Functional Properties of the Individual Thioredoxin-like Domains of Protein Disulfide Isomerase

Nigel J. Darby and Thomas E. Creighton*

European Molecular Biology Laboratory, Meyerhofstrasse 1, D-69012 Heidelberg, Germany

Received April 20, 1995; Revised Manuscript Received July 5, 1995[⊗]

ABSTRACT: The two thioredoxin-like domains of human protein disulfide isomerase (PDI) have been produced in bacteria as individual soluble, folded protein molecules, and their functional properties have been compared to those of intact PDI. The two individual domains were very similar in their functional properties, and there were no indications of synergy between them, so it is unlikely that they have intrinsically different functions in PDI. Both domains efficiently introduced disulfide bonds into unfolded model proteins and peptides but were less efficient than PDI with folded substrate protein molecules. Relative to PDI, neither domain had substantial activity in catalyzing disulfide bond isomerization. This pattern of activities is very similar to that of the bacterial catalyst DsbA and probably reflects similarities in the catalytic mechanisms of these proteins. The differences in activity between PDI and its thioredoxin-like domains suggest that other features of the PDI molecule are also required for its complete range of thiol−disulfide exchange activities.

Disulfide bond formation is an important co- and post-translational event in the biosynthesis of many extracellular proteins that is usually coupled to the process of protein folding. In eukaryotes, it occurs in the endoplasmic reticulum, where disulfide formation probably involves the endogenous oxidized and reduced forms of glutathione (Hwang et al., 1992) and is catalyzed by protein disulfide isomerase [reviewed by Freedman (1992) and Freedman et al. (1994)]. PDI[1] catalyzes the formation, breakage, and rearrangement of disulfide bonds, which are often slow steps in the protein-folding process (Givol et al., 1964; Creighton et al., 1980, 1993; Darby et al., 1994). In addition, PDI has been found to be part of complexes that involve several polypeptide chains (Freedman, 1992).

A major advance in understanding how PDI functions came with the determination by Edman et al. (1985) of its primary structure and the discovery that it contains two segments homologous to thioredoxin. Thioredoxin is a small protein that is involved in many reductive reactions in the cell. It functions by cyclic formation and reduction of a disulfide bond between two cysteine residues that are separated in its sequence by two other residues (Holmgren, 1985). There is substantial evidence that the corresponding cysteine residues of the two thioredoxin domains of PDI are involved in its protein disulfide isomerase activity; chemical modification or mutational substitution of these cysteine residues diminishes that activity (Hawkins & Freedman, 1991; Vuori et al., 1992b; LaMantia & Lennarz, 1993; Lyles & Gilbert, 1994). Protein disulfide rearrangements catalyzed by PDI are thought to occur through a disulfide-linked PDI/protein complex, probably formed by the attack of a thiol group of one on a disulfide bond of the other (Creighton et al., 1980; Darby et al., 1994). Such disulfide-linked complexes between proteins and PDI have been reported (Hu & Tsou, 1991; Huth et al., 1993).

Catalysis of formation of disulfide bonds by PDI is probably of equal or greater importance than catalysis of their rearrangement. For example, PDI caused disulfide bonds that normally are formed primarily by disulfide rearrangements to be formed directly, in effect bypassing the disulfide rearrangements that predominate in its absence (Creighton et al., 1980). PDI can transfer its own disulfide bonds, presumably from each of the two thioredoxin-like domains, directly into other proteins by thiol−disulfide exchange reactions analogous to those with low-molecular weight disulfide reagents (Gilbert, 1989; Lyles & Gilbert, 1991b). The redox state of these PDI cysteine residues, whether they occur as free thiols or inter- or intramolecular disulfides, is presumably determined in the ER by the relative levels of GSH and GSSG. The [GSH]:[GSSG] ratio in the ER has been estimated to be about 3:1, with the overall concentration of glutathione being in the millimolar range (Hwang et al., 1992). Under these redox conditions, PDI can produce *in vitro* disulfide-linked folding of BPTI and of its biosynthetic precursor at rates approaching those observed in the ER (Creighton et al., 1980, 1993; Zapun et al., 1992). PDI can also catalyze thiol−disulfide exchange events involving GSSG. This was observed in a previous study on the effects of PDI on the folding of the BPTI precursor, pro-BPTI, which contains an extra cysteine residue in the pro-sequence; PDI increased the rate of formation of a mixed disulfide between glutathione and this extra cysteine residue (Creighton et al., 1993). Recently, PDI has also been reported to have the expected activity of reducing a protein mixed disulfide species (Hayano et al., 1993). These catalytic

[⊗] Abstract published in *Advance ACS Abstracts,* September 1, 1995.

[1] Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; DTT, dithiothreitol; ER, endoplasmic reticulum; GSH and GSSG, the oxidized and reduced forms of glutathione, respectively; HPLC, high-pressure liquid chromatography; TFA, trifluoroacetic acid; P_{2SH}^{27SH} and P_{S}^{S}, peptide substrate containing cysteine residues at positions 2 and 27 in the dithiol and disulfide forms, respectively; P_{2OH}^{27SH}, peptide substrate with a single cysteine residue at position 27 and a Ser residue at position 2; the mixed disulfide forms of the cysteine residues of these peptides with glutathione are indicated by SSG sub- and superscripts; PAGE, polyacrylamide gel electrophoresis; PDI, protein disulfide isomerase; SDS, sodium dodecyl sulfate.

0006-2960/95/0434-11725$09.00/0

activities have been confirmed and characterized using a simple model peptide as substrate (Darby et al., 1994).

The thioredoxin motif is present in many, if not all, catalysts of protein disulfide formation. It has been found in DsbA, a protein involved in protein disulfide bond formation in the bacterial periplasm (Bardwell et al., 1991; Martin et al., 1993). Its thioredoxin-like structure is interrupted by another, helical domain of about 76 residues. DsbA has been shown to function by introducing disulfide bonds directly into proteins, cycling between the dithiol and disulfide forms of the two active site cysteine residues homologous to those of thioredoxin (Wunderlich et al., 1993; Zapun & Creighton, 1994; Darby & Creighton, 1995). In thioredoxin, PDI and DsbA, the N terminal active site cysteine residue has an unusually high reactivity and a pK_a value that is lower than normal, while the second cysteine residue is buried and unreactive, only forming a disulfide bond with the first (Kallis & Holmgren, 1980; Hawkins & Freedman, 1991; Nelson & Creighton, 1994). A second bacterial protein, DsbC (Missiakas et al., 1994; Shevchik et al., 1994), which seems to function *in vivo* primarily in disulfide rearrangements, also has two cysteine residues with very similar properties (Zapun et al., 1995). Although the three-dimensional structure of DsbC is not known, and there is no substantial homology to thioredoxin in its primary structure, all the indications are that it also contains the thioredoxin motif (Zapun et al., 1995).

The active site Cys-X-Y-Cys sequences of PDI, thioredoxin, and a variety of other related redox proteins are similar, yet they fulfill very different redox functions (Krause et al., 1991; Freedman, 1992; Martin et al., 1993). They are highly oxidizing in the case of DsbA (Zapun et al., 1993), DsbC (Zapun et al., 1995), and PDI (Hawkins et al., 1991a; Lyles & Gilbert, 1991a; Lundström & Holmgren, 1993) but much more reducing in the case of thioredoxin (Lundström & Holmgren, 1990). The relative stabilities of the homologous disulfide bonds differ by more than a factor of 10^6. The versatility of this active site sequence appears to result from the protein structure being able to regulate the redox properties of the cysteine residues.

The thioredoxin-like domains account for only about 40% of the primary structure of PDI. The remainder is comprised largely of two segments that are homologous to each other (Edman et al., 1985), plus a short segment similar to the estrogen receptor (Tsibris et al., 1989) and a very acidic segment at the carboxyl terminus. The thioredoxin-like segments are designated *a* and *a'*, while the two other homologous segments are *b* and *b'*. Their order in the primary structure is: *a-b-b'-a'*. The functional roles of the *b* and *b'* segments are not known, and it is not clear why PDI has two thioredoxin-like domains, *a* and *a'*. Do they have different functional roles? Or, are there two domains simply because acting on two disulfide bonds simultaneously in a protein substrate molecule is most efficient for catalyzing disulfide rearrangements? The latter suggestion arises from the observation that PDI and dimeric DsbC are efficient at catalyzing disulfide rearrangements, whereas monomeric DsbA is not (Zapun et al., 1995).

The primary structure of PDI indicates that it is probably a modular, multidomain protein, and this is supported by analysis of exon−intron boundaries in the PDI gene and by some evidence from restricted proteolysis (Freedman et al., 1994). If so, the individual domains are likely to be independent structural and functional units, and it should be possible to prepare and study them individually. They are being produced individually and in various combinations by protein engineering in *Escherichia coli*, so as to understand their roles in PDI. Here, we characterize the PDI-*a* and -*a'* domains and show that they are indeed independent structural and functional units and that they account for some, but not all, of the activities of PDI in protein disulfide bond formation, breakage, and rearrangement.

MATERIALS AND METHODS

Materials. The cloned gene for human PDI (Pihlajaniemi et al., 1987) was very kindly provided as cDNA clone S-138 by K. Kivirikko (University of Oulu, Finland). Thioredoxin from *E. coli* was from USB. Disulfide-scrambled ribonuclease A was prepared as described by Hawkins et al. (1991b). BPTI was a generous gift of Bayer AG. The peptide substrate used in this study, P_{2SH}^{27SH}, has the sequence FCLEPPYTGPSKARIIRYFYNAKAGLCQ, with the N and C terminal groups acetylated and amidated, respectively. A second peptide, P_{2OH}^{27SH}, differs only in that Cys2 was replaced by a Ser residue. Their properties have been described previously (Darby et al., 1994; Darby & Creighton, 1995). The individual BPTI folding intermediates (30−51, 14−38) and (5−55,14−38) were isolated from refolding mixtures by HPLC and freeze-dried, as were various forms of the peptide, including mixed disulfides with glutathione; they were resuspended in 10 mM HCl just prior to use.

Cloning the a and a' Domains and PDI. The appropriate DNA sequences were amplified by polymerase chain reaction from cDNA clone S-138 for human PDI. The protein sequence chosen for the *a* domain was Asp1 to Ala120 and for the *a'* domain Asp348 to Gly462 of mature human PDI. The primers to the DNA sequence for the N terminus of each protein contained an *Nde*I restriction site, which allowed an initiator ATG methionine codon to be placed in-frame with the rest of the coding sequence. The primers for the DNA sequence that encoded the C terminus of each protein contained an in-frame stop codon and a *Bam*H1 site. The polymerase chain reaction products were initially blunt end cloned into the EcoRV site of pBluescript. The final clones obtained had the expected DNA sequences.

For expression of PDI-*a*, PDI-*a'*, and full length PDI, each gene segment was excised as the *Nde*I−*Bam*HI fragment from the pBluescript clones and cloned into the corresponding sites of expression vector pET 12a (Studier et al., 1990).

Gene expression. Protein production was carried out in *E. coli* strain BL21(DE3), which also contained the pLysS plasmid to control leak-through expression (Studier, 1991). Cells containing the appropriate pET 12a vector were grown at 37 °C in LB media supplemented with 50 μg mL^{-1} carbenicillin and 40 μg mL^{-1} chloramphenicol at 37 °C to an absorbance of 0.4 at 600 nm. Gene expression was induced by addition of isopropyl thiogalactoside to a final concentration of 0.5 mM. After 4−6 h, the cells were harvested by centrifugation and frozen.

Cell pellets from 5 L of culture were thawed into 40 mL of 50 mM Tris (pH 8), 1 mM EDTA, 10 μM leupeptin, and 0.1 mM phenylmethanesulfonyl fluoride. Lysis occurred immediately because of the presence of the lysozyme that is expressed from the pLysS plasmid. The lysed cells were treated briefly with DNAseI and then centrifuged at 15000*g* for 30 min.

Purification of PDI. PDI was purified from the cell lysates by ammonium sulfate precipitation, heat treatment, and chromatography on DEAE-Sephacel as described by Lambert and Freedman (1983) for the natural bovine protein.

Purification of PDI-a Domain. The PDI-*a* domain was apparent in the cell lysate as a new predominant protein band on SDS−PAGE, and its purification was monitored in this way. Ammonium sulfate was added to the cell lysate at 4 °C to 50% of saturation; the precipitated material was removed by centrifugation and discarded. Further ammonium sulfate was added to the supernatant to 95% of saturation and the precipitated protein recovered by centrifugation. The protein pellet was resuspended in 10 mL of sodium phosphate (pH 7) and 1 mM EDTA, loaded onto a 15 × 1.5 cm phenyl Sepharose column equilibrated with 50 mM sodium phosphate (pH 7), 1 mM EDTA, and 5 mM DTT, and saturated with ammonium sulfate. The protein was eluted using a linear gradient from this buffer to the same one without ammonium sulfate. Pooled fractions that contained the expressed protein were dialyzed against 20 mM Tris (pH 8). DTT was added to a concentration of 5 mM and the mixture loaded onto a 15 × 1.5 cm column of Q-Sepharose equilibrated with 20 mM Tris (pH 8) and 5 mM DTT. The protein was eluted with a linear gradient to 0.3 M NaCl in the same buffer.

The reduced protein obtained by the above procedure was converted to the disulfide form by dialysis against 50 mM Tris (pH 7.4) and 0.1 mM GSSG until formation of the disulfide bond was complete as judged by reverse phase HPLC. The oxidized protein was dialyzed against water and then stored frozen at −80 °C.

Purification of PDI-a′ Domain. The PDI-*a′* domain was apparent in the cell lysate as a new predominant protein band on SDS−PAGE, and its purification was monitored in this way. The cell lysate was supplemented with DTT to a concentration of 20 mM and was loaded directly onto a 30 × 1.5 cm Q-Sepharose column equilibrated with 50 mM Tris (pH 8) and 2 mM DTT. The protein was eluted by a linear gradient to 0.2 M NaCl in the same buffer. The protein was oxidized by repeated dialysis against 0.1 mM GSSG in 50 mM Tris (pH 8) until analysis by reverse phase HPLC indicated that all the reduced protein had disappeared. This process produces a proportion of the protein as the disulfide-linked dimer, so the mixture was chromatographed for a second time on the Q-Sepharose, which readily separated the proteins with inter- and intramolecular disulfide bonds. The isolated protein was dialyzed against 50 mM Tris (pH 8) and 50 mM NaCl and stored at −80 °C. Samples of the reduced protein were stored in the same way, but with 5 mM DTT added to the buffer.

Electrophoresis. SDS−PAGE was the system of Laemmli (1970). Native PAGE was performed using the high-pH gel system of Davis (1964).

Concentration Measurements. The concentration of PDI was determined using a molar absorbance coefficient of 56 399 cm^{-1} M^{-1} determined for the bovine enzyme (Hawkins et al., 1991a), which has 95% sequence identity to human PDI and the same content of tyrosine and tryptophan residues. The concentrations of the PDI-*a* and -*a′* domains were determined from their absorbance at 280 nm, using the molar absorbance coefficients of 19 060 and 15 220, respectively, calculated by the method of Gill and von Hippel (1989). All such concentrations refer to the individual polypeptide chains, irrespective of their state of aggregation.

Spectral Analysis. Circular dichroism spectra were measured with a Jasco J710 spectrometer in a 1 mm pathlength cell at 25 °C; spectra were averaged from five scans. The proteins were dissolved in 20 mM Tris-HCl (pH 7.4) in the presence or absence of 0.2 mM DTT. Mass spectrometry was performed by the EMBL service of M. Mann.

Preparation of the Catalysts. When the reaction to be studied occurred in the presence of GSH and GSSG, the catalysts were incubated in this redox buffer for 15 min prior to the reaction being initiated by the addition of the peptide or protein substrate. In the absence of a redox buffer, the catalysts were in either the reduced or disulfide forms at the start of the reaction. PDI and its domains were reduced by addition of DTT to a concentration of 5 mM; after 20 min, the DTT was removed by gel filtration into 10 mM Tris-HCl (pH 7.4). PDI was converted to the putative disulfide form by adding GSSG to 0.1 mM and subsequently removing it by gel filtration in the same way.

Reactivation of Disulfide-Scrambled Ribonuclease A. Reactivation of scrambled bovine ribonuclease A in the presence of catalysts was determined by the method of Hawkins et al. (1991b) but using cyclic cytidine 3′,5′-monophosphate as substrate (Lyles & Gilbert, 1991a).

Disulfide Folding of BPTI. The effects of catalysts on the refolding of reduced BPTI were determined as previously described (Creighton et al., 1993). Disulfide formation in reduced BPTI (30 μM) was monitored in a mixture of 0.1 M Tris-HCl (pH 7.4), 0.2 M KCl, 1 mM EDTA, 0.5 mM GSSG, and 2 mM GSH at 25° C. At the appropriate times, portions of the reactions were quenched by addition of 390 μL of reaction mixture to 50 μL of 1 M HCl; the trapped BPTI species were analyzed by reverse phase HPLC.

Effects of the catalysts on the individual intermediates (30−51,14−38) and (30-51,5−55) were examined in the same way in either the presence or absence of GSH and GSSG.

Disulfide Formation in the Model Peptides. All reactions between peptide, GSH, GSSG, and PDI or its domains were carried out in 0.1 M Tris-HCl (pH 7.4), 0.2 M KCl, and 1 mM EDTA at 25° C, as described previously (Darby et al., 1994; Darby & Creighton, 1995). Reactions were quenched by the addition of 0.2 volumes of 1 M HCl, and the peptide species present were analyzed by reverse phase HPLC.

RESULTS

Expression of the PDI Gene. Full length human PDI was produced in *E. coli* as a soluble protein, with the additional initial methionine residue retained. It was readily purified by a modification of the method of Lambert and Freedman (1983) for the natural bovine protein. Others have produced active PDI in *E. coli* (Gilbert et al., 1991; Vuori et al., 1992a) and in insect cells (Vuori et al., 1992b). The purified protein gave a mass of 55 425 ± 6 by mass spectrometry, compared to an expected mass of 55 425 for the fully reduced form. Five cycles of Edman degradation gave the correct N terminal sequence of Met-Asp-Ala-Pro-Glu. It was essentially homogeneous as judged by SDS−PAGE; however, native gel electrophoresis gave up to three bands that appeared to be different redox forms of the protein. Reduc-

tion and alkylation of the protein with iodoacetamide generated a single band.

Expression of the PDI-a and PDI-a′ Domains. The protein sequence chosen for the PDI-*a* domain was Asp1 to Ala120 of mature human PDI and Asp348 to Gly462 for the PDI-*a′* domain. These sequences were chosen to include the entire segment homologous to thioredoxin, plus all adjoining polar residues; at the C terminus of the PDI-*a′* domain, the limit was taken as the very acidic segment beginning at Asp463. Each of these two polypeptide chains contains an initial methionine residue and a single pair of cysteine residues, corresponding to the active site of thioredoxin.

The PDI-*a* and PDI-*a′* domains were produced as soluble proteins in *E. coli*, with respective final yields of about 6 and 60 mg from 1 L of culture. Each was obtained in greater than 90% homogeneity, as judged by SDS−PAGE and reverse phase HPLC. Native PAGE of the PDI-*a* domain produced a single sharp band of either the disulfide form or the dithiol form blocked by reaction with iodoacetamide. A single sharp band was also obtained for the PDI-*a′* domain in the reduced, alkylated form, but the disulfide form of the protein gave a much more diffuse band. Electrospray ionization mass spectrometry measured the mass of the disulfide form of the PDI-*a* domain to be 13 386.6 (expected: 13 385.8) and that of the PDI-*a′* domain to be 13 042.6 (expected: 13 043.5). Gel filtration on a Superose 12 HR 10/30 column (Pharmacia) in 0.1 M NaCl−50 mM Tris-HCl (pH 7.4) indicated that the PDI-*a* and -*a′* domains were essentially single species, with very little polymeric material present. The elution volumes of the dithiol and disulfide forms of the PDI-*a* domain indicated apparent molecular weights of about 16 000 and 15 000, respectively, very similar to the value of 16 000 measured in the same way for *E. coli* thioredoxin; as the domains are about 30% larger than thioredoxin, the PDI-*a* domain appeared to be monomeric. The disulfide form of the PDI-*a′* domain eluted as a broader peak, with an apparent molecular weight of about 22 000, which became sharper upon reduction of the disulfide bond and indicated an apparent molecular weight of about 20 000; therefore, the PDI-*a′* domain may have some tendency to aggregate. Attempted hybridization of two forms of the PDI-*a′* domain, in which the two cysteine residues had been reacted with either acidic iodoacetate or neutral iodoacetamide to give them different net charges and electrophoretic mobilities on native PAGE (Zapun et al., 1995), gave no indication of hybrid bands; therefore, any oligomerization is unlikely to be stable.

Both domains appeared from their circular dichroism spectra (Figure 1) to be folded, globular proteins when in the reduced form. Their spectra were similar to that of PDI, even though they make up less than half of that molecule. The spectra of both domains differed significantly from that of thioredoxin, even though both undoubtedly have the thioredoxin motif, which has been confirmed for the PDI-*a* domain by nuclear magnetic resonance analysis (J. Kemmink et al., unpublished results). The CD spectrum of thioredoxin does not change when its disulfide bond is formed (Reutimann et al., 1981), but there was a small change in that of the PDI-*a* domain (Figure 1). Upon forming its disulfide bond, the spectrum of the PDI-*a′* domain underwent a much greater change to one approaching that of an unfolded protein. The disulfide bond of the PDI-*a′* domain appears to destabilize its folded conformation; this would be expected

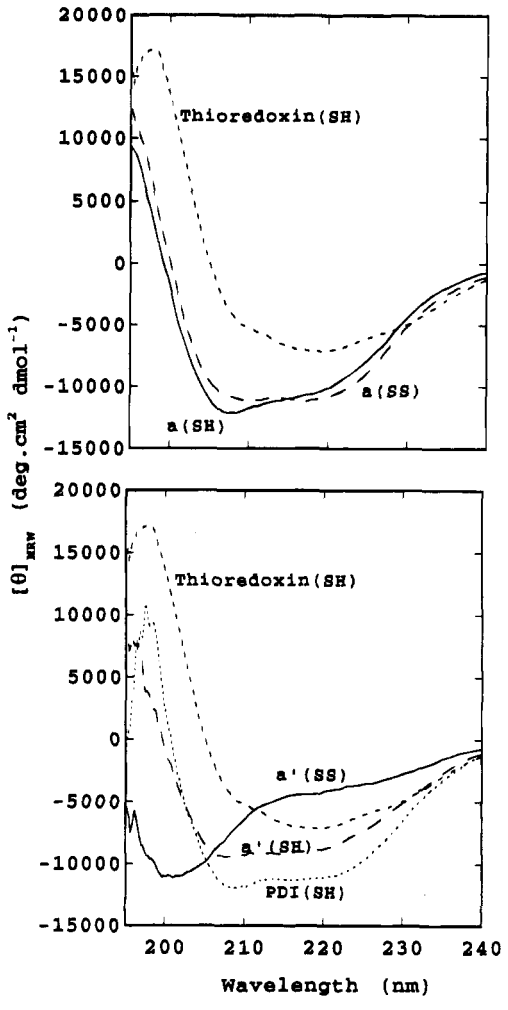


FIGURE 1: Far-UV CD spectra of recombinant human PDI, the PDI-*a* and -*a′* domains, and thioredoxin. The reduced forms of each are indicated by (SH) and the disulfide forms by (SS). The magnitude of the dichroism is expressed as the mean residue ellipticity.

with a very unstable disulfide bond and has been observed with DsbA (Zapun et al., 1993). In the case of the PDI-*a′* domain, the destabilization seems to be sufficient to unfold the protein to a substantial extent.

Reactivation of Disulfide-Scrambled Ribonuclease A. The classical activity assay for PDI is the reactivation of disulfide-scrambled ribonuclease A. This is a complex system, using a very heterogeneous substrate. It requires a sequence of disulfide bond breakage, rearrangement, and reformation steps, and it is not clear which step is rate-determining. The recombinant human PDI had an activity in this assay (Table 1) that was comparable to that of natural PDI purified from bovine liver observed by others (Hawkins et al., 1991b), indicating that the PDI produced here was functional. The activities of the PDI-*a* and -*a′* domains were much lower. Those of high concentrations of the PDI-*a′* domain were only just detectable. The PDI-*a* domain had greater activity, but only about 14% of that of the same concentration of PDI polypeptide chains. The two domains in combination were no more active than expected from the sum of their individual activities, when they had about 17% of the activity of an equivalent concentration of PDI.

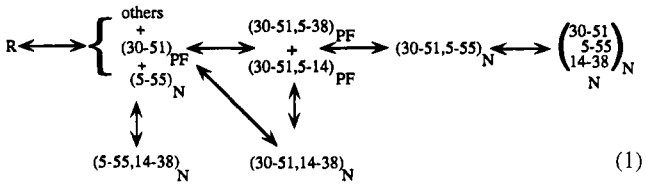
Disulfide Folding of Reduced BPTI. The disulfide folding pathway of BPTI is an ideal subject for studying the effects

Table 1: Catalytic Effects of PDI and Its Domains on the Rate of Regenerating Active Ribonuclease A from Its Disulfide Scrambled Form[a]

catalyst	rate produced by each catalyst (μM ribonuclease min^{-1}/μM catalyst)
PDI	0.27
PDI-*a* domain	0.038
PDI-*a'* domain	0.023
PDI-*a* and -*a'* domains	0.046

[a] The initial rate of appearance of active ribonuclease A was measured with varying concentrations of the reduced forms of the catalysts. In the case where both PDI domains were present simultaneously in equal amounts, the concentration of catalyst was taken to be just that of a single domain.

of catalysts such as PDI; it has been extensively characterized, is well-understood, and includes known steps of both disulfide formation and rearrangement involving unfolded, partly folded, and fully folded protein conformations (Creighton, 1992; Darby et al., 1995). It can be summarized as



(1)

R is the fully reduced and unfolded protein with six free thiol groups. The disulfide species are depicted by the residue numbers of the cysteine residues paired in disulfide bonds. Those that adopt unfolded conformations are designated with the subscript U, partly folded conformations by PF, and fully folded conformations by N. Forming the first disulfide bond in R is essentially random, but it then undergoes rapid disulfide interchange. At neutral pH, as used in these studies, two one-disulfide species predominate: $(30-51)_{PF}$ and $(5-55)_N$. As a result, subsequent disulfide formation is largely restricted to these species, as depicted in the pathway. The quasi-native species $(5-55,14-38)_N$ and $(30-51,14-38)_N$, with two native disulfide bonds, predominate at neutral pH because they are relatively stable and because they are blocked in further disulfide formation. The most productive folding process occurs via intramolecular disulfide rearrangements of the two-disulfide intermediates with non-native second disulfide bonds, (30−51,5−14) and (30−51,5−38), to $(30-51,5-55)_N$ (Darby et al., 1995), which can rapidly form the final disulfide bond. The effects of catalysts can be determined using either the entire pathway or the individual isolated intermediates, in the presence or absence of reagents for making and breaking protein disulfide bonds.

The effects of catalytic quantities of PDI and the PDI-*a* and PDI-*a'* domains on the disulfide folding of reduced BPTI in the presence of thiol and disulfide reagents were examined: 0.5 mM GSSG and 2 mM GSH (Figure 2). These conditions resemble the prevailing redox conditions of the ER (Hwang et al., 1992) and permit a direct comparison to previous studies (Creighton et al., 1993). In the absence of catalyst, disulfide bond formation and rearrangement occur in BPTI by the thiol−disulfide exchange chemical reaction between thiol and disulfide groups of the protein and the glutathione. Generation of the fully refolded protein in this

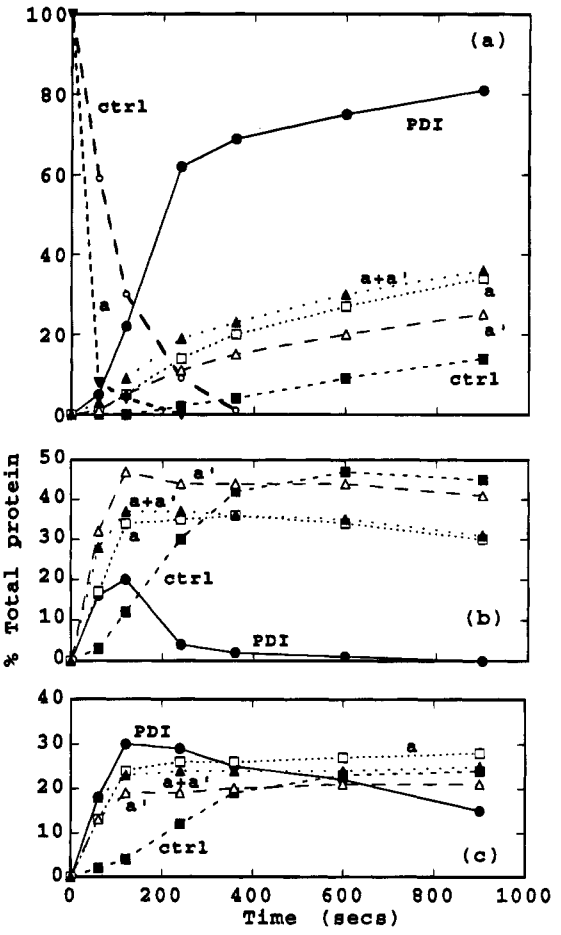


FIGURE 2: Effects of the PDI catalysts on the time course of refolding of reduced BPTI with 0.5 mM GSSG and 2 mM GSH. HPLC separations of the acid-trapped species were used to measure their relative amounts as a function of time: (■) no catalyst (ctrl), (●) 4 μM PDI, (□) 4 μM PDI-*a* domain, (△) 4 μM PDI-*a'* domain, and (▲) 4 μM each of the PDI-*a* and -*a'* domains. (a) Disappearance of R and the appearance of fully refolded N-BPTI. Only a single example of the data for the disappearance of reduced protein in the presence of catalyst is shown, for clarity: (▼) with 4 μM PDI-*a* domain and (○) no catalyst (ctrl). (b) $(30-51,14-38)_N$, irrespective of whether there is a mixed disulfide between a free thiol group and glutathione. (c) $(5-55,14-38)_N$.

way is relatively slow and inefficient under the conditions used here, for many of the molecules accumulate as the quasi-native species $(5-55,14-38)_N$ and $(30-51,14-38)_N$.

A similar but not identical range of BPTI disulfide species was observed to occur during disulfide formation in the presence of the catalysts (data not shown), but the rates of the various steps were increased to varying extents (Figure 2). In the presence of PDI, all the observed steps were increased in rate. The increased rate of disulfide formation is exemplified by the greatly increased rates of disappearance of R and of formation of $(5-55,14-38)_N$ and $(30-51,14-38)_N$; the increased rate of disulfide rearrangement is apparent from the greatly increased rates at which these latter species disappear, especially $(30-51,14-38)_N$, and by the increased rate of appearance of fully refolded BPTI. Studies on the isolated intermediates described below will confirm that PDI increased the rates of rearrangement of $(5-55,14-38)_N$ and $(30-51,14-38)_N$ to the productive intermediate $(30-51,5-55)_N$, as well as inserting the missing disulfide bond directly into them. These two processes largely account for the increased yield of the native protein.

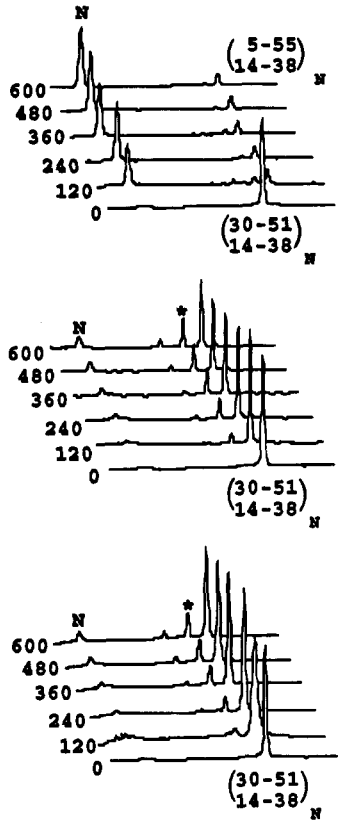


FIGURE 3: Catalytic effects of PDI (top), PDI-*a* domain (center), and PDI-*a'* domain (bottom) on BPTI species $(30-51,14-38)_N$ in the presence of 0.5 mM GSSG and 2 mM GSH. The time course (in seconds) of each reaction is shown by HPLC profiles of acid-trapped aliquots. The initial concentration of $(30-51,14-38)_N$ was 10 μM, and each of the catalysts was present at a polypeptide chain concentration of 2 μM. In the absence of catalyst, no change was observed in the initial $(30-51,14-38)_N$ over the same time span (data not shown). The peak designated with an * is the $(30-51,14-38)_N$ containing a mixed disulfide between one of its free cysteine residues and glutathione.

In the presence of the PDI-*a* and -*a'* domains, the normal intermediates accumulated, but there were also substantial amounts of a species that was not a major intermediate in control folding mixtures. Such a species had been identified previously as being a two-disulfide species, most probably $(30-51,14-38)_N$, with one of the free cysteine residues present as a mixed disulfide with glutathione (Zapun & Creighton, 1994). The same species was found to be generated directly from isolated $(30-51,14-38)_N$ in the presence of GSH plus GSSG and either the PDI-*a* or -*a'* domains (Figure 3). It was not generated in the absence of glutathione or the PDI-*a* or -*a'* domains. When the domains were replaced by PDI, the new species was found only in very small quantities, presumably because the initial $(30-51,14-38)_N$ disappeared so rapidly due to PDI catalysis of the disulfide interchange of this species (Figure 3). The new species was not generated from $(5-55,14-38)_N$ (data not shown). These observations indicate that the species was a mixed disulfide between glutathione and one of the two free cysteine residues of $(30-51,14-38)_N$, and it was included in the quantification of this species in Figure 2.

Each of the PDI domains increased substantially the overall rates of direct disulfide formation in reduced BPTI, as reflected by the increased rates of disappearance of the reduced BPTI and of formation of $(30-51,14-38)_N$ and $(5-55,14-38)_N$ (Figure 2). There was, however, relatively little increase in the rates of conversion of these two intermediates to N-BPTI. The level of accumulation of $(5-55,14-38)_N$ reached a plateau and remained constant thereafter. The level of $(30-51,14-38)_N$ decreased subsequently, but this was largely due to its conversion to the form with the mixed disulfide with glutathione; when this was included (as in Figure 2), the levels of this species also remained relatively constant.

These observations were confirmed with the isolated intermediates $(30-51,14-38)_N$ and $(5-55,14-38)_N$ under the same conditions. PDI caused the rapid disappearance of $(30-51,14-38)_N$; it was converted largely to N-BPTI, but also to $(5-55,14-38)_N$ to a smaller extent (Figure 3). The latter species arises by disulfide rearrangement of $(30-51,14-38)_N$, which also produces $(30-51,5-55)_N$ to about the same extent (Creighton & Goldenberg, 1984). The latter product is rapidly converted to N-BPTI in the presence of GSSG. That only small amounts of $(5-55,14-38)_N$ were generated in Figure 3 indicates that PDI was not producing N-BPTI primarily by catalyzing disulfide rearrangements but by inserting the 5–55 disulfide bond directly into $(30-51,14-38)_N$.

In the presence of the PDI-*a* and -*a'* domains under the same conditions, no significant amounts of $(5-55,14-38)_N$ were produced from $(30-51,14-38)_N$ (Figure 3). Therefore, the domains were not catalyzing disulfide rearrangements in $(30-51,14-38)_N$ to any significant extent. Their primary effect was to introduce the glutathione mixed disulfide on the free cysteine thiol groups. That the individual PDI domains can catalyze the rate of formation of mixed disulfides between the protein and glutathione will be confirmed with a model peptide (see Figure 6 below). Small amounts of N-BPTI were also generated, most probably by insertion of the 5–55 disulfide bond directly into $(30-51,14-38)_N$.

With the more stable $(5-55,14-38)_N$, which has two buried thiol groups, the PDI-*a* and -*a'* domains had essentially no effect under the same conditions (data not shown). Only very small amounts of N-BPTI were generated after 60 min. This could have occurred either by introduction of the 30–51 disulfide bond directly or by disulfide rearrangement to $(30-51,5-55)_N$, followed by rapid formation of the 14–38 disulfide bond; the most significant observation is, however, the very limited magnitude of the effect.

These small effects of the PDI-*a* and -*a'* domains on the two particular quasi-native intermediates can account for at least some of the increased rate of appearance of N-BPTI from reduced BPTI they produced (Figure 2). It is likely, however, that they were also increasing the rates of other paths, such as by causing formation of the 5–55 disulfide bond directly in the intermediate (30–51) (Creighton et al., 1980).

When present together, the two individual domains were no more active than expected from their individual activities, indicating that they were acting independently and in parallel.

The observations of Figures 2 and 3 indicate that the isolated PDI-*a* and -*a'* domains were nearly as active as PDI in catalyzing disulfide formation in reduced BPTI and the early disulfide intermediates, which are relatively unfolded, but less active with the folded quasi-native species $(30-51,14-38)_N$ and $(5-55,14-38)_N$. They could also catalyze formation of mixed disulfides between protein thiol groups

and glutathione. They were, however, essentially inactive in catalyzing disulfide rearrangements in the quasi-native species, where PDI has substantial activity.

Intramolecular Disulfide Rearrangements in the Isolated Intermediates. In the absence of a disulfide reagent, species $(30-51,14-38)_N$ spontaneously rearranges intramolecularly to form either $(5-55,14-38)_N$ or $(30-51,5-55)_N$ (Creighton & Goldenberg, 1984), with a half-time of about 67 min under the conditions used here; the latter species tends to be air-oxidized to N-BPTI. In the presence of 2 μM reduced PDI, the process occurred with a half-time of about 70 s, and the same species were generated. The accumulation of $(5-55,14-38)_N$ was always comparable to, but slightly lower than, the combined accumulation of $(30-51,5-55)_N$ and N-BPTI. In the presence of 2 μM reduced PDI-*a* or -*a*′ domains, or in the presence of both simultaneously, there was no increase in the spontaneous rate of disappearance of $(30-51,14-38)_N$; the levels of all the BPTI species were very similar to those in the control without catalysts (data not shown).

Species $(5-55,14-38)_N$ is very stable to both intramolecular rearrangement and oxidation, and no changes were apparent after 2 h in control mixtures. Addition of 2 μM reduced PDI resulted in rearrangement of $(5-55,14-38)_N$, to generate primarily $(30-51,5-55)_N$, and thence by oxidation to N-BPTI, with an overall half-time of 17 min. In the presence of the same amounts of either the PDI-*a* or -*a*′ domains, or both together, no changes to the $(5-55,14-38)_N$ were apparent (data not shown).

The 14−38 disulfide bond of N-BPTI is reduced rapidly by DTT to produce intermediate $(30-51,5-55)_N$. Subsequent reduction of the remaining two disulfide bonds of this stable species occurs only after their rearrangement through the other two-disulfide intermediates, in the reverse process of eq 1; the spontaneous rate is extremely slow under the conditions used here, with a half-time of several days. In the presence of 5 μM PDI, however, the half-time for reduction of 10 μM $(30-51,5-55)_N$ was reduced to about 1 h (data not shown). In the presence of the same concentration of either the PDI-*a* or -*a*′ domains, no reaction was detectable over a period of 80 min.

These observations indicate that, compared to complete PDI, the domains are unable to catalyze protein disulfide rearrangements.

Direct Disulfide Formation in the Isolated Intermediates. The quasi-native intermediates $(30-51,14-38)_N$ and $(5-55,14-38)_N$ do not form directly their missing disulfide bonds at a detectable rate in the presence of disulfide reagents like GSSG. In the case of $(5-55,14-38)_N$, this is due in the first instance to inaccessibility of the buried thiol groups, but the kinetic block occurs at a later stage in $(30-51,14-38)_N$, as its thiol groups react with GSSG to form the mixed disulfide intermediate (Creighton, 1981).

The third disulfide bond of each quasi-native species could be generated in the presence of stoichiometric amounts of the individual PDI-*a* and -*a*′ domains in the disulfide form. In each case, the rate of the reaction was proportional to the concentrations of both reactants. For example, the 5−55 disulfide bond was incorporated into $(30-51,14-38)_N$ by the disulfide forms of the PDI-*a* and -*a*′ domains with respective bimolecular rate constants of 709 and 89 s^{-1} M^{-1} (Figure 4). These rates of transfer of disulfide bonds from the PDI domains are slower than those observed with a model peptide (see below) but greater than those observed between

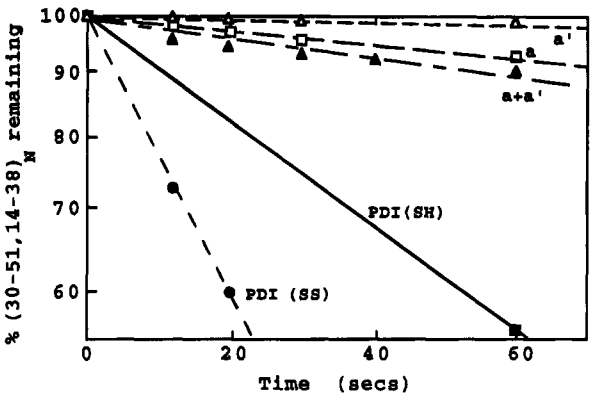


FIGURE 4: Effects of PDI and the PDI-*a* and -*a*′ domains on disulfide formation and rearrangement in BPTI species $(30-51,14-38)_N$ in the absence of thiol and disulfide reagents. The time course of disappearance of the initial 10 μM $(30-51,14-38)_N$ is shown in the presence of disulfide forms of (●) PDI, (□) PDI-*a* domain, (△) PDI-*a*′ domain, and (▲) both the PDI-*a* and -*a*′ domains. Only a single data point is shown with the reduced form of PDI (■), but the indicated rate was constant over at least 6 min. Each catalyst was present at a polypeptide concentration of 2 μM.

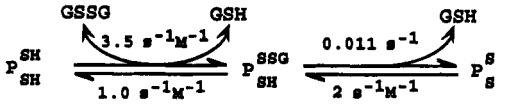
model thiol and disulfide groups under the same conditions. The rate observed with both domains present simultaneously was the sum of the individual rates, indicating that the domains functioned independently. In the case of the more stable $(5-55,14-38)_N$, the buried 30−51 disulfide bond was incorporated by the PDI-*a* and -*a*′ domains more slowly, as would be expected, with respective rate constants of 1.8 and 0.9 s^{-1} M^{-1} (data not shown). N-BPTI was the only product of each reaction.

Comparison of these activities to that of PDI under the same conditions was complicated by the disulfide isomerase activity of the PDI. The rate of formation of N-BPTI from either quasi-native two-disulfide species will reflect both their rearrangement to $(30-51,5-55)_N$, followed by its oxidation to N-BPTI, and the rate of direct incorporation of the final disulfide bond. If the reduced form of PDI is more active in catalyzing disulfide rearrangements, this activity should increase with time as the reduced form is generated by the reaction. To minimize such complications, the relative activities of PDI in disulfide formation and rearrangement were estimated by comparison of the initial rates observed starting with the disulfide and with the dithiol forms of the catalyst (Figure 4). N-BPTI was the expected predominant product in each case, but significant amounts of rearrangement products, $(30-51,5-55)_N$ and $(5-55,14-38)_N$, were apparent even at the early stages of the reaction and even starting with the disulfide form of the catalyst. The initial rates were constant with time. Therefore, the disulfide form of PDI might be active in catalyzing disulfide rearrangements in proteins with both thiol and disulfide groups. The rate of conversion of $(30-51,14-38)_N$ to N-BPTI was about 3-fold greater with the disulfide form of PDI than with its reduced form. This is consistent with the disulfide bonds of PDI being transferred directly to $(30-51,14-38)_N$ at about two-thirds of the observed rate. This rate would be about 10 times greater than that observed with an equivalent mixture of the two isolated domains (Figure 4).

In the case of $(5-55,14-38)_N$, which is more stable and where the thiol groups are buried, the rates with each of the catalysts were about 25-fold lower than had been measured with $(30-51,14-38)_N$ (data not shown). The rate with the

disulfide form of PDI was only 1.6-fold greater than that observed with the reduced form, suggesting that disulfide rearrangements predominated with this two-disulfide species. Nevertheless, the rate of direct transfer of disulfide bonds from PDI appeared to be significant. It occurred about 10 times more rapidly than with the isolated PDI-*a* and -*a*′ domains.

Disulfide Formation in a Model Peptide. A 28-residue synthetic peptide has proven to be a very useful and informative model system for the study of the effects of catalysts on disulfide bond formation (Darby et al., 1994; Darby & Creighton, 1995). It is based on residues 4−31 of BPTI, including Cys5 and Cys30, but with Cys14 replaced by Ser. Consequently, the peptide has two cysteine residues at positions 2 and 27 and is designated P_{2SH}^{27SH}. It is a simple model of an unfolded protein, as it is largely unfolded; only some local nonrandom conformations are apparent (Kemmink & Creighton, 1993), which do not affect formation and breakage of its disulfide bond. This peptide and a variant with Cys2 replaced by Ser, P_{2OH}^{27SH}, were used in previous studies of the effects of PDI and DsbA on disulfide bond formation (Darby et al., 1994; Darby & Creighton, 1995); HPLC could resolve all the possible thiol and disulfide species. The spontaneous chemical thiol−disulfide exchange reactions of the two peptides with GSSG and GSH under the conditions used here were extensively characterized in those studies. The two possible intermediates with mixed disulfides with glutathione on either of the two cysteine residues of P_{2SH}^{27SH} could be distinguished, and that on Cys27 accumulated somewhat more than the other, probably due to weak electrostatic interactions of the acidic glutathione with neighboring charged residues on the peptide. Considering the two intermediates together for kinetic purposes, the apparent rate and equilibrium constants for the noncatalyzed reactions are



(2)

Small, catalytic amounts of PDI were shown previously to catalyze all of these steps, except for interchange of the glutathione moiety between the two cysteine residues (Darby et al., 1994).

The individual PDI-*a* and -*a*′ domains behaved similarly and had just slightly lower activities than PDI in catalyzing formation in P_{2SH}^{27SH} of the mixed disulfide intermediates and of the peptide disulfide bond (Figure 5). The activity of the PDI-*a* domain was slightly greater than that of the PDI-*a*′ domain. The two domains together had virtually the same activity as intact PDI.

That PDI and the PDI-*a* and -*a*′ domains catalyzed formation of the mixed disulfides between glutathione and the peptide was confirmed with the single cysteine peptide P_{2OH}^{27SH} (Figure 6). The increase in the rate of the reaction was directly proportional to the catalyst concentration. The PDI-*a* and -*a*′ domains were nearly as active as PDI, and the two together virtually mimicked PDI. Again, the activity of the PDI-*a*′ domain was about 30% lower than that of the PDI-*a* domain.

That these catalysts increased the rate of the second step, formation of P_S^S from the glutathione mixed disulfide, was

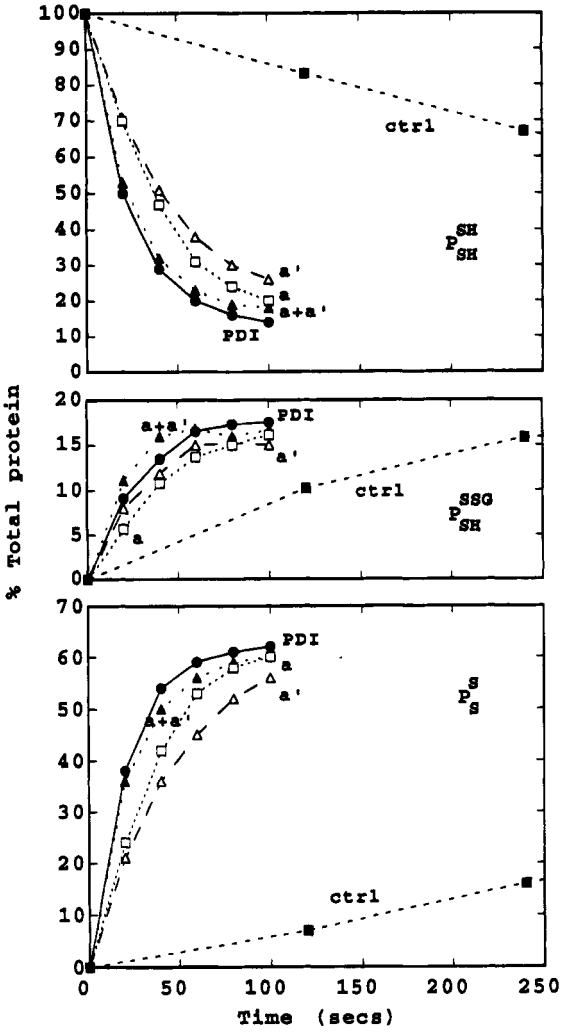


FIGURE 5: Effects of PDI and the PDI-*a* and -*a*′ domains on the reaction between the reduced peptide, P_{2SH}^{27SH}, and glutathione, to generate the two glutathione mixed disulfides (designated collectively as P_{SH}^{SSG}) and the peptide disulfide bond (P_S^S). The relative amounts of the various forms of the peptide were measured by HPLC analysis; the two mixed disulfides of the peptide with glutathione on either of its two cysteine residues were present in their normal ratio in each case, so they were quantified together. The time course of the noncatalyzed reaction with 0.5 mM GSSG and 2.0 mM GSH is depicted by (■). The other reaction mixtures also contained 1 μM each of (●) PDI, (□) PDI-*a* domain, (△) PDI-*a*′ domain, and (▲) both domains together. The catalyzed reactions are approaching the expected equilibrium positions.

confirmed using the two individual isolated mixed disulfide intermediates (Table 2). This reaction differs from the first in that it is intramolecular, normally involving simply the attack of the free cysteine thiol group of the peptide on the mixed disulfide between glutathione and the other cysteine residue. Depending upon which sulfur atom of the mixed disulfide is attacked, the result is either the peptide disulfide bond or transfer of the glutathione to the other cysteine residue; both occur spontaneously with similar rates. PDI has been shown to increase only the rate of forming the peptide disulfide bond and not that of interchange of the glutathione. The PDI-*a* and -*a*′ domains behaved similarly. The increase in rate of the first step was directly proportional to the concentration of each catalyst.

The individual PDI-*a* and -*a*′ domains had substantial activities with both glutathione−peptide mixed disulfides,

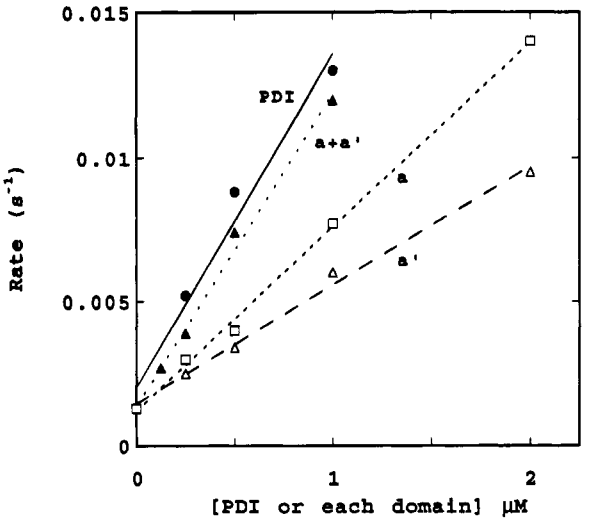


FIGURE 6: Effects of PDI and its isolated domains on the rate of forming the mixed disulfide with glutathione on the cysteine residue of peptide P_{2OH}^{27SH}. The initial rate of the reaction between 10 μM P_{2OH}^{27SH} and 0.5 mM GSSG plus 2.0 mM GSH was measured in the absence and presence of various amounts of the catalysts: (●) PDI, (□) PDI-*a* domain, (△) PDI-*a'* domain, and (▲) the indicated amounts of both domains together.

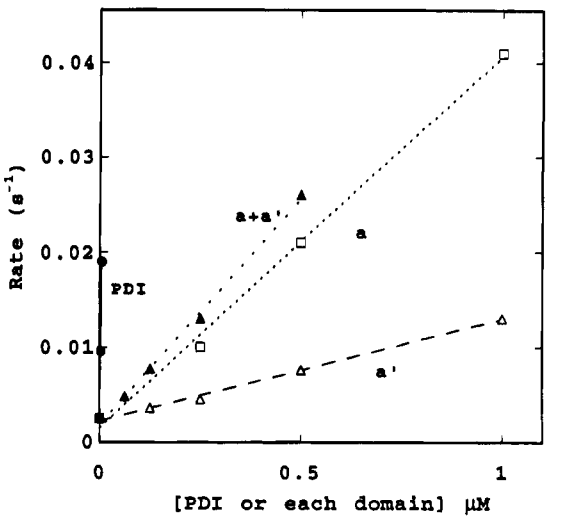


FIGURE 7: Effects of PDI and its isolated domains on the rate of reducing the disulfide of the peptide P_S^S by DTT. The initial rate of the reaction between 10 μM P_S^S and 1 mM DTT was measured in the absence and presence of varying amounts of the catalysts: (●) PDI, (□) PDI-*a* domain, (△) PDI-*a'* domain, and (▲) the indicated amounts of both domains together. The maximum concentration of PDI used was 10 nM.

Table 2: Catalytic Effects of PDI and Its Domains on the Rate of Forming the Disulfide Bond in the Mixed Disulfides between Glutathione and Each of the Peptide Cysteine Residues[a]

	rate produced by each catalyst (s^{-1}/μM catalyst)	
catalyst	P_{2SH}^{27SSG}	P_{2SSG}^{27SH}
PDI	0.30	0.31
PDI-*a* domain	0.024	0.070
PDI-*a'* domain	0.009	0.010
PDI-*a* and *a'* domains	0.041	0.080

[a] The initial rate of the reaction $P_{SH}^{SSG} \rightarrow P_S^S$ was measured with each of the mixed disulfide forms of the peptide and varying concentrations of each of the catalysts, which had been reduced prior to their use. The linear dependence of the observed rate upon the concentration of each catalyst provided the values given here. The observed rate in the absence of catalysts for each mixed disulfide was 0.01 s^{-1}. In the case where both PDI domains were present simultaneously in equal amounts, the concentration of catalyst was taken to be just that of a single domain.

although less than that of PDI. The two domains together produced an additive effect, indicating that they functioned independently. The PDI-*a* domain and the mixture of the two domains was more active with the glutathione mixed disulfide on Cys2 of the peptide, whereas the PDI-*a'* domain was equally active with both. PDI also did not discriminate between the two cysteine residues of the peptide. The most remarkable finding is that PDI was 4- and 8-fold more active with the two substrates than the equivalent mixture of the two isolated domains.

Catalysts must increase the rates in both directions equally; that this was occurring in the above cases was indicated by the reactions between the peptide and glutathione reaching the normal equilibrium positions (Figure 5). The differential effects of PDI and its isolated domains on the second step of forming P_S^S from P_{SH}^{SSG} were nevertheless confirmed by examining the reverse step, reduction of the peptide disulfide bond. DTT was used as thiol reagent, as its attack on the peptide disulfide bond should be rate-limiting. Its mixed disulfides with other molecules are very unstable, for it can form its intramolecular disulfide bond very rapidly. In agreement, no mixed disulfide with the peptide was observed to accumulate. The rate was increased in the presence of small quantities of PDI and its domains (Figure 7). Their efficiencies mimicked those observed in the reverse direction, but the differences were more dramatic, probably as a result of the different redox conditions. In particular, PDI was nearly 100 times more effective than either of its individual domains.

One of the surprising observations made previously (Darby et al., 1994) was that bovine PDI did not catalyze formation of the mixed disulfide in the reduced peptide when cystamine was used in place of glutathione. This observation was confirmed here with the recombinant PDI and was also found to apply to the isolated domains (data not shown).

Neither PDI nor either of the domains increased the rate of disappearance of P_{2SH}^{27SH} or P_{2OH}^{27SH} in the presence of 0.5 mM cystamine and 2 mM cysteamine, indicating that forming the mixed disulfide between the peptide and cysteamine was not catalyzed. But in the presence of PDI and its domains, the mixed disulfide intermediate was converted more rapidly to P_S^S, indicating that the second step of completing the peptide disulfide bond was catalyzed. The magnitude of the effect on this step was greatest with PDI and less with the domains, similar to the observations made with glutathione (Table 2).

Direct Transfer of Disulfide Bonds to the Reduced Peptide. Stoichiometric amounts of the disulfide forms of the PDI-*a* and -*a'* domains reacted directly with P_{2SH}^{27SH} to generate P_S^S (data not shown). No intermediates in the reaction were detectable. The velocity of the reaction was proportional to the concentrations of both reactants. The second-order rate constant for the PDI-*a* domain was 1.1×10^6 s^{-1} M^{-1}, just accessible to manual mixing and trapping methods. That for the PDI-*a'* domain was substantially lower, 9.5×10^4 s^{-1} M^{-1}. The corresponding rate with the oxidized form of PDI was too rapid to measure in this way, with a rate constant of $>10^6$ s^{-1} M^{-1}.

DISCUSSION

The two thioredoxin-like domains of PDI, *a* and *a'*, are shown to have some, but not all, of the functional properties of the complete molecule. The structural and functional properties of the isolated domains confirm that the intact molecule has a modular structure, made up of multiple structural domains. It is not yet clear, however, to what extent those domains are independent; the PDI-*a* domain behaved as a folded globular protein in both its dithiol and disulfide forms, and the structure of the disulfide form has been shown by NMR analysis to be very similar to that of thioredoxin, as expected (J. Kemmink et al., unpublished results). On the other hand, the PDI-*a'* domain appeared to be less stable than expected, as it unfolded at least partly upon formation of its disulfide bond (Figure 1). The low stabilities estimated for the disulfide bonds of complete PDI (Hawkins et al., 1991a; Lyles & Gilbert, 1991a; Lundström & Holmgren, 1993) imply that they should destabilize the folded conformation, for their stabilities will be greater in the unfolded conformation (Siedler et al., 1993; Zapun et al., 1993). This has been confirmed by direct measurement of the stabilities of the disulfide bond of each domain (unpublished data). That the disulfide bond of the PDI-*a'* domain should cause the isolated domain to unfold suggests that its folded conformation is less stable than when part of the complete protein. Other parts of the PDI polypeptide chain, either additional contiguous residues or other domains, are presumably required to enhance its stability.

There was no substantial difference in the catalytic activities of the two PDI domains. The PDI-*a'* domain consistently was somewhat less active than the other, but this could be due simply to its lower conformational stability. The results presented make it unlikely that the two domains have intrinsically different functions in PDI, although they could be modified by their context within the entire PDI molecule. There is evidence that the *a* and *a'* domains have similar and independent activities within PDI (Vuori et al., 1992a), although with some quantitative differences (Lyles & Gilbert, 1994).

Together, the isolated PDI-*a* and -*a'* domains had virtually all the activity of complete PDI in introducing disulfide bonds into reduced BPTI (Figure 2) and into the reduced peptide (Figure 5) in the presence of GSH and GSSG. This property of the domains varied with glutathione and cystamine in the same way as that of the complete PDI. There seems to be little doubt that these two domains are the functional parts of PDI in introducing disulfide bonds into unfolded proteins. This particular activity is likely, however, to arise by the disulfide form of the catalyst reacting with thiol groups of the substrate protein or peptide (Gilbert, 1989), and the rate-limiting step can be regeneration of the catalyst disulfide bond by reaction with disulfide reagent (unpublished data). Therefore, the reaction with the substrate might not be rate-determining (Darby & Creighton, 1995). The process is favorable because the stability of the disulfide bond of each domain is substantially less than that of an unfolded protein; the equilibrium for this process favors the reduced domain and the disulfide protein.

In the case of folded proteins, such as the quasi-native BPTI species $(30-51,14\text{-}38)_N$ and $(5-55,14-38)_N$, the isolated domains had substantial activity in introducing the missing disulfide bonds, but considerably less than that of complete PDI. Some of this difference may be due to the disulfide form of the PDI-*a'* domain tending to be unfolded in isolation (Figure 1), but the PDI-*a* domain was also less active than complete PDI. Introducing a disulfide bond that will be buried in the folded conformation is likely to occur more readily if the protein is unfolded (Darby et al., 1995), so this activity in the catalysts may be enhanced by the catalyst binding the unfolded form of a protein. There are other indications that PDI binds peptides and unfolded proteins (Morjana & Gilbert, 1991; Noiva et al., 1993). Therefore, the activity of PDI being greater than those of its *a* and *a'* domains in this function might result from it having a greater affinity for substrate proteins.

The greatest difference in function of the complete PDI and its *a* and *a'* domains was in their ability to catalyze disulfide rearrangements; the domains were much less active than the complete protein. This was evident in their relative activities with reactivating scrambled ribonuclease A (Table 1), although the identity of the rate-determining step in that assay is not known. It was more dramatic with the disulfide intermediates of BPTI, where the domains were essentially inactive (Figures 2–4). It was also apparent in the lower activity of the domains in catalyzing disulfide formation in the peptide from its mixed disulfide with glutathione (Table 2); this reaction is a type of intramolecular disulfide rearrangement. Clearly, some aspect of complete PDI is missing in its isolated *a* and *a'* domains. The missing aspect may be some other part of the protein, such as the other putative domains, or it could simply be the absence of two active sites on the same molecule.

The isolated PDI-*a* and -*a'* domains have functional properties similar to those of the bacterial catalyst DsbA, whose role *in vivo* appears to be to incorporate disulfide bonds into proteins newly translocated into the periplasm (Bardwell & Beckwith, 1993). The disulfide forms of the domains reacted with the reduced peptide and with $(30-51,14-38)_N$ at about the same rate as did DsbA (Zapun & Creighton, 1994; Darby & Creighton, 1995). Both the PDI domains and DsbA have very limited ability to catalyze disulfide rearrangements (Zapun & Creighton, 1994). DsbA has an extra domain, in addition to the thioredoxin fold, that is not present in the PDI-*a* and -*a'* domains; the present results raise the question of the function of this extra domain of DsbA. The PDI domains were also less active in catalyzing protein disulfide isomerizations than the other known bacterial catalyst, DsbC, which is believed to have this function *in vivo* (Zapun et al., 1995). It is very likely that the PDI-*a* and -*a'* domains function in ways very similar to those determined for DsbA (Darby & Creighton, 1995, and unpublished data).

The similarities and differences between the catalysts of thiol–disulfide exchange should assist in elucidating the mechanistic and structural basis for each of their activities. Although the features of these proteins required to catalyze disulfide formation are now becoming established, those required for the catalysis of disulfide rearrangement remain unclear. Progressive re-engineering of the PDI molecule from its component domains should prove an important approach to understanding these catalytic processes.

ACKNOWLEDGMENT

We thank K. Kivirikko for generously supplying the cloned gene for human PDI, Elke Penka and Renaud Vincentelli for excellent technical assistance, D. Nalis and R. Jacob for synthesizing the peptides, M. Mann for the mass spectroscopy analyses, and Johan Kemmink for the nuclear magnetic resonance analysis.

REFERENCES

Bardwell, J. C. A., & Beckwith, J. (1993) *Cell 74*, 769−771.
Bardwell, J. C. A., McGovern, K., & Beckwith, J. (1991) *Cell 67*, 581−589.
Creighton, T. E. (1981) *J. Mol. Biol. 151*, 211−213.
Creighton, T. E. (1992) in *Protein Folding* (Creighton, T. E., Ed.) pp 301−351, W. H. Freeman, New York.
Creighton, T. E., & Goldenberg, D. P. (1984) *J. Mol. Biol. 179*, 497−526.
Creighton, T. E., Hillson, D., & Freedman, R. B. (1980) *J. Mol. Biol. 142*, 43−62.
Creighton, T. E., Bagley, C. J., Cooper, L., Darby, N. J., Freedman, R. B., Kemmink, J., & Sheikh, A. (1993) *J. Mol. Biol. 232*, 1176−1196.
Darby, N. J., & Creighton, T. E. (1995) *Biochemistry 34*, 3576−3587.
Darby, N. J., Freedman, R. B., & Creighton, T. E. (1994) *Biochemistry 33*, 7937−7947.
Darby, N. J., Morin, P. E., Talbo, G., & Creighton, T. E. (1995) *J. Mol. Biol. 249*, 463−477.
Davis, B. J. (1964) *Ann. N. Y. Acad. Sci. 121*, 404−427.
Edman, J. C., Ellis, L., Blacher, R. W., Roth, R. A., & Rutter, W. J. (1985) *Nature 317*, 267−270.
Freedman, R. B. (1992) in *Protein Folding* (Creighton, T. E., Ed.) pp 455−539, W. H. Freeman & Co., New York.
Freedman, R. B., Hirst, T. R., & Tuite, M. F. (1994) *Trends Biochem. Sci. 19*, 331−336.
Gilbert, H. F. (1989) *Biochemistry 28*, 7298−7305.
Gilbert, H. F., Kruzel, M. L., Lyles, M. M., & Harper, J. W. (1991) *Protein Expression Purif. 2*, 194−198.
Gill, S. C., & von Hippel, P. H. (1989) *Anal. Biochem. 182*, 319−326.
Givol, D., Goldenberger, R. F., & Anfinsen, C. B. (1964) *J. Biol. Chem. 239*, 3114−3116.
Hawkins, H. C., & Freedman, R. B. (1991) *Biochem. J. 275*, 335−339.
Hawkins, H. C., DeNardi, M., & Freedman, R. B. (1991a) *Biochem. J. 275*, 341−348.
Hawkins, H. C., Blackburn, E. C., & Freedman, R. B. (1991b) *Biochem. J. 275*, 349−353.
Hayano, T., Inaka, K., Otsu, M., Taniyama, Y., Miki, K., Matsushima, M., & Kikuchi, M. (1993) *FEBS Lett. 328*, 203−208.
Holmgren, A. (1985) *Annu. Rev. Biochem. 54*, 237−271.
Hu, C. H., & Tsou, C. L. (1991) *FEBS Lett. 290*, 87−89.
Huth, J. R., Perini, F., Lockridge, O., Bedows, E., & Ruddon, R. W. (1993) *J. Biol. Chem. 268*, 16472−16482.
Hwang, C., Sinskey, A. J., & Lodish, H. F. (1992) *Science 257*, 1496−1502.
Kallis, G.-B., & Holmgren, A. (1980) *J. Biol. Chem. 255*, 10261−10265.
Kemmink, J., & Creighton, T. E. (1993) *J. Mol. Biol. 234*, 861−878.
Krause, G., Lundström, J., Barea, J. L., de la Cuesta, C. P., & Holmgren, A. (1991) *J. Biol. Chem. 266*, 9494−9500.
Laemmli, U. K. (1970) *Nature 27*, 680−685.
LaMantia, M., & Lennarz, W. J. (1993) *Cell 74*, 899−908.
Lambert, N., & Freedman, R. B. (1983) *Biochem. J. 213*, 225−234.
Lundström, J., & Holmgren, A. (1990) *J. Biol. Chem. 265*, 9114−9120.
Lundström, J., & Holmgren, A. (1993) *Biochemistry 32*, 6649−6655.
Lyles, M. M., & Gilbert, H. F. (1991a) *Biochemistry 30*, 613−619.
Lyles, M. M., & Gilbert, H. F. (1991b) *Biochemistry 30*, 619−625.
Lyles, M. M., & Gilbert, H. F. (1994) *J. Biol. Chem. 269*, 30946−30952.
Martin, J. L., Bardwell, J. C. A., & Kuriyan, J. (1993) *Nature 365*, 464−468.
Missiakas, D., Georgopoulos, C., & Raina, S. (1994) *EMBO J. 13*, 2013−2020.
Morjana, N. A., & Gilbert, H. F. (1991) *Biochemistry 30*, 4985−4990.
Nelson, J. W., & Creighton, T. E. (1994) *Biochemistry 33*, 5974−5983.
Noiva, R., Freedman, R. B., & Lennarz, W. J. (1993) *J. Biol. Chem. 268*, 19210−19217.
Pihlajaniemi, T., Helaakoski, T., Tasanen, K., Myllylä, R., Huhtala, M.-L., Koivu, J., & Kivirikko, K. I. (1987) *EMBO J. 6*, 643−649.
Reutimann, H., Straub, B., Luisi, P. L., & Holmgren, A. (1981) *J. Biol. Chem. 256*, 6796−6803.
Shevchik, V. E., Condemine, G., & Robert-Baudouy, J. (1994) *EMBO J. 13*, 2007−2012.
Siedler, F., Rudolph-Böhner, S., Doi, M., Musiol, H.-J., & Moroder, L. (1993) *Biochemistry 32*, 7488−7495.
Studier, F. W. (1991) *J. Mol. Biol. 219*, 37−44.
Studier, F. W., Rosenberg, A. H., & Dunn, J. W. (1990) *Methods Enzymol. 185*, 60−89.
Tasanen, K., Parkkonen, T., Chow, L. T., Kivirikko, K. I., & Pihlajaniemi, T. (1988) *J. Biol. Chem. 263*, 16218−16224.
Vuori, K., Myllylä, R., Pihlajaniemi, T., & Kivirikko, K. I. (1992a) *J. Biol. Chem. 267*, 7211−7214.
Vuori, K., Pihlajaniemi, T., Marttila, M., & Kivirikko, K. (1992b) *Proc. Natl. Acad. Sci. U.S.A. 89*, 7467−7470.
Wunderlich, M., Otto, A., Seckler, R., & Glockshuber, R. (1993) *Biochemistry 32*, 12251−12256.
Zapun, A., & Creighton, T. E. (1994) *Biochemistry 33*, 5202−5211.
Zapun, A., Creighton, T. E., Rowling, P. J. E., & Freedman, R. B. (1992) *Proteins: Struct., Funct., Genet. 14*, 10−15.
Zapun, A., Bardwell, J. C. A., & Creighton, T. E. (1993) *Biochemistry 32*, 5083−5092.
Zapun, A., Missiakas, D., Raina, S., & Creighton, T. E. (1995) *Biochemistry 34*, 5075−5089.

BI950883K