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′, ab′, ab′a′ display low disulfide isomerase activity, but all show significant reactivity 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 pdiΔSaccharomyces 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 oxidases 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 pdiΔΔ 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 requirements 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 pdi1Δ 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 PDI1 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 vitro 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, 2,3’ cCMP, NADPH, and carbenicillin 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 Δpdi1 null mutation was obtained from Ron Raines (Univ. of Wisconsin, Madison, WI) (15).

**Methods**—Protein concentrations were determined spectrophotometrically at 280 nm using the extinction coefficients listed in Table I. Reduced RNase (10 mg) was prepared by incubating with DTT (130 mM) in 0.1 M Tris-HCl, 2 mM EDTA, 6 mM guanidine-HCl, pH 8.0, for 1 h at 37 °C. After incubation, the pH was adjusted to 4.0 with glacial acetic acid, and the protein was separated from excess DTT and buffer by gel filtration (PD10, Amersham Pharmacia Biotech) in 10 mM HCl. Reduced RNase was stored at −20 °C in aliquots. Thiol content was determined according to (19). The formation of active RNase was measured spectrophotometrically by monitoring hydrolysis of the RNase substrate, cCMP, at 296 nm (20). Each sample cuvette contained 4.5 mM cCMP, 1 mM GSH, 0.2 mM GSSG, 100 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0–40 μM RNase, and 1 μM PDI or 5 μM PDI variants. The assay was performed at 25 °C and initiated by the addition of RNase. The concentration of native RNase at any time was calculated from the first derivative of the absorbance with respect to time and corrected for the depletion of cCMP and the product inhibition of RNase by CMP. The initial velocity of RNase folding was determined from the steady-state slope of active RNase concentration versus time after the lag period.

Reactions with thioredoxin reductase were carried out essentially as described by Luthman and Holmgren (21). The incubation mixture contained 0.3 mM insulin, 86 mM HEPES, pH 7.6, 3.3 mM EDTA, 0.7 mM NADPH and PDI or PDI domains in a final volume of 120 μl. The reaction was started by the addition of thioredoxin reductase to a final concentration of 9–30 ng. The reaction was stopped after 20 min by the addition of 0.5 ml of 6 μg guanidine HCl, 50 mM Tris-HCl, pH 8.0, containing 1 mM DTNB, and the concentration of SH groups was determined at 412 nm.

**Cloning of the PDI Domains**—DNA sequences corresponding to different domains of PDI were amplified by the polymerase chain reaction (PCR) using the rat PDI gene as template. All primers to the 5’ terminus of the domain sequence contained a BamHI restriction site, and all primers to the 3’ terminus contained a BamHI restriction site. The PCR products were cloned into the pGEM-T vector (CLONTECH, Stockholm, Sweden). The strategy for combining DNA fragments together in an appropriate order is shown in Fig. 1. The pGEM-T vector, carrying the DNA-encoding domain that was to be on the N terminus of the expressed protein, was excised with ApaI and BamHI, while the pGEM-T vector, harboring DNA fragment encoding the domain that was to be at the C terminus of the expressed protein, was excised with BglII and SstI. The fragments were gel-purified pGEM-T vector previously digested with ApaI and SstI. The mixture was incubated with T4 ligase (Promega) at 4 °C overnight and then used to transform Escherichia coli DH5α. Only domain combination linked head to tail will circularize the pGEM-T vector enabling the growth of DH5α in the presence of ampicillin. Such constructs generate proteins with a space of two amino acids, glycine and serine between the two domains. To produce a fusion protein with a’-a, the above procedure was repeated. The sequences of all individual domains were confirmed by dideoxy sequencing and found to be free of mutations. The appropriate linkage of combined domains was verified by diagnostic PCR.

**Protein Expression and Purification from E. coli**—A vector for the expression of PDI domain and domain combinations (pET15xr) was constructed from pET15b (Novagen). Site-directed mutagenesis (QuickChange™ Site-directed Mutagenesis Kit, Stratagene) was used to insert an extra nucleotide, A, into the Xhol site to place the BamHI site in-frame with the rest of the coding sequence. In addition, a stop codon, TAG, was added immediately after the BamHI site. The mutant plasmid was digested with NcoI and EcoRI, and the 390-bp fragment carrying the mutation was ligated back into the corresponding site of a pET15b previously cleaved with NcoI and EcoRI. The sequence of the 390 bp was confirmed by dideoxy sequence analysis.

For expression of individual PDI domains and domain combinations, the constructed gene segment was excised from the pGEM-T vector by BglII–BamHI and cloned into pET15xr previously digested with BamHI. The expression plasmids containing the BglII–BamHI fragment in the proper orientation were identified by diagnostic PCR. Domain borders 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 pET15xr vector were grown at 37 °C in LB media supplemented with 10 μg/ml carbenicillin 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, 1 M NaCl, 0.5% NaCN 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 start buffer and then with 5 ml of elution buffer containing 300 mM imidazole. Fractions of the eluate were analyzed on a 20% SDS-PAGE under reducing condition, containing the protein was pooled and dialyzed against 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 TRP1 yeast-selectable marker. The pRS424 plasmid was digested with BamHI and SstI and those containing the gene were pooled and dialyzed against 50 mM Tris-HCl, pH 8.0, and 1 mM EDTA, which also contained the pLysS plasmid to control leak-through expression. The plasmid was transformed into the yeast strain, which contained the plasmid pRS424, and colonies were selected on plates lacking leucine, tryptophan, and histidine. The plasmid was PCR-amplified to contain a 5′-BamHI site and a 3′-BglII site to permit directional, in-frame insertion into the expression plasmid. The
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).

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, an 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 (TRP selection media) and in the presence of galactose to induce PDI expression. Several individual colonies that survived this selection were streaked onto 5-FOA (1 mg/ml 5-FOA) plates in TRP selection media and in the presence of galactose to lose pCT37 plasmid and grow on 5-FOA-containing plates.

Growth Curves—The transformed cells were selected on plates without tryptophan, then induced with galactose and plated on N-terminal His$_6$ 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 mM 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
TABLE I

Combinations of PDI Domains

| Domain Combination | Activity (%) |
|--------------------|--------------|
| a                  | 100          |
| a'                 | 100          |
| b                  | 100          |
| b'                 | 100          |
| ab                 | 9.7          |
| b'a                 | 10.2         |
| b'a'              | 10.2         |
| ab'                 | 10.2         |
| ab'a                | 10.2        |
| ab'b'              | 10.2         |
| ab'b'c              | 10.2        |

**TABLE II**

| Domain Combination | Activity (%) |
|--------------------|--------------|
| a                  | 100          |
| a'                 | 100          |
| b                  | 100          |
| b'                 | 100          |
| ab                 | 9.7          |
| b'a                 | 10.2         |
| b'a'              | 10.2         |
| ab'                 | 10.2         |
| ab'a                | 10.2         |
| ab'b'              | 10.2         |
| ab'b'c              | 10.2        |

**FIG. 2.** SDS-PAGE analysis of the PDI single and multidomain polypeptide constructs used in this study. Low molecular mass makers (kDa) are indicated on the left.

**FIG. 3.** Effect of PDI and PDI variants on refolding of reduced RNase. Activity is based on the measurements with 8 μM RNase and is divided by concentration of enzymes. 100% corresponds to the activity of wtPDI corrected for the presence of two catalytic sites. XaX represents an active site in which the catalytic CGHC motif has been inactivated by mutation to SGHS. Data from Lyles and Gilbert (34), data from Koivunen et al. (28), data from Walker et al. (9), data from Puig et al. (25), data from Sun et al. (13).

**FIG. 4.** PDI, PDI individual domains, and domain combinations as substrates for thioredoxin reductase. PDIa (○), PDIb’a’c (□), PDIa’ (■), PDIb’ (▲), 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 mM 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 III**

| Domain Combination | Activity (%) |
|--------------------|--------------|
| a                  | 100          |
| a'                 | 100          |
| b                  | 100          |
| b'                 | 100          |
| ab                 | 9.7          |
| b'a                 | 10.2         |
| b'a'              | 10.2         |
| ab'                 | 10.2         |
| ab'a                | 10.2         |
| ab'b'              | 10.2         |
| ab'b'c              | 10.2        |

**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 kcat 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 kcat 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 \( \times \) min\(^{-1}\)) and 4-fold higher than the turnover number of the recombined PDI molecule, abb’a’. 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.

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 \( b’a’c \)
construct (residues 219–491) is based on boundaries defined structurally by Darby (3). The difference compared with weePDI is 88 amino acids. Thus the N-terminal portion of the b’ domains is important for activity. This importance is further supported by data published by Sun et al. (13) who reported that the isomerase activity of b’a’c with a deletion of 33 residues at the N terminus of b’ domain (residue 219–419) is 6% of wild type. Although, their b’a’c construct is less truncated compared with weePDI, it displays weaker isomerase activity. The possible discrepancy may be explained by the difference in method and substrate used to measure activity.

Constructs lacking the c domain (b’a, ab’a’) 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, 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. (13) 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 method and substrate used to measure activity. The possible discrepancy may be explained by the difference in method and substrate used to measure activity.

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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, 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. (13) 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).

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