Three Binding Sites in Protein-disulfide Isomerase Cooperate in Collagen Prolyl 4-Hydroxylase Tetramer Assembly

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Protein-disulfide isomerase (PDI) is a modular polypeptide consisting of four domains, α, β, β', and α'. It is a ubiquitous protein folding catalyst that in addition functions as the β-subunit in vertebrate collagen prolyl 4-hydroxylase (C-P4H) αβb tetramers. We report here that point mutations in the primary peptide substrate binding site in the β' domain of PDI did not inhibit C-P4H assembly. Based on sequence conservation, additional putative binding sites were identified in the α and α' domains. Mutations in these sites significantly reduced C-P4H tetramer assembly, with the α domain mutations generally having the greater effect. When the α or α' domain mutations were combined with the β' domain mutation I272W tetramer assembly was further reduced, and more than 95% of the assembly was abolished when mutations in the three domains were combined. The data indicate that binding sites in three PDI domains, α, β', and α', contribute to efficient C-P4H tetramer assembly. The relative contributions of these sites were found to differ between Caenorhabditis elegans C-P4H αβ dimer and human αβb tetramer formation.

Protein-disulfide isomerase (PDI1; EC 5.3.4.1) is a ubiquitous catalyst of disulfide bond formation and rearrangement during protein folding in the endoplasmic reticulum (ER). In addition, it functions as a subunit in two enzyme complexes, the collagen prolyl 4-hydroxylases (C-P4Hs; EC 1.14.11.2) (1, 2) and microsomal triglyceride transfer protein (3). The C-P4Hs, which are crucial for the synthesis of extracellular collagen macromolecules (4), are tetrameric enzymes consisting of two catalytic α subunits and two β subunits identical to PDI (5). The role of PDI in this tetramer is to keep the highly insoluble α subunits in solution and in a catalytically active, nonaggregated conformation (6, 7). In addition, PDI retains the tetramer for assembly and activity. Surprisingly, no mutations in any of the β' domain residues previously shown to be important in peptide binding prevented C-P4H tetramer assembly or reduced the enzyme activity. We therefore used sequence conservation among the thioredoxin family to identify residues that might form substrate binding sites in the α and α' domains. Efficient assembly of an active C-P4H tetramer was found to be dependent on interaction sites in three PDI domains, α, β', and α', and the same sites were also found to play a role in the

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‡ The abbreviations used are: PDI, protein-disulfide isomerase; ER, endoplasmic reticulum; C-P4H, collagen prolyl 4-hydroxylase; PHY-1, C. elegans collagen prolyl 4-hydroxylase type I α subunit; PHY-2, C. elegans collagen prolyl 4-hydroxylase type II α subunit.
assembly of a Caenorhabditis elegans α subunit-human PDI C-P4H dimer. The relative importance of each site for PDI-protein interactions was greatly dependent on the nature of the interaction partner, and there was clear evidence of cooperativity between the three binding sites in PDI.

**MATERIALS AND METHODS**

**Generation of Mutant PDI Expression Vectors—**Plasmids pLWRP64, an Escherichia coli expression vector encoding mature human PDI with an N-terminal His tag in-frame with the cloned gene; pVL sis, with the minor impurities being at a low level of degradation as seen previously (6, 18, 22). PDI mutants were generated on these vectors using site-directed mutagenesis performed using the QuikChange™ site-directed mutagenesis kit (Stratagene) as recommended by the manufacturer. All of the plasmids generated were sequenced to ensure that there were no errors in the cloned genes.

**Generation of Baculoviruses for PDI Mutants and Expression of the Recombinant Proteins in Insect Cells—**The recombinant PDI mutant plasmids generated were cotransfected into Spodoptera frugiperda SF9 insect cells with a modified Autographa californica nuclear polyhedrosis virus DNA (BaculoGold; PharMingen) by calcium phosphate transfection (23). The resultant viral pools were collected 4 days later, amplified twice, and used for recombinant protein production. Other recombinant baculoviruses used in this work coded for the human C-P4H α(I) and α(II) subunits, the human PDI/β subunit (6, 9), and the C. elegans α subunit PHY-1 (32). SF9 cells were cultured in TNM-FH medium (Sigma) supplemented with 10% fetal bovine serum (BioClear) at 27 °C as monolayers. To produce recombinant proteins, insect cell fractions was assayed by a method based on the hydroxylase activity between the three binding sites in PDI.

**Protein Expression in E. coli and Purification—**Recombinant Proteins in Insect Cells—

**Enzyme Activity Assay—**The P4H activity of the Triton X-100-soluble insect cell supernatants were analyzed by denaturing 8% SDS-PAGE under reducing conditions, non-denaturing 8% PAGE, and Western blotting using an anti-PDI antibody (32). The amount of wild-type and mutant PDI proteins were purified using BD TALONTM single step columns (BD Biosciences). All of the proteins were >95% pure in SDS-PAGE analysis, with the minor impurities being at a low level of degradation as seen in E. coli. The concentration of each protein was determined spectrophotometrically using a calculated absorption coefficient of PDI (45,040 μm⁻¹ cm⁻¹; M, 56,386) at 280 nm.

**Cross-linking and Protease Sensitivity Assay—**Cell extracts from E. coli were prepared by freeze-thawing. Bolton-Hunter 131 labeling of Δ-somatostatin (AGSKNNFWKTFSS) was performed as recommended by the manufacturer (Amersham Biosciences), and cross-linking was performed using the homobifunctional cross-linking reagent disuccinimidyl glutarate (Sigma), as described for Δ-somatostatin or “scrambled” RNase (17). The protease sensitivity assay was carried out by adding proteinase K (0–50 μg/ml) or V8 (0–100 μg/ml) to 2 μl of lysates in a total volume of 10 μl and then incubated on ice for 20 min. After that, 0.5 μl of 20 mM phenylmethylsulfonyl fluoride was added, and the samples were run on a denaturing 12.5% SDS-PAGE under reducing conditions and Western blotted using an anti-PDI antibody (Stressgen). The stringent condition used for screening all of the mutants was 20 μg/ml proteinase K.

**Circular Dichroism Spectrum Analyses—**Far UV circular dichroism spectra were recorded on a Jasco J600 spectrophotometer. All scans were collected at 25 °C as averages of eight scans, using a cell with a path length of 0.1 cm, a scan speed of 20 nm/min, a spectral bandwidth of 1.0 nm, and a time constant of 1 s. The maximal high tension voltage was 750 V. All spectra were corrected for blanks run with no protein added.

**RESULTS**

**Point Mutations in the Substrate Binding Site in the b’ Domain of PDI Do Not by Themselves Impair C-P4H Tetramer Assembly—**We have recently identified several residues in the b’ domain that are important for the binding of peptide substrates (15). Replacement of Ile272 by alanine, tryptophan, asparagine, glutamine, or leucine in the individual b’ domain and full-length PDI greatly impaired binding of the small peptides Δ-somatostatin and mastoparan, whereas replacement of the residues Leu242, Leu244, and Phe258 by various amino acids also reduced peptide binding, but to a lesser extent (15). The I272W mutation had the greatest effect on binding affinity, and in addition to the short peptide substrates it also reduced the affinity of the full-length PDI to “scrambled” RNase (15). We therefore tested whether the mutation of any of these four residues or the spatially adjacent residue Ser256 in the full-length PDI polypeptide would impair the assembly of a C-P4H tetramer.

Recombinant full-length PDI polypeptides containing the single point mutation L42W, L242W, F258A, F258W, I272A, I272W, or S256D in the b’ domain (Fig. 1) were expressed together with the human C-P4H α(I) or α(II) subunits in insect cells. The cells were harvested 72 h after infection, homogenized in a buffer containing Triton X-100, and centrifuged. Assembly of a recombinant human type I α(I), β(II) or type II α(II), β(II) C-P4H tetramer was then analyzed by non-denaturing PAGE of the Triton X-100-soluble proteins (Fig. 2), and P4H activity was assayed by a method based on the hydroxylase-coupled decarboxylation of 2-oxo-[1-14C]glutarate (Table 1). Surprisingly, none of the b’ domain mutations tested was found to inhibit C-P4H tetramer assembly (e.g. see Fig. 2, lanes I–3), and none caused a marked reduction in P4H activity.
measured. There is a good correlation between the effects of the mutations on tetramer assembly and on P4H activity (see Tables I–IV).

Collagen Prolyl 4-Hydroxylase Tetramer Assembly

Collagen prolyl 4-hydroxylase type II tetramers generated in insect cells. Triton X-100-soluble fractions of insect cell homogenates expressing collagen prolyl 4-hydroxylase α(I) and PDI (lane 1), I272W (lane 2), S256D (lane 3), Y99W (lane 4), W111I (lane 5), L386W (lane 6), F452I (lane 7), F452W (lane 8), L1272W/Y99W (lane 9), W111I/I272W (lane 10), I272W/L386W (lane 11), I272W/Y440W (lane 12), W111I/F452I (lane 13), L42W/I272W/L386W (lane 14), Y99W/I272W/Y440W (lane 15), and W111I/I272W/F452W (lane 16). The samples were analyzed by 8% nondenaturing PAGE followed by Coomassie staining. The migration position of the enzyme tetramers is indicated by an arrow, and that of the free PDIs is shown by a bracket. Lanes containing enzyme tetramers with single PDI point mutations are indicated with a solid line above, whereas those containing double and triple mutations are indicated by a dotted and a dashed line, respectively. D, densitometry values of the mutant tetramers compared with the type II wild-type collagen prolyl 4-hydroxylase tetramer obtained using a calibrated imaging densitometer. ND, not determined for the tetramers not visible by Coomassie staining to be measured. There is a good correlation between the effects of the mutations on tetramer assembly and on P4H activity (see Tables I–IV).

TABLE I

| PDI or mutant expressed | Activity of PDI or mutant expressed with α(I) subunit | Activity of PDI or mutant expressed with α(II) subunit |
|-------------------------|------------------------------------------------------|------------------------------------------------------|
|                         | Activity (dpm/100 μg) | Percentage | % | km for (PPG)₁₀₀ | Activity (dpm/100 μg) | Percentage | % | km for (PPG)₁₀₀ |
| PDI                    | 11,600 ± 3080 | 100 | 35 | 17,970 ± 4570 | 100 | 70 |
| L242W                  | 9910 ± 1830 | 85 | 17,060 ± 1630 | 95 |
| L244W                  | 13,410 ± 1090 | 116 | 27,460 ± 1680 | 153b |
| S256D                  | 32,170 ± 1850 | 277b | 44,840 ± 8910 | 250c |
| F258A                  | 22,820 ± 360 | 195b | 33,460 ± 5250 | 186b |
| F225W                  | 10,540 ± 1940 | 91 | 10,210 ± 1450 | 90 |
| I272A                  | 15,080 ± 2280 | 130c | 28,500 ± 2190 | 159c |
| I272W                  | 10,480 ± 480 | 90 | 19,120 ± 730 | 106 |
| PDIbb′a′c′            | <50 | 6220 ± 670 | 35c |
| PDIbb′a′c′/I272W        | <50 | 560 ± 400 | 3c |

- Values are given in dpm/100 μg of extractable cell protein (mean ± S.D. for at least three experiments) and as percentages of the value obtained with the wild-type PDI. The enzyme activity generated by expressing the α(I) subunit alone, 930 ± 120 dpm/100 μg, or α(II) alone, 1160 ± 40 dpm/100 μg, was subtracted from all of the values. Significances of the differences relative to the wild type are indicated by footnotes b–d.

Resides in the a and a′ Domains of PDI Are Important for Efficient C-P4H Tetramer Assembly—Since mutations of the critical residues identified at the primary substrate binding site in the b′ domain of the full-length PDI had no inhibitory effect on C-P4H tetramer assembly, there must be additional binding sites in PDI that contribute to assembly. We have shown previously that the b′ and a′ domains of PDI fulfill the minimum requirement for functioning as the C-P4H β subunit in combination with the α(II) subunit but that the addition of the a and b domains enhances tetramer formation and is essential for the wild-type or the mutant domain construct was co-expressed with the α(I) subunit (Table I). The amount of activity obtained when the bb′a′c′ construct was co-expressed with the α(II) subunit was 35% of that obtained with the full-length PDI, which was again consistent with previous observations (18), whereas the activity generated with the I272W mutant bb′a′c′ construct was only 3% (Table I). The wild-type and mutant bb′a′c′ constructs were expressed at equal levels (Fig. 3A, lanes 10 and 11), and a faint band corresponding to an enzyme tetramer could be detected in nondenaturing PAGE by Western blotting (data not shown). The data therefore indicate that when the PDI α domain is missing, the b′ domain residue Ile²⁷ becomes critical for C-P4H tetramer assembly.

The critical residues identified at the primary substrate binding site in the b′ domain of the full-length PDI had no inhibitory effect on C-P4H tetramer assembly.
sential for PDI to form a tetramer with the α(I) subunit (18). These results, combined with the observations that the isolated a and a’ domains are able to function as efficient catalysts of thiol-disulfide exchange reactions in peptides and proteins (25), suggest that both the a and a’ domains contain substrate binding sites.

To identify such potential sites, we used sequence homology conservation among the members of the thioredoxin family to identify putative protein-protein interaction sites in the two catalytic domains a and a’. The crystal structures of the periplasmic thiol-disulfide oxidase DsbA from a variety of bacterial species reveal a hydrophobic binding groove leading to the active site (26–28). This groove includes a conserved cis-proline lying under the active site -CXX-C- sequence, which is implicated not only in substrate binding by DsbA but also by other superfamily members including thioredoxin and glutaredoxin (29, 30). Multiple sequence alignment of the thioredoxin-like domain of the DsbAs and the catalytic domains of the human PDI family allowed the identification of the residues in PDI equivalent to those that form the hydrophobic binding groove in DsbA. Based on these analyses, Leu<sup>42</sup>, Pro<sup>50</sup>, Ile<sup>68</sup>, Tyr<sup>99</sup>, and Trp<sup>111</sup> were identified in the a domain of PDI, and Leu<sup>386</sup>, Pro<sup>424</sup>, Leu<sup>426</sup>, Tyr<sup>440</sup>, and Phe<sup>452</sup> in the corresponding a’ domain residues. These results, combined with the observations that Leu<sup>42</sup>, Ile<sup>85</sup>, and Trp<sup>111</sup> are occupied by two leucines and a histidine, respectively (21). Since residue Trp<sup>111</sup> was found to be the most important one among the PDI α domain residues studied for C-P4H tetramer assembly, we examined whether the compensatory ability of the ERp57 a domain in tetramer assembly could be increased by mutation of the corresponding histidine, His<sup>103</sup>, to tryptophan. A H103W point mutation generated in the ERpα/PDIβ a’c polypeptide, which was found to be expressed in insect cells at a level corresponding to the nonmutant polypeptide (data not shown) was then co-expressed with the human α(I) and α(II) subunits in insect cells. The H103W mutation did not increase C-P4H activity in the case of the α(I) subunit (Table II), but in the case of the α(II) subunit, this activity increased from 37 to 48% (i.e. by 25%) (Table II). This suggests that the difference between the a domains of ERp57 and PDI in C-P4H assembly can in part be attributed to the Trp<sup>111</sup> position of PDI.

None of the mutations generated in the a’ domain of PDI had as marked an effect on C-P4H tetramer assembly and activity as those in the a domain (Table II), but the L386W mutation did reduce the amount of activity to 44 and 81% of that obtained with the wild-type PDI when co-expressed with the α(I) and α(II) subunits, respectively (Table II). The F452W mutation also lowered the amount of enzyme activity, whereas F452I had the opposite effect (Table II). Corresponding changes were observed in the amounts of the mutant enzyme tetramers (Fig. 2, lanes 6–8). The mutations L426W and Y440W did not cause marked changes in C-P4H assembly and activity (Table II), and none of the a’ domain mutations affected the amount of recombinant PDI polypeptide expressed in insect cells (e.g. see Fig. 3A, lanes 8–9).

### Binding Sites in Three PDI Domains Contribute to C-P4H Tetramer Assembly

None of the single point mutations introduced here into the a, b’, or a’ domains of PDI fully inhibited C-P4H tetramer assembly or enzyme activity. Although the I272W b’ domain mutation alone had no effect on tetramer assembly or activity, it almost completely inhibited the assembly of active C-P4H in the absence of the a domain (Table I), suggesting that this site in the b’ domain is most probably involved in tetramer assembly. We therefore studied the effect of combining the a or a’ domain mutations with the I272W b’ domain mutation.

When the I272W mutation was combined with any of the a or a’ domain mutations, the C-P4H activities obtained were markedly reduced, ranging from 3 to 74% of those of the wild-type C-P4Hs (Table III). Furthermore, in all cases, the activities obtained were reduced by at least 35% relative to those generated with PDI polypeptides harboring only single a or a’ domain mutations (compare Tables II and III). The reductions in enzyme activity were accompanied by corresponding decreases in the amount of the C-P4H tetramers (e.g. see Fig. 2, lanes 9–12), whereas none of the double mutations affected the expression level of the PDI polypeptides (e.g. see Fig. 3B, lanes 1–5). Of the double mutations in the a and b’ domains, W111I/I272W resulted in the lowest activities, 7 and 11% of those generated by the wild-type PDI with the α(I) and α(II) sub-
units, respectively (Table III), and only about one-fifth of those obtained with the W111I mutant PDI (compare Tables II and III), whereas the most effective double mutation in the b’ and a’ domains was I272W/L386W, which reduced the activities to 3% of those in the wild-type cells (Table III).

In contrast with the additive effect seen by combining the mutation in the b’ domain with one of those in the a or a’ domains, the combination of a and a’ domain mutations did not lead to any further decrease in C-P4H activity (compare Tables II and III) or assembly (e.g. see Fig. 2, lane 13). None of the combined a and a’ domain mutations affected the expression levels of the PDI polypeptides (e.g. see Fig. 3B, lanes 6 and 7).

In all cases, except the F452I and Y99W/L426W mutations, the effect on assembly with the a(I) or a(II) subunits showed the same trends, but the effects on a(I) assembly were greater. The significance of the two apparent exceptions is unclear, but it should be noted that the F452I mutation may have a minor effect on assembly with the wild-type subunit.

The data obtained above suggest that residues in three do-

| PDI or point mutant expressed | Prolyl 4-hydroxylase activity<sup>a</sup> | With a(I) subunit | With a(II) subunit |
|------------------------------|------------------------------------------|------------------|------------------|
|                              | Activity | Percentage | Activity | Percentage |
| PDI                          |          |            |          |            |
| a domain mutants             |          |            |          |            |
| L42W                         | 4170 ± 850 | 36<sup>b</sup> | 10,750 ± 4020 | 60<sup>c</sup> |
| I85W                         | 6270 ± 3130 | 54<sup>c</sup> | 16,190 ± 3220 | 90<sup>c</sup> |
| Y99W                         | 17,850 ± 200 | 154<sup>d</sup> | 32,920 ± 710 | 183<sup>d</sup> |
| W111I                        | 3900 ± 120 | 34<sup>b</sup> | 10,320 ± 200 | 57<sup>b</sup> |
| ERp57/PDIb’b’a’c             | 1950 ± 310 | 17<sup>b</sup> | 6590 ± 1980 | 37<sup>b</sup> |
| a’ domain mutants            |          |            |          |            |
| I272W/L386W                  | 5130 ± 860 | 44<sup>b</sup> | 14,530 ± 730 | 81<sup>b</sup> |
| L426W                        | 9520 ± 530 | 82<sup>c</sup> | 18,210 ± 1820 | 101<sup>c</sup> |
| Y440W                        | 10,020 ± 130 | 86<sup>b</sup> | 22,030 ± 1100 | 123<sup>b</sup> |
| F452I                        | 22,710 ± 3750 | 196<sup>b</sup> | 20,380 ± 2830 | 113<sup>b</sup> |
| F452W                        | 7320 ± 1500 | 63<sup>d</sup> | 14,510 ± 3350 | 81<sup>d</sup> |

<sup>a</sup> Values are given in dpm/100 μg of extractable cell protein (mean ± S.D. for at least three experiments) and as percentages of the value obtained with the wild-type PDI. The enzyme activity generated by expressing the a(I) and a(II) subunits alone was subtracted from all of the values. Significances of the differences relative to the wild type are indicated by footnotes b–d.

<sup>b</sup> p < 0.001.
<sup>c</sup> p < 0.05.
<sup>d</sup> p < 0.01.

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Collagen Prolyl 4-Hydroxylase Tetramer Assembly

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Table II
Collagen prolyl 4-hydroxylase activity of Triton X-100-soluble proteins from insect cells expressing the a(I) or a(II) subunit with PDI a or a’ domain single point mutants and ERp57/PDI hybrids

| PDI or point mutant expressed | Prolyl 4-hydroxylase activity<sup>a</sup> | With a(I) subunit | With a(II) subunit |
|------------------------------|------------------------------------------|------------------|------------------|
|                              | Activity | Percentage | Activity | Percentage |
| PDI                          |          |            |          |            |
| a and b’ domain double mutants |          |            |          |            |
| L42W/I272W                   | 2260 ± 420 | 19<sup>b</sup> | 6500 ± 1560 | 36<sup>b</sup> |
| I85W/I272W                   | 3890 ± 1740 | 32<sup>b</sup> | 7680 ± 560 | 43<sup>b</sup> |
| Y99W/I272W                   | 4680 ± 2020 | 40<sup>b</sup> | 8980 ± 800 | 50<sup>b</sup> |
| W111I/I272W                  | 860 ± 300 | 7<sup>b</sup> | 1960 ± 320 | 11<sup>b</sup> |
| b’ and a’ domain double mutants |          |            |          |            |
| I272W/L386W                  | 400 ± 210 | 3<sup>b</sup> | 530 ± 200 | 3<sup>b</sup> |
| I272W/L426W                  | 2350 ± 1890 | 29<sup>b</sup> | 5230 ± 1290 | 29<sup>b</sup> |
| I272W/Y440W                  | 2990 ± 820 | 26<sup>b</sup> | 5930 ± 560 | 33<sup>b</sup> |
| I272W/F452W                  | 4380 ± 410 | 38<sup>b</sup> | 8210 ± 1870 | 46<sup>b</sup> |
| I272W/F452I                  | 7780 ± 530 | 67<sup>b</sup> | 13,210 ± 2460 | 74<sup>d</sup> |
| a and a’ domain double mutants |          |            |          |            |
| L42W/L386W                   | 4910 ± 280 | 42<sup>b</sup> | 9190 ± 2560 | 51<sup>b</sup> |
| I85W/L426W                   | 7820 ± 2430 | 67<sup>d</sup> | 15,530 ± 3870 | 86<sup>d</sup> |
| I85W/F452W                   | 7390 ± 960 | 64<sup>c</sup> | 17,600 ± 3270 | 98<sup>c</sup> |
| Y99W/L426W                   | 9370 ± 320 | 81<sup>d</sup> | 29,160 ± 3320 | 162<sup>d</sup> |
| Y99W/F452W                   | 11,020 ± 930 | 95<sup>d</sup> | 18,220 ± 1980 | 101<sup>d</sup> |
| W111I/F452I                  | 7120 ± 1900 | 61<sup>d</sup> | 12,990 ± 4560 | 72<sup>d</sup> |

<sup>a</sup> Values are given in dpm/100 μg of extractable cell protein (mean ± S.D. for at least three experiments) and as percentages of the value obtained with the wild-type PDI. The enzyme activity generated by expressing the a(I) and a(II) subunits alone was subtracted from all of the values. Significances of the differences relative to the wild type are indicated by footnotes b–d.

<sup>b</sup> p < 0.001.
<sup>c</sup> p < 0.01.
<sup>d</sup> p < 0.05.
I272W/Y440W, and Y99W/I272W/Y440W combinations resulting in lower activities than any of their constitutive double mutant combinations (compare Tables III and IV). The triple mutants including W111I or L386W did not cause any further decrease in the already very low levels of tetramer assembly and activity obtained with the wild-type PDI. The enzyme activity generated by expressing the α(I) and α(II) subunits alone was subtracted from all of the values. *p* < 0.001 (significance of the difference relative to the wild type).

Our results suggest that C-P4H tetramer assembly results from interaction of the α subunit with at least three distinct sites in PDI, in the a, b', and a' domains. The interaction sites found in the a and a' domains appear to act independently from each other but cooperate with that in the b' domain, which is identical to the site identified earlier as being involved in the binding of short peptide substrates (15).

The I272W Mutation in the b' Domain of Human PDI Completely Inhibits Its Assembly with a C. elegans PDI Subunit to Form an Active C-P4H Dimer—The major C-P4H form in the nematode *C. elegans* is a mixed tetramer consisting of two different α subunits, PHY-1 and PHY-2, and two molecules of PDI (31). In the absence of one of the α subunits, the remaining one assembles into an active dimer with PDI, which can either fully or partially compensate for the lack of the mixed tetramer (31). Since recombinant *C. elegans* PHY-1 forms an active C-P4H dimer very efficiently with human PDI when co-expressed in insect cells (32), we next studied the effects of some of the single and double mutations in human PDI on the assembly and activity of the PHY-1/human PDI dimer.

In contrast to the human C-P4H tetramers, assembly of the PHY-1/human PDI dimer was significantly reduced by the mutations F258W and F258A in the b' domain and completely abolished by the I272W mutation (Table V). Interestingly, the mutation I272A, which also significantly reduced 3-somatostatin binding (15), did not significantly reduce the amount of P4H activity generated (Table V). The S256D mutation resulted in a higher amount of C-P4H activity (Table V), as in the case of the human C-P4H tetramers (Table I).

The effects of the single a and a' domain mutations on the assembly of an active PHY-1/human PDI dimer were very similar to those observed with the type I human C-P4H tetramer (compare Tables II and V). The only exceptions were the Y99W mutant, which resulted in no change, and the L426W mutant, which reduced PHY-1/human PDI dimer assembly and activity significantly (Table V). As expected, no C-P4H activity was generated when the PHY-1 polypeptide was co-expressed with double mutant PDI polypeptides that contained the b' domain mutation I272W in addition to a mutation in the a or a' domain, as the former alone abolished dimer assembly (Table V). Most combinations of the a and a' domain point mutations appeared to have an additive effect in reducing the assembly and activity of the PHY-1/human PDI dimer (Table V) by contrast with their effect on the assembly of human C-P4H tetramers (Table II).
Mutant PDI Polypeptides Do Not Show a Significant Alteration in Their Structure—To ensure that the effects on C-P4H assembly observed here were not due to gross structural effects of the PDI mutations, wild-type and mutant polypeptides were compared by means of a variety of biophysical analyses.

It has previously been shown that mutations that destabilize the $a'$ domain of PDI (e.g. F449R) result in a decrease in $\Delta$-somatostatin binding by the $b'$ domain (33). Accordingly, all 15 single and double $a$ and $a'$ domain mutants of PDI generated here were screened for $\Delta$-somatostatin binding. They all showed binding with levels approaching those of the wild-type polypeptide, except for those bearing the W111I mutation, which showed very significant decreases in the levels of binding (Fig. 4, lane 5). Control experiments showed that peptide binding by the $b'$ domain was significantly decreased in all of the polypeptides that included the I272W mutation (data not shown), which is consistent with the reported effects of this mutation on the substrate binding site in the $b'$ domain (15).

The full-length PDI mutants expressed in E. coli were screened for protease stability. Proteinase K digestions under stringent conditions revealed that most mutations had no effect on protease resistance but that four mutations reduced the stability of the protein, the magnitudes of the effects being W111I > F452I > L386W > F452W (data not shown). The destabilizing effects appeared to be additive when these mutations were combined. Even in the case of the most effective destabilizing mutation, W111I, however, some undigested protein was left under these stringent conditions. To examine further the possible degree of destabilization, titration curves of proteinase K concentration versus digestion were created for the W111I and I272W/F452I mutants. The results indicated that the I272W/F452I mutant was only marginally less stable than the wild-type polypeptide and the W111I mutant was only slightly less stable (Fig. 5). Further protease resistance tests performed using V8 protease indicated that only the W111I mutation led to decreased stability and that this effect was marginal relative to the wild-type (data not shown).

The full-length wild-type PDI and three mutants, W111I/I272W/F452I, L42W/I272W/L386W, and Y99W/I272W/Y440W, were then purified from recombinant expression in E. coli by immobilized metal affinity chromatography, exploiting N-terminal His tags (data not shown). CD spectra in the far UV region of the L42W/I272W/L386W and the Y99W/I272W/Y440W were essentially identical to the wild-type protein, whereas the W111I/I272W/F452I showed a slight difference in spectra, suggesting a small change in secondary structure (Fig. 6).

Taken together, these analyses indicate that all of the mutant PDI polypeptides generated, excluding those containing W111I, have essentially the same structure as the wild type and suggest that the structures of the W111I mutants are only marginally different from that of the wild-type protein, as supported by the C-P4H assembly and enzyme activity results (Fig. 2, lane 5; Tables II and III).

**FIG. 4.** Analysis of ability of the PDI single point mutants to bind the 14-amino acid peptide $\Delta$-somatostatin. E. coli expressing wild-type and mutant human PDI were lysed and incubated together with radiolabeled $\Delta$-somatostatin and the homobifunctional cross-linker disuccinimidyl glutarate. Samples were then run on 12.5% SDS-PAGE, and an autoradiograph was taken. The samples on the lanes are as follows: PDI (lane 1), L42W (lane 2), 185W (lane 3), Y99W (lane 4), W111I (lane 5), L386W (lane 6), L426W (lane 7), Y440W (lane 8), F452W (lane 9), and F452I (lane 10).

**FIG. 5.** Titration curves for the proteinase K stability of PDI mutants. E. coli expressing wild-type and mutant human PDI were lysed and incubated together with increasing concentrations of proteinase K. The digestion was then quenched, and the samples were run on 12.5% SDS-PAGE and Western blotted using an anti-PDI antibody. The position of full-length PDI is marked with an asterisk. A, wild-type PDI; B, the W111I mutant; C, the I272W/F452I mutant. The proteinase K concentrations on the lanes were 0 (lane 1), 1 (lane 2), 5 (lane 3), 10 (lane 4), 20 (lane 5), and 30 mg/ml (lane 6).

**DISCUSSION**

It has been shown previously that the $b'$ domain of PDI is sufficient for the binding of short peptide substrates and essential for the binding of larger nonnative protein substrates but that the $a$ and $a'$ domains also contribute to the binding of larger substrates (17). The data suggest that, of the three binding sites, the one in the $b'$ domain has the highest affinity. This is consistent with data on the kinetics of thiol-disulfide oxidation by the isolated $a$ domain (34), which implied that substrate binding by the $a$ domain was of low affinity, and with data showing that the $b'$ domain of PDI is essential for the catalysis of thiol-disulfide isomerization reactions (25). The substrate binding site in the $b'$ domain of PDI has recently been localized by mutagenesis studies, which demonstrated that the single mutation I272W reduced peptide binding very substantially with no discernable effect on the structure of the PDI (15). A reduction in nonnative protein binding by the I272W mutant has been reported, but significant binding was still observed, presumably via the influence of the substrate binding sites in the $a$ and $a'$ domains. Conservation of the protein/peptide-binding site within the $b'$ domain has been reported for ERp57 (35) and Wind, the *Drosophila* homologue of ERp28 (36).

The transient nature of the interaction between PDI and its substrate makes it difficult to investigate the substrate binding sites of PDI. In this work, we exploited the fact that the interaction of PDI with a human C-P4H $\alpha$ subunit to form a stable tetrameric complex can be seen to mimic the interaction of PDI with protein substrates, since the role of PDI in the C-P4H tetramer is to keep the $\alpha$ subunit soluble. We were able to show that mutations in the substrate binding site in the $b'$ domain of PDI did not reduce C-P4H tetramer assembly or activity. We then combined information previously published for the putative substrate binding sites of members of the thioredoxin superfamily to identify presumed substrate binding sites in the $a$ and $a'$ domains of PDI. Single point mutations at either of these sites resulted in a reduction in C-P4H activity and assembly, but a more significant reduction was seen when such mutations were combined with the I272W mutation in the $b'$ domain. A further additive effect was seen when mutations at multiple putative substrate binding sites in the $a$, $b'$, and $a'$ domains were combined, since these reduced P4H activity by more than 95% and reduced tetramer assembly to nondetectable levels. A combination of studies on peptide binding, protease stability, and spectroscopic analysis of protein structure revealed no discernable effect on PDI structure with the exception of a minor effect of the W111I and F452I mutations. These results, in combination with the observation that expression levels of all of the mutant PDI polypeptides in insect cells were comparable with that of the wild-type protein, indicate that the
present observations were due to a direct effect of the mutations on human C-P4H tetramer assembly and not to indirect effects on the structure of PDI.

The combination of three distinct substrate binding sites within PDI resolves a potential dichotomy regarding the functions it has to perform. To act as a catalyst of protein folding, PDI must bind each folding intermediate with relatively low affinity to allow for a bind-release cycle. In contrast, to act as a nonfolded protein. This is supported by the facts that the addition of the disulfide reductant dithiothreitol to C-P4H results in disassembly of the tetramer (37) although the cysteine residues of PDI are not required for tetramer formation (7), and site-directed mutagenesis studies have shown that two intrachain disulfide bonds in the human α1(I) subunit are required for assembly (38, 39). These data imply that PDI binds a structured α subunit with higher affinity than an unstructured one.

In contrast to human C-P4H tetramer assembly, PHY-1/human PDI dimerization was shown to be completely dependent on the substrate binding site in the b′ domain of PDI, with only minor contributions from the sites in the α and α′ domains. The b′ domain is also essential for Δ-somatostatin or mastoparan peptide binding by PDI, with no direct contributions from the sites in α and α′, whereas the binding sites of all three domains contribute to the binding of larger peptides and non-native proteins (17). The picture that emerges is that PDI-substrate binding is a complex process. The previous assumption that, of the three binding sites, the one in the b′ domain has the highest affinity seems to be an oversimplification. Rather, it is likely that for each specific substrate the binding sites in α, b′, and α′ contribute to different extents. This combination not only resolves the potential dichotomy in PDI function, but if each site has a different specificity, it also potentially gives PDI the breadth of binding specificity it needs to act as a folding catalyst for a wide range of protein substrates. In addition, this combination of three substrate binding sites provides an explanation for why ERp57 is still able to act as a thiol-disulfide oxidoreductase on a range of peptide and protein substrates (40–42) although its substrate binding site in the b′ domain has become specialized for interacting with the lectins calreticulin and calnexin (35).

PDI contains a fourth domain, the b domain, which has not been considered here. Previous studies have shown that the b domain does not contribute to the binding of peptides or non-native proteins (17) or to any of the thiol-disulfide oxidoreductase activities of PDI (25). To date, all domains that have a thioredoxin-like fold and interact with proteins whose structure has been resolved include a cis-proline at the beginning of strand βa. This cis-proline has been implicated in substructure binding for several thioredoxin superfamily members, including DsbA, thioredoxin, glutaredoxin, and glutathione-S-transferase (27–30, 43). A proline can be found in a similar position in the model structure for the b′ domain of PDI (16) and is conserved in the α, b′, and α′ domains of all of the catalytically active human PDI family members with the exception of the b′ domain of Erp72 (see Refs. 44 and 45). No modification of these proline residues was undertaken here or in any previous studies on PDI-substrate interactions, since mutation of a cis-proline residue is very likely to have a direct effect on the structure of the protein. The b′ domain is the only domain in PDI that does not have a proline at this position, and the b′ domains of the other members of the human PDI family also lack a proline residue at this position (see Ref. 22). Therefore, the b′ domain may not be involved in protein-substrate interactions. Since the b′ domain also lacks a catalytic site, it is unclear what its role in PDI function may be, unless it plays a structural role (e.g. ensuring the correct orientation of the substrate binding sites and catalytic sites in the α, b′, and α′ domains with respect to each other. Only the three-dimensional structure of a full-length catalytically active PDI family member will eventually enable us to resolve the role of the b′ domain, and since these have been impervious to crystallization attempts for over 30 years, it is likely that PDI will retain a few of its mysteries for some time yet.

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REFERENCES
1. Kivirikko, K. I., and Pihlajaniemi, T. (1998) Adv. Enzymol. Relat. Areas Mol. Biol. 72, 325–398
2. Myllyharju, J. (2003) Matrix Biol. 22, 15–24
3. Gordon, D. A., Wetterau, J. R., and Gregg, R. E. (1995) Trends Cell Biol. 5, 317–321
4. Myllyharju, J., and Kivirikko, K. I. (2004) Trends Genet. 20, 33–43
5. Pihlajaniemi, T., Helaaskoski, T., Tasanen, K., Myllyla, R., Huhtala, M.-L., Koivu, J., and Kivirikko, K. I. (1987) EMBO J. 6, 643–649
6. Vuori, K., Pihlajaniemi, T., Marttila, M., and Kivirikko, K. I. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 7467–7470
7. Vuori, K., Pihlajaniemi, T., Myllyla, R., and Kivirikko, K. I. (1992) EMBO J. 11, 4213–4217
8. Helaaskoski, T., Vuori, K., Myllyla, R., Kivirikko, K. I., and Pihlajaniemi, T. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 4392–4396
9. Annunen, P., Helaaskoski, T., Myllyharju, J., Veijola, J., Pihlajaniemi, T., and Kivirikko, K. I. (1997) J. Biol. Chem. 272, 17342–17348
10. Kuukola, L., Hieta, R., Kivirikko, K. I., and Myllyharju, J. (2003) J. Biol. Chem. 278, 47685–47693
11. Van Der Diepstraten, C., Papay, K., Bolender, Z., Brown, A., and Pickering, J. G. (2003) Circulation 108, 508–511
12. Freedman, R. B., Klappa, F., and Rudder, L. W. (2002) EMBO Rep. 3, 139–140
13. Kemmink, J., Darby, N. J., Dijkstra, K., Nilges, M., and Creighton, T. E. (1996) Biochemistry 35, 7684–7691
14. Kemmink, J., Darby, N. J., Dijkstra, K., Nilges, M., and Creighton, T. E. (1997)
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