Facilitating the Formation of Disulfide Bonds in the Escherichia coli Periplasm via Coexpression of Yeast Protein Disulfide Isomerase

ARTICLE *in* BIOTECHNOLOGY PROGRESS · DECEMBER 1998

Impact Factor: 2.15 · DOI: 10.1021/bp990083r · Source: PubMed

Facilitating the Formation of Disulfide Bonds in the *Escherichia coli* Periplasm via Coexpression of Yeast Protein Disulfide Isomerase

Xiaoming Zhan,[†] Melissa Schwaller,[‡] Hiram F. Gilbert,[‡] and George Georgiou*[,†,§]

Institute of Cell and Molecular Biology and Department of Chemical Engineering, University of Texas, Austin, Texas 78712, and Department of Biochemistry, Baylor College of Medicine, Houston, Texas 77030

Sacchromyces cerevisiae protein disulfide isomerase (yPDI) was expressed in the *E. coli* periplasm by using plasmids encoding the OmpA-yPDI-$(His)_6$ fusion gene under the control of the *araBAD*, *trc*, or *T7* promoter. The expression levels of yeast PDI under these promoters were compared. Our results showed that yeast PDI expressed into the periplasm could catalyze the formation of disulfide bonds in alkaline phosphatase, restoring the *phoA*$^+$ phenotype in *dsbA*$^-$ mutants. The yeast PDI was purified from the *Escherichia coli* periplasm and shown to exhibit catalytic properties comparable to those of the rat enzyme with reduced RNase as substrate. In vivo, coexpression of the yeast PDI increased the yield of bovine pancreatic trypsin inhibitor (BPTI) in *E. coli* by 2-fold, similar to the effect seen previously with the coexpression of the rat enzyme. However yeast PDI was more effective than rat PDI in facilitating the expression of active tissue plasminogen activator (tPA). These results point to differences in the substrate specificity of various PDI enzymes, at least in the context of the *E. coli* periplasm.

Introduction

The formation of disulfide bonds is essential for protein folding and thermodynamic stability (*1*, *2*). Disulfide bonds form predominantly, but not exclusively, after the polypeptide is exported from the mostly reducing environment of the cytoplasm to a more oxidizing compartment such as the periplasmic space of bacteria or the endoplasmic reticulum of eukaryotic cells. Although the oxidation of cysteine thiols and the accompanying formation of disulfide bonds can occur spontaneously in the presence of a suitable oxidant, it is now well-established that, in the cell, these processes are enzymatically catalyzed. The enzymatic machinery responsible for the formation of disulfide bonds in both prokaryotes and eukaryotes is complex and has been the subject of intense study (*2*, *3*). While the overall chemistries of the disulfide bond formation machinery in lower and higher organisms are the same, there is little sequence homology between the respective enzymes, for example, between DsbA and PDI, the proteins that catalyze thiol oxidation in bacteria and yeast, respectively. Because of such differences in the enzymatic machinery as well as in the overall folding environment of the periplasmic space and endoplasmic reticulum (*4*), the expression in *Escherichia coli* of correctly folded eukaryotic proteins containing multiple disulfide bonds is very inefficient. For this reason, numerous recombinant proteins of commercial interest cannot be expressed in active form in appreciable yield. Examples include inhibitors of hydrolytic enzymes (*5*, *6*), enzymes of medical or industrial significance (*7*−*12*), immunological molecules (*13*), and hormones (*14*).

Protein disulfide isomerase (PDI), one of the major proteins in the endoplasmic reticulum, has been shown by both in vitro and in vivo experiments to be the main enzyme responsible for the formation and rearrangement of disulfide bonds during oxidative protein folding in eukaryotes (*3*). PDI is an essential protein and is ubiquitous throughout the eukaryotic world, from yeast to plants to human (*15*). Sequence analysis has revealed that all PDI enzymes contain two domains having homology to thioredoxin. Recently, NMR structures of two of the rat PDI subdomains confirmed that they both fold into conformations very similar to that of thioredoxin (*16*). As with thioredoxin, the active site of PDI contains the conserved sequence CXXC with the more N-terminal cysteine being the catalytic residue. In addition to the two thioredoxin-like domains, PDI contains three other domains that might be important for substrate recognition and binding and for high catalytic efficiency.

Recently, we and others expressed the rat form of PDI in *E. coli* (*17*, *18*). Even though the rat PDI shares little homology with DsbA, it was nonetheless shown to catalyze disulfide bond formation in *E. coli* and could alleviate the physiological defects associated with the inactivation of the *dsbA* gene. The catalytic function of PDI in bacteria was shown to be absolutely dependent on DsbB, a membrane protein that has been shown to be responsible for the recycling of DsbA (*18*). DsbB is responsible for reoxidizing DsbA after the latter becomes reduced by catalyzing the formation of a disulfide in a protein substrate. Importantly, from a practical standpoint, the overexpression of rat PDI in *E. coli* grown in media supplemented with an optimal concentration of reduced glutathione increased the yield of active BPTI

* Corresponding author. Phone: 512-471-6975. Fax: 512-471-7963. E-mail: gg@che.utexas.edu.
[†] Institute of Cell and Molecular Biology, University of Texas.
[‡] Baylor College of Medicine.
[§] Department of Chemical Engineering, University of Texas.

10.1021/bp990083r CCC: $18.00
Published on Web 07/08/1999

(bovine pancreatic trypsin inhibitor), a eukaryotic protein with three disulfide bonds. Similarly, overexpression of the human PDI, which is nearly identical to the rat enzyme, was shown to increase the expression of pectate lyase C in the *E. coli* periplasm and also to have some effect on the expression of Fab antibody fragments (*19*, *20*).

In addition to PDI, the overexpression of other cysteine oxidoreductases has been shown to enhance the yield of various eukaryotic proteins in bacteria. This effect appears to be protein-specific. For example, overexpression of rat PDI or the *E. coli* disulfide isomerase, DsbC, has the same effect on the yield of BPTI (ref *18*, Paul Bassette unpublished). In contrast, the yield of active tPA (tissue plasminogen activator), a large protein with 17 disulfides, is over 20 times greater in cells overexpressing DsbC than in cells overexpressing rat PDI (*9*). Such effects may be a consequence of the different protein specificities of disulfide isomerases from different organisms.

The *Saccharomyces cerevisiae* PDI exhibits only about 30% overall amino acid identity with its mammalian counterparts, though similarity is greater for the two thioredoxin-like domains. In addition, unlike the rat PDI, the yeast enzyme contains five putative glycosylation sites. Given the lower degree of sequence identity between the yeast and rat enzymes and the fact that bacteria and yeast are fast growing unicellular organisms, we decided to investigate the effect of yeast PDI on cell physiology and on heterologous protein expression in *E. coli*. In this work, the yeast PDI gene was expressed from three different strong bacterial promoters. Yeast PDI could complement *E. coli* mutants lacking a functional *dsbA* gene and catalyzed the formation of disulfide bonds in bacterial alkaline phosphatase. Moreover, yeast PDI had a somewhat different effect from the rat enzyme in aiding the expression of active heterologous proteins.

Materials and Methods

Bacterial Strains and Plasmids. The *E. coli* K12 strains used in this study were JCB570 (MC1000 *pho*R zih12::Tn10), JCB571(JCB570 *dsbA*::kan1), BL21(DE3) (F^- *ompT hsdB* ($r_B^-m_B^-$) *gal dcm* (DE3)), and SF110 (F^- Δ*lacX74 galE galK thi rpsL* Δ*phoA degP41* Δ*ompT*) (*21*, *22*). Plasmid pACYCBPTI contains the mature BPTI gene fused to the OmpA leader sequence in a pACYC184-based vector (*18*). Plasmid pBAD-stII-tPA contains the tPA sequence fused to the stII leader peptide under the control of araBAD promoter in a pACYC184 origin vector (*9*). Plasmid pLPPsOmpArPDI contains the gene for mature rat PDI fused to the OmpA signal sequence (*17*). Plasmid pPV10 contains the mature yeast PDI coding sequence amplified from a yeast cDNA library by PCR using the primers 5′-ATATGAATTCTGGTTTTCGCCCAACAAGAAGCTGTGGCC-3′ and 5′-GGACGGAGGATCCTTACAATTCATCATGGTG-3′ and cloned into expression vector pJG105 (*23*). Plasmids pBADyPDI, pTrcyPDI, and pETyPDI contain the mature yeast PDI sequence fused downstream of the OmpA signal sequence with a $(His)_6$ tag sequence at the 3′ end. pBADyPDI and pTrcyPDI were constructed by PCR amplification from pPV10 using primers 5′GCAAATCATGAAAAAGACAGCTATCG-3′ and 5′-CGAATTCTTCTGCAGTTAATGATGATGATGATGATGCAATTCATCGTGAAT-3′. The PCR product was digested with *Bsp*HI/*Pst*I and ligated into expression vectors pBAD24 (*24*) and pTrc99A (Pharmacia). pETyPDI was constructed by digesting PCR products with *Bsp*HI, and the resulting fragment was ligated into the pET28a expression vector (Novagen) which had been digested with *Hind*III, filled-in, and then digested with *Nco*I.

General Methods. Cells were grown at 37 °C either in LB media or in M9 minimal salts media adjusted to pH 7.0 and supplemented with 0.2% glycerol and 0.2% casein hydrolysate. Ampicillin (100 μg/mL), kanamycin (50 μg/mL), and chloramphenicol (70 μg/mL) were added into the growth media as required. When appropriate, the cells were induced with 0.1% arabinose for pBADyPDI, 0.1 mM IPTG for pTrcyPDI and pLPPsOmpArPDI, or 0.4 mM IPTG for pETyPDI. Periplasmic proteins were prepared by osmotic shock as previously described (*25*).

Protein samples were resolved by 12.5% Tris-Glycine SDS−PAGE. Electrophoresis was carried out under reducing conditions using standard procedures. For Western Blotting, proteins were transferred to a poly(vinylidene difluoride) membrane using a MilliBlot-Graphite Electroblotter system (Millipore). The yeast PDI protein was detected with the monoclonal anti-$(His)_6$ tag antibody (1:5000 dilution) (Clontech) followed by horseradish peroxidase-conjugated goat-anti-mouse IgG (1:5000 dilution).

Protein Purification. Yeast PDI was purified from two liter cultures of JCB570 harboring pBADyPDI grown in LB media and induced with 0.2% arabinose at $A_{600} \sim 0.6$. Three hours after induction, the cells were harvested by centrifugation and periplasmic proteins were extracted by osmotic shock. The yeast PDI contains a C-terminal $(His)_6$ tag and was purified by Ni^{2+}-NTA affinity chromatography on a 3 mL bed volume column (Qiagen). The column was washed with 20 bed volumes of washing buffer (20 mM imidazole, 50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl) until the A_{280} of the flowthrough no longer changed. The protein was eluted by flowing 10 mL of elution buffer (200 mM imidazole, 50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl). Densitometric analysis of the Coomassie Blue-stained SDS gel revealed that the purity of the yeast PDI preparation was over 95%.

Activity Assays. Alkaline phosphatase activity assays were performed essentially as previously described (*26*). Briefly, cultures were grown in M9 minimal media, and the expression of yeast PDI was induced as described above. Three hours after induction, 100 μL of cell culture was mixed with 900 μL of 1.5 M Tris pH 8.0 followed by 25 μL of 0.1% SDS and 50 μL of chloroform. The samples were vortexed vigorously for 5 s and then incubated at 30 °C for 5 min. Two-hundred microliters of the alkaline phosphatase substrate, 15 mM *p*-nitrophenyl phosphate (Sigma), was added to the samples, and 10 min later, the reactions were stopped by adding 200 μL of 1 M KH_2PO_4. After the cell debris was pelleted, the alkaline phosphatase activity was quantified by measuring the A_{420}.

For BPTI assays, pBADyPDI or pTrcyPDI was transformed into JCB570 harboring pACYCBPTI. Cells were grown in M9 minimal media and induced at $A_{600} \sim 0.4$. Five hours after induction, the cells were harvested by centrifugation at 5000 rpm for 10 min. The cell pellets were resuspended in 3 mL of 50 mM Tris pH 8.0 and were lysed by French press (20 000 psi). The lysate was fractionated by centrifugation at 12000*g* for 10 min at 4 °C. The protein concentration of the soluble fraction was measured by BCA protein assay (Pierce). ELISA assays were performed as previously described (*18*), except that the TMB peroxidase substrate kit (Bio-Rad) was used as substrate and the A_{450} was measured using an EL311 Microplate Autoreader (Bio-Tek Instruments).

To measure the activity of full-length tPA expressed in *E. coli*, pBADyPDI, we transformed pTrcyPDI or pLPPsOmpArPDI into SF110 harboring pBAD-stII-tPA. Cultures were grown in LB media supplemented with

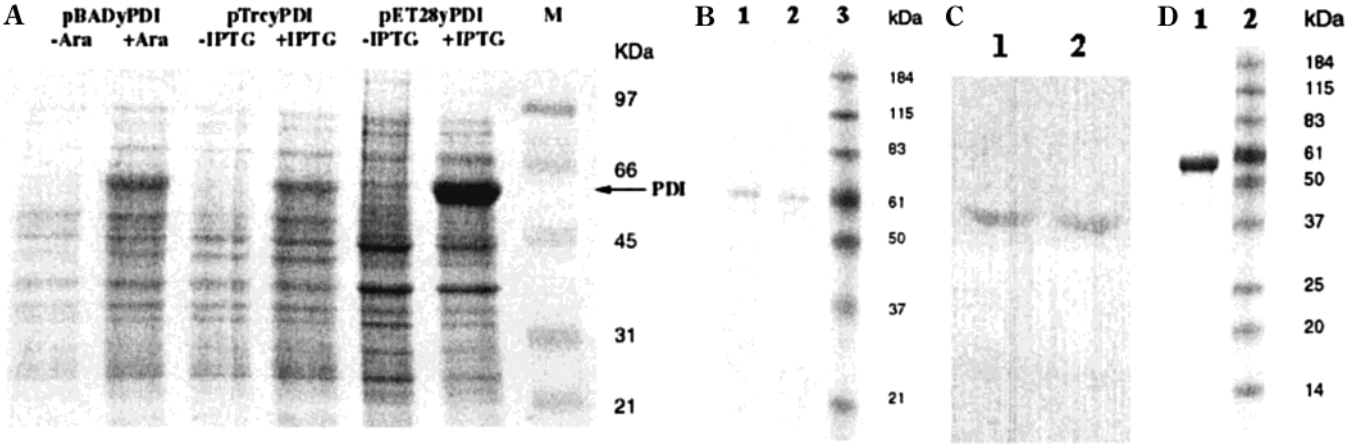

Figure 1. Expression of yeast PDI in *E. coli*. (A) Expression of yeast PDI under three different promoters. Whole cell extracts before induction and 3 h after induction were run on 12.5% SDS−PAGE; M stands for the protein molecular weight marker. (B) Expression of yeast PDI in *E. coli* periplasm. Lanes 1 and 2 are the osmotic shock fractions from JCB570[pBADyPDI] and JCB570-[pTrcyPDI], respectively, and lane 3 is the molecular weight marker. (C) Western Blotting of the osmotic shock fraction from *E. coli* expressing yPDI-$(His)_6$ using anti-$(His)_6$ antibody. (D) SDS−PAGE of yPDI-$(His)_6$ purified from *E. coli*. Lane 1 is the purified protein, and lane 2 is the molecular marker.

ampicillin and chlorophenicol at 30 °C and induced at $A_{600} \sim 0.7$. Three hours after induction, the cells were collected by centrifugation, resuspended in PBS, and lysed in a French press. tPA activity was assayed by using Spectrolyse tPA/PAI activity assay kit (American Diagnostica) in a total assay volume of 270 μL.

The ability of PDI to refold reduced RNase was measured by monitoring the increase in absorbance at 296 nm due to hydrolysis of cCMP by RNase. Assays contained 4.5 mM cCMP, 1 mM GSH, 0.2 mM GSSG, 100 mM Tris-HCl, 2 mM EDTA, 1.4 μM PDI, and 0−40 μM reduced RNase. The amount of RNase refolding that occurs in the absence of PDI was subtracted. The concentration of native RNase was calculated from the first derivative of the absorbance versus time data after correction for the depletion of cCMP and CMP inhibition of RNase. The initial velocity was determined from the slope of RNase concentration versus time.

Results and Discussion

Expression and Function of Yeast PDI Protein in the *E. coli* Periplasmic Space. The yeast PDI gene was cloned by PCR amplification from a *Saccharomyces cerevisiae* cDNA library. For expression in the periplasmic space of *E. coli*, the authentic leader peptide of yeast PDI was replaced with the leader peptide of the bacterial outer membrane protein A (OmpA). Previous studies have demonstrated that the OmpA leader peptide can successfully direct the export of the rat PDI into the periplasm (*17*, *18*). In addition, a sequence encoding a $(His)_6$ peptide tag was added to the C-terminus of yeast PDI for easy detection and purification.

In earlier studies, large amounts of an OmpA-rat PDI protein could be expressed in *E. coli* from the strong, leaky *lpp-lac* promoter which is inducible by IPTG (*17*, *23*). However, when the yeast PDI was expressed from the *lpp-lac* promoter, we found that the corresponding plasmid was highly unstable and that, upon induction with IPTG, the viability of the cells was severely compromised (data not shown). This result indicates that expression of yeast PDI may be more toxic to *E. coli* relative to the mammalian enzyme. To overcome the toxicity problem, we compared the expression of OmpA-yPDI-$(His)_6$ from the *trc* promoter, the *T7* promoter, and finally the *araBAD* promoter. *trc* is an engineered strong promoter that is inducible by IPTG (*27*). The *T7* promoter is transcribed by the T7 RNA polymerase, which in turn is expressed from an IPTG inducible promoter (*28*). The T7 RNA polymerase gene is presented by the λ phage derivative DE3 that is lysogenized into various *E. coli* hosts (*28*). Finally the *araBAD* promoter is also tightly regulated and can be readily induced by adding arabinose to the growth media (*24*). To compare the expression levels from *trc*, *araBAD*, and *T7* promoters, we grew cells in LB media and induced them with arabinose or IPTG at $A_{600} \sim 0.5$. Three hours after induction, the cells were collected and whole-cell extracts were run on SDS−PAGE. Coomassie Blue staining of the gel showed a 57 kDa band corresponding to mature $(His)_6$-tagged yeast PDI. Not surprisingly, the accumulation of yeast PDI expressed from the *T7* promoter was substantially greater (Figure 1A). However, expression from the *T7* promoter requires the presence of lysogenized phage λ DE3 in the host and was very toxic to *E. coli*. Therefore, it was not suitable for studying the in vivo functions of yeast PDI (see below).

Western blotting using a mouse anti-$(His)_6$ antibody confirmed that the 57 kDa band indeed corresponds to the yeast PDI gene product. No lower molecular weight bands could be detected, indicating the absence of N-terminal degradation products. However, C-terminal degradation products cannot be detected with the anti-$(His)_6$ antibody since the $(His)_6$ tag is located at the C-terminal end of the protein. The *araBAD* and *trc* promoters under fully induced conditions gave comparable expression levels in both whole-cell extracts and periplasmic fractions as judged by the band intensity in Coomassie-stained gels (Figure 1).

The *E. coli dsbA* gene encodes a periplasmic protein that is responsible for the formation of disulfide bonds in the periplasm. In *dsbA*-deficient strains the formation of disulfide bonds in the periplasm is severely affected. *dsbA*$^-$ mutants exhibit a number of phenotypic defects, including low levels of alkaline phosphatase activity, impaired motility and pilus formation, and slow growth in minimal media (*21*). Alkaline phosphatase activity is routinely employed as a reporter of the degree of protein oxidation in the periplasm and DsbA function. Overexpression of rat PDI complements the *dsbA*$^-$ phenotypes and restores the alkaline phosphatase activity (*18*). Similarly, we found that the yeast PDI expressed from either the *araBAD* or *trc* promoters could restore the

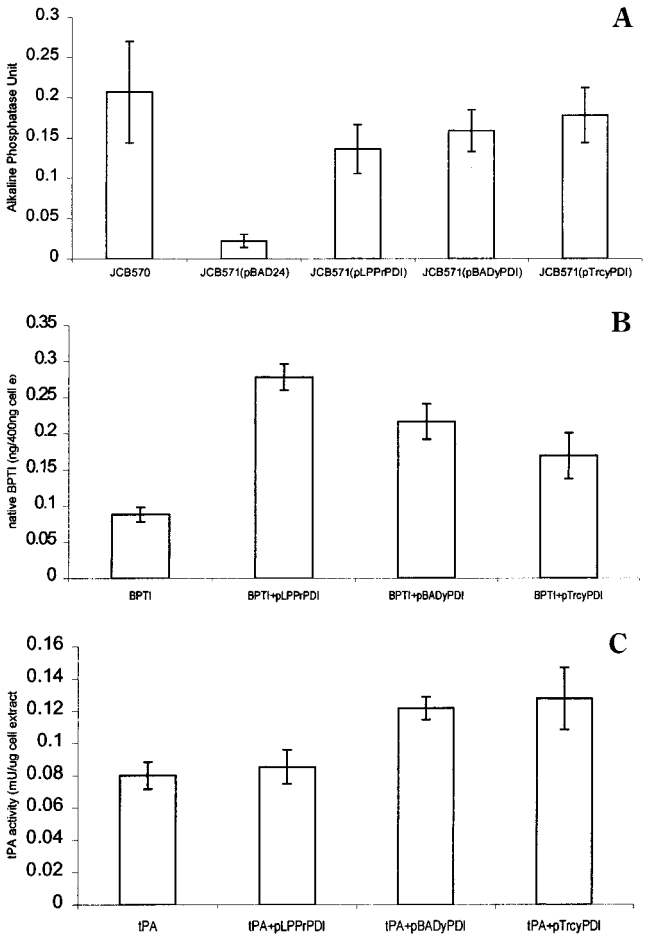

Figure 2. (A) Restoration of *E. coli* alkaline phosphatase activity in vivo with periplasmic expressed yeast PDI. Alkaline phosphatase activities were determined as described by Humphreys et al. (*19*). (B) Expression of yeast PDI increases the amount of correctly folded BPTI. The amount of native BPTI in 400 ng of total cell extracts was reported. (C) Yeast PDI assists tPA folding. tPA activities were reported as milliunits per microgram of total cell protein.

activity of alkaline phosphatase in the *dsbA*$^-$ mutant *E. coli* strain JCB571. In JCB571, alkaline phosphatase is expressed constitutively. However, because of the defect in disulfide bond formation, the enzymatic activity obtained in whole cells is barely above background and is significantly lower than the level observed in the isogenic *dsbA*$^+$ strain JCB570 (Figure 2A). Expression of yeast PDI in the periplasm almost fully restored the alkaline phosphatase activity in JCB571. The slight difference between the alkaline phosphatase activity obtained with rat PDI and yeast PDI is not statistically significant. Thus, yeast PDI is able to function as an oxidant in *E. coli* and complement at least one of the phenotypes of *dsbA*$^-$ mutants. Moreover, the fact that yeast PDI is catalytically active as an oxidant implies that it must be capable of being recycled by a bacterial component. When expressed in the periplasm of *dsbA*$^-$ *dsbB*$^-$ strain JCB758, yeast PDI could not facilitate the formation of disulfides in alkaline phosphatase (data not shown), which suggests that yeast PDI is recycled by DsbB. Likewise, rat PDI is also recycled by DsbB (*18*). It is quite remarkable that, in addition to the bacterial DsbA enzyme, DsbB can also recognize and react with two eukaryotic protein disulfide isomerases that exhibit a fairly high degree of evolutionary divergence. On the other hand, DsbB is not capable of recycling DsbC or thioredoxin (*29*), despite the fact that the latter is structurally very similar to the thioredoxin-like domains of both DsbA and of PDI family members. Evidently, DsbB must be able to recognize subtle yet conserved conformational features common to a variety of enzymes that can serve as protein thiol oxidants.

Effects of Yeast PDI Coexpression on the Yield of Exogenous Secreted Proteins. BPTI is a 6.5 kDa eukaryotic protein containing three disulfide bonds. In *E. coli*, the folding of BPTI is absolutely dependent on the presence of a functional cysteine oxidation catalyst such as DsbA (*5*). However, in wild type bacteria overexpressing DsbA, the yield of correctly folded BPTI is still quite low. Ostermeier et al. (*18*) presented evidence indicating that the rate-limiting step in the folding of BPTI is the isomerization of intermediates containing two of the three disulfides of the native protein. In vitro studies have shown that DsbA is an extremely rapid catalyst of BPTI oxidation, but as a result, it tends to catalyze the formation of numerous incorrect disulfides (*30*). For the protein to be able to reach its correct three-dimensional structure, such non-native disulfides must undergo a process of rearrangement. However, under physiological conditions, disulfide isomerization in the *E. coli* periplasm appears to be rate-limiting.

Coexpression of yeast PDI either from the *araBAD* or from the *trc* promoter increased the expression of correctly folded BPTI. For this study, the formation of native BPTI was monitored by ELISA using antibodies that recognize the correctly folded form of the protein. The accumulation of native BPTI was increased by 2−3-fold upon overexpression of yeast PDI (Figure 2B). As reported previously (*18*) the rat PDI also facilitates the folding of BPTI. In fact the coexpression of rat PDI appears to be slightly more effective in assisting BPTI folding. However, this may be due to slight differences in expression level; the rat PDI is expressed from the *lpp-lac* promoter which, as was mentioned above, could not be employed with the yeast enzyme because of toxicity issues.

We also examined the effect of yeast PDI on the folding of human tissue plasminogen activator (human tPA), a much more complex protein with 17 disulfides. Human tPA secreted in the periplasm of *E. coli* is misfolded and completely inactive. To study the effect of yeast PDI on human tPA folding, we transformed yeast PDI under *araBAD* or *trc* promoter into strain SF110 which harbors the human tPA gene on a compatible expression vector. tPA activity assays showed that coexpression of yeast PDI increased the tPA activity by 50% over background in a statistically significant manner (Figure 2C). In contrast, rat PDI coexpression had barely any effect on the expression of active full-length tPA. While the yeast PDI allowed the formation of some tPA activity, the level obtained was substantially lower than that from cells overexpressing DsbC (*9*).

It was of interest to determine whether the *E. coli*-expressed yeast PDI has similar in vitro functional properties to yPDI isolated from *Saccharomyces cerevisiae* and to the rat enzyme. For this purpose, the yPDI-$(His)_6$ was purified from the osmotic shock fraction using Ni^{2+}-NTA immobilized metal affinity chromatography. SDS−PAGE revealed that the purity of the yPDI in the eluant was over 95%. The ability of *E. coli*-produced yeast PDI to refold reduced RNase was measured by monitoring the increase in absorbance at 296 nm due to the hydrolysis of the RNase substrate cCMP by the correctly folded, catalytically active enzyme. The rate of RNase refolding was measured at 12 different concentrations of

reduced RNase. RNase refolding which occurred in the absence of PDI was subtracted from the data. In this assay the kinetic parameters for the *E. coli*-produced yPDI were very similar to those of the rat enzyme (K_m = 5.1 ± 2.5 μM, k_{cat} = 0.66 ± 0.09 min^{-1} for the *E. coli*-expressed yPDI compared to K_m = 6.9 ± 0.8 μM, k_{cat} = 0.76 ± 0.02 min^{-1} for the rat protein (*31*)). The *E. coli*-produced yPDI was also found to have in vitro properties similar to those of the protein purified from *S. cerevisiae* (*32*). Thus, the recombinant yPDI purified from *E. coli* is similar from the authentic enzyme and from the rat PDI with RNase as the substrate. Nonetheless, as shown in Figure 2C, the yPDI was slightly more effective than the rat enzyme in facilitating the folding of human tissue plasminogen activator. Unlike RNase, human tPA is a complex protein containing five distinct subdomains. The most likely explanation for these results is that the yPDI exhibits different catalytic rates with various substrates. At present, there is very little information on the determinants of substrate specificity in protein disulfide isomerases and on possible differences in the substrate specificity between PDIs from different sources.

It is interesting to note that, among the three cysteine oxidoreductases examined so far in this work and in Qiu et al. (*9*), the one of bacterial origin, DsbC, was found to be more effective than those of fungal or mammalian origin. The reason for this is most likely related to the way in which these enzymes can interact with the rest of the disulfide bond formation machinery. Specifically, DsbC is maintained in the predominantly reduced state through the action of the membrane protein reductant DsbD. Even though DsbC is probably not optimal for catalyzing disulfide isomerization in eukaryotic substrates, the large amount of enzyme present in the periplasm in the reduced, catalytically active form is nonetheless able to facilitate the folding of proteins such as tPA. In contrast, PDI, which should be a better catalyst for disulfide isomerization in eukaryotic proteins is maintained primarily in the oxidized form (*18*) and, as a result, is less effective. If such is the case, then extensive reengineering of the mechanism that maintains the redox state of the periplasm may have to be undertaken to enable a larger fraction of the heterologous PDI to be maintained in an active state to facilitate the folding of heterologous proteins.

Acknowledgment

We would also like to thank Paul Bessette for reading this manuscript and Pascal Valax and Ji Qiu for preliminary experiments. Financial support was provided by NSF Grant BES-9634036 to G.G. and James R. Swartz.

References and Notes

(1) Raina, S.; Missiakas, D. Making and breaking disulfide bonds. *Annu. Rev. Microbiol.* **1997**, *51*, 179−202.
(2) Rietsch, A.; Beckwith, J. The genetics of disulfide Bond formation. *Annu. Rev. Genet.* **1998**, *32*, 163−184.
(3) Gilbert, H. F. Protein disulfide isomerase. *Methods Enzymol.* **1998**, *290*, 26−50.
(4) Georgiou, G.; Valax, P.; Ostermeier, M.; Horowitz, P. M. Folding and aggregation of TEM beta-lactamase: analogies with the formation of inclusion bodies in *Escherichia coli*. *Protein. Sci.* **1994**, *3*, 1953−1960.
(5) Ostermeier, M.; Georgiou, G. The folding of bovine pancreatic trypsin inhibitor in the *Escherichia coli* periplasm. *J. Biol. Chem.* **1994**, *269*, 21072−21077.
(6) Wunderlich, M.; Glockshuber, R. In vivo control of redox potential during protein folding catalyzed by bacterial protein disulfide-isomerase (DsbA). *J. Biol. Chem.* **1993**, *268*, 24547−24550.
(7) Shevchik, V. E.; Condemine, G.; Robert-Baudouy, J. Characterization of DsbC, a periplasmic protein of *Erwinia chrysanthemi* and *Escherichia coli* with disulfide isomerase activity. *EMBO J.* **1994**, *13*, 2007−2012.
(8) Beck, R.; Crooke, H.; Jarsch, M.; Cole, J.; Burtscher, H. Mutation in dipZ leads to reduced production of active human placental alkaline phosphatase in *Escherichia coli*. *FEMS Microbiol. Lett.* **1994**, *124*, 209−214.
(9) Qiu, J.; Swartz, J. R.; Georgiou, G. Expression of active human tissue-type plasminogen activator in *Escherichia coli*. *Appl. Environ. Microbiol.* **1998**, *64*, 4891−4896.
(10) Obukowicz, M. G.; Gustafson, M. E.; Junger, K. D.; Leimgruber, R. M.; Wittwer, A. J.; Wun, T. C.; Warren, T. G.; Bishop, B. F.; Mathis, K. J.; McPherson, D. T.; et al. Secretion of active kringle-2-serine protease in *Escherichia coli*. *Biochemistry* **1990**, *29*, 9737−9745.
(11) Lu, H. S.; Hsu, Y. R.; Lu, L. I.; Ruff, D.; Lyons, D.; Lin, F. K. Isolation and characterization of human tissue kallikrein produced in *Escherichia coli*: biochemical comparison to the enzymatically inactive prokallikrein and methionyl kallikrein. *Protein Expression Purif.* **1996**, *8*, 215−226.
(12) Rudolph, R.; Lilie, H. In vitro folding of inclusion body proteins. *FASEB J.* **1996**, *10*, 49−56.
(13) Wulfing, C.; Pluckthun, A. Correctly folded T-cell receptor fragments in the periplasm of *Escherichia coli*. Influence of folding catalysts. *J. Mol. Biol.* **1994**, *242*, 655−669.
(14) Joly, J. C.; Swartz, J. R. In vitro and in vivo redox states of the *Escherichia coli* periplasmic oxidoreductases DsbA and DsbC. *Biochemistry* **1997**, *36*, 10067−10072.
(15) Freedman, R. B.; Hirst, T. R.; Tuite, M. F. Protein disulphide isomerase: building bridges in protein folding. *Trends Biochem. Sci.* **1994**, *19*, 331−336.
(16) Kemmink, J.; Darby, N. J.; Dijkstra, K.; Nilges, M.; Creighton, T. E. The folding catalyst protein disulfide isomerase is constructed of active and inactive thioredoxin modules. *Curr. Biol.* **1997**, *7*, 239−245.
(17) De Sutter, K.; Hostens, K.; Vandekerckhove, J.; Fiers, W. Production of enzymatically active rat protein disulfide isomerase in *Escherichia coli*. *Gene* **1994**, *141*, 163−170.
(18) Ostermeier, M.; De Sutter, K.; Georgiou, G. Eukaryotic protein disulfide isomerase complements *Escherichia coli* dsbA mutants and increases the yield of a heterologous secreted protein with disulfide bonds. *J. Biol. Chem.* **1996**, *271*, 10616−10622.
(19) Humphreys, D. P.; Weir, N.; Mountain, A.; Lund, P. A. Human protein disulfide isomerase functionally complements a dsbA mutation and enhances the yield of pectate lyase C in *Escherichia coli*. *J. Biol. Chem.* **1995**, *270*, 28210−28215.
(20) Humphreys, D. P.; Weir, N.; Lawson, A.; Mountain, A.; Lund, P. A. Coexpression of human protein disulphide isomerase (PDI) can increase the yield of an antibody Fab′ fragment expressed in *Escherichia coli*. *FEBS Lett.* **1996**, *380*, 194−197.
(21) Bardwell, J. C.; McGovern, K.; Beckwith, J. Identification of a protein required for disulfide bond formation in vivo. *Cell* **1991**, *67*, 581−589.
(22) Baneyx, F.; Georgiou, G. In vivo degradation of secreted fusion proteins by the *Escherichia coli* outer membrane protease OmpT. *J. Bacteriol.* **1990**, *172*, 491−494.
(23) Ghrayeb, J.; Kimura, H.; Takahara, M.; Hsiung, H.; Masui, Y.; Inouye, M. Secretion cloning vectors in *Escherichia coli*. *EMBO J.* **1984**, *3*, 2437−2442.
(24) Guzman, L. M.; Belin, D.; Carson, M. J.; Beckwith, J. Tight regulation, modulation, and high-level expression by vectors containing the arabinose pBAD promoter. *J. Bacteriol.* **1995**, *177*, 4121−4130.
(25) Neu, H. C.; Heppel, L. A. The release of enzymes from *Escherichia coli* by osmotic shock and during the formation of spheroplasts. *J. Biol. Chem.* **1965**, *240*, 3685−3692.
(26) Brickman, E.; Beckwith, J. Analysis of the regulation of *Escherichia coli* alkaline phosphatase synthesis using deletions and phi80 transducing phages. *J. Mol. Biol.* **1975**, *96*, 307−316.
(27) Amann, E.; Ochs, B.; Abel, K. J. Tightly regulated *tac* promoter vectors useful for the expression of unfused and fused proteins in *Escherichia coli*. *Gene* **1988**, *69*, 301−315.

(28) Studier, F. W.; Rosenberg, A. H.; Dunn, J. J.; Dubendorff, J. W. Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.* **1990**, *185*, 60−89.
(29) Debarbieux, L.; Beckwith, J. The reductive enzyme thioredoxin 1 acts as an oxidant when it is exported to the *Escherichia coli* periplasm. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 10751−10756.
(30) Zapun, A.; Creighton, T. E. Effects of DsbA on the disulfide folding of bovine pancreatic trypsin inhibitor and alpha-lactalbumin. *Biochemistry* **1994**, *33*, 5202−5211.
(31) Lyles, M. M.; Gilbert, H. F. Mutations in the thioredoxin sites of protein disulfide isomerase reveal functional non-equivalence of the N- and C-terminal domains. *J. Biol. Chem.* **1994**, *269*, 30946−30952.
(32) Mizunaga, T.; Katakura, Y.; Miura, T.; Maruyama, Y. Purification and characterization of yeast protein disulfide isomerase. *J. Biochem. (Tokyo)* **1990**, *108*, 846−851.

Accepted June 2, 1999.

BP990083R