Complementation of DsbA deficiency with secreted thioredoxin variants reveals the crucial role of an efficient dithiol oxidant for catalyzed protein folding in the bacterial periplasm

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The thiol/disulfide oxidoreductase DsbA is the strongest oxidant of the thioredoxin superfamily and is required for efficient disulfide bond formation in the periplasm of *Escherichia coli*. To determine the importance of the redox potential of the final oxidant in periplasmic protein folding, we have investigated the ability of the most reducing thiol/disulfide oxidoreductase, *E.coli* thioredoxin, of complementing DsbA deficiency when secreted to the periplasm. In addition, we secreted thioredoxin variants with increased redox potentials as well as the catalytic a-domain of human protein disulfide isomerase (PDI) to the periplasm. While secreted wild-type thioredoxin and the most reducing thioredoxin variant could not replace DsbA, all more oxidizing thioredoxin variants as well as the PDI a-domain could complement DsbA deficiency in a DsbB-dependent manner. There is an excellent agreement between the activity of the secreted thioredoxin variants *in vivo* and their ability to oxidize polypeptides fast and quantitatively *in vitro*. We conclude that the redox potential of the direct oxidant of folding proteins and in particular its reactivity towards reduced polypeptides are crucial for efficient oxidative protein folding in the bacterial periplasm.

Keywords: disulfide bond formation/DsbA/redox potential/disulfide oxidoreductases/thioredoxin

Introduction

Thiol/disulfide oxidoreductases are ubiquitous in prokaryotes and eukaryotes and catalyze important redox reactions in the cell (for reviews, see Gilbert, 1995; Holmgren and Björnstedt, 1995; Raina and Missiakas, 1997; Rietsch and Beckwith, 1998). These enzymes possess the thioredoxin fold as a common structural motif and a catalytic disulfide bond at the N-terminus of an α -helix with the consensus sequence Cys–Xaa–Xaa–Cys (Xaa: any amino acid) (Martin, 1995). Despite these similarities, the individual enzymes have different cellular functions. While the cytoplasmic members of this family such as thioredoxin and glutaredoxin (Grx) catalyze reductive processes such as the transfer of electrons to ribonucleotide reductase and

reduction of disulfide bonds, the members from oxidizing cellular compartments, such as protein disulfide isomerase (PDI) from the endoplasmic reticulum (ER) and DsbA and DsbC from the bacterial periplasm, are catalysts of disulfide bond formation during folding of secretory proteins. There is a remarkable correlation between the standard redox potentials (E_o') of these enzymes and their physiological role, in that the members with the lowest redox potentials catalyze reductive processes *in vivo* [thioredoxin: -270 mV (Lin and Kim, 1989; Krause *et al.*, 1991; Mössner *et al.*, 1998); Grx: -233 to -198 mV (Åslund *et al.*, 1997)] while the protein folding catalysts are strong oxidants [PDI: -147 to -175 mV (Lundström and Holmgren, 1993; Darby and Creighton, 1995a); DsbC: -130 mV (Zapun *et al.*, 1995); DsbA: -122 mV (Wunderlich and Glockshuber, 1993a; Zapun *et al.*, 1993; Huber-Wunderlich and Glockshuber, 1998)].

In the periplasm of *Escherichia coli*, catalysis of disulfide bond formation and isomerization is performed by at least two different enzymes. While DsbA is the predominant dithiol oxidant in the periplasm which randomly and rapidly introduces disulfide bonds into folding polypeptides (Wunderlich *et al.*, 1993; Zapun *et al.*, 1994), DsbC constitutes the main periplasmic disulfide isomerase activity (Missiakas *et al.*, 1994; Zapun *et al.*, 1995). Reoxidation of DsbA occurs via disulfide exchange with the inner membrane protein DsbB (Bardwell *et al.*, 1993; Missiakas *et al.*, 1994; Guilhot *et al.*, 1995; Kishigami *et al.*, 1995a; Kishigami and Ito, 1996), which itself appears to be reoxidized indirectly or directly by molecular oxygen (Kobayashi *et al.*, 1997; Bader *et al.*, 1998). The catalytically active, reduced state of DsbC is maintained by DsbD (DipZ), another protein of the inner membrane, which in turn is kept in the reduced state by cytoplasmic thioredoxin (Missiakas *et al.*, 1994; Rietsch *et al.*, 1996, 1997). The DsbA–DsbB and DsbC–DsbD redox systems appear to be independent of each other as disulfide exchange between DsbA and DsbC is very slow and deletions of the *dsbA* or the *dsbB* genes do not alter the redox state of DsbC in the periplasm (Zapun *et al.*, 1995; Rietsch *et al.*, 1997). Two other periplasmic disulfide oxidoreductases are known in *E.coli*, DsbE and DsbG. DsbE (also called CcmG) is membrane anchored and important for cytochrome *c* biogenesis (Fabianek *et al.*, 1998). DsbG is a soluble periplasmic protein with homology to DsbC (Andersen *et al.*, 1997; van Straaten *et al.*, 1998). Its biological function is not yet fully understood.

Although DsbA is the strongest oxidant of all thiol/disulfide oxidoreductases ($E_o' = -122$ mV), the *in vivo* importance of the high redox potential of DsbA is not entirely clear. In particular, all the more reducing DsbA variants investigated so far, with redox potentials as low as -220 mV, were capable of complementing DsbA deficiency (Grauschopf *et al.*, 1995; Hennecke *et al.*,

1999), indicating that the redox potential of DsbA may be less critical than initially assumed. This impression is supported further by the recent finding that secretion of the most reducing thiol/disulfide oxidoreductase, *E.coli* thioredoxin, into the periplasm of a *dsbA*⁻ strain could recover 6–8% of the DsbA-dependent oxidative folding of alkaline phosphatase in the periplasm, and even ~40% of alkaline phosphatase folding in a *dsbA*⁻*dsbC*⁻*dsbD*⁻ strain (Debarbieux and Beckwith, 1998).

The aim of this study was to clarify the *in vivo* importance of the redox potential of the catalyst that acts as the final oxidant of polypeptides in the bacterial periplasm, as well as the rates at which the catalyst directly oxidizes folding proteins. For this purpose, we tested the ability of wild-type thioredoxin and four thioredoxin variants with increased redox potentials (E_0' range: –222 to –195 mV) that harbor the Xaa–Xaa dipeptides from the active sites of DsbA, PDI, Grx and thioredoxin reductase (TR) (Mössner *et al.*, 1998) to replace DsbA as oxidant during protein folding *in vivo*. Similarly, we tested the ability of the catalytic a-domain of human PDI (residues 1–120; $E_0' = -147$ mV) to complement the activity *in vivo*. In contrast to wild-type thioredoxin and the TR-type thioredoxin variant, the three most oxidizing thioredoxin variants and the PDI a-domain can substitute for DsbA. Moreover, the biologically active thioredoxin variants and the PDI a-domain are recycled as oxidants by DsbB *in vivo*. We found excellent agreement between the *in vivo* activity of the thioredoxin variants and their activities in oxidative protein folding experiments *in vitro*. Our results demonstrate that the efficiency with which the final catalyst of disulfide bond formation oxidizes polypeptides is crucial for oxidative protein folding in the bacterial periplasm.

Results

Secretion via the DsbA signal sequence yields authentic thioredoxin and the authentic PDI a-domain in the periplasm

In order to achieve secretion of *E.coli* thioredoxin ($E_0' = -270$ mV) and variants thereof into the *E.coli* periplasm, we constructed exact fusions between the thioredoxins and the DsbA signal sequence and expressed the constructs under control of the *trc* promoter/*lac* operator. SDS–PAGE analysis (Figure 1A) shows that thioredoxin overproduced with the secretion construct is found exclusively in the periplasmic fraction and has the same apparent molecular mass as natural thioredoxin purified from the cytoplasm. The correct cleavage of the DsbA signal sequence by signal peptidase I was proven by Edman degradation of secreted thioredoxin which yielded the authentic N-terminus of the cytoplasmic protein (see Materials and methods). A control experiment in which thioredoxin was overexpressed in the cytosol without the signal sequence showed that ~90% of the protein remained in the cytosolic fraction (Figure 1A), and that only a small amount (~10%) was found in the periplasmic fraction. Such a partial extraction of cytoplasmic thioredoxin into the periplasmic fraction has been reported previously (Lunn and Pigiet, 1982; Ajouz *et al.*, 1998).

The human PDI a-domain was fused to the DsbA signal sequence in exactly the same way as thioredoxin, and was

Fig. 1. Transport to the periplasm of thioredoxin and levels of the periplasmically expressed thioredoxin variants, the PDI a-domain and wild-type DsbA. Coomassie-stained SDS–polyacrylamide gels (15% w/v) are shown. (A) Wild-type thioredoxin fused to the DsbA signal sequence is transported to the periplasm and correctly processed to the authentic protein. Lane 1, purified thioredoxin; lanes 2 and 3, periplasmic extracts of *E.coli* BL21(DE3) expressing thioredoxin without a signal sequence in the cytoplasm and cells expressing thioredoxin with DsbA signal sequence (lanes 4 and 5) before (lanes 2 and 4) and 4 h after (lanes 3 and 5) induction with IPTG; lanes 6–9, same as 2–5, but the corresponding spheroblast fractions with all cytoplasmic proteins; S, molecular mass standard. (B) Secretory expression of the thioredoxin variants under control of the *trc* promoter/*lac* operator in the *dsbA*⁻ strain THZ2 in the absence of IPTG. Periplasmic extracts of cells grown at 37°C to the mid-log phase in DYT medium are shown. Lane 1, purified thioredoxin; lane 2, THZ2 without plasmid, lanes 3–7, THZ2 expressing wild-type thioredoxin (lane 3), PDI-type thioredoxin (lane 4), DsbA-type thioredoxin (lane 5), TR-type thioredoxin (lane 6) and Grx-type thioredoxin (lane 7); S, molecular mass standard. (C) Comparison of expression levels of secreted thioredoxin, the secreted PDI a-domain (arrow) and DsbA in THZ2 at 37°C in the absence of IPTG (DYT medium). Periplasmic extracts as described in (B) are shown. Lane 1, purified DsbA; lane 2, secreted DsbA in the periplasm; lane 3, secreted PDI a-domain; lane 4, secreted wild-type thioredoxin; lane 5, purified thioredoxin; S, molecular mass standard. Quantitative densitometric evaluation of the band intensities yielded $6.5 \pm 2.2 \times 10^4$ DsbA molecules and $2.9 \pm 0.5 \times 10^4$ secreted thioredoxin molecules per *E.coli* cell (a Coomassie-stained band of DsbA is 6-fold more intense than a band with equimolar quantities of thioredoxin).

also obtained in a soluble and processed form in the periplasm. Edman sequencing of the band corresponding to the processed PDI a-domain after SDS-PAGE (cf. Figure 1C) again proved the correct cleavage of the DsbA signal peptide, yielding the natural N-terminus of human PDI.

Only the most oxidizing thioredoxin variants and the PDI a-domain can complement DsbA deficiency

The relative expression levels of all secreted thioredoxin variants and of the secreted PDI a-domain were analyzed in the *dsbA*⁻ strain THZ2 in both rich (DYT) and cysteine- and cystine-free M63 medium in the absence of the inducer isopropyl- β -D-thiogalactopyranoside (IPTG) at 37°C. These growth conditions were used in all subsequent DsbA complementation experiments (see below). Figure 1B and C shows that wild-type thioredoxin, all thioredoxin variants and the PDI a-domain are expressed in comparable amounts in the periplasm, while DsbA, produced with the analogous expression construct (pDsbA3) is obtained in ~2-fold higher amounts.

The ability of the thioredoxin variants to complement DsbA deficiency in DYT rich medium and thiol/disulfide-free M63 medium was analyzed by two phenotypic characteristics, i.e. a blue/white screening assay that is based on oxidative inactivation of periplasmically oriented β -galactosidase (Froshauer *et al.*, 1988; Bardwell *et al.*, 1993) and a motility assay based on the DsbA-dependent folding of the P-ring subunits from the flagellar motor (Dailey and Berg, 1993). In both of the *dsbA*⁻ strains THZ2 and JCB817, the ability of the bacteria to swarm on top of a soft agar plate and form a bacterial lawn was restored by plasmid-encoded periplasmic expression of DsbA, the PDI a-domain and the three most oxidizing thioredoxin variants with the Xaa-Xaa dipeptide sequences of Grx, DsbA and PDI (Figure 2). In contrast, motility according to the above criteria could not be restored with wild-type thioredoxin and the most reducing, TR-like thioredoxin variant (Figure 2).

Analogous results were obtained with complementation experiments in THZ2 and JCB817 using the blue/white screening assay that probes the introduction of inactivating disulfide bonds into periplasmically oriented β -galactosidase (Table I).

Secreted thioredoxin is recycled as oxidant by DsbB

Importantly, all complementing thioredoxin variants and the PDI a-domain are directly or indirectly recycled as oxidants by the inner membrane protein DsbB *in vivo*, as evidenced by their inability to restore disulfide bond formation in the *dsbB*⁻ mutant JCB819 and the *dsbAB*⁻ double mutant JCB818. The oxidation of thioredoxin by DsbB could also be confirmed *in vitro* with a fluorescence assay that has recently been established for the oxidation of DsbA by DsbB (Bader *et al.*, 1998). Reduced wild-type thioredoxin was not oxidized when incubated with membrane preparations of the *dsbB*⁻ strain JCB819, whereas membrane fractions of JCB819 transformed with a DsbB expression plasmid efficiently catalyzed the oxidation of thioredoxin (Figure 3A). The latter reaction was performed at different initial concentrations of reduced

Fig. 2. Recovery of motility of the *dsbA*⁻ strain JCB817 by thioredoxin variants, the PDI a-domain and DsbA secreted into the periplasm. *Escherichia coli* JCB817 cells harboring the corresponding expression plasmids were placed in the center of DYT or thiol/disulfide-free M63 soft agar plates and incubated for exactly 24 h at 37°C in the absence of IPTG. Cells with the DsbA⁺ phenotype spread over the culture plate and form a bacterial lawn, and cells lacking DsbA only grow at the point of inoculation. The lengths of the bars give the mean values from five experiments of the colony diameters (in percent) relative to that of JCB817 harboring the analogous expression plasmid for DsbA (pDsbA3). The colony diameter of JCB817/pDsbA3 after 24 h was exactly the same as that of the isogenic *dsbA*⁺ strain JCB816 grown under these conditions, demonstrating that the 65-fold overexpression of DsbA in JCB817/pDsbA3 had no effect on motility. When wild-type thioredoxin or the TR-like thioredoxin variant were secreted into the periplasm of JCB817, cells were not capable of forming a bacterial lawn. Their colony sizes were, however, reproducibly larger than that of JCB818 and had the appearance of several single colonies. The maximum diameter of these colonies was always <5% of the diameter of JCB817/pDsbA3.

thioredoxin and yielded an apparent K_m value of $20 \pm 7 \mu\text{M}$. Control experiments with reduced DsbA (Figure 3B) yielded a similar value ($13 \pm 2 \mu\text{M}$) for DsbA, which is in good agreement with the value of $10 \mu\text{M}$ reported by Bader *et al.* (1998).

Surprisingly, wild-type DsbA expressed via pDsbA3 proved able to restore motility of the *dsbB*⁻ strains JCB819 and JCB818 in DYT medium and in the absence of IPTG. However, the motility of the strains transformed with pDsbA3 was abolished in the presence of the catabolite repressor glucose (10 g/l). Obviously, the *trc* promoter/*lac* operator system in pDsbA3 is rather leaky, already leading to high periplasmic DsbA concentrations in the absence of IPTG. A quantitative densitometric analysis of the levels of plasmid-encoded DsbA and thioredoxin in the periplasm yielded values of $6.5 \pm 2.2 \times 10^4$ DsbA molecules and $2.9 \pm 0.5 \times 10^4$ thioredoxin molecules per *E. coli* cell. This corresponds to a 65- and 29-fold overexpression, respectively, compared with $\sim 10^3$ mole-

Table I. *In vivo* complementation of DsbA by thioredoxin variants in the DsbA-deficient *E.coli* strain JCB817 and *in vitro* properties of the thioredoxin variants

Variant	Xaa-Xaa dipeptide	E_0^{a} (mV)	Complementation of DsbA ^b (blue/white screen)	$t_{1/2}$ of hirudin refolding ^c (min)
Wild-type DsbA	Pro-His	-122	+	3.0
PDIA	Gly-His	-147 ^d	+	ND
Grx-type Trx	Pro-Tyr	-195	+/-	0.6
DsbA-type Trx	Pro-His	-204	+	0.6
PDI-type Trx	Gly-His	-221	+	1.3
TR-type Trx	Ala-Thr	-222	-	25
Wild-type Trx	Gly-Pro	-270	-	1200 ^e

^aFrom Mössner *et al.* (1998).^bcf. Materials and methods for experimental conditions.^cExperimental error was $\leq 10\%$ in all measurements.^dDetermined at pH 7.4 (Kortemme *et al.*, 1996).^eApparent half-life of refolding, using the initial velocity of formation of native hirudin and assuming 100% yield of refolding. **Fig. 3.** Reduced thioredoxin is a substrate of DsbB. (A) Oxidation of reduced wild-type thioredoxin (7.0 μM) catalyzed by a DsbB-containing membrane preparation from JCB819/pDsbB-DsbA at pH 8.0 and 25°C. The reaction was started by addition of reduced thioredoxin to the membrane preparation and followed by the decrease in the specific thioredoxin fluorescence at 345 nm. (1) Control: reduced thioredoxin mixed with a membrane preparation of the *dsbB* null strain JCB819; (2) reduced thioredoxin mixed with the equivalent amount of a membrane fraction of JCB819 transformed with the DsbB expression plasmid pDsbB-DsbA; (3) membrane fraction from (2) alone. (B) Same as (A), but with 4.6 μM reduced DsbA instead of 7.0 μM reduced thioredoxin.

cules of DsbA in the K12 wild-type strain W3110 (see Materials and methods and Akiyama *et al.*, 1992) (Figure 1C). A plausible explanation for the observed activity of overexpressed DsbA in JCB819 and JCB818 appears to be air oxidation of a small fraction of DsbA, generating sufficient amounts of the oxidized protein to allow complementation. Indeed, the motility in the absence of IPTG

and glucose could be almost fully repressed when the motility assay was performed under anaerobic conditions. The 65-fold overexpression of DsbA with the plasmid pDsbA3 had no influence on the DsbA⁺ phenotype in the motility assay, as colonies of JCB817/pDsbA3 and the isogenic, non-transformed *dsbA*⁺ strain JCB816 reached exactly the same diameters on DYT soft agar plates after 24 h at 37°C.

Only the most oxidizing thioredoxin variants are efficient dithiol oxidants during folding of the model substrate hirudin

The ability to oxidize polypeptides and the disulfide isomerase activity of wild-type thioredoxin and its more oxidizing active site variants were then compared *in vitro* with the activities of wild-type DsbA, using the thrombin inhibitor hirudin as substrate. Hirudin, a 65 residue protein with three disulfide bonds in the native state, is a well established model substrate of DsbA (Wunderlich *et al.*, 1993, 1995; Hennecke *et al.*, 1999). Completely reduced and unfolded hirudin was mixed with three molar equivalents of oxidized DsbA, oxidized wild-type thioredoxin or the respective oxidized thioredoxin variant at pH 7.0, and the reaction was analyzed by reversed-phase HPLC separation of the disulfide-bonded hirudin folding intermediates after quenching of the reactions at different times with acid (Figure 4).

DsbA quantitatively and randomly oxidizes hirudin, followed by catalysis of disulfide isomerization (Wunderlich *et al.*, 1993). At pH 7.0, a half-life of 3 min was measured for the formation of native hirudin (Figure 4). In contrast, wild-type thioredoxin proved to be an extremely poor stoichiometric oxidant of hirudin. After a reaction time of 1 h, the main fraction of hirudin was still in the completely reduced state, and <10% of all hirudin molecules were in the native disulfide conformation (Figure 4). The most reducing, TR-type thioredoxin variant which, like wild-type thioredoxin, failed to complement DsbA deficiency *in vivo* only showed a slightly higher activity as oxidant (25 min half-life of hirudin refolding). In contrast, the three most oxidizing thioredoxin variants that restored the DsbA⁺ phenotype oxidized hirudin very efficiently *in vitro*. Specifically, refolding of hirudin by oxidation with the Grx-type, the DsbA-type and the PDI-type thioredoxin variants proved to be even faster than

Fig. 4. HPLC analysis of oxidative folding of hirudin (three disulfide bonds in the native state) at pH 7.0 and 25°C, initiated by stoichiometric oxidation of completely reduced hirudin with three molar equivalents of oxidized DsbA, oxidized wild-type thioredoxin or the oxidized thioredoxin variants. The reaction was performed in 0.1 M sodium phosphate pH 7.0, 0.2 M KCl, 1 mM EDTA, with an initial concentration of 28 μ M for reduced hirudin and 84 μ M for the corresponding disulfide oxidoreductase. Aliquots of the reactions were removed at the times indicated, disulfide exchange was quenched by addition of formic acid (final pH <2) and the hirudin folding intermediates were separated at 55°C on a Vydac 218TP54 C18 reversed-phase HPLC column using a gradient from 20 to 24% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid. The absorbance of the eluate was monitored at 230 nm. The dashed lines labeled R and N denote the HPLC retention times of fully reduced and native hirudin, respectively.

refolding with wild-type DsbA, with five times faster hirudin folding ($t_{1/2} = 0.6$ min) compared with DsbA ($t_{1/2} = 3$ min) for the Grx- and the DsbA-type variants (Figure 4, Table I).

Figure 4 also shows that the distribution of hirudin folding intermediates in the presence of the thioredoxin variants is clearly different from the pattern of folding intermediates observed in the presence of DsbA. As the detectable intermediates in the reaction with DsbA after 20 s exclusively comprise fully oxidized, three-disulfide intermediates (Wunderlich *et al.*, 1993), the hirudin inter-

mediates with longer HPLC retention times observed in the reactions with the thioredoxin variants must be one- and two-disulfide species. Thus, hirudin folding in the presence of the thioredoxin variants involves consecutive formation and isomerization of disulfide bonds, where non-native three-disulfide species are obviously much less populated. In contrast, hirudin refolding by stoichiometric oxidation with DsbA essentially starts with the mixture of all 15 possible three-disulfide species. The consecutive formation of disulfide bonds in the presence of the thioredoxin variants thus appears to improve the folding

Fig. 5. HPLC analysis of the reaction of native, oxidized hirudin with three molar equivalents of reduced wild-type thioredoxin or the glutaredoxin-type thioredoxin variant at pH 7.0 and 25°C. Native hirudin was incubated with reduced wild-type thioredoxin or the variant at 25°C at pH 7.0 under the same conditions as described in Figure 4. The lower panel shows a control experiment with fully reduced hirudin in the absence of enzyme, proving that no air oxidation occurred during the experiment.

process of hirudin relative to an immediate random oxidation followed by disulfide isomerization. Overall, there is an excellent correlation between the redox potentials of the thioredoxin variants, their ability to complement the DsbA⁻ phenotype and their reactivity towards reduced polypeptides *in vitro*.

In contrast to the stoichiometric reaction with DsbA, significant amounts of fully reduced hirudin and its one- and two-disulfide intermediates were still present after 1 h when the oxidation was performed with wild-type thioredoxin and the thioredoxin variants (Figure 4). As the HPLC profiles after 30 min and 1 h did not differ in any of the refolding reactions (Figure 4), we investigated whether the profiles after 1 h corresponded to the equilibrium states of the reactions. For this purpose, we analyzed the reverse reaction, i.e. the reduction of native, fully oxidized hirudin with three molar equivalents of reduced wild-type thioredoxin or its most oxidizing Grx-like variant. Indeed, after 1 h of incubation, practically the same HPLC profiles were observed as for hirudin oxidation (Figure 5). It follows that wild-type thioredoxin is not only a poor oxidant of hirudin, but also is such a strong reductant that the native disulfide bonds in hirudin simply cannot be formed completely under the applied *in vitro* conditions. This is in agreement with our finding that secreted wild-type thioredoxin cannot complement DsbA deficiency. As expected from its higher redox potential, the Grx-like thioredoxin variant proved to be a poor reductant of hirudin, and ~80% of all hirudin molecules stayed fully oxidized after treatment with the reduced variant for 1 h (Figure 5). To exclude the possibility that

unspecific air oxidation interfered with the analysis of hirudin oxidation, reduced hirudin alone was incubated in the reaction buffer. No air oxidation was detectable within 1 h (Figure 5).

Discussion

Disulfide bond formation in the bacterial periplasm requires the flow of electrons from the thiols of a folding polypeptide through DsbA to the membrane protein DsbB, and then possibly via components of the respiratory chain to molecular oxygen as final electron acceptor (Kobayashi *et al.*, 1997; Bader *et al.*, 1998; Rietsch and Beckwith, 1998). As DsbA is a catalyst of electron transfer which is oxidized and reduced continuously, its *in vivo* function should in principle be independent of its intrinsic redox potential as long as the rate-limiting step of catalysis is not affected significantly. This may either be the oxidation of the folding polypeptide or the recycling of oxidized DsbA. As a lowered redox potential should increase rather than decrease the rate of DsbA oxidation by DsbB, one would expect that a more reducing redox potential can affect the *in vivo* function of DsbA mainly through a decreased rate of polypeptide oxidation.

As even the most reducing known DsbA variant with a redox potential of -220 mV can still complement DsbA deficiency (Grauschopf *et al.*, 1995), we addressed the question of the *in vivo* relevance of the redox potential of the enzyme that acts as final disulfide bond donor to folding polypeptides by secreting the most reducing thiol/disulfide oxidoreductase thioredoxin and variants thereof

($E'_0 = -270$ to -195 mV) into the *E. coli* periplasm. In accordance with the above consideration, we find that only wild-type thioredoxin and its TR-like variant failed to complement DsbA deficiency. Both proteins are the poorest oxidants during *in vitro* folding of the model protein hirudin and also have the lowest redox potentials of all secreted proteins investigated (-270 and -222 mV, respectively). In contrast, all more oxidizing thioredoxin variants (-221 to -195 mV) oxidized hirudin efficiently *in vitro* and restored the DsbA⁺ phenotype in *dsbA*⁻ strains. The same was accomplished by the secreted a-domain of human PDI, which is also an efficient oxidant. We conclude that the ability to oxidize folding polypeptides rapidly is the crucial property of the final catalyst of disulfide bond formation in the *E. coli* periplasm.

Regarding our *in vitro* experiments with hirudin, this ability is in part a direct consequence of the catalyst's redox potential. The stabilities of structural disulfide bonds in proteins vary over an enormous range, i.e. their equilibrium constants with glutathione (K_{eq}) have values between 1 and 10^7 M (Gilbert, 1990). In the absence of excess oxidant, quantitative stoichiometric oxidation of a substrate protein by a disulfide oxidoreductase can only occur when the structural disulfide bond in the protein is more stable than the catalytic disulfide bond of the enzyme. This appears to be no longer the case for the system hirudin-wild-type thioredoxin where the equilibrium is mainly on the side of reduced hirudin and oxidized thioredoxin (Figures 3 and 4). The equilibrium is, however, clearly on the side of native, oxidized hirudin when the more oxidizing thioredoxin variants are used as oxidants ($K_{eq} = 0.027$ – 0.24 M, compared with 11 M for wild-type thioredoxin). This is not in contrast to the fact that wild-type thioredoxin can function principally as a protein folding catalyst in the presence of glutathione redox buffers *in vitro* (Pigiet and Schuster, 1986) and thioredoxin reductase-deficient *E. coli* strains *in vivo* (Derman *et al.*, 1993), as an excess of, for example, a low molecular weight disulfide or molecular oxygen, can shift the overall equilibrium towards the native, fully oxidized protein.

The redox potential of the catalyst of disulfide bond formation in the periplasm is certainly not the only important factor that determines efficient disulfide bond formation *in vivo*. This is already obvious from the different *in vivo* activities of the TR-type and PDI-type variants of thioredoxin which have almost identical redox potentials (Table I). Regarding the catalytic cycle of the enzyme that catalyzes disulfide bond formation in the periplasm, the question arises of whether its reoxidation by DsbB or its reaction with reduced polypeptides is limiting for oxidative protein folding *in vivo*. As even secreted wild-type thioredoxin, which was not capable of complementing DsbA deficiency according to our criteria, is recycled by DsbB with rates and K_m values similar to those measured for DsbA and, like DsbA, is essentially oxidized *in vivo* (Kishigami *et al.*, 1995b; Debarbieux and Beckwith, 1998), it appears that the rate of polypeptide oxidation was the limiting factor for disulfide bond formation in all our complementation studies. Indeed, wild-type thioredoxin and its TR-like variant, which both were inactive *in vivo*, were by far the least efficient oxidants of hirudin *in vitro* compared with all the thioredoxin variants that were active *in vivo* (Figure 4, Table I). We

thus conclude that the slow rate of polypeptide oxidation by wild-type thioredoxin prevents the complementation of DsbA deficiency by these proteins in the *E. coli* periplasm.

The fact that the thioredoxin variants were recycled as oxidants by DsbB *in vivo* suggests that DsbB is rather promiscuous with respect to possible substrate enzymes, even though it interacts with DsbA with a K_m of ~ 10 μ M (Bader *et al.*, 1998). Moreover, the DsbA⁺ phenotype in *E. coli* is not only restored by DsbA homologs from many other bacteria (Rodríguez-Peña *et al.*, 1997, and references therein), but also by rat and human PDI when secreted into the periplasm (Humphreys *et al.*, 1995; Ostermeier *et al.*, 1996). In accordance with the complementing activity of the human PDI a-domain, DsbA complementation by full-length human PDI was also DsbB dependent (Ostermeier *et al.*, 1996). The activity of the catalytic PDI a-domain in the periplasm is also consistent with the function of DsbA as primary oxidant in the periplasm, since the multidomain architecture of eukaryotic PDI is only required for its disulfide isomerase activity (Kemink *et al.*, 1997), whereas this task is fulfilled by DsbC in *E. coli*. The relatively low specificity of DsbB raises the question of why it does not oxidize DsbC and how the DsbA–DsbB and DsbC–DsbD redox systems can function independently. As the active sites of DsbA, thioredoxin and the PDI a-domain have comparable solvent accessibilities (Katti *et al.*, 1990; Kemink *et al.*, 1996; Guddat *et al.*, 1998), the simplest explanation would be a steric hindrance of the attack of the active site in DsbC by DsbB. Clearly, the determination of the three-dimensional structure of DsbC is required to answer the intriguing question of how the periplasmic redox systems can co-exist.

Our experiments are in principle similar to the DsbA complementation study performed with secreted wild-type thioredoxin in the laboratory of J. Beckwith. Debarbieux and Beckwith have re-examined their initial observation that thioredoxin was not reoxidized by DsbB (Debarbieux and Beckwith, 1998) and could now also confirm our result that DsbB can recycle thioredoxin as an oxidant (L. Debarbieux and J. Beckwith, personal communication). The small recovery of motility and folding of alkaline phosphatase in the periplasm reported for wild-type thioredoxin secreted with the *phoA* signal sequence (Debarbieux and Beckwith, 1998) may correspond to the small apparent motility due to clusters of single colonies that we observed in the assays with secreted wild-type thioredoxin (cf. legend of Figure 2). As secretion of wild-type thioredoxin in *dsbA*-deficient strains did not, however, restore formation of a bacterial lawn on soft agar plates (Figure 2), we defined wild-type thioredoxin and its TR-like variant as non-complementing. Other reasons for the small differences between our results and those of Debarbieux and Beckwith may be the different signal sequences used for thioredoxin secretion and different levels of mature thioredoxin in the periplasm.

In contrast to the bacterial periplasm where disulfide bond formation and isomerization are catalyzed by DsbA and DsbC, respectively, a single enzyme, PDI, is responsible for both activities in the ER of eukaryotes (Freedman *et al.*, 1995). These activities are essential for yeast (Laboisserie *et al.*, 1995; Frand and Kaiser, 1998; Pollard *et al.*, 1998). The question of the importance of redox

potential for the catalyst of oxidative protein folding in the ER has been addressed by an approach similar to that used in our study. After random mutagenesis of the Xaa–Xaa sequence in *E.coli* thioredoxin, the resulting variants were tested for their ability to complement *PDI* null mutants of *Saccharomyces cerevisiae* (Chivers *et al.*, 1996). As in our study on DsbA complementation in *E.coli*, only thioredoxin variants with increased redox potentials (–235 to –200 mV) could functionally replace PDI, whereas wild-type thioredoxin failed to complement PDI deficiency (Chivers *et al.*, 1996). Conversely, random mutagenesis of the Xaa–Xaa sequence of PDI yielded *S.cerevisiae* mutants with decreased rates of protein folding in the ER (Holst *et al.*, 1997). Overall, the data on the functional replacement of the direct oxidant of secretory proteins by more oxidizing thioredoxin variants in the bacterial periplasm and in the eukaryotic ER are in agreement. This supports the emerging view that the flow of oxidizing equivalents to folding polypeptides may indeed be similar in bacteria and eukaryotes. Specifically, the yeast glycoprotein Ero1p which is associated with the ER membrane and essential for disulfide bond formation in the ER may constitute the functional equivalent of bacterial DsbB (Fränd and Kaiser, 1998; Pollard *et al.*, 1998). Moreover, despite the high relative concentrations of oxidized glutathione in the ER (Hwang *et al.*, 1992), disulfide bond formation in the ER is independent of glutathione (Fränd and Kaiser, 1998). Thus, as in the bacterial periplasm that lacks oxidized glutathione and other low molecular weight redox compounds, disulfide bond formation and isomerization appear to occur exclusively by disulfide exchange reactions with enzymes. In this context, it is not surprising that similar redox potentials of secreted thioredoxin variants are required to achieve complementation of DsbA deficiency in *E.coli* and PDI deficiency in yeast.

Using the stoichiometric oxidation of hirudin by the different thioredoxin variants as a model reaction for thioredoxin-dependent disulfide bond formation in the periplasm, we found that folding in the presence of the three most oxidizing variants was significantly faster than in the presence of DsbA (Figure 4). HPLC analysis of the distribution of the hirudin folding intermediates (Figure 4) showed that hirudin folding through oxidation by the thioredoxin variants is characterized essentially by consecutive formation of disulfide bonds, whereas DsbA immediately and randomly oxidizes hirudin completely so that intramolecular disulfide isomerization cannot occur without intermolecular attack by a reductant. Indeed, extremely rapid and random oxidation of substrates appears to be the main ‘disadvantage’ of DsbA for folding of proteins with multiple disulfide bonds. The high disulfide isomerase activity of DsbC relative to that of DsbA (Darby *et al.*, 1998) thus ideally compensates for this ‘disadvantage’ in the periplasm. We cannot judge from the HPLC profiles in Figure 4 whether faster hirudin folding in the presence of the most oxidizing thioredoxin variants is a consequence of disulfide isomerization catalyzed by the variants or simply due to uncatalyzed, intramolecular disulfide rearrangements that become possible through slower oxidation. However, it is very likely that at least the PDI-like thioredoxin variant with the Xaa–Xaa sequence Gly–His is as catalytically active as

disulfide isomerase, because the same variant proved to complement PDI deficiency in yeast (Chivers *et al.*, 1996), where the isomerase activity of PDI is essential (Laboisserie *et al.*, 1995). Thus, the three most oxidizing thioredoxin variants analyzed in this study may become useful tools for future applications in biotechnology as *in vitro* folding catalysts.

In summary, the reactivity towards reduced polypeptides of the final oxidant during folding of secretory proteins is crucial for efficient disulfide bond formation in the bacterial periplasm. The excellent agreement between the ability of thioredoxin variants with increased redox potential to complement DsbA deficiency in *E.coli* and PDI deficiency in yeast provides further evidence that the principles underlying the electron flow from the reduced polypeptide through a series of specialized proteins to the final oxidant of disulfide bond formation may be similar in bacteria and eukaryotes.

Materials and methods

Materials

5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB), 1,4-dithio-DL-threitol (DTT) and 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-Gal) were from Sigma (Deisenhofen, Germany). IPTG was purchased from Biomol (Hamburg, Germany), and the Vydac™ 218TP54 HPLC column was from Vydac (Hesperia, USA). HPLC-purified oligodeoxynucleotides were purchased from EuroGentech (Seraing, Belgium). Restriction enzymes were obtained from New England Biolabs (Schwalbach, Germany) and Axon Lab (Baden, Switzerland). Anti-DsbA rabbit antibodies were obtained from Drs Rooskopf and Fraefel, Zurich, Switzerland. All other chemicals were from Merck (Darmstadt, Germany) and of the highest purity available. Recombinant hirudin was kindly provided by R.Seckler (University of Potsdam, Germany). The following *E.coli* strains were kindly provided by T.Zander and J.C.A. Bardwell (University of Michigan): THZ2 (*dsbA::kan*, *recA::cam*, λ malFlacZ-102) (see also Jacobi *et al.*, 1997), JCB816 (JCB570 λ malFlacZ-102) [JCB570 is MC1000 *phoR*-, *zih12::Tn10tetR* (Bardwell *et al.*, 1993)], JCB817 (JCB571 λ malFlacZ-102) [JCB571 is MC1000 (Bardwell *et al.*, 1993) *phoR*-, *zih12::Tn10tetR*, *dsbA::kan1* (Grauschopf *et al.*, 1995)], JCB818 (JCB758 λ malFlacZ-102) (JCB758 is MC1000 *phoR*-, *zih12::Tn10tetR*, *dsbA::kan1*, *dsbB::kan*) and JCB819 [JCB789 λ malFlacZ-102) (JCB789 is MC1000 *phoR*-, *zih12::Tn10tetR*, *dsbB::kan* (Bader *et al.*, 1998)].

Construction of plasmids for periplasmic expression of the thioredoxin variants and the PDI a-domain

Molecular cloning techniques were based on Sambrook *et al.* (1989). The plasmid pDsbA3 is a derivative of pDsbA2 (Jacobi *et al.*, 1997) and was obtained by introduction of a singular *NheI* restriction site at the end of the DsbA signal sequence without changing the amino acid sequence (Hennecke *et al.*, 1999). The genes of wild-type thioredoxin and its active site variants were amplified by PCR from the respective plasmids for cytoplasmic expression (Mössner *et al.*, 1998) with the following primers: N-terminal primer, 5'-CGCATGGAATTCGCTAGC-GCGAGCGATAAAATTATTC-3'; C-terminal primer, 5'-GCCGGAATTCGGATCCTTACGCCAGGTTAGCGT C-3'. The N-terminal primer lacks the methionine start codon of thioredoxin because the N-terminal methionine is cleaved off in the natural protein. The gene coding for the human PDI a-domain (Asp1–Ala120) was amplified from the plasmid pET12PDI (Darby and Creighton, 1995b) with the following primers: N-terminal primer, 5'-CGGCATGGAATTCGCTAGCGCGGACGCC-CCGAGGAGG-3'; C-terminal primer, 5'-GCCGGAATTCGGATCCTT-AGGCAGCCGGGCCCGTGC-3'. All amplified genes were cloned into pDsbA3 behind the genetic sequence of the DsbA signal peptide via the single *NheI* and *BamHI* restriction sites after removal of the segment coding for mature DsbA with the same enzymes. All cloned genes were verified by complete dideoxy sequencing. In the resulting plasmids for secretory expression, all genes coding for the mature proteins are fused to the DsbA signal sequence and are under control of the *trc* promoter/*lac* operator.

For construction of a DsbB expression plasmid, the *dsbB* gene was amplified from the genome of *E.coli* W3110 (Bachmann, 1972) with the

following primers: N-terminal primer, 5'-GCGCACTCTATTCTAG-ATGCAGGGAAATGATTATGTTGCGAT-3'; C-terminal primer, 5'-CC-GCAGGAGCGATTCAAGCTTAGCGACCGAACAGATCACGTTT-3'. The amplified gene was digested with *Xba*I and *Hind*III and cloned into the vector pRBI-PDI (Wunderlich and Glockshuber, 1993b) that had been cut with the same enzymes. The resulting vector pDsbB-DsbA contains a dicistronic operon for co-expression of DsbB and DsbA under *lac* promoter/*lac* operator control. Expression of functional DsbB was verified by complementation of DsbB deficiency of *E. coli* JCB819 as described below.

The complete nucleotide sequences of all newly constructed plasmids described here have been deposited on our website (<http://odin.ethz.ch/glockshuber/>).

In vivo complementation assays

Complementation assays were performed in both DYT rich medium (Sambrook *et al.*, 1989) and M63 minimal medium (Rietsch *et al.*, 1996) supplemented with glucose (2 g/l) as carbon source and all free amino acids (50 mg/l each) except cysteine, cystine and methionine. Complementation of DsbA deficiency was assayed by oxidative inactivation of periplasmically oriented β -galactosidase during growth on DYT or M63 agar plates, containing the chromogenic β -galactosidase substrate X-Gal (40 μ g/ml), yielding blue colonies in the case of inactive DsbA (Bardwell *et al.*, 1991, 1993). Cells of *E. coli* JCB817, JCB818, JCB819 and THZ2 were transformed with the respective expression plasmids, plated on DYT agar (Sambrook *et al.*, 1989) supplemented with 0.4% maltose and 40 μ g/ml X-Gal, and incubated for 24 h at 37°C and 6 h at 4°C. DsbA complementation was classified as follows: white colonies (+), bluish-white colonies (+/-) and blue colonies (-) (cf. Table I). DsbA complementation was analyzed additionally by the recovery of motility (Dailey and Berg, 1993). DYT or thiol/disulfide-free M63 plates containing 0.3% (w/v) agar were inoculated in the middle of the plates. After incubation at 37°C for exactly 24 h, the diameters of JCB817 colonies harboring the different thioredoxin secretion plasmids were measured and compared with the diameters of JCB817 colonies harboring pDsbA3 and *E. coli* JCB816 as positive controls, and the diameters of colonies of JCB818 harboring the same plasmids as negative control.

Export and processing of thioredoxin and the PDI a-domain, protein expression levels

Transport to the periplasm and processing of thioredoxin with and without the DsbA signal sequence were analyzed in *E. coli* BL21(DE3) by cell fractionation and comparison of the cytosolic and the periplasmic fractions via SDS-PAGE. BL21(DE3) harboring the pDsbA3 derivative for secretion of wild-type thioredoxin or the cytoplasmic expression plasmid pTrx with the T7 promoter (Mössner *et al.*, 1998) was grown at 37°C in LB medium containing 200 μ g/ml ampicillin. Protein overexpression was induced with 1 mM IPTG at an OD₅₇₈ of 1.7, the cells were grown further for 4 h and harvested by centrifugation. Periplasmic proteins were extracted by stirring the cells for 1 h at 4°C in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mg/ml polymyxin B sulfate. After centrifugation, the supernatant (periplasmic extract) was removed and the spheroblasts were washed and lysed by incubation at 95°C for 15 min in 1 M Tris-HCl pH 8.0 containing SDS-PAGE loading buffer. Periplasmic and spheroblast fractions were prepared in the same way for non-induced cells, which were harvested immediately before addition of IPTG. The extracts were separated by reducing SDS-PAGE, and correct processing of secreted wild-type thioredoxin was verified by blotting onto a PVDF membrane and N-terminal sequencing (sequenced residues: SDKIIHLTDDSFDTDLKA-DGAILV...). Expression of the secreted thioredoxin variants and the secreted PDI a-domain was analyzed in *E. coli* THZ2 and JCB817 cells in the same way as described above. Correct processing of the PDI a-domain was verified by N-terminal sequencing of the corresponding band (cf. Figure 1C; sequenced residues of the PDI a-domain: DAPEEEDHVL...).

The levels of DsbA and thioredoxin in periplasmic extracts were quantified by densitometric evaluation of Coomassie-stained SDS gels using a Hirschmann Elscript 400 densitometer. Different amounts of purified DsbA and purified thioredoxin were applied as standards onto the same gel. Coomassie-stained DsbA bands proved to be 6-fold more intense than bands with equimolar quantities of thioredoxin. The expression level of DsbA in the wild-type strain W3110 was analyzed by quantitative immunoblots after SDS-PAGE of total cell extracts and DsbA standards using polyclonal anti-DsbA rabbit antibodies as described (Hennecke *et al.*, 1999). For calculation of the number of thioredoxin

and DsbA molecules per *E. coli* cell, a value of 6×10^8 cells per OD₅₇₈ was used.

Protein preparation and purification

Purification of oxidized DsbA and the oxidized thioredoxin variants was performed as described previously (Wunderlich and Glockshuber, 1993a; Huber-Wunderlich and Glockshuber, 1998; Mössner *et al.*, 1998). Protein concentrations were determined by the specific absorbance at 280 nm ($A_{280 \text{ nm}}$, 1 cm, 1 mg/ml) with the following values: 1.24 for wild-type thioredoxin and all thioredoxin variants except for Grx-type thioredoxin (1.37), 1.10 for DsbA, 0.44 for native, oxidized hirudin and 0.37 for reduced, unfolded hirudin (Wunderlich and Glockshuber, 1993a; Wunderlich *et al.*, 1993; Mössner *et al.*, 1998).

Oxidative folding of hirudin

Refolding of hirudin was performed at 25°C in 0.1 M sodium phosphate pH 7.0, 0.2 M KCl, 1 mM EDTA with initial protein concentrations of 28 μ M for reduced hirudin and 84 μ M for the respective oxidized enzyme, corresponding to a dithiol/disulfide ratio of 1:1. Aliquots of 120 μ l were removed after different reaction times and quenched with formic acid [final concentration: 10% (v/v), pH <2]. Hirudin folding intermediates were separated by reversed-phase HPLC on a VydacTM 218TP54 C18 column at 55°C. A 20–24% (v/v) acetonitrile gradient in 0.1% (v/v) trifluoroacetic acid was applied, and the absorbance of the eluate was recorded at 230 nm.

Oxidation of reduced DsbA and reduced thioredoxin by DsbB in vitro

Cells of *E. coli* JCB819 and JCB819 harboring pDsbB-DsbA were grown in LB medium (1.5 l each) at 37°C and IPTG was added at an OD₅₇₈ of 1.0 to a final concentration of 0.1 mM. The cells were grown further for 2 h (final OD₅₇₈ = 1.4 for both cultures), harvested by centrifugation and disrupted by sonification. Membrane fractions of both cultures were prepared exactly as described by Bader *et al.* (1998) and finally suspended in 20 ml of 50 mM Tris-HCl pH 8.0, 300 mM NaCl. The oxidation of reduced DsbA or thioredoxin by DsbB contained in the membrane fractions was followed by the decrease in the specific protein fluorescence (DsbA: excitation at 280 nm, emission at 330 nm; thioredoxin: excitation at 280 nm, emission at 345 nm). The reactions were performed at 25°C in 1 ml of 50 mM Tris-HCl pH 8.0, 300 mM NaCl containing 50 μ l of the above membrane preparations and started by addition of different amounts of reduced DsbA or reduced thioredoxin (final concentrations: 1–40 μ M). Reduced DsbA and reduced thioredoxin were prepared as described (Mössner *et al.*, 1998).

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