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Conserved Residues Flanking the Thiol/Disulfide Centers of Protein Disulfide Isomerase Are Not Essential for Catalysis of Thiol/Disulfide Exchange†

Xijie Lu, Hiram F. Gilbert, and J. Wade Harper*
Verna and Marrs McLean Department of Biochemistry, Baylor College of Medicine, Houston, Texas 77030
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ABSTRACT: Protein disulfide isomerase (PDI) catalyzes the oxidative folding of proteins containing disulfide bonds by increasing the rate of disulfide bond rearrangements which normally occur during the folding process. The amino acid sequences of the N- and C-terminal redox active sites (PWCGHCK) in PDI are completely conserved from yeast to man and display considerable identity with the redox-active center of thioredoxin (EWCGPCK). Available data indicate that the two thiol/disulfide centers of PDI can function independently in the isomerase reaction and that the cysteine residues in each active site are essential for catalysis. To evaluate the role of residues flanking the active-site cysteines of PDI in function, a variety of mutations were introduced into the N-terminal active site of PDI within the context of both a functional C-terminal active site and an inactive C-terminal active site in which serine residues replaced C379 and C382. Replacement of non-cysteine residues (W34 to Ser, G36 to Ala, and K39 to Arg) resulted in only a modest reduction in catalytic activity in both the oxidative refolding of RNase A and the reduction of insulin (10–27%), independent of the status of the C-terminal active site. A somewhat larger effect was observed with the H37P mutation where ~80% of the activity attributable to the N-terminal domain (~40%) was lost. However, the H37P mutant N-terminal site expressed within the context of an inactive C-terminal domain exhibits 30% activity, approximately 70% of the activity of the N-terminal site alone. While this mutation mimics the active site of thioredoxin, this PDI mutant is several orders of magnitude more active than thioredoxin in catalysis of the oxidative folding of RNase. The strict sequence conservation of residues flanking the active-site cysteines is surprising in light of very modest changes in catalytic properties observed with mutation. This suggests the possibility that the strict conservation of this sequence through evolution reflects an additional function for PDI.

Protein disulfide isomerase (PDI)[1] is a M_r 56 300 protein found in the lumen of the endoplasmic reticulum (Lambert & Freedman, 1983). The finding that purified PDI can both catalyze reduction of disulfide bonds and enhance the rate of oxidative renaturation of disulfide bond containing proteins (Anfinsen & Scheraga, 1975; Freedman, 1984; Morin & Dixon, 1985) has led to the proposal that the enzyme functions to assist oxidative refolding of proteins during translation and/or translocation. Experiments of Bulleid and Freedman (1988) in which the oxidative folding capacity of a reconstituted in vitro translation/translocation system was shown to depend on the presence of PDI also support a role for PDI in oxidative folding in the endoplasmic reticulum. Somewhat paradoxically, PDI has also been implicated in a number of other cellular processes (Yamauchi et al., 1987; Cheng et al., 1987; Boado et al., 1988; Geetha-Habib et al., 1988; Obata et al., 1988; Wetterau et al., 1990). In particular, PDI has been identified as the β-subunit of prolyl hydroxylase, an enzyme involved in collagen biosynthesis (Pihlajaniemi et al., 1987).

The complete sequence of rat PDI cDNA (Edman et al., 1985) revealed several intriguing features of the protein. First, PDI contains two sets of internally homologous domains: residues 1–100 display 37% sequence identity with residues 340–440, and residues 150–245 show over 55% identity with residues 250–345. Second, a segment of sequence found in both the N-terminal and C-terminal domains (PWCGHCK) displays extensive sequence identity with the thioredoxin active center (EWCGPCK). This finding suggested that the catalytic centers of PDI are contained within this thiol/disulfide-containing segments. Sequencing PDI cDNAs from a number of different species ranging from *Saccharomyces cerevisiae* (Scherens et al., 1991) to human (Morris & Varandani, 1988) has shown that these thiol/disulfide centers are completely conserved through evolution. Notably, while yeast and human PDI display only 29% sequence identity overall, the region containing the thiol/disulfide center is absolutely conserved. Interestingly, this sequence motif has also been found in proteins such as form I phosphoinositide-specific phospholipase

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* Correspondence should be addressed to the author at the Department of Biochemistry, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030.

[1] Abbreviations: PDI, protein disulfide isomerase; GSH, glutathione; GSSG, glutathione disulfide; RNase A, ribonuclease A; DTT, dithiothreitol; cCMP, cytidine cyclic 3′,5′-monophosphate; CMP, cytidine monophosphate.

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C (Bennett et al., 1988), a developmentally regulated *Trypanosome* gene BS2 (Hsu et al., 1989), and another ER-resident protein, ERp72 (Mazzarella et al., 1990), where there is no clear role for a thiol/disulfide center in their function.

Mechanistic studies examining the oxidative folding of RNase A indicate that PDI functions by enhancing the rate of disulfide bond rearrangements which normally occur during the folding process (Lyles & Gilbert, 1991a). Catalysis depends critically on the redox state of the buffer employed (Lyles & Gilbert, 1991a), and PDI catalysis is optimal under conditions where the uncatalyzed reaction is most efficient. Thus, the thiol/disulfide centers of PDI apparently are capable of multiple rounds of reduction and oxidative during catalysis, although at high concentration PDI can function as both a stoichiometric oxidant and a rearrangement catalyst (Lyles & Gilbert, 1991b). Mutagenesis data indicate that the two thiol/disulfide centers of PDI can function independently in the isomerase reaction and the cysteine residues in each active site are essential for catalysis (Vuori et al., 1990).[2]

While there is no structural information yet available for PDI, structural data are available for both the reduced (Dyson et al., 1990) and oxidized (Katti et al., 1991) forms of *Escherichia coli* thioredoxin. The Cys-Gly-Pro-Cys sequence forms a loop structure which links the β_2-strand with the α_2-helix. In the oxidized protein, the disulfide bridge is partially buried between the edges of a β-sheet and the α_2-helix. In addition, the indole group of Trp-31 is located above the plane of the disulfide and may shield it from solvent (Eklund et al., 1991). While the Lys-36 side chain in thioredoxin is located near the disulfide where it could potentially stabilize the thiolate anion in the reduced protein, mutagenesis studies in which this residue was replaced by Glu indicate that this is not the case (Gleson, 1990).

Despite the thiol/disulfide sequence conservation, the biochemical properties of PDI and thioredoxin are quite distinct. The oxidation potential of PDI (Lyles & Gilbert, 1991a; Hawkins et all, 1991) is approximately 10^4 lower than thioredoxin (Lundstrom & Holmgren, 1990), indicating that the disulfides of PDI are vastly superior oxidants compared to thioredoxin. In addition, PDI is a substrate for thioredoxin reductase. However, PDI is a far better disulfide isomerase than is thioredoxin (Hawkins et al., 1991). The striking conservation of amino acid sequence in regions flanking the active-site cysteines in PDI implies that maintenance of a particular conformation of this presumed loop structure is crucial for PDI function. Krause et al. (1991) noted that the mutation P34H in *Escherichia coli* thioredoxin, which mimics the PDI active-site sequence, caused a 10-fold decrease in the oxidation potential and resulted in a 10-fold decrease in the second-order rate constant for insulin reduction, suggesting that differences in the sequences around the active site were indeed critical, at least for insulin reduction. In this study, we have used site-specific mutagenesis to explore the structural features required for oxidative refolding of ribonuclease A by PDI. Surprisingly, we find that mutation of several conserved residues in the N-terminal reaction center has little impact on the capacity of PDI to perform redox functions. This suggests that the high degree of conservation in this region may be a reflection of other functions of PDI.

Experimental Procedures

Materials. The T7 expression plasmid for rat PDI (pET-PDI.2) is from a previous study (Gilbert et al., 1991). Strains and reagents for mutagenesis were from Bio-Rad. Glutathione disulfide (GSSG), glutathione (GSH), glutathione reductase (yeast, type III), cytidine cyclic 2',3'-monophosphate (cCMP), bovine RNase A, and NADPH (type III A) were from Sigma Chemical Co. Dithiothreitol (DTT) was obtained from Boehringer-Mannheim.

Enzyme Assays. Catalysis by PDI mutants was examined in two types of assays: the GSH-dependent reduction of insulin and the oxidative refolding of RNase A (Lyles & Gilbert, 1991a,b).

(*A*) *Insulin Reduction.* The steady-state reduction of insulin by PDI was monitored by coupling the formation of GSSG to the oxidation of NADPH by glutathione reductase (Chandler & Varandani, 1975). Glutathione reductase (16 units) was preincubated with GSH (10 mM) and NADPH (0.2 mM) in reaction buffer (0.2 M potassium phosphate and 5 mM EDTA, pH 7.5) for 60 s to remove contaminating GSSG. Insulin (25 μM) was added, and the velocity of nonenzymatic insulin reduction was measured by following the decrease in NADPH absorbance at 340 nm for 2 min. The catalytic reaction was then initiated by the addition of PDI (220 nM). The velocity observed for the catalyzed reaction was corrected for the nonenzymatic background reaction. Measurements were performed in triplicate with an average standard deviation of 7 μmol min^{-1} (μmol of PDI)$^{-1}$.

(*B*) *RNase A Refolding.* Fully reduced RNase A was prepared in 6 M guanidine/0.1 M DTT as described (Lyles & Gilbert, 1991) and purified immediately before use by centrifugal gel filtration employing G-25 Sephadex equilibrated with 0.1% acetic acid. Oxidative renaturation of reduced RNase A (8 μM) by PDI was followed continuously at 296 nm by coupling the generation of active RNase to hydrolysis of cCMP. Typical conditions were 4.5 mM cCMP, 1 mM GSH, 0.2 mM GSSG, and 440 nM PDI or PDI mutant in Tris–acetate buffer (pH 8.0) at 25 °C. The concentration of active RNase A at any time could be calculated from the first derivative of the absorbance versus time trace after correction for the depletion of cCMP and for the competitive inhibition of RNase A by the hydrolysis product, CMP (Lyles & Gilbert, 1991). The initial velocity for active RNase formation was then obtained from the initial slope of this curve after a short lag (<5 min). The uncatalyzed reaction was monitored simultaneously in a parallel sample, and this rate was subtracted from the PDI-catalyzed reaction. Measurements were performed in triplicate with an average standard deviation of 0.04 μmol of RNase refolded min^{-1} (μmol of PDI)$^{-1}$.

Site-Directed Mutagenesis. An 1160 bp *Xba*I–*Eco*RI fragment of pET-PDI.2 (Gilbert et al., 1991), encoding residues 1–372 of PDI, was subcloned into M13mp18, and mutagenesis was performed using the method of Kunkel (1985). The following oligonucleotides were used: W34S, 5'-TATGCCCCATCTTGTGGCCAC; G36A, 5'-CCATGGTGTGCCCACTGCAAAG; H37P, 5'-TGGTGTGGCCCCTGCAAAGCA; K39R, 5'-GGCCACTGCCGAGCACTGGCC. Mutant *Xba*I–*Eco*RI fragments (identified by DNA sequencing) were then subcloned into either wild-type pETPDI.2 or a vector backbone containing a C379S:C382S double mutation (provided by M. Lyles). Plasmid DNA was sequenced to verify the desired mutation in the expression vector. The P377A mutant was generated using PCR. A mutagenic oligonucleotide containing the 5' *Eco*RI site, 5'-TGTTGAATTCTATGCTGCCTGGTGTGGTCACTGCAAGC, was employed as a forward primer, and a C-terminal primer, 5'-CTACAGTTCATCCTTCACGGCTTTC, span-

[2] M. Lyles and H. F. Gilbert, unpublished results.

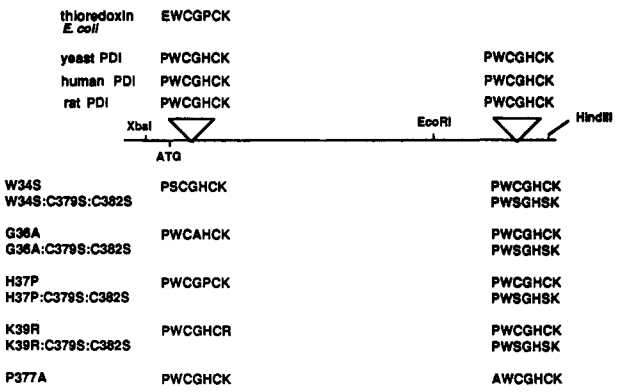

FIGURE 1: Schematic representation of the PDI coding sequence emphasizing the internally duplicated thiol/disulfide sequences and the mutant proteins examined. The sequences of the thiol/disulfide centers from the indicated PDI and thioredoxin proteins are indicated above. The restriction sites employed for cloning mutant DNA fragments are also shown (not to scale).

ning the PDI stop codon located 3′ of a unique *Hin*dIII site was used as reverse primer. After amplification, digestion with *Eco*RI and *Hin*dIII, and gel purification, the 292 bp fragment containing the P377A mutation was ligated into pET-PDI.2 previously digested with *Eco*RI and *Hin*dIII, and mutants were identified by DNA sequence analysis. The *Eco*RI and *Hin*dIII sites located in the pBR322 backbone of the parent pET8c plasmid were deleted by reclosing plasmid DNA which had been digested with *Eco*RI and *Hin*dIII and filled in with DNA polymerase.

Expression and Purification of PDI Mutants. Mutant and wild-type PDI proteins were expressed in *E. coli* strain BL21(DE3) (Studier et al., 1990) essentially as described (Gilbert et al., 1991). Briefly, induced cells were harvested and lysed, and the crude homogenate was dialzyed against 25 mM potassium phosphate, pH 6.3. PDI was purified by successive chromatography on DEAE-Sephacel and zinc chelate resin (Pharmacia/LKB). For wild-type PDI and the H37P mutants, HPLC ion-exchange chromatography (Gilbert et al., 1991) was employed as a final purification step. Peak PDI fractions were concentrated to 1–1.5 mg/mL, dialyzed, and stored in aliquots at −20 °C. The protein concentration was determined by the absorbance at 280 nm, and purity was verified on SDS–PAGE stained by Coumassie blue.

Results

Rationale for Mutagenesis. Figure 1 shows the rationale for creating mutations in the N-terminal active site and lists the mutant proteins generated. We have focused on conserved amino acids directly flanking the catalytic cysteine residues as these were suspected to be involved in controlling the structure and redox properties of the Cys^{35}–Cys^{38} thiol/disulfide. Since the N-terminal and C-terminal active sites can function independently, N-terminal mutations were also examined within the context of a nonfunctional C-terminal reaction center in which C379 and C382 were replaced by serine. Mutations were generated using a 1160 bp *Xba*I–*Eco*RI cassette containing the N-terminal reaction center (Figure 1) which was subsequently placed in either a wild-type PDI backbone or a PDI backbone containing a C379S:C382S double mutation. Mutant PDI proteins were purified from *E. coli* with yields of 10–20 mg/L. Proteins were 95% homogeneous as assessed by SDS–PAGE.

Ribonuclease Refolding by the PDI Mutants. Oxidative refolding of reduced RNase A by wild-type and mutant PDI proteins was measured at RNase concentrations near K_m (Table I). A representative activity trace for wild-type PDI, as well as that for the C379S:C382S, W34S, and W34S:C379S:C382S mutants, is shown in Figure 2. With the W34S, G36A, and K39R mutants, the initial velocity of RNase refolding was reduced by 12–27% while with the H37P mutant activity was reduced by 50%. In general, the relative activity observed in mutants containing an inactive C-terminal domain paralleled that found when the C-terminal domain was left intact (Table I). However, the H37P:C379S:C382S triple mutant displayed 30% activity in this assay, somewhat higher than that expected on the basis of results obtained with the H37P mutant containing a functional C-terminal active site. In all cases, RNase refolding proceeded to completion within the appropriate time period, indicating that the mutant proteins were not defective in redox cycling and were capable of multiple turnover. The K_m and k_{cat} values for RNase refolding by selected PDI mutants are given in Table II. There is no significant difference in apparent K_m values, suggesting that alterations in activity reflect rates of disulfide bond interchange.

Table I: Kinetic Parameters of Wild-Type and Mutant PDI[a]

	RNase A refolding		insulin reduction	
enzyme	sp act. [μmol min^{-1} (μmol of PDI)$^{-1}$]	% act.	sp act. [μmol min^{-1} (μmol of PDI)$^{-1}$]	% act.
wild type	1.28	100	172	100
C379S:C382S	0.52	40	70	41
G36A	0.94	73	138	80
G36A:C379S:C382S	0.51	40	79	46
H37P	0.67	52	156	91
H37P:C379S:C382S	0.41	32	121	70
K39R	1.13	88	149	87
K39R:C379S:C382S	0.39	30	41	24
W34S	0.96	75	142	83
W34S:C379S:C382S	0.37	29	35	20
P377A	1.38	108	138	80

[a] RNase refolding assays were carried out at pH 8.0, 25 °C, using 1 mM GSH, 0.2 mM GSSG, and 8 μM RNase A as described under Experimental Procedures. Specific activity refers to the quantity of RNase A refolded or insulin reduced per minute per micromole of PDI in a 400-μL reaction volume. The concentrations of PDI used were 440 or 220 nM for RNase refolding and insulin reduction, respectively.

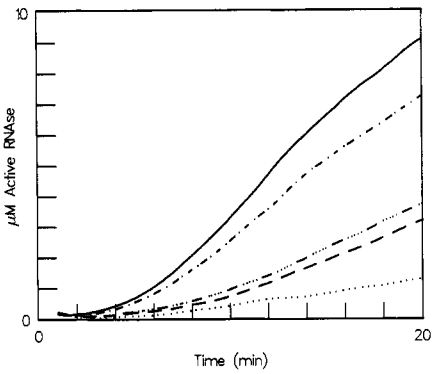

FIGURE 2: Catalysis of RNase refolding by PDI and mutant PDI proteins. Assays were performed at pH 8.0, 25 °C, using 8.9 μM reduced RNase A and 440 nM PDI. (—) Wild-type PDI; (–·–) W34S PDI; (– –) W34S:C379S:C382S; (–···–) C379S:C382S; (···) uncatalyzed reaction in the absence of PDI.

Previous data indicate that thioredoxin is a very poor catalyst of the thiol/disulfide rearrangements required to convert scrambled RNase to the native structure (Hawkins et al., 1991b). In order to establish the activity of thioredoxin for

Table II: Kinetic Constants for RNase A Refolding by Selected PDI Mutants[a]

enzyme	K_m (μM)	k_{cat} [μmol min^{-1} (μmol of PDI)$^{-1}$]
wild-type	4.9 (±1.6)	1.8 (±0.2)
W34S	5.4 (±0.8)	1.7 (±0.1)
W34S:C379S:C382S	7.9 (±1.9)	0.6 (±0.1)
H37P	5.1 (±0.9)	1.0 (±0.1)
H37P:C379S:C382S	6.4 (±1.3)	0.6 (±0.1)

[a] Assays at each RNase A concentration (2, 4, 8, 10, 12, 16, and 24 μM) were performed in duplicate using 440 nM PDI under conditions described under Experimental Procedures. The data were nonlinear least-squares fit to an equation describing a rectangular hyperbola. The standard deviation is given in parentheses.

catalysis of both disulfide bond formation and rearrangement and for the purpose of comparison, RNase oxidative folding assays were performed using thioredoxin at 5 μM, 10-fold higher than the concentration of PDI employed. The velocity of RNase oxidative refolding was indistinguishable from the nonenzymatic rate measured in the absence of PDI. Given the limits of detection, thioredoxin activity is >500-fold lower than that of PDI in this assay.

Insulin Reduction by PDI Mutants. In addition to oxidative refolding, PDI can catalyze the reduction of disulfide bonds in proteins such as insulin. Presumably, this reflects one of the steps normally involved in the oxidative folding process where "non-native" bonds are reduced by PDI prior to rearrangement. As with RNase oxidative folding, removal of the C-terminal reaction center reduces activity to 40% (Table I), indicating that the two active sites can also function independently in this reaction. In general, mutations of residues flanking the N-terminal disulfide center reduce activity to an extent similar to that found in the RNase refolding assay, ~20% for single mutants. An exception to this is again the H37P and H37P:C379S:C382S mutants which display 90% and 70% activity, respectively.

Mutation of Pro-377 in the C-Terminal Reaction Center. While the collection of mutations described above does not include mutations at the conserved residue Pro-33, we have examined the effects of mutating the corresponding residue in the C-terminal domain, Pro-377 (Table I). This mutation results in an insignificant (8%) increase in isomerase activity and a slight (20%) reduction in disulfide bond reduction activity.

Discussion

Strict conservation of amino acid sequences through evolution is generally thought to reflect an essential role directly related either to biological function or to the maintenance of secondary or tertiary structure within the protein. The complete conservation of amino acids within the catalytic dithiol/disulfide center of PDI from species as diverse as yeast and human would argue for an essential conserved structure and function for these regions of the molecule. To examine the specific roles played by residues flanking the essential cysteine residues of PDI in its oxidative refolding function, mutations were introduced at each position and the effects on catalysis assessed. Trp-34 is adjacent to the disulfide centers of all PDI's. In the structures of thioredoxin, the analogous Trp residue partially covers the dithiol/disulfide center, reducing solvent exposure in both the reduced and oxidized molecules (Eklund et al., 1991). It was, therefore, surprising that replacement of Trp-34 by serine reduced the catalytic activity of this active site only marginally in both RNase refolding and insulin reduction assays. This indicates that if the PDI structure in this region is similar to that of thioredoxin, the indole ring plays no specific role in the oxidative refolding process and access to the active site does not significantly impede the normal reaction. With thioredoxin, replacement of Trp-31 by alanine diminishes insulin reduction activity by 4-fold (Krause & Holmgren, 1991) and increases the K_m in the thioredoxin reductase reaction by 2-fold. In contrast, functions of thioredoxin involving protein–protein interactions such as the stimulation of T7 DNA polymerase activity in vitro and the propagation of M13 in vivo are diminished by at least 100-fold (Krause & Holmgren, 1991). Our results with W34S PDI and analogies to thioredoxin mutations at this position would suggest a nonessential role for W34 in the redox activity of the thiol/disulfide center.

On the basis of analogy with the thioredoxin structure, Gly-36 in PDI is expected to be located on the surface of the protein and may provide the flexibility necessary for dithiol/disulfide interconversion during catalysis. Introduction of bulkier amino acid side chains at this position would not be expected to substantially alter the loop structure since the side chain would project into solvent, but could potentially block access to the thiol/disulfide active site for certain substrates. In both the insulin reduction and RNase refolding assays, the mutation Gly to Ala lowers activity only by ~20%. Attempts to express the G36V mutant have been unsuccessful, so we have not been able to assess how more dramatic mutations may affect activity. However, it is clear that Gly-36 is not essential for oxidative protein folding. Interestingly, the corresponding residue in thioredoxin appears to be important for interaction with thioredoxin reductase, but not for maintenance of redox potential (Eklund et al., 1991).

The only difference between PDI and thioredoxin in the active-site dithiol region is the replacement of His-37 between the two active-site cysteines by Pro. Thus, it was of interest to determine if this replacement is responsible for the dramatically increased isomerase activity of PDI. When His-37 was replaced by Pro, isomerase activity in the otherwise unmodified PDI was reduced by 50%. This corresponds to a loss of 80% of the activity attributable to the N-terminal domain. However, the PDI triple mutant in which the H37P mutation is expressed in the context of an inactive C-terminal domain displays 30% activity, demonstrating that this residue is not essential for oxidative protein folding by PDI. Assays carried out using high concentrations of thioredoxin under otherwise identical conditions demonstrated that PDI is at least 500-fold more active than thioredoxin in catalysis of renaturation of fully reduced RNase. Hawkins et al. (1991b) found that thioredoxin was only 40-fold less active than PDI in catalysis of the thiol-dependent rearrangement of the disulfide bonds in scrambled RNase.

The disulfide bond in thioredoxin has a reduction potential of −270 mV and is not readily reduced by GSH (E_0 ~−250 mV), contributing to thioredoxin's low activity in the oxidation and rearrangement of RNase in a GSH/GSSG redox buffer (Krause et al., 1991). When Pro-34 in thioredoxin is replaced by His to mimic PDI, the reduction potential is increased to −235 mV (Krause et al., 1991), making the mutant thioredoxin a 15-fold better oxidizing agent than the wild type. However, this simple change in sequence does not account for the 10^4-fold difference in redox potential of PDI (−0.12 V) (Lyles & Gilbert, 1991a; Hawkins et al., 1991a) compared to thioredoxin. The finding that the H37P:C379S:C382S triple mutant retains substantial activity in both the oxidative folding of RNase and the reduction of insulin indicates that the oxidation potential of the N-terminal disulfide is not greatly affected as it is readily reduced by GSH during the catalytic

cycle. This implies that some other structural component in PDI is critical for maintaining the unusually low oxidation potential of the active-site dithiols.

While it was initially thought that Lys-39 in thioredoxin functions to stabilize the thiolate anion, mutation to Glu had little effect on activity as a reducing agent at pH 7 (Gleason et al., 1990) but increased the K_m for thioredoxin reductase by 3.5-fold. Given the obvious differences in function, it is possible that this residue in PDI performs a specific role in oxidative refolding. To test this, Lys-39 was replaced by Arg. Any *specific* function requiring the ϵ-amino group of Lys would not be expected to be fulfilled by the guanidino group of Arg. However, alterations in activity were only minor, suggesting that the identity of this residue is not crucial to catalysis. Of course, it is possible that lysine is still involved in stabilization of a negative charge and arginine can partially fulfill this role.

In all PDI's sequenced to date, a proline residue precedes the Trp residue at the thiol/disulfide center. This residue was replaced by Ala in the C-terminal active site of PDI. As with the other mutants, activity was affected only modestly. Thus, it appears that this residue is also not required to maintain the structure of the active site, at least in terms of oxidative protein folding.

The active site of PDI can accommodate a surprising variety of changes in primary structure with only minor alterations in its ability to catalyze oxidative and reductive reactions. With the exception of the active-site cysteines, mutation of highly conserved amino acid residues near the thiol/disulfide center has only minor effects on the catalytic activity of PDI. This being the case, it is intriguing that the sequence of the active site has been completely conserved from yeast to man while large differences exist in other parts of the molecule for lower and higher eukaryotes. PDI has been implicated in a variety of other processes: it is a component of the glycosylation apparatus of the ER-golgi (Geetha Habib et al., 1988), it can bind thyroid hormones (Yamauchi et al., 1987; Cheng et al., 1987; Boado et al., 1988; Obata et al., 1988), and it is a subunit of multifunctional enzyme systems such as microsomal triglyceride transferase (Wetterau et al., 1990) and the β-subunit of prolyl hydroxylase (Kivirikko et al., 1989). This being the case, it is plausible that the maintenance of sequence identity in this critical region of PDI reflects alternative functions of PDI not related to redox reactions. One possibility is that this exposed loop constitutes a site for protein–protein interaction important in the formation of multimeric complexes. One potential consequence of the use of such a motif for interactions is that association could be regulated by the redox state of the environment (Gilbert, 1990). The availability of these and other PDI mutants should allow these and other possibilities to be examined experimentally.

Acknowledgments

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Registry No. PDI, 37318-49-3; Cys, 52-90-4; RNase, 9001-99-4.

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^{31}P NMR Spectroscopic Studies on Purified, Native and Cloned, Expressed Forms of NADPH–Cytochrome P450 Reductase†

Ramani Narayanasami,‡ James D. Otvos,§ Charles B. Kasper,‖ Anna Shen,‖ Jayanthi Rajagopalan,⊥ Timothy J. McCabe,‡ Janice R. Okita,# Donald J. Hanahan,‡ and Bettie Sue Siler Masters*,‡

Department of Biochemistry, The University of Texas Health Science Center—San Antonio, San Antonio, Texas 78284-7760, Department of Biochemistry, North Carolina State University, Raleigh, North Carolina 27695, McArdle Laboratory for Cancer Research, University of Wisconsin—Madison, Madison, Wisconsin 53706, Department of Biochemistry, School of Dental Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104, and College of Pharmacy, Washington State University, Pullman, Washington 99164-6510

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ABSTRACT: ^{31}P NMR spectroscopy has been utilized in conjunction with site-directed mutagenesis and phospholipid analysis to determine structural aspects of the prosthetic flavins, FAD and FMN, of NADPH–cytochrome P450 reductase. Comparisons are made among detergent-solubilized and protease (steapsin)-solubilized preparations of porcine liver reductases, showing unequivocally that the ^{31}P NMR signals at ~0.0 ppm in the detergent-solubilized, hydrophobic form are attributable to phospholipids. By extraction and TLC analysis, the phospholipid contents of detergent-solubilized rat liver reductase, both tissue-purified and *Escherichia coli*-expressed, have been determined to reflect the membranes from which the enzyme was extracted. In addition, the cloned, wild-type NADPH–cytochrome P450 reductase exhibits an additional pair of signals downfield of the normal FAD pyrophosphate resonances reported by Otvos et al. [(1986) *Biochemistry 25*, 7220–7228], but these signals are not observed with tissue-purified or mutant enzyme preparations. The $Tyr_{140} \rightarrow Asp_{140}$ mutant, which exhibits only 20% of wild-type activity, displays no gross changes in ^{31}P NMR spectra. However, the $Tyr_{178} \rightarrow Asp_{178}$ mutant, which has no catalytic activity and does not bind FMN, exhibits no FMN ^{31}P NMR signal and a normal, but low intensity, pair of signals for FAD. The latter experiments, taking advantage of mutations in residues putatively on either side of the FMN isoalloxazine ring, suggest subtle to severe changes in the binding of the flavin prosthetic groups and, perhaps, cooperative interactions of flavin binding to NADPH–cytochrome P450 reductase.

Microsomal NADPH–cytochrome P450 reductase (EC 1.6.2.4) is one of only two mammalian enzymes known to contain both FAD and FMN (Iyanagi & Mason, 1973; Masters et al., 1975; Dignam & Strobel, 1975), the other enzyme being nitric oxide synthase from rat cerebellum (Bredt & Snyder, 1990; Bredt et al., 1991). NADPH–cytochrome P450 reductase (hereafter referred to as reductase) is a component of the cytochrome P450 monooxygenase system which is responsible for the oxidative metabolism of various xenobiotics, as well as many endogenous compounds (Conney, 1967; Gillette et al., 1972; Masters & Okita, 1980). The reductase is also capable of transferring reducing equivalents to several other heme proteins, such as heme oxygenase (Schacter et al., 1972; Yoshida, et al., 1980), cytochrome *c* (Horecker, 1950), and cytochrome b_5 (Enoch & Strittmatter, 1979). The major function of the reductase is to transfer electrons provided by NADPH to the heme iron of the cytochrome P450 molecule. The electron-transfer sequence is known to occur in the following manner (Vermilion et al., 1981):

$$\text{NADPH} \rightarrow [\text{FAD} \rightarrow \text{FMN}] \rightarrow \text{cytochrome P450}$$

Reductase has been purified from several species with reported molecular masses varying between 76 and 81 kDa (Masters, 1980). The amino acid sequence has been determined for several of the reductases, such as rat (Porter & Kasper, 1985), rabbit (Katagiri et al., 1986), pig (Haniu et al., 1986; Vogel & Lumper, 1986), yeast (Yabusaki et al., 1988; Sutter et al., 1990), trout (Urenjak et al., 1987), and human (Yamano et al., 1989; Haniu et al., 1989). Very high sequence homology exists among the various reductases, consistent with the im-

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* Author to whom correspondence should be addressed.
‡ The University of Texas Health Science Center—San Antonio.
§ North Carolina State University.
‖ University of Wisconsin—Madison.
⊥ University of Pennsylvania.
Washington State University.

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