Combinations of Protein-disulfide Isomerase Domains Show That There Is Little Correlation between Isomerase Activity and Wild-type Growth*

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Protein-disulfide isomerase (PDI) has five domains: a, b, b', a' and c, all of which except c have a thioredoxin fold. A single catalytic domain (a or a') is effective in catalyzing oxidation of a reduced protein but not isomerization of disulfides (Darby, N. J., and Creighton, T. E. (1995) Biochemistry 34, 11725–11735). To examine the structural basis for this oxidase and isomerase activity of PDI, shuffled domain mutants were generated using a method that should be generally applicable to multidomain proteins. Domains a and a’ along with constructs ab, aa’, aba’, ab’a’ display low disulfide isomerase activity, but all show significant reactive with mammalian thioredoxin reductase, suggesting that the structure is not seriously compromised. The only domain order that retains significant isomerase activity has the b’ domain coupled to the N terminus of the a’ domain. This b’a’c has 38% of the isomerase activity of wild-type PDI, equivalent to the activity of full-length PDI with one of the active sites inactivated by mutation (Walker, K. W., Lyles, M. M., and Gilbert, H. F. (1996) Biochemistry 35, 1972–1980). Individual a and a’ domains, despite their very low isomerase activities in vitro, support wild-type growth of a pdi1Δ Saccharomyces cerevisiae strain yeast. Thus, most of the PDI structure is dispensable for its essential function in yeast, and high-level isomerase activity appears not required for viability or rapid growth.

The correct formation of disulfides is essential for proper folding and export of secreted proteins from the endoplasmic reticulum. In vitro, chemical steps involving the formation and rearrangement of disulfides are usually rate-limiting during uncatalyzed oxidative folding (1, 2). Protein-disulfide isomerase (PDI), a major 55-kDa protein of the endoplasmic reticulum, catalyzes thiol-disulfide exchange reactions during protein folding that introduce disulfides and rearrange those that are incorrectly paired (1). Structurally, PDI consists of five discrete domains, each of which is structurally related to thioredoxin (3). The catalytically active domains, like thioredoxin, also contain a CXXC motif that is responsible for the active site chemistry.

The multidomain nature of PDI was initially recognized from an analysis of its amino acid sequence in which four domains, denoted a, b, b’, and a’, were identified. These four structural domains are followed by a stretch of acidic residues at the C terminus (designated c) (4). The domain boundaries were confirmed and refined by proteolytic mapping and heterologous expression of individual domains (3). The a and a’ domains are homologous both to each other (47% identity) and to thioredoxin (27% identity). Each of these domains contains an active site CGHC motif that can be reduced by mammalian thioredoxin reductase, which further supports a close structural relationship to thioredoxin (5). The b and b’ domains have similar sequences (28% identical), but show no or very low sequence relatedness to thioredoxin.

NMR structures of the isolated a and b domains provided the first detailed structural information about PDI. As expected, the structure of the a domain resembles that of thioredoxin (6), but surprisingly, the b domain also has a thioredoxin-like fold (7). Sequence homology between the b and b’ and a and a’ imply that PDI consists of four tandem domains with thioredoxin structure. Both the active sites contribute to PDI activity (8), although the two domains are not equivalent in catalytic properties (9). The isolated a and a’ domains are folded and catalytically active as oxides with activities that approach that of the full-length molecule. However, neither domain by itself exhibits significant catalysis of disulfide isomerization during the refolding of bovine pancreatic trypsin inhibitor (10).

Reconstruction of the PDI molecule from the isolated a and a’ domains results in a progressive increase in isomerase activity as more domains are added (11). A recent study shows that the b’ domain provides the principal peptide-binding site of PDI, but other domains must contribute to the binding of larger protein substrates (12). Sun et al., by contrast, report very low isomerase activity (~10%) for all constructs with one or more domains deleted, including constructs that contain the b’ domain (13). They suggest that all of the domains contribute significantly to the interactions between PDI and protein substrates.

In yeast, haploid pdi1Δ mutants are not viable (14). How-

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ever, this lethal mutation can be rescued by the expression of full-length, wild-type PDI, or a mutant PDI in which one of the two active site cysteines is replaced with serine (CGHS) (15).

Because CGHS mutants of PDI have very low oxidase activity (<2.5%) but reasonable isomerase activity (~25%), it was concluded that the isomerase activity is essential. However, recent findings involving a pathway of electron transfer from substrate proteins to PDI and then to the ERO1 protein of the ER indicate that the oxidase function of PDI dominates kinetically over the isomerase activity in yeast (16, 17).

A dominant role for PDI in ER protein oxidation would suggest that the requirement for high isomerase in vitro may not parallel structural and functional requirements for the features of the molecule that are required for efficient in vivo function.

To clarify the relationships between the domain structure of PDI, the isomerase activity, and the ability to rescue the lethal deletion of PDI in yeast, we developed a new, general method for combining protein domains that may be useful in other systems with well defined domain boundaries. We have directly compared the catalytic properties of new and previously reported constructs under the same conditions. Our data show that the catalytically inactive domains, b' and c', contribute significantly to isomerase activity only when they recapitulate the natural organization of the b'-a'-c' domains, suggesting that the a and b domains contribute little to the interactions of protein substrates with the a' domain. The inactive b and b' domains may function to ensure that the catalytic domains can function independently. Although PDI mutants with low oxidase activity rescue the pdiΔ mutation, no attempt has been made to rescue species that have normal oxidase activity but no isomerase activity. Surprisingly, the a and a' domains not only rescue the lethal PDI deletion in yeast but support wild-type growth rates, despite a very low in vitro isomerase activity. There is no correlation between in vitro isomerase activity and the ability to support wild-type growth in vivo. Either a very small amount of isomerase activity is required to support substrate isomerization in vivo or the ER environment provides additional mechanisms to supplement substrate isomerization.

**EXPERIMENTAL PROCEDURES**

**Materials**—The cloned gene for rat PDI was kindly provided by Dr. Jeffrey Edman (University of California, San Francisco, CA). Recombinant rat thioredoxin reductase containing selenocysteine was a kind gift of Dr. Elias Arner (18).

Pancreatic ribonuclease A, GSH, GSSG, 2 mM cCMP, NADPH, and carbencillin were obtained from Sigma. Isopropyl-β-D-thiogalactoside and dithiothreitol (DTT) were from Saveen Biotech AB. All other chemicals were of analytical grade or better. The yeast strain carrying the pLysS plasmid also contained the pLysS plasmid to control leak-through expression. Domain boundaries and presence of amino acid linker sequence in constructs are shown in Table I. Protein production was carried out in E. coli strain BL21 (DE3), which also contained the pLysS plasmid to control leak-through expression. Cells transformed with the appropriate pET15x vector were grown at 37 °C in LB media supplemented with 100 μg/ml carbencillin to an absorbance of 10 at 600 nm and induced by adding isopropyl-β-D-thiogalactoside to a final concentration of 1 mM. After 4 h, the cells were harvested by centrifugation, suspended in ice-cold start buffer (pH 7.5, 50 mM phosphate, 0.5 mM NaCl, and 100 mM imidazole) and disrupted by sonication. After centrifugation at 12,000 × g for 15 min at 4 °C, the supernatant was applied to a HiTrap chelating column (Amersham Pharmacia Biotech) precharged with nickel and equilibrated with start buffer. The column was washed with 10 ml of 50 mM Tris-HCl, pH 8.0, and 1 mM EDTA to remove the imidazole. Purified protein was concentrated by Diaflo (Amicon, Inc.) under N2.

**Expression of PDI Domains in Saccharomyces cerevisiae**—A PDI expression cassette was inserted into the plasmid pRS424 (22). This plasmid is a 2-μm-based multicopy yeast-bacterial shuttle vector with a TRPI yeast-selectable marker. The pRS424 plasmid was digested with NheI and EcoRI and the fragment containing the oar1 signal sequence was inserted into the vector. This vector was transformed into S. cerevisiae (21). The insertion of the plasmid was confirmed by PCR amplification using specific primers. The expression plasmids containing the BgII/BamHI fragment in the proper orientation were identified by diagnostic PCR. Domain boundaries and presence of amino acid linker sequence in constructs are shown in Table I. Protein production was carried out in E. coli strain BL21 (DE3), which also contained the pLysS plasmid to control leak-through expression. Cells transformed with the appropriate pET15x vector were grown at 37 °C in LB media supplemented with 100 μg/ml carbencillin to an absorbance of 1.0 at 600 nm and induced by adding isopropyl-β-D-thiogalactoside to a final concentration of 1 mM. After 4 h, the cells were harvested by centrifugation, suspended in ice-cold start buffer (pH 7.5, 50 mM phosphate, 0.5 mM NaCl, and 100 mM imidazole) and disrupted by sonication. After centrifugation at 12,000 × g for 15 min at 4 °C, the supernatant was applied to a HiTrap chelating column (Amersham Pharmacia Biotech) precharged with nickel and equilibrated with start buffer. The column was washed with 10 ml of 50 mM Tris-HCl, pH 8.0, and 1 mM EDTA to remove the imidazole. Purified protein was concentrated by Diaflo (Amicon, Inc.) under N2.
Combinations of PDI Domains

Correct sequence of the entire cassette including the PDI insert was verified by dideoxy sequencing.

Complementation of the pdi1ΔD Mutation in S. cerevisiae—The viability of the yeast strain YPH274 with the chromosomal copy of the PDI gene disrupted by deletion is maintained by an episomal plasmid, pCT37, that expresses wild-type yeast PDI and contains a URA3 selection marker. This strain is not viable on media containing 5-FOA, a compound that is toxic to cells expressing the URA3 gene (15). After transformation with a plasmid containing the PDI or its domains, colonies were grown on minimal medium with all amino acids except tryptophan, then induced with galactose and replated on plates to lose pCT37 plasmid and grow on 5-FOA-containing plates.

Expression of PDI Domains and Multidomain Constructs—Individual catalytic domains of PDI (a and a') and various domain combinations were constructed using a systematic genetic engineering approach that allows the directed combination of any of the PDI domains in any order. We have combined such domains in a controlled manner, but the same principle could be used to randomly reorganize DNA pieces. The system involves bordering each domain construct with in-frame BglII/BamHI sites and then linking two domains in a directed fashion that creates a hybrid BglII/BamHI site at the fusion of the domains but leaves a BamHI site in the C-terminal domain and a BglII site in N-terminal domain. Repeating this cycle of cleavage/ligation enables the combination of any of the PDI domains in a directed fashion. The in-frame, hybrid BglII/BamHI site at the domain junctions inserts six extra nucleotides, GGATCT, resulting in a glycine-serine linker between domains when the protein is expressed. Domain boundaries were based on the experiments of Darby and Creighton (10) who used a combination of expression and proteolysis to accurately establish the structural domains. In addition to those individual domains and domain constructs that have been reported previously (a, a', ab, b'a' and b'a'c (11) and abc, a'c (13)), we also produced several new, multidomain constructs, aba', ab'a', and aa' as well as the reconstruction of the entire molecule with the substitution of Gly-Ser in the linker region (abb'a'c) (Table I). The validity of the method is demonstrated by the similar activity of the reassembled PDI, abb'a'c (nPDI), compared with that of wtPDI (see below).

Recombinant proteins were expressed in E. coli and purified using a N-terminal His₆ tag (Table I). Each of the proteins could be isolated in a soluble and sufficiently pure form to characterize their catalytic properties. Fig. 2 shows the SDS-PAGE analysis of the purified single and multidomain PDI constructs used in this study.

Oxidative Folding of Reduced RNase—For each of the constructs, the activity was measured for catalysis of disulfide bond isomerization during the refolding of reduced, denatured RNase. Native disulfide formation was followed as the rate of hydrolysis of 2',3'cCMP catalyzed by the native RNase that is formed (20). Darby and Creighton failed to detect isomerase activity of the individual a and a' domains with bovine pancreatic trypsin inhibitor as the substrate (23). Although, the activity with RNase refolding is low, it is real and measurable over background isomerization at a concentration of 5 μM active sites. Low but significant isomerase activities could be measured for all of the constructs including those with multiple domains and P34H thioredoxin, a mutant thioredoxin with a PDI-like active site sequence (24).

The activities of the various domain combinations that were examined are shown in Fig. 3. The b'a'c construct is the only

FIG. 1 Cloning strategy for combining individual domains of PDI. In step 1, DNA pieces encoding two protein domains are excised from the pGEMT vector by cleavage with ApaI/BamHI or BglII/SalI. Equimolar amounts of the DNA fragments are introduced into a ligation mixture. This could generate three possible dimers (step 2), but only one with desirable orientation can be inserted into pGEMT previously digested with ApaI and SalI (step 3).

Growth Curves—The transformed cells were selected on plates without tryptophan, then induced with galactose and replated on plates containing 5-FOA. Individual colonies were picked and cultured overnight in liquid media. An equal number of cells (approximated by absorbance at 600 nm) were diluted into fresh media and the absorbance at 600 nm was measured at different time intervals.

RESULTS

Expression of PDI Domains and Multidomain Constructs—Individual catalytic domains of PDI (a and a') and various domain combinations were constructed using a systematic genetic engineering approach that allows the directed combination of any of the PDI domains in any order. We have combined such domains in a controlled manner, but the same principle could be used to randomly reorganize DNA pieces. The system involves bordering each domain construct with in-frame BglII/BamHI sites and then linking two domains in a directed fashion that creates a hybrid BglII/BamHI site at the fusion of the domains but leaves a BamHI site in the C-terminal domain and a BglII site in N-terminal domain. Repeating this cycle of cleavage/ligation enables the combination of any of the PDI domains in a directed fashion. The in-frame, hybrid BglII/BamHI site at the domain junctions inserts six extra nucleotides, GGATCT, resulting in a glycine-serine linker between domains when the protein is expressed. Domain boundaries were based on the experiments of Darby and Creighton (10) who used a combination of expression and proteolysis to accurately establish the structural domains. In addition to those individual domains and domain constructs that have been reported previously (a, a', ab, b'a' and b'a'c (11) and abc, a'c (13)), we also produced several new, multidomain constructs, aba', ab'a', and aa' as well as the reconstruction of the entire molecule with the substitution of Gly-Ser in the linker region (abb'a'c) (Table I). The validity of the method is demonstrated by the similar activity of the reassembled PDI, abb'a'c (nPDI), compared with that of wtPDI (see below).

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domain combination with high isomerization activity. At increasing concentrations of reduced RNase, the reaction reaches saturation at 30 μM (data not shown), which is similar to the behavior of a 21-kDa C-terminal PDI fragment (25). The reconstructed PDI molecule in which the natural linker between the domains has been replaced by Gly-Ser (ab’ab’c) has the same activity as wtPDI.

### Table I

**Combinations of PDI Domains**

The nomenclature adopted for the polypeptide constructs is based upon the domain structure established for human PDI but the sequence numbers refer to the rat PDI sequence. Extinction coefficient for each polypeptide was calculated using the method of Gill and von Hippel (33).

<table>
<thead>
<tr>
<th>Fragment</th>
<th>PDI residues</th>
<th>ε</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>Glu-4 to Arg-115</td>
<td>19900</td>
</tr>
<tr>
<td>a’</td>
<td>Val-352 to Gly-462</td>
<td>15460</td>
</tr>
<tr>
<td>b</td>
<td>Ala-120 to Leu-217</td>
<td>1280</td>
</tr>
<tr>
<td>b’</td>
<td>Val-219 to Lys-350</td>
<td>8490</td>
</tr>
<tr>
<td>ab</td>
<td>Glu-4 to Leu-217</td>
<td>20580</td>
</tr>
<tr>
<td>b’ab’</td>
<td>Val-219 to Gly-462</td>
<td>23850</td>
</tr>
<tr>
<td>b’ab’c</td>
<td>Val-219 to Leu-491</td>
<td>23850</td>
</tr>
<tr>
<td>a’b’</td>
<td>Val-4 to Arg-115</td>
<td>34780</td>
</tr>
<tr>
<td>a’b’c</td>
<td>Val-4 to Leu-491</td>
<td>34780</td>
</tr>
<tr>
<td>ab’b’</td>
<td>Glu-4 to Lys-350</td>
<td>38640</td>
</tr>
<tr>
<td>ab’b’c</td>
<td>Glu-4 to Leu-491</td>
<td>43250</td>
</tr>
<tr>
<td>abb’b’c</td>
<td>Glu-4 to Arg-115</td>
<td>44530</td>
</tr>
</tbody>
</table>

| Gly Ser  | Val-219 to Lys-350 Gly Ser Val-352 to Leu-491 |

### Table II

The activities of PDI individual domains and domain combinations as substrates for thioredoxin reductase. PDIa (○), PDIb’ac (●), PDIb’bc (■), PDIab (□), and PDI (▲) were incubated for 20 min at 37 °C in 120 μl of assay mixture containing 0.3 mM insulin and 0.7 mM NADPH with 30 nM of bovine thioredoxin reductase. The reactions were stopped by addition of 0.5 ml of 6 M guanidium hydrochloride, 50 mM Tris, pH 8.0, and 1 mM DTNB, and the absorbance at 412 nm was measured against an identical blank with thioredoxin reductase.

<table>
<thead>
<tr>
<th>Kₘ (μM)</th>
<th>kₐₜ (min⁻¹)</th>
<th>kₐₜ/Kₘ (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDIa</td>
<td>13.9 ± 1.4</td>
<td>3000</td>
</tr>
<tr>
<td>PDIb’ac</td>
<td>29.6 ± 1.7</td>
<td>770</td>
</tr>
<tr>
<td>PDIab</td>
<td>9.7 ± 2.2</td>
<td>1900</td>
</tr>
<tr>
<td>PDI</td>
<td>60.5 ± 8.0</td>
<td>670</td>
</tr>
<tr>
<td>PDIb’ac</td>
<td>77.6 ± 10.2</td>
<td>1100</td>
</tr>
<tr>
<td>PDI</td>
<td>13.3 ± 1.6</td>
<td>1400</td>
</tr>
<tr>
<td>PDIab’ac</td>
<td>13 ± 2.5</td>
<td>800</td>
</tr>
</tbody>
</table>

* 9 nM thioredoxin reductase was used.

**Activities of PDI and PDI Variants with Thioredoxin Reductase**—PDI is a substrate for mammalian thioredoxin reductase in vitro and will replace thioredoxin in the NADPH-dependent reduction of the disulfides of insulin (5). This assay depends on interactions between thioredoxin reductase and PDI so that high activity can be used to verify that the domain constructs have wild-type active site conformations (Fig. 4). The Kₘ and kₐₜ values were determined using 9 or 15 nM thioredoxin reductase and are summarized in Table II. The formation of SH groups of insulin in the presence of thioredoxin reductase is the result of two serial reactions: the reduction of PDI by thioredoxin reductase, and the reduction of insulin by PDI. Because the rate of the reaction was dependent on the thioredoxin reductase concentration, the reduction of PDI by thioredoxin reductase is rate-limiting. Thus, the apparent Kₘ and kₐₜ values in Table II reflect interactions with thioredoxin reductase and structural similarity to thioredoxin. All of the constructs with an a or a’ domain have activity that is very similar to wtPDI. All constructs involving the a domains have higher catalytic activity than PDI, suggesting that the a domain may be partially shielded when it is a part of a whole PDI molecule or that the presence of other domains may inhibit the binding to thioredoxin reductase.

The a domain and its extended form, ab, both have Kₘ
values for thioredoxin reductase that are similar to PDI, but the a', b'a', and b'a'c have $K_m$ values that are 2.1, 4.7, and 6.0-fold greater than PDI, respectively. The turnover number of a is around 3000 min$^{-1}$ and close to the $k_{cat}$ of rat liver thioredoxin reductase with thioredoxin (21). This is 2-fold higher than the turnover for intact PDI (1400 × min$^{-1}$) and 4-fold higher than the turnover number of the recombined PDI molecule, abb'a'c. The $k_{cat}$ for a' reveals substantial activity (55%) when compared directly to the intact PDI.

Rescue of the Lethal pdi1ΔD Mutation in S. cerevisiae—Full-length rat PDI is very efficient in rescuing yeast cells with a deleted PDI gene. PDI mutants with undetectable (<5%) oxidase activity (9) are capable of complementing the lethal null mutation, leading to the conclusion that the isomerase activity is the essential function of PDI in vivo (26). However, the converse experiment has not been performed with PDI species that have significant oxidase activity but low isomerase activity. Given the very low isomerase activity of the individual a and a' domains, it is surprising to find that these isolated domains provide sufficient activity to support viability when overexpressed. Control experiments show that all tested strains grow on Trp-deficient medium (Fig. 5), but only wtPDI, a, and a' support growth on 5-FOA medium. The vector alone or the vector containing inserts of b or b' fail to grow when the plasmid-expressing yeast PDI is selected against in 5-FOA medium.

PDI and the a and a' domains are overexpressed under the control of a galactose-dependent promoter (Gal1–10) that can be repressed with increasing concentrations of glucose. The concentration of glucose that is required to inhibit cell growth is almost identical for wild-type PDI and the a and a' domains (Fig. 6), suggesting that a comparable activity of Gal1–10 promoter is needed to supply the proper amounts of either wtPDI or the a and a' domains. Single copy plasmids yield the same results; the a and a' domains alone provide all of the essential activity of wtPDI and support wild-type growth rates.

**DISCUSSION**

PDI is thought to have evolved via duplications of genes encoding structures similar to thioredoxin. An overall structure with repetitions of thioredoxin domains is also present in several PDI homologues (27). It is clear that the multidomain structure of PDI is essential for substrate binding and for high isomerase activity. In this study, except for the b'-a'-c construct, all of the new PDI constructs with one or two domains have less than 10% of the activity of the wild-type protein similar to the thioredoxin variant P34H Trx. Thus, the presence of one or two domains is not sufficient to generate high isomerase activity in vivo. In contrast, the same constructs have activities similar to PDI or higher in reactions with thioredoxin reductase and insulin, suggesting that they are folded and have reactive active sites.

The b'a'c construct is the only one with significant isomerase activity. In fact, the isomerase activity of the b'a'c fragment is equivalent to that observed with a full-length PDI in which the N-terminal active site is inactivated by mutation (Fig. 3), suggesting that the a' active site must function locally and derive little from the presence of the a and b domains. By contrast, the a and b domains rely heavily on the C-terminal portion of the molecule. The activity of a full-length mutant, abb'a'c in which the a' active site has been inactivated by mutation is considerably higher than that of the constructs ab or abc. The assistance to the a domain is derived principally from the b' and c domains because aba', ab'a' and abc all have low activity.

The b' domain is thought to contain a peptide/protein binding site; however, other sites may contribute to the binding of larger unfolded protein substrates (11, 12). Our data suggest that the b' domain by itself provides insufficient substrate interactions to support high isomerase activity because the constructs b'a' and ab'a' have low activity.

Puig et al. reported that "weePDI" (residues 307–491) has 16% of wild-type PDI isomerase activity (25) (Fig. 3). Our b'a'c...
that the isomerase activity of b deletion of the yeast PDI1 gene (15) showing that a redox cysteines and no redox-related activity will not complement the possible discrepancy may be explained by the difference in method and substrate used to measure activity.

Constructs lacking the c domain (b’ a’ c’) also have lower isomerase activity. This is somewhat surprising because the c domain in wtPDI has been reported to contribute insignificantly to the isomerase activity of PDI (28). At least for RNase as a substrate, the acidic c region contributes substantially to substrate interactions in truncated PDI variants. Because the a-b-b’ a’ construct has wild-type activity, the interactions with c that are important to the activity of b’ a’ c’ must be replaced by other interactions that are available in the a and b domains. It is clear that the ability of various PDI domains to contribute to high isomerase activity is a function of context rather than the property of any individual domain.

The multidomain structure of PDI is important to the ability of the molecule to provide high isomerase activity in vitro. Surprisingly, this conclusion does not extend to the molecule in vivo. Because pdiΔ yeast transfected with either the a or a’ domain grow at rates that are comparable with strains transfected with the wild-type PDI, most of the PDI structure is dispensable for fulfilling its essential function at least in S. cerevisiae under conditions of laboratory culture. It is unlikely that the amounts of a and a’ that are expressed greatly exceed the amount of wtPDI because all are repressed at approximately the same level of glucose. If a higher expression level of one of the constructs were required to support growth, all growth inhibition would be expected at lower concentration of glucose (Fig. 6). Thus, the a and a’ domains with only 3–4% of the isomerase activity of wtPDI can provide the essential function(s) of PDI and support wild-type growth rates. Except for full-length wtPDI with at least one intact active site, no other PDI variants support normal growth rates (29). Chivers et al. found that the thioredoxin variant P34H Trx, which has similar isomerase activity as the a or a’, would rescue the PDI-null mutation; however, the growth rate is significantly below wild-type (30).

PDI is a very abundant ER protein; its concentration in the ER is estimated at 0.2–0.5 mM (31). PDI with no active site cysteines and no redox-related activity will not complement the deletion of the yeast PDI1 gene (15) showing that a redox function of PDI is required. If only a small fraction (<5%) of the isomerase activity is necessary for wild-type growth, the requirement for high ER concentration of PDI is likely to be related to the capacity of the oxidative pathway where electrons are passed from substrate protein thiols via PDI to the protein ERO1 (17). Both a and a’ are as active as wtPDI in incorporating a disulfide bond into a model peptide corresponding to a natural disulfide in bovine pancreatic trypsin inhibitor (10), implying that a and a’ could fulfill the substrate oxidase requirements in the ER. That a and a’ support wild-type growth rates also suggests that they can be oxidized by ERO1 at a rate that is sufficiently high to meet the oxidative requirements encountered in protein secretion.

Reoxidation of PDI by ERO1 seems to be a kinetically efficient reaction because all of the PDI is in the oxidized form when trapped in vivo with thiolalkylating agents (17). This would promote fast incorporation of disulfide bonds and might be equally well fulfilled by individual a or a’ domain as by wtPDI. However, because isomerization requires a reduced PDI active site (9), extensive oxidation of a or a’ would decrease the available isomerase activity in vivo even more. It has been shown that cellular glutathione does not play a direct role in protein disulfide bond formation but contributes net reducing equivalents to the ER (32). These reducing equivalents might reduce oxidized PDI to the dithiol state thus regenerating its isomerase activity. The low (<5%) in vitro activities of a and a’ were measured under optimal redox conditions where PDI is present in nearly equal proportions of oxidized and reduced active sites. How the in vivo requirements for isomerase and oxidase activities are balanced with the amount of PDI protein and the redox states of the PDI active sites will require further experiments.

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