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review

The human protein disulphide isomerase family: substrate interactions and functional properties

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The process of disulphide bond formation in the endoplasmic reticulum of eukaryotic cells was one of the first mechanisms of catalysed protein folding to be discovered. Protein disulphide isomerase (PDI) is now known to catalyse all of the reactions that are involved in native disulphide bond formation, but despite more than 40 years of study, its mechanism of action is still not fully understood. This review discusses recent advances in our understanding of the human PDI family of enzymes and focuses on their functional properties, substrate interactions and some recently identified family members.

Keywords: disulphide bond formation; endoplasmic reticulum; protein disulphide isomerase; protein folding; thiol-disulphide oxidoreductase

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Introduction

Native disulphide bond formation is a complex process. Disulphide bonds must not only be formed (oxidation), but also incorrect bonds must be broken (reduction) or rearranged (isomerization). During the past couple of years there have been several significant advances in our understanding of these processes—for instance, concerning the role of the endoplasmic reticulum (ER) oxidase Ero1 and the source of oxidizing equivalents (for a review, see Tu & Weissman, 2004). Although there has been a strong focus on oxidation reactions, isomerization rather than oxidation is rate limiting during the *in vitro* refolding of many disulphide-containing proteins. As isomerization reactions are thought to be catalysed only by members of the protein disulphide isomerase (PDI) family, a better knowledge of their mechanisms of action is crucial to our understanding of native disulphide bond formation.

The enzyme PDI is a multi-domain, multi-functional member of the thioredoxin superfamily (for reviews, see Freedman *et al,* 2002; Ferrari & Söling, 1999). PDI can catalyse thiol-disulphide oxidation, reduction and isomerization, the last of which occurs directly through intramolecular disulphide rearrangement or through cycles of reduction and oxidation (Schwaller *et al,* 2003). PDI comprises two thioredoxin-like catalytic domains, **a** and **a′**, which are separated by two non-catalytic domains, **b** and **b′**. The catalytic domains contain a characteristic CXXC active-site motif, with the two amino acids that lie between the cysteine residues having a major role in determining the redox potential of the enzyme and hence its function as a thiol-disulphide reductase, oxidase or isomerase. Despite attempts for more than 30 years to crystallize PDI, the structure of this protein, or of any other catalytically active eukaryotic PDI-family member, has not yet been determined. The structures of the human PDI **a** and **b** domains have been solved by nuclear magnetic resonance (NMR), and both have a thioredoxin-fold (Kemmink *et al,* 1996, 1997). Given the homology between **a** and **a′**, and **b** and **b′**, it is probable that PDI consists of four domains each with a thioredoxin-fold, plus a short acidic carboxy-terminal extension; however, it is unknown how these domains are orientated with respect to each other. Recently, a 19-amino-acid linker region was identified between the **b′** and **a′** domains (Pirneskoski *et al,* 2004), which would potentially allow more flexibility between these domains than between the other domains. However, the physiological relevance of this finding is unknown.

The PDI family

During the past two years, several new human PDI-family members have been reported: ERp18 (Alanen *et al*, 2003; Knoblach *et al*, 2003), ERp44 (Anelli *et al*, 2002, 2003), ERp46 (Knoblach *et al*, 2003; Sullivan *et al*, 2003), ERdj5 (Cunnea *et al*, 2003; Hosoda *et al*, 2003), thioredoxin-related transmembrane protein 2 (TMX2; Meng *et al*, 2003) and PDILT (van Lith *et al*, 2004). When added to the previously known family members PDI, PDIp, ERp57, ERp72, P5, PDIr, ERp28 (Freedman *et al*, 2002; Ferrari & Söling, 1999) and TMX (Matsuo *et al*, 2001), there are now 14 human PDI-family members in the ER, with a wide range of domain architectures and active-site chemistries (Fig 1; Table 1). For the new PDI-family members, the ability to catalyse thiol-disulphide exchange reactions has only been shown for ERp18. In addition to these 14 proteins, three unpublished PDI-family members (ERp27, TMX3 and TMX4), the ER localization of which has been confirmed (L.W.R. and L.E., unpublished data), can be found in public databases (for accession numbers, see Table 1).

Substrate interactions

To function as catalysts of protein thiol-disulphide exchange, PDI-family members must be able to interact with their substrates. In PDI, the **b′** domain provides the primary peptide- or non-native protein-binding site, but other domains also contribute to binding (Klappa *et al*,

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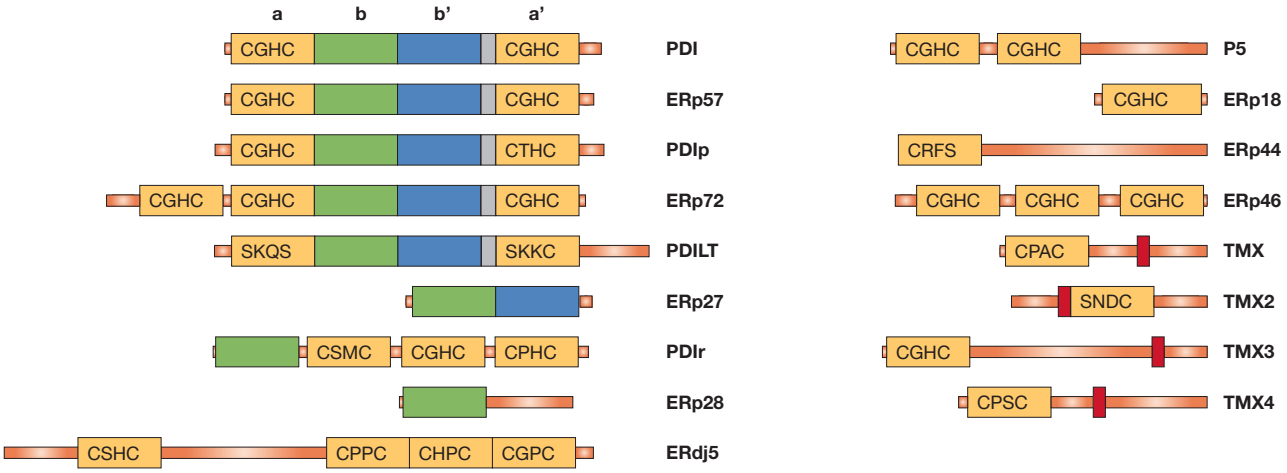

Fig 1 | Schematic overview of the human protein disulphide isomerase family. Thioredoxin-like domains are represented by rectangles with the active-site sequence added for catalytic domains (yellow). The catalytically inactive **b´** domain is shown in blue and other catalytically inactive domains are in green. The linker region between **b´** and **a´** domains is coloured in grey, and transmembrane regions are shown in red. Signal sequences are not shown. Note that the catalytic domain of ERp18 contains a putative 23-amino-acid insert between β3 and α3 (Alanen *et al*, 2003) that is not shown in the figure.

Table 1 | Sequence features of members of the human protein disulphide isomerase family

Name*	SwissProt accession	Length	ER retention	*N*-glycosylation sites (putative)	a-like domains	Charge pair sequence	Conserved arginine
PDI	P07237	508	KDEL	0	2	E47–K81, E391–K424	Yes, yes
ERp57	P30101	505	QEDL	0	2	E51–K82, E400–K433	Yes, yes
PDIp	Q13087	525	KEEL	3	2	E65–K99, K412–E445	Yes, yes
ERp72	P13667	645	KEEL	0	3	E85–K119, E200–K234, E549–K582	Yes, yes, yes
ERp65	Q8N807	584	KEEL	8	2	L66–K100, M411–K444	No, no
ERp27	Q96DN0	273	KVEL	1	0	–	–
PDIr	Q14554	519	KEEL	0	3	M176–N209, M299–A334, M420–A453	No, yes, no
ERp28	P30040	261	KEEL	1	0	–	–
ERdj5	Q8IXB1	793	KDEL	1	4	N152–A183, D474–T505, D582–S613, D694–K725	Yes, no, yes, yes
P5	Q15084	440	KDEL	0	2	E49–A80, E184–A219	Yes, yes
ERp18	O95881	172	EDEL	1	1	I60–N93	No
ERp44	Q9BS26	406	RDEL	0	1	N52–R89	Yes
ERp46	Q8NBS9	432	KDEL	0	3	M83–K118, K211–K244, K344–E378	Yes, yes, yes
TMX	Q9H3N1	280	Unknown	0	1	E50–K82	Yes
TMX2†	Q9Y320	296	KKDK	2	1	E161–K193	?‡
TMX3	Q96JJ7+	454	KKKD	2	1	D47–K81	Yes
TMX4	Q9H1E5	349	Unknown	1	1	K58–K90	No

*This table lists the 17 human protein disulphide isomerase (PDI)-family members the endoplasmic reticulum (ER) location of which has been confirmed. In addition, Q96MT2 is probably an ER-located human PDI-family member. †The version of TMX2 included here represents the consensus version from human cDNA databases and is consistent with homologous proteins found in a wide range of species. A longer version of TMX2 (Q8NBP9, 372 amino acids, of which the first 281 are identical to the sequence listed here) has been published (Meng *et al*, 2003). ‡The alignment of TMX2 with other family members in this region is ambiguous. It is possible that the conserved arginine is present in the sequence. +TMX3 starts at M33 of Q96JJ.

1998). Detailed *in vitro* enzymology on linear combinations of PDI domains has shown that the isolated **a** and **a′** domains can catalyse thiol-disulphide exchange reactions in peptide and protein substrates, but that a combination of a catalytic domain and the **b′** domain is required for simple isomerization reactions. Moreover, all of the thioredoxin-like domains of PDI are required for isomerization reactions that involve substantial changes in structure in the substrate (Darby *et al*, 1998). The implications from these studies are that the **a** and **a′** domains contain as-yet-unidentified, low-affinity binding sites for non-native proteins, whereas the **b′** domain contains a higher affinity binding site by which PDI holds substrates during isomerization reactions. The **b** domain of PDI has neither been implicated in substrate binding (Klappa *et al*, 1998) nor does the addition of the **b** domain add to the catalytic ability of PDI domain constructs (Darby *et al*, 1998), which suggests that this domain may have a structural role in PDI rather than a direct catalytic role.

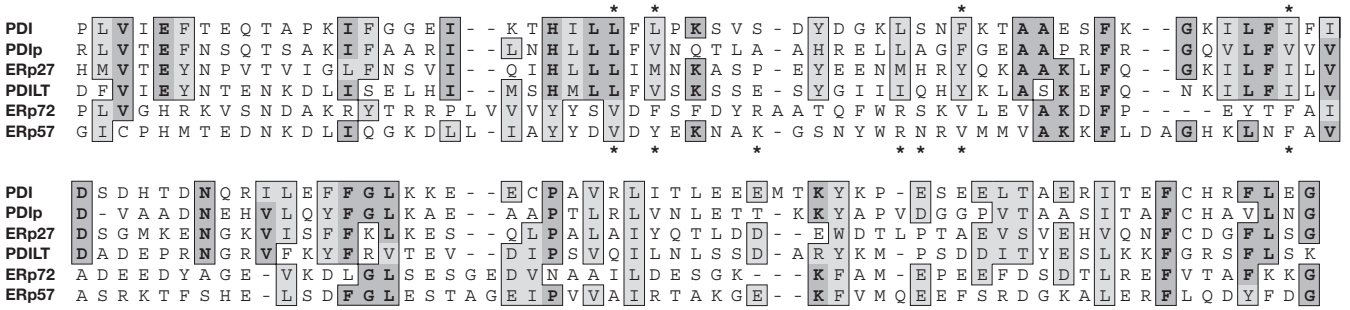

Fig 2 | Multiple sequence alignment of the **b′**-like domains of the human protein disulphide isomerase family. The alignment was constructed from a large number of single and multiple alignments and adjusted by hand, taking secondary structure predictions into account. Amino acids identical or similar to each other in four or more proteins are highlighted. Residues in which mutation inhibits the interaction of ERp57 with calreticulin (Russell *et al*, 2004) are indicated with an asterisk below the alignment, whereas those in which mutation inhibits the interaction of protein disulphide isomerase (PDI) with the peptide substrate Δ-somatostatin (Pirneskoski *et al*, 2004) are indicated with an asterisk above the alignment. At six of the seven sites implicated in ERp57–calreticulin interactions, ERp57 and ERp72 share identity or similarity with each other but not with the PDI/PDIp/PDILT/ERp27 cluster.

Using structural models of the **b′** domains of PDI and PDIp in combination with the known specificity of substrate binding by PDIp (Ruddock *et al*, 2000), the substrate-binding site in the **b′** domain of PDI was recently mapped to a small hydrophobic binding pocket located where the active site is found in catalytic thioredoxin -like domains (Pirneskoski *et al*, 2004). Mutations designed to partially fill or occlude this pocket resulted in a significant reduction in substrate binding by PDI.

Whereas PDI interacts directly with and also folds non-native proteins, ERp57 is known to act *in vitro* and *in vivo* on glycosylated substrates through its interaction with the ER-resident lectins calnexin and calreticulin (Zapun *et al*, 1998; Oliver *et al*, 1997). The primary interaction site on calreticulin has been localized to the highly acidic tip of the P-domain (Frickel *et al*, 2002), which forms an elongated hairpin loop (Ellgaard *et al*, 2001). The regions of ERp57 that are important for this interaction are the **b′** domain and, to a lesser extent, the C-terminal positively charged region (Russell *et al*, 2004; Silvennoinen *et al*, 2004; Urade *et al*, 2004; Pollock *et al*, 2004). The binding site for calreticulin in the **b′** domain of ERp57 has been mapped to residues that are equivalent to those that form the substrate-binding site in PDI (Russell *et al*, 2004).

The picture that emerges from these studies—supported by a recent study on the Wind protein (Barnewitz *et al*, 2004), which is the *Drosophila* homologue of ERp28—is that PDI-family members use a conserved binding pocket located in a non-catalytic thioredoxin-like domain for high-affinity binding of substrates or substrate-interacting cofactors. This, in turn, is likely to be important for the proteins' ability to catalyse isomerization reactions. In most cases described so far, this binding site is located in **b′**-like domains, which are found in PDI, PDIp, ERp57, ERp72, ERp27 and PDILT. An alignment of these domains (Fig 2) reveals that whereas PDIp, ERp27 and PDILT are more PDI-like and are therefore probably able to bind non-native proteins, ERp72 is more ERp57-like, and many of the residues that are implicated in ERp57–calreticulin interactions are conserved in ERp72. However, there is no experimental evidence yet to suggest an ERp72–calreticulin interaction.

The specificity of substrate binding by the **b′** domains of the human PDI-family is poorly understood. The binding site in ERp57 is specialized for interacting with the tip of the P-domain of calreticulin and calnexin (Frickel *et al*, 2002; Russell *et al*, 2004; Pollock *et al*, 2004), whereas the binding of peptides to PDIp is dependent on the presence in the substrate of a single tyrosine or tryptophan residue with no adjacent negative charge (Ruddock *et al*, 2000). In the structural model of the PDI **b′** domain (Pirneskoski *et al*, 2004), the hydrophobic binding pocket is large enough to accommodate only one or two hydrophobic amino-acid side chains, which is consistent with the single amino-acid specificity of PDIp. In addition, several unsatisfied backbone and side-chain hydrogen bonds were found in the immediate vicinity of the binding pocket. Therefore, this binding site seems well suited for interaction with non-native substrates that contain exposed hydrophobic groups and unsatisfied backbone hydrogen bonds; that is, proteins that might require the assistance of a protein-folding catalyst such as PDI.

Predicted functional properties

Although the name of the family implies that all members have a role in protein disulphide isomerization, only a subset are able to catalyse this reaction efficiently, whereas others are probably not directly involved in native disulphide bond formation.

There are four prominent determinants of the enzymatic activity of PDI-family members: the active-site sequence; the presence or absence of additional residues that also modulate the pK_a of the active-site cysteines; the presence or absence of a glutamic acid–lysine charge pair that is involved in proton transfer reactions; and, for the ability to catalyse isomerization reactions, a high-affinity substrate-binding site in a non-catalytic domain (discussed above). The sequences of individual PDI proteins therefore allow predictions to be made about the types of reaction that they perform.

The most common active-site motif in human PDI-family members is CXHC, which is found in efficient thiol-disulphide oxidants of the ER and bacterial periplasm. This motif is present in PDI, PDIp, ERp57, ERp72, P5, ERp46, TMX3, one of the catalytic domains of ERdj5 and two domains of PDIr (Fig 1). By contrast, three of the active sites of ERdj5 contain a CXPC motif that is found in the thioredoxins, which are thiol-disulphide reductants.

The surface-exposed amino-terminal cysteine of the CXXC motif of PDI is essential for any thiol-disulphide reaction and is missing from ERp27, ERp28, PDILT and TMX2. ERp44 lacks the C-terminal active-site cysteine that is required for many thiol-disulphide exchange reactions to proceed efficiently and whose presence makes mixed disulphide intermediates with substrates very transient. The N-terminal active-site cysteine of ERp44 thus forms more stable mixed disulphides, and by this mechanism it mediates the ER retention of proteins, including Ero1 and nascent secretory proteins (Anelli *et al*, 2002, 2003). The function of the non-thioredoxin-like C-terminal two-thirds of ERp44 is unknown, as are the features that determine its substrate-binding specificity.

Recently, a conserved arginine that is present in many members of the PDI family has been reported to modulate the pK_a of the active-site cysteine residues by moving into and out of the active-site locale (Lappi *et al*, 2004). This motion has been implicated in the timing mechanism that allows a single catalyst to act as an efficient isomerase and oxidase of protein substrates, and to allow for the release of non-productive folding substrates. This arginine is important for the catalysis of oxidation by PDI, ERp57, ERp72 and P5 and is also conserved in most of the other PDI-family member **a**-like domains (Table 1).

In addition to a CXXC active site and a modulation of the pK_a values of the active-site cysteines, efficient completion of the catalytic cycle for oxidation or reduction requires numerous proton transfer reactions both within the catalyst and to and from the substrate (Lappi *et al*, 2004). In the thioredoxins, a buried, charged glutamic acid–lysine pair that is located under the CXXC active site has been shown to be important for the catalytic activity of thioredoxin (Dyson *et al*, 1997) and for the oxidative activity of PDI and ERp57 (L.W.R., unpublished data). The glutamic acid—the presumed proton acceptor—of this charged pair is conserved in many of the PDI-family members (Table 1).

The presence of a CXHC active site in combination with the three other determinants of enzymatic activity suggests that PDI, PDIp, ERp57 and ERp72 are involved in disulphide bond oxidation and isomerization, which has been confirmed *in vitro* for PDI, ERp57 and ERp72 (see, for example, Darby *et al*, 1998; Frickel *et al*, 2004; Rupp *et al*, 1994). By contrast, ERp27, ERp28, PDILT and TMX2, which lack the CXXC active-site motif, are probably not involved directly in native disulphide bond formation. P5, TMX3 and ERp46, which all lack a **b′**-like domain but retain the other features and CXHC active sites, would be expected to be efficient oxidases, and ERdj5, which contains CXPC active sites, an efficient reductase. The unusual active site of TMX makes it difficult to predict its functional role, but the protein contains the other features that are required for the efficient catalysis of thiol-disulphide exchange reactions. The remaining family members, PDIr, ERp18 and TMX4, lack the glutamic-acid proton acceptor and therefore would be expected to be relatively inefficient catalysts of these reactions. This latter prediction is confirmed by *in vitro* data showing that ERp18 has only 15% of the oxidase activity of the **a** domain of PDI (Alanen *et al*, 2003), and PDIr has only 2% of the isomerase activity of PDI (Horibe *et al*, 2004) and 6% of its oxidase activity (L.W.R., unpublished data). It has been reported that the low isomerase activity of PDIr might be due to it acting on only a subset of proteins, including α1-antitrypsin (Horibe *et al*, 2004); however, it is noteworthy that a mutant PDIr with all of its active-site cysteines mutated to serine retained 57% of the activity of wild-type PDIr in the reactivation of α1-antitrypsin. This finding implies a reaction mechanism that is independent of thiol-disulphide exchange.

Future directions

Despite the large body of work on the activities of PDI-family members *in vitro* and *in vivo* there are still numerous unanswered questions regarding the physiological functions of the individual proteins and their mechanisms of action, especially within the complex environment of the ER.

The intransigence of catalytically active PDI-family members to crystallization is a major obstacle towards our understanding of them, as is the lack of assays with endogenous substrates for which the rate-limiting steps are well defined. Although some data on the relative activities of PDI-family members are appearing in the literature, a systematic comparison of the different activities that each family member from a single organism has towards a range of substrates is required. It will also be interesting to compare the set of PDI-family members present in different organisms, as this might teach us something about the co-evolution of these proteins and the need for individual organisms to catalyse specific processes.

The known presence of nearly 20 members of the PDI family in humans implies a complexity to the system that we are far from understanding. In particular, it will be necessary to determine whether the different enzymes either have overlapping or separate and distinct substrate specificities. Unfortunately, no gene knockdown studies of PDI-family members in mammalian tissue culture cells have been published. Whether this reflects that no effects have been observed or that it is too difficult at present to evaluate the effects of such experiments is not clear. Other obvious questions to address include a systematic evaluation of the transcriptional regulation of all of these proteins, the cellular mechanisms for regulation of their redox state and the physiological relevance of some unusual locations that have been reported for PDI-family members (for a review, see Turano *et al*, 2002).

There is also a need to understand how PDI-family members interact with each other and with other ER-resident folding catalysts and chaperones. The presence of a J-domain in ERdj5 suggests that this protein cooperates with ER chaperones of the heat-shock protein 70 (Hsp70) family (Cunnea *et al*, 2003; Hosoda *et al*, 2003). There is already evidence for chaperone organization within the ER, and PDI, ERp72, P5 have been reported to form a complex with several ER chaperones and folding factors (Meunier *et al*, 2002). However, much more work is needed to determine the dynamics of such complexes, how they are formed, and what their effects are on the folding process. Other interaction partners that act as modulators also need to be identified.

PDI has been studied for more than 40 years, but the list of what we do not know about this enzyme is still long. With the discovery of novel proteins that belong to the PDI family, the field faces exciting challenges in the years to come.

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