Dissecting the Mechanism of Protein Disulfide Isomerase: Catalysis of Disulfide Bond Formation in a Model Peptide

Nigel J. Darby,‡ Robert B. Freedman,§ and Thomas E. Creighton*,‡

European Molecular Biology Laboratory, Meyerhofstrasse 1, D-69012 Heidelberg, Germany, and The Biological Laboratory, University of Kent, Canterbury CT2 7NJ, England

Received March 7, 1994; Revised Manuscript Received April 22, 1994®

ABSTRACT: As a model for understanding how protein disulfide isomerase (PDI) catalyzes disulfide bond formation in proteins, its action on a 28-residue disordered peptide containing only two cysteine residues has been examined. Disulfide formation in the peptide using the chemical reaction with small molecule thiol/disulfide reagents, such as oxidized and reduced glutathione or cystamine and cysteamine, occurs in two steps, via two alternative intermediate mixed disulfides between the reagent and either peptide cysteine residue. All thiol/disulfide forms of the peptide could be trapped and quantified, so the rates of their interconversion could be measured. Catalytic amounts of PDI increased the rates of these reactions. All rate enhancements were independent of the concentration of the peptide, indicating that it bound to PDI with an apparent K_m of less than 3 μM. In the presence of glutathione, PDI accelerated the formation of both single mixed disulfide species, plus their subsequent rearrangement to form the peptide disulfide bond, but not interchange of the mixed disulfide glutathione between the two cysteine residues. In contrast, PDI did not catalyze the reaction of the reagent cystamine with the reduced peptide to form the mixed disulfide, nor the interchange of this mixed disulfide between cysteine residues, but it did catalyze the subsequent intramolecular step of peptide disulfide bond formation to a similar extent as with the glutathione mixed disulfide. These effects on the two steps involving the mixed disulfides with glutathione or cystamine accounted for much of the overall catalytic effect of PDI on disulfide bond formation in the peptide, indicating that direct transfer of disulfide bonds from PDI to the peptide occurred less frequently. These findings demonstrate the utility of using such peptides as PDI substrates and have implications for the mechanism of action of PDI.

Protein disulfide isomerase catalyzes the disulfide-coupled folding of proteins in the endoplasmic reticulum [reviewed by Freedman (1992)]. The detailed mechanism of PDI[1] action is unknown but is thought to involve acceleration of thiol–disulfide interchange reactions at either or both of the two -Cys-Gly-His-Cys- sequences that occur in each of the two thioredoxin-like sequences in the PDI polypeptide chain (Edman et al., 1985; Hawkins & Freedman, 1991). The two catalytic sites are able to act independently of each other (Vuori et al., 1992).

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). One reason for the versatility of this active site sequence is that the redox properties of these cysteine residues can be regulated by the protein structure to be highly oxidizing in the case of catalysts like DsbA (Zapun et al., 1993) and PDI (Hawkins et al., 1991; Lyles & Gilbert, 1991a; Lundström & Holmgren, 1993) or much more reducing, as in the case of thioredoxin (Lundström & Holmgren, 1990). In each of these proteins there is evidence that one active site cysteine residue has an unusually high reactivity and a pK_a value that is lower than normal (Kallis & Holmgren, 1980; Hawkins & Freedman, 1991; Nelson & Creighton, 1994). This undoubtedly facilitates thiol–disulfide interchange processes but is unlikely to be the sole factor accounting for catalytic activity.

The redox state of the PDI cysteine residues, whether they occur as free thiols or inter- or intramolecular disulfides, is probably 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 (Hwang et al., 1992), with the overall concentration of glutathione being in the millimolar range. Under these redox conditions, PDI is required for the *in vitro* disulfide-linked folding of BPTI and of its natural precursor to occur at rates approaching those observed in the ER (Creighton et al., 1980, 1993; Zapun et al., 1992). PDI catalyzes formation, breakage, and rearrangement of disulfide bonds in this folding reaction, but the catalysis of rearrangements is particularly important in this case to minimize the accumulation of stable quasinative intermediates (Creighton et al., 1980, 1993; Weissman & Kim, 1993). Rearrangement processes are thought to occur through a disulfide-linked PDI/protein complex, probably formed by the attack of a thiol group of PDI on a disulfide bond of the refolding protein (Creighton et al., 1980). The observation of such complexes has been reported (Huth et al., 1993; Hu & Tsou, 1991).

Compared to rearrangements, the catalysis of disulfide bond formation by PDI has received less attention and is not routinely assayed, but it is probably of equal or greater importance. For example, PDI caused the 5–55 disulfide bond to be formed directly in the BPTI (30–51) single-disulfide intermediate, thereby bypassing the disulfide rearrangements

‡ European Molecular Biology Laboratory.

§ University of Kent.

® Abstract published in *Advance ACS Abstracts,* June 1, 1994.

[1] Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; CSSC, cystamine; CSH, cysteamine; ER, endoplasmic reticulum; GSSG and GSH, the oxidized and reduced forms of glutathione, respectively; HPLC, high-pressure liquid chromatography; IAEDANS, *N*-iodoacetyl-*N'*-(8-sulfo-1-naphthyl)ethylenediamine; TFA, trifluoroacetic acid; P^{2SH}_{27SH} peptide substrate containing cysteine residues at positions 2 and 27; 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 these peptides are indicated by the sub- and superscripts -SSG (glutathione) and -SSC (cysteamine); PDI, protein disulfide isomerase.

0006-2960/94/0433-7937$04.50/0

by which the resulting native-like two-disulfide species is normally generated (Creighton et al., 1980). PDI can transfer its own disulfide bonds directly into proteins by thiol–disulfide exchange reactions analogous to those with low molecular weight disulfide reagents (Lyles & Gilbert, 1991b). It is also possible that PDI could catalyze thiol–disulfide exchange events involving the main endogenous oxidant of the endoplasmic reticulum, GSSG. This latter possibility was raised in our 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. It was evident that PDI increased the rate of formation of a glutathione mixed disulfide with this extra cysteine residue (Creighton et al., 1993). Recently, PDI has been reported to reduce a protein mixed disulfide species (Hayano et al., 1993).

Although PDI activity is normally assayed with large protein substrates, such as disulfide-scrambled ribonuclease A, there is considerable evidence and growing interest in its ability to bind and utilize small peptides as substrates (Welply et al., 1991; Morjana & Gilbert, 1991; Gilbert, 1989; Noiva et al., 1993). The ability to bind a peptide substrate would be likely to contribute significantly to the catalytic activity of the protein. PDI has been shown to catalyze reduction of the disulfide bond of a hexapeptide by GSH (Gilbert, 1989), but the full advantages of using small peptides as substrates to investigate the catalytic action of PDI have not been exploited. Such simple substrates potentially allow the action of the catalyst on individual cysteine residues and disulfide bonds to be examined. Because the chemistry of thiol–disulfide interchange processes and the factors that affect them are well understood, it is likely that such analysis will result in a greater understanding of how PDI functions. In this report we exploit these analytical possibilities by using a 28-residue model peptide containing two cysteine residues at positions 2 and 27, designated P_{27SH}^{2SH}, to examine disulfide bond formation catalyzed by PDI.

MATERIALS AND METHODS

Materials. The peptide substrate used in this study, P_{27SH}^{2SH}, has the sequence FCLEPPYTGPSKARIIRYFYNAKAGLCQ, with the N- and C-terminal groups acetylated and amidated, respectively. A second peptide, P_{27SH}^{2OH}, in which Cys2 was replaced by a Ser residue, was also used. The peptides were synthesized using 9-fluorenylmethoxycarbonyl chemistry and purified by HPLC, and their identities were confirmed by mass analysis. Peptide concentrations in solution were determined using the molar extinction coefficient at 280 nm of 3840 cm^{-1} calculated according to Gill and von Hippel (1989), which was verified by the assay of free thiol groups in the peptides by the method of Ellman (Creighton, 1989). PDI was purified from bovine liver as previously described (Lambert & Freedman, 1983).

Thiol–Disulfide Exchange Reactions. All reactions between peptide, thiol and disulfide reagents, and PDI were carried out in 0.1 M Tris-HCl (pH 7.4), 0.2 M KCl, 1 mM EDTA at 25 °C. Reactions were quenched by addition of an aliquot of this mixture to 0.05 volumes of 6 M HCl.

Analysis of Reaction Products. Peptide species in the quenched reaction mixtures were analyzed on a Vydac 25 cm × 0.46 cm 218TP54 column using a linear gradient of 27%–33% (v/v) acetonitrile in 0.1% (v/v) TFA in 25 min at a flow rate of 1 mL min^{-1}. At the end of this gradient the acetonitrile concentration was increased to 60% (v/v) to elute the adsorbed PDI. Peptides were detected and quantified by their absorbance at 220 nm.

Peptide Mapping. The particular cysteine residues of P_{27SH}^{2SH} involved in mixed disulfides with the reagent were identified by peptide mapping. Each lyophilized mixed disulfide species was resuspended in 0.1 M IAEDANS (Kenyon & Bruice, 1977) dissolved in 0.1 M Tris (pH 8.0), and 6 M guanidinium chloride to irreversibly block all free thiol groups, and the alkylation reaction was allowed to proceed 30 min at 25 °C. The AEDANS derivative was isolated by chromatography on a 10 × 0.46 cm Aquapore RP300 column using a 45-min gradient of 5–45% acetonitrile (v/v) in 0.1% (v/v) TFA at a flow rate of 1 mL/min. The freeze-dried peptide was resuspended in 10 mM HCl, and 5 μg of peptide was digested with 0.6 μg of Arg-C endopeptidase in a final volume of 50 μL of 0.1 M Tris-HCl (pH 8) at 37 °C for 3 h. The two mixed disulfide species obtained with each reagent were digested in parallel with the AEDANS derivatives, $P_{27SAEDANS}^{2SAEDANS}$ and $P_{27SAEDANS}^{2OH}$. The peptides were separated by HPLC, and the eluate was monitored at 220 and 340 nm. Only the peptides containing an AEDANS alkylated cysteine residue have substantial absorbance at 340 nm. The peptide maps of $P_{27SAEDANS}^{2SAEDANS}$ and $P_{27SAEDANS}^{2OH}$ produced AEDANS-peptides with both Cys2 and Cys27 and just Cys27, respectively. The peptide digest from the two mixed disulfide species trapped with IAEDANS each contained peptides that corresponded to the labeling of only Cys2 or Cys27 with IAEDANS, identifying the other cysteine residue as being involved in the mixed disulfide.

RESULTS

The Model Peptide. The model peptide used in this study, designated P_{27SH}^{2SH}, is based on residues 4–31 of BPTI, but with Cys14 replaced by Ser. The two cysteine residues of P_{27SH}^{2SH} correspond to Cys5 and Cys30 of BPTI, which do not form a disulfide bond in the native protein but do so transiently at the one-disulfide stage of folding (Darby & Creighton, 1993). Reduced BPTI, with no disulfide bonds, is very disordered, with only some local interactions evident (Kemmink & Creighton, 1993), so it forms the initial disulfide bonds randomly. One of these, the Cys5–Cys30 disulfide bond is formed at about the rate expected for a disordered polypeptide chain (Darby & Creighton, 1993). The resulting intermediate remains unfolded and flexible, except for the disulfide cross-link (Darby et al., 1992).

As would be expected, the thiol and disulfide forms of the peptide, P_{27SH}^{2SH} and P_S^S, respectively, gave ^1H nuclear magnetic resonance spectra typical of random coil peptides, although the weak local interaction involving Tyr10 and the amide of Gly12 observed in reduced BPTI and in short peptides was also evident from the anomalous chemical shift of the Gly12 amide and was similar in both forms (Kemmink & Creighton, 1993); therefore, this interaction does not stabilize the disulfide bond and *vice versa*. The many resonances expected from minor forms with *cis* peptide bonds preceding the three proline residues were also evident. The peptide therefore is a model of an unfolded protein and adopts no stable nonrandom conformations that would affect formation and breakage of its disulfide bond.

Noncatalyzed Thiol–Disulfide Exchange between the Peptide and Glutathione. An HPLC separation system was devised that allowed the separation of all possible peptide products of thiol–disulfide interchange between P_{27SH}^{2SH} and GSSG (Figure 1). Disulfide bond formation in P_{27SH}^{2SH} under these conditions followed the normal chemical mechanism, proceeding through intermediate mixed disulfide species (eq

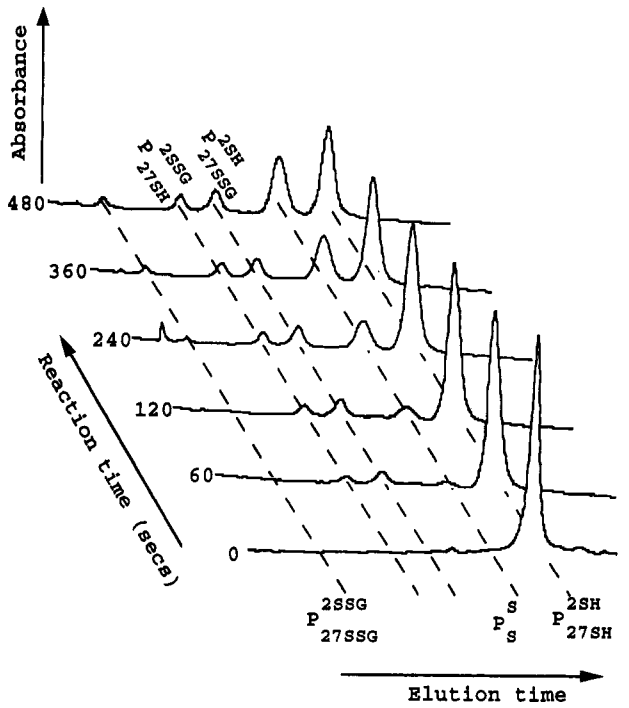


FIGURE 1: HPLC analysis of the products of thiol disulfide interchange between 10 μM P_{27SH}^{2SH} and a mixture of 0.5 mM GSSG and 1.4 mM GSH. At various times, aliquots were removed, acidified, and analyzed by HPLC to quantify the levels of the trapped species. The individual species were identified by measurement of their molecular weights by mass spectroscopy: double mixed disulfide species, 3858 (expected 3858.8); single mixed disulfides, 3553 and 3554 (expected 3553.4); intramolecular disulfide peptide, 3246 (expected 3246.7); reduced peptide, 3248 (expected 3248.7). The cysteine residues involved in the mixed disulfide species were identified by peptide mapping as described under Materials and Methods.

1). The two possible single mixed disulfide species were resolved by the separation system, and the cysteine residues involved in each of the mixed disulfide species were identified by peptide mapping. The first single mixed disulfide eluted was determined to be P_{27SH}^{2SSG} and the second P_{27SSG}^{2SH}. The double mixed disulfide P_{27SSG}^{2SSG} was also resolved but was not a major species under the conditions used here.

Using this separation system, the kinetics of appearance and disappearance of each species were determined during disulfide bond formation and breakage in the peptide using varying concentrations of GSH and GSSG. The rate constants for the individual steps were determined by comparison of the observed kinetics to simulations of the process in which the values of the individual rate constants were varied (data not shown). The data were entirely consistent with the following values for all the rate constants:

$$\text{GSSG} + P_{27SH}^{2SH} \underset{1.0\ s^{-1}M^{-1}}{\overset{3.5\ s^{-1}M^{-1}}{\rightleftharpoons}} P_{27SH}^{2SSG} + P_{27SSG}^{2SH} + \text{GSH} \underset{2\ s^{-1}M^{-1}}{\overset{0.011\ s^{-1}}{\rightleftharpoons}} P_S^S + 2\ \text{GSH}$$

$K_1 = 3.5 \qquad K_2 = 5.5\ \text{mM} \qquad (1)$

The rate constants were also consistent with the equilibrium constants for the two half-reactions measured independently. The single mixed disulfide species were treated together in eq 1. The equilibrium constant for forming the single mixed disulfide species is close to the ideal value of 4 predicted from the statistics of thiol–disulfide interchange (Darby & Creighton, 1993), although the mixed disulfide on Cys27 accumulated to somewhat greater levels than the other. The double mixed disulfide species, P_{27SSG}^{2SSG}, only accumulated to a significant extent (maximum 4% of total peptide) with the highest concentrations of reagent, 0.5 mM GSSG and 1.4 mM GSH (Figure 1), as expected, and could be ignored in the kinetic analyses performed here. The rate constants for its formation and reduction were not measured accurately but were estimated to be approximately 1 s^{-1} M^{-1} in both directions. This reaction is expected to have an equilibrium constant of unity.

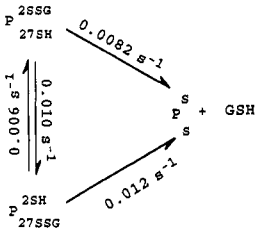
To study formation of the mixed disulfide species in isolation, a variant peptide with a single cysteine residue, with Cys2 replaced by Ser, P_{27SH}^{2OH}, was studied in the same way. The single mixed disulfide peptide could be separated from the reduced protein by HPLC, and the kinetics of formation and reduction of P_{27SSG}^{2OH} at varying concentrations of GSH and GSSG could be determined. The following single set of rate constants for this process were derived by simulation of the experimental data (Figure 2a) and were also consistent with the measured equilibrium constant:

$$\text{GSSG} + P_{27SH}^{2OH} \underset{1.5\ s^{-1}M^{-1}}{\overset{3.0\ s^{-1}M^{-1}}{\rightleftharpoons}} P_{27SSG}^{2OH} + \text{GSH}$$

$K_{eq} = 2 \qquad (2)$

These rate constants are compatible with those found for P_{27SH}^{2SH} (eq 1) in that the rate of forming the single mixed disulfide in P_{27SH}^{2OH} was less than the rate of forming both single mixed disulfides in P_{27SH}^{2SH}, while the rate constant for the reverse step was very similar.

Thiol–disulfide interchange is quenched at acid pH but resumes upon increasing the pH to 7.4. Consequently, the intramolecular conversion of each mixed disulfide peptide to the intramolecular disulfide peptide could be followed using acid trapped P_{27SH}^{2SSG} or P_{27SSG}^{2SH}. Two reactions are possible in each case: formation of the peptide disulfide bond or exchange of the mixed disulfide glutathione between the two cysteine residues. Both processes were observed in experiments that started with each of the isolated mixed disulfides, in the absence of GSH and GSSG (Figure 2b,c). The rate constants for the individual processes were determined by simulation of the kinetic data from eight individual experiments using a single set of rate constants:



(3)

Each of these interconversions involves very similar steps, in which the two cysteine residues of the peptide, with one present as a mixed disulfide, come into suitable proximity for thiol–disulfide exchange to occur. Which reaction occurs depends upon which sulfur atom of the mixed disulfide is the site of attack by the free peptide thiol group (see eq 6). Consequently, the various steps are expected to occur with very similar rate constants; they are observed to vary by no more than a factor of 2. The intramolecular rate constants for making the peptide disulfide bond from each of the mixed disulfides are similar to the overall average value obtained when they were treated as a single kinetic species (eq 1).

By combining these data on the rearrangement of the mixed disulfides of P_{27SH}^{2SH} with those for the formation and reduction

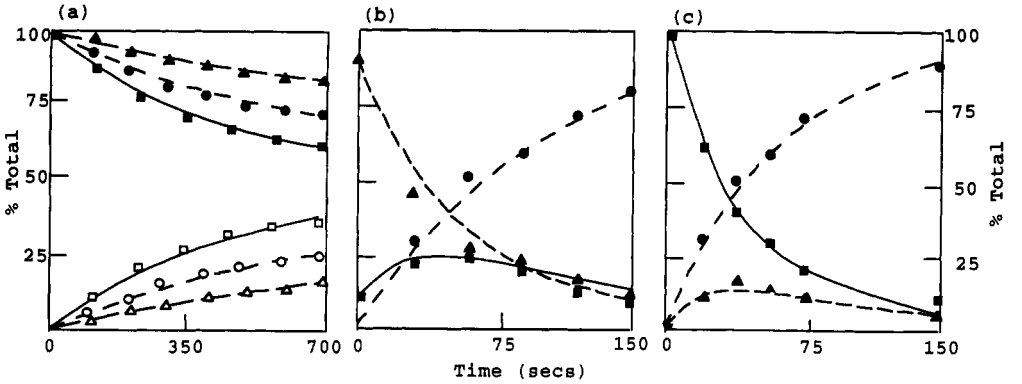
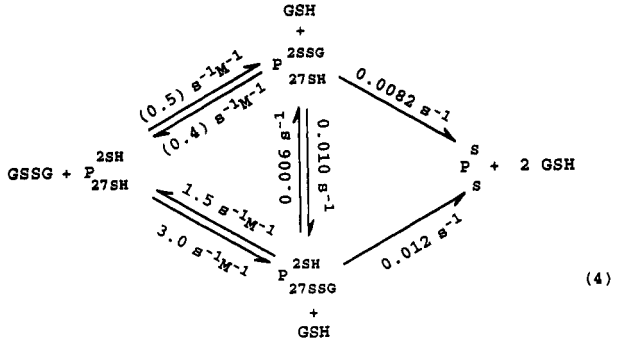


FIGURE 2: Dissecting the process of disulfide bond formation in P^{2SH}_{27SH} with GSSG. (a) Formation of the mixed disulfide species of P^{2OH}_{27SH}. The reaction was carried out using 0.125 mM GSSG/0.7 mM GSH (▲), 0.25 mM GSSG/1 mM GSH (●) or 0.5 mM GSSG/1.4 mM GSH (■) and a peptide concentration of 10 μM. The open points indicate concentrations of the mixed disulfide species and the closed points the reduced peptide. The curves were generated by computer simulations using the rate constants of eq 2 of the text. (b and c) Rearrangement of the acid-trapped mixed disulfide peptides P^{2SSG}_{27SH} (b) and P^{2SH}_{27SSG} (c) at a concentration of 5 μM and in the absence of GSH and GSSG. The curves were generated by computer simulations using the rate constants of eq 3. (▲) P^{2SSG}_{27SH}; (■) P^{2SH}_{27SSG}; (●) P^{S}_{S}. Species P^{2SSG}_{27SH} could not be completely purified, so reactions that started with this species always contained some P^{2SH}_{27SSG}, which was accounted for in the simulation.

of the mixed disulfide of P^{2OH}_{27SH} and for the overall kinetics of mixed disulfide formation in P^{2SH}_{27SH}, the following overall scheme could be established:



(4)

The rate constant for making P^{2SSG}_{27SH} was not determined experimentally but was estimated by subtracting that for formation of P^{2OH}_{27SSG} from the overall rate of mixed disulfide formation in P^{2SH}_{27SH}. Formation and interconversion of the mixed disulfides is a closed cycle, and the net free energy change around it must be zero, so the rate of reduction of P^{2SSG}_{27SH} was calculated to be 0.4 s^{-1} M^{-1}. This value, and that for the rate of reduction of P^{2SH}_{27SSG}, give a weighted estimate of 1.1 s^{-1} M^{-1} for the overall rate of mixed disulfide reduction, which is consistent with the value of 1.0 s^{-1} M^{-1} obtained from simulations of the overall process (eq 1). The two rate constants that were not determined directly are not likely to be very accurate, so they are depicted in brackets; they are not used in the subsequent analysis. Similarly, the rates of breaking the peptide disulfide by attack of GSH on the sulfur atoms of either Cys2 or Cys27 could be extracted from the data and were predicted to be 0.6 and 1.4 s^{-1} M^{-1}, respectively, but these reactions also were not used directly in the subsequent analysis.

The thiol and disulfide groups of the unfolded model peptide would be expected to be accessible and normally reactive in thiol–disulfide exchange, and these expectations were largely confirmed. The rates of intermolecular exchange between the thiol and disulfide groups of the peptide and of glutathione occurred at about the expected rate, 1 s^{-1} M^{-1} for reaction between one thiol group and one sulfur atom of a disulfide bond. The intramolecular rates for interchanging each of the mixed disulfides and for forming the intramolecular peptide disulfide bond, relative to the intermolecular rate for their reduction by GSH, indicated a consistent effective concentration between the two cysteine residues of the peptide (one in the thiol form, the other as a mixed disulfide) of 14 ± 8 mM, which is close to the value measured in the corresponding BPTI intermediate and about that expected for forming a disordered disulfide loop of this size (Darby & Creighton, 1993). The only exception was that the mixed disulfide P^{2SH}_{27SSG} was somewhat favored over P^{2SSG}_{27SH} both in rate of formation and in stability. This is probably due to the presence of an acidic residue, Glu4, near Cys2 and a basic residue, Lys23, near Cys27. Glu4 would diminish the ionization and reactivity of Cys2 and might interact unfavorably with acidic glutathione, while Lys23 would have the opposite effect on Cys27. Nevertheless, these interactions produce only a slight difference in stabilities of the mixed disulfide intermediates.

Catalysis by PDI of Disulfide Bond Formation in P^{2SH}_{27SH} Using Glutathione as Reagent. The catalytic effects of PDI were determined simply upon adding substoichiometric amounts of purified PDI to the same reactions between the peptide and glutathione and monitoring them in the same way. The relative concentrations of GSH and GSSG were chosen to keep the ratio $[GSH]^2$:[GSSG] constant at a value of 4 mM, in the expectation that the relative proportions of the presumed disulfide and thiol forms of PDI would also be kept relatively constant. This ratio and the absolute concentrations were also chosen to be similar to those of GSH and GSSG in the ER (Hwang et al., 1992). Under these conditions, the peptide reaches an equilibrium (calculated from the most accurate equilibrium constants of eq 1) in which the ratio of P^{S}_{S} to P^{2SH}_{27SH} is 4.8. The equilibrium concentration of the mixed disulfide forms depends upon the actual concentrations of GSH and GSSG; with those used here, the mixed disulfides are expected to comprise between 10% and 18% of the molecules. Therefore, the equilibrium concentration of P^{S}_{S} should vary from 68% to 75% of the peptide molecules, and that of P^{2SH}_{27SH} from 14% to 16%.

The presence of PDI increased the rates of all the steps of forming the disulfide bond in P^{2SH}_{27SH}. The rates of disappearance of the reduced peptide and appearance of the disulfide form were both increased about 10-fold by 1.6 μM PDI monomer (Figure 3). The rates at which each of the single mixed disulfide species accumulated were also increased

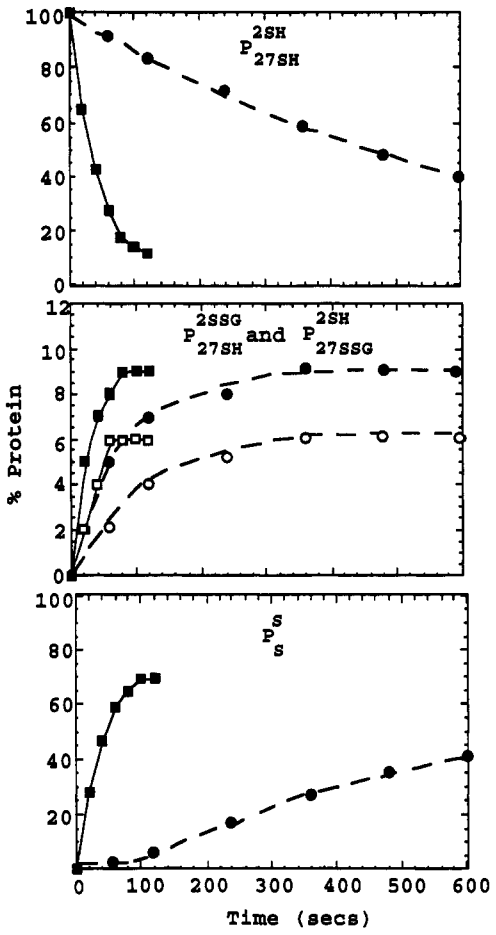


FIGURE 3: Catalysis by PDI of disulfide bond formation in P^{2SH}_{27SH}. The reaction between 10 μM peptide and 0.5 mM GSSG/1.4 mM GSH was measured as described in the legend to Figure 1 in the presence (squares) or absence (circles) of 1.6 μM PDI monomer. In the case of the single mixed disulfide species, the closed symbols refer to P^{2SH}_{27SSG} and the open symbols to P^{2SSG}_{27SH}.

substantially and to about the same extent; the kinetics were consistent with each being formed directly from P^{2SH}_{27SH} at very similar rates. The normal equilibrium mixture of species was attained, although much more rapidly, indicating that PDI also increased, to the same extents, the rates of both reverse reactions, breakage by GSH of the intramolecular peptide disulfide and the mixed disulfide bonds. PDI has long been known to catalyze the reaction between GSH and other peptide disulfide bonds (Freedman, 1992).

The catalyzed rate of disappearance of P^{2SH}_{27SH} was directly dependent on the concentration of PDI and on the concentration of GSSG in the range 0.125–0.5 mM. The catalyzed rate was independent of the concentration of P^{2SH}_{27SH} over the range 3–30 μM (data not shown), indicating that PDI was saturated by binding of the peptide; its K_m must be less than 3 μM. Exact simulation of the time course of this type of kinetic situation is difficult without knowledge of the relative K_m values for the different forms of the peptide, which could not be measured. The relative rates of the forward and reverse reactions individually do not depend directly upon the concentrations of the relevant form of the peptide, but they will in practice during the course of the reaction when the various forms are present and competing for the catalyst. If the substrate and product form of the peptide have similar affinities for the catalyst, a reasonable approximation of the actual situation during the approach to equilibrium is to treat the forward and reverse rates as being proportional to the concentration of the appropriate form of the peptide; this will be used in the following.

The ability of PDI to catalyze formation of the peptide mixed disulfide was confirmed by using P^{2OH}_{27SH} as substrate (eq 2; data not shown), where the forward and reverse rate constants could be measured accurately. The increase in the observed reaction rate was proportional to the concentrations of PDI and of glutathione. The kinetics of mixed disulfide formation and reduction could be simulated well using an approximate model in which the catalyzed contribution to the rates depended upon the concentrations of PDI, of either GSSG or GSH, and of the peptide (solely to correct for the reverse reaction); the rate constants used are given in Table 1. PDI at a concentration of 1.6 μM increased the rate of each step by a factor of 10. If each of the two thioredoxin-like domains of PDI were acting on a bound peptide molecule, the bimolecular reaction between the peptide and glutathione would be occurring about 30 times more rapidly than in solution, and at twice that rate if only a single peptide molecule is bound per PDI monomer.

PDI was also observed to increase the intramolecular rate at which each mixed disulfide species formed the peptide disulfide bond, in the absence of GSH and GSSG (Figure 4). The PDI was first equilibrated with 0.5 mM GSSG and 1.4 mM GSH to fix its redox state and then isolated by gel filtration. Only a limited range of PDI concentrations could be examined because of the rapidity of the uncatalyzed reaction. With 0.6 μM PDI monomer, the reaction was essentially complete within 20 s, with almost complete conversion of the mixed disulfide to the peptide disulfide; even concentrations of PDI as low as 40 nM caused significant rate enhancements. In contrast, there was no corresponding increase in rate of interchange of the mixed disulfide glutathione. The catalyzed rate of conversion of P^{2SH}_{27SSG} to P^{S}_{S} was proportional to the amount of PDI, but independent of the concentration of P^{2SH}_{27SSG} over the range 2.5–15 μM, indicating that PDI was saturated by binding of the mixed disulfide species; its K_m must be less than 2.5 μM.

The kinetic data from several experiments with both acid trapped P^{2SSG}_{27SH} and P^{2SH}_{27SSG} could be simulated using the approximate procedure of treating the rate of each step as being proportional to the total concentration of the relevant form of the peptide, solely to correct for the effect of the reverse reaction. In this way, consistent rate constants could be determined with both P^{2SSG}_{27SH} and P^{2SH}_{27SSG}, but only the primary data starting with P^{2SH}_{27SSG} are presented (Figure 4). PDI increased the rates of the intramolecular steps in forming the peptide disulfide bond more than it did those of formation of the mixed disulfides. For example, 1.6 μM PDI increased the two second steps 33- and 39-fold, while the first steps were increased only 10-fold (Table 1). If PDI is catalyzing the intramolecular reaction to form the disulfide bond in one peptide molecule bound to each of the two thioredoxin domains, the reaction is occurring 100–120 times more rapidly than in solution, but twice that if only one site is active.

Do these effects of PDI on the individual steps involving the mixed disulfide intermediates account for its effect on the overall reaction of forming the disulfide bond in P^{2SH}_{27SH}, or is there a contribution to the overall rate from other pathways? The above measurements of the rates of forming the single mixed disulfide species and converting them to the peptide disulfide can be used to predict the rate via this pathway in the presence of 1.6 μM PDI (Table 1). These estimates assume that 1.6 μM PDI increases the rate of forming and reducing both mixed disulfides by 10-fold, which is the rate increase

Table 1: Apparent Rate Constants for Forming Disulfide Bonds in 10 μM Peptide

reaction	noncatalyzed				with 1.6 μM PDI[a]	
			ratio[b]			
	R = G	R = C	observed	expected[c]	R = G	R = C
P_{SH}^{SH} + RSSR ↔ P_{SH}^{SSR} [d] + RSH						
forward	3.5 s^{-1} M^{-1}	28 s^{-1} M^{-1}	8.0	7.8	35 s^{-1} M^{-1}	28 s^{-1} M^{-1}
reverse	1.0 s^{-1} M^{-1}	4 s^{-1} M^{-1}	4.0	5.0	10 s^{-1} M^{-1}	4 s^{-1} M^{-1}
P_{SH}^{SSR} [d] ↔ P_S^S + RSH						
forward	0.011 s^{-1}	0.025 s^{-1}	2.3	3.4	0.38 s^{-1}	0.50 s^{-1}
reverse	2 s^{-1} M^{-1}	3 s^{-1} M^{-1}	1.5	1.8	72 s^{-1} M^{-1}	60.7 s^{-1} M^{-1}
P_{SH}^{SSR} [d] + RSSR ↔ P_{SSR}^{SSR} + RSH						
forward	1 s^{-1} M^{-1} [e]	15 s^{-1} M^{-1}	15[e]	7.8	1 s^{-1} M^{-1} [e]	15 s^{-1} M^{-1}
reverse	1 s^{-1} M^{-1} [e]	7 s^{-1} M^{-1}	7[e]	4.0	1 s^{-1} M^{-1} [e]	7 s^{-1} M^{-1}
P_{27SH}^{2OH} + RSSR ↔ P_{27SSR}^{20H} + RSH						
forward	3.0 s^{-1} M^{-1}	13 s^{-1} M^{-1}	4.3	7.8	30 s^{-1} M^{-1}	13 s^{-1} M^{-1}
reverse	1.5 s^{-1} M^{-1}	5.0 s^{-1} M^{-1}	3.3	4.0	15 s^{-1} M^{-1}	5.0 s^{-1} M^{-1}
P_{27SH}^{2SSR} ↔ P_{27SSR}^{2SH}						
forward	0.010 s^{-1}	0.021 s^{-1}	2.1	2.3	0.010 s^{-1}	0.021 s^{-1}
reverse	0.006 s^{-1}	0.028 s^{-1}	4.7	2.3	0.006 s^{-1}	0.028 s^{-1}
P_{27SH}^{2SSR} ↔ P_S^S + RSH						
forward	0.0082 s^{-1}	0.020 s^{-1}	2.4	3.4	0.33 s^{-1}	0.56 s^{-1}
P_{27SSR}^{2SH} ↔ P_S^S + RSH						
forward	0.012 s^{-1}	0.018 s^{-1}	1.5	3.4	0.40 s^{-1}	0.48 s^{-1}

[a] The initial rate of the reaction catalyzed by PDI is independent of the concentration of the peptide, but treating the overall reaction as being first-order in peptide concentration accounts approximately for the effect of the reverse reaction. [b] Ratio of observed rate with cystamine (R = C) to that with glutathione (R = G). [c] Ratio of rate with R = C to that with R = G expected on the basis of eq 38 of Szajewski and Whitesides (1980), with pK_a values for the thiol groups of the peptide, GSH, and CSH of 8.7, 8.8, and 7.9, respectively. [d] Considering the two cysteine residues together. [e] Approximate value.

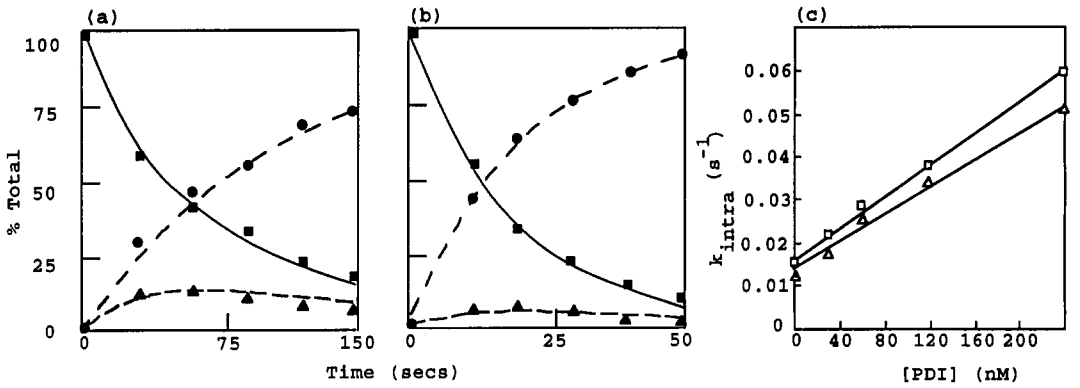


FIGURE 4: Rearrangement of the mixed disulfide P_{27SSG}^{2SH} in the presence of PDI. Rearrangements were initiated by rapidly diluting the acid-trapped mixed disulfide to a final concentration of 10 μM into 0.1 M Tris -HCl (pH 7.4), 0.2 M KCl, and 1 mM EDTA that contained PDI monomer at a concentration of (a) 0 or (b) 120 nM. The PDI had been equilibrated with 0.5 mM GSH/1.4 mM GSH for 15 min before being desalted by gel filtration into 10 mM Tris-HCl (pH 7.4). (■) P_{27SSG}^{2SH}; (▲) P_{27SH}^{2SSG}; (●) P_S^S. The curves were generated by computer simulations using the rate constants of Table 1. The dependence of the intramolecular rate of peptide disulfide formation by rearrangement of (□) P_{27SSG}^{2SH} and (△) P_{27SH}^{2SSG} on the PDI concentration is shown in (c).

measured with P_{27SH}^{2OH}; in agreement, both mixed disulfide species were observed to be generated directly at about the same increased rate (Figure 3). The estimated rate constant for the intramolecular step in forming the peptide disulfide bond is the weighted average of the two values determined from the rearrangement of the individual mixed disulfide species in the presence of PDI. The effects of the reverse reactions were approximated by treating each rate as being proportional to the concentration of the appropriate form of the peptide. This will only approximate the time course of the approach to equilibrium, but the satisfactory fit of similar types of simulations to those shown in Figure 4 demonstrate the accuracy of the approximation.

Using these estimated rates in simulations, the effects of PDI on the rates of formation of the mixed disulfide with glutathione and on the intramolecular step to form the protein disulfide bond could largely account for the overall effect of PDI on the rate of formation of the peptide disulfide bond (Figure 5). The overall rate of forming the peptide disulfide bond was consistently underestimated somewhat, however, which is probably due to its direct formation from PDI, without the involvement of the glutathione mixed disulfide intermediates.

Noncatalyzed Disulfide Bond Formation in P_{27SH}^{2SH} Using Cystamine as the Reagent. Disulfide bond formation in the peptide was also analyzed using cystamine (CSSC) and cysteamine (CSH) as the disulfide and thiol reagents, keeping their relative concentrations the same as those used with glutathione. Exactly the same HPLC separation and peptide mapping techniques could be used to resolve and identify all the possible peptide species (data not shown). Using the types of experiments described above for glutathione, rate constants for the overall kinetic process were determined by simulation of data from experiments at three different concentrations of CSSC and CSH and are given in Table 1. Again, the mixed disulfides accumulated to somewhat different extents, but in

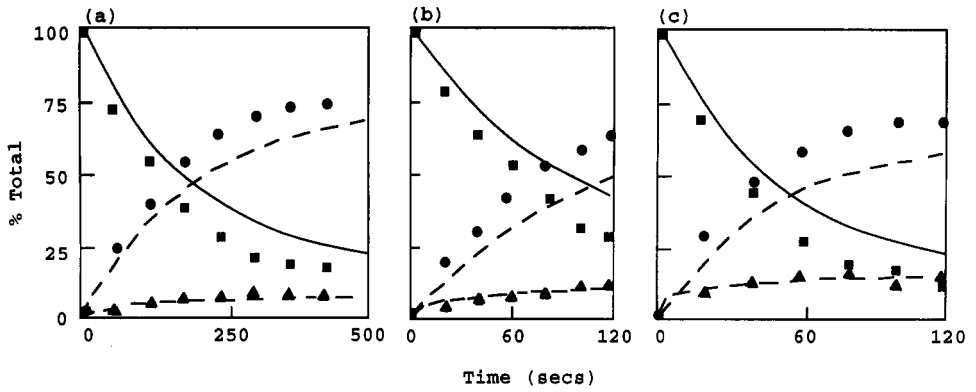
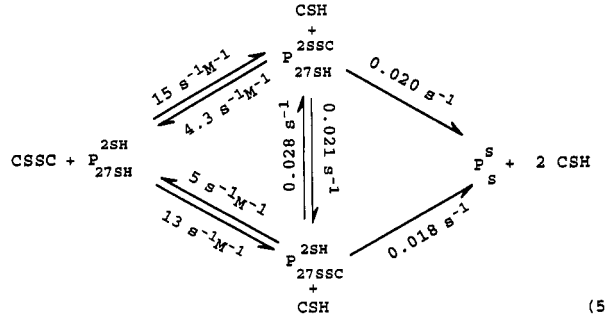


FIGURE 5: Comparison of the observed kinetics of disulfide bond formation in P^{2SH}_{27SH} in the presence of glutathione and 1.6 μM PDI with those expected if the reaction proceeds only through the glutathione mixed disulfide intermediate and there is no direct oxidation of the reduced peptide by PDI. The observed levels of the peptide species in the presence of (a) 0.125 mM GSSG/0.7 mM GSH; (b) 0.25 mM GSSG/1 mM GSH; (c) 0.5 mM GSSG/1.4 mM GSH are given by the points: (■) P^{2SH}_{27SH}; (▲) $P^{2SH}_{27SSG} + P^{2SSG}_{27SH}$; (●) P^{S}_{S}. The curves were generated with the rate constants of Table 1.

this case with P^{2SSC}_{27SH} being slightly favored over P^{2SH}_{27SSC}, the opposite to that observed with glutathione. Cystamine is basic, whereas glutathione is acidic, so this would be consistent with the previous proposal that weak electrostatic interactions between the reagent and Glu4 and Lys23 are responsible for the slight differences in stabilities of the two mixed disulfide forms of the peptide. The rates of thiol–disulfide exchange involving cystamine and cysteamine were up to 8-fold greater than the corresponding reactions involving oxidized and reduced forms of glutathione. Similar observations have been made with these reagents in thiol–disulfide exchange reactions with low molecular weight thiol compounds (Creighton, 1975), and the increased rates are due to the pK_a of the cysteamine thiol group being nearly one pH unit lower than that of glutathione (Creighton, 1975). The different rate constants observed with the two different reagents are almost exactly those predicted from the empirical relationship of Szajewski and Whitesides (1980), which correlates the rate of thiol–disulfide exchange with the pK_a values of the sulfur atoms when present as thiol groups. The expected rate constants were calculated using the symmetrical eq 38 of Szajewski and Whitesides (1980) and pK_a values of the thiol groups of the peptide, GSH, and CSH of 8.7, 8.8, and 7.9, respectively. The predicted ratios of the rate constants with CSSC and CSH to those with GSSG and GSH for each step are compared to the observed ratios in Table 1. The excellent agreement indicates that the differences in rates of reaction of the two reagents with the peptide are totally explicable on the basis of their different properties.

Using P^{2OH}_{27SH} and each of the acid–trapped mixed disulfide species, P^{2SSC}_{27SH} and P^{2SH}_{27SSC}, the overall process was dissected as above for glutathione:



(5)

Catalysis by PDI of Disulfide Bond Formation Using Cystamine as Reagent. Using various concentrations of CSSC and CSH, 1.6 μM PDI monomer produced no substantial increase in the rate of disappearance of the reduced peptide, while there was a modest increase in the rate of appearance of the disulfide form (Figure 6). The levels of the one- and two-mixed disulfide species were substantially reduced. Data similar to those shown in Figure 6 were obtained with 0.125 mM CSSC/0.7 mM CSH and 0.25 mM CSSC/1 mM CSH. These observations indicate that PDI increased the rate of the second, intramolecular step in forming the peptide disulfide bond but did not increase the rate of the first step, formation of the mixed disulfide intermediate.

This interpretation was confirmed by measuring the individual steps in isolation. The presence of 1.6 μM PDI did not increase the rate at which the mixed disulfide P^{2OH}_{27SSC} was formed from P^{2OH}_{27SH} with three different concentrations of CSSC and CSH (Figure 7a). Neither did it increase the rate of interchange of the two mixed disulfide intermediates P^{2SH}_{27SSC} and P^{2SSC}_{27SH}. PDI did, however, increase the rate at which both mixed disulfide species rearranged to the peptide disulfide bond (Figure 7b). The experimental data could be simulated using the same procedures as in the case of glutathione and the rate constants of Table 1. The catalytic effects of PDI on forming the peptide disulfide bond were quantitatively very similar to those measured with the glutathione mixed disulfide intermediates.

The observed overall process of disulfide bond formation in the peptide was compared to that expected if all the effects of PDI were those involving the mixed disulfides with cystamine. Using the same approach as in the case of glutathione, the expected rate constants for the reaction via the mixed disulfide are given in Table 1. Once again, the effects of PDI were largely accounted for by its increase in the rates of the reaction proceeding through the mixed disulfides (Figure 8). The difference between the observed and expected rates was even smaller than in the case of glutathione (Figure 5), indicating an even smaller contribution of other pathways, such as direct transfer of a disulfide bond from PDI.

DISCUSSION

PDI appears to be the primary catalyst of protein disulfide bond formation in eukaryotes and also to have additional functions (Pihlajaniemi et al., 1987; Noiva & Lennarz, 1992; Freedman, 1992), yet little is known about its mechanism of action. Its observed effects on proteins indicate that it can catalyze both rearrangement and the net formation and

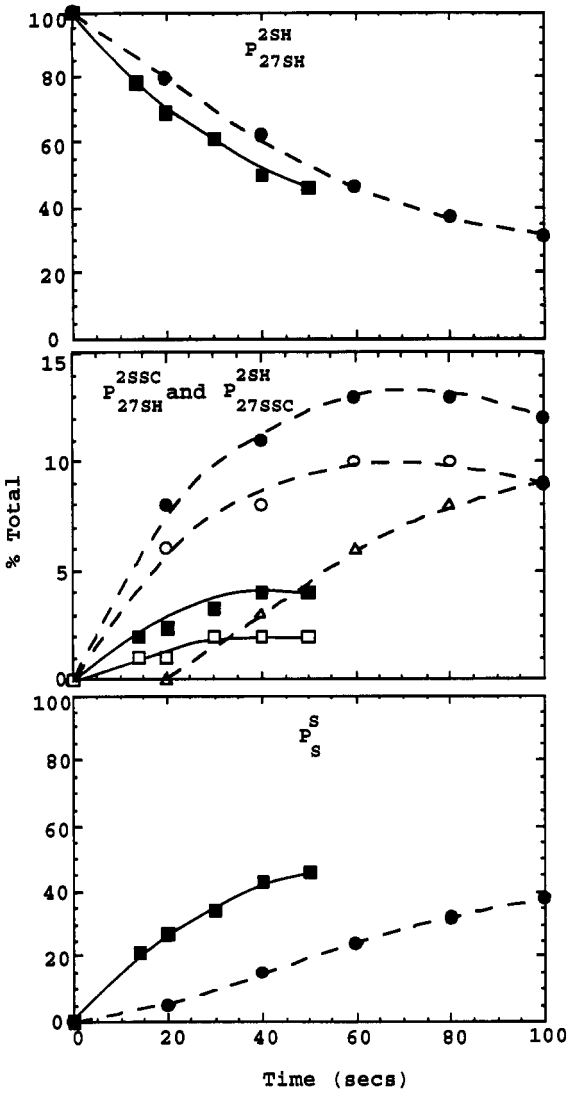


FIGURE 6: Effects of PDI on thiol–disulfide interchange reactions of P^{2SH}_{27SH} with cystamine. The reaction between 10 μM P^{2SH}_{27SH} and 0.5 mM CSSC/1.4 mM CSH in the presence (squares) or absence (circles) of 1.6 μM PDI monomer was monitored as a function of time. In the case of the single-mixed disulfide species, the closed squares and circles refer to P^{2SSC}_{27SH} and the open squares and circles to P^{2SH}_{27SSC}. The open triangles refer to the double-mixed disulfide species in the absence of PDI; this species did not accumulate in the presence of PDI.

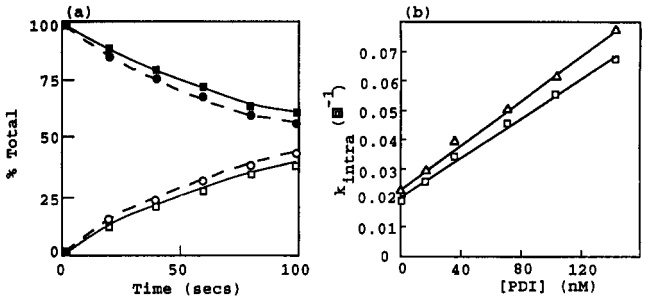


FIGURE 7: Dissecting the effects of PDI on thiol–disulfide interchange between P^{2SH}_{27SH} and cystamine. (a) Mixed disulfide formation in 10 μM P^{2OH}_{27SH} with 0.5 mM CSSC/1.4 mM CSH, in the presence (squares) or absence (circles) of 1.6 μM PDI. The closed symbols refer to P^{2OH}_{27SH} and the open symbols to P^{2OH}_{27SSC}. (b) The dependence on PDI concentration of the intramolecular rate of rearrangement of 10 μM (□) P^{2SH}_{27SSC} and (△) P^{2SSC}_{27SH} to the peptide disulfide. The PDI had been pre-equilibrated with 0.5 mM CSSC/1.4 mM CSH for 15 min before being desalted by gel filtration into 10 mM Tris-HCl (pH 7.4).

breakage of protein disulfide bonds, but studies on complex proteins are not likely to elucidate the mechanism by which PDI functions. This requires the use of a model system in which the effect of PDI on individual steps can be determined directly. The well-behaved model peptide studied here fulfilled that role, for each of the steps involved in forming, breaking, and rearranging disulfide bonds could be monitored. Furthermore, the peptide showed no evidence of stable secondary or tertiary structure in either the dithiol or disulfide forms, so the process of disulfide formation could be studied in isolation and modeled without reference to folding and stabilization of the disulfide bond. PDI was found to catalyze some steps, but not all, and the results give some significant insight into how PDI functions.

There are many indications that the catalytic effects of PDI on disulfide bonds involve thiol–disulfide exchange reactions between the cysteine residues of the protein substrate and those of PDI found in the two -Cys-Gly-His-Cys- sequences in the two segments homologous to thioredoxin. In analyzing the catalytic effects, it is important that the thiol/disulfide status of these groups be maintained constant. This should have been accomplished here by keeping the ratio of $[GSH]^2$/[GSSG] constant at 4 mM, when any PDI intramolecular disulfide bonds should be present at constant amounts at equilibrium. Under these conditions, these PDI cysteine residues will be overwhelmingly (Hawkins et al., 1991; Lyles & Gilbert, 1991a) or substantially (Lundström & Holmgren, 1993) in the dithiol state, depending upon the value taken for their redox potentials. The conditions were also selected to mimic those in the lumen of the ER where PDI functions; in this compartment there are millimolar concentrations of GSH and GSSG, in a ratio of between 3:1 and 1:1 (Hwang et al., 1992). The same concentrations of the nonphysiological CSH and CSSC were used, although the equilibrium constant for forming protein disulfide bonds with this reagent should be about 4-fold greater (Table 1). For experimental reasons, the concentration of PDI used was generally much less than that *in vivo*, which is likely to be at least 0.1 mM. The modest effects of small quantities of PDI observed here in all the *in vitro* assays of thiol/disulfide exchange are therefore likely to be potentially much greater under *in vivo* conditions. On the other hand, the intrinsic catalytic effects of PDI are small by enzymatic standards. Rate enhancements of 10–40-fold were observed with 10 μM peptide and 1.6 μM PDI, which probably corresponds to an active site concentration of 3.2 μM. In this case, the reactions on PDI occurred only 30–120-fold more rapidly than in solution; similar rate enhancements have been measured by others with PDI in other systems (Lyles & Gilbert, 1991a; Freedman, 1992).

In considering how PDI might function, it is useful to consider the less complex but related proteins thioredoxin and glutaredoxin (Holmgren, 1989) and DsbA (Zapun et al., 1993, 1994; Martin et al., 1993), which are structurally and functionally homologous to the two thioredoxin-like domains of PDI. In particular, they each have at their active sites two cysteine residues, separated in the sequence by two other residues, that reversibly form a disulfide bond. These proteins differ dramatically in the stability of that disulfide bond (Martin et al., 1993), but they have the common property of undergoing thiol–disulfide exchange reactions at their active site much more rapidly than do comparable small molecules in solution. Their active sites appear to stabilize the transition state for thiol–disulfide exchange (Nelson & Creighton, 1994). This contribution to catalysis can be augmented by them

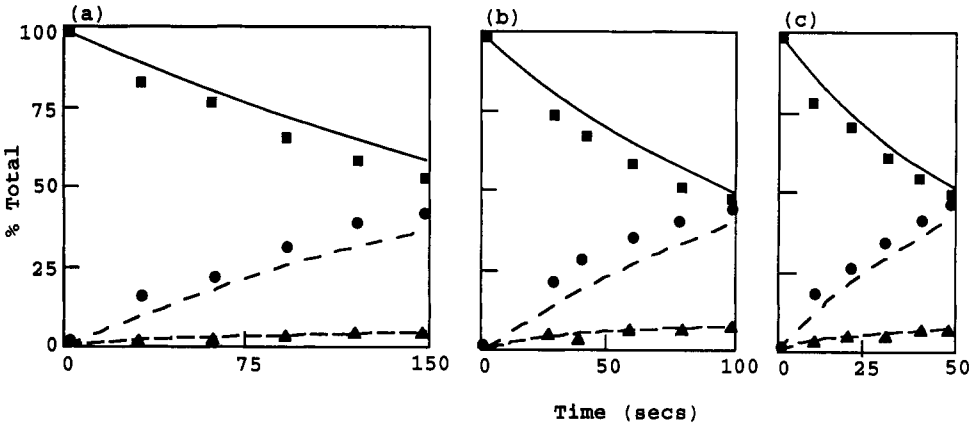


FIGURE 8: Comparison of the observed kinetics of disulfide bond formation in P_{27SH}^{2SH} in the presence of cystamine and 1.6 μM PDI with those expected if the reaction proceeds only through the cystamine mixed disulfide intermediate and there is no direct oxidation of the reduced peptide by PDI. The observed levels of the peptide species in the presence of (a) 0.125 mM CSSC/0.7 mM CSH; (b) 0.25 mM CSSC/1 mM CSH; (c) 0.5 mM CSSC/1.4 mM CSH are given by the points: (■) P_{27SH}^{2SH}; (▲) $P_{27SSG}^{2SH} + P_{27SH}^{2SSG}$; (●) P_S^S. The curves were generated with the rate constants of Table 1.

binding their substrates, which can increase dramatically the effective concentrations of the thiol and disulfide moieties in the active site (Page & Jencks, 1971). PDI appeared to bind the peptide used here, with an apparent K_m value of less than 3 μM. As with any enzyme, binding or release of the substrate could be the rate-limiting step in catalysis. Binding some molecules and not others can also impart specificity to the catalytic activity. For example, the effects of PDI on protein folding would be assisted if it binds to, and acts on, unfolded polypeptide chains in preference to folded proteins.

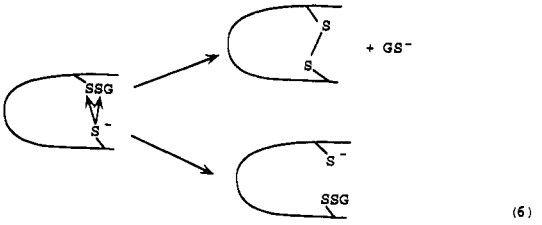
In spite of binding the peptide tightly, there were no indications of selectivity of PDI between its two cysteine residues, for PDI appeared to catalyze formation of the mixed disulfide with glutathione at each cysteine residue to the same extent. Likewise, the intramolecular conversion of each mixed disulfide to the peptide disulfide bond was catalyzed to about the same extent. This is to be expected with a promiscuous catalyst, yet it raises the question how it is possible structurally with tight binding of the peptide to PDI.

In catalysis of mixed disulfide formation by PDI there appeared to be a remarkable specificity for glutathione, which is believed to be the physiological cofactor for PDI (Hwang et al., 1992). Mixed disulfide formation with cysteamine was not catalyzed to any detectable extent. The noncatalyzed reaction with cystamine was more rapid than with glutathione, but catalysis would have been apparent even if PDI increased the rate to that observed with glutathione. No catalysis was apparent at low cystamine concentrations, where the uncatalyzed rates were comparable to those at the higher glutathione concentrations. The absence of catalysis with cystamine suggests either a repulsion of cystamine or a specific interaction of PDI with both GSH and GSSG. On the other hand, such an interaction must be weak, for the catalytic effects of PDI in this reaction were proportional to the free concentrations of GSSG in the range 0.125–0.5 mM and of GSH in the range 0.7–1.4 mM; this suggests that the apparent K_m for each must be greater than these concentrations. Similar observations have been made on PDI catalysis of breakage of peptide disulfide bonds by GSH (Gilbert, 1989; Morjana & Gilbert, 1991), although the redox state of PDI was not kept constant and the individual steps were not measured. Consequently, the thiol/disulfide reagent that is present at highest concentrations in the ER, glutathione, appears also to be intimately involved in the function of PDI and does not serve just to maintain its thiol/disulfide status.

Once the mixed disulfide peptide was formed, PDI exhibited no specificity between those with glutathione or cysteamine; both forms of each cysteine residue were converted intramolecularly to the peptide disulfide bond at very similar rates by PDI. This strongly suggests that there were no specific interactions between PDI and the mixed disulfide of the peptide.

The mechanism by which PDI catalyzes the formation of the mixed disulfide between the peptide and glutathione is not revealed by these experiments. A cysteine thiol group of the reduced peptide bound to PDI could react with either GSSG or a mixed disulfide between PDI and glutathione. Conceivably, an unstable mixed disulfide between the peptide and PDI could also be intercepted by reaction with GSH (otherwise, it should be converted rapidly to the peptide disulfide bond by intramolecular reaction with the second cysteine thiol group of the peptide; see eq 8). With each of these mechanisms, however, it is difficult to account for the observed kinetic behavior and for the specificity for glutathione.

The experimental observations give a clearer indication of how PDI catalyzes the second step, intramolecular formation of the peptide disulfide bond; this involves intramolecular thiol–disulfide exchange between the mixed disulfide on one cysteine residue and the free thiol group of the other cysteine residue of the peptide. In this case, the reaction was catalyzed to very similar extents with both the glutathione and cysteamine mixed disulfides. PDI did not, however, detectably catalyze the transfer of either glutathione or cysteamine of the mixed disulfide to the other cysteine residue. The transfer process and intramolecular disulfide formation are very similar reactions and differ only in which sulfur atom of the mixed disulfide bond is attacked by the free cysteine thiol group in the thiolate anion form:



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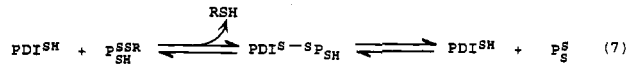
A mechanism for catalysis by PDI of only one of the steps of eq 6 that can account for all of these observations is that

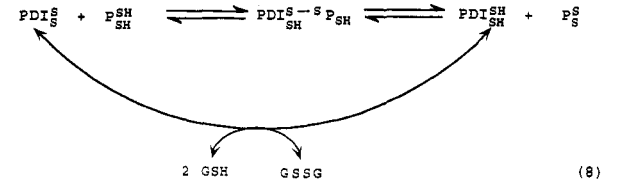
where a thiol group of PDI reacts with the mixed disulfide of the peptide to form specifically the PDI–peptide mixed disulfide:



The reaction would have to be constrained sterically by the active site of PDI, for reaction of the PDI thiol group with the other sulfur atom of the mixed disulfide would release reduced peptide; only very small quantities of reduced peptide (less than 2% of the total) were observed to be generated in the presence of PDI (data not shown). The glutathione or cysteamine of the mixed disulfide would be released in the first step, so there would be no possibility of it being transferred to the other cysteine thiol group. The cysteamine mixed disulfide is much more reactive than that of glutathione, yet both were utilized by PDI at very similar rates, suggesting that the initial step was not rate-limiting. In the second step, the second cysteine residue of the peptide would undergo thiol–disulfide exchange with the PDI mixed disulfide on the first cysteine residue to form the intramolecular peptide disulfide bond. This intramolecular step is the same, irrespective of the initial mixed disulfide, which would explain why PDI catalyzed it to the same extent with both the glutathione– and cysteamine–peptide mixed disulfides. Alternatively, release of the disulfide peptide could be rate-limiting in each case.

A very similar reaction is likely to be involved in rearranging protein disulfide bonds (Creighton et al., 1980). In this case, reaction between a PDI thiol group and a protein disulfide bond, to form a mixed disulfide between them, would release a protein thiol group. This protein thiol group would still be part of the complex, in contrast to that of cysteamine or glutathione in eq 7, and would be available to react again with the PDI–peptide mixed disulfide to regenerate a protein disulfide bond. Net rearrangement of the protein disulfide bonds would, however, require that a different protein thiol group react with the mixed disulfide. If the original protein had no free thiol groups, an intervening interchange of the liberated thiol group with another disulfide bond of the protein would be required. Whether this intervening thiol–disulfide exchange reaction occurs chemically or is catalyzed by the second active site on PDI remains to be determined.

A plausible alternative mechanism for PDI to incorporate disulfide bonds into a substrate protein would have been to transfer its disulfide bonds (Lundström & Holmgren, 1990) directly,



thus acting as a disulfide reagent that would subsequently be regenerated by reaction with GSSG, both in these experimental conditions and *in vivo*. Direct transfer of disulfide bonds from PDI to a reduced protein has been demonstrated when stoichiometric quantities of PDI are used in the absence of any other oxidant, both with reduced ribonuclease A (Lyles & Gilbert, 1991a,b) and with the peptide used here (unpublished observations). Even though this reaction is very fast, it appeared not to predominate with catalytic amounts of PDI in the presence of the peptide and disulfide reagent. Under these conditions, the catalytic effects of PDI could be largely accounted for by the pathway involving the mixed disulfides of the peptide with glutathione (Figure 5) or cystamine (Figure 8). There was only a marginally greater rate of disappearance of P^{2SH}_{27SH} and of appearance of P^{S}_{S} than would be expected from the mixed disulfide pathway, which might be attributed to direct formation from PDI disulfides. The reason why the direct reaction was not utilized to a greater extent remains to be elucidated, for very little is known about the interconversions of the thiol and disulfide groups of PDI. A similar phenomenon has been observed with the related DsbA, however, which has been shown directly to be a result of the bound peptide blocking the reaction between the DsbA active site cysteine residues and GSSG to regenerate the disulfide form of DsbA (N.J.D. and T.E.C., unpublished observations). This and other questions about the mechanism of action of PDI should be answered by studies on the individual thioredoxin-like domains of PDI, which have activities similar to those demonstrated here (N.J.D. and T.E.C., unpublished observations).

An important question with a catalyst like PDI that catalyzes reactions involved in protein folding is whether it catalyzes only the specific reactions, in this case thiol–disulfide exchange, or whether it participates in folding also. The question can be answered in the case of PDI by comparing its catalysis of intermolecular thiol–disulfide exchange reactions that do not involve conformational changes in the peptide to its catalysis of intramolecular steps that do. An example of the first is formation of the mixed disulfide on each cysteine residue, while the second is the intramolecular step in forming the peptide disulfide bond. With glutathione as disulfide reagent, the first steps were catalyzed 33–39-fold, while the second steps were catalyzed 100–120-fold. Therefore, PDI appeared to affect the steps involving conformational changes only slightly more than the others. A very different answer is obtained, however, if the data using cystamine are analyzed in the same way. No catalysis of formation of the mixed disulfides was observed, whereas the second, intramolecular steps were greatly increased, as with glutathione. The results with glutathione are probably the more significant, as it is believed to be the physiological reagent. However, the mechanism of catalysis of formation of the mixed disulfide and the basis of the difference between the two disulfide reagents need to be elucidated before an unequivocal answer to this question can be given.

ACKNOWLEDGMENT

We thank D. Nalis and R. Jacob for synthesizing the peptides, M. Mann for the mass spectroscopy analyses, Johan Kemmink for the nuclear magnetic resonance analysis, and H. C. Hawkins and S. L. M. McLaughlin for the supply of purified PDI. R.B.F. gratefully acknowledges the support of the Science and Engineering Research Council (Grant GR/G/09030).

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