Mutations That Destabilize the a’ Domain of Human Protein-disulfide Isomerase Indirectly Affect Peptide Binding*

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Protein-disulfide isomerase (PDI), an abundant protein of the eukaryotic endoplasmic reticulum, is a catalyst of disulfide-bonded proteins and also a multifunctional polypeptide that acts as the β-subunit in the prolyl 4-hydroxylase αβ2-tetramer (P4H) and the microsomal triglyceride transfer protein αβ-dimer. The principal peptide-binding site of PDI is located in the b’ domain, but all domains contribute to the binding of misfolded proteins. Mutations in the C-terminal part of the a’ domain have significant effects on the assembly of the P4H tetramer and other functions of PDI. In this study we have addressed the question of whether these mutations in the C-terminal part of the a’ domain, which affect P4H assembly, also affect peptide binding to PDI. We observed a strong correlation between P4H assembly competence and peptide binding; mutants of PDI that failed to form a functional P4H tetramer were also inactive in peptide binding. However, there was also a correlation between inactivity in these assays and indicators of conformational disruption, such as protease sensitivity. Peptide binding activity could be restored in inactive, protease-sensitive mutants by selective proteolytic removal of the mutated a’ domain. Hence we propose that structural changes in the a’ domain indirectly affect peptide binding to the b’ domain.

Protein-disulfide isomerase (PDI), an abundant protein of the eukaryotic endoplasmic reticulum, is a catalyst of disulfide bond formation and rearrangement in the course of protein folding (for review see Ref. 1). A molecular interpretation of PDI activity is made difficult by the fact that PDI is multifunctional in the cell. In addition, it has a wide range of actions in vitro and consists of multiple domains. Furthermore, there is as yet no high resolution structure of full-length PDI. Current efforts are focused on analyzing the domain structure of PDI and in establishing the roles of specific domains in specific functional activities.

It is now clear (2, 3) that PDI has a structural organization based on duplicated sequence modules (Fig. 1). The full-length protein is constructed of four structural domains with homologous thioredoxin folds plus a C-terminal acidic extension. The homologous a and a’ sequence modules contain the active site motif -WCGHC- and show significant sequence identity to thioredoxin; a high resolution NMR analysis of the recombinant a domain confirms that it has the thioredoxin fold. The homologous b and b’ modules do not show significant sequence similarity to the a domain, but NMR analysis has revealed that the b domain also exhibits the thioredoxin fold (2, 4). Analysis of the properties of individual domains as catalysts of simple thiol/disulfide oxidoreduction and of more complex protein folding linked to disulfide isomerization shows that the a and a’ domains function effectively as simple thiol/disulfide oxidoreductases but that the remaining domains are required for full activity in catalyzing protein folding associated with the formation of native disulfide bonds (5). No specific function has yet been ascribed to the b domain, but cross-linking studies demonstrate that the b’ domain provides the principal peptide-binding site (6).

In addition to its role in catalyzing protein folding, PDI has also been described as the β-subunit of two hetero-oligomeric proteins, namely prolyl-4-hydroxylase (P4H) (7) and the microsomal triglyceride transferase complex (MTP) (8, 9). MTP is obligatory for the assembly of apoB-containing lipoproteins, whereas P4H is important in the post-translational formation of 4-hydroxyproline in collagen in the endoplasmic reticulum (10, 11). It appears, both from attempts at reassociating these complexes after dissociation in vitro (11) and from studies on assembly of the complexes at biosynthesis, that PDI is required to prevent the aggregation of its partner subunits during either initial folding in the cell or refolding in vitro (12–15). Site-directed mutagenesis of the β-subunit/PDI demonstrated that the active site cysteine residues of PDI were not essential for the assembly and activity of active P4H tetramer or of MTP (12, 15).

The question then arises as to which domains or regions of PDI are involved in or required for its interaction with the other subunits in these hetero-oligomeric complexes. Analysis of deletion and point mutants of PDI recently demonstrated that the acidic C-terminal extension is not critical for the disulfide isomerase activity of PDI or for its ability to assemble into active P4H (16). However, deletion and point mutations in the C-terminal end of the preceding a’ domain led to the identification of several residues that are apparently critical for the assembly of P4H.

The interaction between PDI and the α-subunits of the P4H complex and MTP may be related to the interaction between PDI and the incompletely folded substrates on which PDI acts as an isomerase to facilitate folding and native disulfide bond formation. We have demonstrated previously that such misfolded protein substrates bind to PDI, and this binding is competitive with the binding of peptides; by using individual domains and truncated forms of PDI, we showed that the b’
domain constitutes the principal binding site for peptides but that additional domains are implicated in binding misfolded proteins (6).

By using chemical cross-linking of a model peptide, we show here that mutations in the C-terminal part of the a’ domain, which affect the assembly of P4H, can also affect the peptide binding activity of PDI, a function associated with the b’ domain. We also demonstrate that the mutations influence the conformation and/or dynamics of the protein and infer that these changes in the a’ domain indirectly influence the binding properties of the b’ domain.

EXPERIMENTAL PROCEDURES

Materials—The homobifunctional cross-linking reagent disuccinimidyl glutarate (DSG) was obtained from Sigma. Bolton-Hunter 125I-labeling reagent and x-ray films were purchased from Amersham Pharmacia Biotech. Somatostatin (AGSKNFKWFTSS) was synthesized as described for other peptides (17). The polyclonal antibody raised against bovine liver PDI was from Stressgen. The purification of bovine liver PDI was as described previously (18). The construction of PDI deletion mutants, site-directed mutagenesis, and generation of recombinant baculoviruses have been reported elsewhere (16).

Methods—Bolton-Hunter 125I labeling of Somatostatin—Somatostatin—Chemical cross-linking is a powerful tool for studying peptide interactions with proteins available only in small amounts in crude cell extracts. We have shown previously that peptides and non-native proteins, after radiolabeling, could be cross-linked specifically to PDI and fragments of PDI expressed in E. coli (6). Competitive binding studies showed that peptides and misfolded proteins interact with the same site, implying that the site identified in these peptide-binding studies is that at which misfolded protein substrates are bound in the enzymic activities of PDI. Here we employ cross-linking as a technique to investigate the interactions between a model peptide, specifically Somatostatin, and recombinant mutants of human PDI.

The PDI mutants, specifically tripeptide deletions, more extensive deletions, and single residue substitutions, were expressed in E. coli (Fig. 2A). 125I-Labeled Bolton-Hunter Somatostatin was added to these cell extracts, and the mixture was cross-linked by using the homobifunctional amino-specific cross-linking reagent DSG (Fig. 2B). Single cross-linking products could be detected with cell extracts expressing wild type PDI, the deletion mutants Δ436–439, Δ440–443, Δ446–451, and Δ452–454 or the point mutants R444A, F449W, F449Y, and G448R. No cross-linking products, however, were detectable with cell extracts expressing the deletion mutants Δ436–439, Δ440–443, Δ446–448, Δ449–451, and Δ452–454 or the point mutants F448R and L453E despite the fact that these mutants were expressed in the soluble fraction to nearly comparable levels. To confirm that variations in expression levels did not influence the results, mutants that were expressed to lower levels were tested for peptide binding at equivalent PDI loading levels, instead of equivalent cell density loading. No
tracts expressing the deletion mutants were detectable with cell extracts expressing wild type PDI, the deletion mutant \( \Delta 449–451 \), and \( \Delta 449–451 \) was also expressed in both bacterial and insect cells and tested for peptide binding in both backgrounds. No cross-linking was observed in the bacterial cell lysate (Fig. 2B, lane 4), but interestingly, a cross-linking product with an increased mobility was seen in the insect cell lysate (Fig. 3B, track 12). We thought it most likely that this was due to degradation of the mutant protein, and this was confirmed by the Western blot analysis of various mutants of PDI shown in Fig. 3A. Clearly, degradation products related to PDI were observed in cell extracts expressing the point mutant F449R and the deletion mutants \( \Delta 452–465 \) and \( \Delta 449–451 \). Western blot analysis showed that these mutant proteins were stable when expressed in \( E. coli \) (data not shown).

In the case of the \( \Delta 449–451 \) deletion mutant, when it is expressed in \( Sf9 \) cells only the degraded fragment appears to bind radiolabeled peptide, and a similar result is observed with mutant F449R (Fig. 3B, tracks 12 and 3). We did not observe any interaction between radiolabeled \( \Delta \)-somatostatin and the full-length forms of these mutants or with either form of the deletion mutant \( \Delta 452–465 \).

**Peptide Binding of PDI Mutants Correlates with Their Sensitivity to Proteinase K**—The partial proteolysis of this subset of mutant PDIs in insect cell backgrounds is presumably due to the presence of endogenous proteases, but it suggests that these mutants are intrinsically protease-sensitive. It is noteworthy that the full-length forms of all these mutants are all inactive in peptide binding. To test whether the stability of a PDI mutant correlates with its ability to bind peptides, sensitivity to Proteinase K was tested, initially for one mutant. Wild type PDI and the point mutant F449R were expressed in \( Sf9 \) insect cells, the cell lysates were treated with various concentrations of Proteinase K, and the samples were analyzed by Western blotting and immunodecoration with an anti-PDI antibody (Fig. 4A). Wild type PDI was stable up to 30 \( \mu \)g/ml of Proteinase K, whereas the F449R point mutant showed a degradation product even in the absence of Proteinase K. Upon addition of Proteinase K, this degradation product was further degraded and produced a fragment that was stable up to 30 \( \mu \)g/ml of Proteinase K. Similar Proteinase K susceptibilities were obtained when wild type PDI and the F449R point mutant were expressed in \( E. coli \) (data not shown). However, we noticed that the F449R point mutant was not degraded when expressed in \( E. coli \) in the absence of Proteinase K (Fig. 4, compare A, lane 7, with B, lane 7). From these results we conclude that the F449R point mutant is more sensitive to Proteinase K than the wild type, suggesting that this mutant exhibited either some altered structural features or increased structural mobility or a decrease in conformational stability.

To test whether there is a wider correlation between stability (defined by protease-resistance) and peptide binding, various mutants were expressed in \( E. coli \), the lysates were treated with 20 \( \mu \)g/ml Proteinase K, and the samples were analyzed by Western blotting and immunodecoration with an anti-PDI antibody. Fig. 4B demonstrates that wild type PDI, the deletion mutants \( \Delta 458–461 \) and \( \Delta 462–491 \), and the F449W point mutant were stable under these conditions, whereas the deletion mutant \( \Delta 452–454 \) and the F449R point mutant were degraded in the presence of Proteinase K. The former group of mutants are all active in binding peptide (Fig. 2B, tracks 3, 10, and 15), whereas the latter two mutants are inactive (Fig. 2B, tracks 2 and 14). Clearly, changes in conformation and/or dynamics that variation in peptide binding was observed (data not shown).

To compare the results obtained in \( E. coli \) lysates with recent findings using mutants of human PDI expressed in \( S. frugiperda \) (\( Sf9 \) insect cells) (16), an overlapping set of mutants was constructed and expressed in \( Sf9 \) insect cells. Equal amounts of the crude insect cell lysates were loaded onto a SDS gel and were probed for the expression of various mutants of PDI by immunodecoration with a polyclonal antibody raised against bovine liver PDI (Fig. 3A). Iodinated Bolton-Hunter \( \Delta \)-somatostatin was added to these cell extracts, and the mixture was cross-linked with DSG (Fig. 3B). A \( Sf9 \) wild type cell line expressing no human PDI or mutants of it served as a control. Single cross-linking products were detected with cell extracts expressing wild type PDI, the deletion mutant \( \Delta 466–478 \), and the point mutants K450A, K451A, F449Y, and E454A. No cross-linking products, however, were detectable with cell extracts expressing the “inactive” deletion mutants and point mutants were compatible with the cross-linking procedure (data not shown).

To demonstrate that those cell lysates that did not show any cross-linking products were not blocking cross-linking or otherwise incompatible with the assay, these cell lysates were supplemented with purified bovine liver PDI, and cross-linking to \( \Delta \)-somatostatin was performed. Cross-linking products were detected in all supplemented lysates, indicating that cell lysates expressing the “inactive” deletion mutants and point mutants were compatible with the cross-linking procedure (data not shown).

Three single-site substitutions were common to the two sets of mutants. The mutant F449Y retained peptide binding activity whether expressed in the bacterial or the insect cells, whereas the mutants F449R and L453E were inactive in peptide binding in both backgrounds. Hence we conclude that the two expression systems give comparable results with respect to the peptide binding properties of recombinant PDIs.

**Limited Proteolysis of Mutant Recombinant PDI in Sf9 Cell Lysates**—The deletion mutant \( \Delta 449–451 \) was also expressed in both in bacterial and insect cells and tested for peptide binding in both backgrounds. No cross-linking was observed in the bacterial cell lysate (Fig. 2B, lane 4), but interestingly, a cross-linking product with an increased mobility was seen in the insect cell lysate (Fig. 3B, track 12). We thought it most likely that this was due to degradation of the mutant protein, and this was confirmed by the Western blot analysis of various mutants of PDI shown in Fig. 3A. Clearly, degradation products related to PDI were observed in cell extracts expressing the point mutant F449R and the deletion mutants \( \Delta 452–465 \) and \( \Delta 449–451 \). Western blot analysis showed that these mutant proteins were stable when expressed in \( E. coli \) (data not shown).

In the case of the \( \Delta 449–451 \) deletion mutant, when it is expressed in \( Sf9 \) cells only the degraded fragment appears to bind radiolabeled peptide, and a similar result is observed with mutant F449R (Fig. 3B, tracks 12 and 3). We did not observe any interaction between radiolabeled \( \Delta \)-somatostatin and the full-length forms of these mutants or with either form of the deletion mutant \( \Delta 452–465 \).

**Peptide Binding of PDI Mutants Correlates with Their Sensitivity to Proteinase K**—The partial proteolysis of this subset of mutant PDIs in insect cell backgrounds is presumably due to the presence of endogenous proteases, but it suggests that these mutants are intrinsically protease-sensitive. It is noteworthy that the full-length forms of all these mutants are all inactive in peptide binding. To test whether the stability of a PDI mutant correlates with its ability to bind peptides, sensitivity to Proteinase K was tested, initially for one mutant. Wild type PDI and the point mutant F449R were expressed in \( Sf9 \) insect cells, the cell lysates were treated with various concentrations of Proteinase K, and the samples were analyzed by Western blotting and immunodecoration with an anti-PDI antibody (Fig. 4A). Wild type PDI was stable up to 30 \( \mu \)g/ml of Proteinase K, whereas the F449R point mutant showed a degradation product even in the absence of Proteinase K. Upon addition of Proteinase K, this degradation product was further degraded and produced a fragment that was stable up to 30 \( \mu \)g/ml of Proteinase K. Similar Proteinase K susceptibilities were obtained when wild type PDI and the F449R point mutant were expressed in \( E. coli \) (data not shown). However, we noticed that the F449R point mutant was not degraded when expressed in \( E. coli \) in the absence of Proteinase K (Fig. 4, compare A, lane 7, with B, lane 7). From these results we conclude that the F449R point mutant is more sensitive to Proteinase K than the wild type, suggesting that this mutant exhibited either some altered structural features or increased structural mobility or a decrease in conformational stability.

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make some mutant PDIs susceptible to degradation also interfere with their peptide binding activity.

Interestingly, the degradation products observed with the protease-sensitive mutants co-migrated with the a-b-b' fragment of human PDI (D1-N370 of mature human PDI) (Fig. 4B). Because N-terminal sequencing of the protease-generated F449R fragment (Fig. 4B, track 8) showed the genuine N terminus of PDI, we propose that Proteinase K treatment of the F449R mutant generates a fragment corresponding to the a-b-b' fragment of PDI. This indicates that it is specifically the a' domain of this mutant that is sensitive to Proteinase K.

The Inactive Full-length F449R Mutant Regains Peptide Binding Activity upon Treatment with Proteinase K—The work above has demonstrated that mutations in the a' domain of PDI can affect peptide binding, a property identified with the b' domain. It has also shown that inactivating mutations lead to protease sensitivity of the a' domain. Furthermore, in one case of substitution and one deletion mutant, it was shown that spontaneous proteolytic degradation products but not the full-length proteins are active in peptide binding (Fig. 3A). As a direct test of whether changes arising from mutations in the a' domain can interfere with peptide binding to the peptide binding site in the b' domain, we expressed the F449R mutant in E. coli and pretreated it with Proteinase K prior to cross-linking with Δ-somatostatin.

As shown in Figs. 4B and 5, treatment of the F449R mutant with Proteinase K (20 μg/ml) resulted in a fragment that co-migrated with the a-b-b' fragment of human PDI. Under these conditions, wild type PDI or the a-b-b' fragment of PDI (data not shown) were not sensitive to Proteinase K. In contrast to the untreated full-length F449R mutant, the Proteinase K fragment of this mutant could be cross-linked to radiolabeled Δ-somatostatin (Fig. 5, panel c, tracks 3 and 4). Proteinase K pre-treated wild type PDI (track 2) or the untreated a-b-b' fragment of PDI (track 5) served as controls.

Our results clearly demonstrate that structural changes in the a' domain arising from mutations in that domain can affect the peptide binding site in the b' domain. Once the mutant a' domain is removed, peptide binding to the b' domain can be restored.

PDI in the Prolyl 4-Hydroxylase αβγ-Tetramer Does Not Interact with Radiolabeled Δ-Somatostatin—As mentioned earlier, PDI acts as the β-subunit in the P4H and mutations in the a' domain have been reported to prevent assembly into a functional P4H complex (16). There is a clear correlation between the results reported for assembly into a functional P4H complex and those presented here on peptide binding (Table I), suggesting that the peptide binding site of PDI is involved in the assembly of the holoenzyme. To test this suggestion we asked whether the peptide binding site of PDI in the prolyl 4-hydroxylase αβγ-tetramer can interact with radiolabeled Δ-somatostatin. After chemical cross-linking the samples were loaded onto a native gel with subsequent electrotransfer onto a PVDF membrane. Unassembled PDI and PDI in the prolyl 4-hydroxylase αβγ-tetramer were detected by immunodecoration with an anti-PDI antibody, and the interaction with radiolabeled Δ-somatostatin was detected by autoradiography. As shown in
Fig. 6, we observed an interaction between radiolabeled Δ-somatostatin and unassembled PDI but not with PDI in the prolyl 4-hydroxylase \( \alpha_2\beta_2 \)-tetramer.

**DISCUSSION**

A key issue in the molecular analysis of PDI is to understand its multifunctionality. In catalyzing protein folding associated with native disulfide bond formation, PDI acts on structured folding intermediates to facilitate disulfide isomerization linked to conformational change (21, 22). As a component of the P4H and MTP complexes its essential function appears to be to interact with nascent or newly synthesized proteins is specific, saturable, reversible, and independent of the presence of cysteine residues in the bound peptide (20).

**Mutations in the C-terminal Part of the \( \alpha' \) Domain of PDI Affect Peptide Binding**—We found that the 14-amino acid peptide Δ-somatostatin, after radiolabeling and precipitation, can be chemically cross-linked to wild type PDI and overexpressed in \( E. coli \) or \( Sf9 \) insect cells. Interestingly, we did not observe any interaction of Δ-somatostatin with endogenous insect PDI. When we compared our results obtained with \( Sf9 \) insect cells and \( E. coli \) lysates, we found them to be identical, i.e. mutants that showed peptide binding when expressed in \( Sf9 \) cells also exhibited peptide binding activity after expression in \( E. coli \) cells. Likewise, mutants that failed to bind the peptide in one cell lysate did not show peptide binding in the other cell lysate. We therefore conclude that both cell lysates are compatible with the peptide binding assay, leading to comparable and reproducible results.

We observed that mutations in the C-terminal extension of PDI (465–491) or the deletion of this region did not affect the peptide binding activity. However, certain mutations in the preceding \( \alpha' \) domain, specifically all the deletion mutants from residues 436 to 454 and the point mutations F449R and L453E, were completely inactive with respect to peptide binding. There is no current high resolution structure for this domain, but modelling by homology to the \( \alpha \) domain suggests that this region comprises a loop (434–442) and the C-terminal \( \alpha \)-helix (445–454) of the \( \alpha' \) domain. This result is consistent with recent observations that deletion of the entire C-terminal extension has no inhibitory effect on P4H assembly or function or on the disulfide isomerase activity of PDI (16), whereas the
C-terminal α helix of the α′ domain seems to be a critical region for these functions (Table I). This was also confirmed by recent work of Dai and Wang who showed that truncation of the last 50 amino acids of PDI, comprising part of the C-terminal α helix of the α′ domain, led to inhibition of the chaperone and peptide binding activities (24).

Our results clearly demonstrate that mutations in the C-terminal part of the α′ domain that interfere with the assembly of PDI into a functional α2β2 P4H tetramer also affect peptide binding. From this strong correlation we infer that the generation of a functional P4H tetramer requires the peptide-binding site of PDI.

Peptide Binding Can Be Restored upon Removal of the Mutated α′ Domain—Although the CD spectra of the point mutant F449R showed no major differences compared with wild type PDI (16), we noticed that this mutant was much more protease-sensitive than the wild type both in cell lysates and when probed by added protease. This clearly indicates that the replacement of phenylalanine 449 by an arginine leads to mobility and/or conformational changes that are not detected by CD spectra and that therefore may be fairly local. Protease treatment of the F449R mutant led to a fragment that co-migrated with the a-b-b′ construct of PDI and had the genuine N terminus of PDI. It appears probable that the F449R replacement destabilized the α′ domain, rendering this part of the molecule protease-sensitive. Indeed, structure predictions and homology modeling, based on the structure of the α domain suggest that the side chain of Phe449 is buried in the hydrophobic core of the molecule; replacement by Arg would be expected to have significant effects, whereas substitutions by Tyr and Trp would be more conservative.

Once the protease-sensitive α′ domain was removed by treatment with Proteinase K, peptide binding could be restored to the residual a-b-b′ fragment. We therefore infer that the inhibition of peptide binding by mutations in the α′ domain is an indirect effect. Mutations in the α′ domain might induce subtle (but reversible) structural changes in the b′ domain, leading to disruption of the peptide-binding site. Alternatively, the altered conformation of the mutated α′ domain may physically prevent the reporter peptide from entering the peptide-binding site in the b′ domain. It is also possible that the mutated α′ domain mimics a misfolded polypeptide substrate on which PDI acts. This then would act as a ligand for binding by the peptide-binding site in the b′ domain. Such intramolecular interactions would have a high effective concentration, thus competing strongly for the binding of small peptides. All of the data presented here are fully consistent with the model for intramolecular occupancy of the peptide-binding site, but it should be noted that the F449R mutant shows no major differences in CD spectra to the wild type (16).

The last two scenarios suggest that the α′ domain is in close proximity to the peptide-binding site in the b′ domain, and this model could explain the results obtained previously by Noiva and co-workers (25, 26), who showed that a radiolabeled tripeptide could be cross-linked to residues within the C-terminal 50 amino acid residues of rat liver PDI (25, 26) and therefore assigned the peptide-binding site of rat liver PDI to the C terminus of the α′ domain. We speculate that the tripeptide may have bound to the peptide-binding site within the b′ domain and that cross-linking occurred to the C-terminal part of the α′ domain, which is in close proximity to the peptide-binding site.

From the results presented in this study it is tempting to speculate that there is a common polypeptide-binding site of PDI involved in all its functions. The b′ domain alone being essential and sufficient for the binding of small peptides, but a more extended region involving other domains is required for binding misfolded polypeptides and might be involved in the interaction between PDI and the α-subunit of P4H.

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