Molecular Characterization of the Principal Substrate Binding Site of the Ubiquitous Folding Catalyst Protein Disulfide Isomerase*

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Disulfide bond formation in the endoplasmic reticulum of eukaryotes is catalyzed by the ubiquitously expressed enzyme protein disulfide isomerase (PDI). The effectiveness of PDI as a catalyst of native disulfide bond formation in folding polypeptides depends on the ability to catalyze disulfide-dithiol exchange, to bind non-native proteins, and to trigger conformational changes in the bound substrate, allowing access to buried cysteine residues. It is known that the b’ domain of PDI provides the principal peptide binding site of PDI and that this domain is critical for catalysis of isomerization but not oxidation reactions in protein substrates. Here we use homology modeling to define more precisely the boundaries of the b’ domain and show the existence of an intradomain linker between the b’ and a’ domains. We have expressed the recombinant b’ domain thus defined; the stability and conformational properties of the recombinant product confirm the validity of the domain boundaries. We have modeled the tertiary structure of the b’ domain and identified the primary substrate binding site within it. Mutations within this site, expressed both in the isolated domain and in full-length PDI, greatly reduce the binding affinity for small peptide substrates, with the greatest effect being I272W, a mutation that appears to have no structural effect.

Native disulfide bond formation in the endoplasmic reticulum is a complex process that is rate-limiting in the biogenesis of many outer membrane and secreted proteins. Native disulfide bond formation can occur via multiple parallel pathways, and there is evidence that a large number of different gene families and redox carriers may play a role in the supply of redox equivalents for protein disulfide bond formation. What is clear is that the rate-limiting step for native disulfide bond formation in proteins that contain multiple disulfides is late-stage isomerization reactions, where disulfide bond formation is linked to conformational changes in protein substrates with substantial regular secondary structure. These steps are thought to be catalyzed only by proteins belonging to the protein disulfide isomerase (PDI) family.

PDI1 was the first catalyst of protein folding identified over 40 years ago (1), but despite probably being the most widely studied protein folding catalyst, significant details of the mechanisms of action of this critical enzyme are still unclear. In all eukaryotes, there exists a species-dependent PDI family of enzymes; for example, in humans (2), ERp72, ERp57, P5, PDIp, PDIr, ERp44 (3), ERp28/29 (4), ERdj5 (5), and ERp18 (6) have been reported to date. Functional characterization and differentiation between these family members is far from complete. PDI is a multifunctional, multidomain enzyme. The domain structure of PDI has been determined by theoretical (7) and experimental (8–11) procedures and comprises two catalytic domains, a and a’, separated by two homologous non-catalytic domains, b and b’, plus a C-terminal region designated as c. In addition, it has been proposed (Ref. 11 and references therein), but not substantiated, that there is a short linker region between the b’ and a’ domains.

In vitro studies indicate that PDI catalyzes all of the steps in native disulfide bond formation but that catalysis of disulfide bond isomerization is most significant over the non-catalyzed rate in a glutathione buffer that mimics the redox potential of the ER. The greatest enhancement of rate is for late-stage isomerizations, i.e. the rate-limiting steps for native disulfide bond formation. PDI is remarkable in that, to our knowledge, it appears to be able to catalyze all of the steps in native disulfide bond formation for all substrate proteins reported. It is still unclear how PDI recognizes all of these different folding states, from essentially unfolded through to substrates with quasi-native conformations but lacking specific disulfide bonds, and yet does not appear to interact with correctly folded and disulfide-bonded substrate proteins. Some details have been elucidated of the roles of individual domains of PDI in the different aspects of the overall activity of PDI. It has been reported that oxidation reactions require only a single catalytic domain, simple isomerization reactions require a linear combination of a catalytic domain and the b’ domain, whereas all of PDI excluding the c region is required to catalyze isomerization reactions where disulfide bond formation is linked to conformational changes in protein substrates with substantial regular secondary structure (12). The requirement for the non-catalytic b’ domain was subsequently shown to arise due to the fact that the b’ domain contains the principal peptide or non-native protein binding site (13). For small peptide substrates, the b’ domain alone is sufficient for binding, whereas for larger pro-

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The abbreviations used are: PDI, protein disulfide isomerase; scRNase, scrambled RNase.

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tein substrates, the b' domain is essential, but other domains contribute to substrate binding. No specificity has yet been reported for substrate binding by PDI, nor has the binding site been located within the b' domain. However, for the homologue PDip, the substrate specificity is now well defined (14, 15), a fact that will greatly assist in the localization and definition of the binding site of the PDI family.

Here we confirm the existence of the putative linker region FIG. 1. Multiple sequence alignment for homology modeling including secondary structure assignments and predictions. To emphasize the structural alignment, each domain is split between panel A (N-terminal half of the domain) and panel B (C-terminal half of the domain). X-ray, secondary structure assignment as known from x-ray structure; NMR, secondary structure assignment as known from NMR structure; Pred.Prot., PredictProtein secondary structure prediction; PREDATOR 1, PREDATOR secondary structure prediction using MaxHom derived sequence data; PREDATOR 2, PREDATOR secondary structure prediction using specialized sequence data of b and b'/H11541 domain homologues. Panel A, Human TRX, human thioredoxin-(1–59); E. coli TRX, E. coli thioredoxin-(2–60); PDI a, human PDI a domain (7–67); PDI b, human PDI b domain (119–171); PDI b', human PDI b' domain (218–276); PDI a', human PDI a' domain (352–410). Panel B, human TRX, human thioredoxin-(60–105); E. coli TRX, E. coli thioredoxin-(61–108); PDI a, human PDI a domain-(68–118); PDI b, human PDI b domain (172–217); PDI b', human PDI b' domain (277–351); PDI a', human PDI a' domain (411–462).
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between the b' and a' domains and report the identification and characterization of the binding site within the b' domain. Mutations within this site greatly reduce the binding affinity of PDI for small peptide substrates.

EXPERIMENTAL PROCEDURES

Secondary Structure Prediction—The sequence of mature human PDI was submitted to the PredictProtein server (16). PredictProtein uses the subprogram MaxHom (17) to perform a data base search for homologous sequences and to align those sequences into a multiple alignment file. The MaxHom-derived multiple alignment file contained 53 sequences, and this was used as an input to PredictProtein. The sequence data from the MaxHom-derived multiple alignment file was also used as an input for the secondary structure prediction program PREDATOR (18). The secondary structure assignments for the b and b' domains of human PDI were also predicted with PREDATOR using a different input file that was specially created using the sequences of just the b and b' domains as input for the generation of a multiple alignment file, which contained 35 sequence fragments.

Multiple Sequence Alignment and Homology Modeling of the b' Domain—The published alignments of Escherichia coli thioredoxin and the a domain of human PDI (8), of human thioredoxin and the a' domain of PDI (9), and of the a and a' domains of PDI (19) were combined to produce a multiple sequence alignment of E. coli human thioredoxin and the a, b, and a' domains of human PDI. In cases of conflicting data from different alignments, priority was given to the alignments from Creighton and co-workers (8, 9), which are based on the structural data for PDI. The b' domain of human PDI was added to this multiple sequence alignment after an initial alignment of the b and b' domains using a hierarchical clustering algorithm, comparison table: BLOSUM62, gap opening penalty: 8, gap extension penalty: 1 (20). The resulting multiple sequence alignment was further refined considering experimentally derived and predicted secondary structure assignments. Homology modeling of the b' domain was performed using version 4 of the homology modeling program MODELLER (21). From 10 constructed models, the one with the lowest value of the objective function was selected as the representative model.

Generation of Expression Vectors—A gene insert for an expression vector for the isolated b' domain (Pro-218–Gly-332) of PDI was generated by PCR from an existing vector encoding PDI (11), using primers that included an in-frame NdeI site 5' to the first codon of the gene and a BamHI site after a TAA stop codon at the 3'-end. The insert was cloned into pLWEP91, a modified version of pET23b (Novagen) that encodes for an N-terminal His tag in-frame with the cloned gene. The resulting gene products included the sequence MHHHHHHM- prior to and an in-frame NdeI site 5' to the TAA stop codon at the 3'-end. The resulting multiple sequence alignment was further refined considering experimentally derived and predicted secondary structure assignments. Homology modeling of the b' domain was performed using version 4 of the homology modeling program MODELLER (21). From 10 constructed models, the one with the lowest value of the objective function was selected as the representative model.

RESULTS

Definition of the Boundaries of the b' (Primary Substrate Binding) Domain of PDI—Previous theoretical (7) and experimental (for example, Ref. 11) studies have defined the domain boundaries for the a, b, and a' domains of the human PDIs. The domain boundaries of the b' domain are less clear. The experimental work to date have defined the boundaries based on sequence alignments of the homologous domains. Based in part on partial proteolysis of bovine PDI (10), there has been a suggestion that a structurally linked region exists between the b' and a' domains (2), but there has been no reported experimental confirmation of this.

To define the domain boundaries of the b' domain of human PDI, a sequence alignment of the homologous b and b' domains of human PDI was performed. This alignment was then refined using the results from secondary structure prediction using PredictProtein (16) and PREDATOR (18) to move gaps into loop regions and to align corresponding helix and strand regions without misaligning regions of good sequence similarity. The resulting alignment, together with observed and predicted secondary structure assignments, is shown in Fig. 1. It is clear from this alignment that the b' domain defined previously (11) includes 19 residues at the C terminus that form an unaligned extension of the aligned thioredoxin-like section. This apparent linker region between the b' and a' domains we designate as x.

To confirm the proposed domain boundaries, a PDI b' construct (residues Pro-218–Gly-332 of mature human PDI) was made. This construct and the original construct (residues Lys-213–Pro-351 of mature human PDI, now termed b'x), with an N-terminal hexa-His tag to aid purification, could be expressed soluble in the cytoplasm of E. coli and purified to apparent homogeneity by a combination of immobilized metal affinity chromatography and anion exchange chromatography (data not shown). The ability to generate significant amounts of...
soluble protein in *E. coli* from both of these domain constructs strongly indicates that both define compact stable structures; thus, the domain boundaries of b' defined here are correct, and region x does not form part of this domain.

**Characterization of the b' Domain of PDI**—To confirm that the new b' domain construct is structured and retains the biological activity associated with it in the full-length protein, a variety of biophysical analyses were undertaken. Previously, the b' domain of PDI was reported to include the primary substrate binding site of PDI, binding to small peptides and the region x of PDI—thioredoxin-like domain of PDI. Since the construct contains one tryptophan, and fluorescence spectra of native (0.2 M sodium phosphate, pH 7.0; solid line) and denatured (0.2 M sodium phosphate, 4 M guanidinium chloride, pH 7.0; dotted line) human PDI b' are arbitrary, the representative fluorescence spectra of native (0.2 M sodium phosphate, pH 7.0; solid line) and denatured (0.2 M sodium phosphate, 6 M guanidinium chloride, pH 7.0; dotted line) human PDI b'x. D) guanidinium chloride denaturation curves for human PDI b' (C), left axis, fluorescence intensity is shown) and b'x (○, right axis, the ratio of the average fluorescence intensity 2 nm to either side of the λ<sub>max</sub> for native protein to the average fluorescence intensity over the range 320–400 nm is shown). Each data point represents an average of at least two data points of four scans each.

The b' construct under non-denaturing conditions showed a λ<sub>max</sub> of 304 nm, consistent with the fact that the protein contains 2 tyrosine residues and no tryptophans (Fig. 3B). Upon addition of 4 M guanidinium chloride, a significant decrease in fluorescence intensity was observed with no shift in λ<sub>max</sub>. The b'x construct contains one tryptophan, and fluorescence spectra of b'x, under non-denaturing conditions, gave a λ<sub>max</sub> of 338 nm (Fig. 3C), indicative of a tryptophan residue being in a hydrophobic environment. Upon addition of 6 M guanidinium chloride, the fluorescence spectra of b'x had a λ<sub>max</sub> of 356 nm, indicative of a denatured protein. Fluorescence-based denaturation curves for b' and b'x showed a single phase transition from the native to denatured state. From the data presented in Fig. 3D, the midpoints for denaturation were 1.65 and 2.32 M for b' and b'x, respectively.

The b' domain of PDI contains two thiol residues (Cys-295 and Cys-326) that may be involved in modulating the binding affinity depending on redox potential (see Ref. 22, but also see Ref. 23). An Ellmans assay on purified b' and b'x under native and denaturing conditions indicated that both cysteines exist as free thiols and that both are relatively inaccessible to low molecular weight thiol-reactive reagents in the native state.
This result was confirmed by examining the reaction of b′ with iodoacetate in the presence and absence of 4 M guanidinium chloride (data not shown). These data indicate that neither of the cysteine residues are likely to participate in substrate binding, nor are they likely to exchange between the dithiol and disulfide states upon the modulation of the redox potential.

Identifying the Primary Substrate Binding Site within the b′ Domain of PDI—Previous data (13) demonstrate that the b′ domain of PDI contains the primary substrate binding site of the enzyme, but there are no published data on the localization of the substrate binding site within this domain. Work in this area is hampered by the lack of a structure for any PDI or for any isolated PDI b′ domain. The structures of the a and b domains have been solved by NMR (8, 9, 24), and both show a thioredoxin fold. Since b′ is homologous to b, we sought to construct a model of b′ based on the known structure of b and to use this to identify the primary substrate binding site.

Although the NMR structure of the b domain of human PDI has been determined (9, 24), its coordinates had not been released at the time this modeling work was performed. Therefore, only the structures of E. coli thioredoxin (25), human thioredoxin (26), and the a domain of human PDI (8) could be used as template structures for homology modeling. These structures, combined with the sequence alignments for the individual domains of PDI, were used to build a model of the b′ domain of PDI using the homology modeling program MODELLER (21). Only the thioredoxin-like section of the b′-(218–334) domain was homology-modeled.

Unsurprisingly, the final modeled structure for the b′ domain was very similar to that of the b domain with an overall thioredoxin-like fold. The two cysteine residues that b′ contains are not surface-exposed in the model. The two sulfur atoms are 6.4 Å apart, and thus, they are unlikely to form a disulfide; this is consistent with the experimental data that showed a lack of reactivity of the cysteine residues to iodoacetamide in the native state.

Although the substrate specificity of substrate binding by PDI is not known, there are strong indications (13, 14) that the recognition motifs are primarily hydrophobic in nature, and substrate binding can be inhibited by small molecular weight molecules such as 2-propylphenol. Furthermore, the binding specificity of the homologous protein PDIp has been reported (14, 15), and since it binds single amino acid methyl esters of tyrosine and tryptophan, the substrate binding pocket must be relatively small.

Examination of the model of the b′ domain of PDI revealed...
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TABLE I

Effects of mutations on Δsomatostatin binding

| Plasmid name | Protein produced | Effect on Δsomatostatin binding |
|--------------|------------------|-------------------------------|
| pLWRP64      | PDI mature (Asp-1-Leu-491), H6 | + + + |
| pOLR69       | PDI mature V220I H6             | + + + |
| pOLR5        | PDI mature L242A               | + + + |
| pOLR70       | PDI mature L242T H6            | + |
| pOLR26       | PDI mature L242W               | + + |
| pOLR10       | PDI mature L244A               | + |
| pOLR9        | PDI mature L244W               | + |
| pOLR32       | PDI mature P245A               | + |
| pOLR38       | PDI mature K246A               | + |
| pOLR31       | PDI mature S249A               | + + + |
| pOLR24       | PDI mature S249K               | + + + |
| pOLR6        | PDI mature L255A               | + + + |
| pOLR39       | PDI mature L255R               | + + |
| pOLR17       | PDI mature S256D               | + + |
| pOLR15       | PDI mature F258A               | + |
| pOLR115      | PDI mature F258W               | + |
| pOLR1        | PDI mature D259A               | ++ + |
| pOLR2        | PDI mature K259A               | ++ + |
| pOLR3        | PDI mature I272A               | ++ + |
| pOLR4        | PDI mature I272W               | ++ + |
| pKEHS31      | PDI mature I272W, H6           | ++ + |
| pOLR59       | PDI mature I272N, H6           | ++ + |
| pOLR64       | PDI mature I272Q, H6           | ++ + |
| pOLR65       | PDI mature I272L, H6           | ++ + |
| pLWRP99      | PDI b′ domain (Pro-218–Gly-332) H6 | + + + |
| pAP16        | PDI b′ domain L244A, H6        | + + + |
| pAP8         | PDI b′ domain L244W, H6        | + + + |
| pAP19        | PDI b′ domain S249A, H6        | + + + |
| pAP4         | PDI b′ domain S249K, H6        | + + + |
| pAP6         | PDI b′ domain S256D, H6        | + + + |
| pAP7         | PDI b′ domain F258W, H6        | + + + |
| pAP13        | PDI b′ domain K259A, H6        | + + + |
| pAP9         | PDI b′ domain I272A, H6        | + + + |
| pAP15        | PDI b′ domain I272W, H6        | + + + |
| pLWRP64      | PDI b′x domain (Lys-213–Pro-351), H6 | + + + |

no large distinct hydrophobic pockets. However, a small hydrophobic pocket could be identified close to where the active site would be in the a domain (comprising primarily the side chains of Leu-242, Leu-244, Phe-258, and Ile-272). Furthermore, a larger, more distinct pocket could be identified at the same position in a model of the b′ domain of PDI.6 Since the non-catalytic domains are thought to have arisen by gene duplication of catalytic domains, it is probable that the primary substrate binding site in the b′ domain would have arisen from the catalytic site and thus would be located in the same part of the thioredoxin fold. These two independent strands of evidence suggest the localization of the binding site to the same region of the b′ domain of PDI.

Defining the Primary Substrate Binding Site within the b′ Domain of PDI—To validate the localization of the binding site, a large number of mutations were made in the putative hydrophobic pocket and in spatially adjacent residues both in the isolated b′ domain and in full-length PDI. These mutants were expressed in E. coli. All but one of the mutants made in full-length PDI produced soluble proteins of the expected molecular size and equivalent yields. The exception was the P245A mutation, for which no expressed protein could be seen by SDS-PAGE, suggesting that this residue is structurally important. Even under optimal expression conditions, a significant proportion (~20%) of the wild type b′ domain was found in the insoluble fraction, and this fraction increased in some mutants to the extent that some showed no soluble expression. All of the soluble expressed mutants in PDI and in the b′ domain were screened for their ability to bind Δsomatostatin. The results indicated that mutations in the putative hydrophobic pocket significantly decreased peptide binding (Fig. 4A and Table I), whereas mutations in many juxtaposed residues did not (Fig. 4B). Of special note was Ile-272, where all mutations made (I272W, I272A, I272Q, I272N, and I272L) significantly decreased peptide binding (Fig. 4C). Furthermore, a correlation was seen between the ability of mutant proteins to bind Δsomatostatin and to bind the unrelated 14-amino-acid peptide mastoparan (Fig. 4C), although the effects on binding were more pronounced for mastoparan, consistent with the lower affinity of wild type PDI for this substrate (27).

PDI not only binds small peptides but also binds non-native proteins such as “scrambled” RNase (scRNase). Previously, we have shown that the b′ domain is essential for this binding process, but that it is not sufficient, with other domains, especially a and a′, contributing to binding. The ability of the I272W mutant of full-length PDI to bind scRNase was tested by a well established cross-linking-based assay. The results (Fig. 4D) show that this mutant is still able to bind scRNase but with reduced affinity when compared with the wild type protein.

Previously, mutations in the a′ domain of PDI have been shown to decrease peptide binding (28), but this was due to destabilization of the a′ domain and the probable intramolecular binding of the partially unstructured domain by the adjacent b′ domain (29). To ensure that the effects on substrate

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binding observed here were not due to gross structural effects, wild type and mutant proteins were compared in a variety of biophysical analyses. Screening the mutant forms of full-length PDI expressed in E. coli for protease stability revealed that although some showed a decrease in stability (implying a structural change), others including I272W behaved as per wild type (see Fig. 5A for examples).

The full-length wild type protein and I272W mutant were then purified to apparent homogeneity by a combination of immobilized metal affinity chromatography and anion exchange chromatography (data not shown). The far UV CD spectra of both proteins were essentially identical (Fig. 5B).

Discussion

The ability of PDI to act as an efficient catalyst of disulfide bond formation in folding polypeptides is primarily dependent on three factors: the ability to catalyze disulfide-dithiol exchange, the ability to bind non-native proteins, and the ability to trigger conformational changes in the bound substrate to allow access to buried cysteine residues. To date, much of the focus has been on dithiol-disulfide exchange, which depends on the CXXC thioredoxin-like active site motif. This motif cycles through the disulfide, mixed disulfide, and dithiol states during many of the reactions that PDI catalyzes, and thus, both active site thiols are required for efficient catalysis of native disulfide bond formation, although some reactions may proceed with just the N-terminal thiol. The residues that lie between the two cysteines play a crucial role in determining the redox potential of the enzyme (for references, see Ref. 30) and thus influence the nature of the reactions catalyzed, e.g., oxidation, reduction, or isomerization. Other juxtaposed residues have also been implicated in modulating the redox potential and/or activity of the superfamily (see Ref. 30). Furthermore, we have recently demonstrated that the dynamical changes in the tertiary structure of the isolated catalytic domain are required to complete the oxidative catalytic cycle (31).

The other two factors, the ability to bind non-native proteins and the ability to trigger conformational changes in the bound substrate, have received less attention. It is known that the b' domain of PDI provides the principal peptide binding site of PDI but that the a and a' domains also contribute to binding of misfolded proteins (13). Furthermore, although isomerization reactions require a linear combination of one catalytic domain plus b’, catalysis of isomerization reactions where disulfide bond rearrangement is linked to conformational changes in the protein substrate requires all of PDI excluding the c region (12).

Here we have defined more precisely the boundaries of the b’ domain, localized the primary substrate binding site within this domain, and identified residues contributing significantly to the binding of peptide ligands. The alignment of b’ against b (Fig. 1) and the modeling of the b’ structure both suggested that there is a region of 19 residues between the C terminus of the b’ domain proper and the N terminus of the a’ domain, a suggestion made previously on the basis of partial proteolysis data (10). We have now expressed this better defined b’ domain and shown that it constitutes a well defined domain, on the basis of expression yield and the spectroscopic properties and protease resistance of the purified recombinant domain. We have not characterized the “linker” or x region in any detail,
but the properties of the b'x construct (e.g. the fluorescence denaturation titration and wavelength of the maximum fluorescence emission) support previous suggestions that x is a region of defined structure, not an exposed protease-sensitive loop.

Within the b' domain as now more rigorously defined, the proposed ligand binding site is a small hydrophobic pocket, defined by the residues Leu-242, Leu-244, Phe-258, and Ile-272. Mutation of any of these residues influences peptide binding, with the greatest effect being seen for Ile-272. Surprisingly, substrate binding is very sensitive to mutations at this position with even the highly conservative mutation I272L having an effect on peptide binding. Furthermore, substantive biophysical analysis of full-length PDI and the PDI 1272W could reveal no indications of any alterations in the structure or stability of the protein, indicating that the result is a direct effect on the substrate binding site and not an indirect structural effect. The effect on the binding of small peptides is much greater than the effect on the binding of non-native protein substrates (such as scRNase), consistent with the previous observation that the a and a' domains also contribute to the binding of misfolded proteins (13).

The binding site in the b' domain is located in a position homologous to the position of the active site in the catalytic domains. Specifically, Leu-242 is in an analogous position within the thioredoxin fold as Glu-30 in the a domain, whereas Ile-272 is in an analogous position to Lys-64 in the a domain. Glu-30 and Lys-64 form a salt bridge that is buried under the substrate binding site in the NMR structure. Glu-30 is analogous to Asp-26 10. Freedman, R. B., Gane, P. J., Hawkins, H. C., Hlodan, R., McLaughlin, S. H., 11. Alanen, H. I., Salo, K. E. H., Pekkala, M., Siekkinen, H. M., Pirneskoski, A., Freedman, R. B., and Ruddock, L. W. (2000) Protein Sci. 9, 758–764.

Small structural perturbations of even the a' domain can have an effect on peptide binding. Furthermore, substantive studies on the specificity of PDI suggest that again a non-native protein site arises through gene duplications of catalytic domains (34).

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