CONSERVED ROLE OF THE LINKER \( \alpha \)-HELIX OF THE BACTERIAL DISULFIDE ISOMERASE DsbC IN THE AVOIDANCE OF MISOXIDATION BY DsbB

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The abbreviations used are: tPA, tissue plasminogen activator; IPTG, isopropyl-\( \beta \)-D-thiogalactopyranoside; DTT, dithiothreitol; PDI, protein disulfide isomerase.

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Abstract

In the bacterial periplasm the coexistence of a catalyst of disulfide bond formation (DsbA) that is maintained in an oxidized state and of a reduced enzyme that catalyzes the rearrangement of mispaired cysteine residues (DsbC) is important for the folding of proteins containing multiple disulfide bonds. The kinetic partitioning of the DsbA/DsbB and DsbC/DsbD pathways partly depends on the ability of DsbB to oxidize DsbA at rates >1,000 times greater than DsbC. We show that the resistance of DsbC to oxidation by DsbB is abolished by deletions of one or more amino acids within the \( \alpha \)-helix that connects the N-terminal dimerization domain with the C-terminal thioredoxin domain. As a result, mutant DsbC carrying \( \alpha \)-helix deletions could catalyze disulfide bond formation and complemented the phenotypes of \( \text{dsbA} \) cells.

Introduction

Disulfide bonds formation is a critical step in protein folding. By covalently cross linking amino acids far apart in the protein primary structure, the formation of native disulfides is associated with the increased stability and structural complexity typical of many secreted proteins. In bacterial cells disulfide bond formation occurs in the periplasmic space. The soluble enzyme DsbA is maintained in an oxidized state by the membrane protein DsbB, and catalyzes the rapid, but rather indiscriminate oxidation of cysteines in substrate proteins. DsbB recycles DsbA by transferring electrons to molecular oxygen or other electron acceptors through quinones and the membrane enzymes of the respiratory chain. Non correctly paired cysteines are rearranged by the soluble enzyme DsbC, which is maintained in a reduced state by the membrane protein DsbD which transfers electrons from thioredoxin in the cytoplasm to DsbC in the periplasm (1-3).

The DsbA-DsbB and DsbC-DsbD systems transfer electrons in opposite
directions, i.e. from and into the periplasm, respectively. Cross talking between the oxidation and isomerization pathways would be expected to result in a wasteful, futile cycle that would be detrimental for the cell. For this reason, large kinetic barriers must have evolved to prevent non physiologic interactions between the Dsb enzymes; therefore allowing the coexistence of the two pathways in the same cellular environment (4).

The structural features of DsbC that prevent its misoxidation by DsbB are not well understood. The catalytic domain of DsbC and DsbA are structurally similar and are both thioredoxin motif proteins (1). However, while DsbA is a monomer, DsbC is a homodimer with each subunit comprising the catalytic thioredoxin domain connected via a long \( \alpha \)-helical linker to a domain responsible for dimerization and substrate binding (5). Earlier genetic and biochemical studies by Bardwell and coworkers suggested that the dimerization of the thioredoxin domains is responsible for preventing oxidation by DsbB (6). Recently we constructed a series of chimeric proteins comprising the DsbC dimerization domain linked to either TrxA or to DsbA. Several of these chimeras catalyzed disulfide isomerization and oxidation \textit{in vivo}, suggesting that dimerization of thioredoxin domains is not sufficient to confer protection from the action of DsbB (7).

Comparison of the structure of the \textit{E. coli} DsbC with that of its homolog DsbG suggests that the \( \alpha \)-linker that connects the dimerization and the catalytic domains may play a role in determining the enzyme’s specificity for substrate proteins (8). Interestingly, in DsbG as well as in the DsbCs from both \textit{E. coli} and \textit{H. influenza} (9), the \( \alpha \)-helical linker serves to place the CXXC active sites within the two thioredoxin domains directly facing each other, indicating that this is a conserved feature of bacterial disulfide isomerase enzymes. These observations, together with our data using DsbC chimeras (7), suggested that \( \alpha \)-helical linker may play a pivotal role in the function of DsbC enzymes. Here we show that deletions in the \( \alpha \)-helical linker abolish the ability of DsbC to avoid oxidation by DsbB, and that this phenomenon is conserved among members of this enzyme family. However, the DsbC mutant enzymes retained the ability to catalyze the rearrangement of disulfide bonds in substrate proteins expressed in the \textit{E. coli} periplasm, indicating that the reduction of the enzyme by DsbD is not compromised by the deletions.

Materials and Methods

\textbf{Strains and plasmids.} The bacterial strains and plasmids used in this study are listed in Table 1. The putative \textit{dsbC} from \textit{Haemophilus influenzae}, \textit{Pseudomonas aeruginosa}, \textit{Vibrio cholerae}, \textit{Erwinia chrysanthemi}, and \textit{Yersinia pseudotuberculosis} were amplified from the chromosome of respective bacterial cells, and cloned into pBAD33 (10). The \( \alpha \)-helix deletion constructs of DsbC from \textit{E. coli}, \textit{H. influenzae}, and \textit{Y. pseudotuberculosis} were created by overlap extension PCR and cloned into pBAD33. All the constructs contain a C-terminal hexahistidine tag. For protein purification purposes, the putative \textit{dsbC} genes and DsbC deletion constructs were digested with XbaI and HindIII, ligated into pET28(a), and transformed into \textit{E. coli} BL21 cells.

\textbf{In vivo enzyme assays.} To determine the effect of the DsbC deletion constructs and the \textit{dsbC} homologous gene on the folding yield of a truncated version of the human tissue plasminogen containing 9 disulfide bonds, (vtPA, comprising of the catalytic and kringle 2 domains of the full length protein), \textit{E. coli} DHB4 (araD139 (araA-leu)7679 (codB-lac)X74 galE15 galK16 rpsL150 relA1 thi phoA (PvuII) phoR malF3 F'[lac (lacI) pro]) and \textit{E. coli} PB351 (SF100 \( \Delta \textit{dsbC} \)) were co-transformed with pBAD33 derivatives encoding the \textit{dsbC} genes, and with pTrcStIvtPA. Cultures were grown at 30°C in 15 ml of LB medium with 50 µg/ml of ampicillin and 25 µg/ml of chloramphenicol. Cells were diluted 1:100 from overnight cultures, grown to OD\textsubscript{600} of 0.8, and arabinose was added to a final concentration of 0.2%; 30 min later, vtPA synthesis was induced with 1 mM isopropyl-\( \beta \)-D-thiogalactopyranoside.
Following growth for three additional hours, 6 ml aliquots were pelleted by centrifugation and stored at −20°C. The pellets were resuspended in cold PBS as needed to normalize the number of cells per sample, and lysed with a French press. Following the removal of insoluble material by centrifugation (12,000 × g, 10 min at 4°C), the soluble protein concentration was determined by the Bio-Rad protein assay. tPA activities were obtained by first diluting the samples in 50 mM Tris-HCl (pH 7.4) with 0.01% Tween 80 to a final protein concentration of 0.5 µg/µl. 30 µl of the diluted cell lysates were added to 250 µl of the same buffer containing 0.04 µg of human Glu-type plasminogen (American Diagnostica, Greenwich, CT) per µl, and 0.4 mM Spectrozyme PL (American Diagnostica), incubated at 37°C, and the change in A 405 was monitored.

Expression, Purification, and Biochemical Assays. For the purification of the E. coli and homologous DsbCs, or the α-helix deletion mutants, the appropriate genes were cloned behind the T7 promoter in pET28(a), and the resulting plasmids were transformed into E. coli BL21(DE3). Protein expression and purification was performed as previously described (7). All proteins used in this study were more than 95% pure as judged by Coomassie Brilliant Blue-stained SDS-PAGE gels.

DsbB was purified as previously described (13,14), with the exception that a HiTrap Chelating HP column (Amersham Biosciences, Piscataway, NJ) charged with nickel was used in place of a Ni-nitrilotriacetic acid agarose column (Qiagen, Valencia, CA). The final purity of the DsbB preparation was >95%. For the purification of DsbA, E. coli HK317 (15) carrying pCH3, a pBAD18 derivative encoding dsbA (15), was grown in NZ medium with 200 µg/ml of ampicillin, induced with 0.2% w/v arabinose when the OD600 reached 0.05, and the cells were harvested at OD600=1.3. DsbA was purified from the periplasmic extract by a HiTrap Q FF anion exchange column (Amersham), followed by hydrophobic chromatography on a HiTrap Phenyl HP column (Amersham) as described (16).

Reduced DsbA, DsbCs, or α-helix deletion mutants were prepared by incubation in 20 mM dithiothreitol (DTT) for 20 min at 4°C. Excess DTT was removed by gel filtration on PD-10 Sephadex columns (Amershams) pre-equilibrated with 0.5 mM EDTA pH 8.0.

To determine the oxidation of DsbC by DsbB in vitro, 10 µM reduced wild type DsbC or DsbC mutants in 50 mM sodium phosphate pH 6.0 were incubated at 30°C with 20 µM ubiquinone-1 (coenzyme Q1;
Sigma) in 300 mM NaCl, 0.5 mM EDTA, 0.1% n-dodecyl-β-D-maltoside. The reaction was started by the addition of DsbB to a final concentration of 1 μM. A control reaction between DsbA and DsbB was carried out using 10 μM reduced DsbA and 10 nM DsbB. At time intervals, aliquots were withdrawn and the reaction stopped by immediate mixing with trichloroacetic acid (TCA) to a 10% final concentration. The denatured proteins were collected by centrifugation, and washed with cold acetone. Free thiols in reduced DsbC were modified with 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS) in 100 mM Tris-HCl (pH6.8), 1% SDS, 18 mM AMS as described (17). Reduced and oxidized forms of the proteins were separated by 12% SDS-PAGE without any reducing agents, and detected with Coomassie Brilliant Blue.

The rate of insulin reduction in the presence of DTT was determined as previously described (18). The change of A650 in the reaction was monitored as a direct measure of the aggregation of reduced insulin, and the activity is expressed as the ratio of the initial slope of the turbidity curve to the lag time (19). The renaturation of reduced, denatured RNase A was determined as described by Lyles and Gilbert (20). The protection of citrate synthase from thermal inactivation was monitored according to (32). The rate of thermal inactivation obtained with or without 4 μM of each of the DsbC variants was determined.

Results

Construction of DsbC α-helix deletion mutants. A 12 aa long α-helix linker (aa 60-72) joins the dimerization domain to the catalytic domain in the E. coli DsbC, maintaining the two protein domains structurally and functionally distinct (Figure 1) (5). We constructed a series of mutant genes encoding DsbC deleted of one, two, three, or four amino acids within the α-helical linker (positions 66, 66-67, 66-68, and 66-69) giving rise to the proteins DsbCaΔ1, DsbCaΔ2, DsbCaΔ3, and DsbCaΔ4, respectively. Because 3.6 amino acidic residues are contained in a α-helical turn, the deletion of each amino acid in the DsbC linker would be expected to cause the catalytic domain to be rotated by 100° with respect to the dimerization domain. As a result, the active site cysteines, which in wild type DsbC are oriented facing each other, in the deletion constructs would be expected to be rotated by an angle whose magnitude is determined by the number of amino acid deleted (Fig. 1).

The genes encoding the dsbC deletions were cloned in the medium copy number plasmid pBAD33 (10), under the control of the arabinose promoter. Under the conditions used in these experiments, the wild type E. coli DsbC and the α-helix deletion mutants all accumulated to nearly identical levels, as determined by Western blotting with a polyclonal antibody specific for the C-terminal His tag (Figure 2A).

Disulfide bond isomerization in vivo.

The oxidation of protein cysteines by DsbA is very rapid and may favor the formation of disulfides between adjacent cysteins in the polypeptide, even though these residues may not be necessarily connected in the final conformation of the protein, i.e. the native state (11,21). For this reason the folding of multidisulfide proteins is often limited by the isomerization of non-native bonds. The folding of three native proteins, the periplasmic acid phosphatase AppA or phytase, the peptidoglycan amidase MepA, and RNase I have been shown to depend on the action of DsbC. In addition, several complex heterologous proteins are known to require isomerization in order to attain their active conformation (11). Earlier, we had shown that expression of DsbC from pBAD33 results in a 25-fold increase in the yield of active vtPA, a truncated version of human tissue plasminogen activator containing 9 disulfides, relative to cells expressing DsbC from the chromosomal gene ((22), Figure 2A). We now found that all of the DsbC mutants containing deletions in the α-helix linker support the folding of vtPA. However, DsbCaΔ2 and DsbCaΔ4 result in about 40% of the active vtPA relative to the wild type DsbC. On the other hand, deletion of 1
(DsbCαΔ1) or 3 (DsbCαΔ3) amino acids resulted in active vtPA levels comparable and slightly lower, respectively, to those obtained with intact DsbC (Figure 2B and S1). Similar results were obtained with the native substrate AppA, i.e. DsbCαΔ1 and DsbCαΔ3 supported a higher level of AppA activity relative to DsbCαΔ2 and DsbCαΔ4 (Figure 2C).

Because of the role of DsbA as the primary oxidant of extracytoplasmic proteins, many proteins fail to fold properly in dsbA mutants. As a result, dsbA mutants exhibit pleiotropic phenotypes such as low alkaline phosphatase activity or loss of motility (2). The kinetic barrier between the reductive and oxidative pathways, as we described earlier, prevents DsbC to work as an oxidant of the extracytoplasmic proteins. To further examine the roles of the α-helical linker of DsbC in the properties of this enzyme, the DsbC mutants were expressed in a strain lacking dsbA and their ability to suppress the defects associated with lack of disulfide bond formation were tested. In E. coli MC1000 dsbA::kan grown in low phosphate media, the PhoA activity was 30-fold lower than that of its isogenic parent (Figure 2D). Expression of wild type DsbC from a multicopy plasmid did not result in a significant increase in PhoA activity, consistent with the earlier finding that even under conditions of overexpression, the enzyme is maintained in a reduced form by DsbD and thus it cannot act as a protein oxidant (23). However, deletion of one or more amino acids within the α-helical linker of DsbC gave rise to enzymes that could support the formation of active PhoA. We noted that the ability of the mutants to support the production of alkaline phosphatase, was inversely correlated with the disulfide isomerase activity, as determined in the vtPA and AppA assays. In other words, DsbCαΔ2 and DsbCαΔ4, the mutant proteins that gave a lower yield of active vtPA and AppA, could restore PhoA activity in MC1000dsbA to 60% of the level obtained from the chromosomally expressed DsbA, whereas DsbCαΔ1 and DsbCαΔ3 reproducibly displayed the opposite profile (Figure 2D and S2). The ability of the DsbCαΔ2 and DsbCαΔ4 mutants to support PhoA folding in the absence of DsbA suggests that these DsbC mutants are capable of supplying at least some of the oxidase function of DsbA. Consistent with this, PhoA activity was not detected in a dsbB mutant background (data not shown), revealing that the oxidase activity of the DsbC deletion mutants is dependent on their recycling by DsbB.

The size of the motility halo produced by cells growing on soft agar plates depends on the oxidase activity in the periplasm; MC1000dsbA cells were completely non-motile, and overexpression of DsbC from pBAD33 did not restore motility. However, consistent with the PhoA activity results, the DsbC α-helix deletion mutants restored motility, with about 2-fold larger halos observed with DsbCαΔ2 or DsbCαΔ4 compared to DsbCαΔ1 and DsbCαΔ3 (Figure 2E).

**Biochemical characterization.** Because the in vivo data suggest that the oxidase activity of DsbC deletion mutants is dependent on the presence of DsbB, we investigated the ability of DsbCαΔ1, DsbCαΔ2, DsbCαΔ3, and DsbCαΔ4 to be oxidized by DsbB in vitro. All proteins contained C-terminal hexahistidine tags and could be purified to >90% homogeneity by IMAC chromatography. Gel filtration FPLC analysis confirmed that the DsbC mutants are dimeric (Fig S1). CD spectroscopy indicated that the secondary structure of the mutant proteins was not perturbed by the deletions within the α-helix (data not shown). Quinones in the respiratory chain act as a direct recipient of electrons from DsbB. Oxidation of the reduced form of each DsbC variant by DsbB was performed in the presence of ubiquinone-1 as the recycling agent, and initiated by the addition of DsbB (17). Following alkylation of free thiols with AMS, the oxidized and reduced proteins were resolved by SDS-PAGE (Figure 3). In this assay DsbA got completely oxidized after 5 minutes of incubation with 10 nM DsbB. In contrast, DsbC resisted oxidation by DsbB almost completely: > 80% of the protein was found in the reduced state even after one hour of incubation with 1 µM DsbB. Deletion of
one or more amino acids in the α-helix greatly increased its susceptibility to oxidation by DsbB. Consistent with the in vivo results presented in Figure 2, deletions of two or four amino acids resulted in greater susceptibility to oxidation. Specifically, after 30 minutes, only 28% and 30% of respectively DsbCαΔ1 and DsbCαΔ3 was found in the oxidized state compared to 58% and 65% of DsbCαΔ2 and DsbCαΔ4, respectively.

The disulfide isomerase activity of the DsbC α-helix deletion mutants in the refolding of reduced RNase A was evaluated by monitoring the increase in cyclic AMP hydrolysis rate by active RNase A, as a function of time. In this reaction an initial lag phase that corresponds to the oxidation of RNase A is observed, followed by an increase in the rate of hydrolysis of cyclic AMP, as more native enzyme accumulates (20). In this assay, DsbC deletion mutants exhibited significantly lower isomerase activity relative to the wild type enzyme (Table 2). The reductase activity of the purified enzymes was determined by monitoring insulin reduction in the presence of DTT which results in the aggregation of insulin and a concomitant increase in turbidity (18). The deletion mutants exhibited substantial reductase activity, although lower than that of the wild type enzyme (Table 2). In contrast to the data presented in Figure 2, deletions of 1 or 3 amino acids for the most part did not result in different disulfide isomerase and reductase activities relative to the DsbCαΔ2 and DsbCαΔ4 mutants. Thus, it appears that the differential oxidase and isomerase activities observed in Figure 2 do not reflect the intrinsic catalytic properties of the enzymes per se, but rather the outcome of modified in vivo interactions with DsbB, and possibly DsbD. The chaperone activity of the chimeras was evaluated based on the protection of citrate synthase from thermal inactivation (Table 2). The DsbC deletion mutants delay the inactivation of citrate synthase to an extent comparable to the wild type DsbC suggesting that mutations in the α-helical linker do not affect the chaperone activity.

In vivo and in vitro properties of DsbC homologues. DsbC proteins are widespread among gram-negative bacteria. Currently there are more than 50 DsbC homologues in the NCBI database which exhibit between 6% (Clostridium acetobutilicum) and 100% identity (Shigella flexneri) with the Escherichia coli enzyme. We wondered whether the ability of DsbCs to be reduced by DsbD, yet avoid oxidation by DsbB, is conserved among members of the enzyme family. Five dsbC genes from the γ-proteobacteria _H. influenzae_, _P. aeruginosa_, _E. chrysanthemi_, _Y. pseudotuberculosis_, and _V. cholerae_, each sharing with _E. coli_ DsbC an amino acid sequence identity from 36 to 66%, were cloned by PCR amplification of genomic DNA. The genes encoding the mature proteins, and their respective leader peptides, were fused to a C-terminal His₆ tag, and expressed from pBAD33. With the exception of the _Vibrio cholerae_ protein, the other four DsbCs accumulated in the periplasmic space at a level comparable to the _E. coli_ enzyme (data not shown). As observed in Figure 4B, the DsbC homologues were as efficient as the _E. coli_ enzyme in supporting the folding of vtPA into its active state. The _Vibrio cholerae_ DsbC gave a 50% lower yield of active vtPA activity, but this effect is probably due to the lower expression of that enzyme in the _E. coli_ periplasm. None of the five DsbCs could complement the loss of PhoA activity or motility in LM106 (MC1000 dsbA) (data not shown, see also Figure 5B), suggesting that the ability to avoid oxidation by DsbB must be a conserved property among DsbC enzymes.

The recombinant _H. influenzae_, _P. aeruginosa_, _E. chrysanthemi_, _Y. pseudotuberculosis_ and _V. cholerae_ enzymes were purified to near homogeneity (>95% purity as determined by SDS-PAGE) by IMAC and size exclusion chromatography from the _E. coli_ osmotic shock fraction. The _Vₘₐₓ_ and _Kₘₐₚₐₚₜₐₜ_ values for the refolding of RNase A were determined (Figure S4). Apart from the _Vibrio cholerae_ enzyme, which did not exhibit saturation kinetics even with high substrate concentrations, the six enzymes exhibited comparable _Vₘₐₓ_ values and _Kₘₐₚₐₜₐₜ_ in the range of 20-40 μM. As a result, the
enzymes display comparable isomerase activity with RNase as substrate (kcat/Km), with only the *E. chrysanthemi* enzyme exhibiting a somewhat higher (2-fold) V_max /Km relative to the *E. coli* DsbC. Nonetheless, the rat Protein Disulfide Isomerase (PDI) is at least a 5-fold better isomerase even compared to the fastest bacterial enzyme, due to a substantially lower Km (6.9±0.8 µM) as shown in Table 3. The *Vibrio cholerae* DsbC was shown to be an exceptional reductase with insulin as the substrate, while the other enzymes exhibited similar activities in that assay (Table 3 and Figure S5). The chaperone activity of the homologous DsbCs was evaluated based on the protection of citrate synthase from thermal inactivation and aggregation (Figure S6 and S7). The citrate synthase half life during thermal inactivation is an indication of the chaperone activity of the enzymes. Interestingly, the *Vibrio* as well as the *Pseudomonas* DsbCs had lower chaperone activities (Table 3).

Among the homologous bacterial oxidoreductases analyzed in this study, DsbCs from *H. influenzae* and *Y. pseudotuberculosis* were selected to investigate the effect of mutations in the α-helical linker on its ability to resist oxidation by DsbB, and on the catalysis of disulfide bond isomerization in vivo and in vitro. In contrast to the full length enzyme, even a single amino acid deletion in the α-helical linker enabled DsbC to complement the loss of disulfide bond formation in *dsbA* cells. The level of PhoA activity and cell motility varied depending on the α-helical linker deletion (Fig. 2 D, and E, and Fig. S2, see below). This effect was completely dependent on recycling by DsbB in vivo. Examination of the kinetics of oxidation of DsbCs by DsbB in the presence of a suitable electron acceptor in vitro revealed that any deletion in the linker renders DsbC significantly more susceptible to oxidation (Fig. 3).

We also investigated the ability of *H. influenzae* and *Y. pseudotuberculosis* DsbC deletion mutants to complement the phenotype of a *dsbA* strain. As shown in figure 5B, all of the DsbC mutants restored motility upon overexpression in a *dsbA* strain and, therefore, served as catalysts of oxidation in the periplasm of *E. coli*. For both *Haemophilus* and *Yersinia* the deletion of two and four amino acids gives rise to motility halos considerably larger than the deletion of one or three amino acids. Clearly, the inverse correlation in the *E. coli* DsbC deletion mutants’ isomerase and oxidase activity described above is also observed in the homologous DsbCs analyzed.

**Discussion:**

In this study we examined the effects of deletions in the α-helical linker of the DsbC on its ability to resist oxidation by DsbB, and on the catalysis of disulfide bond isomerization in vivo and in vitro. In contrast to the full length enzyme, even a single amino acid deletion in the α-helical linker enabled DsbC to complement the loss of disulfide bond formation in *dsbA* cells. The level of PhoA activity and cell motility varied depending on the α-helical linker deletion (Fig. 2 D, and E, and Fig. S2, see below). This effect was completely dependent on recycling by DsbB in vivo. Examination of the kinetics of oxidation of DsbCs by DsbB in the presence of a suitable electron acceptor in vitro revealed that any deletion in the linker renders DsbC significantly more susceptible to oxidation (Fig. 3).

This gain of oxidase activity did not abolish the disulfide isomerase activity of the mutant DsbC enzymes. All the mutant enzymes catalyzed disulfide isomerization of both vtPA and the native *E. coli* protein AppA, in a DsbD-dependent manner, albeit generally with reduced efficiency (Fig. 2B and C, and Fig. S1). As expected, catalysis of disulfide bond rearrangement by the mutant DsbCs was completely dependent on reduction by DsbD. The enzymes that displayed a higher ability to assist the folding of proteins with multiple disulfides in a *dsbA* background were those that exhibited a lower oxidase...
activity in *dsbA* cells. A lower oxidase activity indicates that a smaller fraction of the enzyme is oxidized by DsbB at steady state, therefore lowering the relative amount of reduced DsbC that is able to function as an isomerase.

Remarkably, all the α-helical linker truncations examined here were capable of serving as the sole catalyst for both protein thiol oxidation in a *dsbA* background and for disulfide bond isomerization. For this to occur, enzyme that has been oxidized by DsbB must co-exist at steady state with enzyme that has been reduced by DsbD. In this scenario a portion of oxidized DsbC molecules would be expected to be directly reduced by DsbD before they can transfer their disulfides into periplasmic protein substrates. Conversely, some of the reduced DsbDs may undergo direct oxidation by DsbB without having participated in disulfide isomerization. This process would be expected to expend energy in an unproductive manner that could drain the cell’s resources. However, expression of the mutant DsbCs in a *dsbA* background did not impair cell growth compared to the parental strain (data not shown). Perhaps the unproductive electron flux in the NAPDH-DsbD-DsbC-DsbB-O₂ cycle is small, and therefore does not pose a significant burden on the cell, at least under laboratory conditions.

To further probe the interactions of DsbC family enzymes with DsbD and DsbB we examined the *in vivo* and *in vitro* function of homologues from *H. influenzae*, *P. aeruginosa*, *V. cholerae*, *E. chrysanthemi*, and *Y. pseudotuberculosis*. Despite the considerable degree of amino acid divergence among these proteins, all five enzymes could be recycled by *E. coli* DsbD, and supported the folding of vtPA to levels essentially indistinguishable from those obtained with the *E. coli* DsbC expressed under identical conditions (Fig. 4B). The crystal structure of the *E. coli* DsbC-DsbDa complex has revealed that the interface between the two molecules involves only residues from the thioredoxin domain of DsbC, and appears to have been optimized for rapid disulfide exchange (24). Arg97, Thr125, Gln126, Thr182, and Pro198 are the contact residues responsible for the binding interaction between *E. coli* DsbC and DsbDa (24). Sequence alignment revealed that these five residues are highly conserved among all the putative DsbCs reported in the NCBI database (Fig 4A, residues highlighted in yellow, and data not shown).

In addition, the bacterial disulfide isomerases appear to have employed a similarly conserved mechanism for evading aberrant interactions with DsbB. DsbB seems to react specifically with thioredoxin family proteins (6,25,26), and as such it can readily oxidize the active site of DsbC in the monomeric state (6). However, the dimeric, wild type forms of the enzymes from all six organisms used in this study were strongly resistant to oxidation by the *E. coli* DsbB (Fig. 2B, Fig. 5B and data not shown). In complete accordance with our findings regarding the *E. coli* DsbC, deletions in the α-helical linker of the *H. influenza* and *Y. pseudotuberculosis* DsbCs allowed the mutant enzymes to complement the phenotypes displayed by *dsbA* cells (Fig. 5B). These results, argue that the linker region and presumably the geometry of the active sites in DsbC enzymes (see below), has evolved to evade improper oxidation in the periplasm of bacterial cells.

Interestingly, the effect of sequential deletions in the linker of the *E. coli*, *H. influenza* and *Y. pseudotuberculosis* gave the same pattern of *in vivo* oxidation and isomerization activities. Deletion of either 1 or 3 amino acids resulted in higher *in vivo* disulfide isomerase activities and lower oxidase activities. In contrast, truncation of 2 or 4 amino acids gave better complementation of *dsbA* phenotypes, but lower yields or vtPA which folding depends on disulfide isomerization (Fig. 5). This dependence of *in vivo* isomerization and oxidation activity on the linker length does not stem from the intrinsic catalytic properties of the enzymes *per se*. Biochemical analysis of the *E. coli*
DsbCαΔ1- DsbCαΔ4 enzymes revealed relatively small differences in isomerization activity, as determined in the well established RNAse A refolding assay. All the DsbC deletion mutants showed comparable activity in the RNAse A assay (which however was lower than the wild type E. coli enzyme (between 15-26%)) and did not correlate with the ability of the enzymes to support disulfide bond isomerization in vivo. For example, DsbCΔ1 and DsbCΔ2 showed identical specific activities (Table 2), yet the former resulted in a 2-fold higher yield of vtPA and AppA in vivo. Similarly, reductase activities, determined from the rate of reduction of insulin, could not explain the trends observed in vivo.

Given that the dimerization region and the thioredoxin catalytic domain of DsbC are able to fold independently, and are stable on their own (27), which is also supported by our CD analysis of DsbC variants, it is unlikely that a truncation in the linker results in any major conformational changes in either of these domains. Rather, deletion of an amino acid in the linker would be expected to (a) slightly shorten the α-helix and (b) cause a rotation of the end of the helix and possibly the entire catalytic domain, including the CGYC active site by 100° per amino acid deleted, relative to the axis of symmetry of the molecule (Figure 1). The reduction in the length of the α-helix per amino deleted is small (1.5 Å) and would result in an equal shortening of the distance between the peptide binding cleft (in the cleft formed by the dimerization domain) (5) and the catalytic center. While it is possible that the shortening of the linker by itself may be the main reason for the observed differences in the in vivo and in vitro properties of the DsbCαΔs, it should be noted that the two DsbC family enzymes whose structures are known (E. coli DsbC and DsbG) have different linker lengths. However, in all three enzymes the catalytic sites are oriented parallel to each other and at 90° with respect to the long axis of symmetry. Thus we believe that the effect of linker deletions is more likely to arise from the rotation of the two active sites with respect to each other. Even a slight deviation of the active sites from the perfect orientation seen in wild type DsbC (for example a rotation of the CGYC motif by -60° would be expected to occur in the DsbCαΔ3 mutant, relative to the wt enzyme (Figure 1)) seems to impact the interaction of the proteins with DsbB and DsbD. Still, it is not clear why a rotation of the active sites by 100° in DsbCαΔ1 would give rise to a protein that is more readily reduced by DsbD and more resistant to oxidation by DsbB compared to the DsbCαΔ4 mutant where the active sites would be expected to be rotated by only 40°. Similarly, it would be interesting to determine whether the two misaligned active sites in the DsbCαΔs can both interact simultaneously with DsbDα, as is the case with the wt DsbC dimer. The CD spectra of the DsbC mutants suggest no significant changes in their secondary structures. However, we can not rule out a possibility that, in addition to the effect on the angle of the whole catalytic domain, the deletion might cause the α-helix to be more flexible or affect the ability of the protein to undergo conformational changes in the presence of its substrates. Then the altered angle of the whole catalytic domain, in conjunction with these effects, might allow the DsbC mutants to interact with both DsbD and DsbB. Delineation of these effects will have to await the high resolution structures of DsbCαΔ and DsbCαΔ-DsbDα complex, which is currently in progress.

In bacteria, large kinetic barriers prevent the flow of electrons between enzymes of the Dsb pathway. Non-physiological interactions between DsbA-DsbD, DsbC-DsbA and DsbC-DsbB are in fact three to seven orders of magnitude slower than biologically relevant interactions (4). Bader et al. showed that mutations that abolish the dimerization of DsbC allow the protein to serve as a weak oxidant in a DsbB dependent manner, leading to the suggestion that the dimeric structure of the enzyme is responsible for its resistance to oxidation by DsbB (6). However, it now appears that it is not the dimeric nature of the enzyme per se that prevents the interaction of the DsbC active site with the catalytic center of DsbB, but rather...
more subtle conformational features that probably relate to the geometry and orientation of the active sites in the overall structure of the molecule.

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### Table 1. Strains and plasmids

| Strains and Plasmids | Relevant Genotype | Reference |
|----------------------|-------------------|-----------|
| DH5α                | F- (80MlacZ-M15) - (lacZYA-argF) U169 deoR recA1 endA1 hsdR17 (rk-,mk+) supE44, thi-1 gyrA96, relA1 | Laboratory Collection |
| DH10B               | araD139 (araA-leu)7679 (codB-lac I)X74 galE15 galK16 rpsL150 relA1 thi phoA (PvuII) phoR malF3 F' [lac I] proc | Laboratory Collection |
| MC1000              | araD139 (araA-leu)7679 (codB-lac I)X74 galE15 galK16 rpsL150 relA1 thi F' [lac I] proc | Ref. 29 |
| LM106               | arabinose (araA-leu)7679 (codB-lac I)X74 galE15 galK16 rpsL150 relA1 thi phoA (PvuII) phoR malF3 F' [lac I] proc | Ref. 30 |
| LM102               | arabinose (araA-leu)7679 (codB-lac I)X74 galE15 galK16 rpsL150 relA1 thi F' [lac I] proc | Ref. 31 |
| PB351               | arabinose (araA-leu)7679 (codB-lac I)X74 galE15 galK16 rpsL150 relA1 thi F' [lac I] proc | Ref. 32 |
| BL21(DE3)           | arabinose (araA-leu)7679 (codB-lac I)X74 galE15 galK16 rpsL150 relA1 thi F' [lac I] proc | Ref. 33 |
| pET-28(a)           | T7 expression vector, C-terminal 6x histidine tag | Novagen |
| pBADdsbC            | DsbC from *Escherichia coli* in pBAD33 | Laboratory Collection |
| pTeCStlVPA          | tPA(D6-175) with Stl leader in pTZ99A | Ref. 23 |
| pBADHinfdsbC        | DsbC from *Haemophilus influenzae* in pBAD33 | This work |
| pBADYpsedsbC        | DsbC from *Yersinia pseudotuberculosis* in pBAD33 | This work |
| pBADPaedsbC         | DsbC from *Pseudomonas aeruginosa* in pBAD33 | This work |
| pBADVchodsbC        | DsbC from *Vibrio cholerae* in pBAD33 | This work |
| pBADEchdsbC         | DsbC from *Erwinia chrysanthemi* in pBAD33 | This work |
| pET-28Ecoliα1       | DsbC from *Escherichia coli* in pET-28(a) | This work |
| pET-28Ecoliα2       | DsbC from *Escherichia coli* in pET-28(a) | This work |
| pET-28Ecoliα3       | DsbC from *Escherichia coli* in pET-28(a) | This work |
| pET-28Ecoliα4       | DsbC from *Escherichia coli* in pET-28(a) | This work |
| pET-28Hinfα1        | DsbC from *Haemophilus influenzae* in pET-28(a) | This work |
| pET-28Ypsα1         | DsbC from *Yersinia pseudotuberculosis* in pET-28(a) | This work |
| pBADEcoliα1         | DsbC from *Escherichia coli* in pBAD33 | This work |
| pBADEcoliα2         | DsbC from *Escherichia coli* in pBAD33 | This work |
| pBADEcoliα3         | DsbC from *Escherichia coli* in pBAD33 | This work |
| pBADEcoliα4         | DsbC from *Escherichia coli* in pBAD33 | This work |
| pBADYpsα1           | DsbC from *Yersinia pseudotuberculosis* in pBAD33 | This work |
| pBADYpsα2           | DsbC from *Yersinia pseudotuberculosis* in pBAD33 | This work |
| pBADYpsα3           | DsbC from *Yersinia pseudotuberculosis* in pBAD33 | This work |
| pBADHinfα1          | DsbC from *Haemophilus influenzae* in pBAD33 | This work |
| pBADHinfα2          | DsbC from *Haemophilus influenzae* in pBAD33 | This work |
| pBADHinfα3          | DsbC from *Haemophilus influenzae* in pBAD33 | This work |
| pBADHinfα4          | DsbC from *Haemophilus influenzae* in pBAD33 | This work |
Table 2. *In vitro* activities of purified deletion constructs

| Enzyme | RNAse refolding*† | Insulin reduction* | Citrate Synthase* |
|--------|------------------|-------------------|------------------|
| -      | μM/min/μM Enz    | *10⁻³ ΔA₆₅₀nm/min⁻² | Inactivation | Aggregation |
| DsbC   | 0.028 ± 0.003    | 6.01 ± 0.11       | 0.94 ± 0.01     | 0.51 ± 0.01 |
| DsbCαΔ1| 0.0042 ± 0.0004  | 4.48 ± 0.27       | 3.15 ± 0.02     | 0.22 ± 0.02 |
| DsbCαΔ2| 0.0044 ± 0.0003  | 2.03 ± 0.16       | 2.45 ± 0.06     | 0.23 ± 0.05 |
| DsbCαΔ3| 0.0064 ± 0.0006  | 3.72 ± 0.15       | 2.29 ± 0.08     | 0.24 ± 0.07 |
| DsbCαΔ4| 0.0073 ± 0.0001  | 5.03 ± 0.22       | 3.01 ± 0.05     | 0.22 ± 0.04 |

nd, not determined.

*Data are expressed as mean ± SD (n=3-8)
†The activities were determined from a plot of isomerization velocity against enzyme concentrations.
Table 3. *In vitro* activities of purified DsbCs from different bacterial species

|                   | RNAse refolding*† | Insulin reduction* | Citrate Synthase* |
|-------------------|-------------------|-------------------|-------------------|
|                   | V_{max} \mu M RNase/min/\mu M | K_{m} (\mu M) | V_{max}/K_{m} | Inactivation | Aggregation |
| DsbC              |                   |                  |                  | CS t_{1/2} (min) | \mu (min^{-1}) | K_i (\mu M) |
| E. coli           | 0.68 ± 0.19       | 32 ± 16          | 1.2             | 6.01 ± 0.11     | 0.94 ± 0.01    | 0.51 ± 0.01  |
| H. influenzae     | 0.53 ± 0.09       | 29 ± 3           | 1.8             | 16.87 ± 0.15    | 3.15 ± 0.02    | 0.22 ± 0.02  | 9.5 ± 1.4    |
| P. aeruginosa     | 0.3 ± 0.05        | 20 ± 7           | 1.5             | 4.02 ± 0.11     | 5.12 ± 0.03    | 0.12 ± 0.03  | 11.0 ± 2.0   |
| V. cholerae       | -                 | -                | 1.3*            | 88.24 ± 0.84    | 7.36 ± 0.01    | 0.09 ± 0.02  | nd           |
| E. chrysanthemi   | 0.98 ± 0.7        | 42 ± 5           | 2.5             | 6.65 ± 0.17     | 3.12 ± 0.01    | 0.22 ± 0.02  | 8.8 ± 1.1    |
| Y. pseudotubercolis | 0.5 ± 0.1        | 30 ± 11          | 1.6             | 5.03 ± 0.14     | 2.65 ± 0.04    | 0.23 ± 0.05  | 7.3 ± 1.2    |
| PDI (rat)         | 0.76 ± 0.02       | 6.9 ± 0.8        | 11              | -               | -             | -             | -            |

nd, not determined.

* Saturation kinetics were not observed using RNase concentrations up to 40 \mu M. *Data are expressed as mean ± SD (n=3-8)

† The activities were determined from a plot of isomerization velocity against enzyme concentrations. V_{max}/K_{m} is the slope of the linear plot of velocity as a function of RNase concentration.
Figure Legends

Fig. 1. Helical wheel plots of the linker helices from DsbC and DsbC deletion constructs. The C-terminal amino acid of the linker in each helical wheel plot is highlighted in grey.

Fig. 2. In vivo activity of E. coli DsbC deletion constructs. (A) Protein expression level, determined by Western blotting. 1, DsbC; 2, DsbCαΔ1; 3, DsbCαΔ2; 4, DsbCαΔ3; 5, DsbCαΔ4. (B) Yield of active vtPA. DHB4 transformed with pTrcStIIvtPA and pBAD derivatives encoding the respective DsbC deletion proteins were grown in LB medium. Protein synthesis was induced as described in Materials and Methods, and the yield of active vtPA at 3 h after induction was determined. Relative activities were obtained by dividing the ΔA405 (absorbance of each strain subtracted of the background consisting of a strain not expressing vtPA) by the A405 of a strain expressing vtPA alone, represented by the last bare (-) in the graph (C) AppA activity. MB69 (DHB4 ΔdsbC) transformed with pAppA and pBAD derivatives encoding the respective DsbC deletion proteins were grown in LB media until mid-log phase. Assays were performed as described in the Materials and Methods. The AppA activity was determined by measuring the A410. One unit is defined as 1,000× A410 per min/A600 per ml as described (11). (D) Effect of the expression of the deletion proteins on alkaline phosphatase activity in the periplasm of MC1000 dsbA (grey bars). The alkaline phosphatase activity of the parental isogenic strain MC1000 is shown by the black bar. Cells were induced with 0.2% arabinose, harvested in mid-log phase, and lysed, and activity assays were conducted as described. (E) Cell-motility assays. Motility of MC1000 dsbA cells transformed with the following: (1) pBADdsbC; (2) pBADcoliα1; (3) pBADcoliα2; (4) pBADcoliα3; (5) pBADcoliα4; 6 and 7 represent MC1000 dsbA and MC1000 respectively. Cultures were grown in low-phosphate media described in the material and methods, diluted to the same cell density, and 3-µl aliquots were spotted on the center of each plate.

Fig. 3. Oxidation of E. coli DsbA, DsbC, and DsbC deletion mutants by DsbB in vitro. Purified proteins (5 µM) were reduced and incubated at 30°C with ubiquinone-1 and purified DsbB as described in Materials and Methods. The reaction was stopped by mixing sample aliquots withdrawn at different times with TCA, and DsbB oxidation was detected by AMS alkylation and separation of reduced and oxidized protein on a 12 % SDS page gel stained with Coomassie Brilliant Blue. Left and right panels represent the reaction performed respectively without and with incubation with DsbB for each protein. Each lane corresponds to different times of incubation as indicated in the figure. The amount of oxidized protein present in the different reactions was quantified using ImageJ (28).

Fig. 4. Analysis of putative dsbC from different bacterial species. (A) Multiple alignment and phylogenetic tree of DsbC from E. coli, H. influenza, P. aeruginosa, V. cholera, E. chrysanthemi, and Y. pseudotuberculosis obtained with ClustalW. Contact residues involved in the interaction with DsbD are highlighted in yellow. (B) Yield of active vtPA. PB351 (SF100 ΔdsbC) transformed with pTrcStIIvtPA and pBAD derivatives encoding putative dsbC genes from H. influenza, P. aeruginosa, V. cholerae, E. chrysanthemi, and Y. pseudotuberculosis were grown in LB medium, and protein synthesis was induced as described in the Materials and Methods. The yield of active vtPA was determined as described in the legend to figure 2B.

Fig. 5. In vivo activity of H. influenzae and Y. pseudotuberculosis DsbC deletion constructs. (A) Yield of active vtPA. DHB4 transformed with pTrcStIIvtPA and pBAD derivatives encoding the
respective H. influenzae (grey bars) and Y. pseudotuberculosis (black bars) DsbC deletion proteins were grown in LB medium and vtPA activity was determined as described in Fig 2B. (B) Cell-motility assays. Motility of MC1000 dsbA cells transformed with the following: (1) pBADHinfdsbC; (2) pBADHinfa1; (3) pBADHinfa2; (4) pBADHinfa3; (5) pBADHinfa4; (6) pBADYpsedsbC; (7) pBADYpsefa1; (8) pBADYpsefa2; (9) pBADYpsefa3; (10) pBADYpsefa4; (11) MC1000 dsbA; and (12) MC1000 grown in low-phosphate media. Cultures were diluted and plated as described in Fig 2E.
Figure 1

Dimerization domain --- V T N K M L L K Q L N A L --- Catalytic domain

α-helical linker
(aa 62-74)

DsbC wild type

DsbCαΔ1  DsbCαΔ2  DsbCαΔ3  DsbCαΔ4
Figure 2

A

B

C

Relative tPA Activity

-DsbC-
α∆

-DsbC-
α∆

-DsbC-
α∆

-DsbC-
α∆

-DsbC-
α∆

Relative AppA Activity

-DsbC-

-DsbC-cΔ1

-DsbC-cΔ2

-DsbC-cΔ3

-DsbC-cΔ4

Relative AppA Activity

0

20

40

60

80

100

120

0

5

10

15

20

25

123456

123456

123456
Figure 3

![Figure 3](image_url)

- **DsbA**
- **DsbC**
- **DsbCαΔ1**
- **DsbCαΔ2**
- **DsbCαΔ3**
- **DsbCαΔ4**

**DsbbB**

Time points: 15'' 1' 5' 10' 30' 60'
| Protein       | Amino Acid Sequence                                                                 | Length |
|--------------|------------------------------------------------------------------------------------|--------|
| DsbC_ERWCH   | MKKRVFLSLLTLALSG-----VARADDAAIKQTLNLG--LQSAEVKDSPIGGMK                            | 49     |
| DsbC_YERPS   | MKKSSLMLMLMAALSG-----VARADDAAIKQTLNLG--LQSAEVKDSPIGGMK                            | 49     |
| DsbC_ECOLI   | M KKGFMLFTL-AAFSG-----FAQADDAAAQTLNLG--IKSDPFAPIAGRMK                             | 48     |
| DsbC_HAEIN   | M KKGFMLFTL-AAFSG-----FAQADDAAAQTLNLG--IKSDPFAPIAGRMK                             | 48     |
| DsbC_VIBCH   | MSVLRLWLFLSLLMANNVQANTAPQNLKAELEXQRFAKLG----LQVEEIKTDGKLL                         | 58     |
| DsbC_PSEAE   | M RVTRFLAALGIMS----LALADNADQNIKTLQALQGELPDIASSPLQGQY                            | 53     |
| DsbC_ERWCH   | TVLTENG-VLYITEDGKHLLQGPLYDVSGKT-PVNVTNH----ILNERLD-ALKDMIVY                      | 102    |
| DsbC_YERPS   | TVMTESG-VLYISADGKHLLQGPLYDVSGQ-PINVTNQ----ALLKQKL-ALSSLVMY                       | 102    |
| DsbC_ECOLI   | TVLTNG-VLYITDGKHIIQGPMTYDVSGSTA-PVNVTNH----MLLQLLM-ALQLKQVM                      | 101    |
| DsbC_HAEIN   | TVLTNG-VLYITDGKHIIQGPMTYDVSGSTA-PVNVTNH----MLLQLLM-ALQLKQVM                      | 101    |
| DsbC_VIBCH   | TVMTESG-VLYISADGKHLLQGPLYDVSGQ-PINVTNQ----ALLKQKL-ALSSLVMY                       | 102    |
| DsbC_PSEAE   | TVLTENG-VLYITEDGKHLLQGPLYDVSGKT-PVNVTNH----ILNERLD-ALKDMIVY                      | 102    |
| DsbC_ERWCH   | KAP-EEKHVTVFDITCVCYCHKLLHEQMDYNALGTVRLAFPLFQGGQLAEKMKAIW                        | 160    |
| DsbC_YERPS   | KAP-KEKHVTVFDITCVCYCHKLLHEQMDYNALGTVRLAFPLFQGGQLAEKMKAIW                        | 160    |
| DsbC_ECOLI   | KAP-CEKHVTVFDITCVCYCHKLLHEQMDYNALGTVRLAFPLFQGGQLAEKMKAIW                        | 160    |
| DsbC_HAEIN   | KAP-KEKHVTVFDITCVCYCHKLLHEQMDYNALGTVRLAFPLFQGGQLAEKMKAIW                        | 160    |
| DsbC_VIBCH   | KAP-KEKHVTVFDITCVCYCHKLLHEQMDYNALGTVRLAFPLFQGGQLAEKMKAIW                        | 160    |
| DsbC_PSEAE   | KAP-KEKHVTVFDITCVCYCHKLLHEQMDYNALGTVRLAFPLFQGGQLAEKMKAIW                        | 160    |
| DsbC_ERWCH   | CVADRNKAFDAAMKGD-----DDVSPATCKTDIGAHQYLFGQEVNLATGQWWY                          | 216    |
| DsbC_YERPS   | CVADRNKAFDAAMKGD-----DDVSPATCKTDIGAHQYLFGQEVNLATGQWWY                          | 216    |
| DsbC_ECOLI   | CVADRNKAFDAAMKGD-----DDVSPATCKTDIGAHQYLFGQEVNLATGQWWY                          | 216    |
| DsbC_HAEIN   | CVADRNKAFDAAMKGD-----DDVSPATCKTDIGAHQYLFGQEVNLATGQWWY                          | 216    |
| DsbC_VIBCH   | CVADRNKAFDAAMKGD-----DDVSPATCKTDIGAHQYLFGQEVNLATGQWWY                          | 216    |
| DsbC_PSEAE   | CVADRNKAFDAAMKGD-----DDVSPATCKTDIGAHQYLFGQEVNLATGQWWY                          | 216    |
| DsbC_ERWCH   | YQPPKEMMAMLDAHKASLKG-----DDVSPATCKTDIGAHQYLFGQEVNLATGQWWY                      | 238    |
| DsbC_YERPS   | YQPPKEMMAMLDAHKASLKG-----DDVSPATCKTDIGAHQYLFGQEVNLATGQWWY                      | 238    |
| DsbC_ECOLI   | YQPPKEMMAMLDAHKASLKG-----DDVSPATCKTDIGAHQYLFGQEVNLATGQWWY                      | 238    |
| DsbC_HAEIN   | YQPPKEMMAMLDAHKASLKG-----DDVSPATCKTDIGAHQYLFGQEVNLATGQWWY                      | 238    |
| DsbC_VIBCH   | YQPPKEMMAMLDAHKASLKG-----DDVSPATCKTDIGAHQYLFGQEVNLATGQWWY                      | 238    |
| DsbC_PSEAE   | YQPPKEMMAMLDAHKASLKG-----DDVSPATCKTDIGAHQYLFGQEVNLATGQWWY                      | 238    |

A

E. coli
E. chrysanthemi
Y. pseudotuberculosis
H. influenzae
P. aeruginosa
V. cholerae
Fig. S1 Yield of active vtPA. DHB4 transformed with pTrcStllvtPA and pBAD derivatives encoding DsbC from *E. coli*, *H. influenzae*, and *E. chrysanthemi*. Protein synthesis was induced as described in *Materials and Methods*. Relative activities were obtained by dividing the ΔA405 (absorbance of each strain subtracted of the background consisting of a strain not expressing vtPA) by the A405 of a strain expressing vtPA alone.

Fig. S2. Effect of the expression of the deletion proteins on alkaline phosphatase activity in the periplasm of MC1000 *dsbA*. The alkaline phosphatase activity of MC1000 *dsbA* and of the parental isogenic strain MC1000 are also represented. The experiment and activity measurements were performed as described in the *Materials and Methods*.

Fig. S3 Gel filtration traces of DsbC, DsbCα1, and dsbCα2 obtained with size exclusion chromatography.

Fig. S4. *In vitro* refolding of RNAse in the presence of increasing concentration of DsbC from *E. coli* (A), *H. influenzae* (B), and *E. chrysanthemi* (C). The activity assay was performed as described in the *Materials and Methods*.

Fig. S5. Rate of insulin reduction in the presence of DsbC from different bacterial species.

Fig. S6. *In vitro* aggregation of thermally denaturated citrate synthase in the presence of increasing concentration of DsbC from *E. coli* (A), *H. influenzae* (B), and *E. chrysanthemi* (C). The experiment was performed as described in the *Materials and Methods*.

Fig. S7. *In vitro* activity of thermally denaturated citrate synthase in the presence of DsbC from *E. coli* (A), *H. influenzae* (B), and *E. chrysanthemi* (C). The activity assay was performed as described in the *Materials and Methods*. 
Figure S1

[Graph showing relative tPA activity over time for control, E. coli, H. influenzae, and E. chrysanthemi.]
Figure S2

- MC1000
dsbA
DsbC

MC1000
dsbA
Dsb\(\alpha\)1

MC1000
dsbA
Dsb\(\alpha\)2

MC1000
dsbA
Dsb\(\alpha\)3

MC1000
dsbA
Dsb\(\alpha\)4

MC1000
dsbA
Figure S3

UV (mAU)
Flow 0.5 ml/min

Superdex 200

-5 0 5 10 15 20 25 ml

-500 0 500 1000 1500 2000 2500

DsbC
DsbCa2
DsbCa4

DsbC
DsbCa2
DsbCa4

DsbCα2
DsbCα4
Figure S4

E. coli

H. influenzae
Figure S5

The figure shows the OD$_{650}$ over time for various bacterial strains:

- **control**
- **E. coli**
- **H. influenzae**
- **P. aeruginosa**
- **V. cholerae**
- **E. chrysanthemi**
- **Y. pseudotuberculosis**

The x-axis represents time in minutes (0 to 20), and the y-axis represents OD$_{650}$ values from 0.00 to 2.00.
Figure S6

A

E. coli  $K_i$ 9.5 $\pm$ 1.4

B

H. influenzae  $K_i$ 11 $\pm$ 2

C

E. chrysanthemi  $K_i$ 8.8 $\pm$ 1.1

Relative Aggregation Rate

[DsbC] (µM)
Figure S7

A

E. coli

Activity (%) vs. time (min)

B

H. influenzae

Activity (%) vs. time (min)

C

E. chrysanthemi

Activity (%) vs. time (min)
Conserved role of the linker α-helix of the bacterial disulfide isomerase DsbC in the avoidance of misoxidation by DsbB
Laura Segatori, Lori Murphy, Silvia Arredondo, Hiroshi Kadokura, Hiram Gilbert, Jon Beckwith and George Georgiou

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