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Eukaryotic Protein Disulfide Isomerase Complements *Escherichia coli dsbA* Mutants and Increases the Yield of a Heterologous Secreted Protein with Disulfide Bonds*

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Eukaryotic protein disulfide isomerase (PDI) is a 55-kDa enzyme with cysteine oxidoreductase, chaperone, and antichaperone activities that catalyze disulfide formation and rearrangement in the eukaryotic endoplasmic reticulum. In Gram-negative bacteria, the formation of disulfide bonds in the periplasm is mediated by DsbA, a strong cysteine oxidase but an inefficient catalyst of disulfide bond isomerization with no known chaperone activity. We show that rat PDI (rPDI) secreted in the periplasmic space of *Escherichia coli* can catalyze the formation of disulfide bonds and complement several of the phenotypes of *dsbA* mutants. The function of rPDI was dependent on the *dsbB* gene, suggesting that the reoxidation of this eukaryotic enzyme involves direct interactions with bacterial redox proteins. Co-expression of rPDI increased the yield of bovine pancreatic trypsin inhibitor (BPTI) severalfold, an effect that was enhanced when reduced glutathione was added to the growth medium. Whereas PDI is thought to function primarily as an isomerase in the eukaryotic endoplasmic reticulum, rPDI failed to decrease the accumulation of two-disulfide folding intermediates of BPTI and thus did not appear to appreciably catalyze the rate-limiting step in the oxidative folding pathway of BPTI. These results demonstrate that expression of eukaryotic foldases in *E. coli* can be exploited to better understand their function *in vivo* and also to increase the yield of biotechnologically valuable proteins.

Most exported proteins contain disulfide bonds that confer increased thermodynamic stability to the folded polypeptide chain. The sequence of events involved in cysteine oxidation and correct pairing to form native disulfide bonds is a critical step in protein folding. Due to constraints related to the reactivity or structural accessibility of cysteine thiols in proteins, disulfide bonds often form very slowly. A complex cellular machinery, whose components and mode of action are only now beginning to be understood, has evolved to catalyze these processes *in vivo*. In Gram-negative bacteria such as *Escherichia coli*, the cytoplasm is highly reducing, and therefore disulfide formation normally occurs after a polypeptide chain has been translocated across the inner membrane (1, 2). Genetic analysis has identified at least six genes coding for cell envelope proteins that play a role in disulfide bond formation.[1] Four of these proteins have been characterized in some detail (2, 3). DsbA is a 21.5-kDa enzyme having a thioredoxin-like subdomain with an extremely reactive and highly oxidizing disulfide bond but poor disulfide isomerization activity (4–8). DsbB is a cytoplasmic membrane protein that is required for the reoxidation of DsbA (9–12). DsbC is another soluble cysteine oxidoreductase and has much higher disulfide isomerase activity than DsbA (2, 13). Finally, the recently discovered DsbD is an inner membrane protein that has been proposed to function as a reducing source in the periplasm and to be required for maintaining proper redox conditions (3).

Bacterial proteins become oxidized and fold rapidly soon after export from the cytoplasm. However, the formation of native disulfide bonds in heterologous proteins with multiple cysteines is often very inefficient (7, 14). Partially folded molecules are highly susceptible to degradation, thus resulting in very low yields (1). The shortcomings of the disulfide bond formation machinery of *E. coli* with respect to eukaryotic proteins have been illuminated by analyzing the folding pathway of the bovine pancreatic trypsin inhibitor (BPTI)[2] expressed in the periplasmic space (15). BPTI is a 6.5-kDa protease inhibitor with three disulfide bonds. In *E. coli*, just as *in vitro*, the rate-limiting step in folding is the isomerization of two-disulfide intermediates. The bacterial periplasmic space is thought to be strongly oxidizing (7, 16, 17) and appears to lack sufficient disulfide isomerase activity required for the folding of heterologous multidisulfide proteins. In sharp contrast to the bacterial periplasm, disulfide bond formation in eukaryotes occurs in the endoplasmic reticulum, a compartment that is maintained at more reducing conditions than the periplasm (18). Disulfide bond formation and isomerization in the endoplasmic reticulum is catalyzed by protein disulfide isomerase (PDI), a very abundant 55-kDa enzyme that, apart from its thioredoxin-like active sites, shares little homology with prokaryotic proteins. PDI contains two active sites that are not functionally equivalent, has been shown to both promote and inhibit protein aggregation, and can exist in different oligomerization states (19–22).

Here we show that rat PDI (rPDI) expressed in the *E. coli* periplasmic space can substitute for DsbA to catalyze the formation of disulfide bonds in bacterial proteins and to complement several of the phenotypes of *dsbA* mutants. Interestingly, rPDI function is nearly abolished in *dsbB* mutants, suggesting that this eukaryotic protein becomes reoxidized via disulfide

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[1] S. Rainas and D. Missiakas, personal communication.

[2] The abbreviations used are: BPTI, bovine pancreatic trypsin inhibitor; IPTG, isopropyl-β-D-thiogalactopyranoside; PDI, protein disulfide isomerase; rPDI, rat PDI; ELISA, enzyme-linked immunosorbent assay.

exchange with a component of the prokaryotic oxidoreductase machinery. We also demonstrate that expression of rPDI in the *E. coli* periplasmic space is beneficial for the expression of the recombinant multidisulfide trypsin inhibitor BPTI.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—The *E. coli* K12 strains used in this study were JCB570[MC1000 *phoR* zih12::Tn10], JCB571[JCB570 *dsbA*::kan1], JCB789[JCB570 *dsbB*::kan], JCB758[JCB570 *dsbA*::kan *dsbB*::kan], JCB502[(*lD69*), *lacZ*::Tn10 (tet^s by fusa-ric acid)], and JCB572 [JCB502 *dsbA*::kan1] (4, 10). The last two strains contained F′[*proAB, lacIq, lacZ ΔM15, Tn10*]. Plasmid pTI103 contains the OmpA leader-BPTI gene fusion and has been described previously (23). Plasmid pLPPsOmpArPDI contains the gene for the mature rat PDI, fused to the OmpA signal sequence, under the control of the *lpp-lac* promoter (24). pACYCBPTI contains the OmpA leader-BPTI gene fusion and the origin of replication of pACYC184.

General Methods—Unless otherwise specified, cells were grown at 37 °C in M9 minimal salts media, adjusted to pH 7.0, and supplemented with 0.2% glucose and 0.2% casein hydrolysate. For labeling experiments, cultures were supplemented with 50 μg/ml L-amino acids (except cysteine and methionine) instead of casein. Ampicillin (50 μg/ml) and/or chloramphenicol (40 μg/ml) was added as required. In the BPTI production experiments, 100 μg/ml ampicillin and 170 μg/ml chloramphenicol were used to maintain the co-transformants.

Fractionation by osmotic shock was carried out essentially as described by Neu and Heppel (25). Sensitivity to filamentous phages was tested by diluting overnight cultures, grown without IPTG, to an $A_{600\,nm}$ = 0.005, followed by infection with phage JB4 (a Cm^r, M13 derivative). Subsequently the cells were plated on LB with 0.2% glucose and 20 μg/ml chloramphenicol. Conjugation experiments using SF103 (F$^-$ Δ*lacX74 glaE galK thi rpsL*(*strA*) Δ*phoA*(*Pvu*II) *ptr-32*::ΦCm^r) as the recipient strain were conducted as described (26). Rabbit polyclonal antisera against native BPTI (Boehringer-Mannheim) and rPDI were prepared using standard protocols (27).

Pulse-Chase Experiments, Immunoprecipitation, and Electrophoresis—For monitoring the oxidative state of alkaline phosphatase and OmpA, mid-exponential phase cells were labeled with 100 μCi/ml Trans^{35}S Label (ICN Biomedicals Inc.) for 45 s and chased with 20 mM methionine and 3 mM cysteine. Samples (1 ml) were withdrawn at various times and added to trichloroacetic acid on ice at a final concentration of 10%. The protein pellets obtained by centrifugation at 12,000 × *g* for 10 min were resuspended in 0.5 ml of 100 mM Tris-HCl (pH 9.0), 1.5% SDS, 5 mM EDTA, and 35 mM iodoacetamide. Samples were then diluted 4-fold in immunoprecipitation buffer (10 mM Tris-HCl, pH 8.0, 0.1% Triton X-100, 0.14 M NaCl, and 0.025% NaN_3) and immunoprecipitated with antisera to alkaline phosphatase (from 5 Prime → 3 Prime, Boulder, CO) and OmpA as described previously (15). Oxidized and reduced proteins were resolved by SDS-polyacrylamide gel electrophoresis in 20-cm nonreducing gels essentially as described (28). Pulse-chase experiments for following the kinetics of folding of BPTI were carried out as described previously (15) except that the chase contained 3 mM cysteine.

Detection of BPTI by ELISA—Cells were induced with 0.1 mM IPTG at $A_{600\,nm}$ = 0.3–0.35, and for some samples GSH and/or GSSG was added 20 min later at the concentrations indicated in the text. Five hours after induction, samples were frozen at −70 °C and then thawed to 4 °C, lysed by French press (20,000 p.s.i.a.), and fractionated into insoluble and soluble fractions by centrifugation at 12,000 × *g* for 10 min. The protein concentration of the soluble fraction was measured by the Bio-Rad Protein Assay. Next, 100 μl of soluble protein diluted to a concentration of 2.5 μg protein/ml in ELISA coating buffer (32 mM Na_2CO_3, 68 mM $NaHCO_3$) was added to 96-well plates. After incubation overnight at 4 °C, the wells were washed three times with washing buffer (0.5% Tween 20 in phosphate-buffered saline) and three times with double distilled H_2O, blocked with 200 μl of 2% bovine serum albumin (Boehringer Mannheim) in phosphate-buffered saline for 1 h at 37 °C and then washed again. Subsequently, 100 μl/well of BPTI antisera (diluted 1:1000 in phosphate-buffered saline with 0.05% Tween 20 and 0.25% bovine serum albumin) was added to the plate and incubated for 1 h at 37 °C. The plate was then washed again as before, and 100 μl of goat anti-rabbit horseradish peroxidase conjugate (diluted 1:1000 in phosphate-buffered saline with 0.05% Tween 20 and 0.25% bovine serum albumin) was added to each well. After 30 min at 37 °C and a final wash, 100 μl of peroxidase substrate ABTS (Bio-Rad) was added. Developing was stopped with 100 μl of 2% oxalic acid after 5 min, and the $A_{410\,nm}$ was measured on a MR300 MicroElisa Reader (Dynatech Laboratories Inc., Chantilly, VA). The soluble fraction of cells without plasmid was spiked with known amounts of BPTI and used as a standard.

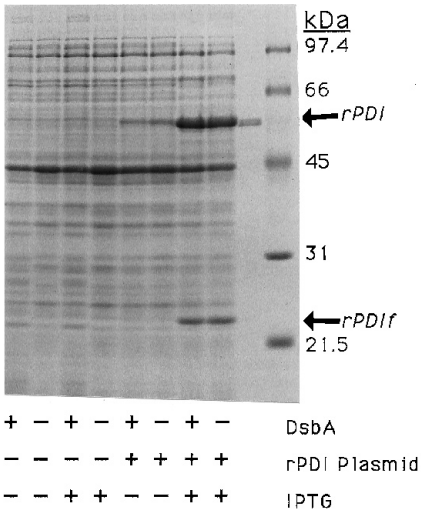


FIG. 1. **Expression of rPDI in a *dsbA* mutant.** Bacterial cultures JCB570 (*dsbA*$^+$) and JCB571 (*dsbA*$^-$), with or without the rPDI expression plasmid pLPPsOmpArPDI, were grown overnight at 37 °C and diluted 100-fold into fresh media. After 30 min, the cultures were divided in two equal parts, one of which received IPTG at a final concentration of 0.5 mM. Cells were harvested at $A_{600\,nm}$ = 0.4 and fractionated by osmotic shock. Electrophoresis on the osmotic shock supernatant was carried out on 12.5% acrylamide gels under reducing conditions. The rPDIf fragment, which represents a C-terminal fragment of rPDI including the second active site, arises due to an internal translation initiation within *rpdi* (24).

Affinity Precipitation of BPTI with Trypsin-Agarose—Soluble fractions from 1.5 ml of culture volume were mixed with 1.5 ml of 50 mM Tris-HCl (pH 8.0) buffer and 12.5 μl of trypsin-agarose beads (Sigma, 20 units/ml) and incubated on a rotator at 4 °C overnight. The beads were pelleted by centrifugation, and the pellet was washed twice for 30 min in 50 mM Tris-HCl (pH 8.0) and then for another 30 min at 10 mM Tris-HCl (pH 6.8) on a rotator at 4 °C. Subsequently, the beads were resuspended in SDS loading buffer, boiled for 5 min, and centrifuged, and the soluble fractions were loaded onto 16% Tricine SDS-polyacrylamide gel electrophoresis (Novex, San Diego). Electrophoresis was carried out under reducing conditions. The proteins were then transferred to a polyvinylidene difluoride membrane for 45 min at 2.5 mA/cm^2 using the MilliBlot-Graphite Electroblotter System (Millipore, Corp., Bedford, MA) and immunologically detected with the anti-BPTI primary antibody (1:1000 dilution) followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (1:3000 dilution) (Bio-Rad) (27).

RESULTS

The gene encoding the complete sequence of mature rat protein disulfide isomerase has been fused to the bacterial OmpA leader peptide and expressed from the strong *lac-lpp* promoter (24). Even in the absence of the inducer IPTG, a 55-kDa band corresponding to the mature rPDI was readily visible in SDS-polyacrylamide gels of the osmotic shock fraction of *Escherichia coli* (Fig. 1) and was detected by Western blot analysis using rPDI-specific sera. Upon induction with 0.5 mM IPTG, rPDI was overexpressed and became the most prominent protein in the periplasmic space. In addition to the intact rPDI monomer, a lower molecular weight product, designated rPDIf, was evident in induced but not in uninduced cultures (Fig. 1). De Sutter *et al.* (24) have shown that rPDIf corresponds to a polypeptide synthesized from an internal translation initiation codon in the rPDI gene. Although rPDIf is found predominantly in the spheroplast fraction, a portion is released by osmotic shock, as is evident from Fig. 1. Western blotting of samples from induced cultures also revealed several minor lower molecular weight species, presumably degradation products. The level of expression was identical in both the *dsbA*

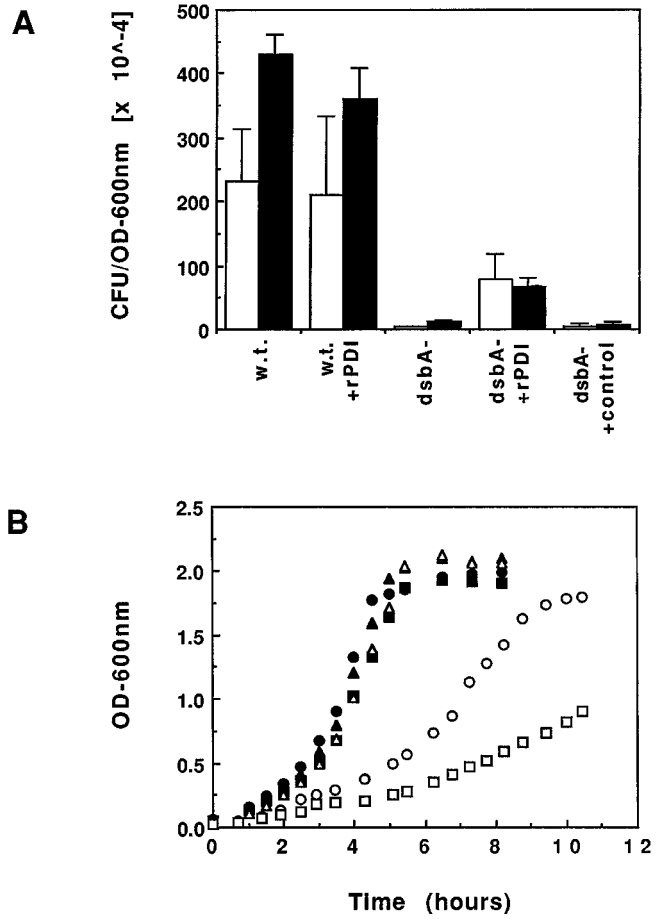


FIG. 2. **rPDI rescues phenotypes of *dsbA* mutants.** *A*, F-pilus assembly. Uninduced cells were either infected with the filamentous phage JB4 (Cm^r M13) or used as the donor for conjugation with SF103 as the recipient. Strains are as follows: *w.t.*, JCB502 F′; *dsbA*$^-$, JCB572 F′. Plasmids are as follows: *rPDI*, pLPPsOmpArPDI; *control*, pTI103. Data represent the average from three independent experiments. *B*, growth in M9 minimal media. *Solid symbols*, JCB570 (*dsbA*$^+$); *open symbols*, JCB571 (*dsbA*−); ● and ○, no plasmid; ▲ and △, pLPPsOmpArPDI; ■ and □, pTI103 (control plasmid).

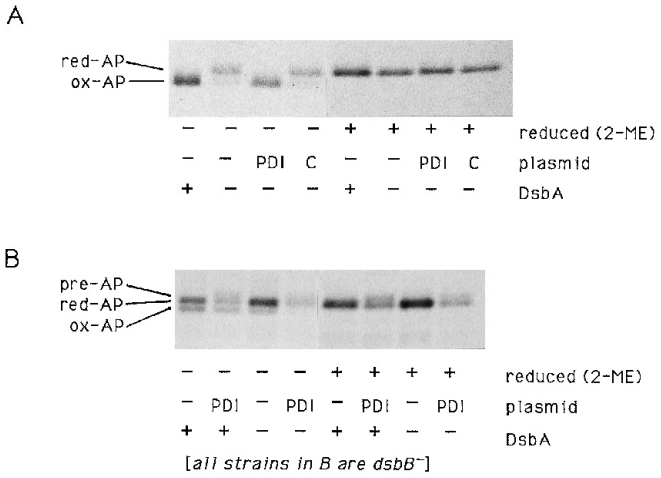


FIG. 3. **Oxidation of alkaline phosphatase in *dsbA*$^-$ and *dsbB*$^-$ cells with or without rPDI.** Bacterial cultures JCB570 (*dsbA*$^+$ *dsbB*$^+$), JCB571 (*dsbA*$^-$ *dsbB*$^+$) JCB789 (*dsbA*$^+$ *dsbB*$^-$), and JCB758 (dsbA$^-$ *dsbB*$^-$) were labeled with Tran^{35}S-label for 45 s. followed by a 10-min chase with excess methionine and cysteine. Proteins were precipitated with 10% trichloroacetic acid, treated with iodoacetamide, and immunoprecipitated with antisera against alkaline phosphatase. Immune complexes were resolved by electrophoresis under nonreducing or reducing conditions and visualized by autoradiography. The positions of reduced (*red-AP*), oxidized (*ox-AP*), and precursor (*pre-AP*) are indicated. *A*, rPDI rescue of disulfide formation in dsbA$^-$ cells. *B*, effect of rPDI on disulfide formation in dsbB$^-$ cells.

mutant strain JCB571 and in the isogenic control strain JCB570. Furthermore, Western blots demonstrated that rPDI production was not substantially affected by the *dsbA*, *dsbB*, or *dsbAdsbB* mutations under either induced or uninduced conditions (data not shown). This is in contrast to other disulfide bond-containing secreted proteins such as alkaline phosphatase, β-lactamase, urokinase and BPTI (2, 15). The production of these proteins is substantially reduced in *dsbA* and *dsbB* mutants, presumably because inefficient formation of disulfide bonds results in increased susceptibility to proteolytic degradation.

While *dsbA* is not essential for cell viability, null mutants exhibit pleiotropic phenotypes including resistance to filamentous phages, impaired motility and conjugation, poor growth in minimal media, and formation of mucoid colonies when grown with sublethal concentrations of antibiotics (2, 4). As shown in Fig. 2, transformation with pLPPsOmpArPDI resulted in complementation of several *dsbA* phenotypes. Basal expression of rPDI in cells grown without inducer was sufficient to restore conjugation competence and sensitivity to f1 phage to about 20 and 35% of the level in the parental strain, respectively. Expression of rPDI also restored the growth rate of *dsbA*$^-$ cells in minimal media to that of dsbA$^+$ cells (Fig. 2*B*). Complementation of the *dsbA* phenotypes is not merely due to the expression of a heterologous secreted protein since it was not observed in cells producing preOmpA-BPTI, which is also exported in the *E. coli* periplasmic space via the OmpA leader peptide and, like rPDI, contains six cysteines. It should be noted that in these experiments, cells were not induced with IPTG because (i) rPDI was already expressed at significant levels without induction and (ii) the overproduction of rPDI in induced cultures was found to negatively affect the efficiency of conjugation in wild type cells.

In some genetic backgrounds a null *dsbA* allele confers sensitivity to dithiothreitol (11). However, the growth of JCB570 and JCB571 were similarly affected by the presence of reduced dithiothreitol or GSH in both rich and minimal media (data not shown). Thus, in the JCB570 genetic background, it was not possible to determine whether the expression of rPDI affects the sensitivity of *dsbA*$^-$ cells to reducing agents.

The ability of rPDI to complement the phenotypes of dsbA null mutants suggested that it must be able to catalyze the formation of disulfide bonds in the periplasmic space. Direct evidence for the function of rPDI *in vivo* was obtained by examining the kinetics of oxidation of two bacterial exported proteins, alkaline phosphatase and OmpA. Cultures were radiolabeled with 100 μCi/ml Trans^{35}S Label for 45 s, and then samples were added to iodoacetamide at different times to carboxymethylate free cysteine residues. Subsequently, reduced and oxidized alkaline phosphatase were resolved electrophoretically in 20-cm polyacrylamide gels. The formation of disulfide bonds in *dsbA*$^+$ cells was very rapid and was largely completed within 1 min, whereas in *dsbA*$^-$ cells, no oxidized alkaline phosphatase was detectable even after 10 min (Fig. 3*A*). However, in cells expressing basal levels of rPDI, the formation of disulfide bonds was restored, and oxidized alkaline phosphatase was the only species detectable after 10 min of chase. Transformation with a control plasmid (pTI103) did not have any effect on the oxidation state of alkaline phosphatase. Similar results were observed with the oxidation of the outer membrane protein OmpA, which contains a single disulfide bond (data not shown).

For PDI to be functional as a direct oxidase, its active site must be regenerated through disulfide exchange with an ap-

propriate donor/acceptor. Whereas in the endoplasmic reticulum the redox state of PDI is determined by the ratio of reduced to oxidized glutathione, there is no evidence for an analogous low molecular weight redox buffer in the periplasmic space. In *E. coli*, the reoxidation of DsbA is thought to be mediated by DsbB, a cytoplasmic membrane protein that contains at least four, and possibly five, cysteines within two periplasmic exposed loops (9–12, 29). *dsbB* mutants exhibit a defect in disulfide bond formation, although not as severe as that of *dsbA* mutants. To determine whether the active state of rPDI might also be dependent on DsbB, we monitored the oxidation of alkaline phosphatase in *dsbB* mutants transformed with pLPPsOmpArPDI. In *dsbB* mutants less than 30% of the alkaline phosphatase was found in the oxidized form even after 10 min postchase (Fig. 3*B*). Expression of rPDI was largely unable to rescue the formation of oxidized alkaline phosphatase, as only 50% of the alkaline phosphatase was oxidized after 10 min. Furthermore, no oxidized protein was detected in *dsbAdsbB* double mutants with or without pLPPsOmpArPDI. Further evidence of rPDI's dependence on DsbB came from studying the production of the heterologous protein BPTI. As discussed in greater detail below, although rPDI could rescue the formation of BPTI in *dsbA* mutants, rPDI could not rescue the formation of BPTI in *dsbB* mutants. Thus, the catalysis of disulfide formation by rPDI in *E. coli* is dependent on a functional *dsbB* gene.

In *dsbB* mutants, but not in *dsbA* mutants or in wild type cells, the expression of rPDI appeared to mildly interfere with the processing of the leader peptide as evidenced by the presence of a band corresponding to the alkaline phosphatase precursor 1 min after the chase (data not shown). A faint band corresponding to the precursor was also evident even after 10 min (Fig. 3*B*). The reason for precursor accumulation in *dsbB* mutants bearing pLPPsOmpArPDI is not understood.

Under physiological conditions, the periplasmic space of *E. coli* is rather poor in disulfide isomerase activity, a function that is thought to be mediated primarily by DsbC (2). Since a major role of PDI in the endoplasmic reticulum appears to be the catalysis of disulfide bond isomerization (30, 31), we reasoned that the presence of rPDI in the *E. coli* periplasm may facilitate the expression of heterologous proteins whose folding requires the rearrangement of disulfide bonds. The rate-limiting step in the *in vitro* folding of bovine pancreatic trypsin inhibitor (BPTI), a protein containing three disulfides, is the isomerization of two-disulfide intermediates (32–34). *In vitro*, the presence of PDI modestly increases the rate of formation of two-disulfide intermediates but greatly increases their rate of intramolecular rearrangement (35, 36). Expression of secreted BPTI in *E. coli* results in low levels of native protein and is accompanied by the accumulation of two-disulfide intermediates in the periplasmic space (15). To measure the effect of rPDI on BPTI expression, cells were co-transformed with pLPPsOmpArPDI and pACYCBPTI, a compatible plasmid carrying the BPTI gene. The cells were grown in minimal media supplemented with chloramphenicol and ampicillin to maintain both plasmids. Because the standard assay for BPTI, which is based on trypsin inhibition, is not very sensitive and suffers in part from interference from endogenous proteases and trypsin inhibitors,[3] BPTI was quantified by ELISA using a primary polyclonal antibody raised against native BPTI.

Coexpression of rPDI in wild type cells resulted in a 6-fold increase in BPTI in the absence of glutathione and a 15-fold increase in its presence (Fig. 4). The increased yield with rPDI coexpression was not due to a higher rate of BPTI synthesis. If

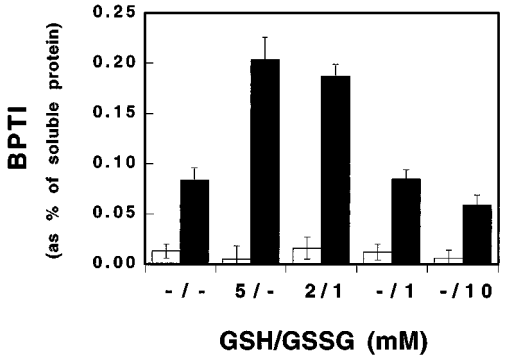


FIG. 4. **Coexpression of rPDI improves the yield of BPTI as detected by ELISA.** Bacterial cultures JCB570 containing pACYCBPTI (*open bars*) or pLPPsOmpArPDI and pACYCBPTI (*solid bars*) were induced with 0.1 mM IPTG at $A_{600\,nm}$ = 0.3–0.35. GSH and/or GSSG as indicated was added 20 min after induction. Five hours after induction the cells were harvested, and the concentration of BPTI in the soluble fraction was detected by ELISA. The data reported here represent the average of three independent experiments; for each sample, ELISAs were performed at least in triplicate.

anything, co-expression of rPDI resulted in a slightly lower rate of BPTI synthesis as determined by radiolabeling experiments. This slight reduction in the rate of protein synthesis cannot account for the increased efficiency of native BPTI formation, since in the absence of rPDI, BPTI production was not found to improve in cells where the synthesis of BPTI was reduced by lowering the amount of inducer, IPTG.

To confirm that the increased level of BPTI detected by ELISA was due to the production of native protein, BPTI was affinity-precipitated with trypsin immobilized on agarose beads and detected by Western blotting. The interaction between trypsin and native BPTI is exceedingly strong (dissociation constant 6×10^{-14} M), and the complex is stable for weeks at 4 °C (37). Reduced, carboxymethylated BPTI does not bind to trypsin. Coomassie staining of trypsin-precipitated samples from wild type cells not bearing any plasmid detected a band at approximately 16 kDa, the molecular weight of the *E. coli* trypsin inhibitor ecotin, which has no homology with BPTI (38). Several other faint bands were also visible, but none of these bands cross-reacted with anti-BPTI sera on Western blots (Fig. 5*A*, *lane 2*). When such *E. coli* extracts were spiked with high levels of purified BPTI and trypsin affinity-precipitated, more than one band was detected by Western blotting (Fig. 5). At lower protein loadings, however, only a single band was visible.

Western blots showing the level of BPTI in wild type, *dsbA*, and *dsbB* cultures with or without co-expression of rPDI and in the presence of various amounts of reduced or oxidized glutathione are shown in Fig. 5. These experiments confirmed that coexpression of rPDI increases the level of native BPTI production severalfold and that production could be further enhanced by supplementing the growth media with moderate amounts of GSH. In the absence of rPDI co-expression, glutathione alone did not increase the production of BPTI. A modest increase was observed for some cultures supplemented with 5 mM GSH, 6.2 mM GSSG, but this effect was somewhat variable. The presence of high concentrations of GSH (25 mM) was found to adversely affect the production of BPTI both with and without rPDI co-expression.

Cells lacking a functional DsbA or DsbB were found to be completely impaired in BPTI production, a deficiency that could not be alleviated by the addition of reduced or oxidized glutathione or a mixture thereof (Fig. 5*B*). Although exogenous oxidized glutathione can partially oxidize DsbA and thus complement some of the phenotypes of *dsbB* mutants, in this case it was not sufficient to rescue BPTI production. Coexpression of

[3] M. Ostermeier and D. Sharp, unpublished data.

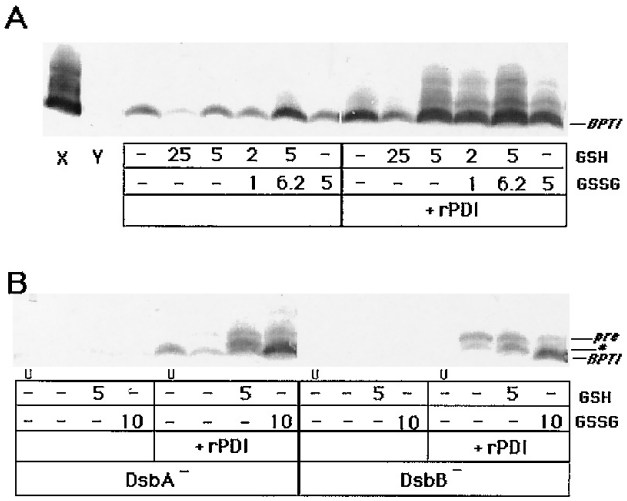


FIG. 5. **Western blot showing expression of BPTI with and without coexpression of rPDI.** Bacterial cultures JCB570 (wild type), JCB571 (*dsbA*⁻), and JCB789 (*dsbB*⁻) carrying pACYCBPTI were induced (except those marked with a *U*) with 0.1 mM IPTG at $A_{600\ nm}$ = 0.3–0.35. GSH and GSSG at the indicated mM concentrations were added 20 min after induction. Five hours after induction the cells were harvested, and the soluble fraction was affinity-precipitated with trypsin-agarose beads, electrophoresed through 16% SDS-polyacrylamide Tricine gels, and detected by Western blot (see "Experimental Procedures"). *A*, wild type cells. *Lanes 1* and *2*, soluble fraction of non-plasmid-containing wild type cells with (*X*) and without (*Y*) an addition of 2 μg of BPTI. *B*, Dsb mutant cells. *U*, uninduced cells; *pre*, preOmpABPTI; #, degradation product of preOmpArPDI; *rPDI*, carrying plasmid pLPPsOmpArPDI.

rPDI was able to restore BPTI production in *dsbA* mutants but not *dsbB* mutants, further illustrating that rPDI complements the *dsbA* mutation and that rPDI's oxidase activity is dependent on a functional DsbB protein. In *dsbB* mutants, BPTI was detected only in cells coexpressing rPDI and supplemented with 10 mM oxidized glutathione. The addition of 10 mM cystamine to cultures expressing rPDI could also complement the *dsbB* mutation (data not shown). Since PDI has been shown to have a specificity for glutathione in forming the mixed disulfide in dithiol-mediated oxidation (39), these results suggest that the likely role of the added dithiols is to oxidize the active site of rPDI, which then carries out direct oxidation of the protein substrate. Finally, it should be mentioned that 10 mM oxidized dithiothreitol, which is a much weaker oxidizer that GSSG or cystamine, could not rescue BPTI *dsbB* mutants coexpressing rPDI.

In mutant cells, particularly *dsbB* mutants, the induction of rPDI resulted in the accumulation of two higher molecular weight species, one of which migrated with an electrophoretic mobility identical to the BPTI precursor. These species are not likely to represent cross-reacting *E. coli* proteins, as both were recognized by an antiserum against BPTI obtained from a different laboratory and were not detected in cells lacking the BPTI expression plasmid. As was discussed above, the expression of rPDI in *dsbB* mutants results in some retardation of precursor processing. Therefore, it is possible that the higher molecular weight species detected in the *dsbB* mutants where rPDI was overproduced correspond to preOmpA-BPTI species. Such preOmpA-BPTI species must contain at least some of the native disulfides, since otherwise they could not have been bound by the immobilized trypsin.

To elucidate the role of PDI in the folding of BPTI, the kinetics of folding were monitored in pulse-chase experiments where folding intermediates were trapped by blocking free cysteines with iodoacetamide and separated by nonreducing gel electrophoresis. In the electrophoretic system used here, all

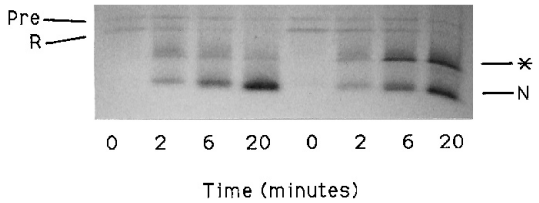


FIG. 6. **Accumulation of two-disulfide folding intermediates of BPTI in *E. coli* coexpressing rPDI as monitored by nonreducing electrophoresis.** Proteins immunoprecipitated from cultures labeled with L-[^{35}S]cysteine for 1 min and quenched with 100 mM iodoacetamide were resolved on nonreducing gels containing 8 M urea. Times indicated are minutes after the chase. *Lanes 1–4*, no addition of glutathione. *Lanes 5–8*, 2 mM GSH added 30 min before pulse. The positions of preOmpA-BPTI (*Pre*), reduced carboxymethylated BPTI (*R*), native BPTI (*N*), and two-disulfide intermediates (*) are indicated.

two-disulfide intermediates (designated as *) are well resolved from the native protein and from other folding species (15). Without coexpressing rPDI, these two-disulfide intermediates accumulate during folding, and the rate-limiting step in the formation of native protein is their isomerization. In cells expressing rPDI, two-disulfide intermediates were still observed to accumulate (Fig. 6). Furthermore, when reduced glutathione is added to the cells, two-disulfide intermediates accumulated to an even greater extent. In this case over 50% of the BPTI is found as two-disulfide intermediate species after 20 min of chase. It appears that under these conditions rPDI in the *E. coli* periplasm does not have a noticeable effect on the rearrangement and/or direct oxidation of kinetically trapped two-disulfide intermediates. This does not preclude the possibility of rPDI having isomerase activity in the periplasm, as rPDI's isomerase activity could conceivably be masked by an existing isomerase activity in the periplasm (*e.g.* that of DsbC). However, rPDI's isomerase activity can not be the cause for the improved yield of BPTI. The exact fate of the * band is not known at present, but it must be degraded and/or eventually fold to native protein. When lysates of wild type cells or *dsbA* mutants expressing rPDI (which had been labeled with ^{14}C-L-amino acids for 5 h and free cysteines blocked with 100 mM iodoacetamide) were immunoprecipitated and resolved on nonreducing Reisfeld urea gels, only native BPTI with three disulfides was found to be present (data not shown).

In cells co-expressing rPDI the half-life for the formation of the native protein was approximately 6–7 min, independent of whether GSH/GSSG had been added. This is experimentally indistinguishable from the rate of BPTI folding without rPDI (15) and consistent with rPDI not having a significant effect on the rate-limiting step, disulfide isomerization. For comparison, the folding of BPTI in eukaryotic microsomes, which are rich in PDI, can occur with a half-life of less than 1 min at 30 °C and is accompanied by relatively little accumulation of two-disulfide intermediates, depending on the redox conditions employed (40).

DISCUSSION

Rat protein disulfide isomerase is expressed at a high level in the *E. coli* periplasm even in *dsbA* mutants. Besides restoring normal growth in minimal media, expression of rPDI restores conjugation competence and sensitivity to filamentous phages. These phenotypes are dependent on the presence of correctly assembled F pili, a process which is impaired when disulfide bond formation is compromised in *dsbA* mutants. Ostensibly, rPDI restores pili assembly by facilitating disulfide bond formation. Indeed, rPDI could catalyze the oxidation of native *E. coli* proteins such as alkaline phosphatase and OmpA in cells lacking DsbA, albeit at a rate somewhat slower than DsbA's.

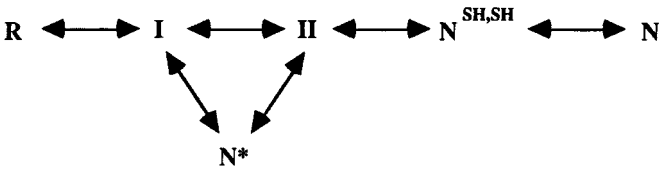
Although our results strongly suggest that rPDI functions as

a cysteine oxidase in the periplasmic space of Gram-negative bacteria, another explanation is that rPDI does not function catalytically but instead somehow induces the synthesis of other *E. coli* proteins that are responsible for disulfide bond formation. For example, Missiakas *et al.* (13) have shown that overexpression of DsbC can complement *dsbA* mutations, and it may be possible that the phenomena we observed arose indirectly due to the induction of DsbC. We believe that this is not the case for the following reasons. (i) The oxidation of alkaline phosphatase in *dsbA* mutants expressing rPDI is dependent on DsbB. However, high levels of DsbC in fact complement *dsbB* mutations (13). Thus, the function of rPDI cannot merely be due to the induction of DsbC, since then it would not be expected to be *dsbB*-dependent. (ii) As shown in Fig. 5 and discussed further below, the expression of rPDI is essential for the formation of native BPTI in *dsbA* mutants. In the absence of rPDI, no BPTI is formed in *dsbA* mutants even when the cells are grown with a wide range of concentrations of exogenous thiols and disulfides. Thus it cannot be argued that rPDI simply changes the redox state of the periplasm; rather, its enzymatic activity *per se* catalyzes the formation of disulfide bonds in BPTI.

Given the presence of millimolar concentrations of glutathione in the endoplasmic reticulum (18) and PDI's specificity for glutathione (39) it appears that in eukaryotes the active state of PDI is maintained by glutathione. Since there is no evidence for the presence of glutathione or other low molecular weight thiols in the bacterial periplasmic space (1), it is reasonable to assume that for rPDI to be functional in the periplasm, it must be able to interact directly with a component of the prokaryotic disulfide-forming machinery. Indeed, the rPDI-mediated formation of disulfide bonds in alkaline phosphatase and BPTI was found to be partially dependent on DsbB. This is interesting given that rPDI, apart from its thioredoxin active site, shows little homology to DsbA or to other bacterial proteins. It may be that the active site of rPDI, whose three-dimensional structure has not yet been solved, conforms to the thioredoxin fold as is the case with DsbA. If the active sites of the two proteins are structurally similar, then it is reasonable to expect that a protein such as DsbB, which normally interacts with DsbA, may also be able to interact with rPDI. Alternatively, rPDI's dependence on DsbB may be an indirect effect.

In catalytic amounts and in the presence of glutathione or other low molecular weight thiols, PDI primarily catalyzes the formation of mixed disulfides with model peptide substrates (39). However, the lack of a periplasmic low molecular weight redox couple in bacteria and rPDI's dependence on DsbB imply that rPDI catalyzes disulfide bond formation by direct transfer of its own disulfide bonds to protein substrates. Direct formation of disulfide bonds from PDI to reduced proteins has been observed *in vitro* with stoichiometric quantities of the enzyme and in the absence of other oxidants (39, 41). It should be noted that DsbA also normally transfers its disulfide bond directly to reduced protein substrates (42), but unlike with PDI, this reaction is not enhanced by the addition of glutathione redox buffers (8).

Gram-negative bacterial proteins fold faster under very oxidizing conditions. For example, the optimal rate for folding of alkaline phosphatase *in vitro* occurs in 6 mM GSSG and proceeds at 50% the maximal rate in 30 mM GSSG (16). Accordingly, there is evidence that the periplasmic space is indeed a highly oxidizing environment. Most, if not all, of the DsbA molecules have been shown to be in the oxidized form in the periplasm of wild type cells (17). The highly oxidized state of the periplasm can in part explain why eukaryotic proteins containing multiple disulfides are often poorly expressed. In



SCHEME I

the endoplasmic reticulum, these proteins normally fold in a relatively reduced environment, which affords the opportunity for reduction of incorrect disulfides and disulfide rearrangement, processes catalyzed by PDI (19, 31). Disulfide bond isomerization is likely to be relatively unimportant to *E. coli*, as periplasmic and outer membrane proteins with more than two disulfides are rare (8).

For rPDI to be efficient in the oxidation of alkaline phosphatase in *dsbA* mutants, it must provide a redox environment comparable with that afforded by DsbA, an equivalent $[GSH]^2/[GSSG]$ equilibrium constant of around 20 μM (16). The redox potential of PDI at near physiological pH is equivalent to $[GSH]^2/[GSSG]$ of around 40–80 μM. Thus, a significant fraction, if not the majority, of the rPDI molecules in the periplasm must be present in the oxidized form in order to effectively facilitate disulfide formation. This is quite different from the endoplasmic reticulum, where the redox state is believed to be $[GSH]^2/[GSSG] = 0.5–3.3$ mM and PDI would be present almost exclusively in reduced form (18).

To examine whether rPDI can catalyze disulfide bond isomerization in the periplasm, we chose to monitor the oxidative folding of BPTI. BPTI is a three-disulfide protease inhibitor that is very poorly expressed in *E. coli* and whose *in vitro* folding pathway involves disulfide rearrangement as described in Scheme I (34).

In Scheme I, R is the fully reduced protein, I represents the various one-disulfide species, II represents the native-like intermediate N′(30–51, 14–38) as well as two-disulfide species with one non-native and one native disulfide, N* represents the kinetic trap (5–55, 14–38), $N^{SH,SH}$ is the native-like species (30–51, 5–55), and N is the native protein with three disulfides. The rate-limiting step in folding is the conversion of II to $N^{SH,SH}$, which is then rapidly oxidized to the native protein. In the two native-like disulfide intermediates N′ and N*, the unpaired cysteines are buried within the interior of the protein. N* and to a lesser extent N′ have a native-like conformation, and therefore the activation energy for the conformational change required to position the two disulfides in close proximity is high, and thus the rate of rearrangement to $N^{SH,SH}$ is slow. Thus, two-disulfide intermediates accumulate as has been observed *in vitro* with small molecular weight thiols (43) and with DsbA (42), and *in vivo* in the periplasm of *E. coli* (15).

PDI has been shown to catalyze virtually all of the steps in the *in vitro* folding pathway of BPTI (35, 36). In the presence of catalytic amounts of protein disulfide isomerase and a suitable redox buffer, the rate of formation of N′ and N* from reduced protein is increased by about 3-fold, whereas the subsequent folding of these two kinetically trapped intermediates to N is accelerated by more than 3,000-fold (35), resulting in a dramatic decrease in the amount of two-disulfide intermediates observed during folding. Catalysis also appears to occur *in vivo* as evidenced by the fact that relatively small amounts of two-disulfide intermediates were detected during the folding of BPTI in microsomes (40).

Co-expression of rPDI in wild type *E. coli* increased the steady-state level of BPTI by 15-fold in the presence of glutathione and 6-fold in its absence. However, pulse-chase experiments revealed that the presence of rPDI does not decrease the accumulation of two-disulfide intermediates. In fact, in the

presence of glutathione, two-disulfide intermediates accumulated to a greater extent. Thus, in wild type *E. coli*, rPDI does not facilitate the isomerization of N′ and N* to $N^{SH,SH}$ any more than the formation of N′ and N*.

The above results suggest the following. (i) In wild type cells, rPDI does not function as an appreciable isomerase to rearrange the two-disulfide intermediates in order to form native BPTI. This is supported further by experiments in our lab in which mutants of rPDI devoid of direct oxidase activity, but not disulfide isomerase activity, were unable to improve the yield of BPTI or rescue its production in *dsbA* mutants, even with added glutathione.[4] rPDI's lack of appreciable isomerase activity in the periplasm is not surprising, since evidence suggests that the active sites of rPDI in the periplasm are predominantly oxidized and therefore can only catalyze direct oxidation and not disulfide rearrangement. Attempts to improve BPTI production *in vivo* by making the periplasm more reducing (*i.e.* by adding reducing agents or by using *dsb* mutants) in order to elicit rPDI isomerase activity were unsuccessful but should not be construed as evidence of an inherent inability of rPDI to exhibit isomerase activity in the periplasm. In a more reducing periplasm, the rates of formation of the first and second disulfides in BPTI should decrease, and thus the competing process of proteolysis is likely to limit the yield of correctly folded protein. It may be that the conditions for eliciting sufficient rPDI isomerase activity to appreciably increase the conversion of two-disulfide intermediates to native protein leave the periplasm too reducing for any BPTI molecules to form disulfides efficiently in order to avoid proteolysis. (ii) The increase in two-disulfide intermediates with added glutathione indicates that rPDI is functioning as an oxidase that supplements DsbA in forming the two-disulfide intermediates. *In vitro*, PDI's oxidase activity has been found to be enhanced by the addition of a redox buffer of glutathione (39). (iii) Increased steady state levels of BPTI appear to be the result of the faster formation of two-disulfide intermediates from reduced protein, thus avoiding proteolysis. It is also conceivable that the chaperone-like activity of PDI may play a role in the increased levels of BPTI (21, 44). It should be also noted that we have found that co-expression of rPDI can enhance the correct folding of at least one other eukaryotic protein, tissue plasminogen activator[5] and thus may be a general tool for improving protein expression.

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Addendum—While this paper was under review, Peter Lund and co-workers (45) showed that the expression of human PDI expressed to the *E. coli* periplasm improved the yield of PelC, a pectate lyase from *Erwinia carotovora* that contains two disulfides. In *dsbA* mutants, hPDI restored wild type alkaline phosphatase activity but required GSSG to restore wild type levels of PelC.

[4] P. Bessette, M. Ostermeier, H. F. Gilbert, and G. Georgiou, manuscript in preparation.

[5] J. Qiu, J. R. Swartz, and G. Georgiou, unpublished data.

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