Protein disulfide isomerases exploit synergy between catalytic and specific binding domains

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Protein disulfide isomerases (PDIs) catalyse the formation of native disulfide bonds in protein folding pathways. The key steps involve disulfide formation and isomerization in compact folding intermediates. The high-resolution structures of the a and b domains of PDI are now known, and the overall domain architecture of PDI and its homologues can be inferred. The isolated a and a' domains of PDI are good catalysts of simple thiol–disulfide interchange reactions but require additional domains to be effective as catalysts of the rate-limiting disulfide isomerizations in protein folding pathways. The b' domain of PDI has a specific binding site for peptides and its binding properties differ in specificity between members of the PDI family. A model of PDI function can be deduced in which the domains function synergically: the b' domain binds unstructured regions of polypeptide, while the a and a' domains catalyse the chemical isomerization steps.

Introduction

The lumen of the endoplasmic reticulum (ER) is a compartment specialized for protein folding; proteins destined for secretion, or for compartments accessed via the secretory pathway, enter the ER in an unfolded state and only exit once they are correctly folded and assembled. Protein folding in this context is associated with the formation of native disulfide bonds, facilitated by the ubiquitously expressed enzyme protein disulfide isomerase (PDI, EC 5.3.4.1) (Freedman *et al.*, 1994; Ferrari and Soling, 1999; Freedman and Klappa, 1999) and by its more recently discovered homologues, including P5, Erp72, Erp57, PDlp and PDlr. PDI was the first catalyst of protein folding to be identified (Goldberger *et al.*, 1963), but many questions remain unanswered about its mechanism of action. These are difficult to address because of the complexity of the physiological substrates and the absence of a complete three-dimensional

structure for any PDI. This review highlights advances made within the past 3–4 years; these arise primarily from improved structural information, from the resultant ability to generate domains or domain combinations as recombinant constructs and from a better analysis of catalytic function derived from using assays reflecting different aspects of the enzyme's activity.

The structural organization of PDI

Since the first PDI cDNA was sequenced (Edman *et al.*, 1985), internal sequence homologies within the protein have been recognized and a multi-domain protein architecture proposed. When recent experimental data (proteolysis of native PDI and characterization of recombinant fragments) is combined with bioinformatic approaches, the model that emerges is of PDI comprising four structural domains, **a**, **b**, **b'** and **a'**, plus a linker region between **b'** and **a'** and a C-terminal acidic extension (Figure 1) (Kemink *et al.*, 1997; Freedman *et al.*, 1998). The homologous **a** and **a'** domains of PDI, which contain the active-site motif -WCGHC-, show significant sequence identity to thioredoxin, a small protein involved in many cytoplasmic redox functions (Martin, 1995). The single domain members of the thioredoxin superfamily (thioredoxins and glutaredoxins) all have the same characteristic fold, an α/β fold, with the structure $\beta\alpha\beta\alpha\beta\beta\alpha$. The central core is made up of a five-stranded β -sheet, with all of the strands except β_4 being parallel, surrounded by four α -helices. The WCxxC active-site motif, which is redox active and converts between the dithiol and disulfide forms, is found in an exposed turn linking β_2 to α_2 . The sulfur atom of the more N-terminal Cys residue is at the N-terminal pole of the α -helix and is exposed at the surface of the molecule, while the sulfur atom of the more C-terminal Cys residue is buried behind it. The core thioredoxin structure, $\beta\alpha\beta\alpha\beta\beta\alpha$, can also be detected in other members of the thioredoxin superfamily,

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Fig. 1. Domain structure of PDI. The structure and boundaries presented are based on NMR-derived structural information for domains **a**, **b** and **a'** (Kemink *et al.*, 1996, 1997; Dijkstra *et al.*, 1999), homology modelling of domain **b'**, data on the limited proteolysis of intact PDI (Freedman *et al.*, 1998) and data on the properties of a range of 'putative domain' recombinant constructs. Residue numberings are for mature human PDI. Properties of the **a**, **a'** and **b'** domains are described in the text; the role of the **b** domain is not yet fully specified. The **c** region is highly acidic and contains the -KDEL motif for ER retention, while the **x** region is a linker of undefined structure and function.

including DsbA (a bacterial periplasmic oxidase), and in a number of enzymes involved in glutathione or sulfur metabolism (Martin, 1995). In all members of the thioredoxin superfamily, the redox potential of the active site, i.e. the equilibrium between the dithiol and disulfide states, is dependent mainly on the stabilization of the thiolate forms of the dithiol species, that is on the pK_a values for the two cysteine residues in each active site. Since the redox equilibrium of the active site in cellular conditions determines the physiological capability of the enzyme (oxidase, reductase or isomerase), the residues that influence these pK_a values will directly influence function. In the case of DsbA, there has been considerable focus on the residues which lie between the active-site cysteines, and it is clear that these influence redox potential (Grauschopf *et al.*, 1995). However, residues and other structural features that lie distant in the primary structure may be close in the tertiary structure, and these may also have a direct influence on the pK_a values of the active-site cysteines (Gane *et al.*, 1995). Such effects may be intra- or interdomain, but no detailed analysis has yet been carried out for PDI because its full-length structure is not known.

NMR studies on the recombinant **a** domain of human PDI have confirmed that it is a genuine structural domain with the thioredoxin fold (Figure 2) (Kemink *et al.*, 1996). In addition to adopting the same overall fold, the **a** domain shares many common features with thioredoxin: (i) the active-site motif is located at the N-terminus of helix α_2 , which is distorted by a proline residue; (ii) the peptide bond before the proline residue at the N-terminus of β_4 is in the *cis* formation; and (iii) there is a buried acidic residue, Glu30, in an analogous position in the **a** domain of PDI to that of Asp26 in thioredoxin, which has been postulated to play a role in the redox properties of the protein (Martin, 1995). No high-resolution structure of the **a'** domain has yet been derived, although preliminary NMR data and secondary structure assignment confirm the similarity of the overall fold to that of **a** (Dijkstra *et al.*, 1999).

The **b** and **b'** domains show significant sequence similarity to each other but no obvious similarity to thioredoxin or to the **a** domain. Nevertheless, NMR analysis has revealed that the **b** region also forms a domain with the thioredoxin fold (Kemink *et al.*, 1997), but the characteristic thioredoxin-like active site has been deleted and other residues associated with redox properties have been replaced. The sequence similarity of **b'** to **b** implies that **b'** also has the thioredoxin fold.

Functional characterization of PDI and its domains

Thioredoxin and some of its simple homologues have been thoroughly characterized as dithiol-disulfide oxidoreductants and catalysts of thiol-disulfide interchange. Redox assays using simple peptide substrates show that the isolated **a** and **a'** domains of PDI are characteristic members of the thioredoxin superfamily, intermediate in properties between the strongly reducing thioredoxin and the strongly oxidizing DsbA, enabling them to act both as reductases and as oxidases depending on the substrate and on the redox environment (Zapun *et al.*, 1993; Darby and Creighton, 1995a,b). In contrast, the activity of full-length PDI is not simply the sum of the activities of the isolated **a** and **a'** domains, suggesting that other parts of PDI are required for its full range of activities. This effect is most pronounced for reactions involving complex disulfide isomerizations in substrates with substantial structure; these reactions involve both thiol chemistry and conformational change. To examine these effects in detail, assays have exploited the *in vitro* folding pathway of the bovine pancreatic trypsin inhibitor (BPTI), a small single domain protein with three intramolecular disulfides (Goldenberg, 1992; Weissman and Kim, 1993; Creighton *et al.*, 1995). The folding pathway of BPTI involves isomerizations between a number of protein species containing two disulfide bonds (Weissman and Kim, 1993; Creighton *et al.*, 1995). In these isomerizations, the substrate undergoes partial unfolding of a compact conformation, thiol-disulfide interchange in the partially unfolded state and relaxation to a more native-like state. Similar isomerizations are rate-determining in the folding pathways of other model disulfide-bonded proteins such as lysozyme and ribonuclease. While PDI is able to catalyse these complex isomerizations dramatically (Darby *et al.*, 1998), the isolated **a** and **a'** domains are not able to do so (Darby and Creighton, 1995c).

Using a combination of these assays for the study of defined transitions (oxidation, reduction and isomerization) and a series of PDI constructs that included nearly every linear combination of domains, the contribution of individual domains to the activities of PDI was defined (Darby and Creighton, 1995c; Darby *et al.*, 1998). This showed that simple thiol-disulfide

Fig. 2. Structures of the isolated **a** (A) and **b** (B) domains of human PDI. Ribbon diagrams of the backbone structures as determined by NMR (Kemink *et al.*, 1996, 1997). Both domains show the characteristic thioredoxin fold despite a low degree of sequence similarity. For the **a** domain, the active-site cysteine residues are shown in grey and yellow.

chemistry only requires the **a** or **a'** domains, that simple isomerization requires one of these in a linear combination of domains that includes **b'**, while complex isomerization (isomerizations that would require substantive conformational change in the substrate as well as thiol–disulfide chemistry) requires all four of these PDI domains (but not the C-terminal acidic extension). From these results it is clear that there is synergic action in catalysis between the **a** and **a'** domains, and indeed with the **b'** domain, despite the fact that **b'** cannot participate directly in thiol–disulfide exchange since it does not contain a CxxC active-site.

Catalysis of these complex isomerizations implies that full-length PDI must be able to stabilize a partially unfolded conformation of the protein substrate, presumably by binding effectively to side chains or the backbone of one or more regions of polypeptide that are exposed in the partially unfolded state. We have explored this ability of PDI by developing binding assays based on chemical cross-linking for peptides and unfolded polypeptides; preliminary work showed that these assays reflect saturable and reversible binding to a site specific for peptides, unstructured polypeptides and misfolded proteins—presumably a recognition site for unfolded regions of protein substrates (Klappa *et al.*, 1997). Only domain constructs of PDI that included the **b'** domain were found to bind these substrates. The isolated **b'** domain of PDI was essential and sufficient for the binding of small peptides (Klappa *et al.*, 1998a). However, larger peptide or non-native protein substrates required at least **b'a'c** in order to bind these substrates (Klappa *et al.*, 1998a). Thus, it appears that the **b'** domain of PDI provides the principal peptide binding site of PDI, but that all domains contribute to the binding of larger substrates such as non-native proteins. These observations explain the apparent importance of the **b'** domain in the catalysis of complex isomerization reactions by PDI, suggesting that it holds the substrate protein in a partially unfolded conformation while the catalytic sites act synergically to perform the chemical processes of thiol–disulfide exchange.

Specificity of function analysed by comparison between PDIs

During the past decade, several gene products with clear similarity to PDI have been described in higher eukaryotes, specifically PDlp (Desilva *et al.*, 1996), ERp57 (Oliver *et al.*, 1997), ERp72 (Mazzarella *et al.*, 1990), PDlr (Hayano and Kikuchi, 1995) and ERp5 (Lundstrom-Ljung *et al.*, 1995). All of the members of the PDI family identified to date are probably localized to the ER, and all of those tested have activity in thiol–disulfide exchange assays. No systematic comparative study has been conducted to quantify the relative abundance of all of the PDI family members, but from the numerous reports on individual family members or tissue types it is clear that (i) tissues that actively secrete large amounts of disulfide-bonded proteins have the highest overall expression of PDIs; (ii) the relative levels of the PDI family members vary greatly between different tissue types, between different cell types within the same tissue and with the physiological state of the cell; and (iii), overall, PDI appears to be the most highly expressed member of the family.

Although no direct structural information is available, the sequence homologies between PDI family members allow prediction of their domain organization (Figure 3). PDI, PDlp and ERp57 all share a similar organization, with a linear

sequence of four thioredoxin-like domains in an **a-b-b'-a'** pattern (Figure 3). The strong overall homology between these three proteins suggests that they share a similar function, but there are significant sequence differences in the **b'** domains, suggesting that PDlp and ERp57 are isoforms of PDI with specialized substrate binding properties. This hypothesis is supported by data now emerging (Klappa *et al.*, 1998b; High *et al.*, 2000; Ruddock *et al.*, 2000; Lindquist *et al.*, 2001).

In contrast to other members of the PDI family found in higher eukaryotes, PDlp shows very specific tissue distribution, being exclusively expressed in the exocrine pancreas (Desilva *et al.*, 1997). This suggests that PDlp may be involved in the folding of only a subset of secreted proteins (e.g. pancreatic zymogens) and might show a restricted substrate specificity *in vitro*. Using cross-linking and competitive binding studies, we have identified the motif defining the specificity of binding of peptides and other small ligands by PDlp (Ruddock *et al.*, 2000; Klappa *et al.*, 2001). PDlp binds specifically to peptides that include either a Tyr or a Trp residue (except where the Tyr or Trp is C-terminal or where there is an adjacent negatively charged residue).

The sequence motif for binding to PDI is less clearly defined than that for PDlp. However, its binding specificity may be reflected in another property of PDI, namely that, in addition to its direct role in native disulfide bond formation, PDI is an obligatory component of the hetero-oligomeric enzymes prolyl-4-hydroxylase (P4H) and microsomal triglyceride transfer protein (Wetterau *et al.*, 1991). P4H is an $\alpha_2\beta_2$ tetrameric enzyme involved in the post-translational modification of procollagen in the ER (Kivirikko and Pihlajaniemi, 1998). In the P4H holoenzyme, the β subunits (PDI) function to maintain the α subunits in an active form within a soluble complex. We have shown recently that a PDI **b'-a'** domain construct fulfils the minimum requirement for function as a subunit of P4H (Pirneskoski *et al.*, 2001). Since there is good evidence that the active-site cysteines in the **a** and **a'** domains of PDI are not essential for the assembly or the activity of the holoenzyme (Vuori *et al.*, 1992), this strongly suggests that it is the **b'** domain of PDI, and specifically its binding site for polypeptide substrates, that plays the key role.

ERp57, like PDI, shows a ubiquitous distribution in a wide variety of cell types (Marcus *et al.*, 1996). However, ERp57 cannot substitute for PDI as the β subunit of P4H (Pirneskoski *et al.*, 2001), and expression of the human ERp57 gene, unlike that of human PDI, does not rescue a *PD11*-null strain of *Saccharomyces cerevisiae* (Gunther *et al.*, 1993). Both these findings show that ERp57 and PDI, although similar with respect to their overall domain architecture and active-site motifs, have different functions *in vivo*. ERp57 interacts specifically with N-glycosylated secretory proteins and membrane proteins (Oliver *et al.*, 1997), and it is now clear that the specificity of this interaction with N-glycosylated proteins is not intrinsic to ERp57, but instead is due to a long-term association between ERp57 and calnexin or calreticulin. These glycoprotein-specific chaperones of the ER (Zapun *et al.*, 1998; High *et al.*, 2000) would be responsible for substrate recognition/binding in the multi-subunit chaperone/Erp57 complex. Hence, it appears that ERp57 does not possess a general binding site for non-native proteins, but rather that it has a specialized binding site for specific partner proteins (calnexin and calreticulin).

Among these three members of the PDI family, binding specificity—defined principally by the **b'** domain—can be for a

Fig. 3. Domain structures of PDI family members. The overall domain structures of PDI family members are shown for comparison with that of human PDI. The left-hand column shows members of the PDI family found in mammalian genomes (discussed in more detail in the text). The right-hand column shows members of the PDI family found in the *S. cerevisiae* genome. Homologous domains are coloured as for the corresponding domains in PDI. Regions of unknown function or structural homology are in blue.

local-sequence-defined motif in unfolded proteins (PDIp), for a very specific long-term protein partner (Erp57) or for both types of substrate (PDI). Within the family of proteins containing a PDI-like **a** or **a'** domain, it is clear that not all contain a **b'** (or **b**) domain (Figure 3), but the absence of such a domain gives us information on putative function. Yeast Mpd1p and Mpd2p both contain a single active site and so are unlikely to act as isomerases and probably function either as oxidases or reductases or to shuffle redox equivalents between proteins within the ER. P5 contains two active sites and therefore may act as an isomerase, but this would require either interaction with an as yet unreported partner protein, as for Erp57 with calnexin/calreticulin, or for the C-terminal portion of P5 to be an alternative type of binding domain for misfolded proteins. Interestingly, the C-terminal regions of unknown homology of these three proteins are all predicted to be completely α -helical.

Conclusion: binding, catalysis and 'chaperone' activity

In this model of PDI function, individual domains with specialized roles contribute different activities to enable the catalysis of complex disulfide isomerizations in substantially folded protein substrates. This modular synergic model is a significant advance in our understanding of PDI, but it is not unique. Trigger factor, a ribosome-bound member of the peptidyl-prolyl isomerase (PPI) family, consists of distinct catalytic and peptide/protein binding domains (Scholz *et al.*, 1997; Zarnt *et al.*, 1997), as does the periplasmic PPI, surA (Behrens *et al.*, 2001; Webb *et al.*, 2001). In both cases it is likely that all domains act synergically in the enzymatic action on protein substrates. The model also clarifies one area of possible confusion, namely whether PDI can itself be described as a molecular chaperone as well as a folding catalyst. In some *in vitro* assays (Cai *et al.*, 1994; Song and Wang, 1995; Wang *et al.*, 1997), and in some *in vivo* analyses (McLaughlin and Bulleid, 1998; Gillece *et al.*, 1999), PDI has properties expected of a molecular chaperone. However, it is now clear that this does not represent an activity unrelated to its role as a catalyst of disulfide-associated protein folding, rather that it seems

to represent deployment of a subset of the multiple, associated actions required to enable PDI to bring about rate-limiting disulfide re-arrangements in intermediates in protein folding pathways.

Further structural information will certainly clarify and broaden our functional model, but it appears that we are already beginning to understand how PDI and its homologues perform their remarkable function.

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