table>
2. Molecular cloning techniques

Molecular cloning procedures were based on those described in the Reference (Sambrook J., 1989)

2.1 Bacterial strains, plasmids and oligodesoxynucleotides

**E coli strains**

*E. coli* AAEC189 (Blomfield *et al*., 1991)

*E. coli* BL21(DE3)  
*F*,*sds,gal::IDE3 del (int) lacUV5PO T7RNApol,rB', mB', lon, omT (Dubendorff and Studier, 1991b),(Dubendorff and Studier, 1991a)

*E. coli* HM125 KS272 degP eda rpoH15 (Meerman and Georgiou, 1994)

*E. coli* JBC 816 MC1000, *phoR*, zih12::Tn10tetR, λmalFlacZ-10 (Jonda *et al*., 1999)

*E. coli* JBC 817 MC1000, *phoR*, zih12::Tn10tetR, dsbA::kan1, λmalFlacZ-102 (Jonda *et al*., 1999)

*E. coli* JBC 818 MC1000, *phoR*, zih12::Tn10tetR, dsbA::kan1,dsbB::kan, λmalFlacZ-10 (Jonda *et al*., 1999)

*E. coli* JBC 819 MC1000 *phoR*, zih12::Tn10tetR, dsbB::kan, λmalFlacZ-102 (Jonda *et al*., 1999)

*E. coli* FED 117 MC1000; F. Katzen personal communication

*E. coli* FED 126 MC1000, ΔdsbD; F. Katzen personal communication

*E. coli* FED 215 MC1000, dsbC::mini-Tn10Kan'; F. Katzen personal communication

*E. coli* FED 266 MC1000, ΔdsbD, dsbC::mini-Tn10Kan'; F. Katzen personal communication

**Oligodesoxynucleotides**

Cloning primers

PF 1  
5’-GGGAATTCC*CATATG*GATGACGCGGCAATTCAACAA

ACG-3’

NH₂–terminal primer for the amplification of the dimerisation domain of DsbC without signal sequence. NdeI restriction site is indicated in *bold-italics.*
PF 2
5'-GGGAATTCCATATGAAAGAAGGTGATTGTTGTTT
ACT-3’
NH₂-terminal primer for the amplification of the dimerisation domain of DsbC with DsbA signal sequence. NdeI restriction site is indicated in \textit{bold-italics}.

PF 3
5'-TACCG\textbf{GCTGAG}CGCATTCAACTGCTTTAACAG-3’
COOH–terminal primer for the amplification of the dimerisation domain of DsbC for fusion protein with DsbA. XhoI restriction site is indicated in \textit{bold-italics}.

PF 6
5'-\textbf{ATTGACTAGCTAGCG}CGTCACCAATAAGATGCTGTTTA
AAGCAG-3’
NH₂-terminal primer for the amplification of the catalytic domain of DsbC with DsbA signal sequence. NheI restriction site is indicated in \textit{bold-italics}.

PF 7
5'-\textbf{GCGGATCC}TTAGTGATGATGATGATGATGATGCCTTTT
ACCGCTTGTCAATTTTTG-3’
COOH–terminal primer for the amplification of the catalytic domain of DsbC with a COOH-terminal His₆ tag. BamHI restriction site is indicated in \textit{bold-italics}.

PF 8
5'-\textbf{CACTACTCGAG}AAAGAAGGCGCGCAGTATGAAGATGGTAAA-3’
NH₂-terminal primer for the amplification of DsbA for fusion protein with DsbC dimerisation domain. XhoI restriction site is indicated in \textit{bold-italics}.

PF11
5'-CGCG\textbf{GATCC}TTAGTGATGATGATGATGATGCTTTTTT
CTCGGACAGATATTT-3’
COOH–terminal primer for the amplification of DsbA with a COOH-terminal His₆ tag. BamHI restriction site is indicated in \textit{bold-italics}.

PF 15
5'-GCGCCTGTAAGCTGCTGGCAAAAGACAGTTCTGACT-3’
Forward primer for site-directed mutagenesis of DsbC. Exchange of Met 24 to Lys nucleotides is shown in \textit{bold-italics}.

PF 16
5'-AGTCAGAAACTGTCTTTTGGCCAGCTACAGGGCGC-3’
Backward primer for site-directed mutagenesis of DsbC. Exchange of Met 24 to Lys nucleotides is shown in \textit{bold-italics}.
Materials and Methods

PF 17 5'-ACCGATGATGGTAAAGATACTATTCAAGGGGCCA-3'
Forward primer for site-directed mutagenesis of DsbC. Exchange of His 45 to Asp nucleotides is shown in bold-italics.

PF 18 5'-TGGCCCTGAATGATACTTTACCATCATCGGT-3'
Backward primer for site-directed mutagenesis of DsbC. Exchange of His 45 to Asp nucleotides is shown in bold-italics.

PF 19 5'-GATGGTAAACATATCAAACAGGGGCCAATGTAT-3'
Forward primer for site-directed mutagenesis of DsbC. Exchange of Ile 47 to Lys nucleotides is shown in bold-italics.

PF 20 5'-ATACATTGGCCCCTGTTTGATATGTTTACCATC-3'
Backward primer for site-directed mutagenesis of DsbC. Exchange of Ile 47 to Lys nucleotides is shown in bold-italics.

PF 21 5'-AAACATATCATTCAGCGGCAAATGTATGACGTT-3'
Forward primer for site-directed mutagenesis of DsbC. Exchange of Gly 49 to Arg nucleotides is shown in bold-italics.

PF 22 5'-AACGTCATACATTGGGCCTGCTGAATGATATGTTT-3'
Backward primer for site-directed mutagenesis of DsbC. Exchange of Gly 49 to Arg nucleotides is shown in bold-italics.

PF 23 5'-AAACATATCATTCAGGAAACCAATGTATGACGTT-3'
Forward primer for site-directed mutagenesis of DsbC. Exchange of Gly 49 to Glu nucleotides is shown in bold-italics.

PF 24 5'-AACGTCATACATTGGTTCTGCTGAATGATATGTTT-3'
Backward primer for site-directed mutagenesis of DsbC. Exchange of Gly 49 to Glu nucleotides is shown in bold-italics.

PF 25 5'-GGGCCAATGTATGACAAAGTGCCACGGCTCCG-3'
Forward primer for site-directed mutagenesis of DsbC. Exchange of Val 54 to Lys nucleotides is shown in bold-italics.

PF 26 5'-CGGAGCCGTGCCACTTTGTCATACATTGGCCC-3'
Backward primer for site-directed mutagenesis of DsbC. Exchange of Val 54 to Lys nucleotides is shown in bold-italics.

PF 47 5'-ATTGACACTAGCTACGGCGGAAGGTATGCAGTTCGAC-3'
NH₂-terminal primer for the amplification of the apical domain of GroEL. NheI restriction site is indicated in bold-italics.

PF 48 5'-TAACGTATCCGCCGGGCACCGGGCTGGATGC-3'

43
AGC-3’
COOH–terminal primer for the amplification of the apical domain of GroEL for fusion protein with DsbA. *NarI* restriction site is indicated in *bold-italics*.

**PF 51**
5’-GGGAATGGCCGCGCCTGAGTATGAAGATGGT
NH2–terminal primer for the amplification of DsbA for fusion protein with apical domain of GroEL. *NarI* restriction site is indicated in *bold-italics*.

**PF 58**
5’-CGCGATGCTTAAGTGTGATGGGTATGGCACCACG
GCCCCTGAGTGCGCAG-3’
COOH–terminal primer for the amplification of GroEL with a COOH-terminal His6 tag. *BamHI* restriction site is indicated in *bold-italics*.

**PF 59**
5’-GGGAATTCAATATGGCACCAGCGACGCCAAATG-3’
NH2–terminal primer for the amplification of the catalytic domain of DsbG without signal sequence. *NdeI* restriction site is indicated in *bold-italics*.

**PF 61**
5’-CGCGATGCTTAAGTGTGATGGGTATGGCACCACG
GCCCCTGAGTGCGCAG-3’
COOH–terminal primer for the amplification of the catalytic domain of DsbG with a COOH-terminal His6 tag. *BamHI* restriction site is indicated in *bold-italics*.

**PF 62**
5’-CCGGGCCATGCTGATGCTGATGGGTATGGCACCACCACGCCTG-3’
Forward primer for site-directed mutagenesis to extend pG7D to pG12D. Elongation is shown in *bold-italics*.

**PF 63**
5’-ACCGGCCATGCTGATGCTGATGGGTATGGCACCACCACGCCTG-3’
Backward primer for site-directed mutagenesis to extend pG7D to pG12D. Elongation is shown in *bold-italics*.

**PF 68**
5’-GCCGGCGCCGCGCGGTGGGTGGGTGGGTGGCAGTA
TGAAGATGCG-3’
Forward primer for site-directed mutagenesis to extend pG12D to pG17D. Elongation is shown in *bold-italics*.

**PF 69**
5’-CCATCTTCATACTGCGCGCCACCGACCGACCGACCGACCGACCGACCGACCGACCGA
CGCGGCCCGC-3’
Backward primer for site-directed mutagenesis to extend pG12D to pG17D. Elongation is shown in bold-italics.

Sequencing primer

ptrc99for 5’-GTTGACAATTAATCATCGGCTC-3’
NH2-terminal sequencing primer for pDsbA3 or pDsbAcyt

EP3-IR 5’/TGCAAGTGTAGCGGTAC-3’
COOH-terminal sequencing primer for pDsbA3 or pDsbAcyt

Plasmids

pDsbA3 general periplasmic expression vector containing a trc-promotor and a ampicillin-resistance gene
(Hennecke et al., 1999)
pDsbAcyt general cytoplasmic expression vector containing a T7-promotor and an ampicillin-resistance gene

pRBI-DsbC periplasmic expression plasmid for DsbC
(Maskos et al., 2003)
pDsbC_His periplasmic expression plasmid for DsbC with His6 tag
pDsbC CCAA periplasmic expression plasmid for inactive DsbC
pDsbC-C periplasmic expression plasmid for DsbC-C
pD-DsbA periplasmic expression plasmid for D-DsbA
pD-DsbAcyto cytoplasmic expression plasmid for D-DsbA
pDsbC M24K periplasmic expression plasmid for DsbC M24K
pDsbC H45D periplasmic expression plasmid for DsbC H45D
pDsbC I47K periplasmic expression plasmid for DsbC I47K
pDsbC G49R periplasmic expression plasmid for DsbC G49R
pDsbC G49E periplasmic expression plasmid for DsbC G49E
pDsbC V54K periplasmic expression plasmid for DsbC V54K
pDsbG periplasmic expression plasmid for DsbG
pDsbG_His cytoplasmic expression plasmid for DsbG with His6 tag
pDsbG-C cytoplasmic expression plasmid for DsbG-C with His6 tag
Materials and Methods

<table>
<thead>
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<th>Plasmid</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pDsbB</td>
<td>expression plasmid for DsbB</td>
<td>lab collection</td>
</tr>
<tr>
<td>(Grauschopf et al., 2003)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pnDsbD</td>
<td>cytoplasmic expression plasmid for nDsbD</td>
<td>lab collection</td>
</tr>
<tr>
<td>(Rozhkova et al., 2004)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pSSG7D</td>
<td>periplasmic expression plasmid for G7D</td>
<td>this thesis</td>
</tr>
<tr>
<td>pSSG12D</td>
<td>periplasmic expression plasmid for G12D</td>
<td>this thesis</td>
</tr>
<tr>
<td>pSSG17D</td>
<td>periplasmic expression plasmid for G17D</td>
<td>this thesis</td>
</tr>
<tr>
<td>pSSGroEL-AD</td>
<td>periplasmic expression plasmid for GroEL-AD</td>
<td>this thesis</td>
</tr>
<tr>
<td>pGroE</td>
<td>periplasmic expression plasmid for GroEL and GroES</td>
<td>lab collection</td>
</tr>
</tbody>
</table>

2.2 Transformation of E. coli with plasmid DNA

Chemical transformation

Aliquots of 30 μl of TSS E. coli strain BL21(DE3) or HM125 were mixed with 1 μl of plasmid solution (~ 4 μmol/ml) and incubated at 4 °C for 30 min. LB medium (0.27 ml) was then added, and the cells were grown at 37 °C for 30 min to allow formation of the antibiotic-resistance. Transformants were selected by plating cells on LB agar plates containing ampicillin (Chung et al., 1989).

Electroporation

The transformation was performed by electroporation, using an E. coli pulser (Bio-Rad Laboratories, Richmond, United States). 2 μl of DNA ligation reaction was added to 50 μl of E. coli AAEC129 cells. The mixture was then subjected to an electric field (1.8 kV, 1 mm cuvette). LB medium was added to 300 μl; the cells were incubated at 37 °C for 30 min to allow formation of the antibiotic-resistance and grown on LB agar plates containing ampicillin.

2.3 Isolation of plasmid DNA from E. coli

Plasmid DNA from 6 ml of bacterial culture was isolated using the Wizard Plus SV Minipreps DNA purification System.
2.4 Polymerase chain reaction

The primers, mutating the wild type genes, introduce restriction enzyme recognition sites, which are used for restriction analysis and ligations to the desired gene. The restriction enzymes recognition sites for BamHI, NarI, NdeI, Nhel or XhoI at the 5’- or 3’- end and the His6 tag at the 3’- end of the wild type gene were introduced by PCR (Mullis and Faloona, 1987).

<table>
<thead>
<tr>
<th>PCR construct</th>
<th>description</th>
<th>5’- primer</th>
<th>3’- primer</th>
<th>template</th>
</tr>
</thead>
<tbody>
<tr>
<td>DsbA His</td>
<td>DsbA modified for dimeric DsbA fusion protein</td>
<td>PF 8</td>
<td>PF 11</td>
<td>pDsbA3</td>
</tr>
<tr>
<td>DsbC-D</td>
<td>DsbC dimerisation domain for dimeric DsbA fusion protein</td>
<td>PF 1</td>
<td>PF 3</td>
<td>pRBIDsbC</td>
</tr>
<tr>
<td>DsbC-DSS</td>
<td>DsbC dimerisation domain with DsbA signal sequence for dimeric DsbA fusion protein</td>
<td>PF 2</td>
<td>PF 3</td>
<td>pRBIDsbC</td>
</tr>
<tr>
<td>DsbC-CSS</td>
<td>coding for DsbC catalytic domain with DsbA signal sequence</td>
<td>PF 6</td>
<td>PF 7</td>
<td>pRBIDsbC</td>
</tr>
<tr>
<td>DsbG-C</td>
<td>DsbG catalytic domain</td>
<td>PF 59</td>
<td>PF 61</td>
<td>pDsbG</td>
</tr>
<tr>
<td>DsbA Fus</td>
<td>DsbA modified for fusion protein with GroEL apical domain</td>
<td>PF 51</td>
<td>PF 11</td>
<td>pDsbA3</td>
</tr>
<tr>
<td>apDGroEL Fus</td>
<td>apical domain of GroEL modified for fusion protein with DsbA</td>
<td>PF 47</td>
<td>PF 48</td>
<td>pGroE</td>
</tr>
<tr>
<td>apDGroEL – SS</td>
<td>the apical domain of GroEL with DsbA signal sequence</td>
<td>PF 47</td>
<td>PF 58</td>
<td>pGroE</td>
</tr>
</tbody>
</table>

PCR Protocol
see Appendix

Purification of the PCR products was performed using the PCR Purification kit QIAquick.
2.5 Site-directed mutagenesis

To disrupt the dimerization interface of DsbC, different amino acid residues that are involved in DsbC dimerization were exchanged. The mutations for the monomeric DsbC variants were introduced by sequence specific site-directed mutagenesis using the site-directed mutagenesis kit *QuikChange II*. Additionally the linker in the apical domain of GroEL DsbA fusion protein was elongated by site-directed mutagenesis.

**Table 2**  
*List of the site-directed mutagenesis construct for periplasmic expression with the corresponding amplification primers and genetic templates.*

<table>
<thead>
<tr>
<th>Site-directed mutagenesis construct</th>
<th>description</th>
<th>forward primer</th>
<th>backward primer</th>
<th>Template plasmid</th>
</tr>
</thead>
<tbody>
<tr>
<td>pDsbC M24K</td>
<td>monomeric DsbC with Met 24 to Lys exchange</td>
<td>PF 15</td>
<td>PF 16</td>
<td>pDsbChis</td>
</tr>
<tr>
<td>pDsbC H45D</td>
<td>monomeric DsbC with His 45 to Asp exchange</td>
<td>PF 17</td>
<td>PF 18</td>
<td>pDsbChis</td>
</tr>
<tr>
<td>pDsbC I47K</td>
<td>monomeric DsbC with Ile 47 to Lys exchange</td>
<td>PF 19</td>
<td>PF 20</td>
<td>pDsbChis</td>
</tr>
<tr>
<td>pDsbC G49R</td>
<td>monomeric DsbC with Gly 49 to Arg exchange</td>
<td>PF 21</td>
<td>PF 22</td>
<td>pDsbChis</td>
</tr>
<tr>
<td>pDsbC G49E</td>
<td>monomeric DsbC with Gly 49 to Glu exchange</td>
<td>PF 23</td>
<td>PF 24</td>
<td>pDsbChis</td>
</tr>
<tr>
<td>pDsbC V54K</td>
<td>monomeric DsbC with Val 54 to Lys exchange</td>
<td>PF 25</td>
<td>PF 26</td>
<td>pDsbChis</td>
</tr>
<tr>
<td>pG12D</td>
<td>GroEL apical domain and DsbA fusion protein connected via 12 amino acid linker</td>
<td>PF 62</td>
<td>PF 63</td>
<td>pSSG7D</td>
</tr>
<tr>
<td>pG17D</td>
<td>GroEL apical domain and DsbA fusion protein connected via 17 amino acid linker</td>
<td>PF 68</td>
<td>PF 69</td>
<td>pSSG12D</td>
</tr>
</tbody>
</table>
2.6 Cleavage of DNA with restriction endonucleases

For cloning and restriction analysis, 55 µl and 2 µl of plasmid DNA (0.1 µg/µl), respectively, were mixed with the restriction enzyme (0.1 U/µl solutions) in the buffer recommended by the manufacturers of the enzymes. The reaction mixture was incubated for 2 hours at the temperature optimum for the enzyme. The cleavage products were analyzed by agarose gel electrophoresis.

2.7 Gel electrophoresis of DNA fragments

Double-stranded DNA molecules were separate by 1 % (w/v) agarose gel electrophoresis according to their molecular size. Agarose was dissolved by heating in TAE buffer (40 mM Tris/Acetic acid, 1 mM EDTA pH 8.0). After the solution was cooled to < 50 °C, ethidium bromide (1.5 µg/ml) was added.

The samples were mixed with one fifth of their volume with agarose gel sample buffer (0.6 g/ml Saccharose, 2.5 mg/ml Bromphenol blue, 2.5 mg/ml Xylenecyanol) and subjected to electrophoresis in TAE buffer at 10 V/cm (~50 min). DNA bands were visualized with UV light.

2.8 Extraction of DNA fragments

After separation on an agarose gel, cleaved DNA fragments were excised and extracted using the Gel Extraction kit QIAquick.

2.9 Ligation of DNA fragments

PCR constructs were ligated into expression plasmid with vector DNA, PCR construct (molar ratio vector: insert = 1:2) and 1 µl T4 DNA ligase in 20 µl ligase buffer according to the manufacturer’s instructions.

The ligation mixture was incubated using following program (Lund et al., 1996)

\[
\begin{align*}
10 ^\circ C & \quad 30 \text{ sec} & \text{temperature increase by 0.1 °C/sec} \\
30 ^\circ C & \quad 30 \text{ sec} & \text{temperature decrease by 0.1 °C/sec}
\end{align*}
\]
### Materials and Methods

repeated 99 times

#### Table 3

Ligation products listed with the corresponding PCR construct and ligation vector.
The corresponding restriction sites are indicated by their PCR constructs.

<table>
<thead>
<tr>
<th>Expression plasmid</th>
<th>Description</th>
<th>PCR construct 1</th>
<th>PCR construct 2</th>
<th>Vector</th>
</tr>
</thead>
<tbody>
<tr>
<td>pD-DsbA</td>
<td>periplasmic expression of dimeric DsbA</td>
<td>DsbC-DSS (Ndel and XhoI)</td>
<td>DsbA His (XhoI and BamHI)</td>
<td>pDsbA3 (Ndel and BamHI)</td>
</tr>
<tr>
<td>pD-DsbAcyt</td>
<td>cytoplasmic expression of dimeric DsbA</td>
<td>DsbC-D (Ndel and XhoI)</td>
<td>DsbA His (XhoI and BamHI)</td>
<td>pDsbAcyt (Ndel and BamHI)</td>
</tr>
<tr>
<td>pDsbC-C</td>
<td>periplasmic expression of the catalytic domain of DsbC</td>
<td>DsbC-CSS (NheI and BamHI)</td>
<td></td>
<td>pDsbA3 (NheI and BamHI)</td>
</tr>
<tr>
<td>pDsbG-C</td>
<td>cytoplasmic expression of the catalytic domain of DsbG</td>
<td>DsbG-C (NdeI and BamHI)</td>
<td></td>
<td>pDsbA3 (NdeI and BamHI)</td>
</tr>
<tr>
<td>pSSG7D</td>
<td>periplasmic expression of GroEL apical domain and DsbA fusion protein connected via 7 amino acid residue linker</td>
<td>apDGroEL Fus (NheI and NarI)</td>
<td>DsbA Fus (NarI and BamHI)</td>
<td>pDsbA3 (NheI and BamHI)</td>
</tr>
<tr>
<td>pSSGroEL-AD</td>
<td>periplasmic expression of apical domain of GroEL</td>
<td>apDGroEL-SS (NheI and BamHI)</td>
<td></td>
<td>pDsbA3 (NheI and BamHI)</td>
</tr>
</tbody>
</table>

#### 2.10 DNA Sequencing

All double-stranded DNA plasmids were verified by DNA sequencing. The principle of this procedure is chain-termination with dideoxynucleotides, using the Vistra DNA System kit.
Materials and Methods

1 μl of sequencing primer (3.2 pmol/μl) was added to 800 ng DNA and the reaction mix was filled up to 26 μl with distilled water. Then 6 μl of the mixture was placed in 4 different PCR wells and 2 μl of A-, C-, G- and T-reagent, respectively, were added. 20 μl of Wax Chill-out 14TM were added in each reaction mix and the assay was submitted to a PCR reaction. After denaturation for 2 min at 95 °C, the following reaction was performed:

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>95 °C</td>
<td>30 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>58 °C</td>
<td>30 sec</td>
</tr>
<tr>
<td>Elongation</td>
<td>72 °C</td>
<td>60 sec</td>
</tr>
<tr>
<td></td>
<td></td>
<td>repeated 30 times</td>
</tr>
</tbody>
</table>

The reaction was stopped by adding 2 μl of formamide loading dye and subsequent incubated at 80 °C for 2 min.

The sequencing was done on a LI-COR DNA Sequencer 4000 (LI-COR Biosciences, Bad Homburg, Germany) where the cycle sequencing products were separated by polyacrylamide gel (6 %) electrophoresis and visualized by a laser beam (785 nm). TBE buffer (89 mM Tris / Boric acid, 2 mM EDTA pH 8.5) was used as running buffer.

Alternatively, sequencing was done by Synergene (Schlieren, CH).
3. Biochemical techniques

3.1 Expression and purification of proteins

DsbC

*E. coli* HM125 cells harboring the plasmid pRBIDsbC were grown overnight at 25 °C in DYT medium containing 100 μg/l ampicillin. Then 5 l flasks containing 1.5 l DYT and 100 μg/l ampicillin were inoculated with the cell culture (1:100). The cell culture was shaken with 150 rpm at 25 °C. After induction with IPTG (1 mM) at an OD_{600} of 1.0, the cells were grown at 25 °C for further 16 hours. Cells were harvested by centrifugation (4000 x g, 4 °C, 15 min). The cell pellet was resuspended in 50 mM sodium phosphate/NaOH, 300 mM NaCl pH 8.0 and 1 mg/ml Polymixin B sulfate (10 ml/l of bacterial culture) and the outer membranes were lysed by gentle shaking at 4 °C for 1.5 h. After centrifugation (39000 x g, 4 °C, 45 min) the periplasmic extract was dialyzed against 10 mM Bis-Tris/HCl pH 7.0 and centrifuged (39000 x g, 4 °C, 45 min). The supernatant was applied to a DE52 anion exchange column (40 ml) equilibrated with 10 mM Bis-Tris/HCl pH 7.0. The DE52 column was washed with 10 mM Bis-Tris/HCl pH 7.0 and the protein was eluted with a linear gradient (V = 800 ml) from 0 mM to 400 mM NaCl. DsbC-containing fractions were pooled and dialyzed against 40 mM Tris/HCl, 150 mM NaCl pH 7.5. After addition of ammonium sulfate to the end concentration of 1.5 M, the proteins were applied to a Phenyl Sepharose hydrophobic interaction column (12 ml) that was equilibrated with 40 mM Tris/HCl, 150 mM NaCl, 1.5 M ammonium sulfate pH 7.5. Proteins were eluted with a linear gradient (V = 300 ml) from 1.5 M to 0 M ammonium sulfate. DsbC-containing fractions were pooled, dialyzed against 100 mM Tris/HCl pH 8.0 and centrifuged (39000 x g, 4 °C, 45 min). DsbC was concentrated with an Amicon 82000 YM 10 (Millipore, Volketswil, Switzerland) to a concentration of 0.5 mM. To achieve a complete oxidation of DsbC, the protein was incubated with 100 mM GSSG at room temperature (RT) for 1 hour. GSSG was then removed from DsbC on a Superdex 75 HiLoad 16/26 size-exclusion column equilibrated with H_{2}O (2.5 ml/min).

DsbC-C

*E. coli* BL21(DE3) harboring pDsbC-C were grown overnight at 30 °C in DYT medium containing 100 μg/ml ampicillin to an OD_{600} of 1.0 and induced with 1 mM IPTG as described above. After further growth at 30 °C for 16 hours cells were harvested by
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centrifugation (4000 x g, 4 °C, 15 min). The cell pellet was resuspended in 50 mM sodium phosphate/NaOH, 300 mM NaCl pH 8.0 and 1 mg/ml Polymixin B sulfate (10 ml/l bacterial culture) and the outer membranes were lysed by gentle shaking at 4 °C for 1.5 h. After centrifugation of the periplasmic extract (39000 x g, 4 °C, 45 min) the supernatant was loaded to a Ni-NTA column (40 ml) equilibrated with 50 mM sodium phosphate/NaOH, 300 mM NaCl pH 8.0. The Ni-NTA column was washed with 50 mM sodium phosphate/NaOH, 300 mM NaCl pH 8.0 and the protein was eluted with a linear gradient (V = 300 ml) from 0 mM to 300 mM imidazole. DsbC-C-containing fractions were pooled and concentrated. DsbC-C was applied to a Superdex 75 HiLoad 26/60 size-exclusion column equilibrated with 20 mM sodium phosphate, 115 mM NaCl, 0.1 mM EDTA pH 7.5 and separated by size-exclusion chromatography (2.5 ml/min). Fractions with pure monomeric DsbC-C were pooled, dialyzed against H2O and concentrated to 4 mg /ml.

D-DsbA

*E. coli* BL21(DE3) harboring pD-DsbAcyto were grown at 30 °C in DYT medium containing 100 μg/ml ampicillin to an OD<sub>600</sub> of 1.5 and induced with 1 mM IPTG as described above. After further growth at 30 °C for 16 hours the cells were harvested by centrifugation (4000 x g, 4 °C, 15 min), resuspended in 50 mM sodium phosphate/NaOH, 300 mM NaCl, 1 mg lysozyme pH 8.0 and 1 μl benzonase (30 ml/l cell culture) at 4 °C, lysed with the *EmulsiFlex* C5 high pressure homogenisator (Avestin, Ottawa, Canada) and centrifugated (39000 x g, 4 °C, 45 min). The supernatant was directly loaded to a Ni-NTA column (40 ml) equilibrated with 50 mM sodium phosphate/NaOH, 300 mM NaCl pH 8.0. The Ni-NTA column was washed with 50 mM sodium phosphate/NaOH, 300 mM NaCl pH 8.0 and the protein was eluted with a linear gradient (V= 300ml) from 0 mM to 300 mM imidazole. D-DsbA-containing fractions were pooled, dialyzed against 10 mM acetic acid/NaOH pH 5.0 after addition of 1 mM EDTA and applied to a CM52 cation exchange column (40 ml) equilibrated with 10 mM acetic acid/NaOH pH 5.0. After elution with a linear gradient (V = 400ml) from 0 mM to 400 mM NaCl, D-DsbA-containing fractions were pooled, dialyzed against 20 mM sodium phosphate/NaOH, 115 mM NaCl, 0.1 mM EDTA pH 7.5 and concentrated to 0.1 mM. D-DsbA was oxidized by addition of 20 mM GSSG (1 h, RT). The mixture was applied to a Superdex 75 HiLoad 26/60 size-exclusion column equilibrated with 20 mM sodium phosphate/NaOH pH 7.5, 115 mM NaCl, 0.1 mM EDTA and separated (2.5 ml/min). Fractions with pure, dimeric D-DsbA were pooled, dialyzed against H2O and concentrated.
Monomeric DsbC variants

_E. coli_ BL21(DE3), harboring a plasmid for a monomeric DsbC variant expression, were grown overnight at 30 °C in DYT medium containing 100 μg/ml ampicillin to an OD₆₀₀ of 1.0 and induced with 1 mM IPTG as described above. After further growth at 30 °C for 16 hours cells were harvested by centrifugation (4000 x g, 4 °C, 15 min). The cell pellet was resuspended in 50 mM sodium phosphate/NaOH, 300 mM NaCl pH 8.0 and 1 mg/ml Polymixin B sulfate (10 ml/l bacterial culture) and the outer membranes were lysed by gentle shaking at 4 °C for 1.5 h. After centrifugation of the periplasmic extract (39000 x g, 4 °C, 45 min) the supernatant was loaded to a Ni-NTA column (40 ml) equilibrated with 50 mM sodium phosphate/NaOH, 300 mM NaCl pH 8.0. The protein was eluted with a linear gradient (V = 300 ml) from 0 mM to 300 mM imidazole (Bader et al., 2001).

The fractions of the monomeric DsbC variants were pooled, dialyzed against 10 mM Tris/HCl, 1 mM EDTA pH 8.0 and applied to a Resource Q anion exchange column (V = 6 ml) equilibrated with 10 mM Tris/HCl, 1 mM EDTA pH 8.0. After elution with a linear gradient (V = 48ml) from 0 mM to 500 mM NaCl, the fractions with monomeric DsbC were pooled and dialyzed against 100 mM sodium phosphate/NaOH, 500 mM NaCl, 1 mM EDTA pH 7.0. The monomeric DsbC variants were concentrated, applied to a Superdex 75 HiLoad 16/60 size-exclusion column equilibrated with 100 mM sodium phosphate/NaOH, 500 mM NaCl, 1 mM EDTA pH 7.0 and separated (1.5 ml/min). Fractions with pure, monomeric DsbC variants were pooled, dialyzed against H₂O and concentrated.

DsbG

_E. coli_ BL21(DE3) harboring pDsbG were grown overnight at 30 °C in DYT medium containing 100 μg/ml ampicillin to an OD₆₀₀ of 1.0 and induced with 1 mM IPTG as described above. After further growth at 30 °C for 16 hours cells were harvested by centrifugation (4000 x g, 4 °C, 15 min). The cell pellet was resuspended in 50 mM sodium phosphate/NaOH, 300 mM NaCl pH 8.0 and 1 mg/ml Polymixin B sulfate (10 ml/l bacterial culture) and the outer membranes were lysed by gentle shaking at 4 °C for 1.5 h. After centrifugation of the periplasmic extract (39000 x g, 4 °C, 45 min) the supernatant was dialyzed against 30 mM Tris/HCl, 0.5 mM EDTA pH 8.5. The protein was applied to a QA52 anion exchange column (V = 40 ml) equilibrated with 30 mM Tris/HCl, 0.5 mM EDTA pH
Materials and Methods

8.5 and eluted with a linear gradient (V = 250 ml) from 0 mM to 300 mM NaCl. DsbG-containing fractions were pooled, dialyzed against 30 mM Tris/HCl, 0.5 mM EDTA pH 8.5, concentrated and applied to a SE52 cation exchange column (V = 40 ml) equilibrated with 30 mM Tris/HCl, 0.5 mM EDTA pH 8.5. After elution with a linear gradient (V = 300 ml) from 0 mM to 300 mM NaCl, DsbG-containing fractions were pooled, dialyzed against 30 mM Tris/HCl, 0.5 mM EDTA pH 8.5 and concentrated. The protein was again applied to a QA52 anion exchange column (V = 40 ml) equilibrated with 30 mM Tris/HCl, 0.5 mM EDTA pH 8.5 and eluted with a linear gradient (V = 400 ml) from 0 mM to 300 mM NaCl. DsbG-containing fractions were pooled, concentrated and applied to a Superdex 75 HiLoad 16/60 size-exclusion column equilibrated with 30 mM Tris/HCl pH 8.5, 50 mM NaCl, 0.5 mM EDTA buffer and separated (1.5 ml/min). Fractions with pure DsbG were pooled, dialyzed against H₂O and concentrated.

DsbG<sub>His</sub>

The growing of the E. coli BL21(DE3) harboring the plasmid pDsbG<sub>His</sub> and the first centrifugation step was done as in the expression and purification of DsbG described. The cells were resuspended in 50 mM sodium phosphate/NaOH, 300 mM NaCl, 1 mg lysozyme pH 8.0 and 1 μl benzonase (30 ml/l cell culture) at 4 °C, lysed with the EmulsiFlex C5 high pressure homogenisator and centrifugated (39000 x g, 4 °C, 45 min). The supernatant was directly loaded on to a Ni-NTA column (40 ml) equilibrated with buffer containing 50 mM sodium phosphate/NaOH, 300 mM NaCl pH 8.0. The Ni-NTA column was washed with 50 mM sodium phosphate/NaOH, 300 mM NaCl pH 8.0 and the protein was eluted with a linear gradient (V= 300ml) from 0 mM to 300 mM imidazole. Fractions with pure DsbG<sub>His</sub> were pooled, dialyzed against H₂O and concentrated.

DsbB

E. coli HM125 harboring the plasmid pDsbB were grown overnight at 30 °C in DYT containing 100 μg/l ampicillin. Then 5 l flasks containing 1.5 l DYT and 100 μg/l ampicillin were inoculated with the cell culture (1:100) and the cells were grown further on (150 rpm, 30 °C). After induction with IPTG (1 mM) at an OD<sub>600</sub> of 1.0, the cells were grown at 30 °C for 4 hours. Cells were harvested by centrifugation (4000 x g, 4 °C, 15 min). The cells were resuspended in 10 ml/l cell culture of 50 mM Tris/HCl, 300 mM NaCl, 1 mM PMSF, one
Materials and Methods

Protease inhibitor cocktail tablet, 3 μl benzoase pH 8.0 and 15 μl 1M MgSO₄ lysed with the EmulsiFlex C5 high pressure homogenisator and cell debris was removed by centrifugated (39000 x g, 4 °C, 45 min). Membranes were collected by centrifugated (100'000 x g, 4 °C, 60 min), resuspended in 10 ml 20 mM sodium phosphate/NaOH, 115 mM NaCl pH 7.5 and stored after shock freezing in liquid nitrogen at -80 °C. Membrane proteins were solubilized in 50 mM sodium phosphate/NaOH, 1% (w/v) Triton X100, 1 M NaCl pH 8.0 by six strokes with a Dounce homogenisator (VWR, Dietikon, Switzerland) and incubated on ice for 1 hour. The insoluble part of the preparation was removed by centrifugation (100’000 x g, 4 °C, 60 min) and the supernatant was applied to a Ni-NTA column (15 ml) equilibrated with 50 mM sodium phosphate/NaOH, 1% (w/v) Triton X100, 1 M NaCl pH 8.0 by continuous circulation at 4 °C for 16 hours. The Ni-NTA column was washed extensively with 50 mM sodium phosphate/NaOH, 0.1% (w/v) DDM, 1 M NaCl pH 8.0. To remove the bound coenzyme the column was washed with 12 ml of 50 mM sodium phosphate/NaOH, 1% (w/v) LS, 300 mM NaCl pH 8.0 and again with 50 mM sodium phosphate/NaOH, 0.1% (w/v) DDM, 300 mM NaCl pH 6.2. DsbB was eluted with a linear gradient (V = 250 ml) from 0 to 500 mM imidazole. DsbB-containing fractions were pooled, applied onto a hydroxypatite column (12 ml) equilibrated with 50 mM sodium phosphate/NaOH, 0.1% (w/v) DDM, 300 mM NaCl pH 6.2 and eluted with a linear gradient (V = 50 ml) from 50 to 500 mM sodium phosphate/NaOH, 300mM NaCl pH 6.2. Fraction of pure DsbB were pooled and dialyzed against 100 mM sodium phosphate/NaOH, 0.1 mM EDTA pH 7.0 (Grauschopf et al., 2003).

nDsbD

E. coli BL21(DE3) harboring pnDsbD were grown at 37 °C in DYT medium containing 100 μg/ml ampicillin to an OD₆₀₀ of 1.5 and induced with 1 mM IPTG as described above. After further growth at 37 °C for 16 hours the cells were harvested by centrifugation (4000 x g, 4 °C, 15 min), resuspended in 50 mM sodium phosphate/NaOH, 300 mM NaCl, 1 mg lysozyme pH 8.0 and 1 μl benzonase (30 ml/l cell culture) at 4 °C, lysed with the EmulsiFlex C5 high pressure homogenisator (Avestin, Ottawa, Canada) and centrifugated (39000 x g, 4 °C, 45 min). The supernatant was dialyzed against 10 mM Tris/HCl, 1 mM EDTA pH 8.0 and applied to a QA52 anion exchange column equilibrated with 10 mM Tris/HCl, 1 mM EDTA pH 8.0. Proteins were eluted by a linear gradient (V = 300 ml) from 0 M to 1 M NaCl. After addition of ammonium sulfate to the end concentration of 1 M, the proteins were applied to a Phenyl Sepharose hydrophobic interaction column equilibrated with 10 mM Tris/HCl, 1 M
ammonium sulfate, 1 mM EDTA pH 8.0 and eluted by a linear gradient (V = 500 ml) from 1 M to 0 M ammonium sulfate. The fractions containing nDsbD were pooled and dialyzed against 100 mM sodium phosphate/NaOH, 1 mM EDTA pH 7.0 (Rozhkova et al., 2004).

### Apical domain of GroEL and its DsbA fused variants

*E. coli* BL21(DE3) harboring either of the plasmids pGroEL-AD, pSSG7D, pSSG12D or pSSG17D were grown overnight at 37 °C in DYT medium containing 100 μg/ml ampicillin to an OD₆₀₀ of 1.0 and induced with 1 mM IPTG as described above. After further growth at 37 °C for 16 hours cells were harvested by centrifugation (4000 x g, 4 °C, 15 min). The cell pellet was resuspended in 50 mM sodium phosphate/NaOH, 300 mM NaCl pH 8.0 and 1 mg/ml Polymixin B sulfate (10 ml/l bacterial culture) and the outer membranes were lysed by gentle shaking at 4 °C for 1.5 h. After centrifugation of the periplasmic extract (39000 x g, 4 °C, 45 min) the supernatant was directly loaded to a Ni-NTA column (40 ml) equilibrated with 50 mM sodium phosphate/NaOH, 300 mM NaCl pH 8.0. The Ni-NTA column was washed with 50 mM sodium phosphate/NaOH, 300 mM NaCl pH 8.0 and the protein was eluted with a linear gradient (V= 300ml) from 0 mM to 300 mM imidazole in the same buffer. The protein containing fractions were pooled, dialyzed against 50 mM Tris/HCl pH8.0 and applied to a Resource Q anion exchange column (V = 6 ml) equilibrated with 50 mM Tris/HCl pH 8.0. After elution with a linear gradient (V = 48ml) from 0 M to 1 M NaCl, the fractions with protein were pooled, concentrated and applied to a Superdex 75 HiLoad 26/60 size-exclusion column equilibrated with 50 mM Tris/HCl, 500 mM NaCl pH 8.0 and separated (2.5 ml/min). Fractions with pure proteins were pooled, dialyzed against H₂O and concentrated.

### Other proteins

DsbA and Trx were a gift of Björn Philipps, DsbC₄₅₅ and DsbC CCAA were a gift of Valentin Meraldi and DsbG-C was purified by Anna Rozhkova. RBI was purified as described before (P. Frei, Diploma thesis).

### 3.2 Gel electrophoresis of proteins
Materials and Methods

The discontinuous sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Schägger and coworkers (Schagger et al., 1988). Protein samples were mixed with one quarter volume of the loading buffer (250 mM Tris/HCl pH 8.0, 0.4 mg/ml glycerol, 120 mg/ml SDS, 6 mg/ml bromphenol blue, 50 mg/ml β-ME), heated at 95 °C (membrane proteins were heated for 37 °C) for 10 min, centrifuged and loaded on a gel.

SDS-PAGE was performed at constant voltage of 20 V/cm for 45-55 min with 50 mM Tris/HCl, 190 mM glycine pH 8.3 and 0.1 % (w/v) SDS as running buffer. After electrophoresis, the stacking gel was removed, and the separating gel was shortly heated up in Coomassie staining solution (2 mg/ml Coomassie Brilliant Blue R-250, 40 % (v/v) methanol, 10 % (v/v) acetic acid) and stained for at least 30 min. The background was destained by boiling in H₂O for 30 min.

3.3 Spectroscopic methods

UV absorption spectroscopy

Absorption spectra were used to determine the protein concentrations, to detect non-protein impurities and aggregated material and for biochemical assays. Proteins absorb light in the UV range. The absorbance is caused by the peptide groups, by the aromatic side chains and, to a small extent, by disulfide bonds. Apart from small contributions of the peptide bonds, which strongly absorb light below 230 nm, the absorption of proteins in the 230-300 nm range is determined by the aromatic side chains of tyrosine, tryptophan and phenylalanine and the disulfide bridges (Gill and von Hippel, 1989; Schmid F.X., 1989). All protein spectra were recorded from 230 nm to 350 nm and corrected for the corresponding buffer.

\[ \varepsilon_{280} = n_{\text{Trp}} \varepsilon_{\text{Trp}} + n_{\text{Tyr}} \varepsilon_{\text{Tyr}} + n_{\text{Cys}} \varepsilon_{\text{Cys}} \]

\( n_x \) = quantity of the corresponding amino acid

\( \varepsilon_x \) = molecular extinction coefficient of the corresponding amino acid at \( \lambda = 280 \) nm

(\( \varepsilon_{\text{Trp}} = 5690 \text{ M}^{-1}\text{cm}^{-1}, \varepsilon_{\text{Tyr}} = 1280 \text{ M}^{-1}\text{cm}^{-1}, \varepsilon_{\text{Cys}} = 60 \text{ M}^{-1}\text{cm}^{-1} \))

Using the known amino acid composition of a protein, a molecular extinction coefficient was calculated for all proteins and no differences between the proteins with or without His-tag could be determined.
Table 4

*Calculated molecular extinction coefficient of the proteins*

The molecular extinction coefficient of DsbC was used for DsbC<sub>CCAA</sub> and DsbC<sub>His</sub> and that one of DsbG for DsbG<sub>His</sub>.

<table>
<thead>
<tr>
<th>Protein</th>
<th>ε&lt;sub&gt;280&lt;/sub&gt;</th>
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<tbody>
<tr>
<td>DsbC</td>
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</tr>
<tr>
<td>DsbA</td>
<td>21260 M&lt;sup&gt;-1&lt;/sup&gt; cm&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>DsbC H45D</td>
<td>16050 M&lt;sup&gt;-1&lt;/sup&gt; cm&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>DsbC V54K</td>
<td>16050 M&lt;sup&gt;-1&lt;/sup&gt; cm&lt;sup&gt;-1&lt;/sup&gt;</td>
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<tr>
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</tr>
<tr>
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<tr>
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<tr>
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<tr>
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<td>24300 M&lt;sup&gt;-1&lt;/sup&gt; cm&lt;sup&gt;-1&lt;/sup&gt;</td>
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<tr>
<td>G17D</td>
<td>24300 M&lt;sup&gt;-1&lt;/sup&gt; cm&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

The absorbance is linearly related to the concentration by the Beer–Lambert law:

\[ A_\lambda = \varepsilon \cdot c \cdot l \]

A = absorbance at the wavelength λ

\( c = \text{molar concentration (M)} \)
l = path length (cm)
\( \varepsilon = \) molar extinction coefficient (M\(^{-1}\)cm\(^{-1}\))

Protein concentration was determined using a Cary 3E (Varian, Basel, Switzerland) spectrophotometer.

**Fluorescence spectroscopy**

The change of the intrinsic fluorescence under different condition was used to measure either kinetic reactions or chemical protein stabilities. Measurements were performed on a PTI Fluorescence Spectrometer (PhotoMed, Seefeld, Germany). Differential emission spectra from 295 nm to 450 nm were measured with excitation of tyrosine and tryptophan at 280 nm for both type of measurement. The wave length with the biggest difference in spectra was used for the continuous monitoring of the chemically induced protein folding and unfolding transition or for the kinetic reaction. All spectroscopic measurements were performed with filtrated and degassed solutions.

**Circular dichroism spectroscopy**

Far-UV circular dichroism (CD) spectra were recorded on a JASCO J–710 spectropolarimeter (Jasco, Gross-Umstadt, Germany). The residue ellipticity \([\Theta]_{MRW}\) (degrees x cm\(^2\) x dmol\(^{-1}\)) was calculated from the measured ellipticity \([\Theta]\) (in degrees) by using the following equation (Schmid F.X., 1989)

\[
[\Theta]_{MRW} = \Theta * 100 / c * d * n
\]

\([\Theta]_{MRW}\)  = residue ellipticity
\(\Theta\)  = measured ellipticity
\(c\)  = protein concentration in M
\(d\)  = path length in cm
\(n\)  = number of amino acids of the protein

The change in far-UV CD was used to follow the thermal unfolding of proteins. All spectroscopic measurements were performed with filtrated and degassed solutions.
3.4 Immunoblotting

For immunospecific detection, proteins were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane for 1.5 h (10 W, 100 mA/gel) (Harlow E. and Lane D., 1988) in a semidy blotting apparatus with 7 mM Tris/HCl, 50 mM glycine pH 8.35 after separation on a SDS–polyacrylamide gel. Immunblots were blocked with 5% nonfat-dry milk powder, 20 mM Tris/HCl, 0.1% (v/v) Tween 20, 75 mM NaCl pH 7.6 for 1 h at RT, treated for 1 h with specific polyclonal antibodies from rabbits (1/1000 dilution in the same buffer), washed 3 times with 5% nonfat-dry milk powder, 20 mM Tris/HCl, 0.1% (v/v) Tween 20, 75 mM NaCl pH 7.6 and incubated with alkine phosphate conjugated anti rabbit antibodies (1/2500 dilution in the same buffer) for another 30 min. The membrane was washed twice with the same buffer, then twice with 20 mM Tris/HCl, 0.1% (v/v) Tween 20, 75 mM NaCl pH 7.6, 3 times with 20 mM Tris/HCl, 75 mM NaCl pH 7.6 and was stained with 300 μl BCIP (5% (w/v) in DMF) and 40 μl NBT (7.5% (w/v) in 70% DMF) in 100 ml 100 mM Tris/HCl, 150 mM, 15 MgCl₂ pH 8.8. The reaction was stopped by washing the attained PVDF-membrane with H₂O.

3.5 Quantification of free sulfhydryl groups

To determine the redox state of the proteins, the Ellman’s assay was carried out at a concentration where the absorption at 412 nm was expected to be around 1 for the fully reduced protein. 4 mg/ml DTNB, 80 mM sodium phosphate/NaOH, 1 mM EDTA pH 8.0, 2% (w/v) SDS was added to the protein solution at RT and the absorbance was recorded at 412 nm after incubation for 15 min. The concentration of the free sulfhydryl groups were calculated using $\varepsilon_{412nm} = 13600 \text{ M}^{-1}\text{cm}^{-1}$ (Ellman, 1959).

3.6 Size-exclusion chromatography

To determine the oligomeric state of proteins, size-exclusion chromatography (0.25 ml/min) was performed at RT using an analytical Superdex 75 HR 10/30 size exclusion column equilibrated with 100 mM sodium phosphate/NaOH, 1 mM EDTA pH 7.0. The protein concentrations used are shown in Results. As a control, the mixture of the following proteins
was applied: Serum albumin (67.0 kDa), Ovalbumin (43.0 kDa), Chemotrypsinogen A (25.0 kDa) and Ribonuclease A (13.7 kDa).

3.7. Chemically induced unfolding and refolding of DsbC-C

To determine the stability of DsbC-C, guanidinium chloride (GdmCl) induced unfolding and refolding transition were measured. The concentration of GdmCl in each samples was determined from the refraction index measurements using the following equation (Pace, 1986):

\[
[GdmCl] = 57.147(\Delta N) + 38.68(\Delta N)^2 - 91.60(\Delta N)^3
\]

\[\text{[GdmCl]} \quad \text{= concentration of GdmCl} \]
\[\Delta N \quad \text{= difference between the refractive index of the denaturant solution in buffer and buffer alone} \]

For unfolding measurements samples of DsbC-C (1 μM) were directly incubated in 20 mM HEPES/NaOH, 170 mM NaCl, 1 mM EDTA pH 7.0 containing various concentration of GdmCl at RT for 24 hours. For refolding measurements samples were first unfolded with 7 M GdmCl in 20 mM HEPES/NaOH, 170 mM NaCl, 1 mM EDTA pH 7.0 at RT for 16 hours and then diluted 1 to 50 for the refolding transition in the same buffer containing various concentration of GdmCl and incubated at RT for 24 hours. The unfolding or refolding of DsbC-C was followed by change in the intrinsic fluorescence at 320 nm (excitation 280 nm).

3.8 Thermal unfolding

Thermal unfolding of DsbA, DsbC, DsbG, GroEL-AD, G7D, G12D and G17D was monitored by the change of the far-UV CD ellipsity. 5 μM of the protein was used in filtrated and degassed 100 mM sodium phosphate/NaOH, 1 mM EDTA pH 7.0. To determine the wavelength where the largest change of signal can be observed, differential spectra between 250 and 200 nm were recorded at 25 °C and 95 °C. The thermal unfolding was recorded between 25 °C and 95 °C with continuous increase of the temperature (1 °C/min) on a JASCO J-710 spectropolarimeter using a 1 mm cuvette.
3.9 Subunit exchange of DsbC and DsbG

The strength of the binding of His6-tagged proteins to Ni-NTA resin depends on the amount of His residues per protein molecule. In the DsbC or DsbG subunit exchange assays, this fact was used. DsbC dimer with and without a His6 tag were mixed and incubated at RT for different times. When a subunit exchange occurs, a mixture of DsbC dimers without a His6 tag, with one His6 tag or with two His6 tag can be found after certain time. This mixture can be separated afterwards by their binding strength to the Ni-NTA resin.

25 μM of wild type DsbC and its variant with a COOH-terminal His6 tag (DsbCHis) were mixed in 100 mM sodium phosphate/NaOH pH 7.0 and applied to a Ni-NTA column after different periods of incubation at RT. The formation of heterodimers was analyzed by elution from Ni-NTA column with a gradient from 0 mM to 200 mM imidazole. To determine the retention time of DsbCWT and DsbCHis on the Ni-NTA column, the elution profiles of both proteins were determined independently.

Wild type DsbC and DsbCHis (25 μM each) were unfolded together in the presence of 6 M GdmCl in 100 mM sodium phosphate/NaOH pH 7.0 at RT for 3 hours and dialyzed excessively against 100 mM sodium phosphate/NaOH pH 7.0. The mixture was applied to a Ni-NTA column to analyze the statistical formation of the different DsbC dimers by a linear gradient from 0 mM to 200 mM imidazole.

The same experiment was done with wild type DsbG and a variant with a COOH-terminal His6-tag (DsbGHis) to study differences in the behavior of DsbC and DsbG. In addition the possibility to detect heterodimers either of wild type DsbC and a variant of DsbCHis with active site Cys exchange to Ala (DsbCCCA) at different ratios or between DsbGHis and DsbC was analyzed.

To determine the relative amount of eluted protein, the elution profile was subjected to integration analysis of the peak (peak fit, seasolve, San Jose CA, USA).

3.10 Oxidative refolding of RBI

To assess the disulfide isomerase activity of DsbC, DsbC-C, DsbA and D-DsbA, the kinetics of oxidative refolding of denatured, reduced RBI was investigated. RBI refolding in the presence or absence of DsbA or DsbC was already done before in a more reducing, an optimal and more oxidizing state of the buffer. Additionally pH 7.5 and pH 8.7 was also tested for the refolding of RBI (Maskos et al., 2003).
For the application as a model for disulfide oxidoreductases assisted refolding of RBI, the condition at higher pH and more oxidizing environment show in the uncatalyzed reaction fast building of intermediates, but slow reshuffling of non-native disulfide. These conditions are perfect to test the disulfide isomerase activity of DsbA and DsbC and their variants D-DsbA and DsbC-C.

Denatured, reduced RBI was obtained by incubation of 0.5 mM active, native RBI in 50 mM Tris/HCl, 6 M GdmCl, 100 mM DTT pH 8.7 at RT for 16 hours, followed by gel filtration on a PD 10 cartridge equilibrated with 10 mM HCl. The fully reduced state of RBI was verified by Ellman’s assay. Denatured, reduced RBI in 10 mM HCl proved to be stable against air oxidation and aggregation at 4 °C for at least one week.

The RBI refolding assay was performed with RBI and catalyst proteins (4.6 μM each) in 100 mM Tris/HCl, 200 mM NaCl, 1 mM EDTA pH 7.5 containing 2 mM GSH and 40 mM GSSG to favor oxidation of scrambled RBI. The mixture without RBI was incubated at 15 °C for 20 min and the assay was started by addition of the denatured, reduced RBI. At different time points samples were removed, mixed with 0.4 volumes of 30% (w/v) formic acid (pH 2) and kept at 4 °C prior to HPLC analysis. RBI without any catalyst was used as negative control.

The separation of fully folded RBI and folding intermediates thereof was performed at 55 °C on a C18 reversed phase HPLC column. The acid–quenched samples (0.7 ml, 3.3 μM RBI) were applied to the column and separated with a linear gradient from 28 % to 45 % of acetonitrile in 0.1 % TFA with a flow rate of 0.5 ml/min. The absorbance was recorded at 275 nm and individual peaks were quantified by integration.

### 3.11 Insulin reduction assay

**Catalyzed reduction of insulin by DTT**

To assess the reductase activity of different oxidoreductases, the insulin reduction assay was used (Holmgren, 1979).

Insulin was dissolved in 10 mM HCl to a concentration of 520 μM. 250 μl of this solution was mixed with 250 μl of assay buffer containing 200 mM potassium phosphate, 4 mM EDTA, pH 7.1. The catalytic disulfide oxidoreductases and their variants were diluted in 250 μl solutions with water to a concentration of 4 μM and incubated at RT 15 min after adding 250 μl assay buffer containing additionally 4 mM DTT. The reaction was started by addition
of the insulin solution. The reduction of insulin was followed by measuring the increase in absorbance at 650 nm for 60 min.

**Monitoring the DsbG chaperone activity**

To test the ability of DsbG to prevent insulin from aggregation the insulin reduction assay was performed as described above with different increasing amounts of DsbG and adapted time scale. Additionally the GroEL-AD was tested for chaperone activity too.

**HPLC analysis of insulin reduction**

The insulin reduction assay was additionally used to analyze the chaperone-like activity of DsbG in more detail. Insulin was dissolved in 10 mM HCl to a concentration of 520 μM. 100 μl of this solution was mixed with 100 μl of assay buffer containing 200 mM potassium phosphate, 4 mM EDTA pH 7.1. DsbC (1 μM monomer concentration) and DsbG (65 μM monomer concentration) were adjusted to 100 μl with water and incubated 15 min at RT after adding 100 μl assay buffer containing additionally 4 mM DTT. The reaction was started by addition of the insulin solution to the catalyst-containing solution and after 0 min, 60 min, 120 min, 180 min and 240 min the reduction was stopped by mixing with 0.4 volume of 30 % (w/v) formic acid (pH 2). The samples were kept at 4 °C.

The separation of the samples was performed at 55 °C on a C18 reversed phase HPLC column. The acid-quenched samples (0.56 ml, 93 μM Insulin) were applied to the column and separated with a linear gradient from 0 % to 50 % of acetonitrile in 0.1 % TFA. The absorbance was recorded at 280 nm and individual peaks were quantified by integration.

**3.12 RNaseA isomerization assay**

**Formation of scrambled RNaseA**

Production of scrambled RNaseA (scRNaseA) using the wild type RNaseA from bovine pancreas was performed as already described (Hillson *et al.*, 1984). 2.2 mM RNaseA was incubated in the presence of 6 M GdmCl and 150 mM DTT in 50 mM Tris/HCl pH 8.0 at RT for 16 hours. The reaction was stopped by addition of acetic acid to the final pH 4.0 and the reduced protein was isolated by desalting with a PD 10 cartridge using degassed 0.1 M acetic
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acid/NaOH pH 4.0. Protein concentration was determined spectrophotometrically and the reduced state of RNaseA was confirmed by Ellman’s assay (Ellman, 1959). The reduced RNaseA was diluted to a concentration of 0.5 mg/ml with 0.1 M Tris/HCl pH 8.5 and GdmCl was added to a final concentration of 6 M. The reaction mixture was incubated for reoxidation at RT for at least 3 days protected from light. The randomly reoxidized protein was isolated as described above and the redox state of the scRNaseA was tested by Ellman’s assay.

Continuous monitoring of scRNaseA reactivation

Reactivation of scRNaseA can be measured by increasing RNaseA cleavage activity. The cleavage of the cyclic-2′,3′-Cytidinemonophosphate (cCMP) to the 3′-cytidine-monophosphate (3′CMP) increases the absorption at 296 nm. Therefore the increase in RNaseA activity can be followed spectroscopically. The reactivation of scRNaseA is a disulfide bond isomerization. This allows to use the scRNaseA reactivation as a disulfide isomerization assay. First measurements for a disulfide isomerization assay were done as continuous monitoring, where all components were mixed together.

RNaseA or scRNaseA was diluted in degassed and filtrated 50 mM Tris/HCl, 25 mM KCl, 5 mM MgCl₂ pH 7.5 to final concentration of 2.6 μM and incubated at 30 °C for 15 min. The reaction was started by addition of cCMP (3 mM) and followed spectroscopically at 296 nm (Crook et al., 1960).

The data from the assay described above showed some disadvantages (see Results), which leads to the decision to uses another assay already described in the literature. RNaseA or scRNaseA was diluted in 100 mM Tris/acetic acid pH 8.0 to a final concentration of 8.4 μM and the reaction was started by addition of cCMP (4.5 mM) (Lyles and Gilbert, 1991a; Lyles and Gilbert, 1991b). The reaction was followed by the increase in the absorption at 296 nm.

To test disulfide isomerase activity in the scRNaseA assay, DsbC was used additionally in the modified assay described above. DsbC and cCMP was diluted in the assay buffer containing 0.2 mM GSSG and 1.0 mM GSH to a final concentration 1.4 μM and 4.5 mM respectively. By adding scRNaseA (8.4 μM) the assay was started and monitored spectroscopically at 296 nm. ScRNaseA without DsbC or wild type RNaseA were used as negative or positive controls, respectively.
The use of the redox buffer (100 mM Tris/acetic acid, 0.2 mM GSSG, 1.0 mM GSH pH 8.0) in the assay had increased the reactivation of the negative control, which is therefore not usable for the application as a disulfide isomerase activity assay. The reactivation of scRNaseA therefore was finally measured by using reduced DsbC instead of the redox buffer. 5 μM DsbC, 5 μM DTT and 3 mM cCMP in 100 mM Tris/acetic acid pH 8.0 was incubated at 25 °C for 15 min and scRNaseA (5 μM) was added to start the assay. The reactivation of scRNaseA was monitored by RNaseA catalyzed cleavage of cCMP at 296 nm at 25 °C. The fraction of reactivated enzyme was calculated using the following equation (Lyles and Gilbert, 1991a; Lyles and Gilbert, 1991b)

\[
[E]_t = \frac{(v_t)}{([cCMP] * k_{cat}) / ([cCMP] + K_M * (1 + [3'CMP] / K_i))}
\]

- \([E]_t\) = wild type RNaseA concentration at time \(t\)
- \(v_t\) = velocity at time \(t\)
- \([cCMP]\) = cCMP concentration at time \(t\)
- \([3'CMP]\) = 3’CMP concentration at time \(t\)
- \(k_{cat}\) = turnover number of wild type RNaseA (196 min\(^{-1}\)) (Rupp et al., 1994)
- \(K_M\) = Michaelis–Menten–constant (8 mM) (Lyles and Gilbert, 1991a)
- \(K_i\) = inhibition constant of 3’CMP (2.1 mM) (Lyles and Gilbert, 1991a)

**scRNaseA isomerization assay**

40 μM scRNaseA was added to a solution of 10 μM (calculated for active sites) of the tested proteins (see Results) in 100 mM sodium phosphate/NaOH, 1 mM EDTA, 10 μM DTT pH 7.0 at 25 °C. At different time points samples of 100 μl were removed. These samples were added to 100 mM sodium phosphate/NaOH, 1 mM EDTA pH 7.0 containing cCMP (3 mM). Ribonuclease activity was followed by measuring the increase in absorbance at 296 nm at 25 °C for 3 min (Frickel et al., 2004). scRNaseA with DTT or wild type RNaseA were used as negative or positive control, respectively.
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3.13 DsbB catalyzed ubiquinone reduction

Ubiquinone 1 (Q1) (15 μM) was preincubated with either reduced DsbC, DsbC-C, DsbC V54K, DsbG, DsbG-C, DsbA or D-DsbA (concentration of the active site 15 μM) in 100 mM sodium phosphate/NaOH, 0.1 % (w/v) DDM pH 6.0 for 5 min at 30 °C. The assay was started by addition of DsbB (1 μM) and the reduction of Q1 was followed spectroscopically at 275 nm for at least 10 min (Bader et al., 2001; Bader et al., 2000). Additionally, 25 nM and 200 nM DsbB was tested as catalyst of this reaction.

To determine the KM and kcat for the DsbB catalyzed reduction of Q1 by D-DsbA, the reaction was performed as described above. The concentration of D-DsbA or as a control DsbA was varied from 0 μM to 25 μM. The data were fitted according to the Michaelis–Menten equation (Bader et al., 2000).

3.14 Continuous monitoring of nDsbD oxidation

Oxidized DsbC, DsbC V54K or DsbC-C (5 μM) was incubated at 25 °C for 5 min in 100 mM sodium phosphate/NaOH, 1 mM EDTA pH 7.0 and the reduction of the proteins was started by addition of reduced nDsbD (5 μM). After different time points sample were removed, mixed with 0.4 volume of 30% (w/v) formic acid (pH 2) and kept at 4 °C prior to analysis by HPLC.

The proteins were separated at 55 °C on a C18 reversed phase column. The acid–quenched samples (0.7 ml, 3.5 μM protein) were applied to the column and separated with a linear gradient from 30 % to 55 % of acetonitrile in 0.1 % TFA with a flow rate of 0.5 ml/min. The absorbance was recorded at 280 nm and peaks were quantified by integration (Rozhkova et al., 2004). The % of reduced protein was calculated from peak area and plotted against time. The rate was calculated assuming a second order kinetic.

The fluorescence of reduced DsbA is significant higher then that of oxidized DsbA. In the D-DsbA, the dimerisation domain has no influence on the intrinsic fluorescence change depending on the redox state. The reduction of DsbA and D-DsbA by reduced nDsbD can therefore be directly measured in a fluorescence spectrophotometer. In addition, DsbG shows also an intrinsic fluorescence change depending on the redox state and the reduction of DsbG or DsbG-C by nDsbD were also measured in a fluorescence spectrophotometer.
Oxidized DsbA, D-DsbA, DsbG or DsbG-C (5 μM) was incubated for 5 min at 25 °C in 100 mM sodium phosphate/NaOH, 1 mM EDTA pH 7.0 and the reduction of the proteins was started by addition of reduced nDsbD (5 μM). The reduction was followed by increase of the intrinsic fluorescence at 320 nm (excitation 280 nm). The rate was calculated assuming a second order kinetic.

3.15 Continuous monitoring of DsbB reduction

The result of the DsbB catalyzed reduction of Q1 was not expected. To estimate the velocity of the electron transfer between DsbC, DsbG or their monomerized variant and DsbB, continuous monitoring of the oxidation of these disulfide oxidoreductases by DsbB was preformed with fluorescence spectroscopy.

DsbC, DsbC-C, DsbC H45D, DsbG or DsbG-C, were reduced with 50 mM DTT at RT for 1 hour in 100 mM sodium phosphate/NaOH, 1 mM EDTA, 0.1 % (w/v) DDM pH 7.0 and the DTT was removed with a PD10 cartridge equilibrated in the same buffer. Reduced protein were diluted to a final concentration of 5 μM in 100 mM sodium phosphate/NaOH, 1 mM EDTA, 0.1 % (w/v) DDM pH 7.0 and the reaction was started by addition of oxidized DsbB (5 μM). The reaction was followed by fluorescence at 332 nm (excitation 280 nm) and the data were evaluated assuming a second order kinetic.

3.16 In vivo complementation assay

E. coli strains lacking the dsbA gene show no motility on soft agar plates (0.3 % w/v agar). This is due to the fact that the correct anchoring of the flagella in the outer membrane is prohibited. In the p-subunit of this anchor an essential disulfide bridge is missing. The ability for dsbA complementation in vivo can be tested by oxidizing this essential disulfide bridge and therefore it was analyzed by the recovery of the motility of E.coli (Jonda et al., 1999).

The assay was performed using the dsbA deficient strain JCB 817 on either LB medium plates containing 0.3 % agar and 100 μg/l ampicillin or M63 minimal medium plates supplemented with 40 mg/l free amino acids except Cys and Met, 0.3 % agar, 2 g/l glucose as carbon source, 0.1 g/l Thiamin and 100 μg/l ampicillin. A colony was plated at the center of the plate. The complementation of dsbA was analyzed visually after incubation at 37 °C for 24 hours. Additionally the dsbA/dsbB deficient strain JCB 818 was tested in this assay.
3.17 Determination of the DsbC redox potential

The redox potential of DsbC was determined based on the procedure as described (Zapun et al., 1995). The analysis was performed in 100 mM sodium phosphate/NaOH, 1 mM EDTA buffer pH 7.0. (Rozhkova et al., 2004)

3.18 Further methods

Catalyzed Oxidation of GSH

Oxidation of GSH to GSSG catalyzed by DsbC, DsbC-C, DsbG, DsbG-C, DsbA or D-DsbA can be followed indirectly by glutathione reductase catalyzed electron transfer from NADPH to GSSG, whereby NADPH gets oxidized and GSSG reduced. The oxidation of NADPH can be followed spectroscopically at 340 nm (Nagai and Black, 1968). The concentration of the disulfide oxidoreductase was at 10 μM. The measurement was performed in 50 mM HEPES/NaOH, 1 mM EDTA pH 8.0 containing 0.25 mM NADPH, 0.1 u glutathione reductase, 2.5 mM L-Cystin. The reaction was started by addition of GSH (1 mM) and followed spectroscopically.

Disulfide oxidoreductase-dependent expression of RBI

The expression level of RBI depends on DsbC and is increased by 40 times when DsbC is coexpressed (Maskos et al., 2003). A trypsin inhibition assay using RBI as inhibitor was developed in our lab. To use trypsin inhibition by RBI as a tool for an in vivo disulfide isomerase assay the expression plasmids pRBI and pRBIDsbC were transformed either into FED 117 (wild type strain), FED 126 (dsbD deficient strain), FED 215 (dsbC deficient strain) or FED 266 (dsbC/dsbD deficient strain). The strains containing the expression plasmid were grown in either DYT medium or M63 supplemented minimal medium (see Appendix) at 25 °C until they reached an OD$_{600nm}$ = 1.0. Protein expression was induced with IPTG (1 mM). After further growth at 25 °C for 16 h, cells were harvested by centrifugation (4000 x g, 4 °C, 15 min). The cell pellet was resuspended in 50 mM sodium phosphate/NaOH, 300 mM NaCl pH 8.0 and 1 mg/ml Polymixin B sulfate (10 ml/l bacterial culture) and the outer membranes were lysed by gentle shaking at 4 °C for 1.5 h. After centrifugation (39000 x g, 4 °C, 45 min) the supernatant was diluted to absorption of 0.6 at 280 nm with 10 mM MOPS/NaOH, 150
mM NaCl, 5 mM EDTA pH 7.0. The trypsin inhibition assay was performed with different amounts of periplasmic extract in 200 mM Tris/HCl, 100 mM NaCl, 20 mM CaCl₂, 0.005 % (v/v) Triton X100 pH 8.0. The periplasmic samples were preincubated with trypsin (60 nM) at 25 °C for 15 min. The assay was started by addition of 0.2 mM L-BAPA as a trypsin substrate and followed spectroscopically at 405 nm for 15 min (Maskos et al., 2003).
Results

1. Protein expression and purification

1.1 DsbC

DsbC was expressed in the periplasm of *E. coli* and was subsequently purified (Maskos *et al.*, 2003). 18 mg of DsbC were obtained per liter cell culture. The size of the protein was verified by MALDI-TOF spectrometry and NH$_2$-terminal Edman sequencing. No degradation was detected even after storage at -20 °C for one year. Far-UV CD confirmed the presence of the folded protein (data not shown).

1.2 DsbC-C

DsbC-C was expressed and purified as described in Materials and Methods. 1.5 mg of pure DsbC-C was obtained per liter cell culture. MALDI-TOF spectrometry gave a mass of 16.2 kDa (expected mass 18.0 kDa) and NH$_2$-terminal Edman sequencing revealed that the amino acid sequence started with LKQLNA instead of the expected sequence VTKMLLKQ. The results showed that the NH$_2$-terminus of the DsbC-C was shortened by 5 amino acid residues. No further degradation was observed after storage at -20 °C for at least one year. Far-UV CD spectrum confirmed that the protein was in the folded state (data not shown).

1.3 D-DsbA

D-DsbA was expressed in the cytoplasm and purified as described in Materials and Methods. The fractions that had been eluted from the Ni-NTA column contained degradation products. The degradation products were successfully removed by additional purification steps as described in Materials and Methods. 60 mg of pure D-DsbA were obtained per liter cell culture. The correct size of D-DsbA was verified by MALDI-TOF spectrometry and NH$_2$-terminal Edman sequencing. The folded state of the protein was confirmed by far-UV CD spectroscopy (data not shown).
1.4 Monomeric DsbC variants

The proteins were expressed in the periplasm of *E. coli* and purified as described in the Materials and Methods. The monomeric DsbC variants were degraded and a separation of the degradation products on the Ni-NTA column was not possible. Additional purification steps to purify the monomeric DsbC variants were necessary. Only in the case of the DsbC H45D and DsbC V54K, the degradation products could be separated using 2 connected Superdex 75 HiLoad 16/60 preparative size-exclusion columns. For the other monomeric DsbC variants separation of the degradation products was not possible (see Figure 19). MALDI-TOF spectrometry and NH$_2$–terminal Edman sequencing indicated cleavage in the dimerization domain of DsbC.

![Figure 19: Specific expression image of monomeric DsbC variants.](image)

DsbC H45D and DsbC V54K were verified by MALDI-TOF spectrometry and NH$_2$–terminal Edman sequencing. 9 mg of pure DsbC H45D and 5 mg of pure DsbC V54K were obtained per liter cell culture.
1.5 DsbG

DsbG or DsbG_{His} were expressed and purified as described in the Materials and Methods. 40 mg of DsbG_{His} and 15 mg of DsbG were obtained per liter cell culture. The proteins were verified by MALDI-TOF spectrometry and NH_2–terminal Edman sequencing. Far-UV CD spectroscopy confirmed the presence of folded protein (data not shown).

1.6 nDsbD and DsbB

The nDsbD and DsbB were expressed and purified as described in Materials and Methods. 2.5 mg of DsbB and 15 mg of nDsbD were obtained per liter cell culture.

1.7 GroEL-AD, G7D, G12D and G17D

The expression and purification of the proteins were done in parallel as described in Materials and Methods. In all cases, about 8 mg of pure protein was obtained per liter cell culture. The size of the proteins was verified by MALDI-TOF spectroscopy and NH_2–terminal Edmann sequencing. The folded state of the proteins was confirmed by far-UV CD spectroscopy (data not shown).
2. Characterization of the proteins

2.1 Size–exclusion chromatography

Analytical size-exclusion chromatography was used to determine the oligomeric state of DsbC, DsbG and D-DsbA at different concentrations.

![Graph](image)

**Figure 20: Analytical size-exclusion chromatography.** Elution profile of DsbC (A), DsbG (B) and D-DsbA (C) applied with concentration of either 460 μM (●), 46 μM (■) or 4.6 μM (♦) in 100 mM sodium phosphate/NaOH pH 7.0, 1 mM EDTA on an analytical size-exclusion column. Black lines indicate the elution positions of the marker proteins, which were used as control (serum albumin 67.0 kDa, ovalbumin 43.0 kDa, chemotrypsinogen A 25.0 kDa and ribonuclease A 13.7 kDa).

All three proteins were in the dimeric state at all three concentrations. All activity assays with the dimeric proteins, which were tested during this thesis, were performed in a concentration of at least 1 μM. The dilution during the analytical size-exclusion chromatography is about 7 - 10 fold, which clearly indicates that the tested proteins were in the dimeric state at concentration of 1 μM.

2.2 Chemically induced unfolding and refolding of DsbC-C

The chemical stability of DsbC-C was measured in the oxidized and reduced state of DsbC-C. The measurement of the reduced DsbC-C was performed in the presence of 0.5 mM DTT. For measuring the refolding of the reduced DsbC-C DTT was present at every stage of the
experiment. The data were evaluated according to a two state model (equation to fit the measured data see Appendix) (Bolen and Santoro, 1988; Santoro and Bolen, 1988).

![Chemical stability of oxidized DsbC-C](image)

**Figure 21: Chemical stability of oxidized DsbC-C.** GdmCl induced unfolding (●) and refolding (■) of 1 μM oxidized DsbC-C in 20 mM HEPES/NaOH, 170 mM NaCl, 1 mM EDTA pH 7.0 and various concentration of GdmCl. The unfolding measurement was started with native protein and the refolding experiment with unfolded DsbC-C. The data were evaluated according a two state model and normalized. The unfolding transition is shown in a solid line, whereas the dashed line indicates the refolding transition. The concentration of GdmCl was directly determined from refraction index (Pace, 1986).

The midpoint of the transition is at 2.2 M GdmCl for unfolding and 2.1 M GdmCl for refolding of oxidized DsbC-C. The free energy ΔG° of the unfolded oxidized DsbC-C was -37.7 kJ mol⁻¹ and for the refolding -35.5 kJ mol⁻¹, whereas the cooperativity (m) for the unfolding was 17.1 kJ mol⁻¹ M⁻¹ and 16.7 kJ mol⁻¹ M⁻¹ for the refolding of oxidized DsbC-C (see Figure 21). The results of the unfolding and refolding transition were in the same interval and the small differences in the value can be explained due to the experimental errors. The unfolding and refolding of oxidized DsbC-C is reversible and the cooperativity is close to the calculated value (16.0 kJ mol⁻¹ M⁻¹) (Myers et al., 1995).
Figure 22: Chemical stability of reduced DsbC-C. GdmCl induced unfolding (●) and refolding (■) of 1 μM reduced DsbC-C in 20 mM HEPES/NaOH, 170 mM NaCl, 0.5 mM DTT, 1 mM EDTA pH 7.0 and various concentration of GdmCl. The unfolding measurement was started with native protein and the refolding experiment with unfolded DsbC-C. The data were evaluated according a two state model and normalized. The unfolding transition is shown in a solid line, whereas the dashed line indicates the refolding transition. The concentration of GdmCl was directly determined from refraction index (Pace, 1986).

The midpoint of the transition is at 2.2 M GdmCl for unfolding and at 1.9 M GdmCl for refolding of reduced DsbC-C. The free energy \( \Delta G^0 \) of the unfolded reduced DsbC-C was -35.4 kJ mol\(^{-1}\) and for the refolding -22.9 kJ mol\(^{-1}\), whereas the cooperativity (m) for the unfolding of reduced DsbC-C was 16.5 kJ mol\(^{-1}\) M\(^{-1}\) and 12.5 kJ mol\(^{-1}\) M\(^{-1}\) for the refolding of reduced DsbC-C (see Figure 22). The differences of the unfolding and refolding measurement are too big to be explained by experimental errors during the measurement. DsbC-C contains additionally to the catalytically active disulfide bond a structural disulfide bond. This structural disulfide bond is normally buried and stabilizes the catalytic domain of DsbC. During the unfolding of the DsbC-C for the refolding experiment, this structural disulfide bond was exposed to the DTT and was also reduced. The absence of this stabilizing disulfide bridge can explain the differences between the unfolding and refolding measurement.

The unfolding of oxidized and reduced DsbC-C is in the same range and when the cooperativity of the measurement is averaged, the free energy \( \Delta G^0 \) of oxidized DsbC-C was
Results

calculated to be -37.0 KJ mol\(^{-1}\) and for the reduced -36.2 KJ mol\(^{-1}\). Therefore, no difference in stability was detected and the stability of DsbC-C is redox-state independent.

2.3 Thermal unfolding

The thermal unfolding of DsbA, DsbC, DsbG, GroEL-AD, G7D, G12D and G17D was measured to identify the thermal stability of these proteins.

![Graph A](image1.png)  ![Graph B](image2.png)

**Figure 23: Thermal stability of DsbC.** The thermally induced unfolding of oxidized (A) and reduced (B) DsbC (5 \(\mu\)M) was measured in 100 mM sodium phosphate/NaOH, 1 mM EDTA pH 7.0. The thermal unfolding of reduced DsbC was measured in the presence of 0.1 mM DTT. The data were evaluated according a two state model and normalized. The thermal transition was followed by far-UV CD using a 1 mm cuvette.

The analysis of the data from the thermally induced unfolding of DsbC was done according to a two-state model (equation to fit the measured data see Appendix). The two-state model proved to be the best fitting model to determine the thermal stability for DsbC. Other tested models did not fit to the measured data. The melting temperature for oxidized DsbC is 345.3 K (72.2 °C) and for reduced DsbC 344.7 K (71.6 °C). DsbC thermal stability is redox state independent (see Figure 23).
Figure 24: Thermal stability of DsbG. The thermally induced unfolding of oxidized (A) and reduced (B) DsbG (5 μM) was measured in 100 mM sodium phosphate/NaOH, 1 mM EDTA pH 7.0. The thermal unfolding of reduced DsbG was measured in the presence of 0.1 mM DTT. The thermal transitions were followed by far-UV CD using a 1 mm cuvette. The data were evaluated according a model, where a folding intermediate is present and normalized. The measured data of the first transition of oxidized (C) and reduced (D) DsbG were fitted according a two state model independently and normalized.

The continuous monitoring of the thermal unfolding has shown that DsbG has two temperature transitions. The measured data were therefore evaluated using an equation, which is more applicable when an additional intermediate is present (equation to fit the measured data see Appendix). The melting temperature for the oxidized DsbG is 331.9 K (58.8 °C) for the first transition and 352.7 K (79.6 °C) for the second, whereas for the reduced DsbG the first transition melting point is 348.7 K (75.6 °C) and the second 352.9 K (79.8 °C) (see Figure 24). The differences in the first transition melting temperature comes from the
different redox state of the protein, whereas the second transition melting temperature shows no significant differences in the two redox states. This observation indicates that thermal stability of DsbG depends on the redox state and the first transition of DsbG shows the thermal unfolding of the catalytic domain.
Figure 25: Thermal stability of the fusion proteins. Thermally induced unfolding of GroEL-AD (A), DsbA (B), G7D (C), G12D (D) or G17D (E) was measured with a concentration of 5 μM in 100 mM sodium phosphate/NaOH pH 7.0, 1 mM EDTA. The data were evaluated according a two state model and normalized. The thermal transition was followed by far-UV CD using a 1 mm cuvette.

The analysis of the data from the thermally induced unfolding was done according to a two-state model (equation to fit the measured data see Appendix). The melting temperature of DsbA was 342.3 K (69.2 °C), whereas the melting temperature of GroEL-AD was 344.2 K (71.1 °C). With this small difference in the melting temperature of these two proteins, evaluation of the thermal unfolding of the individual domains in the fusion proteins between the apical domain of GroEL and DsbA is difficult. The melting temperatures of the single domains of the fusion proteins are superimposed and were evaluated according to a two state model (equation to fit the measured data see Appendix). The melting temperature of the G7D was 339.5 K (66.4 °C), for G12D 341.6 K (68.5 °C) and 339.9 K (66.8 °C) for the fusion protein with the longest linker, G17D (see Figure 25). The linker length of the fusion protein between the apical domain of GroEL and DsbA has no influence on the thermal stability of the fusion protein.

2.4 Subunit exchange of DsbC and DsbG

To verify the possibility that a DsbC heterodimer with only one active site could be formed by mixing the homodimeric DsbC WT with the inactive homodimeric DsbC variant (DsbC_{CCAA}) DsbC WT and DsbC_{His} were mixed, incubated and applied to a Ni–NTA column. DsbC dimers, containing two, one or no His_{6} tag, eluted from the Ni–NTA column at different concentrations of imidazole.
Figure 26: Subunit exchange in DsbC dimers. (A) DsbC WT and DsbC_{His} (each 25 μM) were mixed together at RT in 100 mM sodium phosphate/NaOH pH 7.0. The subunit exchange of DsbC after different incubation time or combined refolding was followed by absorption at 280 nm after separation on a Ni-NTA column. (B) Kinetic of the subunit exchange in DsbC. The peak areas of the individual proteins from the elution profile were calculated and plotted against time.

The three different species were separated on a Ni–NTA column with a gradient from 0 mM to 250 mM of imidazole, whereas DsbC WT dimer without His_{6} tag did not bind to the column, DsbC heterodimer with one His_{6} tag eluted with 85 mM imidazole and DsbC_{His} with two His_{6} tags needed 120 mM imidazole to elute. The peak area of the individual proteins were calculated and plotted against the incubation time. The distribution of the DsbC species after incubation for 240 min shows already 64 % of the DsbC species in the heterodimeric form and only 36 % in the homodimeric DsbC_{His} form. From statistics one can calculate a distribution of one DsbC WT homodimer, two DsbC heterodimer and one DsbC_{His} homodimer, which is in a good correlation to the measured distribution. Additionally, DsbC WT and DsbC_{His} were unfolded and refolded together to see if the His_{6} tag has an influence on this measurement. This was not the case. The subunit exchange reached 66.7 % of the DsbC species in the heterodimeric DsbC/DsbC_{His} form and only 33.3 % in the homodimeric DsbC_{His} form (see Figure 26).

The assay was then used to test mixtures of DsbC WT and inactive DsbC_{CCAA} for forming a heterodimer with only one active site (DsbC-S).
Results

Figure 27: Subunit exchanges of DsbC active / inactive dimers. Different concentrations of DsbC WT and DsbC CCAA were incubated in 100 mM sodium phosphate / NaOH pH 7.0 at RT for 16 hours. The subunit exchange was followed by absorption at 280 nm after separation on a Ni-NTA column.

With the method described above, the subunit exchange of DsbC dimers was tested for a mixture of 25 μM DsbC WT and 25 μM DsbC CCAA. The calculated peak area of the individual proteins gave the statistically predicted distribution of one DsbC WT homodimer, two DsbC-S heterodimer and one DsbC CCAA homodimer. From a one to one mixture of DsbC WT and DsbC CCAA still 25 % of the DsbC WT was in DsbC WT dimer form, which was too much for testing the disulfide isomerase activities of the DsbC-S dimer. A mixture of 5 μM DsbC WT and 50 μM DsbC CCAA was tested therefore and about 95 % of the wild type DsbC subunits were in the heterodimeric DsbC-S (see Figure 27).
Figure 28: Subunit exchange of DsbG. DsbG WT and DsbG_{His} (each 25 μM) were mixed together at RT in 100 mM sodium phosphate/NaOH pH 7.0. The subunit exchange of DsbG was followed after different incubation time or combined refolding by absorption at 280 nm after separation on a Ni-NTA column.

DsbG dimers were also tested for their ability to exchange the subunits as described for DsbC dimers before. The assay was performed as for DsbC, but no real subunit exchange was detected even after 72 hours. Only unfolding and refolding of DsbG WT and DsbG_{His} together could yield the expected distribution of the species. DsbG showed, in contrast to DsbC, no dynamic subunit exchange (see Figure 28).
Figure 29: Subunit exchange of DsbC/DsbG dimers. DsbC WT and DsbG_{His} (each 25 μM) were mixed together at RT in 100 mM sodium phosphate/NaOH pH 7.0. The subunit exchange of DsbC and DsbG was followed after different incubation time or combined refolding by absorption at 280 nm after separation on a Ni-NTA column.

In addition, mixtures of DsbC WT and DsbG_{His} were tested for subunit exchange and the ability to form DsbC/DsbG heterodimer. Even after the combined unfolding and refolding no heterodimers were detected. The dimerization interfaces of DsbC and DsbG are too different to build heterodimers from DsbC and DsbG monomers (see Figure 29).

2.5 Determination of the DsbC redox potential

The redox titration of DsbC was done to quantify the redox potential in the standard buffer condition (100 mM sodium phosphate/NaOH, 1 mM EDTA pH 7.0).
Figure 30: Redox equilibrium between DsbC and glutathione. Redox potential determination of 1 \( \mu \text{M} \) DsbC was done in 100 mM sodium phosphate/NaOH, 1 mM EDTA pH 7.0 containing 20 mM GSSG and different amounts of GSH. The peak area of reduced DsbC was determined, normalized and as fraction of reduced DsbC plotted against the ratio of \([\text{GSH}]^2/\text{[GSSG]}\).

The separation of oxidized and reduced DsbC was done on a C18 reversed-phase HPLC column and the different species of DsbC were detected by absorption at 280 nm. The peak area of the reduced DsbC was calculated and plotted against the ratio of \([\text{GSH}]^2/\text{[GSSG]}\). The data evaluation was done according to the standard determination (equation to fit the measured data see Appendix) (Zapun et al., 1995) (see Figure 30).

The redox titration of DsbC with glutathione gave an equilibrium constant (K) of 393 \( \mu \text{M} \) at pH 7.0, whereas in the literature it is published 200 \( \mu \text{M} \) at pH 7.5 (Zapun et al., 1995). The redox potential of DsbC, calculating with the Nernst equation, is –140 mV, which is more reducing than for DsbA (-122 mV) (Wunderlich and Glockshuber, 1993) and DsbG (-127 mV) (Bessette et al., 1999).
3. Biochemical assays

3.1 Oxidative refolding of Ragi bifunctional inhibitor (RBI)

To test the ability of dimerized DsbA and monomerized DsbC to act as disulfide isomerase, an appropriate assay has to be used to allow time-dependent characterization of the isomerization activity.

RBI was chosen as a model substrate for the first isomerase assay, because it has 5 disulfide bonds in a complex disulfide pattern and the assay was well established in this lab (Maskos et al., 2003).

![Figure 31: Uncatalyzed refolding of RBI. HPLC profile of the uncatalyzed oxidative RBI refolding after indicated time points. N = native RBI, I = folding intermediates of RBI.](image)

**Figure 32: DsbC-catalyzed RBI refolding.** HPLC profile of the DsbC-catalyzed oxidative RBI folding after indicated time points. **N** = native RBI, **I** = folding intermediates of RBI, **O** = oxidized DsbC, **R** = reduced DsbC.

**Figure 33: DsbA-catalyzed RBI refolding.** HPLC profile of the DsbA-catalyzed oxidative RBI folding after indicated time points. **N** = native RBI, **I** = folding intermediates of RBI, whereas DsbA is not shown on this part of the HPLC profile.
Figure 34: DsbC-C-catalyzed refolding of RBI. HPLC profile of the DsbC-C-catalyzed oxidative RBI folding after indicated time points. \( N \) = native RBI, \( I \) = folding intermediates of RBI, C, DsbC-C.

Figure 35: D-DsbA-catalyzed RBI refolding. HPLC profile of the D-DsbA-catalyzed oxidative RBI folding after indicated time points. \( N \) = native RBI, \( I \) = folding intermediates of RBI, whereas D-DsbA is not shown on this part of the HPLC profile.

Native RBI and its folding intermediates can be separated from the proteins used as catalysts on a C18 reversed–phase HPLC column. The peak area of the native RBI was integrated from the HPLC profile and the peak area of the native RBI sample after 48 hours of the DsbC-
catalyzed oxidative refolding was set to 100%. The % of the native RBI in each sample was calculated compared to the 48 hour sample.

**Figure 36: Compilation of the RBI refolding.** Time dependent oxidative refolding of RBI catalyzed by DsbC (●), DsbA (■), DsbC-C (○), D-DsbA (□) or uncatalyzed (◊). The plot shows the amount of native RBI in % against time.

Adding the reduced, denatured RBI to the refolding buffer and removing the first sample was long enough to partially reoxidize RBI. After 15 min incubation, native RBI was already detected in the DsbC-catalyzed refolding reaction, whereas in the other reactions 30 min were necessary. At the end of the measurement 56% of native RBI was formed in the D-DsbA-catalyzed refolding reaction compared to the DsbC-catalyzed refolding reaction and even less (47 %) in the uncatalyzed assay, whereas in the DsbA-catalyzed and DsbC-C-catalyzed refolding reaction of native RBI 43 % and 41 %, respectively, of native RBI were formed. Dimerization of DsbA increases its isomerization activity slightly, while the monomerization of DsbC decreases its isomerization activity to the level of DsbA.
3.2 Catalyzed reduction of insulin

Disulfide oxidoreductase catalyzed insulin reduction

The reduction of a disulfide bond by an attacking thiolate anion is the first step of the disulfide isomerization and therefore a disulfide isomerase should be able to act as a disulfide reductase. To test this, the reduction assay of insulin, where DTT reduces insulin and the aggregation of the reduced β-chain could be monitored by light scattering in a photo spectrometer, is a good tool. The assay was performed in a similar way as already described before (Holmgren, 1979).

As a first attempt to adapt the assay used by Holmgren, DsbC, DsbA and Trx were tested for their ability to catalyze DTT induced reduction of insulin. These three proteins, a natural disulfide isomerase (DsbC), a natural disulfide oxidase (DsbA) and a natural disulfide reductase (Trx), were used as model proteins to determine the ideal concentration for testing different disulfide oxidoreductases later.
**Figure 37:** Catalyzed reduction of insulin. Reduction of insulin (130 μM) by 1 mM DTT catalyzed by either 5.5 μM (◊), 1.2 μM (□), 0.56 μM (○), 0.09 μM (●), 0.01 μM (■) DsbC (A), Trx (B) and DsbA (C) or uncatalyzed (•) was performed in 100 mM sodium phosphate/NaOH, 1 mM EDTA pH 7.0 at 25 °C. The aggregation of reduced insulin was followed spectroscopically at 650 nm.

The first trials already showed that DsbC reduced insulin even faster than the natural disulfide reductase Trx and the disulfide oxidase DsbA showed no considerable acceleration of reduction compared to the uncatalyzed reaction. From the data of these trials, the best concentration of the disulfide oxidoreductases was 1 μM. DsbC-catalyzed reduction of insulin is not too fast and there is enough time in the lag phase to collect data. The differences in the DsbA-catalyzed and the uncatalyzed reaction are large enough to separate these insulin reductions (see Figure 37).

The insulin reduction assay was used to test the behavior of the different proteins as disulfide reductases for disulfide bonds in insulin. The light scattering of the reduced insulin β-chain aggregates was used as an indirect signal for the reductase activity of the catalysts compared to the uncatalyzed reaction. The reduction of insulin was tested with DsbC, DsbC-C, DsbC-S, D-DsbA, DsbA, DsbG and DsbG-C as catalysts.

**Figure 38:** Catalyzed reduction of insulin. Reduction of insulin (130 μM) by 1 mM DTT was catalyzed by 1 μM disulfide oxidoreductases and performed in 100 mM sodium phosphate/NaOH, 1 mM EDTA pH 7.0 at 25 °C. The aggregation of reduced insulin was followed spectroscopically at 650 nm. (A) The insulin reduction assay was performed either in the absence (▲) or presence of DsbC (○), DsbC-C (□), DsbC-S (▼), DsbA (◊) or D-DsbA
Results

(B) The insulin reduction assay was performed either in the absence (▲) or presence of DsbC (♦), DsbG (♦), DsbG-C (◊) or DsbA (▼), respectively.

By testing all the soluble disulfide oxidoreductases from the periplasm of *E. coli* and their constructs, some interesting results were obtained. The time, when the aggregation reached an absorption of 0.025, was set as a reference point to compare the influence of the proteins as catalysts in the assay. No differences between the DsbC WT (7 min) and DsbC-S (7 min) could be detected. Furthermore, DsbG showed only a small increase in time (35 min) compared to the uncatalyzed reaction (37 min). The other dimer, D-DsbA, decreased the time to 23 min, whereas in the case of DsbA aggregation started after 28 min. The monomeric variants of DsbC (DsbC-C) or DsbG (DsbG-C) either increased the time, where aggregation can be detected, to 32 min or showed the same time as the uncatalyzed reaction (37 min). DsbC showed the same ability to catalyze the reduction of insulin independently if one or two active sites are present in the DsbC dimer. DsbG showed no activity as a catalyst in this assay, neither as wild type protein nor as catalytic domain alone (see Figure 38). D-DsbA, DsbA and DsbC-C show the expected activity for insulin reduction as already seen in the oxidative refolding assay of RBI.

Additionally to this measurement, the insulin reduction assay was performed to test the reductase activity of the fusion proteins between the apical domain of GroEL and DsbA.

![Graph A](image1.png)

**Figure 39: Catalyzed reduction of insulin.** Reduction of insulin (130 μM) by 1 mM DTT was catalyzed by 1μM disulfide oxidoreductases and performed in 100 mM sodium phosphate/NaOH, 1 mM EDTA pH7.0 at 25 °C. The aggregation of reduced insulin was
followed spectroscopically at 650 nm. (A) The insulin reduction assay was performed either in the absence (•) or presence of DsbA (■), GroEL-AD (●), a one to one mix of GroEL-AD and DsbA (○) or G7D (□), respectively. (B) The insulin reduction assay was performed either in the absence (•) or presence of DsbA (■), G12D (●) or G17D (○), respectively.

The fusion of DsbA to the apical domain of GroEL did not increase the velocity of the aggregation of the insulin β–chain compared to DsbA in the reduction assay. Even more, no differences between the fusion constructs were detected and the time point of aggregation was at 32 min for all three construct independent from the linker length. The value for DsbA and a one to one mixture of DsbA and the apical domain of GroEL (i.e. not fused) was in this assay at 32 min. The apical domain of GroEL showed the same time point (47 min) as the uncatalyzed reaction (see Figure 39).

**DsbG chaperone activity in the prevention of insulin aggregation**

The results of DsbG and the apical domain of GroEL were surprising and needed a deeper study. To test a possible chaperone-like activity of these two proteins for insulin, increasing amounts of these proteins in the insulin assay were investigated. It has been already shown in the initial experiments with DsbC that increasing amounts of DsbC accelerated aggregation of β–chain and DsbC did not show chaperon-like activity (see Figure 37).

![Figure 40: Chaperone-like activity to prevent insulin aggregation.](image-url) Reduction of insulin (130 μM) by 1 mM DTT was performed in the presence of different concentration of GroEL-
AD or DsbG in 100 mM sodium phosphate/NaOH, 1 mM EDTA pH7.0 at 25 °C. The aggregation of reduced insulin was followed spectroscopically at 650 nm. (A) GroEL-AD catalyzed reduction of insulin by DTT using either 35.0 μM (○), 25.0 μM (♦), 1.0 μM (■) GroEL-AD or uncatalyzed (•). (B) DsbG catalyzed reduction of insulin by DTT using either 260 μM (◇), 130 μM (□), 65.0 μM (○), 10.0 μM (▲), 5.0 μM (♦), 1.0 μM (■) DsbG (calculated as monomer) or uncatalyzed (•).

The apical domain of GroEL (GroEL–AD) binds the substrates of GroEL and shows chaperon activity (Zahn et al., 1996). GroEL-AD showed no quantity-dependent deceleration of the aggregation of β–chain in the reduction of insulin by DTT; it had the same time point of aggregation, independent from the concentration of GroEL-AD, as the uncatalyzed reaction. In contrast, DsbG clearly increased the time of aggregation in a quantity-dependent manner. The time point of aggregation was increased from 36 min for 1 μM DsbG to 106 min for 260 μM DsbG (calculated as monomer).

**HPLC analysis of insulin reduction**

The behavior of DsbG in the insulin reductase assay was not expected. The quantity-dependent increase of the time point of aggregation directed to the question if DsbG inhibits the reduction of the insulin or if DsbG retards the aggregation of the β-chain of insulin. To test these two possibilities, insulin was reduced by DTT, the reaction was then stopped by acidification with 30% (w/v) formic acid (pH 2) and the products were separated on a C18 reversed-phase HPLC column. The HPLC separated peaks were collected and analyzed by MALDI–TOF mass spectroscopy. The peaks of insulin and the β-chain of insulin could be separated and identify on the C18 reversed-phase HPLC column (data not shown). The insulin reduction assay was then performed in the presence of either 65 μM DsbG, 1μM DsbC or uncatalyzed.
Figure 41: HPLC analysis of insulin reduction. Reduction of insulin (130 μM) by 1 mM DTT was uncatalyzed (A) or catalyzed with 1 μM DsbC (B) or 65 μM DsbG (C) and performed in 100 mM sodium phosphate/NaOH, 1 mM EDTA pH 7.0 at 25 °C. Samples after different times were removed and analyzed on a C18 reversed-phase HPLC. The separation of the individual peaks was followed spectroscopically at 280 nm.

The HPLC analysis of the insulin reduction assay has clearly shown that the DsbC-catalyzed reaction was already finished after 60 min, whereas the DsbG-catalyzed and the uncatalyzed reactions showed no significant difference. This analysis and the behavior of DsbG in the quantity-dependent reduction of insulin in the online assay led to the conclusion that DsbG retard the aggregation of the β-chain of insulin like a molecular chaperone. In contrast, DsbC accelerates the insulin reduction.

3.3 Catalyzed scRNaseA isomerization
Spectroscopically followed ScRNaseA reactivation

In addition to the insulin reduction and the oxidative refolding RBI assays, the same proteins were tested for their disulfide isomerization activity in a further disulfide isomerase assay.
RNaseA is a well characterized protein, which could be used for testing the disulfide isomerisation activity. Recovering of wild type RNaseA activity from either reduced, denaturated RNaseA or fully oxidized, folded protein with non-native disulfide bonds (scRNaseA) was already described in the literature (Crook et al., 1960; Lyles and Gilbert, 1991a; Rupp et al., 1994).

RNaseA was prepared as scrambled RNaseA, where all 4 disulfide bonds were randomly oxidized and a stable scRNaseA fraction was achieved with only 4 % RNaseA activity. To test the disulfide isomerization activity of the disulfide oxidoreductases, cyclic CMP (cCMP) was used as a cleavable substrate of RNaseA, whereas the cleavage of cCMP can be followed spectroscopically at 296 nm. First trials were used to test assay conditions, at which the activities of the disulfide oxidoreductases could be differentiated.

![Figure 42: RNaseA activity measurement.](image)

**Figure 42: RNaseA activity measurement.** RNaseA activity of 2.6 μM wild type RNaseA (●) or 2.6 μM scRNaseA (■) tested with 3 mM cCMP in 50 mM Tris/HCl, 25 mM KCl, 5 mM MgCl₂ pH 7.5 at 30 °C. The cleavage of cCMP was followed spectroscopically at 296 nm.

Using the assay conditions according to the publication of Crook and coworkers (Crook et al., 1960), the observed absorption differences were very small during the measurements and with the utmost probability not sufficient for an exact measurement of the disulfide isomerase activities. For the investigation of the influence of different disulfide oxidoreductases, these assay conditions could not be used (see Figure 42). Changing the conditions was a possibility to improve the assay. The cleavage of cCMP by either RNaseA or scRNaseA (8.5 μM each)
was therefore tested in 100 mM Tris/AcOH pH 8.0 and the amount of cCMP as substrate was increased to 4.5 mM.

![Graph showing RNaseA activity determination.](image)

**Figure 43: RNaseA activity determination.** RNaseA activity of 8.5 μM wild type RNaseA (■) or 8.5 μM scRNaseA (●) tested with 4.5 mM cCMP in 100 mM Tris/AcOH pH 8.0 at 25 °C. The cleavage of cCMP was followed spectroscopically at 296 nm.

The primary test using these conditions showed a good differences between the two extreme values, where 100% RNaseA activity as positive control and about 4% RNaseA activity from the scRNaseA as negative control were measured. Additionally, the differences between the start and the end value in the measurement were large enough for the disulfide isomerisation activity measurement (see Figure 43). Adding a reduction buffer as described (Lyles and Gilbert, 1991a) should allow DsbC, used as a test disulfide oxidoreductase, to isomeraze the non-native disulfide bonds in scRNaseA and to reactivate it.
Figure 44: DsbC-catalyzed scRNaseA reactivation. RNaseA activity of 8.5 μM wild type RNaseA (●), 8.5 μM scRNaseA and 1.4 μM DsbC (♦) or 8.5 μM scRNaseA (■) tested with 4.5 mM cCMP in 0.2 mM GSSG, 1.0 mM GSH, 100 mM Tris/AcOH pH 8.0 at 25 °C. The cleavage of cCMP was followed spectroscopically at 296 nm.

The DsbC-catalyzed scRNaseA isomerization led to a time-dependent increase in the cCMP-cleavage activity of RNaseA. As a negative control, scRNaseA alone was also isomerized by glutathione and showed an increasing RNaseA cleavage activity. This behavior disturbed the measurement for the reactivation of scRNaseA by isomerization through disulfide oxidoreductases (see Figure 44). The first step of disulfide isomerization is the reduction of the substrate by an attacking thiolate anion and therefore using reduced disulfide oxidoreductase instead of glutathione in the buffer should be sufficient to reactivate scRNaseA, but prevent the negative control from disulfide bond isomerization. The online measured assay was modified by using RNaseA or scRNaseA, DTT and DsbC (5 μM each) in 100 mM Tris/AcOH pH 8.0 at 25 °C and the activity was followed spectroscopically at 296 nm by cleavage of 3 mM cCMP.
Figure 45: DsbC-catalyzed scRNaseA reactivation. A) Continuous spectroscopic monitoring of the cleavage of 3 mM cCMP by either 5.0 μM wild type RNaseA (●), 5.0 μM scRNaseA and 5.0 μM DsbC (♦) or 5.0 μM scRNaseA (■) at 296 nm. The measurement was performed in 100 mM Tris/AcOH pH 8.0 at 25 °C. B) Decrease of the cCMP concentration in the online measurement of 5.0 μM wild type RNaseA (●), 5.0 μM scRNaseA and 5.0 μM DsbC (♦) or 5.0 μM scRNaseA (■). C) Wild type RNaseA concentration calculated according to the equation form Lyles and coworker (Lyles and Gilbert, 1991a) with wild type RNaseA (●), scRNaseA reactivated by DsbC (♦) or scRNaseA (■).

DsbC reactivates scRNaseA to RNaseA wild type activity at 75 % after about 40 min. The use of DTT as reductant instead of a mixture of GSSG and GSH allow measurements where neither RNaseA nor scRNaseA measurement were affected by DTT. As shown in the calculated RNaseA concentration of the wild type RNaseA measurement, continuous measurements longer than 10 min would be difficult to calculate and direct comparison in
activity would be therefore not allow an explicit statement. In this case, continuous monitoring can only be used for primarily tests to see tendencies.

**ScRNaseA reactivation by disulfide oxidoreductases**

To test the disulfide isomerase activity of different disulfide oxidoreductases and their variants, the scRNaseA assay was modified. The buffer was changed to 100 mM sodium phosphate/NaOH, 1 mM EDTA pH 7.0, which was already used as buffer in the insulin reduction assay. Lowering the pH did not affect the cleavage of cCMP (data not shown). Furthermore, the assay was performed in a test tube without cCMP and samples were taken after different times. Samples were mixed with cCMP containing buffer to test the wild type RNaseA cleavage activity in a spectrophotometer, where the length of the measurement was only 3 min. In this first 2 to 4 min the reaction slope is linear (data not shown) and no product inhibition influences the reaction during the measurement.
Figure 46: Catalyzed RNaseA reactivation. Assay of RNaseA reactivation of 40 μM scRNaseA by 10 μM DTT either catalyzed by 10 μM disulfide oxidoreductases or uncatalyzed in 100 mM sodium phosphate/NaOH, 1 mM EDTA pH 7.0 at 25 °C. Samples of the reactivation assay were taken after different time points and the activity of the RNaseA for cleavage of 3 mM cCMP was tested spectroscopically at 296 nm. The RNaseA activity was tested and compared to the samples of wild type RNaseA, which were set to 100% activity. A) Wild type RNaseA (●), scRNaseA without DTT (■), DsbC as catalyst (♦), DsbC-S as catalyst (○) and DsbC-C as catalyst (□). B) Wild type RNaseA (●), scRNaseA without DTT (■), scRNaseA with DTT (♦), DsbA as catalyst (○), D-DsbA as catalyst (□) and DsbG as catalyst (◊). C) Wild type RNaseA (●), scRNaseA with DTT (■), DsbA as catalyst (♦), G7D as catalyst (○), G12D as catalyst (□) and G17D as catalyst (◊).

DsbC reactivated scRNaseA to 90% wild type RNaseA activity after 8 hours. DsbC-S increased the activity of scRNaseA after 8 hours to 77% wild type RNaseA activity. DsbG reactivated the scRNaseA only to 17% and DsbA reactivated to 13% wild type RNaseA activity. D-DsbA increased the RNaseA activity to 24%, whereas DsbC-C showed a yield of 10% in reactivation of scRNaseA. The fusion proteins between the apical domain of GroEL and DsbA reactivated the scRNaseA only to 10-12% of the wild type RNaseA activity. In the first 5 to 6 hours no differences between DsbC and DsbC-S catalyzed reactivation of scRNaseA were detected. The presence of only one active site in DsbC dimer is enough to act as a disulfide isomerase for scRNaseA. DsbG showed in this assay no disulfide isomerase activity and the yield of reactivation of scRNaseA was lower than the yield obtained for D-DsbA. The dimerization of DsbA increases its disulfide isomerization activity compared to DsbA and the monomerization of DsbC decreased the disulfide isomerase activity dramatically. The fusion of the apical domain of GroEL and DsbA showed no influence on disulfide isomerization activity compared to DsbA, independent from the linker length.

3.4 DsbB-catalyzed electron transfer from disulfide oxidoreductases to ubiquinone

The ability of different disulfide oxidoreductases to be oxidized by DsbB was tested in vitro. The assays described above showed the loss of disulfide isomerase activity of monomeric DsbC-C compared to wild type DsbC. This directed to the question if the DsbC-C or monomerized DsbC, as described by Bader and coworkers (Bader et al., 2001), is turned to a
disulfide oxidase by loosing the dimeric state and therefore the dimerization of DsbA would inhibit the disulfide oxidase activity of DsbA. To test these assumptions, the DsbB catalyzed reduction of ubiquinone 1 (Q1) by DsbA, DsbC, DsbG and their variants was performed.

Figure 47: DsbB-catalyzed reduction of ubiquinone 1. 15 μM ubiquinone 1 was reduced by 15 μM of reduced DsbA (•), D-DsbA (○), DsbC (■), DsbC-C (□), DsbC H45D (x), DsbG (♦) or DsbG-C (◊) in 100 mM sodium phosphate/NaOH, 0.1 % DDM pH 6.0 at 30 °C. The reaction was catalyzed by A) 25 nM DsbB, B) 200 nM DsbB or C) 1 μM DsbB and followed spectroscopically at 275 nm.

The observed reaction curve is linear in the first part of the measurement. The reduction of Q1 with 15 μM DsbA or D-DsbA catalyzed by 1 μM DsbB was too fast to be measured by the absorption spectrometer and therefore it is not shown in the Figure 47C. DsbA and D-DsbA were able to reduce Q1 comparably efficient with an initial velocity of 348 mM min⁻¹ mM⁻¹ DsbB and 283 mM min⁻¹ mM⁻¹ DsbB, respectively. DsbC and its variants, as well as DsbG
and its variant, reduced ubiquinone about 700 to 800 fold slower. DsbC-C, the fastest reaction partner of this group, reduced Q1 with an initial velocity of 0.54 mM min⁻¹ mM⁻¹ DsbB, whereas the other monomeric DsbC variant DsbC H45D reduced Q1 with 0.40 mM min⁻¹ mM⁻¹ DsbB and DsbC 0.11 mM min⁻¹ mM⁻¹ DsbB. DsbG-C reduced Q1 with an initial velocity of 0.072 mM min⁻¹ mM⁻¹ DsbB and DsbG 0.013 mM min⁻¹ mM⁻¹ DsbB, which is even slower than DsbC. The monomerization of either DsbC or DsbG did not result in the excepted increase in the velocity of the disulfide exchange with DsbB. DsbG and its catalytic domain reacted 10 fold slower than DsbC. DsbA and D-DsbA reduced Q1 with similar velocities, which was not expected and therefore the Michaelis-Menten parameters of the DsbB catalyzed reduction of Q1 by these two proteins were measured.

![Graphs](image)

**Figure 48: Determination of the catalytic parameter of the DsbB-catalyzed reduction of ubiquinone 1.** Catalytic parameter determination of DsbB catalyzed ubiquinone 1 reduction with **A)** D-DsbA and **B)** DsbA. The measurement was done with 20 μM ubiquinone 1, 25 nM DsbB and different amount of either D-DsbA or DsbA in 100 mM sodium phosphate/NaOH, 0.1 % DDM pH 6.0 at 30 °C.

The Michaelis – Menten parameters were determined for D-DsbA (k_cat = 9.1 s⁻¹, K_M = 10.5 μM and a k_cat/K_M = 8.8 10⁵ M⁻¹ s⁻¹) and DsbA (k_cat = 8.9 s⁻¹, K_M = 8.4 μM and a k_cat/K_M = 1.1 10⁶ M⁻¹ s⁻¹). The dimerization of DsbA did not inhibit the interaction with DsbB. The catalytic part of D-DsbA was still interacting comparably to DsbA with DsbB.
3.5 Reduction of DsbB by putative disulfide isomerases and their monomeric variants

The reduction of DsbB by DsbC, DsbG, DsbC-C, DsbC H45D or DsbG-C was measured by change of intrinsic fluorescence of DsbB.

![Graphs A, B, C, D, E showing the reduction of DsbB by different isomerases over time.](image)
Figure 49: Electron transfer from putative disulfide isomerases to DsbB. DsbB reduction by DsbC (A), DsbC-C (B), DsbC H45D (C), DsbG (D) or DsbG-C (E) was measured by the change of intrinsic fluorescence of DsbB. The measurement was done with 5μM DsbB and 5μM of disulfide oxidoreductases in 100 mM sodium phosphate/NaOH, 1 mM EDTA, 0.1 % DDM pH 7.0 at 25 °C.

The reduction of DsbB was performed under second order condition. DsbC reduced DsbB with a second-order rate constant of 3626 M⁻¹s⁻¹. DsbC-C and DsbC H45D reduced DsbB faster than DsbC with a second-order rate constant of 5261 M⁻¹s⁻¹ and 5052 M⁻¹s⁻¹, respectively. The monomeric variants of DsbC were faster than DsbC. DsbG was (572 M⁻¹s⁻¹) the slowest tested protein, whereas monomeric DsbG-C reduced DsbB with a second-order rate constant of 1350 M⁻¹s⁻¹.

3.6 Oxidation of nDsbD by disulfide oxidoreductases

To investigate the electron transfer from nDsbD to DsbA, D-DsbA, DsbC, DsbC-C, DsbC H45D, DsbG and DsbG-C, the oxidation of nDsbD by these proteins was measured. The intrinsic fluorescence change upon reduction of DsbA, D-DsbA, DsbG or DsbG-C and the fact that the fluorescence of nDsbD does not change upon oxidation allow following the reaction in a fluorescence spectrometer. Since the change in the redox state of DsbC, DsbC-C or DsbC H45D is spectroscopically silent, the oxidation of nDsbD by these proteins was measured by HPLC.
Figure 50: Reduction of different disulfide oxidoreductases by nDsbD. The reduction of A) DsbC (●), DsbC-C (○) or DsbC H45D (■) and B) DsbA (○), D-DsbA (●), DsbG (■) or DsbG-C (□) by nDsbD was performed. The measurement was done with 3.5 μM nDsbD and 3.5 μM of different disulfide oxidoreductases in 100 mM sodium phosphate/NaOH, 1 mM EDTA pH 7.0 at 25 °C. The amount of reduced proteins were calculated, normalized and plotted against time.

The reduction of DsbA, D-DsbA, DsbC, DsbC-C, DsbC H45D, DsbG and DsbG-C by nDsbD allowed a deeper look into the disulfide isomerase pathway, where DsbG and DsbC are reduced by DsbD. The oxidation of nDsbD was performed under second order condition. DsbC is reduced by nDsbD with a second-order rate constant of 3.9 x 10⁶ M⁻¹s⁻¹, whereas the monomerized DsbC-C or DsbC H45D were reduced by nDsbD with second-order rate constants of 3.6 x 10³ M⁻¹s⁻¹ and 8.8 x 10³ M⁻¹s⁻¹, respectively. DsbA was reduced by nDsbD with a second-order rate of 900 M⁻¹s⁻¹. D-DsbA showed a higher the second-order rate constant (3.8 x 10³ M⁻¹s⁻¹) compared to DsbA. DsbG, the other natural partner of DsbD, showed a second-order rate constant of 1.9 x 10⁶ M⁻¹s⁻¹, where monomerized DsbG-C showed a slightly smaller second-order rate constant (2 x 10⁵ M⁻¹s⁻¹). The monomerization of DsbC had a larger influence on the rate constant of electron transfer from nDsbD than the monomerization of DsbG.

3.7 *In vivo* complementation of a *dsbA* deficient strain

The motility assay was performed to see how the variants of the disulfide oxidoreductase behave *in vivo*. A *dsbA* deficient *E. coli* strain shows no motility on a soft agar plate. It is due to the fact that a structurally important disulfide bond of the *p*-subunit of the flagella anchor is not formed in this strain. Therefore, the *p*-subunit of the anchor is not properly folded and the flagellum is not fixed in the membrane. In this assay, a *dsbA* deficient strain is used to see if plasmids encoding proteins could complement DsbA and the *p*-subunit of the flagella anchor therefore is functional. First, the complementation was tested in the LB media. All tested proteins were cloned in the vector pDsbA3 under the control of a trc promoter. The trc promoter is weak and no induction by IPTG was necessary.
**Figure 51: DsbA complementation in vivo.** The recovery of the motility in the *dsbA* deficient strain JCB 817 was performed on LB soft agar plates. Cells were transformed with (A) pDsbA, (B) pD-DsbA, (C) pDsbC-C or (D) pDsbC.

The *dsbA* deletion in the *E. coli* strain JCB 817 was complemented by the plasmid encoded DsbA, D-DsbA and DsbC-C, whereas DsbC is not able to restore the motility. The monomerized DsbC variant DsbC H45D was not tested, because the periplasmic expression of DsbC H45D led to protein degradation as it was already seen in the expression for protein purification and therefore this plasmid was not used for these measurements (see Figure 19). Additionally, the influence of the plasmid encoding proteins was also tested in the *dsbA/dsbB* deficient *E. coli* strain JCB 818.
Figure 52: DsbA/DsbB complementation in vivo. The recovery of the motility in the 
*dsbA/dsbB* deficient strain JCB 818 was performed on LB soft agar plates. Cells were 
transformed with (A) pDsbA, (B) pD-DsbA, (C) pDsbC-C or (D) pDsbC.

The motility of the bacteria could be partially recovered by DsbA, D-DsbA and DsbC-C, 
which was not expected in a *dsbA/dsbB* deficient *E. coli* strain. The recovery of the motility 
upon the expression of the same proteins as in the *dsbA* deleted strain was surprising and 
raised the question where did the oxidation equivalent come from in the *dsbA*, *dsbB* strain. 
To exclude the LB medium as source of oxidation equivalent, M63 medium supplemented 
with defined amino acids and other ingredients was used further on.
Figure 53: DsbA complementation in vivo. The recovery of the motility in the \textit{dsbA} deficient strain JCB 817 was performed on M63 supplemented soft agar plates. Cells were transformed with (A) pDsbA, (B) pD-DsbA, (C) pDsbC-C or (D) pDsbC.

The \textit{dsbA} deletion in the \textit{E. coli} strain JCB 817 can be complemented by the expression of plasmid encoding DsbA and D-DsbA, whereas DsbC-C showed only partial complementation of DsbA \textit{in vivo}. DsbC was not able to restore the motility.
Figure 54: DsbA/DsbB complementation *in vivo*. The recovery of the motility in the *dsbA/dsbB* deficient strain JCB 818 was performed on M63 supplemented soft agar plates. Cells were transformed with (A) pDsbA, (B) pD-DsbA, (C) pDsbC-C or (D) pDsbC.

In the *dsbA/dsbB* deficient *E. coli* strain JCB 818 no tested plasmid encoded protein was able to complement *dsbA* to restore the motility of the bacteria. This result was expected, since, with the change from LB medium to the supplemented M63 medium, the source of the oxidizing equivalents was the natural one and any influence of the environment was eliminated.

### 3.8 Catalyzed oxidation of GSH

The oxidation of GSH to GSSG catalyzed by DsbA, D-DsbA, DsbC, DsbC-C, DsbG or DsbG-C can be measured indirectly by the glutathione reductase catalyzed oxidation of NADPH by the resulting GSSG. The oxidation of NADPH can be followed spectroscopically at 340 nm.
Figure 55 Catalyzed oxidation of GSH. The oxidation of GSH (1mM) to GSSG catalyzed by 10 μM DsbA (●), D-DsbA (○), DsbC (■), DsbC-C (□), DsbG (♦), DsbG-C (◊) or uncatalyzed (x) was measured indirectly and preformed in 50 mM HEPES/NaOH, 1mM EDTA pH 8.0 containing 0.25 mM NADPH, 0.1 U glutathione reductase and 2.5 mM L-cystin. The reaction was followed spectroscopically by the decrease in absorption at 340 nm at 25 °C due to the oxidation of NADPH by GSSG.

In this assay only preliminary results were obtained and therefore further investigations have to be done. The results showed already that the DsbA and its dimeric variant were the best catalyst. DsbC and DsbC-C were even slower than DsbG or DsbG-C. The catalytic activities of these proteins in this assay increase in the same order as their redox potential (DsbA > DsbG > DsbC).

3.9 Disulfide oxidoreductase dependent expression of RBI

Coexpression of RBI and DsbC increased the yield of RBI about 40 times (Maskos et al., 2003). The expression of RBI was tested by trypsin inhibition activity of RBI.
Figure 56: Expression level of RBI in the periplasm. Expression level of RBI was investigated in the periplasm of different *E. coli* strains expressing either pRBI or pRBIDsbC. The cells were grown in either M63 supplemented minimal medium (A) or DYT medium (B). The periplasmic proteins were separated by SDS-PAGE and RBI was immunostained by the corresponding polyclonal antibodies. In A) Lane 1 indicates: (FED117 pRBI), Lane 2: (FED 117 pRBIDsbC), Lane 3: (FED 126 pRBI), Lane 4: (FED 126 pRBIDsbC), Lane 5: (FED 215 pRBI), Lane 6: (FED 215 pRBIDsbC), Lane 7: (FED 266 pRBI), Lane 8: (FED 266 pRBIDsbC) whereas in B) Lane 1: (FED117 pRBIDsbC), Lane 2: (FED 117 pRBI), Lane 3: (FED 126 pRBIDsbC), Lane 4: (FED 126 pRBI), Lane 5: (FED 215 pRBIDsbC), Lane 6: (FED 215 pRBI), Lane 7: (FED 266 pRBIDsbC), Lane 8: (FED 266 pRBI).
Figure 57: Trypsin inhibition by periplasm of different *E. coli* strains expressing RBI. L-BAPA cleavage by trypsin was followed spectroscopically at 405 nm. After incubation of trypsin with different samples of periplasmic extracts from the *E. coli* strains FED 117 (●), FED 126 (○), FED 215 (■), FED 266 (□) or without periplasmic samples (◊), the assay was performed in 200 mM Tris/HCl, 100 mM NaCl, 20 mM CaCl₂, 0.005 % (v/v) Triton X100 pH 8.0 at 25 °C for 15 min. Trypsin alone is indicated (◊) as base line of the measurement. A) 80 μl of periplasmic extract after expressing RBI in M63 supplemented minimal medium were incubated with trypsin. B) 80 μl of periplasmic extract after coexpressing RBI and DsbC in M63 supplemented minimal medium were incubated with trypsin. C) 10 μl of periplasmic extract after expressing RBI in DYT medium were incubated with trypsin. D) 10 μl of periplasmic extract after coexpressing RBI and DsbC in DYT medium were incubated with trypsin.

The inhibition of trypsin could be clearly detected, when RBI was coexpressed with DsbC in *E. coli*. The western blot showed that the coexpression of RBI and DsbC in DYT medium
increased the yield of RBI in all four tested strains, whereas in the case of the expression in the minimal medium M63 supplemented with amino acids (except Met and Cys), thiamin and glucose, only the coexpression of RBI and DsbC in the \textit{dsbC} deficient or wild type strain increased the yield. The coexpression of DsbC was necessary to get enough native RBI to inhibit trypsin. The \textit{dsbD} deficient strains FED 126 and FED 266 showed no presence of RBI on the western blot or trypsin inhibition even when RBI was coexpressed with DsbC in the minimal medium, whereas in the DYT medium the yield of the coexpression with DsbC was lower, but still RBI was detected by western blot.
Discussion

In this work, proteins involved in the disulfide isomerisation in *E. coli* were studied. In the first part, the two homologous proteins DsbC and DsbG were compared to each other. In the second part, the function of DsbC was studied in more detail. Finally, based on the results of the second part of this work, a fusion protein combining good peptide binding site and disulfide oxidoreductase activity was expressed and studied.

1. **DsbC and DsbG: two homologous proteins are functionally different**

DsbC and DsbG are disulfide oxidoreductases from *E. coli*, which have a lot in common. Both are homodimers, each having a catalytic domain and a dimerisation domain per subunit. Both proteins share a thioredoxin–like fold and the Cys–Xaa–Xaa–Cys active site motif in their catalytic domain. The amino acid sequences of DsbC and DsbG have 26 % sequence identity and 40 % sequence similarity. The sequence alignment of the proteins shows some interesting features.

![Sequence alignment of DsbC and DsbG.](image)

Figure 58: Sequence alignment of DsbC and DsbG. Similar amino acid residues are highlighted in **grey** and identical amino acid residues are highlighted in **black**. Amino acid residues that form part of the dimerization interface are denoted with **asterisks**.

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The dimerization interface in both proteins consists of a symmetric $\beta$-sheet. The amino acid residues of the dimerisation interface are mostly identical in both proteins; however the amino acid pairs, which are responsible for the hydrogen bonds in the dimerization interface, are different. No differences in the oligomeric state between the two proteins could be detected by size exclusion chromatography studies (see Figure 20). Both proteins, even at an applied concentration of 4.6 $\mu$M, are in the dimeric state. In contrast, mixtures of two DsbC variants (with and without a His$_6$ tag) show subunit exchange (see Figure 26), whereas DsbG under the same conditions (with and without His$_6$ tag) has to be refolded from 6 M GdmCl to show the same distribution of homodimers and heterodimers (see Figure 28). Although the dimerization interface of DsbC and DsbG shows high identity, no subunit exchange between DsbC and DsbG$_{His}$ was observed (see Figure 29). The formation of DsbC/DsbG heterodimers is prevented by the differences in the amino acid residue sequences in the dimerization interface of the proteins. The different results in the subunit exchange can be explained using a computer program collection package that analyzes the dimerisation interface for accessible surface area, hydrogen bonds, possible salt bridges, bridging water molecules and other parameters (Http://www.biochem.ucl.ac.uk/bsm/PP/server) (Hubbard, 1992; Laskowski, 1995; Lee and Richards, 1971; McDonald and Thornton, 1994). DsbG has a dimerization interface which is 20% bigger than the dimerization interface of DsbC. The DsbG dimer is stabilized by 10 hydrogen bonds; no bridging water molecules are present. The DsbC dimer is stabilized only by 9 hydrogen bonds, and two bridging water molecules are present. These differences in the dimerization interface give a possible explanation why DsbC shows subunit exchange and DsbG does not.

Furthermore, the thermodynamic stabilities of both proteins differ. The catalytic domain of DsbC shows no differences in the chemically induced unfolding in the oxidized and reduced states (see Figure 21 and 22). In the case of the thermal unfolding of DsbC, the determination of the unfolding transition is more difficult (see Figure 23). The thermal unfolding of the catalytic domain and the dimerisation domain seems to have a similar transition midpoint. The thermal stability of the individual domains could thus not be calculated independently. No differences between the thermal unfolding of oxidized and reduced DsbC can be found. Therefore the thermodynamic stability of DsbC can be assumed to be independent of its redox state. In contrast, DsbG clearly shows differences in thermal unfolding, depending on its redox state (see Figure 24). In thermal unfolding, the dimerisation domain and the catalytic domain can be distinguished and DsbG shows clearly a two step unfolding. The stability difference of oxidized and reduced DsbG in the transition at low temperature is a good
indication that the unfolding of the catalytic domain takes place. The dimerization domain of DsbG thus appears to be more stable than the catalytic domain and the dimerization interface form stronger contacts, thereby stabilizing the DsbG dimerization domain. Reduced DsbG appears to be more stable than oxidized DsbG, as in the case of DsbA. In contrast, the stability of DsbC shows no redox dependency. Furthermore, the stability of the dimerization domain of DsbG seems to be higher than the stability of dimerization domain of DsbC, as indicated by the higher melting temperature. The higher stability of DsbG in the thermal unfolding can be an additional reason for the different behavior in the subunit exchange of DsbC and DsbG. Could the redox-dependent thermodynamic stability differences result from the differences in the amino acid sequences between the two active site cysteines? The active site of DsbG (Cys-Pro-Tyr-Cys) has a Pro residue in the same position as it is present in the active site of DsbA (Cys-Pro-His-Cys) and a Tyr in the same position as it is present in the active site of DsbC (Cys-Gly-Tyr-Cys). It is thus conceivable that DsbG should be functionally similar to DsbA and DsbC. The Pro residue in the active site sequence influences the stability of DsbA and DsbG. The publication of Huber–Wunderlich and coworkers (Wunderlich and Glockshuber, 1993) investigates the influence of the substitution of amino acid residues between the active site cysteins in DsbA. The exchange of the Pro residue diminishes, but does not abolish the difference in stability. It must be thus more than only the amino acid sequence in the active site, which is responsible for the measured differences in the thermodynamic stability of DsbC and DsbG.

Additionally, DsbC and DsbG differ clearly in their disulfide isomerase and reductase properties. DsbG shows no isomerase or reductase activity in these assays (see Figures 38 and 46), where DsbC was the best catalysts in the assays. More interesting is the fact that DsbG prevents the insulin β-chain from aggregation (see Figures 38, 39 and 40) and displays chaperone-like activity as already has been shown with citrate synthase as a substrate (Shao et al., 2000). In contrast, DsbC shows no chaperone-like activity in the insulin reduction assay and the increase in DsbC concentration accelerates the aggregation of the insulin β-chain. In the scRNaseA assay (see Figure 46), DsbG shows a comparable to DsbA (13 % RNaseA activity after 8 hours) isomerase activity (17 % RNaseA activity after 8 hours), whereas DsbC has among the disulfide isomerase tested in the assay the highest yield and activates scRNaseA up to 90 % of wild type RNaseA activity after 8 hours.

Can these disulfide isomerase and reductase properties of DsbC and DsbG be explained by differences in the amino acid sequence of the peptide binding site of these proteins? In DsbC, a hydrophobic surface is located in the cleft between the two subunits, which binds wrongly
folded proteins and exposes them to the reduced active site cysteines of DsbC. The linker helix between the catalytic domain and the dimerisation domain in DsbC is 2.5 turns shorter than in DsbG and the active site is therefore closer to the peptide binding site. Additionally, the active sites of DsbC are facing each other and facing the hydrophobic cleft between the two subunits, which is not the case in DsbG. Moreover, DsbG has a mostly negatively charged surface in the cleft between the subunits and could therefore bind to hydrophilic protein surfaces more efficiently than DsbC; interactions with hydrophobic peptide surfaces are probably disturbed by the charges in the cleft of DsbG. This difference in the cleft between the two subunits of DsbC and DsbG can explain the results from the disulfide exchange assays with either DsbB (see Figure 47 and 49) or the NH2-terminal domain of DsbD (nDsbD) (see Figure 50). In the case of DsbC, the rate of electron exchange with DsbB is slightly improved by monomerization of the dimer, but the rate of electron transfer with nDsbD decreases considerably when DsbC is monomerized. In contrast, no significant differences in both interaction assays could be found for the monomeric catalytic domain and the dimer of DsbG. The mixed disulfide complex of DsbC and nDsbD is formed by a DsbC dimer and one n-DsbD molecule (Haebel et al., 2002). To form the complex with nDsbD, DsbC needs contacts between the second subunit of DsbC and nDsbD at the opposite side of nDsbD in addition to the contacts around the active site mixed disulfide bond (see Figure 59).
Figure 59: Solid ribbon diagram of the X-Ray structure of the mixed disulfide complex between nDsbD and DsbC. α–helices are shown in red and β-sheet in blue. The mixed disulfide bond between Cys99 of DsbC subunit 1 and Cys109 of nDsbD is shown in ball and sticks. (Haebel et al., 2002).

From the results of the electron exchange between DsbG or DsbG-C and nDsbD no such decrease in the rate of disulfide exchange as in the case of monomeric DsbC could be detected. Therefore binding of DsbG to nDsbD does not depend on dimerization of DsbG. The differences between the two homodimeric disulfide oxidoreductases, DsbC and DsbG, discussed above in this part of the work, give a clear indication that their functions are different. At least for DsbG, the substrate specificity must be very narrow, if DsbG is a disulfide isomerase at all. DsbG may also have another function in *E. coli*, which is not yet identified.
2. Role of dimerization for the DsbC function

Two models could explain the function of DsbC as a disulfide isomerase. First, two domains can provide a large and possibly connected peptide binding surface. Second, the formation of a mixed disulfide of one active site cysteine with the substrate could lead to a strong increase in the effective concentration of the second active site in DsbC homodimer independent of a peptide binding site.

Figure 60: Models describing the need for two subunits for the DsbC function. In model 1 two domains build a large, connected peptide binding site for the substrates of DsbC, whereas in model 2 the effective concentration of the second active site cysteine increases after formation of a mixed disulfide between the substrate and the first active site of DsbC. The Cys amino acid residues are shown in yellow circles.

To test these models, DsbC-C (the catalytic domain of DsbC), DsbC H45D (a monomerized variant of DsbC) and DsbC$_{CCAA}$ (an inactive DsbC variant) were expressed and purified. Additionally, DsbA and D-DsbA (a dimeric DsbA variant) were used to test the influence of the dimerization. Furthermore, a mixture of 10 parts of DsbC$_{CCAA}$ and one part of DsbC allows the formation of a DsbC heterodimer with only one active site (DsbC-S) (see Figure 27). DsbC-S can be used to test the models from above. If the model 1 explains the function of DsbC, DsbC-S should show the same disulfide isomerase and reductase activity as DsbC.
Therefore only the large contiguous peptide binding site is responsible for the disulfide isomerase activity of DsbC, whereas, if the model 2 explains the function of DsbC, DsbC-S should be inactive and D-DsbA should show an increased disulfide isomerase activity.

In the aggregation of the β-chain of insulin DsbC-S shows the same catalytic activity as wild type DsbC (see Figure 38). The subunit exchange analysis of DsbC and DsbC_{CCAA} shows that a minor part of wild type DsbC, around 5% of the total amount, stays in DsbC-S (see Figure 27). But this amount of DsbC, as the concentration dependent measurements of insulin reduction (see Figure 37) demonstrate, would not be enough to catalyze the insulin reduction as efficient as measured for DsbC-S. Therefore, it can be concluded that DsbC needs only one active site per dimer for the reduction of insulin. The model 1, where the two domains built a large, connected peptide binding site is a good explanation for the function of DsbC in the insulin reduction assay. D-DsbA accelerates the reduction of insulin comparably to DsbA and DsbC-C.

Similar conclusion can be drawn from scRNaseA reactivation, a disulfide isomerase activity assay. But in the scRNaseA assay the reactivation of scRNaseA by DsbC-S achieves only 77% of wild type RNaseA activity after 8 hours compared to 90% of activity for the DsbC-catalyzed reactivation (see Figure 46). Why does DsbC-S lose part of its disulfide bond reshuffling activity compared to DsbC? In the first 5 hours no differences in the disulfide bond reshuffling activity of DsbC-S and DsbC could be detected, but then DsbC-S shows decreasing disulfide isomerase activity compared to wild type DsbC. It could not be excluded that during the assay a part of the active sites of the proteins would be oxidized. For DsbC, if one of the two active sites is oxidized, the second active site is still active and it has no consequence for the disulfide isomerase activity whereas for DsbC-S, the oxidation of its single active site inhibits the disulfide isomerase function of the protein. For scRNaseA reactivation the model 1 (see Figure 60) with the large, connected peptide binding site explains best the function of DsbC.

It was shown that a heterodimeric DsbC, in which one active site was covalently modified, was inactive as disulfide isomerase (Sun and Wang, 2000). However, the carboxymethylation of one of the active site cysteine pairs disturb, as it was shown in the publication of Sun and coworkers, its native fold and may cause the inactivation. In contrast, exchange of the active site cysteines to alanines does not disturb the native fold, as proved by the far-UV CD (data not shown). DsbC-S shows the same isomerase and reductase activity as wild type DsbC. Thus the model 1, where the DsbC dimers build a large, contiguous peptide binding site, can explain the disulfide isomerase function of DsbC. From the results of these assays with D-
DsbA, it is not possible to distinguish if the two active sites of D-DsbA or the presence of the dimeric interface alone results in the increased in comparison to wild type DsbA isomerase and reductase activities of D-DsbA. In contrast to the increased disulfide isomerase activity of D-DsbA, the loss of the disulfide isomerase and reductase activity of the catalytic domain of DsbC directs to the idea that monomerized DsbC can be turned to a disulfide oxidase according to the findings of Bader and coworkers (Bader et al., 2001). This in turn implicates that the dimerization of DsbA should result in the loss of the oxidase activity of DsbA. To test these hypotheses the interaction between DsbA, DsbC or their variants and DsbB or nDsbD in vitro and in vivo were investigated.

Recent experiments by Bader and coworkers (Bader et al., 2001) showed that monomeric DsbC variants are able to complement DsbA in a dsbA deficient strain. Their result predicted that DsbC-C should be a substrate of DsbB and for this reason complement DsbA in same assay. Plasmid encoded DsbC, DsbC-C, DsbA or D-DsbA was tested for complementation of dsbA in a dsbA deficient strain by recovery of bacterial motility on LB soft agar plates. Except for DsbC, all tested expression plasmids show dsbA complementation not only in the dsbA deficient strain, but also in a dsbA/dsbB deficient strain (see Figure 52 and 53). In a dsbA/dsbB deficient strain no complementation should be detected because the electron transfer to the quinones is disrupted by dsbB deletion. It seems that the LB medium served as a source of redox equivalents. To prevent the influence of the redox conditions outside the bacteria, minimal medium with a defined composition had to be used. In minimal medium D-DsbA complemented DsbA completely, whereas the expression of DsbC did not affect the motility of E. coli and the expression of DsbC-C led to partial complementation (see Figure 54 and 55). The monomeric DsbC variants used by Bader and coworkers could not be tested, because of their degradation during expression (see Figure 19).

Since interaction with DsbB is a prerequisite for the in vivo complementation for a dsbA deficiency, the ability of DsbA, DsbC and their variants to reduce DsbB was tested in vitro. D-DsbA had the same rate as DsbA in DsbB catalyzed reduction of ubiquinone (see Figure 47). The determined catalytic parameters in presence of each of the proteins were in a comparable range (see Figure 48), in contrast to the hypotheses of Segatori and coworkers (Segatori et al., 2004). Here, D-DsbA with a Gly between the linker helix and DsbA was used to endow a certain flexibility that may be crucial to orient the active site.

The results of this work indicate that the dimerisation of DsbA does not prevent or disturb electron exchange with DsbB. In contrast, the monomerization of DsbC increased 5-times the velocity of the electron exchange with DsbB compared to DsbC. But even with this increase,
the reaction is still about 700 times slower than the DsbB-catalyzed reduction of ubiquinone by DsbA or D-DsbA. Therefore, this increase in the rate can not explain the partial complementation of DsbA in the motility assay by DsbC-C (see Figure 49).

Additionally, the reduction of DsbA, DsbC and their variants by nDsbD was tested in vitro (see Figure 50). DsbA shows a 10^5-fold lower rate in the reaction with nDsbD than DsbC and the monomerization of the DsbC decreases the rate by a factor of 10^3, whereas dimerization of DsbA leads to the rate increase by a factor of 10. These data highlighted another interesting aspect of the in vivo results. Apparently during the complementation assay, a competition between both electron transfer pathways takes place. The disulfide bond exchange reactions between DsbA or DsbC and their natural partners, DsbB or DsbD, have so evolved that the natural reactions dominate in this competition. Therefore only the result of the natural disulfide bond exchange reactions could be found in vivo. For DsbC-C, the loss of the ability to be reduced by DsbD and the increased oxidation of the active site by DsbB leads to its oxidation in vivo in contrast to DsbC (Joly and Swartz, 1997). This partial oxidation of DsbC-C in vivo could allow the complementation of DsbA in the motility assay.

In conclusion, it appears that DsbC needs only one active site per dimer, but a contiguous peptide binding site for the disulfide isomerase activity. Furthermore, the second subunit is necessary for the electron exchange with nDsbD and therefore for reduced state of DsbC in vivo. Monomerization of DsbC does not lead to disulfide oxidase activity, but leads to the loss of the disulfide isomerase function. In contrast, dimerization of DsbA does not influence the disulfide oxidase function of DsbA but improves its disulfide isomerase activity. Probably this results from Gly at the COOH-terminal end of the linker helix between the DsbC dimerisation domain and DsbA, which allows a certain flexibility of the active site that may be necessary for its positioning.

3. Fusion of a peptide binding site to DsbA to form a potential disulfide isomerase

The result in the previous part of the thesis led to the idea that the fusion of a good peptide binding site to a thioredoxin like fold may result in a disulfide isomerase. DsbA was fused to the apical domain of GroEL via linker of different lengths. Three tested variants exhibited properties of a folded protein. The stability of the fusion proteins was in the same range as the wild type proteins or the corresponding domain, indicating that the fusion does not influence
the structure of the protein (see Figure 25). The results of the disulfide reductase (see Figure 39) or isomerase (see Figure 46) assays show no improved disulfide isomerase or reductase activities compared to DsbA. Similarly a mixture of DsbA and the apical domain of GroEL (i.e. no-fused) show the same activity as DsbA alone. Using different amounts of the apical domain of GroEL alone in the insulin reduction assay has no influence on the aggregation of insulin, indicating that insulin does not bind to the apical domain of GroEL and is therefore not a substrate of the fusion protein. The fusion of GroEL apical domain to DsbA does not promote disulfide isomerase and reductase activity. There could be different reasons for this behavior. The active site in DsbA is not in the right orientation relative to the binding pocket of the apical domain, although three different linker lengths were tested. Furthermore the tested substrate may not bind to the apical domain at all or the peptide binding site may be inactive. And therefore other peptide binding sites have to be tested.
Outlook

One of the most interesting questions in the field of oxidative protein folding is still unsolved. Why did nature evolve a homolog protein, DsbG, to the disulfide isomerase DsbC from *E. coli*? Both proteins share the V-shape structure and the catalytic domain with a thioredoxin-like fold and a Cys-Xaa-Xaa-Cys active site motif, but they act at least in the tested disulfide isomerase and reductase assays totally different. As it has already shown in various publications (Shevchik *et al.*, 1994; Zapun *et al.*, 1995), DsbC is a periplasmic disulfide isomerase. It was additionally shown that RNAsel I and MepA are *in vivo* substrates of DsbC (Hiniker and Bardwell, 2004b), whereas no substrates of DsbG are known until now. Further studies are necessary to answer the question about the function of DsbG in *E. coli*.

The structure determination of the mixed disulfide complex between DsbG and nDsbD, the last not solved complex between nDsbD and its natural substrates, would show the binding mode of this complex. As already seen from the results of the disulfide exchange between nDsbD and DsbC or DsbG in this work, the mixed disulfide complex between DsbC or DsbG and nDsbD exhibit probably different stoichiometry. This complex can give additional information for the differences in the behavior between DsbC and DsbG in context of its disulfide exchange reaction with nDsbD.

Investigations in the thermodynamic stability of DsbC and DsbG in more details will allow some further conclusion of their different function. The stability differences found by thermal unfolding of oxidized and reduced DsbG, but not found in oxidized and reduced DsbC, have to be confirmed by chemically induced unfolding.

The expression of a DsbG variant, where Cys112 is exchanged to Ala, in a *dsbG* deficient strain can probably lead to the formation of mixed disulfide between DsbG and a substrate protein, which then could be isolated and characterized in more detail. Additionally, it would be interestingly to see if the DsbG active sites are necessary for its chaperone-like activity *in vitro*.

The exact catalytic mechanism of the disulfide isomerase DsbC remains unclear. Does the isomerization of substrate disulfide bonds proceed via cycles of reduction and reoxidation followed by the release of the substrate from the peptide binding site? Or does Cys98 of DsbC form a mixed disulfide bond with one of the cysteine residues of the substrate protein and a substrate internal reshuffling of the disulfide bonds occurs until the native disulfide bond pattern is formed. Identification of a mixed disulfide between DsbC and a substrate protein or
mixtures of reduced and oxidized DsbC during disulfide bond reshuffling would allow discriminating between these two models.

As it has been already shown for DsbC, the large contiguous peptide binding site is required for its disulfide isomerase function. Can this also be an explanation for the increased disulfide isomerase activity of dimeric DsbA in comparison to DsbA? To answer this question, preparation and characterization of a dimeric DsbA, with only one active site per dimer, is necessary.
Appendix

Media

LB

Bacto trypton 10 g/l
Bacto yeast extract 5 g/l
NaCl 5 g/l
Bacto Agar (for culture plates) 15 g/l

DYT

Bacto Trypton 16 g/l
Bacto yeast extract 10 g/l
NaCl 10 g/l

M63 minimal medium (Miller, 1992)

potassium dihydrogen phosphate 13.6 g/l
ammonium sulfate 2.0 g/l
ferrous sulfate 0.5 mg/l
adjusted to pH 7.0 with KOH
Bacto agar (for cultures plates) 3.0 g/l

Autoclave and add the indicated volumes of the sterile filtrated solution
1M magnesium sulfate 1/1000 vol
20% (w/v) glucose 1/100 vol
1% (w/v) thiamine hydrochloride 1/2000 vol
10 mg/ml from every amino acid 1/250 vol
except cysteine and methionine
## PCR Protocol

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<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>template</td>
<td>8.0 μl</td>
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<tr>
<td>NH₂-terminal primer</td>
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<tr>
<td>COOH-terminal primer</td>
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<tr>
<td>dNTP mix (2 mM of each nucleotide)</td>
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<tr>
<td>10x Taq polymerase buffer</td>
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<td>10x BSA</td>
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<td>MgCl₂ solution</td>
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</tr>
<tr>
<td>Taq polymerase</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>Pwo polymerase</td>
<td>1.2 μl</td>
</tr>
<tr>
<td>H₂O</td>
<td>ad 100 μl</td>
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Two drops of mineral oil were added to avoid evaporation. After 5 min at 95°C, 30 of the following temperature cycles were done, followed by 5 min at 4°C in a thermocycler (Biometra Trio-Thermoblock, Biolab, Châtel-St. Denis, Switzerland)

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
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<tr>
<td>Denaturation</td>
<td>95°C</td>
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</tr>
<tr>
<td>Annealing</td>
<td>50°C - 52°C</td>
<td>30 sec</td>
</tr>
<tr>
<td>Elongation</td>
<td>72°C</td>
<td>120 sec</td>
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## SDS-polyacrylamide gel composition

For twelve 15% SDS-polyacrylamide gel

### Separating gel for SDS–polyacrylamide gels (15%)

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<thead>
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<tr>
<td>40 % (w/v) acrylamid/bisacrylamide (37.5 : 1)</td>
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</tr>
<tr>
<td>3 M Tris/HCl pH 8.85, 4 mg/ml SDS buffer</td>
<td>20</td>
</tr>
<tr>
<td>H₂O</td>
<td>30</td>
</tr>
<tr>
<td>TEMED</td>
<td>25 μl</td>
</tr>
<tr>
<td>10 % (w/v) APS</td>
<td>400 μl</td>
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</tbody>
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### Stacking gel for SDS–polyacrylamide gels

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</tr>
<tr>
<td>0.5 M Tris/HCl pH 6.8, 4 mg/ml SDS</td>
<td>10</td>
</tr>
<tr>
<td>H₂O</td>
<td>26</td>
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<tr>
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<td>25 μl</td>
</tr>
<tr>
<td>10 % (w/v) APS</td>
<td>400 μl</td>
</tr>
</tbody>
</table>
Appendix

Equation for data evaluation of measurements

Chemical unfolding and refolding (Bolen and Santoro, 1988; Santoro and Bolen, 1988)

\[
y = \frac{(y_f + m_f [D]) + (y_u + m_u [D]) \exp \left[-(\Delta G^0 / R T + m [D] / R T)\right]}{1 + \exp \left[-(\Delta G^0 / R T + m [D] / R T)\right]}
\]

- \( y \) = observed fluorescence signal
- \( y_f \) = intercept of the folded protein
- \( m_f \) = slope of the pretransitional baseline
- \( y_u \) = intercept of the unfolded protein
- \( m_u \) = slope of the posttransitional baseline
- \([D]\) = concentration of denaturant
- \( m \) = cooperativity
- \( \Delta G^0 \) = free energy change of folding at zero [D]

Thermal unfolding using a two-state model (Pace et al., 1998)

\[
y = \frac{(y_f + m_f T) + (y_u + m_u T) \exp \left[-(\Delta H_m / R T) * ((T - T_m))\right]}{1 + \exp \left[-(\Delta H_m / R T) * ((T - T_m))\right]}
\]

- \( y \) = observed far UV CD signal
- \( y_f \) = intercept of the folded protein
- \( m_f \) = slope of the pretransitional baseline
- \( y_u \) = intercept of the unfolded protein
- \( m_u \) = slope of the posttransitional baseline
- \( T_m \) = midpoint of the thermal unfolding curve
- \( \Delta H_m \) = enthalpy change for unfolding at \( T_m \)
- \( T \) = temperature
Thermal unfolding using a model with an folding intermediate (Ch Puorger, personal communication)

\[ y = \left\{ \left( y_f + m_f T \right) \times \exp\left[ -\left( \frac{\Delta H_{m2}}{R T_{m2}} \right) \times \left( \frac{T - T_{m2}}{T_{m2}} \right) \right] \right\} \times \left( y_i + (y_u + m_u T) \right) \times \left( \exp\left[ -\left( \frac{\Delta H_{m1}}{R T_{m1}} \right) \times \left( \frac{T - T_{m1}}{T_{m1}} \right) \right] \right) / \left( 1 + \exp\left[ -\left( \frac{\Delta H_{m2}}{R T_{m2}} \right) \times \left( \frac{T - T_{m2}}{T_{m2}} \right) \right] \right) \]

- \( y \) = observed far UV CD signal
- \( y_f \) = intercept of the folded protein
- \( m_f \) = slope of the pretransitional baseline
- \( y_u \) = intercept of the unfolded protein
- \( m_u \) = slope of the posttransitional baseline
- \( y_i \) = intercept for the intermediate
- \( T \) = temperature
- \( T_{m1} \) = midpoint of the first thermal unfolding curve
- \( \Delta H_{m1} \) = enthalpy change for unfolding at \( T_{m1} \)
- \( T_{m2} \) = midpoint of the second thermal unfolding curve
- \( \Delta H_{m2} \) = enthalpy change for unfolding at \( T_{m2} \)

Redox potential determination (Zapun et al., 1995)

\[ Y_{\text{obs}} = \left( Y_{\text{ox}} + \left( \frac{M}{K} \right) \times Y_{\text{red}} \right) / \left( 1 + \left( \frac{M}{K} \right) \right) \]

- \( Y_{\text{obs}} \) = observed amount of DsbC_{red}
- \( Y_{\text{ox}} \) = intercept of DsbC_{ox}
- \( Y_{\text{red}} \) = intercept of DsbC_{red}
- \( M \) = ratio of [GSH]^2/[GSSG]
- \( K \) = equilibrium constant
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Tachikawa, H., Takeuchi, Y., Funahashi, W., Miura, T., Gao, X.D., Fujimoto, D., Mizunaga, T. and Onodera, K. (1995) Isolation and characterization of a yeast gene, MPD1, the


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Danksagung

Die vorliegende Arbeit wurde vom Mai 2001 bis Januar 2006 am Institut für Molekularbiologie und Biophysik der ETH Zürich ausgeführt.

Bei Herrn Prof. Rudi Glockshuber möchte ich für die Möglichkeit danken, dass ich die vorliegende Arbeit ausführen konnte. Im Besonderen möchte ich mich für den mir überlassenen Freiraum, die ständige Diskussionsbereitschaft und das Vertrauen, dass mir geschenkt wurde, bedanken.

Frau Prof. Eilika Weber-Ban danke ich für die Übernahme des Korreferates sowie der ständigen Bereitschaft, sich Zeit für aufkommende Fragen während dieser Arbeit zu nehmen.

Den jetzigen wie auch den früheren Laborkollegen möchte ich für das angenehme Arbeitsklima, die zahlreichen Hilfestellungen und Unterstützungen sowie für die anregenden Diskussionen danken. Im Besonderen gilt mein Dank Dr. Ulla Grauschopf für die kritischen Diskussionen, der permanenten Hilfe und Unterstützung während der Zeit. Dr. John Grimshaw möchte ich für das kritische Lesen, aber im Besonderen für seine Geduld während dem Schreiben dieser Arbeit danken. Einen speziellen Dank gilt Dr. Anna Rozhkova für die anregenden Diskussionen, das kritische Korrekturlesen dieser Arbeit, aber auch für die sportlichen Ablenkungen während der Schlussphase dieser Arbeit.

Bei meinen beiden Praktikanten, Maria Chiara Catenazzi und Sebastian Falk, möchte ich für ihre Hilfe während ihrem Vollpraktikum im dritten Teil dieser Arbeit danken.

Ebenso gilt den jetzigen und früheren Mitgliedern, insbesondere Dr. René Brunisholz und Helen Rechsteiner, des Protein Service Labor des Departments Biologie für die MALDI-TOF Messungen und die NH2-terminales Edman Sequenzierungen ein besonderer Dank.
Bei Dr. Björn Philipps möchte ich mich für die Einführung in die Geheimnisse der proteinchemischen Forschung anlässlich meines Vollpraktikums und meiner Diplomarbeit bedanken.
