Characterization of the Active Site Cysteine Residues of the 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 August 21, 1995; Revised Manuscript Received October 16, 1995[⊗]

ABSTRACT: The dithiol/disulfide active sites of each of the two isolated thioredoxin-like domains of protein disulfide isomerase (PDI) expressed in *Escherichia coli* have been characterized in order to understand their catalytic mechanisms and their functions in PDI. In each of the folded domains, as in other proteins of the thioredoxin family, only one of the cysteine residues of the active site sequence -Cys-Gly-His-Cys- is accessible, and its thiol group is highly reactive and has a low p$K_a$value. The kinetics and equilibria have been measured of the reactions between the active site cysteine residues and glutathione, the predominant thiol/disulfide reagent of the endoplasmic reticulum. A disulfide bond can be formed very rapidly between the pair of cysteine residues of each domain, but each disulfide bond is very unstable and reacts rapidly with reduced glutathione. The very low stabilities of these disulfide bonds, which destabilize the protein structures, account for the efficiency with which PDI and each of the isolated domains can introduce disulfide bonds into proteins. These kinetic and equilibrium data go far in helping to understand the catalytic mechanism of PDI and its individual domains.

Protein disulfide bond formation is an important co- and post-translational event in the biosynthesis of many proteins and is often linked to their folding. In the endoplasmic reticulum of eukaryotic cells, in the presence of millimolar concentrations of both GSH[1] and GSSG, this process is catalyzed by protein disulfide isomerase [reviewed by Noiva and Lennarz (1992), Freedman (1992, 1995), and Freedman et al. (1994)]. PDI accelerates the rates of disulfide bond formation, breakage, and rearrangement in proteins, steps that can be rate-limiting in disulfide-coupled folding (Givol et al., 1964; Creighton et al., 1980, 1993; Zapun et al., 1992; Darby et al., 1994). The detailed mechanism of how PDI functions is not known, but its primary structure contains two segments that are homologous to thioredoxin, each with a -Cys-Gly-His-Cys- sequence (Edman et al., 1985). It is clear that the function of PDI involves thiol−disulfide exchange reactions at these cysteine residues (Hawkins & Freedman, 1991; Vuori et al., 1992; LaMantia & Lennarz, 1993; Lyles & Gilbert, 1994). In the course of these reactions, it is likely that the active site cysteine residues function in a way similar to that originally proposed for thioredoxin (Kallis & Holmgren, 1980) and are cycled between their thiol and disulfide forms, via a mixed disulfide with the protein substrate or with glutathione (see eqs 1−3 below).

The thioredoxin structural motif and active site -Cys-X-Y-Cys- sequences are found not only in thioredoxin and PDI but also in a variety of proteins (Martin, 1995). This includes the proteins DsbA and DsbC of the periplasm of Gram-negative bacteria, which are also involved in catalyzing disulfide formation during the folding of exported proteins (Bardwell et al., 1991; Missiakas et al., 1994; Shevchick et al., 1994). There are also a number of homologues of PDI in the eukaryotic cell; their exact functions are unclear, but they also may play some role in the catalysis of protein folding (Freedman et al., 1994). Although all these thioredoxin-like enzymes have similar active site sequences, they can fulfill very different redox functions: the active site cysteine residues can be highly oxidizing in the case of PDI (Hawkins et al., 1991; Lyles & Gilbert, 1991a; Lundström & Holmgren, 1993), DsbA (Zapun et al., 1993), and DsbC (Zapun et al., 1995), or much more reducing in the case of thioredoxin (Holmgren, 1981). This versatility seems to be a consequence of the protein structure regulating the redox properties of the active site cysteine residues. With thioredoxin, DsbA, and DsbC, there is evidence that the first active site Cys residue is solvent-exposed, with a high reactivity and a low pK_a (Kallis & Holmgren, 1980; Hawkins & Freedman, 1991; Zapun et al., 1993; Nelson & Creighton, 1994). The high reactivity of the cysteine thiol and disulfide groups undoubtedly accounts in part for the catalytic activities of these proteins, but other features, such as binding of the substrate molecule to the catalyst, are probably also required (Morjana & Gilbert, 1991; Darby & Creighton, 1995a; Qin et al., 1995).

Considerable recent progress in understanding the mechanisms by which these proteins act has come from studies on thioredoxin, DsbA, and DsbC. These are small proteins with a single active site that are more readily studied than the much larger PDI, which has two active sites, two further cysteine residues, and additional putative domains and activities (Edman et al., 1985; Freedman, 1992, 1995). Each of the small proteins has proved amenable to structural analysis (Martin, 1995), and the properties of their active site cysteine residues are becoming sufficiently well understood to develop detailed mechanisms for their catalytic action.

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

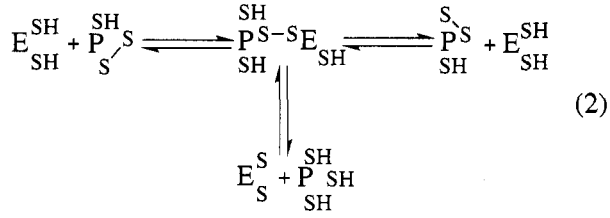
[1] Abbreviations: GdmCl, guanidinium chloride; GSH and GSSG, thiol and disulfide forms of glutathione, respectively; HPLC, high-pressure liquid chromatography; PDI, protein disulfide isomerase; PDI-*a* and PDI-*a'*, individual *a* and *a'* domains of PDI, respectively residues 1−120 and 348−462 of mature human PDI, each with an initiating methionine residue.

0006-2960/95/0434-16770$09.00/0

Disulfide bond formation between two cysteine residues in a reduced protein, P, by a catalyst, E, probably involves the direct transfer of a disulfide bond from the catalyst to the protein, via an intermediate mixed disulfide between the two (Creighton et al., 1980; Lyles & Gilbert, 1991b; Darby & Creighton, 1995a,b):

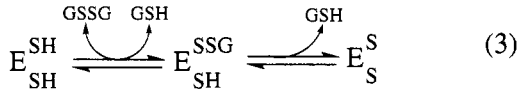
$$E_S^S + P_{SH}^{SH} \rightleftharpoons E_{SH}^{S-S}P_{SH} \rightleftharpoons E_{SH}^{SH} + P_S^S \quad (1)$$

This mixed disulfide complex between the protein and the catalyst is also likely to be involved in the catalysis of rearrangements of disulfide bonds in the protein (Creighton et al., 1980; Hu & Tsou, 1991; Huth et al., 1993):



(2)

In the endoplasmic reticulum, the active site cysteine residues of PDI are probably also reacting with the endogenous redox buffer glutathione, which is present at millimolar concentrations and in a [GSH]:[GSSG] ratio of between 1:1 and 3:1 (Hwang et al., 1992):



(3)

Under these conditions *in vitro*, PDI catalyzes all the required reactions of disulfide bond formation, breakage, and rearrangement at about the same rate as observed *in vivo* (Creighton et al., 1993). There is the additional possibility that PDI catalyzes reactions that more directly involve glutathione in the disulfide bond formation process. For example, PDI catalyzes both the formation and reduction of mixed disulfides between glutathione and the protein (Darby et al., 1994), although this may be explained by sequential reaction of the two reactants (Darby & Creighton, 1995a). The disulfide bond of the catalyst in eq 1 might also be its mixed disulfide form with glutathione. To understand the mechanisms of action of PDI, it is clearly important to determine the energetics of the thiol groups and disulfide bonds of PDI and to understand the way in which glutathione cycles each active site between the disulfide and dithiol forms.

The standard method for measuring the stability of a protein disulfide bond is to compare it to that of GSSG via the equilibrium constant for eq 3. A protein disulfide bond is intramolecular, in contrast to the "intermolecular" disulfide bond of GSSG, which produces two molecules of GSH when reduced. Therefore, the equilibrium constant of eq 3 has the dimensions of concentration, which can be roughly interpreted as the "effective concentration" of the two protein sulfur atoms relative to each other in the protein without a disulfide bond between them. The measured values range between 80 μM for DsbA (Zapun et al., 1993; Wunderlich & Glockshuber, 1993), to about 10 M for thioredoxin (Holmgren, 1981), to up to 10^5 M for very stable, structural disulfide bonds (Creighton & Goldenberg, 1984). Such estimates of the stability of the PDI active site disulfide bonds have necessarily been very indirect, but all the measurements have indicated that they are very unstable, with values ranging from 40 μM to 3 mM (Hawkins & Freedman, 1991; Lyles & Gilbert, 1991b; Lundström & Holmgren, 1993). Nevertheless, the reasons for the large differences in these values are unclear. Additionally, there are few data on the kinetics of the equilibration process. Both factors are needed to formulate a detailed understanding of the PDI reaction mechanism.

Recently the two putative thioredoxin domains of PDI have been expressed as individual proteins in *Escherichia coli*; they are denoted as PDI-*a* and PDI-*a'* (Darby & Creighton, 1995b). Each has only two cysteine residues, those at the active site. PDI-*a* consists of residues Asp1−Ala120 of the mature human sequence, and appears to be fully folded in both the dithiol and disulfide states; the three-dimensional structure of the disulfide form has been determined by nuclear magnetic resonance methods (Kemmink et al., 1995). PDI-*a'* comprises Asp348−Gly462 of intact PDI; it is also folded in the dithiol state but unfolds at least partly on formation of the active site disulfide bond. This destabilizing influence of the disulfide bond is consistent with it having a low stability and appears sufficient to unfold the isolated PDI-*a'* domain. Both isolated domains appear to be less stable than when part of the intact protein (unpublished observations).

The isolated domains had very similar functional properties, but only some of the catalytic activities characteristic of PDI (Darby & Creighton, 1995b). In particular, both had the ability to introduce disulfide bonds into unfolded peptides and proteins at rates similar to those observed with intact PDI. Both also exhibited a specificity for glutathione, compared to cysteamine as the low molecular weight disulfide reagent, similar to that observed with intact PDI. Compared to PDI, however, the individual domains were much less efficient in rearranging protein disulfide bonds and at introducing disulfide bonds into folded substrate proteins. Overall, the catalytic activities of both PDI-*a* and PDI-*a'* were quantitatively very similar to those of the simple bacterial catalyst DsbA, suggesting that all three proteins have a similar mechanism of action. Further characterization of the individual PDI domains should give insight into the mechanisms of the reactions that they catalyze within PDI. By understanding the differences between the domains and PDI, it should be possible to understand the features of the PDI molecule that are required for those processes, such as disulfide bond rearrangement, that are not efficiently catalyzed by the domains or by the catalytically similar DsbA.

In this report, the active site disulfide bonds and thiol groups of PDI-*a* and PDI-*a'* are characterized with regard to their reactions with low molecular weight thiol and disulfide reagents. The results obtained provide insight into the redox and reactivity properties of the active site disulfide bonds of PDI and its catalytic mechanism.

MATERIALS AND METHODS

Materials. The preparation of complete human PDI and of the individual PDI-*a* and PDI-*a'* domains, using an *E. coli* expression system, has been described previously (Darby & Creighton, 1995b).

Mutagenesis of the PDI-a and PDI-a' Genes. The active site cysteine residues were changed to Ser or Ala residues

by oligonucleotide-directed mutagenesis using the "megaprimer" method (Sarkar & Sommer, 1990), with the original expression vectors serving as templates. The changes in the sequence were confirmed by direct sequencing of the mutated genes in the expression vectors. Mutant proteins were expressed and purified as described for the normal proteins. Their identities were confirmed by measurement of their molecular masses by mass spectrometry.

Reactivity of the Active Site Cysteine Residues to Iodoacetic Acid and Disulfide Reagents. The PDI domains were reduced at pH 7.4 by addition of dithiothreitol to a final concentration of 5 mM. After 20 min, the dithiothreitol was removed by gel filtration into 10 mM Tris (pH 7.4). Reactions of 2−4 μM protein in 0.1 M Tris (pH 7.4), 0.2 M KCl, and 1 mM EDTA were initiated by the addition of the various reagents. After different time intervals, the reactions were quenched by addition of HCl to a final concentration of 0.1 M to portions of the reaction mixture. Some reactions were also trapped by addition of iodoacetate, as described in the text and appropriate figure legend.

Reactivity of the protein thiol groups as a function of pH was determined in the same way, but using a composite buffer of final composition 0.2 M KCl, 10 mM sodium acetate, 10 mM 2-(*N*-morpholino)ethanesulfonic acid, 10 mM 3-(*N*-morpholino)propanesulfonic acid, and 10 mM Tris adjusted to the required pH. In these experiments, the reduced protein had also been gel filtered into the same buffer.

Samples of the domains that had been reacted with iodoacetate were analyzed on a Vydac 25 × 0.46 cm 218TP54 column, using a linear gradient of 31−38% (v/v) acetonitrile in 0.1% trifluoroacetic acid (v/v) in 30 min at a flow rate of 1 mL min^{-1} in the case of PDI-*a*, and from 35% to 39% (v/v) acetonitrile in 30 min in the case of PDI-*a*′. Acid-trapped samples were analyzed using a linear gradient of from 31% to 33% (v/v) acetonitrile in the case of PDI-*a* over 50 min, and from 35% to 40% acetonitrile (v/v) in the case of PDI-*a*′.

Reduction of Disulfide Bonds by GSH. The rate of reduction of 2−4 μM of the disulfide forms of each of the domains in 0.1 M Tris (pH 7.4), 0.2 M KCl, and 1 mM EDTA was determined using the acid-trapping and HPLC separations described above.

Determination of the Equilibria between the PDI Domains and Glutathione. The equilibrium between each of the domains and GSSG and GSH was determined in 0.1 M Tris (pH 7.4), 0.2 M KCl, and 1 mM EDTA; 15 μM PDI-*a* or 2 μM PDI-*a*′ was incubated in different redox mixtures of excess GSH and GSSG for 10−20 min. The reaction was then quenched by addition of HCl to a final concentration of 0.1 M. The major portion of the sample was analyzed by HPLC as described above to determine the relative amounts of the dithiol, disulfide, and mixed disulfide forms of each of the domains. A small portion of the reaction mixture was used to determine its total thiol content by reaction with 5,5′-dithionitrobenzoic acid (Creighton, 1990), to permit correction for the small amount of air oxidation that occurred during the reaction. The same method was used to determine the redox properties of the domains in the presence of 8 M urea, but the concentration of the protein was decreased to 2 μM.

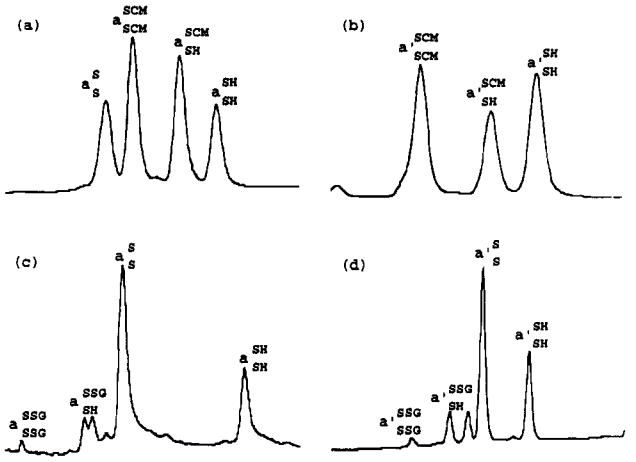


FIGURE 1: Reactivity of the thiol groups of PDI-*a* and PDI-*a*′ with iodoacetic acid and glutathione. (a, b) HPLC separation of the products of reaction of iodoacetate with (a) PDI-*a* and (b) PDI-*a*′. The proteins with the single and double modifications were generated by limited reaction with iodoacetate in the absence and presence of 6 M GdmCl, respectively. The elution of the disulfide form of PDI-*a* with this gradient is also shown in (a), as the separation was used in some equilibrium determinations (e.g., Figure 3). (c, d) HPLC separation of the species of (c) PDI-*a* and (d) PDI-*a*′ at equilibrium with 2 mM GSSG and 10 mM GSH in the presence of 8 M urea. The reactions were trapped by addition of HCl to 0.1 M. In figures (a) and (b), the sulfur atom of the reactive cysteine residue (Cys1) is designated as the superscript, the less reactive cysteine residue (Cys2) as the subscript; "SCM" depicts a sulfur atom that has reacted with iodoacetate. In figures (c) and (d), "SSG" is the mixed disulfide with glutathione; the two isomers with the mixed disulfide on different cysteine residues are not distinguished.

RESULTS

Reactivities of the Cysteine Thiol Groups of PDI-a and PDI-a′. The accessibilities of the two cysteine residues in each of the domains was examined using their reactivity to iodoacetic acid as the probe. Analysis of the products by HPLC (Figure 1a,b) allowed the separation of the reduced protein that had not been alkylated from those forms with one or two cysteine residues that had. In the absence of denaturant, a single product was rapidly generated upon alkylation of either the PDI-*a* or the PDI-*a*′ dithiol domain. A second, further product was generated slowly after prolonged incubation or rapidly in the presence of denaturant. The results were consistent with each folded protein having a single accessible cysteine thiol group and a second that is buried and reacts very much more slowly. In contrast, both thiol groups reacted rapidly if the protein is unfolded. The large difference in the rates of alkylation of the two cysteine residues in the folded protein allowed them to be readily determined with both domains (Table 1). The accessible cysteine residue of each folded domain reacted at a rate about 5-fold faster than a normal cysteine residue thiol group, for example, in an unstructured peptide, and about 1000-fold faster than the second cysteine residue. In each folded domain, the second cysteine residue was reacting in the presence of the modified form of the first residue.

Thiol−Disulfide Equilibrium with Glutathione. The equilibria for thiol−disulfide exchange between the thiol and disulfide forms of glutathione and of each domain, in the absence and presence of 8 M urea, were examined by HPLC separation of the acid-trapped protein species. These equilibria occur through mixed disulfides between glutathione

Table 1: Kinetics of Reaction of Iodoacetic Acid with the Cysteine Residues of PDI-*a* and PDI-*a'*

	Cys1	Cys2[b]
PDI-*a*	5.05 ± 0.05 (4)	(7.5 ± 0.5) × 10^{-3} (5)
PDI-*a'*	5.40 ± 0.6 (4)	(9.0 ± 0.9) × 10^{-3} (3)

[a] The reactivities (s^{-1} M^{-1}) of the individual cysteine residues with excess iodoacetate were determined in 0.1 M Tris (pH 7.4), 0.2 M KCl, and 1 mM EDTA at 25 °C, using the HPLC separation methods shown in Figure 1. The values are the mean ± standard deviation of the number of experiments given in parentheses. Mutant forms of PDI-*a* in which each cysteine residue had been replaced indicate that Cys1 is the first residue and Cys2 the second, in the active site sequence -Cys-Gly-His-Cys- of each domain. [b] Cys2 is reacting in the protein species in which Cys1 has already reacted.

and one or both of the cysteine residues of the protein (eq 3). The reaction is most straightforward with the folded proteins, where only a single mixed disulfide on the accessible cysteine residue was apparent, as indicated in eq 3. When the proteins were unfolded in 8 M urea, both cysteine residues were reactive and accumulated as mixed disulfides with glutathione. All the possible species accumulated and could be resolved by HPLC after trapping, although the separation of the two single mixed disulfide species was incomplete with PDI-*a* (Figure 1c,d). Nevertheless, it was clear that both such species accumulated to similar levels in 8 M urea, as expected, and they were quantified together. In addition, a disulfide-linked dimer was apparent (data not show) when PDI-*a'* was present in the equilibration mixture at a concentration of 50 μM, but not at 2−4 μM; the measurements were made at the lower concentrations. In contrast, no significant amounts of such a dimer were apparent with PDI-*a* under any conditions.

The relative concentrations of the various thiol/disulfide forms of each domain, both in the presence and in the absence of urea, were determined with various [GSH]/[GSSG] ratios; an example of the data is shown in Figure 2. These could be used to determine values (Table 2) for the following equilibrium constants for eq 3:

$$K_1 = \frac{[E_{SH}^{SSG}][GSH]}{[E_{SH}^{SH}][GSSG]},\ K_2 = \frac{[E_S^S][GSH]}{[E_{SH}^{SSG}]},\ K_{SS} = K_1K_2 = \frac{[E_S^S][GSH]^2}{[E_{SH}^{SH}][GSSG]} \quad (4)$$

The equilibrium constants for both unfolded domains in 8 M urea were similar to each other and to those measured with unfolded DsbA (Zapun et al., 1993), which has a similar active site with two cysteine residues that also are separated by two other residues: -Cys-Pro-His-Cys-. With each unfolded domain, the equilibrium constant for formation, from the reduced protein, of a single mixed disulfide with glutathione at either of the cysteine residues had approximately the expected statistical value of about 4, indicating that the mixed disulfide bonds were similar in free energy to that of GSSG. With both domains, the corresponding equilibrium constant for formation of the double mixed disulfide species (data not shown) had the value of 0.7, close to the expected value of 1.

In the absence of urea, both PDI-*a* and PDI-*a'* were found to form extremely unstable disulfide bonds, with overall equilibrium constants of 0.7 and 1.9 mM, respectively (Table 2). Both disulfide bonds were much less stable than when the protein was unfolded in 8 M urea; this implies that the disulfide bond should destabilize the folded conformation, as has been observed for PDI-*a'* (Darby & Creighton, 1995b). The differences in the overall stabilities of the disulfide bonds, between the domains and between the folded and unfolded states, were primarily attributable to differences in stability of the mixed disulfide relative to the dithiol protein (K_1). The stability of the domain disulfide bond relative to the mixed disulfide (K_2) was similar in both proteins; it was relatively unaffected by the presence of 8 M urea, but this is presumed to be coincidental, as the conformations of the proteins were unfolded by urea.

Dependence of the Equilibrium between PDI-a and Glutathione on the Trapping Conditions. The approach of acid-trapping thiol−disulfide exchange reactions is effective because it rapidly protonates ionized thiol groups, to render them unreactive, but also because it simultaneously unfolds a protein catalyst, destroying its catalytic properties. Even with such a rapid trapping reaction, however, there is the potential for equilibria to be distorted during acid trapping if the active site disulfide bond or thiol groups have exceptional reactivities, which is generally the case with the thioredoxin-like proteins. In particular, the mixed disulfide with glutathione of the folded proteins can be difficult to trap, as it can be displaced by a rapid, intramolecular reaction with the second cysteine residue of the protein, to generate the intramolecular disulfide bond (eq 3). This has previously been manifest with other proteins by a progressive increase in the amount of mixed disulfide species trapped as the concentration of acid used to trap the reaction was increased (Darby & Creighton, 1995a; Zapun et al., 1995). This would only marginally affect the value of the overall equilibrium constant K_{SS}, but could have significant effects on the values of the partial equilibrium constants K_1 and K_2. The validity of the acid trapping method for the PDI domains was examined by trapping an equilibrium mixture with concentrations of HCl in the range 0.1−0.3 M. With neither PDI domain was there any significant difference in the relative amounts of dithiol, disulfide, and mixed disulfide species quantified. All the data were consistent with the acid trapping being quantified accurately, but it is difficult to exclude completely the possibility that the unstable mixed disulfide species was underestimated by acid trapping. For that reason, the partial equilibrium constants of Table 2, and all rate constants derived from them (see Table 3 below), must be considered tentative.

Previous attempts to determine the redox properties of the active sites of intact PDI have relied upon the much slower alkylation reaction to block the free thiol groups. One method (A) used the simultaneous addition of a denaturant (Lundström & Holmgren, 1993); the other (B) did not (Hawkins & Freedman, 1991). The first measured the fraction of cysteine residues that had reacted with iodoacetate; the second measured the effects on PDI activity; neither method identified the cysteine residues that were involved. The two methods gave somewhat different results: method B gave a very low overall equilibrium constant of about 40 μM, whereas a greater value of about 3 mM was measured with method A. These different methods for trapping the reaction were examined with the PDI-*a* domain, and the products were analyzed by HPLC to resolve the principal dithiol and disulfide forms of the protein. The conditions

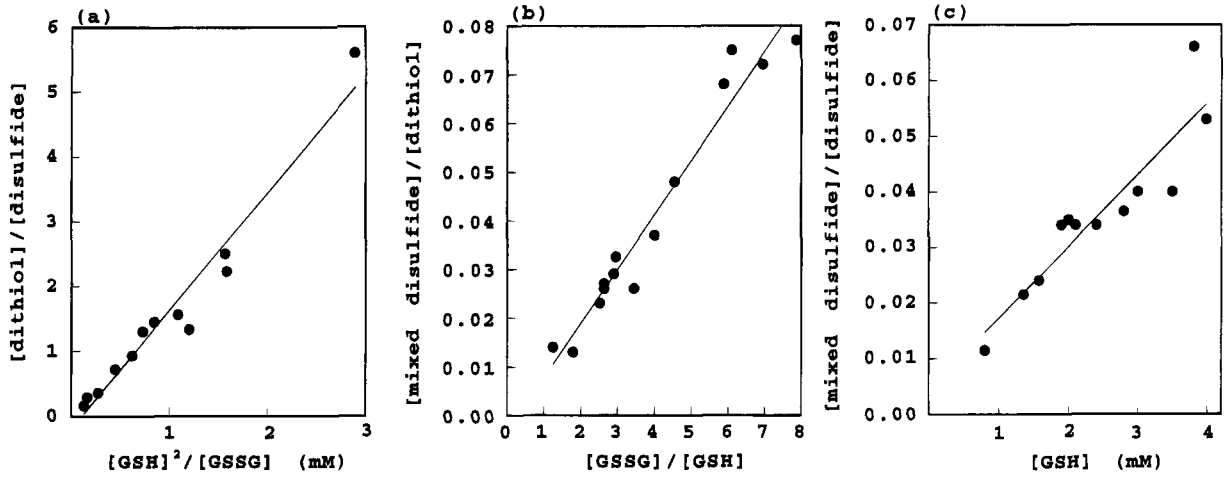


FIGURE 2: Measurement of the thiol−disulfide equilibrium of PDI-*a* with glutathione. PDI-*a* (15 μM) was incubated in 0.1 M Tris (pH 7.4), 0.2 M KCl, and 1 mM EDTA at 25 °C containing 5, 10, or 15 mM GSSG and varying concentrations of GSH. Duplicate aliquots after 10 and 20 min incubations were quenched by addition of HCl to 0.1 M, and the relative amounts of the different species were quantified by reverse phase HPLC as in Figure 1c. (a) The plot of [dithiol]/[disulfide] forms of the protein *vs* $[GSH]^2/[GSSG]$ should be linear, and the slope gives the value of K_{SS}^{-1}. (b) The plot of [mixed disulfide]/[dithiol] forms of the protein *vs* [GSSG]/[GSH] should be linear, and the slope gives the value of K_1. (c) The plot of [mixed disulfide]/[disulfide] forms of the protein *vs* [GSH] should be linear, and the slope gives the value of K_2^{-1}. The equilibrium constants are defined in eq 4 of the text, and the values obtained are presented in Table 2.

Table 2: Equilibrium Constants for the Thiol−Disulfide Exchange Reactions between the PDI Domains and Glutathione[a]

	PDI-*a*		PDI-*a'*	
	no denaturant (12)	8 M urea (11)	no denaturant (16)	8 M urea (15)
K_1	0.01 ± 0.002	3.0 ± 0.4	0.053 ± 0.009	3.97 ± 0.64
K_2 (mM)	69 ± 10	43 ± 9	34 ± 6	42 ± 4
K_{SS} (mM)	0.70 ± 0.13	140 ± 33	1.87 ± 0.23	170 ± 25

[a] The parameters are defined in eq 4. The values are the mean ± standard deviation of the number of experiments given in parentheses. The values of the partial equilibrium constants K_1 and K_2 depend upon the mixed disulfide intermediate being quantified accurately by acid trapping. Only a single mixed disulfide intermediate accumulated in the absence of denaturant, whereas both were present in similar quantities in 8 M urea. The double mixed disulfide species was also significant in 8 M urea, but this equilibrium is not included here. The conditions were 25 °C in 0.1 M Tris (pH 7.4), 0.2 M KCl, and 1 mM EDTA.

used in the original studies were followed closely, except that the equilibration conditions were those used here: 0.2 M KCl, Tris buffer (pH 7.4), and 1 mM EDTA at 25 °C. Trapping such equilibrium mixtures with 0.1 M HCl gave the expected linear dependence of the amount of dithiol form of the protein on the concentrations of GSH and GSSG, which indicated a value of 670 μM (Figure 3).

With method B, the overall amount of glutathione ([GSH] + 2[GSSG]) was kept at 10 mM; the main difference with the previous experiments (Hawkins & Freedman, 1991) was that they used phosphate buffer at pH 7.5 and 20 °C in the equilibrium mixture. The equilibrium was trapped by the addition of iodoacetate to 0.1 M and incubation for 5 min before analysis. This method produced much greater amounts of the dithiol PDI-*a* domain than were trapped with acid, and implies a smaller average equilibrium constant for making the PDI-*a* disulfide bond (K_{SS}) of about 40 μM; the same value was obtained with intact PDI measured in the same way (Hawkins & Freedman, 1991). The amount of dithiol form of the protein trapped did not vary linearly with the concentrations of GSH and GSSG in the expected manner (Figure 3). A similar phenomenon was observed with intact

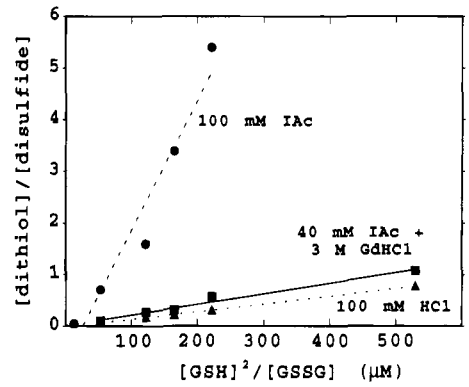


FIGURE 3: Comparison of the different methods of trapping the equilibrium between the PDI-*a* domain and GSH and GSSG. Equilibrium mixtures were prepared as described in Figure 2, but the total amount of glutathione moieties in the mixture was restricted to 10 mM. After 15 min equilibration, the reaction mixtures were trapped and the products analyzed by HPLC, as in Figure 1. The trapping methods used were: (●) 110 μL of reaction mixture was added to 28 μL of 0.5 M iodoacetate (IAc) at pH 7.4 to give a final IAc concentration of 0.1 M. After 5 min at 25 °C, the mixture was analyzed by HPLC. (■) 100 μL of reaction mixture was added to 20 μL of 0.5 M IAc and then immediately added to 120 μL of 6 M GdmCl, 0.2 M Tris (pH 8). After 5 min, the reactions were analyzed by HPLC; the final concentrations were 40 mM IAc and 3 M GdmCl. (▲) 100 μL of reaction mixture was added to 11 μL of 1 M HCl and stored on ice until analysis by HPLC. The ratio of the dithiol to disulfide forms of the protein was measured; the slope of each curve gives the apparent value of K_{SS}^{-1}. Note that the curve with 0.1 M IAc is nonlinear, as expected if the reaction with IAc is pulling the equilibrium toward the trapped dithiol form of the protein.

PDI trapped in the same way and was ascribed to interactions between the two active sites (Hawkins & Freedman, 1991).

The same equilibrium measurements were repeated using method A, in which addition of iodoacetate was followed by the addition of GdmCl to give a final trapping mixture that contained 40 mM iodoacetic acid and 3 M GdmCl; the principal difference with the previous experiments was that Lundström and Holmgren (1993) used phosphate buffer at pH 7.0 and an equilibration temperature of 37 °C. The results obtained with this trapping method were similar to

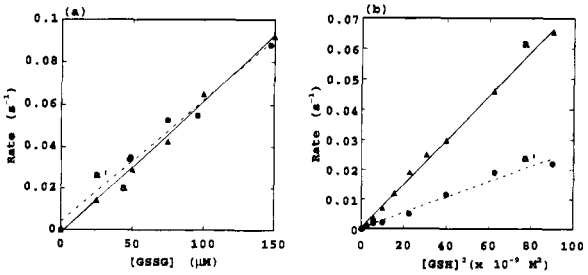


FIGURE 4: Kinetics of reaction of the dithiol (a) and disulfide (b) forms of PDI-*a* (▲) and PDI-*a'* (●) with excess GSSG or GSH, respectively. The dependence of the observed rate on the concentrations of GSSG and GSH is presented. The plots shown are expected to be linear, and the lines are linear fits to the data.

those with acid trapping, although there was consistently a somewhat higher proportion of the protein trapped in the reduced state, which indicated a slightly smaller equilibrium constant of 490 μM.

These data suggest that iodoacetate at practical concentrations alone is insufficient to trap faithfully the dithiol/disulfide exchange reactions of the PDI domains at equilibrium with high concentrations of glutathione. The reason is probably that the rate at which the thiol groups react with iodoacetate is comparable to the rate at which the disulfide bond in each domain is reduced by GSH at the concentrations used (see below). The greater reactivity toward iodoacetate of the accessible thiol group of each of the PDI domains (Table 1) than that of GSH results in the iodoacetate "pulling" the equilibrium to trap artifactually more dithiol protein than was actually present at equilibrium. This would be expected to be more significant at higher concentrations of GSH, and the data of Figure 3 are nonlinear in the expected way. Use of denaturant in addition to the iodoacetate at least partly alleviates the problem in this case, by unfolding the protein, because the disulfide bond of the unfolded protein is substantially more stable to reduction than is that of the folded protein; also, the reactivity of the thiol groups of the unfolded protein is less, reducing the magnitude of the "pulling" effect that iodoacetic acid can exert at equilibrium. In general, though, adding a denaturant to trap specific disulfide species is not desirable with proteins containing more than two cysteine residues, as disulfide rearrangements during unfolding could alter the specific disulfide bonds present.

Kinetics of Reaction of the PDI Domains with Glutathione. The kinetics of the reaction of the PDI domains with GSH and GSSG could be determined using the same acid-trapping technique that was used with the equilibrium mixtures, in combination with HPLC separation to resolve the various species. The rate constants are defined as

$$E^{SH}_{SH} \underset{k_{-1}}{\overset{GSSG,\ k_1}{\rightleftharpoons}} E^{SSG}_{SH} \underset{k_r}{\overset{k_{intra},\ GSH}{\rightleftharpoons}} E^{S}_{S} \qquad (5)$$

Reaction of each of the dithiol domains with excess GSSG, and no added GSH, produced only the disulfide form of the domain, with no substantial quantities of the mixed disulfide between them. The pseudo-first-order rate constant was directly proportional to [GSSG] over the range that was accessible to manual mixing (Figure 4a). These results indicate that the first step, formation of the mixed disulfide, with rate constant k_{+1}, was rate-limiting. This reaction was very rapid, with a rate constant about 600-fold greater than for a normal thiol group at pH 7.4. The expected value for the reverse rate constant, k_{-1}, can be calculated from k_{+1} and the equilibrium constant, K_1.

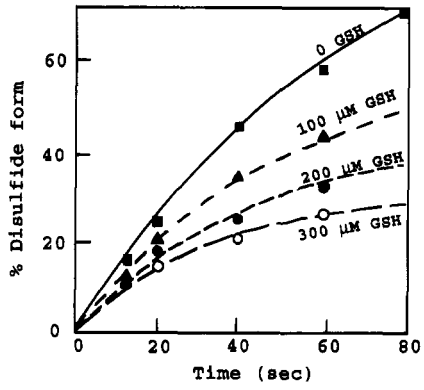


FIGURE 5: Effects of added GSH on the kinetics of reaction of the dithiol form of PDI-*a* with 25 μM GSSG. The points give the experimental measurements with the indicated initial concentration of GSH. Simulations of the data using numerical integration were used to obtain the rate constants of Table 3, which produced the simulated curves presented.

When the mixed disulfide intermediate does not accumulate to substantial levels, the rate of reaction of each of the PDI domain disulfide bonds with GSH, in the absence of added GSSG, is expected to depend upon the concentration of GSH in the following way:

$$\text{rate}_{obs} = \frac{k_r k_{-1}[GSH]^2}{k_{intra} + k_{-1}[GSH]} \qquad (6)$$

The pseudo-first-order rate for reduction was found to be linearly dependent on $[GSH]^2$ (Figure 4b) across the entire concentration range accessible to manual mixing. This indicates that $k_{intra} >> k_{-1}$ [GSH], so the observed rate of reduction is $\text{rate}_{obs} = k_r k_{-1}[GSH]^2/k_{intra}$. Therefore, only minimum values of the rate constants k_{intra} and k_r, plus their ratio, can be obtained from these data and the value of k_{-1}.

For both the PDI-*a* and PDI-*a'* domains, the low stability of the intermediate mixed disulfide with glutathione arises from the very high rate at which it is reduced by GSH. The rate at which equilibrium is reached will depend upon the relative values of k_{-1}[GSH] and k_{intra}. The rates of reaction with GSSG of the dithiol forms of the PDI-*a* and PDI-*a'* domains were decreased by the presence of small amounts of added GSH (Figure 5). The results could be simulated by numerical integration, using the known values for k_{+1} and k_{-1} determined above, to estimate values for k_{intra} within a range of about a factor of 3. The expected values of k_r were then calculated from k_{intra} and the equilibrium constant K_2. The final values consistent with all the data are given in Table 3.

The ratio of the apparent rate constants in the two directions for the overall reaction between the domains and glutathione (eq 3) gives another estimate of the value of the overall equilibrium constant, K_{SS}. The values of 0.8 and 2.35 mM indicated by the kinetic date for PDI-*a* and PDI-*a'*, respectively, are close to the values determined directly at equilibrium (Table 2).

Effects of pH on the Reactivity and Equilibrium of the PDI Domains. The ionization of a thiol group is an important parameter, as only the ionized thiolate anion is

Table 3: Rate Constants for the Reaction of the PDI Domains with Glutathione[e]

	PDI-*a*	PDI-*a'*
k_{+1} (s^{-1} M^{-1})	596 ± 35 (5)	614 ± 95 s^{-1} (7)
$k_r k_{-1}/k_{intra}$ (s^{-1} M^{-2})	(7.45 ± 0.56) × 10^5 (10)	(2.8 ± 0.5) × 10^5 (7)
k_{-1}[a,b] (s^{-1} M^{-1})	6 × 10^4	1 × 10^4
k_{intra}[c] (s^{-1})	10−30	3−8
k_r[b,d] (s^{-1} M^{-1})	145−434	87−235

[a] The rate constant was determined from the value of k_{+1} and the value of the equilibrium constant K_1. [b] The accuracy of this value depends upon the accuracy of the values of K_1 and K_2, which are dependent upon the accuracy of the quantification of the mixed disulfide intermediate by acid trapping (see Table 2). [c] The values of k_{intra} consistent with the observed kinetics were estimated from simulations of the rates of reaction with GSSG of the dithiol forms of each domain in the presence of small amounts of GSH. [d] The value of k_r was determined from the value of k_{intra} and the equilibrium constant K_2 (Table 2). [e] The rate constants are defined in eq 5, and the conditions are described in the legend to Table 2. The values are the mean ± standard deviation of the number of experiments given in parentheses.

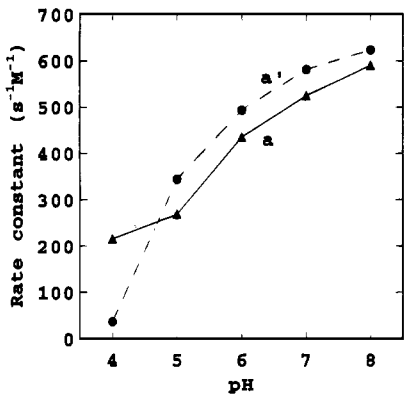


FIGURE 6: pH dependence of the apparent rate constant for reaction of the dithiol forms of PDI-*a* (▲) and PDI-*a'* (●) with excess GSSG. The rate of disappearance of the dithiol protein was determined by HPLC analysis of acid-trapped samples.

normally reactive; moreover, the thiol pK_a value reflects the intrinsic reactivity of the sulfur atom, even when in a disulfide bond (Szajewski & Whitesides, 1980). The pK_a value of a thiol group can usually be determined from the pH dependence of its reactivity, as only the ionized thiolate anion is normally reactive. The rate of reaction with GSSG of the accessible thiol groups of the dithiol forms of each of the domains (k_{+1} of eq 5) was measured over the pH range 4−8. Both domains reacted at significant rates across the entire pH range. In contrast, a normal thiol group with a pK_a of about 8.7 should react at pH 4 at a rate lower than that at pH 8 by nearly a factor of 10^4. For PDI-*a'*, the rate varied by only a factor of about 3 between pH 4 and pH 8 (Figure 6). The rate of reaction of the PDI-*a'* domain altered more substantially across the same pH range, but the rate at pH 4 was about 20-fold greater than that normally expected for a thiol group at pH 7.4. These data cannot be interpreted unambiguously as thiol pK_a values, but they suggest that the reactive thiol groups of both PDI domains are substantially ionized at pH values as low as 5.

If the thiol groups of the PDI domains have pK_a values very different from that of GSH, the value of the equilibrium constant for the reaction with glutathione (eq 3) should be pH-dependent in the pH region where the thiol groups on the two sides of the equation have different ionization properties. The pK_a value of the GSH thiol group is approximately 8.8, so the value of the equilibrium constant K_{SS} should increase at lower pH values, until the thiol groups of the protein are no longer ionized. For this reason, the values of the equilibrium constants for the reaction of PDI-*a* with glutathione were measured at pH 4. The data (not shown) gave the values K_1 = 2.7 ± 0.4; K_2 = 64 ± 7 mM ; K_{SS} = 0.17 ± 0.01 M. The value of K_2 was not changed significantly on reducing the pH, consistent with the buried thiol group of PDI-*a* in the mixed disulfide having a pK_a value greater than 7.4, as expected for a buried thiol group and as observed in DsbA (Nelson & Creighton, 1994). In contrast, the stabilities of the mixed disulfide (K_1) and of the domain disulfide bond (K_{SS}), relative to the dithiol protein, increased about 250-fold at pH 4, as expected if the active site thiol group of PDI-*a* has a very low pK_a value. Similar observations were made previously with DsbA, where the thiol group has an apparent pK_a value of 3.5 (Nelson & Creighton, 1994).

Together, the pH dependence of the reactivity of the accessible thiol group of reduced PDI-*a* and of the equilibrium of this protein with glutathione confirms that this thiol group has a very low pK_a value.

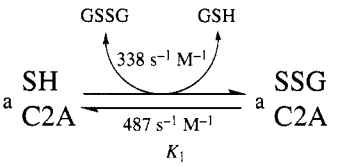
Reactivity with β-Mercaptoethanol and Cystamine. An intriguing feature of the catalytic activity of intact PDI was the observation of some specificity for utilizing glutathione relative to cystamine (Darby et al., 1994). PDI did not increase the rate of protein disulfide formation in the presence of cystamine, whereas it did in the presence of glutathione, and the same behavior was exhibited by the isolated domains (Darby & Creighton, 1995b). For that reason, the rates of reaction of the PDI domains with cystamine were examined. The rate constant for reaction of cystamine with an ordinary thiol group is normally about 8-fold greater than the corresponding reaction of GSSG because of the difference of nearly 1 pH unit in the pK_a of the leaving thiol group (Creighton, 1975). The rate constants for the reaction of cystamine with the dithiol PDI-*a* and PDI-*a'* domains were, however, 7.9 and 10.9 s^{-1} M^{-1}, respectively, 90- and 50-fold lower than the corresponding reactions with GSSG. These rate constants are comparable to that for reaction of cystamine with a normal protein thiol group at pH 7.4. With the disulfide form of neutral β-mercaptoethanol as the reagent, the rate constants for reaction with the dithiol forms of the PDI-*a* and PDI-*a'* domains were, respectively, 136 and 156 s^{-1} M^{-1}. These values are only about 4-fold lower than those for reaction with GSSG (Table 3) and similar to the 2-fold difference expected on the basis of the intrinsic reactivities of the disulfide bonds of these two reagents. This similarity in rates between two structurally different reagents suggests that the rapid rate of reaction of the domains with GSSG is not due to specific interactions between them. Instead, the reaction with cystamine seems to be disfavored in some way. It is remarkable that the reactivities of the two domains toward these three different disulfide reagents changed in parallel, suggesting that the rates vary with the reagent for some fundamental reason.

Active Site Mutations of the PDI Domains. Mutant proteins in which one or both of the active site cysteine residues are replaced by another amino acid have proved to be useful tools for studying both the active site properties and the catalytic mechanisms of thioredoxin, DsbA, and DsbC (Holmgren, 1995; Zapun et al., 1994, 1995; Darby & Creighton, 1995a). For both the PDI-*a* and PDI-*a'* domains, the first cysteine residue of the active site sequence was

replaced with serine (C1S), because it was likely to be accessible to solvent, while the second was replaced with alanine (C2A), because it was expected to be buried.

The C1S and C2A mutant forms of PDI-*a'* had altered physical properties and proved difficult to purify by the usual method. Furthermore, circular dichroism analysis of the available preparations indicated that each mutation had caused a considerable disruption in structure, so they were not characterized further.

In the case of the PDI-*a* domain, both active site mutant proteins could be isolated and their properties examined. Circular dichroism spectra indicated that both proteins retained the normal folded structure (data not shown). The rates and equilibrium of the reaction of glutathione with the C2A mutant protein were determined. Initial rate measurements starting from both the thiol and the mixed disulfide forms of the protein were used to determine the following rate constants:



(7)

The ratio of the rate constants indicates a value of 0.69 for K_1, similar to the value of 0.44 ± 0.05 determined directly at equilibrium.

The value of K_1 measured in the C2A mutant protein is 44-fold greater than that measured with the normal PDI domain (Table 2) and close to the value of 1 expected for reaction with GSSG of a normal, accessible thiol group. The rate of formation of the mixed disulfide by reaction with GSSG is half that determined for the normal domain, but the mixed disulfide is very much more stable than expected, mainly because the value of k_{-1} is only about 1% of that observed in the normal protein (Table 2). It appears that mutation of the second residue alters the properties of the accessible residue, making its thiol group more normal, as has been observed, albeit to a lesser extent, in the cases of DsbA and DsbC. This possibility was examined by measuring the reactivity with GSSG of the C2A mutant at pH 4 and comparing it to that at pH 7.4. Its reactivity at pH 7.4 was only about 2-fold lower than the normal protein, but was about 76-fold lower at pH 4. Again, this pH dependence is indicative of a thiol group with much more normal reactivity and ionization properties.

An additional complication with the C2A mutant form of PDI-*a*, which was not detected in the case of the normal domain, was the formation of disulfide-linked dimers between the peptide chains at the 6 μM protein concentration used. This undoubtedly occurs by reaction between the thiol and glutathione mixed disulfide forms of the protein, and the data indicated an equilibrium constant of

$$\frac{[E^{S-S}E][GSH]}{[E^{SSG}][E^{SH}]} = (8.2 \pm 3.4) \times 10^2 \qquad (8)$$

The large value of this equilibrium constant suggests that there are interactions between the two molecules of PDI-*a* that stabilize the dimeric form.

In the alternate C1S mutant form of PDI-*a*, the rate of reaction of the remaining cysteine residue with GSSG was determined to be 0.026 s^{-1} M^{-1}; this is only 8 × 10^{-5} the value observed after mutation of the other cysteine residue. These data confirm that it is the first cysteine residue at the active site that is reactive and that the second cysteine residue is relatively inaccessible and unreactive.

DISCUSSION

The active site cysteine residues of the individual thioredoxin-like *a* and *a'* domains of PDI have been characterized to elucidate their likely functions within the intact PDI molecule. The results also provide further illustration of how the thioredoxin fold has been adapted to fulfill a variety of redox and catalytic functions and permit direct comparison with other simple proteins of the thioredoxin family.

The present study indicates that the active sites of the isolated PDI domains have many properties in common with the other thioredoxin-related proteins. In particular, each active site has an accessible, highly reactive cysteine residue that has an unusually low pK_a, and a second inaccessible cysteine residue that is normally unreactive. In the case of the PDI-*a* domain, the C1S and C2A mutants established that the more N-terminal cysteine residue in the active site was the more accessible and reactive cysteine residue. This is also the case for thioredoxin, glutaredoxin, DsbA, DsbC, and presumably the PDI-*a'* domain, although the disruptive effects of the corresponding PDI-*a'* mutants did not allow this to be confirmed. Despite the similarity between thioredoxin and the PDI domains that is evident at both the sequence level (Edman et al., 1985) and in the structure of the PDI-*a* domain determined by NMR (Kemmink et al., 1995), the stabilities of their active site disulfide bonds differ by a factor of about 10^4.

The active sites of the isolated domains of PDI are expected to have similar properties to those of intact PDI. Nevertheless, it is impossible to rule out that some aspect, such as the stabilities of the disulfide bonds, will be different in the isolated domains and in the intact protein. This will be the case if further interactions of each of the domains with the rest of the protein molecule stabilize preferentially either their dithiol or their disulfide forms. The individual domains will be comparable to the corresponding parts of the intact protein only if their further interactions with the rest of the protein are the same in the dithiol and disulfide forms.

There are indeed many similarities between the properties of the isolated domains and intact PDI. For example, Hawkins and Freedman (1991) reported that PDI contains two accessible and highly reactive cysteine residues that are essential for catalytic activity and are readily modified by iodoacetate or iodoacetamide. The reactivities of these thiol groups as a function of pH indicated that they had relatively low pK values, and somewhat similar behavior was shown here with the individual domains (Figure 6). Two other members of the thioredoxin family, DsbA (Nelson & Creighton, 1994) and glutaredoxin (Gan et al., 1990; Yang & Wells, 1991; Mieyal et al., 1991), have cysteine thiol groups with pK_a values as low as 3.5. The origin of this anomalous pK_a is complex, but it certainly originates in part from the location of the accessible cysteine residue of the active site near the N-terminus of an α-helix, so that the negative charge of the thiolate anion is stabilized by the positive end of the dipole of the α-helix (Hol, 1985; Kortemme & Creighton, 1995).

The high reactivity of the accessible cysteine residue of each of the domains was apparent from the rapid rate of reaction of its thiol group with reagents such as GSSG and iodoacetate (Tables 1 and 3) and only in the folded structure of the protein. The reactivity of the exposed thiol group with iodoacetate was very similar to that of the corresponding group of thioredoxin (Kallis & Holmgren, 1980) and about 5-fold greater than that of a normal cysteine thiol group (Nelson & Creighton, 1994). In contrast, the same reaction occurs with DsbA about 40-fold faster than normal (Nelson & Creighton, 1994). Although DsbA reacts more rapidly with iodoacetate than do the domains, the reverse is the case with the reactivity toward GSSG. The rate constant for reaction with GSSG of the thiol group of each of the domains was about 600 s^{-1} M^{-1}, which is 15-fold greater than the corresponding rate with DsbA, and about 600-fold greater than the rate of reaction of GSSG with a normal thiol group. This rapid rate of reaction with GSSG should facilitate the rapid equilibration of PDI with the high levels of glutathione in the endoplasmic reticulum (Hwang et al., 1992). There are, however, indications that the rate of regeneration of the active site disulfide bonds of PDI may limit the rate at which disulfide bonds are formed in substrate proteins in the presence of both glutathione and catalytic amounts of PDI (Darby & Creighton, 1995a,b, and unpublished observations).

Although the rate of reaction of the domains with GSSG is rapid, there are no indications from this or previous studies with intact PDI (Gilbert, 1989) that this results from binding of GSSG to the proteins. In the case of glutaredoxin, which is known to bind GSSG, the rate constant for this reaction is even larger, $> 7 \times 10^5$ s^{-1} M^{-1}, while the reactions with other low molecular weight disulfide reagents are very slow (Rabenstein & Millis, 1995). The reaction of the domains with oxidized β-mercaptoethanol occurred at a slightly lower rate than with GSSG, but a large part of this difference is due to the lower reactivity of its disulfide bond (Creighton, 1975), which was also observed with DsbA (Zapun et al., 1993). In contrast, the reactivity of the domains toward cystamine was lower than with GSSG, even though it has intrinsically greater reactivity, due to the very low pK_a of the leaving cysteamine thiol group (Creighton, 1975). The slow rate of reaction with cystamine can explain the very low catalytic activity of both PDI and its isolated domains in assays of disulfide bond formation in which cystamine, rather than GSSG, is present as the redox buffer (Darby et al., 1994; Darby & Creighton, 1995b). The rate of regeneration of the disulfide bonds of the domains and of intact PDI is likely to be the rate-limiting step during catalysis of disulfide bond formation in a substrate protein. Why the reactivity of the domain thiol group toward cystamine is so low is unclear, but, whether by design or by chance, it might be important in preventing the enzyme interacting *in vivo* with cystamine, which is present intracellularly. In contrast, cystamine has been speculated to be present in the endoplasmic reticulum and to be the *in vivo* oxidant of protein disulfide bonds (Ziegler & Poulson, 1977).

Although GSSG reacted very rapidly with the accessible thiol group of both the PDI-*a* and PDI-*a'* domains, the stability of the resulting intermediate mixed disulfide was substantially lower than that of a normal disulfide bond (as when the protein is unfolded) (Darby & Creighton, 1993). The reason for this instability of the mixed disulfides is that their rates of reaction with GSH were very rapid, for the PDI-*a* and PDI-*a'* domains, respectively, 1.2×10^5 and 2×10^4 times more rapid than normal. In contrast, the stability of the disulfide form of each domain, relative to the mixed disulfide, was similar in both the unfolded and folded forms of each domain. The lower stability of a disulfide bond in the folded protein than in the unfolded protein indicates that this disulfide bond destabilizes the folded protein structure. This was seen most clearly in the case of the PDI-*a'* domain, which was only partly folded in the disulfide form. In intact PDI, other stabilizing interactions, from other parts of the protein, presumably increase the stability of the PDI-*a'* domain and keep it folded in the disulfide form.

The stabilities of the disulfide bonds in each domain were measured directly and were also confirmed from the kinetics of disulfide bond formation and breakage. The stabilities measured are toward the higher range of indirect estimates made with the intact PDI molecule (Lundström & Holmgren, 1993). The overall equilibrium constant of 3 mM measured with intact PDI, which would probably pertain to both the *a* and *a'* domains, is just somewhat greater than the values of 0.7 and 1.9 mM measured here for the isolated domains. The values with the domains were considerably different from those measured by Hawkins and Freedman (1991).

The possibility of applying direct analytical techniques to measure the stabilities of the disulfide bonds of the domains allowed examination of the trapping methods that were used previously in less direct studies on the intact PDI molecule. The method used by Lundström and Holmgren (1993) for trapping intact PDI, which included the addition of a denaturant, gave values with the individual domains that were very similar to those obtained with the more reliable acid trapping (Figure 3). The methods used by Hawkins and Freedman (1991) gave very different values, which could be explained by the slowness of the trapping reaction with iodoacetate, relative to the rapid rate at which GSH reacts with the disulfide bonds of the domains. For this reason, this trapping method also gave nonlinear results with the domains (Figure 3) and with intact PDI (Hawkins & Freedman, 1991). Such nonlinearity with intact PDI had been interpreted previously as indicating cooperativity between the two domains, but all other evidence with intact PDI (Vuori et al., 1992; LaMantia & Lennarz, 1993; Lyles & Gilbert, 1994) and with the isolated domains (Darby & Creighton, 1995b) indicates that the domains in intact PDI function similarly and independently.

The relatively good agreement between the values measured for the stabilities of the disulfide bonds of intact PDI and of one or more of the isolated domains using the different methods of trapping suggests that the same phenomena were occurring in the isolated domains and in intact PDI. It is therefore likely that the stabilities of each of the disulfide bonds of the *a* and *a'* domains in the individual domains studied here are the same as in intact PDI. The values measured by acid trapping with the isolated domains are probably the most accurate values for intact PDI. The low stabilities of the active site disulfide bonds observed in the domains are consistent with the ability of both PDI and the domains to convert protein cysteine residues rapidly to the disulfide forms. They are also compatible with the millimolar concentrations of both GSH and GSSG that are believed to determine the redox environment of the endoplasmic reticulum (Hwang et al., 1992); at equilibrium, both

PDI domains would be present in substantial proportions as both their dithiol and disulfide forms.

The overall rates at which the disulfide bonds of the two PDI domains reacted with GSH were very high, and their combined rate of 10^6 s^{-1} M^{-2} is very similar to that reported by Gilbert (1989) for the most rapidly reduced disulfide bonds of intact PDI. Gilbert (1989) also estimated a value of k_r for these disulfides of 333 s^{-1} M^{-1}, which is consistent with the range of values observed with the domains. These data also define the value of k_{-1}/k_{intra} to be 3000, which is similar to the values obtained here. The rate of reduction of the disulfide bond of PDI-*a'* was comparable to that of PDI-*a*, in spite of the former appearing to be predominantly unfolded. Such a high rate of reduction is unlikely to occur for a disulfide bond of an unfolded protein. This suggests that the equilibration between the folded and unfolded disulfide forms of the protein is very rapid and that the rate observed is reflecting the reduction of the disulfide bond in the folded form.

The disruptive effects of the mutations of the active site cysteine residues of PDI-*a* and PDI-*a'* indicate the importance of these cysteine residues in the structural stability of these domains. Similar, but smaller, effects have been observed previously with both DsbA and DsbC (Zapun et al., 1994, 1995). The greater effects with the domains may reflect the relatively low stabilities of the isolated domains. As might be expected, the effects observed were greatest with the PDI-*a'* domain, which is a very unstable protein and tends to unfold on forming its destabilizing disulfide bond. Mutation of each buried cysteine residue to alanine increased the stability of the mixed disulfide with glutathione of PDI-*a*, and also had directly observable structural consequences in the case of PDI-*a'*. The origins of the destabilizing effect in this case are presumably due at least in part to the alteration in packing of the protein structure on replacing an uncharged cysteine residue with a smaller alanine residue. The most substantial effect occurred, however, on replacement of the accessible cysteine residue of PDI-*a'* by serine, which appeared to render the protein insoluble and unfolded. Such a dramatic effect from mutating an exposed side chain is presumably not due to a packing alteration, but is probably indicative that other interactions involving the cysteine residues are an important stabilizing factor. It has been apparent from studies on DsbA that the charge on the accessible cysteine thiol group, as a result of its low pK_a, may be an important factor in stabilizing the reduced state of the protein (Nelson & Creighton, 1994). The recent postulate of Jeng et al. (1995) of proton sharing between the two thiol groups of reduced thioredoxin may also be relevant.

The data presented here provide further insight into the properties of the active sites of PDI and are lent credibility by their similarities to the properties of intact PDI observed indirectly. While further studies on intact PDI will be required to confirm them, the kinetic and equilibrium data go far to help rationalize the catalytic properties that are shared by the isolated domains and by PDI. These include the much lower efficacy of cystamine as an active site oxidant and the possibility that during *in vitro* catalysis the active sites are not at equilibrium with the GSH/GSSG buffer, due to their relatively slow rates of reaction. Finally, while the thioredoxin-like domains of PDI have such striking sequence similarity to thioredoxin, their disulfide bonds are only 10^{-3}–10^{-4} as stable as that of thioredoxin. Therefore, the domains, and particularly the PDI-*a* domain for which the structure has been determined (Kemmink et al., 1995), should be ideal models for understanding how these differences arise.

ACKNOWLEDGMENT

We thank Professor Kari Kivirikko for providing the original cloned gene for human PDI, and Elke Penka and Renaud Vincentelli for excellent technical assistance.

REFERENCES

Bardwell, J. C. A., McGovern, K., & Beckwith, J. (1991) *Cell 67*, 581–589.
Creighton, T. E. (1975) *J. Mol. Biol. 96*, 767–776.
Creighton, T. E. (1990) in *Protein Structure: a practical approach* (Creighton, T. E., Ed.) pp 155–166, IRL Press, Oxford.
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. (1995a) *Biochemistry 34*, 3576–3587.
Darby, N. J., & Creighton, T. E. (1995b) *Biochemistry 34*, 11726–11735.
Darby, N. J., Freedman, R. B., & Creighton, T. E. (1994) *Biochemistry 33*, 7937–7947.
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, New York.
Freedman, R. B. (1995) *Curr. Opin. Struct. Biol. 5*, 85–91.
Freedman, R. B., Hirst, T. R., & Tuite, M. F. (1994) *Trends Biochem. Sci. 19*, 331–336.
Gan, Z.-R., Sardana, M. K., Jacobs, J. W., & Polokoff, M. A. (1990) *Arch. Biochem. Biophys. 282*, 110–115.
Gilbert, H. F. (1989) *Biochemistry 28*, 7298–7305.
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. (1991) *Biochem. J. 275*, 341–348.
Hol, W. G. J. (1985) *Prog. Biophys. Mol. Biol. 45*, 149–195.
Holmgren, A. (1981) *Trends Biochem. Sci. 6*, 26–29.
Holmgren, A. (1995) *Structure 3*, 239–243.
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.
Jeng, M.-F., Holmgren, A., & Dyson, H. J. (1995) *Biochemistry 34*, 10101–10105.
Kallis, G.-B., & Holmgren, A. (1980) *J. Biol. Chem. 255*, 10261–10265.
Kemmink, J., Darby, N. J., Dijkstra, K., Scheek, R. M., & Creighton, T. E. (1995) *Protein Sci.* (in press).
Kortemme, T., & Creighton, T. E. (1995) *J. Mol. Biol. 253*, 799–812.
LaMantia, M., & Lennarz, W. J. (1993) *Cell 74*, 899–908.
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. (1995) *Structure 3*, 245–250.

Mieyal, J. J., Starke, D. W., Gravina, S. A., & Hocevar, B. A. (1991) *Biochemistry 30*, 8883−8891.
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., & Lennarz, W. J. (1992) *J. Biol. Chem. 267*, 3553−3556.
Qin, J., Clore, G. M., Kennedy, W. M. P., Huth, J. R., & Gronenborn, A. M. (1995) *Structure 3,* 289−297.
Rabenstein, D. L., & Mills, K. K. (1995) *Biochim. Biophys. Acta 1249*, 29−36.
Sarkar, G., & Sommer, S. S. (1990) *BioTechniques 8*, 289−297.
Shevchik, V. E., Condemine, G., & Robert-Baudouy, J. (1994) *EMBO J. 13*, 2007−2012.
Szajewski, R. P., & Whitesides, G. M. (1980) *J. Am. Chem. Soc. 102*, 2011−2026.
Vuori, K., Myllylä, R., Pihlajaniemi, T., & Kivirikko, K. I. (1992) *J. Biol. Chem. 267*, 7211−7214.
Wunderlich, M., & Glockshuber, R. (1993) *Protein Sci. 2*, 717−726.
Yang, Y., & Wells, W. W. (1991) *J. Biol. Chem. 166*, 12759−12765.
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., Cooper, L., & Creighton, T. E. (1994) *Biochemistry 33*, 1907−1914.
Zapun, A., Missiakas, D., Raina, S., & Creighton, T. E. (1995) *Biochemistry 34*, 5075−5089.
Ziegler, D. M., & Poulson, L. L. (1977) *Trends Biochem. Sci. 2*, 79−81.

BI951980G