Domains $b'$ and $a'$ of Protein Disulfide Isomerase Fulfill the Minimum Requirement for Function as a Subunit of Prolyl 4-Hydroxylase

THE N-TERMINAL DOMAINS $a$ AND $b$ ENHANCE THIS FUNCTION AND CAN BE SUBSTITUTED IN PART BY THOSE OF ERp57

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Protein disulfide isomerase (PDI) is a modular polypeptide consisting of four domains, $a$, $b$, $b'$, and $a'$, plus an acidic C-terminal extension, c. PDI carries out multiple functions, acting as the $b$ subunit in the animal prolyl 4-hydroxylases and in the microsomal triglyceride transfer protein and independently acting as a protein folding catalyst. We report here that the minimum sequence requirement for the assembly of an active prolyl 4-hydroxylase $\alpha_2\beta_2$ tetramer in insect cell coexpression experiments is fulfilled by the PDI domain construct $b'a'$ but that the sequential addition of the $b$ and $a$ domains greatly increases the level of enzyme activity obtained. In the assembly of active prolyl 4-hydroxylase tetramers, the $a$ and $b$ domains of PDI, but not $b'$ and $a'$, can in part be substituted by the corresponding domains of ERp57, a PDI isoform that functions naturally in association with the lectins calnexin and calreticulin. The $a'$ domain of PDI could not be substituted by the PDI $a$ domain, suggesting that both $b'$ and $a'$ domains contain regions critical for prolyl 4-hydroxylase assembly. All PDI domain constructs and PDI/ERp57 hybrids that contain the $b'$ domain can bind the 14-amino acid peptide $\Delta$-somatostatin, as measured by cross-linking; however, binding of the misfolded protein “scrambled” RNase required the addition of domains $ab$ or $a'$ of PDI. The human prolyl 4-hydroxylase $\alpha$ subunit has at least two isoforms, $\alpha(I)$ and $\alpha(II)$, which form with the PDI polypeptide the $(\alpha(I))_2\beta_2$ and $(\alpha(II))_2\beta_2$ tetramers. We report here that all the PDI domain constructs and PDI/ERp57 hybrid polypeptides tested were more effectively associated with the $\alpha(II)$ subunit than the $\alpha(I)$ subunit.

Protein disulfide isomerase (PDI) EC 5.3.4.1, a major protein within the lumen of the eukaryotic endoplasmic reticulum, is a catalyst of disulfide bond formation and rearrangement in protein folding (for reviews, see Refs. 1 and 2). PDI is a modular protein consisting of four domains, $a$, $b$, $b'$, and $a'$, plus an acidic C-terminal extension, c (3, 4). The $a$ and $a'$ domains show sequence similarity to thioredoxin, contain the catalytic site motif CGHC (1), and have the thioredoxin fold (3, 5). The $b$ and $b'$ domains show no amino acid sequence similarity to thioredoxin and have no catalytic site sequence, but recent NMR studies have indicated that the $b$ domain (and by homology the $b'$ domain) also has the thioredoxin fold (2, 6).

PDI is a multifunctional polypeptide. In addition to its role in protein folding within the endoplasmic reticulum (12–20), PDI serves as the $b$ subunit in the animal prolyl 4-hydroxylase $\alpha_2\beta_2$ tetramers and $\alpha b$ dimers (7–9) and in the microsomal triglyceride transfer protein $\alpha b$ dimer (10, 11). Prolyl 4-hydroxylase plays a central role in the synthesis of all collagens (8, 9, 21), whereas the microsomal triglyceride transfer protein is essential for the assembly of apoB-containing lipoproteins (10, 11). The main function of PDI in both of these proteins appears to be to keep their highly insoluble $\alpha$ subunits in a catalytically active, nonaggregated conformation (8, 9, 11, 22–24). This function is likely to be related to the peptide binding and chaperone functions of PDI, and it does not require the catalytic site cysteine residues (24, 25).

Previous cross-linking studies with various peptides and polypeptides have indicated that the principal peptide binding site of PDI is located in the $b'$ domain and that this domain alone is sufficient for binding peptides of 10–15 residues (26). However, binding of longer peptides requires the additional presence of either the $ab$ domains or the $a'$ domain (26). In agreement with these data, it has been found that the isolated $a$ and $a'$ domains function effectively as simple thiol-disulfide oxidoreductases but that the remaining domains are required for full catalytic activity in assisting protein folding associated with the formation of native disulfide bonds (27). It thus seems likely that the nonnative protein binding region extends beyond the principal peptide binding region present in domain $b'$, through all four domains (26, 28). The C-terminal extension $c$ plays no reported role in any of the functions of PDI (29).

ERp57 is a PDI-related polypeptide (1, 9) that forms complexes with both calnexin and calreticulin, these complexes being specifically involved in the modulation of glycoprotein folding within the lumen of the endoplasmic reticulum (30, 31). ERp57 resembles PDI in size, has thioredoxin-like domains with CGHC catalytic site motifs in positions corresponding to the $a$ and $a'$ domains of PDI, and also shows significant sequence similarity to PDI in regions corresponding to the $b$ and $a$ domains.
b' domains (32). However, it does not substitute for PDI as the β subunit of prolyl 4-hydroxylase (32).

The present work sets out to study which domains of the PDI polypeptide are required for the assembly of a prolyl 4-hydroxylase tetramer and whether some of these domains can be substituted by the corresponding domains of ERp57. The vertebrate prolyl 4-hydroxylase α subunit has at least two isoforms, α(I) and α(II), which form with the PDI polypeptide the (α(I))₂β₂ type I, and (α(II))₂β₂ type II, enzyme tetramers (33, 34). Therefore we also studied whether any differences exist in the assembly of the PDI domains and PDI/ERp57 hybrid polypeptides between the α(I) and α(II) subunits. To correlate the prolyl 4-hydroxylase assembly data with the less specific binding capabilities of PDI for folding substrates, we also analyzed the ability of the various PDI domains and PDI/ERp57 hybrid constructs to bind the peptide Δ-somatostatin and the misfolded protein “scrambled” RNase.

EXPERIMENTAL PROCEDURES

Construction of Baculovirus Expression Vectors and Generation of Recombinant Baculoviruses—PDI domain constructs were synthesized by polymerase chain reaction using a human PDI cDNA (7) as a template. The PDI signal sequence was added in front of the constructs that did not begin at the α domain. Similarly, ER-retention signals were added at the end of constructs that did not include region c. Thus, construct PDIabb’ codes for amino acids 1–350 of the mature PDI polypeptide, PDIabb’a for 119–491, PDIbb’a for 217–491, PDIa’c for 348–491, PDIbb’a for 119–351 and 1–120, and PDIabb’a for 1–352 and 1–120. cDNAs for PDIabb’ and PDIbb’a were ligated into the EcoRI-BamHI site of pVL1392 (Invitrogen), PDIbb’a into the XhoI-BamHI site, and PDIabb’ into the EcoRI-EcoRI site of this plasmid, whereas those for PDIabb’a and PDIa’c were ligated into the EcoRI site of pVL1393. Expression constructs for PDI/ERp57 hybrids were synthesized by polymerase chain reaction using the cDNAs for human PDI (7) and ERp57 (32) as templates. Two polymerase chain reaction products, those coding for the domains of PDI and the other for those of ERp57, were ligated blunt-ended together and into cohesive sites of pVL1392. Thus, PDIabb’ ERp57a’ codes for amino acids 1–350 of PDI, 353–465 of ERp57, and an AVKDEL retention signal; ERp57a’ PDIabb’ codes for amino acids 1–107 of ERp57, an R, and amino acids 117–491 of PDI; ERp57a’ PDIabb’a codes for amino acids 1–218 of ERp57 and 219–491 of PDI; ERp57a’ PDIabb’a codes for amino acids 1–352 of ERp57 and 351–491 of PDI; and PDIabb’ ERp57a’ PDIa’c for amino acids 1–117 of PDI and 110–481 of ERp57. The constructs for PDIabb’ ERp57a’, ERp57a’ PDIabb’a, and PDIabb’ ERp57a’ PDIa’c were ligated into the EcoRI-BamHI site, and those for ERp57a’ PDIabb’a and ERp57a’ PDIa’c were ligated into the EagI-BamHI site of pVL1392.

Spodoptera frugiperda Sf9 insect cells (Invitrogen) were cultured as monolayers in TNM-FH medium (Sigma) supplemented with 10% fetal bovine serum (Biolear) at 27 °C. The recombinant baculovirus transfer vectors were cotransfected into Sf9 insect cells with a modified Autog-rapha california nuclear polyhedrosis virus DNA (BaculoGold, Pharmingen) by calcium phosphate transfection (35). The resultant viral pools were collected 4 days later, amplified twice, and used for recombinant protein production. Other recombinant baculoviruses used in this work were human PDI, human ERp57, and human α(I) and α(II), coding for the corresponding prolyl 4-hydroxylase α subunits (32, 33, 34).

Expression and Analysis of Recombinant Proteins—For the expression of recombinant proteins, Sf9 insect cells were infected with the recombinant baculoviruses at a multiplicity of 5. In coexpression experiments, viruses were used at a ratio of 1:1. The cells were harvested 3 days after infection; washed with a solution of 0.15 M NaCl and 0.02 M phosphate, pH 7.4; homogenized in a solution of 0.1 M glycine, 0.1 M NaCl, 10 μM diithiothreitol, 0.1% Triton X-100, and 0.01 M Tris, pH 7.8; and centrifuged at 10,000 × g for 20 min at 4 °C. The resulting supernatants were analyzed by SDS-PAGE or by nondenaturing PAGE, followed by Coomassie staining or Western blotting, and assayed for prolyl 4-hydroxylase activity. In Western blotting, polyclonal antibodies against human PDI, human α(I) subunit and mouse α(II) subunit were used.

Prolyl 4-Hydroxylase Activity—Prolyl 4-hydroxylase activity was assayed by a method based on the hydroxylation-coupled decarboxylation of 2-oxo[1-14C]glutarate as described previously (36). Total cellular protein concentrations were determined using a Bio-Rad protein assay kit.

Synthesis and Labeling of Δ-Somatostatin and Scrambled RNase—Scrambled RNase, the homobifunctional cross-linking reagent disuccinimidyl glutarate (DSG), and all other chemicals were obtained from Sigma. α(1)-121Labeled Bolton-Hunter labeling reagent, ECL reagent, and x-ray films were purchased from Amersham Pharmacia Biotech. The somatostatin derivative without cysteine residues (Δ-somatostatin, AG-SKNNFKWFSTPS) was synthesized as described previously for other peptides (37). The polyclonal antibody raised against PDI was from Stressegen. α(1)-121Labeled Bolton-Hunter labeling of Δ-somatostatin was performed as recommended by the manufacturer.

Binding and Cross-linking of Δ-Somatostatin and Scrambled RNase—After precipitation with trichloroacetic acid, the radiolabeled Δ-somatostatin was dissolved in distilled water. Labeled Δ-somatostatin (approximately 3 μM) or scrambled RNase (approximately 50 μM) was added to Buffer A (100 mM NaCl, 25 mM KCl, 25 mM phosphate buffer, pH 7.5) containing crude cell extracts (approximately 20 mg/ml). The samples (10 μl) were incubated for 10 min on ice before cross-linking (26). Cross-linking was performed using DSG (26). The samples were supplied with 1/10 volume of cross-linking solution (10 mM DSG in Buffer A). The reaction was carried out for 60 min at 0 °C. Cross-linking was stopped by the addition of SDS-PAGE sample buffer (26). The samples were subjected to electrophoresis in 12.5% SDS-PAGE with subsequent autoradiography. Western blotting was performed using a polyclonal antibody raised against PDI. The detection was carried out with enhanced chemiluminescence (ECL).

RESULTS

Expression of PDI Domain Constructs and PDI/ERp57 Hybrids in Insect Cells—Recombinant baculoviruses coding for the polypeptides described in Fig. 1 were generated and used to
infected Sf9 insect cells. The cells were harvested 72 h after infection, homogenized in a buffer containing Triton X-100, and centrifuged. The 0.1% Triton X-100-soluble proteins were analyzed by 10% SDS-PAGE under reducing conditions, followed by Coomassie staining. Bands corresponding to polypeptides of the expected size were found in the Triton X-100-soluble fractions of the samples (Fig. 2).

Coexpressions of PDI Domain Constructs with Prolyl 4-Hydroxylase α Subunits—PDI domains required for the assembly of a prolyl 4-hydroxylase tetramer were studied by coexpression of the various domain constructs (Fig. 1) with either the α(I) or α(II) subunit of human prolyl 4-hydroxylase in insect cells. The cells were harvested and homogenized as described above. Then the Triton X-100-soluble proteins were analyzed by non-denaturing PAGE followed by Western blotting with a polyclonal antibody against human PDI (Fig. 3) and assayed for prolyl 4-hydroxylase activity by a procedure based on the hydroxylation-decarboxylation of 2-oxo-[1-14C]glutarate (Table I).

When PDAbb' was coexpressed with either the α(I) or α(II) subunit, no enzyme tetramer was detected by Western blotting (Fig. 3, lanes 3 and 4) and no significant level of enzyme activity was generated (Table I). Coexpression of PDAbb'a'c with the α(I) subunit led to assembly of a product with slow mobility in Western blotting (Fig. 3, lane 5), but very little enzyme activity, if any, was generated (Table I). However, when this same construct was coexpressed with the α(II) subunit, an enzyme tetramer with a mobility that corresponded to the reduced size of the PDI polypeptide was produced (Fig. 3, lane 6), and this tetramer was an active prolyl 4-hydroxylase (Table I). Nevertheless, the amount of enzyme activity generated in insect cells using this construct was only about 40% of that obtained with wild-type PDI (Table I), due to either a less efficient assembly level obtained with the mutant or a lower specific activity of the mutant enzyme tetramer. When the PDI construct was shortened further to contain just b'a'c, no assembly was detected with the α(I) subunit (Fig. 3, lane 7). However, assembly was still detected with the α(II) subunit (Fig. 3, lane 8), although the amount of enzyme activity generated was only about 9% of that obtained with the wild-type control (Table I). PDIα'c gave no assembly (Fig. 3, lanes 9 and 10) or activity (Table I) with either the α(I) or α(II) subunit.

PDI domains a and a' both contain the catalytic site motif CGHC, show a high degree of amino acid sequence similarity (7), and have very similar folded structures (3, 5). We therefore investigated whether the a' domain could be substituted by the a domain. A baculovirus coding for PDIbb'a was generated and used to infect a insect cell with a virus coding for either the α(I) or α(II) subunit. No assembly was obtained with this mutant polypeptide with either α subunit (Fig. 3, lanes 11 and 12), and correspondingly, no enzyme activity was generated (Table I). To ensure that the result was not influenced by the missing N-terminal a domain, a baculovirus coding for PDI abb'a was generated and used for additional coexpression experiments. However, no assembly or activity was seen with this construct either (Fig. 3, lanes 13 and 14; Table I). The data thus indicate

![Fig. 2. Analysis of expression of PDI, ERp57, PDI domain constructs, and PDI/ERp57 hybrids in insect cells by SDS-PAGE under reducing conditions. Triton X-100-soluble samples from insect cells infected with baculoviruses coding for human PDI (lane 1), PDIAbb' (lane 2), PDIbb'a'c (lane 3), PDIb'a'c (lane 4), PDIα'c (lane 5), PDAbb'a (lane 6), PDIAbb'a (lane 7), ERp57 (lane 8), PDAbb 'ERp57α' (lane 9), ERp57αPDIb'a'c (lane 10), ERp57αPDIb'a (lane 11), ERp57bb 'PDIαc (lane 12), and PDIεPDI57bb'a'c (lane 13). The samples were analyzed by 10% SDS-PAGE and Coomassie staining. Asterisks indicate migration of the polypeptides.](http://www.jbc.org/)

![Fig. 3. Analysis of coexpression of the human prolyl 4-hydroxylase α(I) or α(II) subunit with PDI domain constructs in insect cells by PAGE under non-denaturing conditions. Triton X-100-soluble samples from insect cells coinfected with baculoviruses coding for human α(I) subunit and PDI (lane 1), PDAbb' (lane 3), PDIb'a'c (lane 5), PDAbb'a (lane 7), PDIα'c (lane 9), PDAbb'a (lane 11), and PDIAbb'a (lane 13). Triton X-100-soluble samples from coinfections with baculoviruses coding for human α(II) subunit and PDI (lane 2), PDAbb'a (lane 4), PDIbb'a'c (lane 6), PDIb'a'c (lane 8), PDIα'c (lane 10), PDAbb'a (lane 12), and PDIAbb'a (lane 14). The samples were analyzed by 8% PAGE under non-denaturing conditions. Western blotting was carried out by using an anti-PDI antibody. Asterisks indicate migration of complexes formed with the α subunits and the PDI domain constructs. Migration of the free monomers is indicated by M.](http://www.jbc.org/)
that the α domain of PDI cannot functionally substitute for the α′ domain in prolyl 4-hydroxylase assembly.

Coexpression of PDI/ERp57 Hybrid Polypeptides with Prolyl 4-Hydroxylase α Subunits—To study whether the PDI domains can be functionally replaced by the corresponding domains of ERp57, baculoviruses coding for several hybrid PDI/ERp57 polypeptides (Fig. 1) were generated. Coexpression of PDIα′β′/ERp57α′ with either the α(I) or α(II) subunit led to no assembly (Fig. 4A, lanes 5 and 6) and generated no enzyme activity (Table I). In contrast, coexpression of ERp57αPDIIαβ′′/α′′ with either the α(I) or α(II) subunit resulted in tetramer assembly (Fig. 4A, lanes 7 and 8) and in the generation of prolyl 4-hydroxylase activity (Table I). In the case of the α(I) subunit, the amount of enzyme activity obtained with ERp57αPDIIαβ′′/α′′ was about 23% of that obtained with the wild-type PDI. In contrast, the construct PDIIαβ′′/α′′ without the ERp57 α domain gave less than 5% of the wild-type activity (Table I). In the case of the α(II) subunit, no difference could be observed between the ERp57αPDIIαβ′′/α′′ and PDIIαβ′′/α′′ constructs, both of which gave about 40% of the activity level seen with the wild-type PDI (Table I). A positive effect of the addition of ERp57 domains was seen even more distinctly with the

![Image](https://via.placeholder.com/150)
construct ERp57aβPDI b′ a′c. Cells expressing this polypeptide formed an active enzyme with both the α(I) and α(II) subunits even though detection of the complex with the α(I) subunit failed by nondenaturing PAGE, most probably due to lability of this tetramer (Fig. 4A, lanes 9 and 10; Table I), whereas the PDI b′ a′c construct showed no tetramer formation with the α(I) subunit and gave less enzyme activity with the α(II) subunit (8.7% versus 17.4%; Table I). The addition of ERp57ab′ in front of PDI a′c did not rescue prolyl 4-hydroxylase assembly (Fig. 4A, lanes 11 and 12; Table I). Similarly, PDIaERp57bb′ a′c resulted in no tetramer assembly (Fig. 4A, lanes 13 and 14; Table I), as confirmed by Western blotting with anti-α(I) and anti-α(II) subunit antibodies (Fig. 4B, lanes 3 and 6).

Binding of Radiolabeled Δ-Somatostatin to PDI Domain Constructs and PDI/ERp57 Hybrids—125I-Labeled Bolton-Hunter labeled Δ-somatostatin was added to the insect cell extracts of PDI domain constructs and PDI/ERp57 hybrids to investigate their interactions. After cross-linking with DSG, single cross-linking products could be detected in cell extracts expressing PDI, PDIab′, PDI b′a′c, PDIb′a, PDI bb′a, PDIab′ERp57α′c, ERp57aPDI bb′a′c, and ERp57abPDI b′a′c (Fig. 5A, lanes 1–4, 6, and 7; Fig. 5B, lanes 2–4). No cross-linking products were detected with Δ-somatostatin when cell extracts expressing PDIa′c (Fig. 5A, lane 5), ERp57, ERp57ab′PDIa′c, or PDIaERp57bb′a′c (Fig. 5B, lanes 1, 5, and 6) were tested. These results (Table II) are consistent with previous data (26) indicating that the minimum sequence requirement for binding of Δ-somatostatin to PDI is fulfilled by the b′ domain, and they extend the data by demonstrating that the b′ domain of ERp57 is not capable of replacing the corresponding PDI domain in this assay.

Interaction of Scrambled RNase with PDI Domain Constructs and PDI/ERp57 Hybrids—When crude cell extracts expressing the PDI domain constructs were incubated in the presence of scrambled RNase and subsequently cross-linked, cross-linking products were seen for PDI, PDIab′, PDI b′a′c, PDIbb′a, and PDIbb′a (Fig. 6A, lanes 2, 4, 6, 10, and 12) but not for PDIbb′c or PDIa′c (Fig. 6A, lanes 8 and 14). The cross-linking product of PDI b′a′c only became visible after a longer exposure time (Fig. 6B, lane 4). When the corresponding polypeptide produced in Escherichia coli was incubated in the presence of scrambled RNase and subsequently cross-linked, a cross-linking product was also obtained (Fig. 6B, lane 2). The result obtained with PDIab′ differs from that previously obtained with the corresponding construct in crude cell extracts of E. coli, in which no binding of biotinylated scrambled RNase was detected using a streptavidin-horseradish peroxidase conjugate (26). This conjugate could not be used in insect cell extracts because the biotinylated scrambled RNase was bound by some polypeptide(s) present in the lysate. When PDIab′ produced in E. coli was analyzed by the method used here, a cross-linking product was also obtained (data not shown).

Studies on the binding of scrambled RNase to the PDI/ERp57 hybrids were limited to those constructs that could be recognized by the anti-PDI antibody used. Cross-linking products were observed in the presence of scrambled RNase for the PDIab′ERp57α′c, ERp57aPDI b′a′c, and ERp57abPDI b′a′c constructs (Fig. 6C, lanes 2, 4, and 6). ERp57aPDI b′a′c exhibited a doublet band on cross-linking, probably resulting from an intramolecular cross-linking event. No cross-linking to the ERp57ab′PDIa′c construct was observed.

DISCUSSION

PDI has been shown to be a multifunctional protein. Because PDI is a modular polypeptide, one important issue in its molecular analysis is to understand the roles played by each of the individual PDI domains in its multiple functions. The data presented here allow us to analyze the roles of the individual domains of PDI in prolyl 4-hydroxylase assembly and the degree to which ERp57 domains can substitute for PDI in assembly and in the binding of peptides and nonnative proteins.

By coexpressing PDI domain constructs with the α subunits of prolyl 4-hydroxylase, we have determined that the minimum sequence requirement for assembly of an active tetramer is fulfilled by the PDI construct b′a′c (Table I). As a previous study (29) has demonstrated that the presence or absence of the C-terminal extension c has no observable effect on any of the main functions of PDI (i.e. the oxidoreductase, disulfide isomerase, chaperone, or protein subunit functions), the minimum requirement for prolyl 4-hydroxylase assembly is in fact fulfilled by the PDI domains b′a′c. However, the addition of the b domain gave a higher level of prolyl 4-hydroxylase activity, and all four domains of PDI were required for the highest level of activity measured. It is not clear whether this represents an increase in the absolute level of active prolyl 4-hydroxylase or in the specific activity of the enzyme tetramer formed using the PDI constructs. In either case, these data indicate that the primary sites of interaction between PDI and the α subunit lie in the b′ and a′ domains of PDI but that both the b and α domains contribute to the structure or stability of the tetramer. Such a contribution may either come from direct interactions between these domains and the α subunit or may come from a
contribution due to interactions between the β subunits (i.e. a dimerization event in PDI). Further evidence for the absolute requirement for the b’ and a’ domains and also the involvement of the b and a domains in tetramerization comes from an analysis of the differences between α(I) and α(II) subunit tetramerization and the use of PDI/ERp57 hybrids.

The two types of human prolyl 4-hydroxylase α subunit, the α(I) and α(II) subunits, readily form the (α(I))$_3$β$_2$ and (α(II))$_3$β$_2$ enzyme tetramers in insect cell coexpression experiments with PDI. The catalytic properties of the two types of enzyme tetramer are highly similar, but there are some distinct differences in the binding properties and binding sites for proline-rich peptide substrates and peptide inhibitors between the two isoenzymes (34, 38). To date, no differences have been reported in the tetramer assembly between these subunits (22, 34). However, the data presented here indicate a major difference between the two types of α subunit, in that the α(II) subunit became more effectively associated with the shortened PDI constructs, as seen by both the appearance of tetramers on native gels and by prolyl 4-hydroxylase activity. Detailed structural data are needed to fully understand the differences found here in the tetramer assembly between the α(I) and α(II) subunits.

PDI and ERp57 have homologous domain structures, the highest degree of identity and similarity being found in the α and a’ domains. It is therefore perhaps not surprising that the role of the α domain of PDI in prolyl 4-hydroxylase assembly could, in part, be substituted by the a domain of ERp57, this effect being more significant with the α(I) subunit (Table I). The degree of amino acid sequence identity between the b domains of human PDI and ERp57 is only 23% (based on the domain boundaries used here), but the ab fragment of PDI could likewise be, in part, substituted by the ab fragment of ERp57 (Tables I and II). Neither PDIαbb’ERp57a’ nor the ERp57αbb’PDιa’c constructs formed prolyl 4-hydroxylase tetramers, further indicating the importance of the b’ and a’ domains of PDI in the assembly process.

The primary peptide binding site of PDI is located in the b’ domain, and this domain alone is sufficient for the binding of peptides of 10–15 residues (26). In agreement with this requirement, Δ-somatostatin could be cross-linked here to all PDI domain and hybrid constructs that contain the PDI b’ domain. In contrast, the b’ domain of ERp57 does not appear to contain a binding site for small peptides such as Δ-somatostatin, as this peptide could not be cross-linked to ERp57 or to any hybrid lacking PDI domain b’ (Table II). Because the b’ domain of PDI could not be substituted by b’ of ERp57 in the assembly of a prolyl 4-hydroxylase tetramer, it seems reasonable to speculate that the primary peptide binding domain of PDI, which is also required for nonnative protein binding, is involved in interacting with the α subunit during the assembly process.

Whereas the primary peptide binding site of PDI is located in the b’ domain, binding of longer substrates (for example, a 28-amino acid fragment derived from bovine pancreatic trypsin inhibitor) has been shown to minimally require either abb’ or b’a’c (26). The binding of nonbiotinylated scrambled RNase was likewise found here to require either PDI abb’ or b’a’c.

A remarkable finding was that the a’ domain of PDI could not be replaced in prolyl 4-hydroxylase assembly by either a’ of ERp57α or α of PDI, even though these three domains are highly similar in their amino acid sequences (7, 32) and folded structures (3, 5). Previously, it has been demonstrated that substitution in PDI of the 78 most C-terminal residues of do-

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main $\alpha'$ and the C-terminal extension $c$ by the corresponding residues of ERp57 totally abolishes the assembly of a prolyl 4-hydroxylase tetramer (32). Similarly, several mutations introduced into the C-terminal region of the PDI domain $\alpha'$ prevent prolyl 4-hydroxylase assembly (29). It thus seems that the $\alpha'$ domain contains a region that is highly critical for the assembly of the prolyl 4-hydroxylase tetramer (Table II), as is the primary peptide binding region present in the $b'$ domain. As no assembly was seen with PDI$\alpha'\beta'\beta''\gamma'$ and either the $\alpha(1)$ or $\alpha(II)$ subunit, the implication is that the requirements for the assembly of a prolyl 4-hydroxylase tetramer are more stringent than those for the binding of nonnative proteins such as scrambled RNase (Table II). Detailed structural comparison between PDI domains $\alpha'$ and $\alpha'$ and ERp57 domain $\alpha'$ and a determination of the relative spatial positions of the domains of PDI in the native structure are needed to understand these functional differences.

The results presented here indicate that in PDI there is a broad binding region for folding substrates and functional partners to which domains contribute to different extents. For small peptides, the $b'$ domain is essential and sufficient. For large peptides, nonnative proteins such as scrambled RNase, and for the $\alpha$ subunit of prolyl 4-hydroxylase, the $\alpha'$ domain contributes significantly to the binding and the $b$ and $a$ domains enhance binding. The inability of the $b'$ and $a'$ domains of ERp57 to substitute for the analogous PDI domains suggests that these domains have very specific binding properties, e.g. to their permanent partners, the lectins calnexin and calreticulin, which are therefore analogous to the $\alpha$ subunit in prolyl 4-hydroxylase.

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Domains $b'$ and $a'$ of Protein Disulfide Isomerase Fulfill the Minimum Requirement for Function as a Subunit of Prolyl 4-Hydroxylase: THE N-TERMINAL DOMAINS $a$ AND $b$ ENHANCE THIS FUNCTION AND CAN BE SUBSTITUTED IN PART BY THOSE OF ERp57

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