ANTIOXIDANTS & REDOX SIGNALING
Volume 8, Numbers 3 & 4, 2006

Forum Original Research Communication

pH Dependence of the Peptide Thiol-Disulfide Oxidase Activity of Six Members of the Human Protein Disulfide Isomerase Family

H.I. ALANEN,[1] K.E.H. SALO,[1] A. PIRNESKOSKI,[1,2] and L.W. RUDDOCK[1]

ABSTRACT

Protein folding in the endoplasmic reticulum is often associated with the formation of native disulfide bonds, a process which *in vivo* is one of the rate limiting steps of protein folding and which is facilitated by the enzyme protein disulfide isomerase (PDI). Higher eukaryotes have multiple members of the PDI family, for example, seventeen human PDIs have been reported to date. With multiple members of the same family being present, even within the same cell, the question arises as to what differential functions are they performing? To date there has been no systematic evaluation of the enzymological properties of the different members of the PDI-family. To address the question of whether different PDI family members have differing thiol-disulfide chemistry, we have recombinantly expressed and purified six members of the family, PDI, PDIp, ERp57, ERp72, P5, and PDIr from a single organism, human. An examination of the pH-dependence and nature of the rate limiting step for the peptide thiol-disulfide oxidase activity of these enzymes reveals that, with the exception of PDIr, they are all remarkably similar. In the light of this data potential differential functions for these enzymes are discussed. *Antioxid. Redox Signal.* 8, 283–291.

INTRODUCTION

NATIVE DISULFIDE BOND formation is one of the rate limiting steps for protein folding for proteins destined for secretion or for the outer membrane. In order to facilitate this process organisms have developed complex networks of protein folding catalysts. In the periplasm of gram negative bacteria there is the Dsb (disulfide bond forming) family of enzymes (for review, see Ref. 4), while in the endoplasmic reticulum of higher and lower eukaryotes there is the PDI (protein disulfide isomerase) family of enzymes (for reviews, see Refs. 8 and10).

Bovine PDI was the first catalyst of protein folding reported, nearly 40 years ago (12) and for many years was thought to be the sole catalyst of disulfide bond formation in the endoplasmic reticulum. However, over the past decade a series of PDI family members have been reported and currently humans are known to have at least seventeen ER-located family members (8).

To date there has been no systematic evaluation of the relative abundance of all of the human PDI family members, nor of their regulation at the transcriptional, translational or protein level nor of the enzymological properties of the different members of the PDI family. To initiate such a study we have initially focused on the basic question of whether different members of the human PDI family have different thiol-disulfide chemistries. We have recombinantly expressed and purified six members of the human PDI family and the peptide thiol-disulfide oxidase activity of each enzyme was tested, including an examination of the pH-dependence of activity and details of the nature of the rate limiting step. These

[1]Biocenter Oulu and Department of Biochemistry, University of Oulu, Oulu, Finland.
[2]Previous address: Department of Biosciences, University of Kent, Canterbury Kent, United Kingdom.

studies reveal that, with the exception of PDIr, these enzymes possess remarkably similar activities in this assay. Potential differential functions for these enzymes in light of these results are discussed.

MATERIALS AND METHODS

Generation of expression vectors:

The constructs used here (Table 1) were generated for a previous study on defining the domain boundaries of the PDIs (1).

Protein expression and purification:

Protein production was carried out in *E. coli* strain BL21 (DE3) pLysS. Purification of the proteins was as per that of ERp18 (2) except that PDIr was applied to a Resource S cation exchanger and not a Resource Q anion exchanger. The concentration of each protein was determined spectrophotometrically using a calculated absorption coefficient at 280 nm.

Oxidase assay:

The method of Ruddock *et al.* (21) using a fluorescent decapeptide (NRCSQGSCWN) was used to determine the oxidase activity of each of the purified human PDI family members. Disulfide bond formation (oxidation) is monitored by the quenching of the intrinsic fluorescence of the single tryptophan in the peptide as the arginine residue is brought into close proximity upon disulfide formation. McIlvaine buffer (0.2 *M* disodium hydrogen phosphate/0.1 *M* citric acid; pH 3.0–7.5) to give a final assay volume of 1 ml was placed in a fluorescence cuvette. Except where noted in the text, to this was added 10 µl of oxidized glutathione (50 m*M* stock solution in buffer A), 20 µl of reduced glutathione (100 m*M* stock solution in buffer A) and 5–10 µl of enzyme. After mixing, the cuvette was placed in a Perkin-Elmer LS50 spectrophotometer for 5 min to allow thermal equilibration of the solution. 20 µl of substrate peptide (170 µ*M* in 30% acetonitrile/0.1% TFA) was added, mixed, and the change in fluorescence intensity (excitation 280 nm, emission 350 nm, slits 5/5 nm) was monitored over an appropriate time (15 min to 1 h) with 600 to 1800 data points being collected. Analysis of the data was as Ruddock *et al.* (21), except where noted in the text.

For all enzyme-catalyzed reactions, the rate of reaction was directly proportional to the enzyme concentration used. Initially all enzymes were tested at near equivalent molar active site concentrations which for PDI enabled the activity to be monitored over the entire pH range. Due to its higher activity the concentration of ERp57 subsequently had to be lowered to enable the reaction to be monitored, the concentration of PDIp raised to determine activity over the noncatalyzed reaction and the concentration of PDIr was raised to the maximal possible to examine the very low oxidase activity this enzyme had.

For P5 in the absence of substrate at pH values of 5 or below, a small linear decrease in fluorescence was observed, possibly representing loss of protein through aggregation. While this represented maximally a loss of 2% of the fluorescence signal of P5 over the course of an assay, the large number of tryptophans that P5 possesses meant that this became significant in analyzing the assay results. This was corrected for by adding back the measured loss of P5 signal to the fluorescent peptide assay results performed at these low pH values. Other than this effect: (a) the average change in fluorescence of the enzymes was maximally 0.5% (probably reflecting photobleaching) and (b) the average decrease in fluorescence during peptide oxidation was constant for enzyme-catalyzed and noncatalyzed reactions. These two controls ensured that the only change in fluorescence being observed was due to that of the substrate peptide.

Table 1. Plasmids Used in This Study

Name	*Construct*
pLWRP64	human PDI D18-L508
pLWRP69	human PDI D18-A137
pKEHS69	human ERp57 S25-L505
pHIA64	human ERp72 V20-L645
pHIA43	human P5 L20-L440
pAP32	human PDIp Q22-L507
pHIA44	human PDIr S22-L519

With the exception of pLWRP69, which generates the isolated **a** domain of human PDI, the remainder generate full-length mature protein, based on analysis of the preferred cleavage site of the signal sequence. All constructs were MHHHHHHM-N-terminally tagged.

RESULTS

Protein production

All of the vectors generated in this study produced soluble proteins of the expected molecular weight, which could be purified to near homogeneity by a simple combination of immobilized metal affinity chromatography and ion exchange chromatography (Fig. 1). The primary source of contamination was proteolytic degradation, but this was very minor except for PDIr. The addition of protease inhibitors during the cell-lysis step decreased the amount of proteolytic product obtained during the purification of PDIr but did not completely eliminate the problem (data not shown). From SDS-PAGE the primary degradation contaminant from the PDIr purification appears to be three domains in size and since this binds to the IMAC column and the his-tag is N-terminal, it must be the C-terminal fourth domain which is lost. This contaminant co-elutes with full-length PDIr from the ion exchange column. The PDIr used for the assays was about 95% pure, with 5% degradation product. To our knowledge this is the first reported purification for recombinant mature (his-tagged) human PDIp, P5, and ERp72.

Peptide oxidase assay for recombinant mature human PDIs

The peptide oxidase-activity of the PDIs can be monitored in real time using a substrate peptide in which quenching of the intrinsic fluorescence of the single tryptophan occurs as

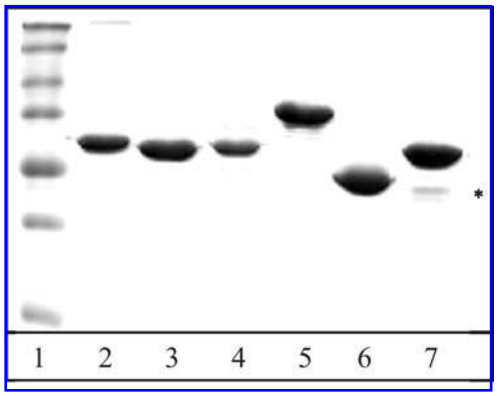

FIG. 1. SDS-PAGE analysis of purified proteins. *Lane 1*, molecular weight markers (250, 150, 100, 75, 50, 37, 25 kDa); *Lane 2*, human PDI; *Lane 3*, human ERp57; *Lane 4*, human PDIp; *Lane 5*, human ERp7; *Lane 6*, human P5; *Lane 7*, human PDIr. The position of the degradation product of PDIr is marked with an *.

an arginine residue is brought into close proximity upon the formation of a disulfide bond (21).

All six of the human PDI family members tested showed catalytic peptide thiol-disulfide oxidase activity at pH 6.5 (Table 2). On a per molar active site basis, ERp57, ERp72, and P5 showed more activity than PDI or PDIp (see Fig. 4). Correcting for the noncatalyzed reaction PDIr exhibited only 6% of the activity of PDI on a per molar active site basis.

Previously it had been shown that for *V. cholerae* DsbA and bovine PDI the rate limiting step for the peptide oxidation reaction was reoxidation of the enzyme by glutathione; consistent with this observation, the decrease in fluorescence was linear for over 50% of the oxidation reaction (21). An initial examination of the results indicated that a) the specific activity of recombinant human PDI was substantially greater than that of the bovine PDI previously reported; b) that the kinetics of oxidation for many of the reactions observed were more complex than those of bovine PDI.

Enzyme-catalyzed peptide oxidation in its simplest form can be broken down into four distinct phases, each of which requires the rearrangement of covalent bonds and hence which could potentially be the rate limiting step in the reaction. In addition to these steps, there will also be association reactions (e.g., to form noncovalently linked substrate-enzyme complexes)

$$\text{Enzyme (ox)} + \text{Peptide (red)} \rightleftarrows \text{Enzyme–peptide} \quad (1)$$

$$\text{Enzyme–peptide} \rightleftarrows \text{Enzyme (red)} + \text{Peptide (ox)} \quad (2)$$

$$\text{Enzyme (red)} + \text{GSSG} \rightleftarrows \text{Enzyme-SG} + \text{GSH} \quad (3)$$

$$\text{Enzyme-SG} \rightleftarrows \text{Enzyme (ox)} + \text{GSH} \quad (4)$$

Initially a mixed disulfide complex is formed between the peptide and the N-terminal cysteine of the enzyme active site CXXC motif (reaction 1). This mixed disulfide can either be subject to nucleophilic attack by the C-terminal cysteine of the enzyme active site resulting in the formation of the starting material (reverse reaction 1) or by a free thiol group on the peptide (reaction 2) to generate reduced enzyme. To complete the catalytic cycle, the enzyme must be reoxidized by oxidized glutathione, which is again a two-step process with the initial formation of a mixed disulfide between the enzyme and the glutathione tripeptide (reaction 3) followed by nucleophilic attack by the C-terminal cysteine to form oxidized enzyme and reduced glutathione (reaction 4). Further complexities arise due to the potential ability of the reduced enzyme to reduce oxidized peptide (reverse reaction 2) and from the potential ability of reduced glutathione to reduce the oxidized peptide or oxidized enzyme or the enzyme–peptide complex (in each case to form a mixed disulfide) or the enzyme-glutathione mixed disulfide (to form reduced enzyme and oxidized glutathione).

Two extreme cases which give rise to simple kinetic schemes are possible in which either peptide oxidation is rate limiting or enzyme reoxidation is rate limiting. In the system being studied only the net effect of the conversion of reduced to oxidized peptide is observed and [GSSG] >> [peptide] > [enzyme]. The outcome of this is that if peptide oxidation is rate limiting (and the reverse reactions are slow) the reaction behaves as a first order reaction with respect to substrate (i.e., exponential decrease in fluorescence), whereas if enzyme reoxidation is rate limiting the reaction behaves as a zero order reaction with respect to substrate (i.e., a linear decrease in fluorescence, as observed previously (21)). For a representative sample of assays we attempted fits of the data to both models. Unsurprisingly, the nonenzyme-catalyzed reaction fitted well to an exponential function with random residuals (Fig. 2A/B), as did several of the enzyme-catalyzed reactions, most notably those for ERp57, while several of the enzyme-catalyzed reactions fitted well to a linear function with random residuals (Fig. 2C/D), most notably those for PDIp. The majority of enzyme-catalyzed reactions could be fitted to a linear function and by eye looked to be reasonable fits, but a plot of the residuals showed nonrandom scatter (i.e., the rates of both stages of the reaction were sufficiently close in magnitude for neither to be truly rate limiting). Due to this the original method for examining the kinetics, the calculation of the half time of the reaction (21) was used for all enzyme-catalyzed reactions.

TABLE 2. HALF-TIME FOR ENZYME-CATALYZED PEPTIDE-OXIDATION REACTIONS

Protein	*Half-time*
None	1285 s
PDI	257 s
ERp57	118 s
PDIp	285 s
ERp72	164 s
P5	157 s
PDIr	640 s

All reactions in McIlvaine buffer pH 6.5, 2 m*M* GSH, 0.5 m*M* GSSG, 3.4 μ*M* substrate, 25°C. Enzyme concentrations used were [PDI] = 81 n*M*; [ERp57] = 63 n*M*; [PDIp] = 162 n*M*; [ERp72] = 60 n*M*; [P5] = 84 n*M*; [PDIr] = 224 n*M*.

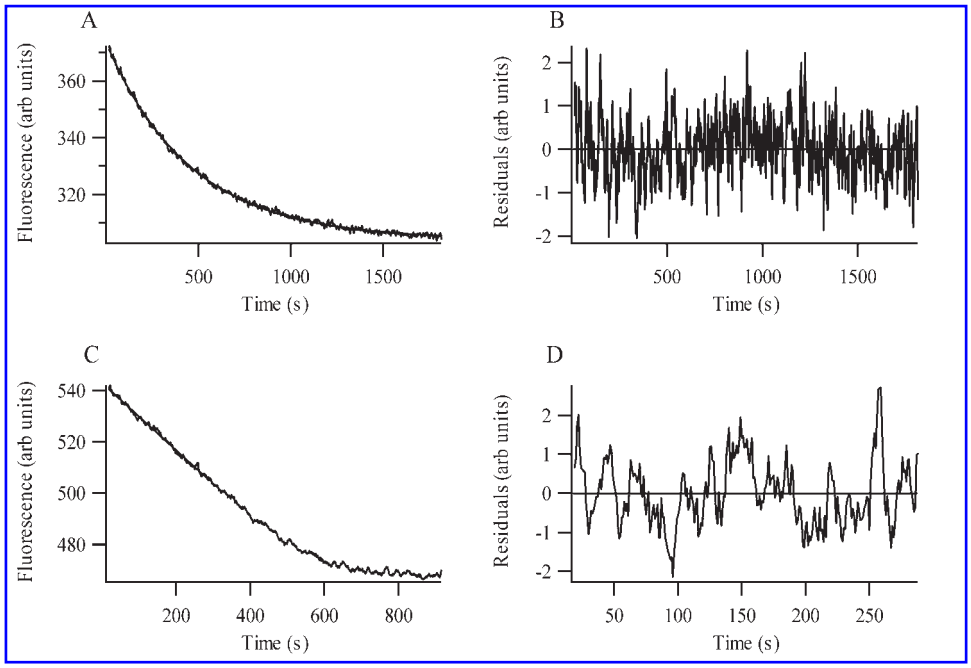

FIG. 2. Representative analysis of the nature of the kinetics of peptide thiol-disulfide oxidation. Both reactions were performed at 25°C, 0.5 m*M* GSSG, 2 m*M* GSH in McIlvaine buffer. (**A**) Nonenzyme-catalyzed reaction at pH 7.5, fitted line is to an exponential function; (**B**) Residuals for **A**; (**C**) PDIp catalyzed reaction at pH 6.5. [PDIp] = 162 n*M*. Fitted line is over the first half of the reaction to a linear function; (**D**) Residuals for **C**.

pH-dependence of the oxidase activity

To further examine potential differences between the activities of the human PDI family, the pH dependence of oxidase activity of each family member was examined. All of the human PDIs, except PDIr, had activity down to pH 4.0, but at this pH the rate was too low to determine accurately in a catalyzed system (where substrate concentration > 10× enzyme concentration) with fluorescence detection. Furthermore, the rate of PDIr peptide thiol-disulfide oxidase activity in this assay was so low that at pH values below 6.0 it was difficult to determine the overall rate of peptide oxidation, while at pH values of 7.0 or above the noncatalyzed rate was so fast that it was difficult to determine the rate of the catalyzed reaction. Accordingly no further detailed studies were undertaken in this assay system with PDIr.

The nonenzyme-catalyzed rate was determined over the pH range 6.0–7.5. Over this pH range the rate of oxidation increased with increasing pH. Below pH 6.0 concerns over fluorescence quenching during prolonged measurements meant that the noncatalyzed rate of reaction became too slow to determine accurately (extrapolation of the data obtained suggested a half time of the order of 12 h at pH 5.0). Extrapolation of the data to pH 5.5 indicated that maximally (for PDIp) the noncatalyzed rate was only 6.6% of the catalyzed rate and that for most reactions it was less than 2% of the overall rate (i.e., that correction for the nonenzyme-catalyzed rate became nonessential).

The pH dependence of the oxidase activity of human PDI (Fig. 3A) was very similar to that previously reported for bovine PDI, with the rate of PDI-catalyzed oxidation increasing with pH and reaching a plateau at higher pH values. The midpoint for the enzyme-catalyzed rate occurred at pH 5.9, c.f. a pK_{app} of 5.6 ± 0.1 for bovine PDI (21). The primary difference was that on a per molar basis, the human PDI used in this study had at pH 6.5, 2.5 times the specific activity of bovine PDI previously reported (21). This difference probably arises due to the complex purification procedure required for bovine PDI, which includes heating the liver homogenate to 50ºC (9).

The peptide–oxidase activity of human PDIp increased with increasing pH over the pH range 4.5–7.5 (Fig. 3B), for both the total activity and enzyme-catalyzed activity. Taking the enzyme-catalyzed activity at pH 7.5 to be the maximal rate, the pH at which half maximal activity was observed was 6.6.

The pH dependence of human ERp57 (Fig. 3C), human ERp72 (Fig. 3D) and human P5 (Fig. 3E) showed a more complex pH dependence, reminiscent of that of *V. cholerae* DsbA (21). In each case the rate of oxidation increased with increasing pH, over the pH range 4.5–7.5. Since the observed rate comprises the sum of the enzyme-catalyzed rate and the noncatalyzed rate, which increases with pH, the enzyme-catalyzed rate for all three enzymes is bell-shaped, with a maximum at pH 7.0 and half maximal activity at pH 5.8, 5.8, and 5.7 for ERp57, ERp72, and P5, respectively.

Each of the PDI family members tested has multiple catalytic domains. To examine the peptide thiol-disulfide oxidase activity of an isolated catalytic domain, the **a** domain of PDI was purified and assayed. At all pH values tested, the isolated **a** domain of PDI showed greater activity than the full-length enzyme on a per molar active site basis. The pH depen-

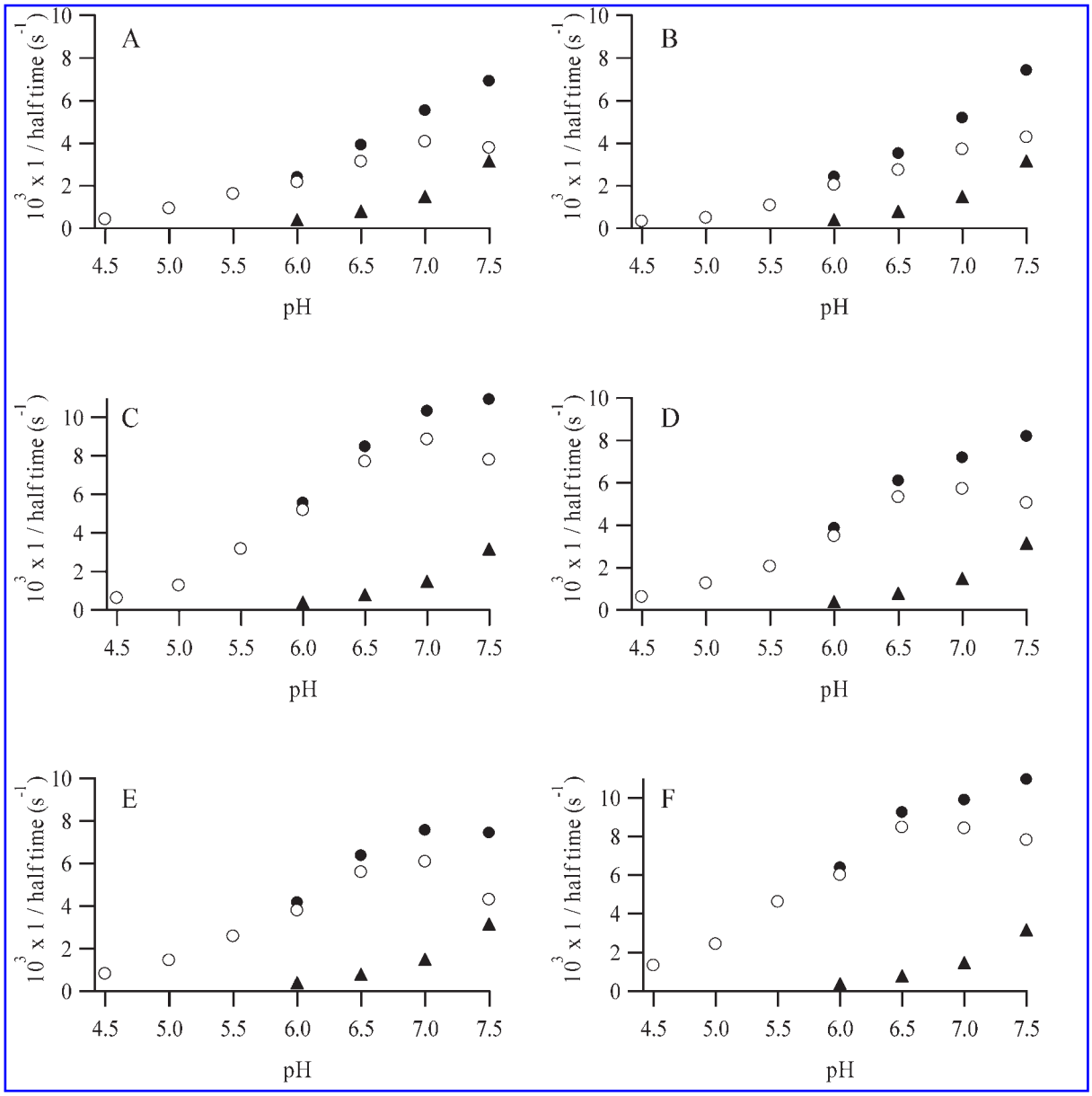

FIG. 3. pH-dependence of the peptide thiol-disulfide oxidase activity of the human PDIs, represented by the inverse half-time for the reaction in seconds. All reactions were performed at 25°C, 0.5 m*M* GSSG, 2 m*M* GSH in McIlvaine buffer, in duplicate or triplicate. For each data point the error was less than 10% of the value of the inverse half-time, for clarity error bars are not shown. Total rates (●), nonenzyme-catalyzed rates (▲), and calculated enzyme-catalyzed component of the rate (○) are shown. (**A**) Human PDI catalyzed reaction, [PDI] = 81 n*M*; (**B**) human PDIp catalyzed reaction, [PDIp] = 162 n*M*; (**C**) human ERp57 catalyzed reaction, [ERp57] = 63 n*M*; (**D**) human ERp72 catalyzed reaction, [ERp72] = 60 n*M*; (**E**) human P5 catalyzed reaction, [P5] = 84 n*M*; (**F**) **a** domain of human PDI catalyzed reaction, [**a** domain] = 181 n*M*.

dence profile for oxidase activity was similar to that of the full-length enzyme, except that the plateau region was reached earlier and there was a small decrease in activity at pH 7.5 compared with pH 7 (Fig. 3F). The pH at which half-maximal activity was observed was slightly lower than for the other members of the family at 5.4.

A comparison of the relative activity at each pH value of the peptide thiol-disulfide oxidase activity for human PDI, PDIp, ERp57, ERp72, P5, and the **a** domain of PDI per molar active site is shown in Figure 4.

Investigation of the rate limiting step for peptide oxidation

It is possible to elucidate some information about the rate limiting step by varying the enzyme, peptide, and glutathione concentrations in the fluorescence based peptide–oxidase activity assay utilized here, but the inability to resolve each step individually prohibits a comprehensive analysis.

For human PDI, ERp57, ERp72, and P5, a series of catalyzed reactions were performed at pH 5.5, a pH at which the

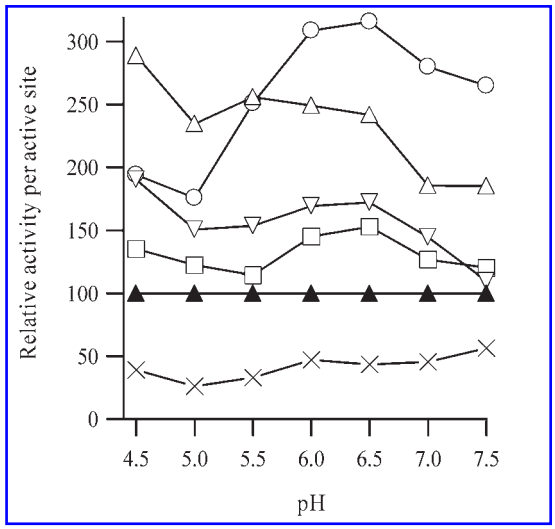

FIG. 4. pH-dependence of the relative activities of the human PDIs per molar active site. PDI (▲), PDIp (X), ERp57 (❍), ERp72 (❑), P5 (▽), and the **a** domain of PDI (△).

nonenzyme-catalyzed rate was very slow, but the enzyme-catalyzed rate could still be determined accurately over a reasonable timescale. The reactions performed included varying the enzyme concentration, halving the substrate concentration, doubling the oxidized glutathione concentration and removing the reduced glutathione from the reaction (Table 3). For human PDIp a similar series of reactions were performed at pH 6.5 (Table 3), as the enzyme-catalyzed rate was very slow at pH 5.5. For all conditions tested the rate of reaction increased proportionally to the enzyme concentration.

Removing the reduced glutathione from the assay conditions resulted in a small increase in activity for PDI, ERp57, ERp72, PDIp, and P5 of between 9% and 30%. Since no difference in the rate of reaction for the nonenzyme-catalyzed reaction at pH 7.5 was observed in the presence or absence of reduced glutathione (data not shown) this increase in rate does not represent a reduction of the oxidized peptide by reduced glutathione, but either reduction of the oxidized enzyme or isomerization of the enzyme-peptide mixed disulfide intermediate (to form an enzyme-glutathione or glutathione-peptide mixed disulfide) or of the enzyme-glutathione mixed disulfide intermediate (to form reduced enzyme and oxidized glutathione).

Increasing the oxidized glutathione concentration in the assay resulted in an increase in activity of between 37% and 57%. If reoxidation of the enzyme had been the rate limiting step, then doubling the concentration of oxidized glutathione would have been expected to double the rate of the reaction, whereas if peptide oxidation had been rate limiting then it would have been expected to have had no effect on the rate of the reaction. This intermediate value suggests that the overall rate of oxidation is governed by both peptide oxidation and enzyme reoxidation (i.e., that the rates are of the same order of magnitude and that neither can be said to be rate limiting). This hypothesis is further supported by the results from halving the substrate concentration, where a decrease in rate of between 23% and 39% was observed. PDIp showed the greatest dependence on oxidized glutathione concentration and the least dependence on substrate concentration, but it should be noted that this was done at pH 6.5, whereas the rest were done at pH 5.5 and each step in the peptide thiol-disulfide oxidation reaction may exhibit a different pH dependence. Previous studies on the substrate and oxidized glutathione dependence of the reaction catalysed by bovine PDI, which showed reoxidation of the enzyme to be rate limiting but only 40% of the activity of recombinant human PDI, were also performed at pH 6.5 (21).

DISCUSSION

In vitro and *in vivo* in the ER the process of native disulfide bond formation is catalyzed by the PDI-family of enzymes, of which 17 have been reported to date in humans (8). With multiple members of the PDI family being present in mammalian tissues, even within the same cell, the question arises as to why should there be so many members of the same family, all with the same putative function and all located in the ER? It is possible that there may be differences in tissue distribution, substrate specificity, induction specificity, reaction specificity, and differential regulation of activity. The resolution of these issues is perhaps best characterized by a comparison between PDI and PDIp, a pancreas specific

TABLE 3. ENZYME-CATALYZED PEPTIDE-OXIDATION REACTIONS UNDER NONSTANDARD CONDITIONS

Enzyme	*1*	*2*	*3*	*4*
PDI	394 ± 7	295 ± 1 (67%)	268 ± 3 (147%)	344 ± 5 (115%)
ERp57	403 ± 13	319 ± 14 (63%)	274 ± 9 (147%)	310 ± 6 (130%)
ERp72	472 ± 1	355 ± 8 (67%)	345 ± 5 (137%)	388 ± 11 (122%)
P5	439 ± 3	361 ± 7 (61%)	321 ± 6 (137%)	357 ± 4 (123%)
PDIp	285 ± 3	202 ± 3 (77%*)	177 ± 4 (150%*)	266 ± 6 (109%*)

*Corrected for nonenzyme-catalyzed rate, note noncatalyzed rate under condition 3 is twice that under "standard conditions."

Half time for enzyme-catalyzed peptide-oxidation reactions and a comparison of the rate compared with "standard conditions" in parentheses, correcting for the half substrate concentration in reaction condition 2. All reactions at pH 5.5, except for PDIp at pH 6.5. Conditions: 1) "standard", McIlvaine buffer, 2 m*M* GSH, 0.5 m*M* GSSG, 3.4 μ*M* substrate; 2) as 1 except 1.7 μ*M* substrate; 3) as 1 except 1 m*M* GSSG; 4) as 1 except no GSH. Enzyme concentrations used were, [PDI] = 162 n*M*; [ERp57] = 63 n*M*; [ERp72] = 60 n*M*; [P5] = 84 n*M*; [PDIp] = 162 n*M*.

homologue. It has been demonstrated that the two proteins show differences in tissue distribution, a putative difference in regulation and most importantly differences in substrate binding (14, 20). Thus although there is now evidence that several of possible reasons for multiple family members may be occurring, the molecular characterization required to fully define the functional differentiation is far from complete.

While very detailed studies have been performed on the enzymatic activity of human PDI (and from other sources) and on the contribution of individual domains in this process (6, 7, 24 as examples), studies on the other family members are more patchy. Human ERp57 (3, 11, 25), rat ERp72 (16, 17, 22, 23), and rat P5 (16, 17, 22) have been shown to possess thiol disulfide oxidoreductase activity, but no systematic studies comparing relative activities have been published.

In this paper we initiate enzymatic studies on recombinantly generated human PDI, PDIp, ERp57, P5, ERp72, and PDIr. The use of enzymes from a single source will, we believe, allow for a detailed comparison of the activities of these enzymes at the molecular level and allow for studies into potential synergistic actions using enzymes which will naturally be co-localized.

With the exception of PDIr, which shows susceptibility to proteases at the flexible linkers between domains, all of the enzymes could be purified to homogeneity and all showed peptide thiol-disulfide oxidase activity. This represents the least-complex activity possessed by PDI with respect to catalysis of the formation of native disulfide bonds. The absolute activity per mole active site and the pH dependence of this activity was remarkably similar for all five enzymes. Furthermore, when alterations were made to the assay conditions, all five showed very similar shifts in activity, that in each case suggests that both peptide oxidation and enzyme reoxidation have near equivalent rates. Taken together these results indicate that, excluding PDIr, the human PDIs have remarkably similar thiol-disulfide chemistries.

The one enzyme which did not show significant peptide thiol-disulfide oxidase activity was PDIr. The structure of PDIr is unique among the PDIs (8) consisting of three domains containing a thioredoxin-like active site and one not, each apparently linked by a short flexible stretch of amino acids. Two of the three –CXXC- active site motifs of PDIr differ from the usual consensus motif (CGHC) found in other human PDIs. While the sequence identity between the catalytic domains of PDIr and the **a** domain of PDI is generally lower than that of other family members (Table 4), it is not significantly different than, for example, the **a'** domain of PDI. PDIr has also been reported to have significantly lower isomerization activity than PDI (13) and it has been speculated that the inability of the enzyme to efficiently catalyze thiol-disulfide exchange reactions arises from the lack of a conserved glutamic acid beneath the active site (8).

It had previously been reported that the catalytic **a** and **a'** domains of PDI possess thiol-disulfide oxidoreductase activity in isolation, but that simple isomerization of a protein substrate requires a linear combination of domains which includes **b'**, while isomerizations which would require a substantive conformational change in the substrate protein as well as thiol-disulfide chemistry requires all four domains of PDI (7). Furthermore, the **b'** domain is implicated in substrate binding (15, 19) and not in thiol-disulfide exchange reactions (7). Consistent with these results the isolated **a** domain of PDI examined here showed peptide thiol-disulfide oxidase activity, indeed on a per molar active site basis it was significantly more active than the wild type enzyme. The 1.9- to 2.9-fold difference between the activity per molar active site of the isolated **a** domain and full length PDI probably does not arise due to the **a** and **a'** domains in full length PDI having significantly different activities, as both domains had previously been shown to be active in thiol-disulfide exchange (5, 7). Instead the most likely explanation is increased steric hindrance in accessing the active site in the full length protein. To further eliminate a role for the **b'** domain in this assay, a ten-fold molar excess of the PDI substrate binding inhibitor 2-propylphenol (14) was added to the peptide oxidase assay. For both PDI and the isolated **a** domain no differences in activity were seen in the presence or absence of 2-propylphenol (data not shown).

From these results it is apparent that the peptide oxidase assay utilized here requires only thiol-disulfide chemistry and that all of the human PDI family members except PDIr possess near equivalent activities per molar active site with respect to this, in terms of both turnover, pH-dependence, and the nature of the rate limiting step. Hence, excluding PDIr, differences between these six PDI family members do not lie in differences in simple thiol-disulfide chemistry. Therefore the differences must lie in interactions with potential substrates (which includes specificities and the ability to catalyze complex-isomerizations) or with other ER resident partner proteins.

The data presented here is consistent with a model whereby PDIp and ERp57 are isoforms of PDI with altered substrate specificity in the **b'** domain, allowing PDIp to act on a subset of folding proteins via its highly specific substrate binding specificity (14, 20) and ERp57 to act in folding glycosylated proteins via its specific interactions with the lectins calnexin

TABLE 4. SEQUENCE IDENTITY OF THE CATALYTIC DOMAINS

Domain	*Sequence identity*
PDI **a'**	36.8% (106)
PDIp **a**	55.0% (109)
PDIp **a'**	34.3% (105)
ERp57 **a**	49.5% (109)
ERp57 **a'**	43.0% (107)
ERp72 domain 1	45.3% (106)
ERp72 domain 2	52.8% (108)
ERp72 domain 5	37.0% (108)
P5 domain 1	44.7% (103)
P5 domain 2	47.1% (104)
PDIr domain 2	33.3% (108)
PDIr domain 3	37.9% (103)
PDIr domain 4	35.8% (107)

Sequence identity of the domains of the human PDI family homologous to PDI **a** and **a'** domains, with the **a** domain of human PDI. The number in parentheses represents the number of amino acids identity was determined over. Sequence identity and similarity is ubiquitously lower over the C-terminal section, especially those regions corresponding to the last β-strand and α-helix of the **a** domain of human PDI.

and calreticulin (18, 25). ERp72 appears to be PDI with an additional catalytic domain, which theoretically should allow it to perform more complex thiol-disulfide isomerizations and hence be involved in the folding of proteins which contain large numbers or complex patterns of disulfides. P5 possesses two **a**-like domains, but elucidation of its physiological function is still required; specifically this will require an understanding of the role of the C-terminal half of the protein, which shows little homology with other PDI family members and which is of yet of unknown function. PDIr, from the data presented here does not appear to be involved in the efficient catalysis of thiol-disulfide oxidation reactions. This result combined with the unusual domain architecture, with three sequential thioredoxin-like domains with putative flexible linkers between each domain, might suggest PDIr plays a critical role in the late complex-isomerization steps that are essential for the generation of native disulfide bonds in nascent polypeptide chains in the endoplasmic reticulum, but this remains to be elucidated.

ACKNOWLEDGMENTS

The authors thank Robert Freedman and Peter Klappa for valuable comments on the manuscript. The work was supported by Biocenter Oulu and by Biotechnology and Biological Sciences Research Council Grant 96/C13322.

ABBREVIATIONS

Dsb, disulfide bond forming; ER, endoplasmic reticulum; GSH, reduced glutathione; GSSG, oxidized glutathione; IMAC, immobilized metal affinity chromatography; PDI, protein disulfide isomerase.

REFERENCES

1. Alanen HI, Salo KE, Pekkala M, Siekkinen HM, Pirneskoski A, and Ruddock LW. Defining the domain boundaries of the human protein disulfide isomerases. *Antioxid Redox Signal* 5: 367–374, 2004.
2. Alanen HI, Williamson RA, Howard MJ, Lappi AK, Jantti HP, Rautio SM, Kellokumpu S, and Ruddock LW. Functional characterization of ERp18, a new endoplasmic reticulum-located thioredoxin superfamily member. *J Biol Chem* 278: 28912–28920, 2003.
3. Bourdi M, Demady D, Martin JL, Jabbour SK, Martin BM, George JW, and Pohl LR. cDNA cloning and baculovirus expression of the human liver endoplasmic reticulum P58: characterization as a protein disulfide isomerase isoform, but not as a protease or a carnitine acyltransferase. *Arch Biochem Biophys* 323: 397–403, 1995.
4. Collet JF and Bardwell JC. Oxidative protein folding in bacteria. *Mol Microbiol* 44: 1–8, 2002.
5. Creighton TE, Zapun A, and Darby NJ. Mechanisms and catalysts of disulfide bond formation in proteins. *Trends Biotechnol* 13: 18–23, 1995.
6. Darby NJ and Creighton TE. Functional properties of the individual thioredoxin-like domains of protein disulfide isomerase. *Biochemistry* 34: 11725–11735, 1995.
7. Darby NJ, Penka E, and Vincentelli R. The multi-domain structure of protein disulfide isomerase is essential for high catalytic efficiency. *J Mol Biol* 276: 239–247, 1998.
8. Ellgaard L and Ruddock LW. The human protein disulfide isomerase family: substrate interactions and functional properties. *EMBO Rep* 6: 28–32, 2005.
9. Freedman RB, Hawkins HC, and McLaughlin SH. Protein disulfide-isomerase. *Methods Enzymol* 251, 397–406, 1995.
10. Freedman RB, Klappa P, and Ruddock LW. Protein disulfide isomerases exploit synergy between catalytic and specific binding domains. *EMBO Rep* 3: 136–140, 2002.
11. Frickel EM, Frei P, Bouvier M, Stafford WF, Helenius A, Glockshuber R, and Ellgaard L. ERp57 is a multifunctional thiol-disulfide oxidoreductase. *J Biol Chem* 279: 18277–18287, 2004.
12. Goldberger RF, Epstein CJ, and Anfinsen CB. Acceleration of reactivation of reduced bovine pancreatic ribonuclease by a microsomal system from rat liver. *J Biol Chem* 238: 628–635, 1963.
13. Horibe T, Gomi M, Iguchi D, Ito H, Kitamura Y, Masuoka T, Tsujimoto I, Kimura T, and Kikuchi M. Different contributions of the three CXXC motifs of human protein-disulfide isomerase-related protein to isomerase activity and oxidative refolding. *J Biol Chem* 279: 4604–4611, 2004.
14. Klappa P, Freedman RB, Langenbuch M, Lan MS, Robinson GK, and Ruddock LW. The pancreas-specific protein disulfide-isomerase PDIp interacts with a hydroxyaryl group in ligands. *Biochem J* 354: 553–559, 2001.
15. Klappa P, Ruddock LW, Darby NJ, and Freedman RB. The b′ domain provides the principal peptide-binding site of protein disulfide isomerase but all domains contribute to binding of misfolded proteins. *EMBO J* 17: 927–935, 1998.
16. Kramer B, Ferrari DM, Klappa P, Pohlmann N, and Söling HD. Functional roles and efficiencies of the thioredoxin boxes of calcium-binding proteins 1 and 2 in protein folding. *Biochem J* 357: 83–95, 2001.
17. Lundstrom-Ljung J, Birnbach U, Rupp K, Söhling HD, and Holmgren A. Two resident ER-proteins, CaBP1 and CaBP2, with thioredoxin domains, are substrates for thioredoxin reductase: comparison with protein disulfide isomerase. *FEBS Lett* 357: 305–308, 1995.
18. Oliver JD, van der Wal FJ, Bulleid NJ, and High S. Interaction of the thiol-dependent reductase ERp57 with nascent glycoproteins. *Science* 275: 86–88, 1997.
19. Pirneskoski A, Klappa P, Lobell M, Willliamson RA, Byrne L, Alanen HI, Salo KE, Kivirikko KI, Freedman RB, and Ruddock LW. Molecular characterization of the principal substrate binding site of the ubiquitous folding catalyst protein disulfide isomerase. *J Biol Chem* 279: 10374–10381, 2004.
20. Ruddock LW, Freedman RB, and Klappa P. Specificity in substrate binding by protein folding catalysts: tyrosine and tryptophan residues are the recognition motifs for the binding of peptides to the pancreas-specific protein disulfide isomerase PDIp. *Protein Sci* 9: 758–764, 2000.

21. Ruddock LW, Hirst TR, and Freedman RB. pH-dependence of the dithiol-oxidizing activity of DsbA (a periplasmic protein thiol:disulfide oxidoreductase) and protein disulfide-isomerase: studies with a novel simple peptide substrate. *Biochem J* 315: 1001–1005, 1996.
22. Rupp K, Bimach U, Lundstrom J, Van PN, and Söling HD. Effects of CaBP2, the rat analog of ERp72, and of CaBP1 on the refolding of denatured reduced proteins. Comparison with protein disulfide isomerase. *J Biol Chem* 269: 2501–2507, 1994.
23. Van PN, Rupp K, Lampen A, and Söhling HD. CaBP2 is a rat homolog of ERp72 with protein disulfide isomerase activity. *Eur J Biochem* 213: 789–795, 1993.
24. Weissman JS and Kim PS. Efficient catalysis of disulfide bond rearrangements by protein disulfide isomerase. *Nature* 365: 185–188, 1993.
25. Zapun A, Darby NJ, Tessier DC, Michalak M, Bergeron JJM, and Thomas DY. Enhanced catalysis of ribonuclease B folding by the interaction of calnexin or calreticulin with ERp57. *J Biol Chem* 273: 6009–6012, 1998.

Address reprint requests to:
Dr. L. W. Ruddock
Biocenter Oulu and Department of Biochemistry
PO Box 3000, FIN-90014
University of Oulu
Oulu, Finland

E-mail: lloyd.ruddock@oulu.fi

Received after revision November 26, 2004; accepted March 17, 2005.

This article has been cited by:

1. Andrew D. Johnston, Paul R. Ebert. 2012. The Redox System in C. elegans, a Phylogenetic Approach. *Journal of Toxicology* **2012**, 1-20. [CrossRef]
2. Guglielmo R. D. Villani, Armando Chierchia, Daniele Napoli, Paola Natale. 2011. Unfolded protein response is not activated in the mucopolysaccharidoses but protein disulfide isomerase 5 is deregulated. *Journal of Inherited Metabolic Disease* . [CrossRef]
3. Xin#Miao Fu, Bao Ting Zhu. 2010. Human pancreas-specific protein disulfide-isomerase (PDIp) can function as a chaperone independently of its enzymatic activity by forming stable complexes with denatured substrate proteins. *Biochemical Journal* **429**:1, 157-169. [CrossRef]
4. Feras Hatahet , Lloyd W. Ruddock . 2009. Protein Disulfide Isomerase: A Critical Evaluation of Its Function in Disulfide Bond Formation. *Antioxidants & Redox Signaling* **11**:11, 2807-2850. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
5. Kensuke Iwasaki, Shinya Kamauchi, Hiroyuki Wadahama, Masao Ishimoto, Teruo Kawada, Reiko Urade. 2009. Molecular cloning and characterization of soybean protein disulfide isomerase family proteins with nonclassic active center motifs. *FEBS Journal* **276**:15, 4130-4141. [CrossRef]
6. Francisco R.M. Laurindo , Denise C. Fernandes , Angélica M. Amanso , Lucia R. Lopes , Célio X.C. Santos . 2008. Novel Role of Protein Disulfide Isomerase in the Regulation of NADPH Oxidase Activity: Pathophysiological Implications in Vascular Diseases. *Antioxidants & Redox Signaling* **10**:6, 1101-1114. [Abstract] [Full Text PDF] [Full Text PDF with Links]
7. Teresa M. Buck, Christine M. Wright, Jeffrey L. Brodsky. 2007. The activities and function of molecular chaperones in the endoplasmic reticulum. *Seminars in Cell & Developmental Biology* **18**:6, 751-761. [CrossRef]
8. Anna-Riikka Karala , Panagiotis Psarrakos , Lloyd W. Ruddock , Peter Klappa . 2007. Protein Disulfide Isomerases from C. elegans are Equally Efficient at Thiol-Disulfide Exchange in Simple Peptide-Based Systems But Show Differences in Reactivity Towards Protein Substrates. *Antioxidants & Redox Signaling* **9**:11, 1815-1824. [Abstract] [Full Text PDF] [Full Text PDF with Links]
9. Catherine E Jessop, Seema Chakravarthi, Natalio Garbi, Günter J Hämmerling, Simon Lovell, Neil J Bulleid. 2007. ERp57 is essential for efficient folding of glycoproteins sharing common structural domains. *The EMBO Journal* **26**:1, 28-40. [CrossRef]
10. Dr. , Adam M. Benham , Roberto Sitia . 2006. The Diversity of Oxidative Protein Folding. *Antioxidants & Redox Signaling* **8**:3-4, 271-273. [Citation] [Full Text PDF] [Full Text PDF with Links]
11. P. Maattanen, G. Kozlov, K. Gehring, D.Y. Thomas. 2006. ERp57 and PDI: multifunctional protein disulfide isomerases with similar domain architectures but differing substrate-partner associations. *Biochemistry and Cell Biology* **84**:6, 881-889. [CrossRef]