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Anna-Kaisa Lappi

MECHANISMS OF PROTEIN DISULPHIDE ISOMERASE CATALYZED DISULPHIDE BOND FORMATION

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Abstract

Protein folding of outer membrane and secreted proteins, including receptors, cytokines and antibodies is often linked to disulphide bond formation. Native disulphide bond formation is complex and is usually the rate limiting step in the folding of such proteins. The enzymes which catalyse the slow steps in disulphide bond formation belong to the protein disulphide isomerase (PDI) family. PDI catalyses formation, reduction and isomerization of newly synthesized disulphide bonds. The mechanisms of action of the PDIs are currently poorly understood and this not only inhibits our understanding of the biogenesis of a range of medically important proteins, and hence associated disease states, but also prevents the effective manipulation of the cellular environment by the biotechnology industry for the production of high value therapeutic proteins. Hence, understanding the mechanism of action of these enzymes is vital for a wide range of medically important processes and therapies.

In this study the role of a conserved arginine residue in the catalytic activity of PDI was shown. The movement of this residue into and out of the active site locale of PDI was shown to modulate the pK_a of the C-terminal active site cysteine of PDI and by that way to allow the enzyme to act efficiently as catalyst both of oxidation and isomerization reactions.

The possible role of hydrogen peroxide produced by sulphydryl oxidases during disulphide bond formation was studied in an oxidative protein refolding assay. Analysis showed that hydrogen peroxide can be used productively to make native disulphide bonds in folding proteins with minimal side reactions.

In addition, the kinetics of oxidation and reduction of the **a** domains of PDI and Pdi1p by glutathione was studied in this thesis. The kinetics obtained with stopped-flow and quenched-flow experiments showed the reactions to be more rapid and complex than previously thought. Significant differences exist between the kinetics of PDI and Pdi1p. This implies that the use of yeast systems to predict physiological roles for mammalian PDI family members should be treated cautiously.

Keywords: disulphide bond formation, endoplasmic reticulum, isomerization, pKa, protein disulphide isomerase, protein folding

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Oulu, June 2010 Anna-Kaisa Lappi

Abbreviations

A	Alanine
ACN	Acetonitrile
Arg	Arginine
Asp	Aspartic acid
BPTI	Bovine pancreatic trypsin inhibitor
C-	Carboxy(terminal)-
CD	Circular dichroism
Cys	Cysteine
D	Aspartic acid
DMSO	Dimethyl sulphoxide
DHA	Dehydroascorbate
DNA	Deoxyribonucleic acid
DNTB	5,5-dithio-bis(2-nitrobenzoic acid)
DTT	Dithiothreitol
EDTA	Ethylenediamidetetraacetic acid
ER	Endoplasmic reticulum
FAD	Flavin adenine nucleotide
Glu	Glutamic acid
Grx	Glutaredoxin
GSH	Reduced glutathione
GSSG	Oxidized glutathione
IAA	Iodoacetamide
kDa	Kilo Dalton
LB	Luria-Bertani
Lys	Lysine
M	Methionine
MHC	Major histocompatibility complex
N-	Amino (terminal)-
PCR	Polymerase chain reaction
PDB	Protein data bank
PDI	Protein disulphide isomerase
PPI	Peptidyl prolyl cis-trans isomerase
Q	Glutamine
QF	Quenched flow
R	Arginine

ROS	Reactive oxygen species
S	Serine
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TFA	Trifluoroacetic acid
Trx	Thioredoxin
UV	Ultraviolet
V	Voltage
W	Tryptophan
X	Any amino acid

List of original publications

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:

I Lappi AK, Lensink M, Alanen HI, Salo KEH, Lobell M, Juffer A & Ruddock LW (2004) A conserved arginine plays a role in the catalytic cycle of the protein disulphide isomerases. J Mol Biol 335: 283–295.

II Karala AR, Lappi AK & Ruddock LW (2010) Modulation of an active site pK_a allows PDI to act both as a catalyst of disulfide bond formation and isomerization. J Mol Biol 396: 883–892.

III Karala AR, Lappi AK, Saaranen MJ & Ruddock LW (2009) Efficient peroxide mediated oxidative folding of protein at physiological pH and implications for oxidative folding in the endoplasmic reticulum. Antioxid Redox Signal 11: 963–970.

IV Lappi A-K & Ruddock LW (2010) Re-examination of the role of interplay between glutathione and protein disulfide isomerase. Manuscript.

Contents

1 Introduction

Protein folding and assembly of native secretory proteins is a complicated process, which is initiated in the lumen of the endoplasmic reticulum (ER). Most secretory proteins require disulphide bond formation as an obligatory post-translational modification to gain their native structure. A disulphide bond is a covalent linkage between cysteine residues in proteins. The formation of disulphide bonds is influenced by multiple factors and is therefore not a simple chemical reaction. Formation of disulphide bonds is required for folding, for stability and for function of a large number of proteins. Protein disulphide isomerase (PDI) is known to catalyse all of the reactions involved in native disulphide bond formation, but despite of years of studies its detailed reaction mechanisms are not known.

In the present study the catalytic activites of PDI were studied at the molecular level. Conservation of an arginine residue located in the loop between β5 and α4 of the catalytic domains of all of the human PDI family, except the first domain of PDIr, was observed and its significant role in the catalytic activity of PDI was shown. Based on molecular simulation studies the movement of the arginine residue into and out of the active-site locale was suggested. The effects of this movement on the pK_a values of the active-site cysteines and hence on the catalytic activity were examined in detail. Modulation of the pK_a of the C-terminal active-site cysteine by the movement of the arginine residue was shown to allow PDI to act efficiently as a catalyst both for oxidation and isomerization reactions.

In vitro oxidation of PDI by hydrogen peroxide was studied in detail in this thesis. Peroxide added directly to folding bovine pancreatic trypsin inhibitor (BPTI) or generated *in situ* by the action of glucose oxidase and glucose resulted in the efficient formation of natively folded protein. At physiological pH the peroxide-mediated oxidation was shown to be faster than glutathione-mediated. Peroxide was shown also to be able to oxidize the active site of PDI or reduced glutathione. The kinetics of the oxidation and reduction of the active site by glutathione was studied and found to be more rapid and complex than was earlier considered.

The rates of glutathione-mediated reactions were compared between the **a** domains of human PDI and yeast Pdi1p. The kinetics of glutathione-based oxidation of the two enzymes was observed to be remarkably similar. In contrast, very significant differences in kinetics were observed when reduction of these

two proteins by reduced glutathione (GSH) was studied. The rate of reduction of the **a** domain of Pdi1p by GSH was found to be remarkably slower than that of the corresponding rate for the human protein.

Taken together the studies presented here elucidate some of the mechanisms of PDI catalyzed disulphide bond formation, but many mechanistic details remain unknown for this remarkable enzyme.

2 Review of the literature

2.1 Protein folding

The production of functional native proteins in the cell is a complex process. During translation, amino acids, encoded by mRNA formed during transcription, are linked by peptide bonds to form a linear polypeptide chain. The polypeptide chains formed need to be folded into their functional form, with native secondary and tertiary and for oligomeric proteins quaternary structure. Folding of polypeptide chains can happen in multiple cellular compartments. In eukaryotes it occurs in the cytoplasm, mitochondria, chloroplasts and the endoplasmic reticulum.

Protein folding can happen spontaneously (Anfinsen 1973), but when non-catalyzed, it can be a slow and inefficient event. Three of the slow steps of protein folding are disulphide bond formation, cis-trans peptidyl prolyl isomerization and oligomerization. Disulphide bond formation is the formation of a covalent linkage between two cysteine residues in a polypeptide chain or between cysteines in two polypeptide chains. The function of disulphide bonds is to stabilize the tertiary and/or quaternary structure of a protein. The other common rate limiting step in protein folding is cis-trans peptidyl prolyl isomerization. Peptide bonds can exist in two geometrical isomers, cis and trans. They are synthesized in the trans form, but to adopt the final folded structure, those peptide bonds required to be in the cis form need to be isomerized. Cis-peptide bonds are generally much less thermodynamically favourable than the trans state. Most cis-peptide bonds found in proteins are found prior to proline residue as cis X-Pro and trans-Pro have only a small difference in Gibbs free energy due to the cyclic nature of proline. The non-catalysed isomerization between the cis and trans states is very slow and hence physiologically a catalyst is required. The reaction is catalysed by enzymes called peptidyl prolyl cis-trans isomerases (PPIs).

Protein folding is assisted both by catalysts that accelerate protein folding and by chaperones that inhibit protein aggregation. Non-punctual folding of proteins can be problematic for the cell. Misfolding of a protein can lead to the loss of that protein's function but it can also lead to protein accumulation as large insoluble aggregates which can interfere with cell function. Misfolded proteins accumulate in several diseases, including Alzheimer's (see review Harper *et al.* 1997), goiter

(Kim *et al.* 1996), cystic fibrosis (see review Thomas *et al.* 1995) and prion infections (DebBurman *et al.* 1997).

In eukaryotic systems a great number of the proteins synthesized in the cell are secreted to the extracellular space. These proteins are translocated to the endoplasmic reticulum (ER), where folding takes place before secretion through the Golgi apparatus. Protein folding in the ER is often associated with the formation of native disulphide bonds (Fig. 1), which stabilize the tertiary structure of the protein and that way ensure the stabilization of the structure of secreted or cell-surface proteins (Freedman 1984). Disulphide bond formation occurs in the lumen of the ER where the redox state is more oxidizing than that of the cytosol (Hwang *et al.* 1992). Formation of disulphide bonds is a complex process and for many secreted and outer membrane proteins also the rate limiting step in their synthesis (for reviews see Freedman *et al.* 2002, Ellgaard & Ruddock 2005, Hatahet & Ruddock 2009). The formation of disulphide bonds in proteins is a reversible reaction in which the thiol groups in two cysteine residues are oxidized to form a covalently linked disulphide bond. This can be achieved by several pathways. Dithiols in folding protein can be oxidized directly by molecular oxygen, however this is probably too slow reaction to have physiological relevance during protein folding. In addition, dithiols can be oxidized by low molecular weight biochemical compounds such as oxidized glutathione (Freedman 1995), by reactive oxygen species (ROS) for example hydrogen peroxide (Cumming *et al.* 2004, Saurin *et al.* 2004), by dehydroascorbate (Bánhegyi *et al.* 1998, Bánhegyi *et al.* 2003, Saaranen *et al.* 2010) or by enzymes belonging to the thioredoxin superfamily, like protein disulphide isomerase (PDI) for example (see reviews Ferrari & Söling 1999, Ellgaard & Ruddock 2005, Hatahet & Ruddock 2009).

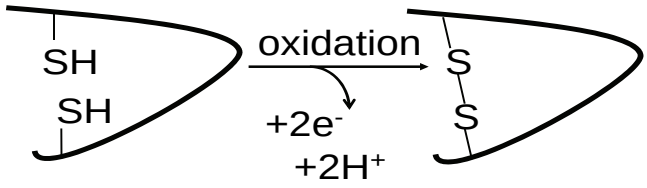

Fig. 1. Schematic presentation of disulphide bond formation.

The current model for the major route for disulphide bond formation *in vivo* is the highly regulated flow of oxidizing equivalents from molecular oxygen to the sulphydryl oxidase ER oxidoreductin (Ero1). Ero1 is a FAD-dependent enzyme that is oxidized by molecular oxygen and then acts as an oxidant for PDI, which then oxidizes dithiols to disulphide bonds in folding proteins (Frand & Kaiser 1998, Pollard *et al.* 1998, Frand & Kaiser 1999, Tu *et al.* 2000, Gross *et al.* 2006, Sevier *et al.* 2007). Humans have two Ero1 family members, Ero1α and Ero1β (Cabibbo *et al.* 2000, Pagani *et al.* 2000). Recent *in vitro* studies have revealed detailed information about the binding between Ero1α and PDI and showed the minimal fragment of PDI needed for the interaction (Kulp *et al.* 2006, Wang *et al.* 2009). Very recent *in vivo* experiments have shown selective function for Ero1β in insulin biogenesis and have raised significant questions as to whether Ero1 is a component of the major route for disulphide bond formation in mammals (Zito *et al.* 2010). The other flavin-linked sulphydryl oxidase family of enzymes that passes through the human ER is quiescin-sulphydryl oxidase (QSOX) (Hoober *et al.* 1999a), which can catalyze the direct oxidation of disulphides in folding proteins (Hoober *et al.* 1999b, Thorpe *et al.* 2002). However, QSOX is a secreted protein or at least a post-ER resident protein (Chakravarthi *et al.* 2007) and hence is not currently thought to play a role in disulphide bond formation in the ER. The use of molecular oxygen as a terminal electron acceptor may generate reactive oxygen species (ROS) that contribute an additional source of cellular oxidative stress. Since the hydrogen peroxide made by Ero1 in each catalytic cycle (Gross *et al.* 2006) is potentially harmful to the cells, Ero1 family members have

complex, redox dependent regulation of their activity (Sevier *et al.* 2007, Appenzeller-Herzog *et al.* 2008, Baker *et al.* 2008).

2.2 Thioredoxin superfamily

Thioredoxins (Trxs) are a family of proteins that contain a similar fold and catalyze oxidoreductase reactions by dithiol-disulphide exchange mechanisms. They form part of a superfamily whose sub-families are defined on the exchange reaction catalyzed and on their sub-cellular location. The thioredoxin fold is defined by the core of three α-helices and four β-strands. The β-strands form a mixed β-sheet which is surrounded by the α-helices. One of these helices contains the Cys-X-X-Cys – active site motif, where X is any amino acid (Holmgren 1985). The physiological capability of individual members of thioredoxin superfamily is determined by the redox equilibrium of the disulphide-dithiol state of the active site cysteines. The thioredoxin superfamily contains thioredoxins, glutaredoxins (Grxs) and protein disulphide isomerases. The main function of thioredoxins is to reduce disulphide bonds in target proteins (Holmgren 1985). The reduction reaction occurs in two steps: the N-terminal active cite cysteine attacks the disulphide bond of the target protein, releases a free thiol and forms a mixed disulphide bond with the second cysteine of the target protein. Then the C-terminal cysteine of the active site of thioredoxin breaks the intramolecular disulphide bond, releases the reduced target protein and thioreoxin becomes oxidized. The physiological capacity of thioredoxin family members is determined by the relative stability of the reduced and oxidized states of the active site, which depends in part on the pK_a values of the active site cysteines. Residues that modulate these pK_a values have direct influence on the function of the enzyme. The first (N-terminal) cysteine of the thioredoxin active site, which is located near the start of an α-helix, is surface exposed and has pK_a of 7.1 (Dyson *et al.* 1997). This is significantly lower than the normal pK_a of a protein cysteine thiol. The pK_a of the N-terminal cysteine is thought to be result from several factors, including the influence of the helix dipole (Kortemme & Creighton 1995) and an influence of partial charge of the buried aspartic acid residue near the active site (Chivers *et al.* 1996, Dyson *et al.* 1997). A buried charged glutamic acid – lysine pair that is located under the active site has shown to influence to the catalytic activity of thioredoxin (Dyson *et al.* 1997).

2.3 The endoplasmic reticulum

The ER is a subcellular compartment responsible for folding and maturation of proteins destined for secretion or to be located at the plasma membrane (Ellgaard & Helenius 2003, Görlach *et al.* 2006). The ER also has a significant role in metabolism and in many signaling processes (for example Tenhunen *et al.* 1968, Kent 1995, Vance & Vance 2004, Miller *et al.* 2005, Cribb *et al.* 2005, Görlach *et al.* 2006). More than one-third of all human proteins are folded in the ER (Chen *et al.* 2005). Hence the ER contains catalysts to help folding of proteins by generating a variety of post-translational modifications, for example disulphide bond formation (Sevier & Kaiser 2002) and N-glycosylation (Helenius & Aebi 2004). The ER provides an ideal oxidizing environment to facilitate proper folding and assembly of newly synthesized exportable proteins. The ER also contains mechanisms to monitor the quality of synthesized proteins and to prevent the export of incompletely folded proteins from the ER (Ellgaard & Helenius 2003). The formation of disulphide bonds is an important step in the maturation of many secreted proteins. A common mechanism for the formation of a covalent linkage between two cysteine residues in protein is a thiol-disulphide exchange reaction of free thiols with disulphide bonded molecules. Thiol-disulphide exchange reactions are catalysed by a class of proteins known as oxidoreductases. The activity of these proteins depends on a CXXC-motif (where X is any amino acid), which usually exists in a domain related to the small redox protein thioredoxin (Trx). The most extensively studied ER-resident thiol-disulphide oxidoreductase is PDI, which is an ER-located protein containing two redox active Trx-domains.

2.4 PDI

PDI is a multifunctional enzyme (EC. 5.3.4.1) catalyzing the formation, reduction and isomerization of newly synthesized disulphide bonds. Mammalian PDI was first identified as a catalyst of oxidative protein folding (Goldberger *et al.* 1964), but has later also been shown to participate in several other cellular processes, for example collagen maturation (Pihlajaniemi *et al.* 1987), antigen processing (Park *et al.* 2006), regulating NAD(P)H oxidase (Janiszewski *et al.* 2005) and ER-associated degradation (Molinari *et al.* 2002). PDIs are found in all eukaryotic species including fungi, plants and animals. The human protein is constitutively expressed in most tissues and organs, and is located in the ER via the combination

of an N-terminal signal sequence and a classical C-terminal KDEL-retrieval motif (Lewis & Pelham 1990). PDI is present in the ER in high concentrations being one of the most abundant proteins there (Lambert & Freedman 1985).

2.5 The structure of PDI

The mature form of human PDI is 491 amino acids, with a molecular weight of about 56 kDa. PDI has a multi-domain structure (Fig. 2), which consists of four distinct domains, **a**, **b**, **b'** and **a'** plus a highly acidic C-terminal extension **c** and a 19 amino acid long inter-domain linker between the **b'** and **a'** domains named **x** (Pirneskoski *et al.* 2004). This domain structure was revealed by a combination of intron-exon analysis, comparison with other superfamily members e.g. thioredoxin, partial proteolysis studies, and the expression and characterization of isolated domains and combinations of domains. The **a** and **a'** domains of PDI, which contain the active site motif –WCGHC-, share 36.8% identity with each other and significant homology with thioredoxin, but not with the **b** and **b'** domains (for review see Hatahet & Ruddock 2009). The active site motifs are involved catalyzing the thiol-disulphide exchange reactions, the **b** domain is probably important for the overall structure of the PDI and **b'** domain is involved in substrate recognition and binding (Darby *et al.* 1998, Klappa *et al.* 1998).

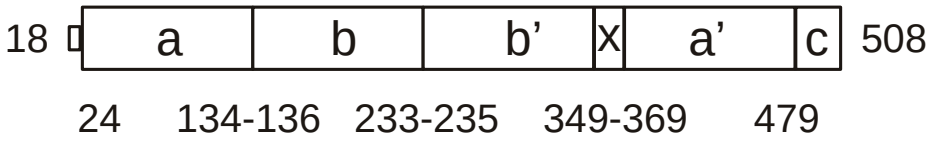

Fig. 2. The domain organization of human PDI. The catalytic domains a and a' contain the active site motifs (WCGHC). Numbering is for the full length protein. (Adapted from Hatahet & Ruddock 2009).

The NMR structures of the isolated human PDI **a** and **b** domains provided the first valuable structural information about PDI and showed that the **a** domain is comprised of a thioredoxin fold and shares other secondary structural elements with thioredoxin. In particular: i) the active site motif is located at the N-terminus of α2-helix, which is distorted by a proline residue, ii) the peptide bond before the proline residue at the N-terminus of β4-sheet is in the cis formation and iii) there is an analogous buried acidic residue Glu47 in the **a** domain of PDI (Kemmink *et al.* 1996) to a Asp26 found in thioredoxins (Dyson *et al.* 1997). Surprisingly, the **b**

domain, which has only little sequence similarity with the catalytic domain, has a thioredoxin-like fold (Kemmink *et al.* 1997) though it lacks the active site, both prolines and the buried charged pair. Also the structures of the **a'** (unpublished, PDB entry code 1x5c) domain, **b'x** (Nguyen *et al.* 2008) and **bb'** (Denisov *et al.* 2009) double domains have been solved. The **b'** domain contains the primary peptide binding site (Klappa *et al.* 1998) and is essential for non-covalent binding of incompletely folded protein substrates. The **a** and **a'** domains are also involved in substrate binding (Klappa *et al.* 1998, Klappa *et al.* 2000, Koivunen *et al.* 2005) though no direct binding activity has observed in the absence of **b'** domain. The substrate binding site in the **b'** domain has been mapped to the small hydrophobic pocket located where the active site is located in thioredoxin-like domains (Pirneskoski *et al.* 2004). The crystal structure of **b'x** and supporting NMR data have revealed that the **x** region can interact with **b'** domain by "capping" the hydrophobic site and therefore inhibit substrate binding (Nguyen *et al.* 2008). The **b'** domain of PDI is in conformational exchange but binding the **x** region stabilizes the structure (Byrne *et al.* 2009). The **b'** domain may adopt different configurations during different functions of enzyme and therefore vary the ability of PDI to interact with its substrates (Nguyen *et al.* 2008).

To date the crystal structure of full-length human PDI is still unsolved but the structures of several PDI family members have been solved during the last few years. Together with the solved human PDI domain structures they provide important knowledge to the field. The crystal structure of yeast PDI, Pdi1p, was a major break-through in the field by showing that the four domains form a "twisted U" structure with two catalytically active thioredoxin-like sites facing each other (Tian *et al.* 2006, Tian *et al.* 2008). The recent crystal structure of ERp57 in complex with tapasin shows significant similarity to that of Pdi1p (Dong *et al.* 2009). In addition, the structures of full-length human ERp44 (Wang *et al.* 2008), yeast Mpd1p (Vitu *et al.* 2008) and human ERp29 (Barak *et al.* 2009) have all been recently reported.

2.6 PDI family

There are around 20 other mammalian PDI-like proteins reported to date (Fig. 3). They are characterized by one or more domains homologous to thioredoxin and ER localization (Ellgaard & Ruddock 2005). PDI-like proteins are not functionally equivalent. The different family members probably catalyze specific reactions or interact with different set of substrates (Ellgaard & Ruddock 2005,

Hatahet & Ruddock 2007, Jessop *et al.* 2009) however the functional characterization of the family is far from complete.

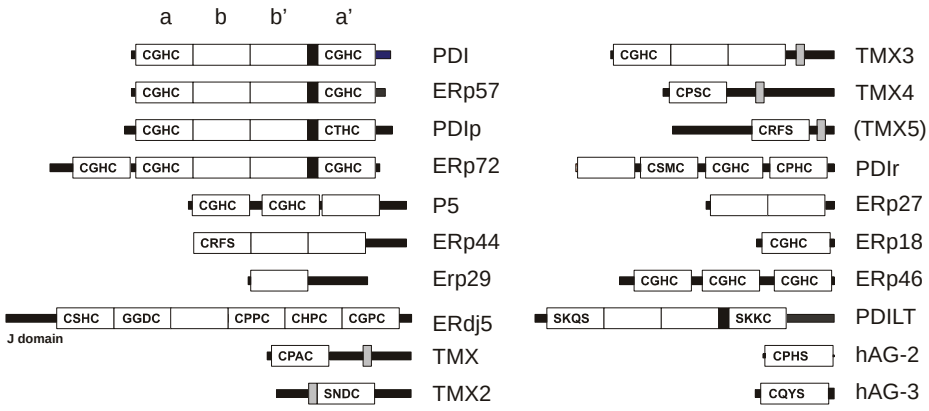

Fig. 3. Domain organization of mammalian PDI family members (adapted from Hatahet & Ruddock 2009). Catalytic domains that propably have a thioredoxin fold are shown as white boxes where the active site amino acid sequence is written. The non-catalytic domains that propably have a thioredoxin fold are shown in white and transmembrane regions are shown in grey.

ERp57, which is one of the best studied family members, shares great similarity in length and in domain architecture (**a**-**b**-**b'**-**a'**-**c**) with PDI (Oliver *et al.* 1997). ERp57 is a glycoprotein specific disulphide isomerase, via association with lectin-like chaperones calnexin or calreticulin, and helps substrates bound to calnexin and calreticulin to fold (Zapun *et al.* 1998). The role of ERp57 in folding has been studied by creating a knockout mice model (Garbi *et al.* 2006, Solda *et al.* 2006) which is embryonically lethal. When the maturation of secreted proteins were studied in ERp57 knockout cells, a significant loss of folding efficiency was observed for hemagluttinin (HA) of influenza virus, which is a protein known to require calnexin for efficient folding (Molinari *et al.* 2004).

PDIp (DeSilva *et al.* 1996) is the other family member that has significant sequence similarity (66%) with human PDI and shares the same domain architecture. It also has a unique active site motif (WCTHC) in it's **a'** domain, a less acidic **c** region and an additional acidic N-terminal extension before the **a** domain (DeSilva *et al.* 1996). Human PDIp is unique also in tissue distribution, being highly expressed in acinar cells in pancreas with limited other expression in humans (DeSilva *et al.* 1996).

ERp72 (Mazzarella *et al.* 1990) is the only family member composed of five thioredoxin-like domains of which three are catalytic domains containing active sites similar to PDI. The recent structure of the **bb'**-domains of ERp72 shows significant similarity with ERp57 but also great differences in surface charges that are responsible ERp72 not binding calnexin as ERp57 does (Kozlov *et al.* 2009).

The smallest member of the PDI family is ERp18 (Alanen *et al.* 2003b), also known as ERp16 (Jeong *et al.* 2008) and ERp19 (Knoblach *et al.* 2003). It has one slightly extended catalytic domain and is capable of catalyzing thiol-disulphide exchange reactions but with relatively slow kinetics (Alanen *et al.* 2003b, Jeong *et al.* 2008).

ERp44 (Anelli *et al.* 2002) has one catalytic domain with an unusual active site motif (CRFS), lacking the C-terminal active site cysteine. The crystal structure of human ERp44 has recently been solved to 2.6 Å and it suggests that the flexible C-terminal tail would have a regulatory role in binding and releasing a substrate during protein quality control in the early secretory pathway (Wang *et al.* 2008) analogous to the role of the **x** region in modulating substrate binding to the **b'** domain of PDI (see above).

ERp29, which is also known as Erp28, has two non-catalytic domains and lacks a catalytic domain (Demmer *et al.* 1997, Ferrari *et al.* 1998). ERp29 is widely expressed (Demmer *et al.* 1997) and is reported to be highly expressed upon ER stress (Mkrtchian *et al.* 1998). The crystal structure of human ERp29 (Barak *et al.* 2009) confirms that the protein consists of two domains. While several putative roles have been proposed (for example Sargsyan *et al.* 2002, Magnuson *et al.* 2005) the physiological function(s) of ERp29 are still unknown.

Other human PDI-family members are less well characterized. ERp27 is another small family member of human PDI that lacks a catalytic domain. It is able to bind peptides and non-native proteins via its **b'**-like domain and via interaction with ERp57 (Alanen *et al.* 2006b) but like Erp29 its physiological function(s) are unknown. PDIr (Hayano *et al.* 1995) and ERp46 (Knoblach *et al.* 2003), known also as endoPDI (Sullivan *et al.* 2003), have three catalytic domains but neither has been well characterized to date. ERdj5 (Cunnea *et al.* 2003), known also as JPDI (Hosoda *et al.* 2003) has four catalytic domains, three of which have active site motifs similar to thioredoxin (CXPC). It has reported to have the most reducing redox equilibrium constant within the PDI family and hence may play a role in reducing disulphide bonds in misfolded proteins destined for degradation (Ushioda *et al.* 2008). P5 has two catalytic domains and it is able to catalyze thiol-disulphide exchange reactions *in vitro* (Kramer *et al.* 2001,

Kikuchi *et al.* 2002, Alanen *et al.* 2006a) and forms a part of a multi-protein complex in the ER (Meunier *et al* 2002.). PDILT (van Lith *et al.* 2005) is the other member of PDI-family with specific tissue distribution reported to date; it is expressed only in the testis. PDILT forms *in vivo* a tissue specific chaperone complex with the calnexin homologue calmegin and provides a system for testis-specific protein folding (van Lith *et al.* 2007).

Most of the mammalian PDI-family members are soluble ER-lumenal proteins but some of the members extend to ER membrane. These include thioredoxin-like transmembrane proteins TMX (Matsuo *et al.* 2001), TMX2 (Meng *et al.* 2003), TMX3 (Haugstetter *et al.* 2005) and TMX4 (Roth *et al.* 2009). All TMX proteins contain one thioredoxin-like domain but are otherwise very different from each other. TMX has shown to interact with misfolded MHC class I heavy chain and protect it from proteasomal degradation. Based on the protective affect TMX is suggested to have relevance in ER quality control (Matsuo *et al.* 2009). Hag2 and Hag3 are the newest members in PDI family (Persson *et al.* 2005) shown to be localized in the ER (Raykhel *et al.* 2007). They are small single domain proteins which show sequence and structural homology with human ERp18 (Persson *et al.* 2005).

2.7 Functions of PDI

The two conserved active sites (CGHC) of PDI, which are located in the **a** and **a**' domains of PDI, enable PDI to catalyze a wide range of thiol-disulfide exchange reactions in protein substrates including oxidation, reduction and isomerization (Fig. 4) as well as having molecular chaperone-like properties (Hayano *et al.* 1993, Cai *et al.* 1994, Song & Wang 1995, Yao *et al.* 1997). The active site of PDI, as well as of other members of the thioredoxin superfamily, exists in the reduced dithiol state and in the oxidized disulphide state or in an intermolecular mixed disulphide state. The oxidized and reduced species are able to catalyze different reactions: oxidation of dithiols in substrate protein and isomerization/reduction of protein disulphides in substrate protein, respectively (Freedman *et al.* 1994, Gane *et al.* 1995, Chivers *et al.* 1997). Isomerization may occur directly through intramolecular disulphide rearrangement or through cycles of reduction and oxidation (Schwaller *et al.* 2003). PDI can also act as a molecular chaperone facilitating the folding of the proteins devoid of disulphide bonds (Wang & Tsou 1993). As well as being a major protein component of the ER, PDI is reported to be present in other cellular compartments including the

nucleus and on the cell surface (reviewed by Turano *et al.* 2002) which suggests it may have additional functions, but how it escapes the ER-targeting mechanism, except by lysis (Reinhardt *et al.* 2008), is currently unclear.

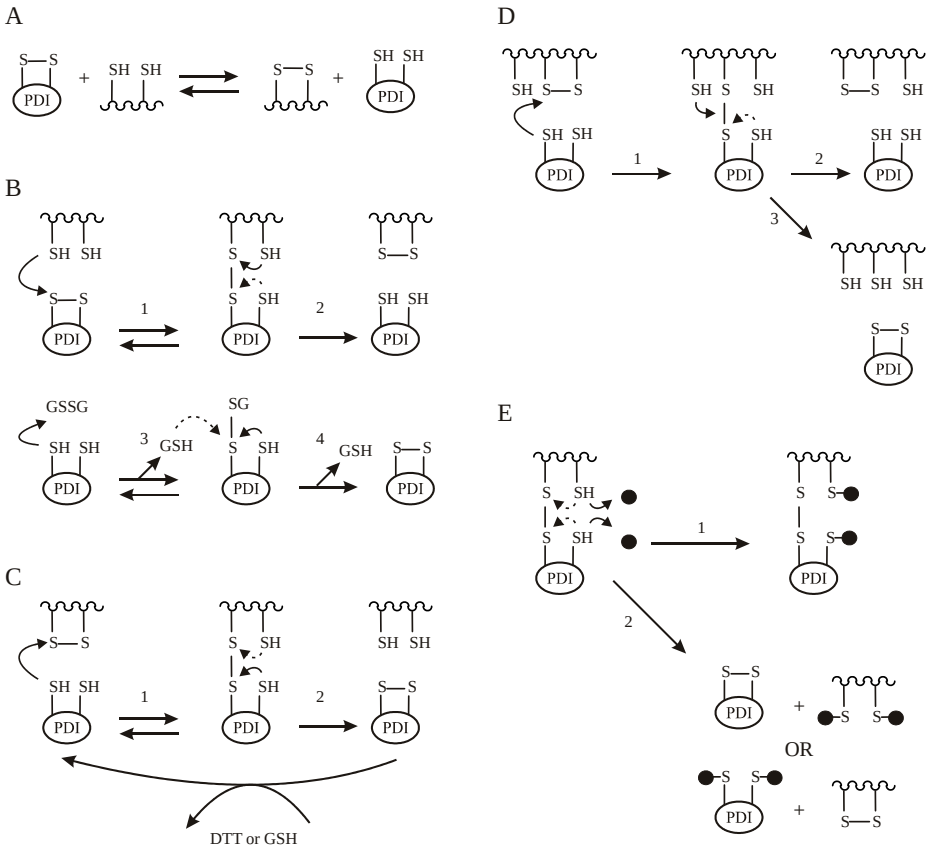

Fig. 4. Schematic presentations of the reactions of PDI. For clarity, only one of the two CGHC active sites in PDI is shown. In all panels, nucleophilic attack is indicated with a continuous curved arrow for the forward reaction and a broken curved arrow for the reverse reaction. For simplicity, the thiol/thiolate status of each cysteine is not marked, but it should be noted that thiolates are much more potent nucleophiles than thiol groups, and hence, the reactions of thiolates are very much faster. A) The thiol-disulphide equilibrium between a thioredoxin-superfamily members, such as PDI, and a substrate protein is determined by the relative stability of their dithiol and disulphide states. The dithiol state of PDI is stabilized relative to the disulphide state, in part by low pK_a of the N-terminal active site cysteine. This promotes the action of PDI in the

introduction of disulphide bonds into folding proteins in the ER. B) To act as a catalyst of disulphide bond formation, four distinct covalent rearrangements are required. After the first step, there is a competition that depends on the relative pK_a values of the second substrate protein cysteine and C-terminal active site cysteine residue. Hence, the pK_a of this C-terminal active site cysteine must be higher than the average value for cysteines in proteins or the reverse reaction will be kinetically favoured. In contrast, after step 3, there is a competition between the reaction of the C-terminal active site cysteine and GSH. Hence, for step 4 to proceed efficiently, the pK_a of the C-terminal active site cysteine must be low. This differential requirement for the pK_a of this group has been proposed to be resolved by the motion of the side chain of an arginine residue into and out of the active site locale (I). C) To act as an efficient catalyst of disulphide bond reduction, the N-terminal active site cysteine must have a low pK_a to favour the formation of the mixed disulphide and the C-terminal active site cysteine must have a low pK_a to favour the second step. The reduction of oxidized PDI by GSH or DTT is not shown in detail but it is the reverse of reactions 3 and 4 in B). D) To act as an efficient isomerase, the N-terminal active site cysteine of PDI must have a low pK_a to allow reaction 1 to proceed efficiently. After the first step, there is a kinetic partioning between the reaction of a cysteine residue from the substrate protein, which would result in isomerisation, and the reaction of the C-terminal active site cysteine, which would result in reduction. While isomerisation can proceed via cycles of oxidation and reduction (Schwaller *et al.* 2003) direct isomerisation is favoured by the C-terminal active site cysteine having higher-than-average pK_a value. The lifetime of the mixed disulphide state is obviously dependent on the pK_a of the C-terminal active site cysteine, since formation of a thiolate promotes the formation of an intramolecular disulphide with concomitant loss of the intermolecular disulphide. However, it is also dependent on the pK_a of the N-terminal acive site cysteine both for formation and for the ability of PDI to act as an efficient leaving group during nucleophilic attack by a second substrate cysteine (Szajewski *et al.* 1980). In addition, the lifetime of the mixed disulphide will be influenced by the affinity of the substrate binding sites of PDI to bind, but this is not directly related to the active site chemistry. E) Trapping mixed disulphide involving PDI with reagents such as iodoacetamide or N-ethylameide involves a competition reaction between intermolecular nucleophilic attack by C-terminal active site cysteine and all free thiol groups on the substrate on the quenching reagent and cysteine protein intramolecular nucleophilic attack. Trapping is only successful if all free thiol groups react with the quenching reagent. The accessibility and reactivity of thiol groups on substrate proteins will be highly variable, while the inaccessibility of the C-terminal active site cysteine will favour reaction 2 over reaction 1. (II, published by permission of Elsevier).

To be able to catalyze the different thiol-disulfide exchange reactions both the thermodynamics and kinetics of the system must be appropriate as well as the redox conditions of the cellular milieu. The sensitivity of cysteine residues in a

protein to oxidation will be affected by a combination of the pK_a and the local environment of the cysteine residue. Since an N-terminal thiolate stabilizes the reduced state of the active site, thioredoxin-superfamily members with a low pK_a for the N-terminal active site cysteine are better at catalyzing oxidation reactions (Fig. 4a). Similarly a low pK_a for the N-terminal active site cysteine favours the kinetics of catalysis of oxidation (Fig. 4b), reduction (Fig. 4c) and isomerization (Fig. 4d). In contrast, the requirement for the C-terminal active site cysteine is more complex. There a low pK_a kinetically favours reduction (Fig. 4c) and the last step of oxidation (Fig. 4b), but inhibits an earlier step in the catalysis of oxidation (Fig. 4b) and inhibits catalysis of isomerization (Fig. 4d). To resolve this paradoxical requirement, to be an efficient catalyst of oxidation, reduction and isomerization PDI must have mechanism in place that modulates the pK_a of the C-terminal active site cysteine.

2.7.1 Oxidation

PDI catalyses the reaction where a reduced protein or a peptide dithiol is oxidized to the disulphide form. During the reaction PDI loses its active site disulphide and becomes reduced. To complete the catalytic cycle PDI must be reoxidised. For *in vitro* oxidation reactions oxidized glutathione (GSSG) is usually used as the terminal electron acceptor. The reaction generates reduced glutathione (GSH) and changes the redox potential and redox buffering capacity of the reaction buffer. If no electron acceptor is present PDI is able to reduce a disulphide bond in one non-native protein molecule in order to form a disulphide bond in another non-native protein. The pathways for disulphide bond formation *in vivo* are complex and not completely understood (Fig. 5, III). The current model for the major route for disulphide bond formation in the ER is the tightly regulated flow of oxidizing equivalents from the sulphydryl oxidase Ero1 to PDI and to substrate proteins (Frand & Kaiser 1998, Pollard *et al.* 1998, Frand & Kaiser 1999, Tu *et al.* 2000, Gross *et al.* 2006, Sevier *et al.* 2007). While Ero1 oxidizes dithiols in substrate protein it makes one molecule of hydrogen peroxide per each disulphide bond formed (Gross *et al.* 2006). Hydrogen peroxide is often regarded as dangerous by-product that results in oxidative stress to cells, but it can potentially be used to form disulphide bonds via a cysteine sulphenic acid intermediate.

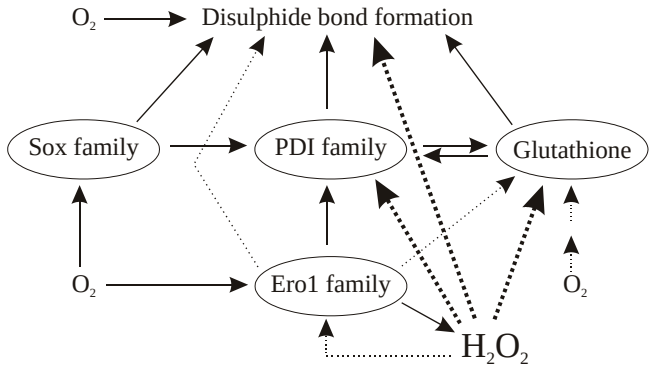

Fig. 5. The pathways for disulphide bond formation in the endoplasmic reticulum. Multiple pathways for dithiol oxidation to a disulphide in a folding protein exist. Direct oxidation by molecular oxygen, while widely used *in vitro*, is too slow to have physiological significance *in vivo*. Whereas members of the sulphydryl oxidase family are extremely efficient at making disulphide bonds in folding proteins (Thorpe *et al.* 2002, Thorpe & Coppock 2007) or transferring them to PDI family members (Sevier *et al.* 2001). PDI family members and GSSG can both introduce disulphide bonds into folding proteins, but both need to be reoxidized to complete the catalytic cycle. PDI is thought to be reoxidized by Ero1 (Pollard *et al.* 1998, Frand & Kaiser 1999, Tu *et al.* 2000, Sevier *et al.* 2007) while the source of GSSG is under debate in the literature. The hydrogen peroxide made by Ero1 each catalytic cycle (Gross *et al.* 2006) has potential to oxidize dithiols in substrate proteins, to oxidize glutathione to GSSG, to oxidize the active site of PDI family members, and to form the regulatory disulphides (Sevier *et al.* 2007, Appenzeller-Herzog *et al.* 2008, Baker *et al.* 2008) in Ero1 and hence shut down peroxide production. All dotted arrows represent hypothetical redox routes which have not been demonstrated experimentally *in vivo*. The bold dotted arrows indicate routes shown in this thesis work by *in vitro* methods (III, published by permission of Mary Ann Liebert Inc).

2.7.2 Reduction

In reduction reactions a protein or peptide disulphide bond is reduced to the dithiol form. During the reaction a disulphide bond is formed in the active site of PDI. To complete the catalytic cycle PDI must be reduced. *In vitro* DTT or GSH are commonly used as the electron donors. The only known protein reduction pathway in the ER is driven by reduced glutathione which acts as a net reductant

in the ER. GSH may play a role in the reduction of misfolded substrates or reshuffling of protein intermediates along the folding pathway. The source of GSH is the cytosol and therefore a continuous flux of reduced glutathione from the cytoplasm into the ER is required to maintain a pool of GSH for protein reduction (Bánhegyi *et al.* 1999, Jessop & Bulleid 2004, Molteni *et al.* 2004). GSH is maintained in reduced form in the cytosol by glutathione reductase, which ultimately obtains electrons from NADPH but these are currently no known glutathione reductases in the ER though membrane protein NADPH oxidases are at least partially resident in the ER and have the active site exposed in the cytosol. GSH is possibly transported from cytosol by a selective small molecule transporter (Banhegyi *et al.* 1999). Reduced glutathione may transfer electrons directly to folding proteins or it may transfer them to a protein reductase for example a PDI-family member, which then catalyzes the reduction of substrate protein (Jessop & Bulleid 2004).

2.7.3 Isomerization

PDI derives its name from the ability to catalyse isomerization reactions where the disulphides and thiols in proteins or peptides are rearranged to give a different disulphide bond pattern. Isomerization can occur via an intramolecular pathway or via cycles of reduction and oxidation (Schwaller *et al.* 2003). The attack of the N-terminal active site cysteine to a substrate disulphide initiates the formation of mixed-disulphide between PDI and a substrate cysteine residue. The N-terminal cysteine in PDI active site CGHC-motif is a strong nucleophile when deprotonated under the physiological conditions of the ER. After the initial reaction two different pathways can result in substrate isomerization: 1) Intramolecular rearrangement may occur when the sulphydryl of the substrate cysteine released by the formation of the mixed PDI reacts with another substrate disulphide (Fig. 4d). In this mechanism PDI enables reactions between the thiols and disulphides of the substrate while the substrate is covalently bound to PDI. In the final step of intramolecular isomerization a substrate cysteine displaces PDI from the covalent complex forming another disulphide in the substrate and reduced PDI for another round of catalysis. 2) In the other mechanism PDI resolves the covalent mixed-disulphide by reducing it by a reaction where the C-terminal active site cysteine reacts with mixed-disulphide to form an intra-molecular disulphide with the N-terminal cysteine. The reduction of the substrate disulphide is followed by reoxidation of the substrate protein in an alternative

configuration. In this mechanism isomerization of a substrate disulphide bond is a chain of reactions of reduction followed by reoxidation until the correct configuration of disulphide bonds is achieved.

2.7.4 Other functions of PDI

PDI is reported to be involved in many other cellular processes. These include being a subunit in two ER-resident complexes. The most intensely studied of these is PDI's function as a subunit of prolyl 4-hydroxylase. Collagen prolyl 4-hydroxylases (P4Hs) are ER located enzymes involved in the biosynthesis of collagens by catalyzing the formation of 4-hydroxyproline. 4-hydroxyprolines are essential for the formation and the stability of collagen triple helix (for recent review Myllyharju 2008). In addition, cytoplasmic P4Hs play a role in the regulation of the hypoxia-inducible factor HIFα (reviewed by Myllyharju 2008). Collagen P4Hs are $\alpha_2\beta_2$ tetramers in which the β-subunit is identical to PDI (Koivu *et al.* 1987, Pihlajaniemi *et al.* 1987). The β-subunit of P4H present in the native human P4H tetramer has about 50% of the wild-type PDI isomerase activity in rearranging the scrambled ribonuclease disulphide bonds (Koivu *et al.* 1987). PDI activity is not essential for tetramer assembly or for P4H activity (Vuori *et al.* 1992). The primary function of PDI in collagen prolyl 4-hydroxylase seems to be to keep the α-subunit in soluble catalytically active form (Veijola *et al.* 1996). In addition, due to the lack of ER retention signal in the α-subunit, PDI might help to retain P4H in the lumen of ER (Vuori *et al.* 1992).

PDI also forms the β-subunit of microsomal triglyseride transfer protein (MTP) (Wetterau *et al.* 1990), which is essential for the assembly of apoB-containing lipoproteins (Kivirikko 1998) PDI's main function in the complex also appears to be to keep highly insoluble MTP α-subunit in catalytically active conformation (Wetterau *et al.* 1991, Lamberg *et al.* 1996).

In addition, PDI is known to bind thyroid hormone and estradiol. The formed complexes may act as cellular hormone reservoirs (Primm & Gilbert 2001). There is evidence that PDI participates in other ER redox systems, for example connected with vitamin K epoxide reductase (VKOR). VKOR is a transmembrane protein of the ER that catalyzes the regeneration of vitamin K from vitamin K1 2,3-epoxide. Vitamin K1 is needed as a redox co-factor for the enzymatic carboxylation of glutamic acid residues. PDI has been proposed to have a role as a reductant of the active site in VKOR (Wajih *et al.* 2007).

3 Aims of the present work

The purpose of this study was to determine the catalytic mechanisms of PDI at the molecular level. Protein folding and especially the formation of disulphide bonds in folding protein is complex series of reactions. Despite years of research, the detailed role of PDI in this process remains unclear. This thesis aims to clarify the role of PDI in disulphide bond formation. More specifically the aims of the study were i) To determine the role of a conserved arginine residue in the PDI-family in the catalytic cycle. ii) To determine the pK_a-values of the active site cysteines of PDI and their effect on PDI catalyzed reactions. iii) To study the reaction kinetic of oxidation and reduction of the active site of PDI including the role of interplay between glutathione and PDI.

4 Materials and methods

4.1 Generation of expression vectors (I, II, III IV)

Expression vectors for the **a** domain of PDI (D18-A137), the first catalytic domains of P5 (L20-G140), ERp72 (N62-P170), ERp57 (S24-P133) and full length PDI with an N-terminal His-tag in-frame with the cloned gene were generated as described earlier (Alanen *et al.* 2003a). The resulting gene products included the sequence MHHHHHHM- before the first amino acid sequence of the domain sequence. Site-directed mutagenesis was performed as recommended by the manufacturer of PfuTurbo™ DNA Polymerase kit (Stratagene, La Jolla, CA, USA). The gene for expression of yeast Pdi1p was generated by PCR from yeast genomic DNA (generous gift from Dr. Angela Dunn, University of Kent, UK) using primers that included in frame NdeI site to 5' to the first codon of the gene and a BamHI site after a stop codon at the 3'-end. The insert was cloned into pLWRP51, a modified version of pET23b (Novagen) which encodes for an N-terminal his-tag in frame with the cloned gene. The resulting gene products included the sequence MHHHHHHM- prior to the first amino acid of the domain sequence. A gene insert for yeast PDI **a** domain (Q23-P141) was subcloned as a NdeI/BamHI fragment from the plasmid encoding full length Pdi1p into pOLR130, a modified version of pLWRP51, with an additional SpeI site in the multi-cloning site. All plasmids generated were sequenced to ensure the correctness of the cloned genes.

4.2 Protein purification (I, II, III, IV)

Proteins were expressed in *E.coli* strain BL21 (DE3) pLysS. Strains were grown in LB medium supplemented with 35 μg/ml of chloramphenicol and 100 μg/ml of ampicillin at 37 °C and induced at an A_{600} of 0.3 for four hours with 1 mM isopropyl β-D-thiogalactoside (Fermentas, Burlington, ON, Canada). Cells were harvested by centrifugation and the pellet was resuspended into 20 mM Sodium phosphate, pH 7.3 (buffer A) with 1 μg/ml DNase (Roche Diagnostics Gmbh, Mannheim, Germany). The cells were lysed by freezing and thawing twice and centrifuged. Filtered supernatants were loaded on an immobilized metal affinity column (HiTrap Chelating HP, Amersham Biosciences, Uppsala, Sweden) precharged with Ni^{2+} and equilibrated with buffer A. The column was washed

with 50 mM imidazole, 0.5 M NaCl in buffer A and bound proteins were eluted with 50 mM EDTA in buffer A and further subjected to anion (Resource Q, Amersham Biosciences) exchange chromatography. The proteins were eluted with linear gradient from buffer A to 0.5 M NaCl in buffer A over nine column volumes. The purity of eluted protein fractions was analysed on Coomassie Brilliant Blue stained sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and pure fractions were pooled and buffer exchanged into buffer A. The purity and size of proteins was also tested by mass spectrometry. The concentrations of proteins were determined spectrophotometrically based on their calculated molar extinction coefficients at 280 nm.

4.3 Circular dichroism (I)

Far-UV circular dichroism spectra of the wild type and R120 mutants of PDI**a** and the first domains of ERp57, ERp72 and P5 were recorded on a Jasco J600 spectrophotometer. All scans were collected as an average of eight scans at 25 °C, using 0.1 cm path length, 50 nm/min scan speed, 1.0 nm spectral band width and 0.5 s time-constant. The maximal HT voltage was 750 V.

4.4 Peptide oxidation assay (I, IV)

The method of Ruddock *et al.* (1996) using a fluorescent decapeptide NRCSQGSCWN (University of Kent, Department of Biosciences, Biomolecular Science Facility, UK) was used to determine the catalytic ability of enzymes to form a disulphide bond in a peptide. McIlvaine buffer (0.2 M disodium hydrogen phosphate / 0.1 m citric acid; pH 4.0 – 7.5) was used, except where stated differently in the original articles, in a fluorescence cuvette to give a final reaction volume of 1 ml. Except where noted in the original articles, the final concentration of 0.5 mM of oxidized glutathione, 2 mM of reduced glutathione, 0.1 – 2 μM of enzyme and 3.2 μM of substrate peptide was added to the reaction buffer. Change in fluorescence intensity was monitored with Perkin-Elmer LS50B spectrophotometer. All the measurements were done at 25 °C over an appropriate time using excitation wavelength of 280 nm, emission wavelength of 350 nm and slit widths of 5 nm.

4.5 Oxidation and reduction assays with stopped-flow (III, IV)

The rates of oxidation by GSSG or H_2O_2 and reduction by GSH of the active site of the **a** domain of PDI and the PDI **a** mutants as well as the **a** domain of Pdi1p were determined by the change in fluorescence of tryptophan 52 (and the corresponding tryptophan 60 in Pdi1p), which is juxtaposed to the N-terminal active site cysteine and hence acts as a useful spectroscopic marker. The other tryptophan 128 in the PDI**a** was mutated to phenylalanine to remove the background signal. The change in fluorescence was monitored by using a KinTek SF-2004 stopped-flow fluorometer (KinTek, Austin, TX, USA), excitation 280 nm and band-pass emission >320 nm at 25 ˚C. The enzyme concentration used was 10 µM, GSSG concentration varied from 1 mM to 50 mM, GSH concentration varied from 2.5 mM to 50 mM and H_2O_2 concentration varied from 0.1 M to 2.5 M. Before monitoring the oxidation or reduction PDI **a** W128F and the mutants were reduced or oxidised with 5-fold molar excess of DTT or GSSG (both from Sigma, St.Louis, MO, USA), respectively for 30 min at RT. The excess of DTT or GSSG was removed and enzymes buffer exchanged into reaction buffer (0.1 M disodium hydrogen phosphate, 0.1M citric acid, 0.1 M boric acid, 1 mM EDTA, pH7) with Biomax Ultrafree® 0.5 centrifugal filter concentrators (Millipore, Bedford, MA, USA).

4.6 Insulin precipitation assay (II)

The reductase activity of PDI was determined by the insulin precipitation assay. The precipitation of B-chain of bovine insulin (Sigma, St.Louis, MO, USA) was initiated by adding insulin to a final concentration of 1 mg/ml into 0.1 m Na-phosphate buffer, pH 7 containing 1 mM EDTA and 10 mM DTT. In enzyme catalysed reactions, PDI or mutant PDI were at 1 µM concentration. As a negative control we used full length PDI where all the active site cysteines were mutated (PDI C53S / C56A / C397S / C400A). The precipitation of the B-chain of insulin was monitored as an apparent time dependent change in absorbance due to light scattering at 540 nm in a Shimatzu BioSpec-1601 E spectrophotometer.

4.7 Determination of pK_a-values (II)

The pH-dependence of the reactivity of the cysteines in PDI **a** mutants were determined by reaction with Ellman’s reagent DNTB (5,5-dithio-bis(2-

nitrobenzoic acid)) at different pHs. Buffer for the experiments was 0.1 M Na_2HPO_4, 0.1 M citric acid (pH 4 -7.75) or 0.1 M Na_2HPO_4, 0.1 M boric acid (pH 8-10). pH from 4 to 10 with 0.25 pH unit intervals was adjusted with NaOH. Stock buffer solutions were prepared and stored frozen in aliquots to avoid day to day variation. Similarly a stock of 4.5 mg/ml Ellman's reagent in DMSO was made up and stored in frozen aliquots. The reactions were performed at 25 °C using 25 μM enzyme with 0.073 mg/ml DTNB and followed by change in absorbance at 412 nm using KinTek SF-2004 stopped-flow. The rate constant for each reaction was determined by fitting the data to a first exponential equation using KinTek SF-2004 software (KinTek, Austin, TX, USA). Then the data from all of the reactions at different pHs was analysed with Igor Pro 3.14 (Wavemetrics Inc., Lake Oswego, OR, USA) by fitting it to pseudo first order kinetics with single pK_a dependence or with two pK_a dependence.

4.8 Quenched-Flow (IV)

Intermediates of the reaction of reduced enzyme with oxidised glutathione or the proportion of oxidized protein in different time points of the reaction were determined by quench flow. The reaction of 40 μM enzyme in reaction buffer and glutathione was quenched after the reaction time of interest with 1.1 M IAA (Sigma Steinheim, Germany) or 200 mM NEM (Sigma). Rapid reactions were done with KinTek RQF3 Quench flow apparatus (KinTek, Austin TX, USA).The excess quencher was removed by purification with PepClean™ C18 Spin column (Pierce, Rockford, IL, USA). The sample was washed two additional times with 0.5% TFA, 5% ACN to remove salts, and eluted from the column with 50% ACN. TFA was added to samples to get 0.1% final concentration. The samples were analysed by electrospray mass spectrometer (Micromass LTC, Machester, UK) using positive ionization. For time points longer than 10 s, manual mixing of the reagents was performed.

4.9 Trapping experiment (II)

The ability of arginine mutant of PDI (R120 / R461) to stabilize PDI-protein mixed disulphide was examined by a trapping experiment. PDI and the R120Q / R461Q and R120D / R461D mutants at a final concentration of 10 μM were allowed to react for 5 minutes with GSSG and then reduced BPTI was added.

After two minutes the reaction was quenched with 100 mM iodoacetamide. The samples were analysed by 10% non-reducing SDS-PAGE.

5 Results

5.1 Conservation in the catalytic domains of PDI family (I)

Multiple alignments were done of the 14 catalytic domains of the known human PDI family members (PDI, PDIp, ERp57, ERp72, P5 and PDIr) using CLUSTAL W (Higgins *et al.* 1994), T-Coffee (Notredame *et al.* 2000), Match-Box (Depiereux *et al.* 1997) and MultAlin (Corpet 1988) (Fig 1, I). The results from all four alignments were different and they were combined into a single alignment based on consensus alignment and using the known structure of **a** domain of PDI to move the gaps into loop regions and to align corresponding helix and strand regions without misaligning regions of good sequence similarity. Of the 12 residues that show >90% identity eight are located around the active site motif. Three of the remaining residues are structural; P61 forms a kink in the α2 helix while the proline residue of the conserved motif (P100-T101) forms a cis-peptide bond located under the active site. D83 is conserved except in the first and the third catalytic domains of PDIr. In the NMR structure of human PDI this residue forms a hydrogen bond with Y49 and hence may play a structural role. The remaining site showing >90% identity is R120 in PDI. All domains except the first catalytic domain of PDIr have this residue and all four multiple alignment programs found the alignment in all domains. The amino acid identity at this position is greater than that of the active site glycine (G54) which is considered to be one of the residues that define PDI and to separate it from the members of thioredoxin superfamily.

5.2 The role of the conserved arginine residue in the oxidative catalytic cycle of the PDI family (I)

The arginine at position 120 in PDI showed high conservation within the human PDI family members. Based on the NMR structure of the **a** domain of PDI the arginine 120 side chain points out into solution far from the active site and makes no significant interactions with other residues and therefore no clear function for it could be deduced. To examine the role of Arg120 in the oxidative catalytic cycle mutations were made. The activities of the mutant proteins were compared with the wild type in the standard peptide oxidase activity assay (Ruddock *et al.* 1996). The R120Q mutant showed 26% and R120D <2% of the activity of the

wild type PDI**a** (Fig. 2A, I). The decrease in activity was not as a result of changes in the structure of the protein, since far-UV CD spectra for the mutants and wild type proteins were identical. To examine if the effect of analogous arginine residues is conserved within PDI family members, mutations were made into the first catalytic domain of ERp57, ERp72 and P5. In each case the mutation of arginine to glutamine clearly decreased the oxidase activity when compared to the wild type. Furthermore, no significant differences were seen between the wild type and mutant far-UV CD spectra (Fig. 2B, I). Based on the peptide oxidation assay (Ruddock *et al.* 1996) with varying concentrations of substrate peptide or oxidized glutathione arginine 120 were shown to have a role in reoxidation of the active-site of PDI, not in interaction with the substrate. When reoxidation of the dithiol form of the enzyme was determined by using oxidized glutathione, PDI **a** domain showed a time–dependent decrease in fluorescence with the rate of reoxidation increasing with increasing concentrations of GSSG and the arginine 120 mutants clearly decreased the rates of this reaction (Fig. 3, I).

5.3 Determination of pK_a values of the active site cysteines (I, II)

The pK_a values of the active site cysteine residues play an important role in determining the function of members of the thioredoxin-superfamily. Molecular simulations of the PDI **a** domain showed movement of side chain of arginine 120 into the active site locale. This motion lowers the pK_a of both active site cysteines, the effect being greatest for the C-terminal cysteine (I). These studies combined with *in silico* calculations showed that the pK_a values of C-terminal cysteine (Cys 56) shifted during the molecular simulation studies consistent with the requirement for changes in pK_a during the catalytic cycle.

Experimentally the pK_a-values of the active site cysteines were determined by studying the pH-dependence of the reactivity of each cysteine, since thiolates are more potent nucleophiles than thiols and hence show much faster rates of reaction. Specifically we studied the reaction with Ellman's reagent DNTB (5,5-dithio-bis(2-nitrobenzoic acid)) (Ellman 1958) since this assay gives a significant change in absorbance at 412 nm as nitrothiobenzoate is formed as a product and since this assay is the standard assay for thiol group determination.

To study the effect of the arginine on each of the active site cysteine residues in the **a** domain of PDI, we worked on single cysteine mutants with the N-terminal cysteine mutated to methionine (C53M) or the C-terminal active site cysteine mutated to serine (C56S). For the C53M mutant, and all mutants made in

this background, the rate of reaction of excess Ellmans's reagent with Cys56 was studied by stopped-flow kinetics at 0.25 pH unit intervals over the pH range 6.5 to 10. At all pH values the rate of change of absorbance at 412 nm fitted to a pseudo first order reaction with random residuals. Visual examination of the data obtained suggested a discontinuity between pH 9.25 and 9.5 consistent with either a significant conformational change in the protein or a denaturation event (Fig. 2, II). Analysis of the data over the pH range 6.5 to 9.25 revealed that the data fitted well to a single pK_a dependent event with a calculated pK_a of 8.60 ± 0.02, with no determinable rate of reaction for the thiol state and a second order rate constant of $9.5 \pm 0.2 \times 10^4$ $M^{-1}s^{-1}$ for the thiolate state. When these experiments were repeated with the arginine mutants R120Q and R120D in the C53M background the data again fitted to pseudo first order reactions with random residuals, there was an apparent discontinuity between pH 9.25 and 9.5 and the data over the range 6.5 to 9.25 fitted well to a single pK_a dependent event (Fig. 2, II). However, there was a clear shift in the calculated pK_a values for Cys56 to 9.14 ± 0.04 (C53M / R120Q) and 9.22 ± 0.06 (C53M / R120D) confirming our hypothesis that arginine 120 modulates the pK_a of the C-terminal active site cysteine of the **a** domain of PDI.

Other residues have been implicated in modulating the pK_a of the active site cysteine residues, specifically the residues involved in the salt bridge buried under the active site (Dyson *et al.* 1997) - Glu47 and Lys81 in the **a** domain of PDI (Ellgaard & Ruddock 2005). When these residues are mutated, the **a** domain of PDI shows >80% decrease in activity in catalysis of the oxidation of a small peptide substrate (data not shown). However, examination of the pH dependence of the rates of reaction of Cys56 with either Glu47 (C53M / E47L) or Lys81 (C53M / K81M) mutated showed no significant differences with the C53M control, with calculated pK_a values of 8.64 ± 0.03 and 8.73 ± 0.04 respectively (data not shown). This result implies that neither Glu47 nor Lys81 significantly modulate the pK_a of Cys56.

Based on the molecular simulation studies we postulated that the effect of Arg120 was greater on the pK_a of Cys56 than on Cys53 (I). To confirm this experimentally the pH dependence of the rate of reaction of Ellman's reagent with Cys53 was examined by using the C56S mutant. Over the pH range 4.0 to 7.5 the rate of change of absorbance at 412 nm fitted to a pseudo first order reaction with random residuals. However, the C56S mutant showed a more complex pH dependence of rate than had previously been seen for the C53M mutant. The simplest reaction scheme that gave random residuals for all constructs, except the C56S / R120D (see below), was a fit to a two pK_a dependent event (Fig. 2, II).

For the C56S construct the first pK_a was 4.81 ± 0.07 and was associated with a second order rate constant of 2.9 ± 0.2 x 10^4 $M^{-1}s^{-1}$, while the second pK_a was 6.61 ± 0.23 and was associated with a further increase in the second order rate constant for the reaction to 4.1 ± 0.1 x 10^4 $M^{-1}s^{-1}$. Examination of the C56S / R120Q mutant showed no effects of the arginine mutation on the pK_a values obtained, with calculated pK_a values of 4.84 ± 0.03 and 6.87 ± 0.18. However there was a small decrease in the second order rate constants associated with both pK_a values to 2.5 ± 0.1 x 10^4 $M^{-1}s^{-1}$ and 3.3 ± 0.1 x 10^4 $M^{-1}s^{-1}$ (Fig. 2, II). In contrast to the results obtained with the C56S and C56S / R120Q mutants, the results from the C56S / R120D mutant fitted to a single pK_a dependent event with a pK_a identical to the first pK_a determined for the C56S construct, with a value of 4.81 ± 0.03. However, the associated second order rate constant was again smaller with a value of 2.3 ± 0.1 x 10^4 $M^{-1}s^{-1}$. These results are consistent with the hypothesis that the effects of Arg120 are larger on the pK_a of Cys56 than on that of Cys53. However, there are clearly effects of Arg120 on the reactivity of Cys53 that are independent of modulating the pK_a.

5.4 Mutating arginine 120 and 461 has different effects on PDI reductase and isomerase activities (II)

Previously we showed that the conserved arginine in the catalytic domains of PDI has an important role in the catalytic cycle of PDI when it is acting as a catalyst for the glutathione dependent formation of a disulfide bond in a small peptide substrate (I). Specifically the R120Q mutant reduced the activity of the **a** domain of PDI by circa 75% while the R120D mutant reduced the activity by >98%. This reduction in activity was linked to the reoxidation of the active site by oxidized glutathione (GSSG).

Catalyzing the oxidation of dithiols to disulfides in peptide and folding proteins is just one of the activities of PDI, with catalysis of the reduction of disulfides and thiol-disulfide isomerization being two of the others. Examination of the reaction scheme for the catalysis of reduction (Fig. 4) suggests that the pK_a of the C-terminal active site cysteine will play a critical role in determining the kinetics of catalysis. Hence, mutation of arginine 120 and arginine 461, the analogous residue in the catalytic **a'** domain, would be expected to have a significant effect on the ability of PDI to catalyze reduction reactions.

The classic assay for examining the ability of thioredoxin-superfamily members to catalyze disulfide bond reduction is the insulin reduction assay (for

example, Matsuo *et al.* 2001, Nørgaard & Winther 2001). Insulin is a small protein which in the mature form comprises two polypeptides, the A- and B-chains, which are linked by two disulfide bonds. In the presence of dithiothreitol (DTT) these inter-chain disulfide bonds are reduced and the isolated B-chain aggregates leading to an increase in light scattering. The non-catalyzed reaction using 10 mM DTT at pH 7 is relatively slow with an average lag-phase before aggregation of 445 seconds (Fig. 3, II). The addition of catalytic amounts of wild-type mature human PDI resulted in a significant increase in the rate of reduction of the disulfide bonds in insulin. Hence wild-type PDI leads to a decrease in the average lag-phase before aggregation to 106 seconds (Fig. 3, II). Mutation of Arg120 and Arg461 resulted in a significant decrease in the ability of PDI to catalyze insulin reduction, with the R120D / R461D double mutant and the R120Q / R461Q double mutant both showing comparable activity to a human PDI mutant in which all four active site cysteine residues had been mutated (Fig. 3, II).

While mutation of arginine 120 and arginine 461 had a significant deleterious effect on the ability of PDI to catalyze reduction reactions and to be reoxidised during the catalysis of oxidation reactions, mutation of these residues would also be expected to stabilize PDI-protein mixed disulfides. This stabilization would increase the time available for direct isomerization (Fig. 4) and hence a mutation in these residues might be expected to increase the ability of PDI to act as an isomerase. The ability of PDI to act as an isomerase was studied by the refolding of bovine pancreatic trypsin inhibitor (BPTI). BPTI is a small protein that contains three disulphide bonds in its native state. It has a complex folding pathway where isomerization of disulphides is the rate limiting step (Weissman & Kim 1991, Creighton 1992, Weissman & Kim 1995). The number of disulphide bonds in different BPTI species as function of time were analyzed by mass spectrometric methods. The non-catalysed refolding of BPTI in a glutathione buffer at pH 7 is slow. The fully reduced state disappears with a second-order rate constant of 7.0 ± 0.7 $M^{-1}s^{-1}$ with the concomitant formation of one-disulphide species, then two disulphide bonds containing species. Finally the three disulphide bonds containing native species is formed. The kinetics of this formation is slow due to the requirement of intramolecular thiol-disulphide isomerization (Weissman & Kim 1993). When catalytic amounts of PDI wild-type is present the reaction proceeds significantly faster (Fig. 4, II), 80% of native three-disulphide species being formed within 10 minutes. The disappearance of the fully reduced species for PDI R120Q / R461Q catalysed reaction had the

second-order rate constant of 19.0 ± 0.5 $M^{-1}s^{-1}$ and for PDI R120D / R461D 13.6 ± 0.8 $M^{-1}s^{-1}$. In contrast to non-catalyzed reaction the formation of the native three-disulphide bond state was fast with 80% being formed within 20 minutes.

To study refolding in more detail, reduced BPTI was refolded for 2 hours in a glutathione refolding buffer to generate a mix containing predominantly two disulphide-containing species that require an isomerization even to reach their native form. Reaching the three disulphide state is slow when a catalyst is not present in the reaction mixture. When a catalytic amount of human wild-type PDI is present, the formation of three disulphide state is rapid. The reaction fits well to a first-order process, with an average rate constant of 0.121 ± 0.0002 min^{-1}. Similarly, when the R120Q / R461Q mutant of PDI was present, the reaction was rapid and fitted to a first-order process with an average rate constant of 0.165 ± 0.018 min^{-1}. In contrast, while R120D / R461D mutant catalyzed the formation of three-disulphide state with the similar speed, the reaction kinetics were more complex.

5.5 Stopped-flow kinetics of oxidation of the active site of PDI by peroxide and glutathione (III, IV)

Peroxide and glutathione oxidation of the active site of PDI was studied directly by fluorescence studies on the active site of the W128F mutant of the catalytic **a** domain of PDI. This mutant contains only single tryptophan, W52, which is juxtaposed to the active site and acts as a useful and direct spectroscopic marker of the redox state of the active site (I). When either GSSG or peroxide was added to the reduced protein a time-dependent decrease in fluorescence was observed, consistent with the difference in fluorescence of the dithiol and disulfide states of the active site. The average decrease in fluorescence was 49 ± 1% for the GSSG-mediated oxidation and 48 ± 1% for the peroxide-mediated oxidation The GSSG-dependent oxidation of PDI fitted to a two step process (Fig. 1, IV). The first rate constant was linearly dependent on GSSG concentration with k = 191 ± 3 $M^{-1}s^{-1}$ and represents the formation of the GSH-PDI mixed disulfide. The second rate constant was independent of GSSG concentration, with k = 0.23 ± 0.03 s^{-1}, and represents the formation of the active site disulfide. These data imply that at physiological concentrations of GSSG the rate limiting step for oxidation of the active site of PDI is the nucleophilic attack by the C-terminal active site cysteine on the GSH-PDI mixed disulfide. In contrast, at peroxide concentrations up to 2.5 M the peroxide-dependent oxidation of PDI fitted to a single exponential function

(Fig. 3, III), with a linear dependence on the concentration of peroxide k = 9.2 $M^{-1}s^{-1}$. These data imply that the rate limiting step for peroxide mediated oxidation of PDI active site is the initial formation of the N-terminal cysteine sulfenic acid and that the rate of the nucleophilic attack by the C-terminal active site cysteine on the N-terminal sulfenic acid must be significantly greater than 40 s^{-1}.

5.6 Quenched-flow studies (IV)

To ensure that the rate constants determined from the stopped-flow experiments represent the predicted steps in the reaction quenched-flow experiments were carried out. Oxidized glutathione was added to the reduced proteins and at set time intervals a large molar excess of a quenching reagent was added to the reactions. Since the reactions studied are based on thiol-disulphide exchange the quencher used were thiol-alkylating agents, N-ethyl maleimide (NEM) or iodoacetamide (IAA). The products were analyzed by electrospray mass spectrometry, which allows the identification of three potential species, the oxidized protein, glutathione mixed disulphide with the C-terminal active site cysteine alkylated and the reduced alkylated protein. For all the experiments only the oxidized and reduced alkylated proteins were detected. The kinetics of the oxidation of reduced enzyme by 10 mM GSSG observed by quenched-flow gave a pseudo first order rate constant consistent with the results derived from the stopped-flow studies of 2.8 s^{-1}.

5.7 Reduction of the active site of PDI by glutathione (IV)

The W128F mutant of PDI **a** domain was used to study the reduction of the active site disulphide by stopped-flow. Glutathione reduction of the active site of PDI was studied by monitoring the change in fluorescence of the tryptophan juxtaposed to the active site cysteines. When GSH was added to oxidized PDI **a** W128F, a time dependent increase in fluorescence was observed (Fig 2, IV). The increase in fluorescence was equivalent to the decrease of fluorescence observed during oxidation studied by stopped-flow. Glutathione dependent reduction of oxidized PDI is thought to be a two-step process. The first step is thought to be the nucleophilic attack of GSH on the actives site disulphide to form a mixed-disulphide while the second step is expected to be the nucleophilic attack of another molecule of GSH on the mixed disulphide to form the reduced active site

and oxidized glutathione. Reduced glutathione was in large excess over oxidized PDI in the experiments and therefore both of the reactions were expected to be pseudo first order reactions with the rate being proportional to the concentration of GSH. An alternative reaction mechanism would be via the formation of a trimolecular complex of two GSH and one protein molecule. Here the reaction would be third order, but due to the large excess of GSH over oxidized PDI the reaction would be observed as a pseudo first order reaction with the rate constant being proportional to the concentration of GSH squared. Analysis of the stopped-flow data fitted best to a single exponential process at all GSH concentrations. The plot of the derived rate constants versus glutathione concentration was not proportional neither to the GSH concentration nor to the concentration of GSH squared (Fig 2b, IV). The kinetics of glutathione based reduction of PDI is complex. The initial mixed disulphide formed form the reaction of reduced glutathione with oxidized PDI undergoes kinetic partioning. Either the mixed disulphide reacts with another molecule of GSH to form a reduced protein and GSSG or the C-terminal active site cysteine is able to initiate an intra-molecular nucleophilic attack to form the starting material (Fig 2c, IV) The kinetic partitioning depends upon the concentration of GSH. When the data obtained for the GSH-dependence was fitted to this reaction scheme, three rate constants were obtained. The first nucleophilic attack by GSH on oxidized PDI **a** domain gave a second order rate constant of 11200 $M^{-1}s^{-1}$. The second nucleophilic attack of GSH on the PDI-GSH mixed disulphide gave a second order rate constant 4600 $M^{-1}s^{-1}$, while the reverse reaction initiated by the C-terminal active site cysteine gave a first order rate constant of 430 s^{-1}.

5.8 Stopped-flow kinetics of oxidation and reduction of the active site of Pdi1p by glutathione (IV)

In contrast to the human PDI-family, which consists of circa 20 members, the yeast *S. cerevisiae* has only five members. Yeast family member Pdi1p and human PDI are considered to be homologous proteins. Previously when the *in vitro* and *in vivo* results obtained from these two proteins are compared, no potential differences are usually considered (see Hatahet & Ruddock 2009 for a review). However, these two proteins are only 29.1% identical and few comparative studies have been done. In this thesis work the kinetics of the **a** domain of yeast Pdi1p and human PDI reacting with glutathione were compared.

The **a** domain of Pdi1p contains only a single tryptophan residue (W60), which is located juxtaposed to the active site in an analogous position to the human PDI **a** W52 and hence can be used as a spectroscopic marker for the redox state of the active site. When oxidised glutathione was added to the reduced Pdi1p **a** domain, a time-dependent decrease in fluorescence was observed, similar to the decrease observed for the reduced human PDI **a**. In addition, similarly to human PDI **a**, the kinetic traces fitted to a sequential two-step process with one rate being dependent on the concentration of GSSG and the other being independent (Fig 3a, IV). The derived second order rate constant for the formation of the Pdi1p-glutahione mixed disulphide was 163 ±5 $M^{-1}s^{-1}$ while the first order rate constant for the loss of the mixed disulphide was 0.13 ± 0.01 s^{-1}. These results suggest that while the mixed disulphide is more stable species for yeast Pdi1p **a** domain than for the human protein, the kinetics of glutathione oxidation of the two proteins are remarkably similar.

In contrast, very significant differences in kinetics were observed when reduction of the two proteins was studied. When reduced glutathione was added to the oxidised **a** domain of yeast Pdi1p, a time-dependent increase in fluorescence was observed, which similarly to the human protein showed a complex dependence on the concentration of GSH (Fig 3b, IV). When the data obtained for the GSH-dependence was fitted to the same kinetic partitioning reaction scheme used for human protein, values for three rate constants were obtained. The first nucleophilic attack of GSH on oxidised yeast Pdi1p **a** domain gave a second order rate constant of 720 $M^{-1}s^{-1}$. The second nucleophilic attack by GSH on the Pdi1p-GSH mixed disulphide gave a second order rate constant of 170 $M^{-1}s^{-1}$, while the reverse reaction intiated by the C-terminal active site cysteine gave a first order rate constant of 6.4 s^{-1}. These rate constants are between 15 and 68 times slower that the corresponding rate constants for the human PDI **a** domain.

5.9 Effect of the C-terminal active site cysteine on the reactivity of the N-terminal cysteine residue (IV)

The data for the glutathione mediated oxidation of both human PDI **a** domain and yeast Pdi1p **a** domain indicated that the rate limiting step of the oxidation is the intra-molecular nucleophilic attack of C-terminal active site cysteine on the mixed disulphide species. This implies that the mixed disulphide is a relatively long living species with a half-time around 3 and 5 seconds respectively. To date it is

has been assumed that the reactive species during the oxidation of a peptide substrate is the oxidised i.e. the intra-molecular disulphide form of PDI. However, there is no reason to expect the PDI-glutathione mixed disulphide form not to be a target for nucleophilic attack by a thiolate on a peptide or a protein substrate (Fig 4a, IV). If it is able to be a target, then C56 mutants of PDI **a** domain would be expected to be active in catalysing the GSSG-mediated oxidation of a peptide substrate.

For *in vitro* and *in vivo* studies on the reaction mechanisms of action of PDI-family members the C-terminal active site cysteine is often mutated to a serine, due to the similarity of serine compared to cysteine in terms of size and polarity. However, serine is also a strong β-sheet and α-helix breaker and therefore the C-terminal cysteine has been mutated to an alanine in some studies. To examine the both possibilities the C56S and C56A mutants of human PDI **a** domain in the W128F background along with the C56S mutant in the wild-type **a** domain were made in this study. The standard peptide oxidation assay (Ruddock *et al.* 1996) but with no GSH in the reaction gave the activity of C56A / W128F mutant of PDI **a** domain about 1% of the activity of the wild-type enzyme (Table 1, IV). The low activity was not due to the W128F mutation as this mutation had the activity >90% of the activity of the wild-type enzyme. While the C56A / W128F mutant had only low activity, it retained some activity while C53M mutant of the PDI **a** domain did not. The residual activity was further examined by increasing the concentration of the C56 mutant enzymes ten-fold. Under these conditions the substrate peptide is only a small excess over the enzyme concentration and due to the nature of the assay the useable concentration range of the peptide substrate in the assay is narrow. Activity of the C56S / W128F mutant under these conditions was only 25% of the activity of the wild-type enzyme, indicating that on molar basis it had only 2.5% of the wild-type enzyme activity. In contrast to these results, the C56A /W128F mutant of the PDI **a** domain mutant showed more complex kinetics. Instead of a change in fluorescence that could be fitted to a single exponential process, a clear double exponential potential was observed (Fig 4b, IV). The relative change in fluorescence indicated that the slower of the two processes was linked to the formation of the oxidised peptide. This would indicate the activity of this mutant to be of 1.2% of the wild-type activity on a per molar basis. The faster rate constant was $4.9 \pm 1.1 \times 10^{-2} s^{-1}$. Due to the high concentration of enzyme in these assays the faster rate constant is suggested to represent the formation of the glutathione-enzyme mixed disulphide.

To examine this in more detail mass spectrometry based quenched flow analysis was carried out. In the reaction of C56 mutants with GSSG two products were observed, the initial reduced species containing a single reactive thiol and the mixed disulphide with glutathione. Quantification of these two species allowed the calculation of the pseudo first order rate constants. The obtained rate constants were 1.24 ± 0.23 s^{-1} for C56A / W128F mutant (Fig. 4c, IV), 0.18 ± 0.01 s^{-1} for C56S /W128F mutant (Fig 4d, IV) and 0.21 ± 0.01 s^{-1} for C56S mutant of the PDI **a** domain (Fig 4e, IV). Since the concentration of the GSSG used was 10 mM these rate constants could be converted into second order rate constants of 124 $M^{-1}s^{-1}$, 18 $M^{-1}s^{-1}$ and 21 $M^{-1}s^{-1}$ respectively, which all are significantly lower than the value obtained for the wild-type protein by stopped-flow and quenched-flow experiments. A second order rate constant of 124 $M^{-1}s^{-1}$ would give an apparent pseudo first order rate constant of 6 x 10^{-2} s^{-1} with the 0.5 mM GSSG used in the peptide oxidation assay. This is comparable with the fast rate constant observed for the C56A / W128F mutant of the PDI **a** domain in that assay, suggesting that this rate represents the formation of the glutathione mixed disulphide. From the quenched-flow studies the expected pseudo first order rate constant for the C56S / W128F mutant reacting with GSSG under peptide oxidation assay conditions would be 9 x 10^{-3} s^{-1} and the similarity of this rate constant with the observed rate of peptide oxidation may explain why the separation of the two rate constants was impossible.

6 Discussion

6.1 Modulation of the pK_a of the active site cysteine residue of PDI allows it to act as catalyst of oxidation and isomerization

The pK_a values of the active site cysteine residues have a significant effect on the physiological function of the whole thioredoxin superfamily. The cysteine residues are able to cycle between dithiol and disulphide states with the equilibrium between these states determining the physiological function of the enzyme. To act as an oxidant, as per PDI, the dithiol state of the enzyme must be stabilized relative to the disulphide state. In part this is achieved by the stabilization of the thiolate state of the N-terminal cysteine residue and the destabilization of the thiolate state of the C-terminal cysteine residue. The pK_a of the N-terminal cysteine of PDI is very low and that of the C-terminal cysteine is very high (Hawkins & Freedman 1991, Nelson & Creighton 1994), which helps the protein to be more oxidizing and increases the nucleophilicity of the N-terminal cysteine and decreases that of the C-terminal cysteine residue. The increased pK_a of the C-terminal cysteine residue promotes oxidation of substrates but on the other hand inhibits the reoxidation of PDI, which requires nucleophilic attack by the C-terminal cysteine residue and hence is promoted by the formation of a thiolate at this position. Reoxidation of PDI is essential to complete the catalytic cycle, which makes the different requirements of the high and low pK_a values of the C-terminal cysteine in the different steps of the reaction important.

Molecular simulation studies resolved the problem of requirements of modulating the pK_a values by showing the movement of the conserved arginine residue of PDI into and out of the active site locale. The majority of time the side chain of arginine 120 is located far from the active site, the pK_a of the C-terminal cysteine is high and the dithiol form of the protein is stabilized, which allows PDI to act as an oxidant. The pK_a of the C-terminal cysteine residue is lowered significantly by the movement of the arginine 120 side chain into the active site locale. The thiolate formed is able to act as a nucleophile on any mixed disulphide formed by the N-terminal cysteine with substrate or glutathione. Experimental determination of pK_a values of the active site cysteine residues of PDI in wild type and R120 mutation background showed a clear shift in the pK_a values for C-terminal cysteine (from 8.60 ± 0.02 for wild type to 9.14 ± 0.04 and 9.22 ± 0.06 for R120Q and R120D mutants, respectively). Hence arginine 120 modulates the

pK_a of the C-terminal active site cysteine residue of the **a** domain of PDI. The effect of arginine was shown to be greater on the pK_a of the C-terminal cysteine than on the N-terminal cysteine residue.

Other residues have been implicated in modulating the pK_a of the active site cysteines, especially the residues involved in the salt bridge buried under the active site (Dyson *et al.* 1997), Glu47 and Lys81 in PDI (Ellgaard & Ruddock 2005). When mutating these residues, the **a** domain of PDI shows >80% decrease in activity in catalysis of the oxidation of a small peptide substrate. However, examination of the effect of these mutations to the pK_a values of the active site cysteine residues showed no significant differences compared to the wild type. These results imply that neither Glu47 nor Lys81 significantly modulate the pK_a of the C- or N-terminal cysteines. Hence, their effect on activity must have a different pathway. Examination of the effects of mutations at R120 and the analogous residue R461 in the catalytic **a'** domain of PDI on the ability of PDI to catalyze the reduction of protein disulphide bonds was studied with the insulin reduction assay. Arginine mutants showed comparable activity in the reduction of insulin to a PDI construct where all the active site cysteines were mutated. This implies that mutating these arginine residues abolishes the catalysis of insulin reduction that arises from the thiol-disulphide chemistry associated with the active sites. The results from the BPTI refolding assay suggest that R120 and R461 mutations increase the rate of catalysis of thiol-disulphide isomerization in a folding protein substrate via stabilization of the PDI-protein mixed disulphide. The different effects of arginine 120 on the activities of PDI studied here imply that the wild type enzyme may not be optimized for individual oxidation or isomerization reactions, but it rather would be in balance to be able to catalyze both reactions. Timing of the motion of the side chain of the arginine residue into the active site locale will influence the reactivity of C-terminal cysteine. Decreasing the pK_a of the Cys56 or increasing the frequency of the motion of R120 into the active site locale will theoretically increase the rate of reoxidation and hence increase the efficiency of PDI to act as a catalyst of disulphide bond formation. However, increasing this rate will also theoretically decrease the length of time that PDI is in mixed disulphides with protein substrates and hence decrease the efficiency of direct isomerization. Data presented here implies that PDI has evolved to be a catalyst both of both disulphide bond formation and isomerization and that both should be considered to be physiological functions in the cell.

6.2 The role of glutathione in oxidation and reduction of protein disulphide isomerase

Disulphide bond formation in the ER is a complex process with multiple possible routes for oxidative protein folding. To date the major route for dithiol oxidation to disulphide is thought to be oxidation of PDI by Ero1 followed the oxidation of substrate proteins by PDI. Since the discovery of Ero1 the potential role of GSSG as a route for disulphide bond formation has been underrated. Indeed, the role of glutathione in the formation of native disulphide bonds is often regarded as either to be solely in the reduction of incorrect disulphide bonds or as a competitor for the formation of protein disulphides (Chakravarthi & Bulleid 2004, Jessop & Bulleid 2004, Molteni *et al.* 2004). However, this overlooks the key facts from decades of *in vitro* studies. Firstly, the ratio of oxidised and reduced glutathione present in the ER (Hwang *et al.* 1992, Bass *et al.* 2004) is optimal for native disulphide bond formation *in vitro* (Lyles & Gilbert 1991). Secondly, previous studies have shown that the rates of reaction of glutathione with PDI are rapid (Darby & Creighton 1995) and therefore could expect to have physiological significance.

In this study, the kinetics of the reactions of the glutathione with the **a** domain of PDI and Pdi1p were shown to be more rapid and more complex than previously had been thought. The concentrations of oxidised and reduced glutathione in the ER are not known and they may vary between species and between cell types. The total cellular glutathione concentration is assumed to be circa 10 mM (Jessop & Bulleid 2004). The probable cellular concentration of glutathione together with the reported ratio of 3:1 of GSH:GSSG (Hwang *et al.* 1992, Bass *et al.* 2004) gives rate constants for the two steps of oxidation of PDI by GSSG of 1.2 s^{-1} and 0.23 s^{-1}. This implies half-times of 0.6 s and 3 s respectively. The rate of reduction is more complex but with the same concentrations of glutathione the calculated rate constant is 3.5 s^{-1}, giving a half-time of 0.2 s. These reactions appear all to be too fast to be discounted but rather suggest GSSG and GSH play a physiological role in oxidative protein folding in the cell.

The analysis of the glutathione-based oxidation and reduction of the **a** domains of human PDI and yeast Pdi1p revealed several other important observations. Firstly, the reaction rates of yeast Pdi1p **a** domain with GSH are more than an order of magnitude slower that the reactions of human PDI **a** domain. The two enzymes are clearly not equivalent. This study shows that the

results from protein folding studies obtained with *S. cerevisiae* should be examined more carefully before extrapolating them into mammalian systems and that previous experiments, performed with *S. cerevisiae*, relating to the physiological role of glutathione in native disulphide bond formation (for example Frand & Kaiser 1998, Cuozzo & Kaiser 1999) should be reconsidered.

Secondly, the results from this study show a clear contradiction. The rate constant obtained for the nucleophilic attack of the C-terminal cysteine on the mixed disulphide during oxidation is 0.23 s^{-1}, while the rate constant for nominally the same reaction during reduction is 430 s^{-1}. Trapping of the mixed disulphide was unsuccesful and therefore confirmation of the reaction steps that the rate constants represent was not possible. The reasons for the difficulties in trapping the mixed disulphide form are shown in Fig 1D (IV). It is possible that one or both of the rate constants actually represent something else, for example an intra-molecular conformational exchange. However, the results are consistent with hypothetical analyses of possible reaction mechanisms. In addition, a closer examination of the reaction schemes suggests that a large difference in these nominally identical reaction steps is not only plausible but also probable. Our previous results showed the pK_a of the C-terminal active site cysteine was regulated by an intra-molecular conformational exchange of the side chain of arginine 120 into the active site locale (I, II). Normally the pK_a of C56 is high and only the infrequent juxtaposition of the positively charged side chain of the arginine residue decreases that allowing the formation of a reactive thiolate. This is the situation occurring during the GSSG-mediated oxidation of PDI (Fig. 6, IV). In contrast, the species formed during the GSH-mediated reduction of PDI is the PDI-GSH mixed disulphide, but not the same mixed disulphide. Instead the leaving group in this reaction is the C56 thiolate (Fig. 6, IV). This is a highly reactive species and hence nucleophilic attack by this group on the mixed disulphide can proceed rapidly. The variation of the pK_a of C56 during the catalytic cycle may explain the great difference in the proportion of the C56 thiolate species at pH 7 between the initially formed mixed disulphide states on the oxidation and reduction pathways. With the calculated pK_a of C56 of 12.8 (I), in the case of Arg120 is located far from the active site locale, the proportion of C56 in the active thiolate form at pH 7 would be only 0.00016% and hence the difference may be up to 630000-fold.

Thirdly, the PDI-glutathione mixed disulphide is a potential species for nucleophilic attack by a peptide cysteine and hence a three covalent step catalytic cycle has not previously been considered. In this study the mutation of the C-

terminal active site cysteine was shown to result in maximally 2.5% activity of the wild-type PDI **a** domain in the peptide oxidation assay. While this is higher than would be expected by the dogma in the field, it is still 40-fold lower than the activity of the wild-type enzyme. The reduction in catalytic activity arises from at least two separate effects. i) The reactivity of the N-terminal cysteine towards oxidised glutathione is reduced by mutating the C-terminal active site cysteine. The effect is greater for the C56S mutants, which show circa 10-fold decrease in the rate of reaction of C53 towards GSSG, while the C56A shows only a two-fold effect. The reduction in the rate of reaction for the C56S mutant may be due to the local conformational changes araising from serine acting as strong breaker of regular secondary structure and/or hydrogen bonding by the serine, for example with C53. ii) Since both C56S and C56A mutants of PDI **a** domain show a greater effect on their activity in peptide oxidation than on their rates of reaction with oxidised glutathione, there must be a difference between the rates of reaction of a peptide cysteine with oxidised PDI and with the PDI-glutathione mixed disulphide, with the rates for the reaction with the mixed disulphide being significantly lower. This effect is also seen in the rates of reaction of GSH with oxidised PDI and with the mixed disulphide reported here (11200 $M^{-1}s^{-1}$ compared with 4600 $M^{-1}s^{-1}$ for human PDI **a** domain). For human PDI **a** domain these rates are circa 2.5-fold different while for yeast Pdi1p **a** domain they are around 4-fold different.

Fourthly, several studies have used C-terminal active site cysteine mutants of PDI-family members to examine their substrate specificity *in vivo* (for example Kadokura *et al.* 2004, Jessop *et al.* 2007). While this method traps only a subset of potential substrates (Hatahet & Ruddock 2007), it is a powerful technique for screening. However, the results presented in this study indicate that such studies should be considered carefully since mutating the C-terminal active site cysteine changes the reactivity of the N-terminal active site cysteine. Furthermore, in other members of the superfamily mutation of the C-terminal active site cysteine alters the substrate specificity of the enzyme as well (Saaranen *et al.* 2009).

6.3 The efficiency of hydrogen peroxide in oxidative refolding of a protein at physiological pH

The current model for the major route for disulphide bond formation in the ER is the highly regulated flow of oxidizing equivalents from molecular oxygen to the sulphydryl oxidase Ero1 and from Ero1 to PDI and from there to substrate protein

(Frand & Kaiser 1998, Pollard *et al.* 1998, Frand & Kaiser 1999, Gross *et al.* 2006, Sevier *et al.* 2007). However, sulphydryl oxidases, including Ero1 make one molecule of hydrogen peroxide per disulphide bond made (Hoober *et al.* 1996, Gross *et al.* 2006). The peroxide generated is regarded as harmful by-product of disulphide bond formation that results in oxidative stress to cells. Our *in vitro* BPTI refolding results clearly show that peroxides are able to efficiently oxidize dithiols in folding proteins to the native state at physiological pH. In addition to the direct oxidation of dithiols in folding proteins, peroxides are also able to oxidize other dithiols, including glutathione and the active site cysteines in PDI **a** domain. Since oxidized glutathione and PDI are able to introduce disulphide bonds to folding proteins, all these oxidation reactions could ultimately lead to oxidative folding of proteins in the ER. In addition to the contribution in oxidative protein folding, hydrogen peroxide made by Ero1 in each catalytic cycle has the potential to provide a regulatory mechanism to decrease disulphide bond formation and peroxide production by forming the regulatory disulphide bonds (Sevier *et al.* 2007, Appenzeller-Herzog *et al.* 2008, Baker *et al.* 2008) in Ero1 family members.

7 Conclusions

Disulphide bond formation is a complex series of reactions and is the critical rate-limiting step for the folding of great number of proteins. The role of protein disulphide isomerase as a catalyst in disulphide bond formation has been under extensive research for decades. However, the molecular mechanisms of some of its catalytic activities have not been determined. This thesis has focused on solving some aspects of the catalysis. In this thesis study the conservation of the arginine residue located in the loop between β5 and α4 of the catalytic domains of the human PDI family was observed and its significant role in the catalytic activity of PDI was shown. Based on molecular dynamic simulation studies the movement of the arginine residue into and out of the active-site locale was suggested. The effects of this movement on the pK_a values of the active-site cysteines and hence on the catalytic activity was studied in detail. The results of this thesis study suggest that the modulation of the pK_a of the C-terminal active-site cysteine by the movement of the arginine residue allows PDI to act efficiently as catalyst both for oxidation and isomerization reactions.

The possible role of hydrogen peroxide produced by sulphydryl oxidases during disulphide bond formation was studied using the BPTI refolding assay. Peroxide-mediated refolding was shown to be more efficient in obtaining the native state at physiological pH than the use of glutathione redox buffer. In addition to direct oxidation of dithiols in folding proteins, peroxides were proven to able to oxidize dithiols to disulphides *in vitro*, including glutathione and the active site of PDI. The kinetics obtained from the stopped-flow data showed clearly hydrogen peroxide to be fast enough to offer physiologically relevant route in disulphide bond formation and provide a productive utilization instead of being a harmful by-product of Ero1 and other sulphydryl oxidases.

In this study the kinetics of oxidation and reduction of **a** domains of PDI and Pdi1p by glutathione were examined. The kinetics obtained with stopped-flow and quenched-flow experiments showed clearly that reactions are more rapid and complex than previously thought. The data generated in this thesis on the reactivity of PDI towards glutathione together with the observations of the differences between kinetics of PDI and Pdi1p implicates that the several aspects of field of catalyzed disulphide bond formation should be re-evaluated and more attention should be paid on the potential physiological role of oxidized glutathione in the ER.

In summary this thesis presents data elucidating some of the molecular mechanisms of this ubiquitous enzyme, but much more needs to be done before its full mysteries are revealed.

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