The b' domain provides the principal peptide-binding site of protein disulfide isomerase but all domains contribute to binding of misfolded proteins

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Protein disulfide isomerase (PDI) is a very efficient catalyst of folding of many disulfide-bonded proteins. A great deal is known about the catalytic functions of PDI, while little is known about its substrate binding. We recently demonstrated by cross-linking that PDI binds peptides and misfolded proteins, with high affinity but broad specificity. To characterize the substrate-binding site of PDI, we investigated the interactions of various recombinant fragments of human PDI, expressed in *Escherichia coli*, with different radiolabelled model peptides. We observed that the b' domain of human PDI is essential and sufficient for the binding of small peptides. In the case of larger peptides, specifically a 28 amino acid fragment derived from bovine pancreatic trypsin inhibitor, or misfolded proteins, the b' domain is essential but not sufficient for efficient binding, indicating that contributions from additional domains are required. Hence we propose that the different domains of PDI all contribute to the binding site, with the b' domain forming the essential core.

Keywords: cross-linking/domain/peptide binding/protein disulfide isomerase

Introduction

The first catalyst of protein folding to be identified was protein disulfide isomerase (PDI), an enzyme located in the endoplasmic reticulum of higher and lower eukaryotes. Before the majority of molecular chaperones had been recognized, the catalytic properties of purified PDI from mammalian liver were described (DeLorenzo *et al.*, 1966; Creighton *et al.*, 1980; for review, see Freedman, 1984), and were consistent with its function in the catalysis of native disulfide bond formation during the folding of nascent secretory proteins. PDI interacts with nascent and newly translocated secretory proteins, as has been shown by using chemical cross-linkers (Roth and Pierce, 1987; Klappa *et al.*, 1995). *In vitro*, PDI increases the rate of disulfide bond formation (oxidation) and of the rearrangement (isomerization) of disulfide bonds during the folding pathway of such secretory proteins (R.B. Freedman and P. Klappa, submitted). In addition to this, PDI has also been described as the β -subunit of prolyl-4-hydroxylase (Pihlajaniemi *et al.*, 1987) and as a component of the

microsomal triglyceride transferase complex (Wetterau and Combs, 1989; Wetterau *et al.*, 1990).

Since rat PDI cDNA was first cloned and sequenced (Edman *et al.*, 1985), internal sequence homologies within the protein have been recognized, and it was suggested that there is a structural organization based on duplicated sequence modules (Figure 1A). The homologous a and a' sequence modules, which contain the active site motif WCGHC, show significant sequence identity to thioredoxin. It was confirmed by NMR that the recombinant a module of human PDI is a genuine structural domain, and that it has the thioredoxin fold (Kemmerling *et al.*, 1995). Although the homologous b and b' modules do not show significant sequence similarity to thioredoxin or to the a domain, NMR analysis recently has revealed that the b region also forms a domain with the thioredoxin fold (Kemmerling *et al.*, 1997). Hence we assume that the full-length protein is constructed of four structural domains with homologous thioredoxin folds, plus a C-terminal acidic extension.

A great deal is known about the catalytic function of PDI (Darby *et al.*, 1994; Freedman *et al.*, 1994, 1995), while the nature of the interaction with its substrates is unclear. The most striking feature of the wide-ranging catalytic activities of PDI is its ability to catalyse steps in protein folding pathways which involve significant conformational change in the protein substrate, associated with protein disulfide bond formation or isomerization. Full-length PDI is much more effective than the individual a and a' domains as a catalyst for such steps in the folding pathway of bovine pancreatic trypsin inhibitor (BPTI), although the isolated domains are equally effective as catalysts of simple thiol:disulfide interchange reactions (Darby and Creighton, 1995b). This suggests that the complex natural substrates of PDI make extensive contacts with the PDI molecule which facilitate transitions between different conformational states of the protein substrate.

To address the question of the nature of the interaction between PDI and its substrates, we used chemical cross-linkers which have been shown to be a powerful tool to study interactions between proteins and which can be applied to proteins available in small amounts even in crude cell extracts. Recently, we demonstrated that purified bovine liver PDI can be cross-linked specifically to peptides and non-native proteins in a manner that is saturable, reversible and independent of the presence of cysteine residues in the bound peptide. We also have observed the cross-linking of these peptides to purified PDI using several distinct cross-linking and detection methods (Klappa *et al.*, 1997).

Ultimately, we want to know whether PDI has a distinct binding site for substrates and which part of PDI is in contact with bound substrates. Furthermore, we want to investigate what role this interaction plays in facilitating

B
 Δ -Somatostatin (Δ -SRIF):
 AGS**K**NFFW**K**TFTSS

Somatostatin (SRIF):
 AGC**K**NFFW**K**TFTSC

Mastoparan (MAS):
 INL**K**ALAAL**K**KIL

Glucocorticoid receptor fragment (GCR):
 A**K**AILGL**K**NLHLLDDQMTLL

Bovine trypsin inhibitor fragment (BPTI 4-31):
 FCLEPPYTGPS**K**ARIIRYFYNA**K**AGLCQ

Fig. 1. PDI fragments and peptides used in cross-linking studies. (A) Recombinant fragments and linear arrangement of the various domains of human PDI. The first and the last amino acid, respectively, are indicated. The numbering is based on the mature sequence of human PDI and does not include the initiating amino-terminal methionine residue of each expressed protein. (B) Amino acid sequence of the peptides used. Residues that can be modified by [125 I]Bolton–Hunter labelling are in bold. Note that in BPTI 4–31 a serine residue replaces the cysteine at position 14 of native BPTI.

folding of natural substrates such as newly translocated secretory proteins. Here we report the results of our recent studies using chemical cross-linking of model peptide substrates (Figure 1B) to recombinant fragments of human PDI in crude cell extracts.

Results

Radiolabelled peptides interact specifically with recombinant fragments of human PDI

Recently, we demonstrated that peptides and non-native proteins can be cross-linked specifically to purified bovine liver PDI (Klappa *et al.*, 1997). We also showed that cross-linkers are a powerful tool to study peptide interactions with proteins available in small amounts, even in crude cell extracts derived from mammalian tissues (P.Klappa, T.Stromer, R.Zimmermann, L.W.Ruddock and R.B.Freedman, submitted). Here we employed cross-linking as a technique to investigate the interactions between model peptides and recombinant fragments of human PDI, expressed in *Escherichia coli*. Figure 2 shows the crude bacterial cell extracts (upper panel) used in this study, probed for the expression of various fragments of human PDI by immunodecoration with a polyclonal antibody raised against bovine liver PDI (lower panel). Interestingly, in contrast to all other fragments, the b domain, although clearly visible after Coomassie staining, was not detected by this antibody.

To address the question of whether recombinant fragments of human PDI interact with model substrates in a crude bacterial cell extract, [125 I]Bolton–Hunter-labelled peptides were added and the mixture cross-linked by using the homobifunctional cross-linking reagent disuccinimidyl

Fig. 2. Expression of various recombinant fragments of human PDI. Equal amounts of total cellular protein, as estimated by Coomassie staining of the SDS gel, were loaded and electro-transferred onto PVDF membrane (upper panel) with subsequent immunodecoration with a polyclonal antibody raised against PDI (lower panel). PDI, full-length human PDI; PDI, full-length human PDI with the mutations C295A and C326A.

Fig. 3. Interaction of various recombinant PDI fragments with small peptides. Equal amounts of total cellular proteins (~15 µg/µl) were incubated with 30 µM radiolabelled Δ -somatostatin (A), somatostatin (B), mastoparan (C) and a fragment derived from the glucocorticoid receptor (D), with subsequent cross-linking. PDI, full-length human PDI; PDI/, full-length human PDI with the mutations C295A and C326A; PDI pur, purified bovine liver PDI; control, cell lysate not expressing PDI or PDI-related fragments. A cross-linking product comprising radiolabelled peptides and an unidentified bacterial protein is indicated (*).

glutarate (DSG). It was shown previously that these peptides—specifically somatostatin, an inhibitor of PDI-dependent insulin reduction (Morjana and Gilbert, 1991), Δ -somatostatin, a somatostatin derivative without cysteine residues, and mastoparan—can be cross-linked to purified PDI (Klappa *et al.*, 1997). Furthermore, in those experiments, we demonstrated by competition that these peptides bind to the same site in purified PDI.

[125 I]Bolton–Hunter-labelled Δ -somatostatin (Figure 1B) was cross-linked to purified bovine PDI (PDI pur), recombinant human PDI (PDI), a PDI mutant without the cysteine residues in the b' domain (PDI/) and various recombinant fragments of human PDI, respectively (Figure 3A). In the presence of purified PDI, a single cross-linking product with an apparent M_r of 55 kDa could be detected. In the absence of DSG, no cross-linking products could be observed (data not shown).

When recombinant human PDI was expressed and the crude cell lysate was subjected to cross-linking, only a single cross-linking product could be observed, indicating that the interaction with PDI was highly specific. A cell

lysate that did not express PDI or PDI-related fragments served as a control. We observed single cross-linking products with cell extracts expressing the a–b–b', a–b–b'–a', b'–a'–c, b–b'–a–c or b' domains, respectively. In contrast, no cross-linking products could be observed when cell extracts expressing the a, a–b, a', a'–c or b domains were subjected to cross-linking (Figure 3A). Identical results were obtained when somatostatin (Figure 3B), mastoparan (Figure 3C) or a fragment of the glucocorticoid receptor (Figure 3D) were used, indicating that the interaction is independent of the amino acid sequence of the peptide. In addition, the presence of a disulfide bond did not change the cross-linking pattern (Figure 3A versus B). In certain cases, we also observed additional cross-linking products (*), which were not related to PDI or fragments of it, but labelling of this endogenous bacterial protein was low compared with that of the recombinant PDI products. We did not detect any significant interactions between the probed peptides and other proteins, e.g. molecular chaperones, either in *E. coli* or in crude mammalian cell extracts (data not shown). However, we have

Fig. 4. The interaction between Δ -somatostatin and the b' domain is reversible and sensitive to detergent. (A) Cell lysates expressing 2 μ M of the b' domain (lanes 1–3) or 2 μ M purified bovine liver PDI (lanes 4–6) were pre-incubated with 10 μ M radiolabelled Δ -somatostatin (S*) (lanes 1 and 4) for 5 min at 0°C. Subsequently a 10-fold excess of unlabelled Δ -somatostatin (S) was added with further incubation for 5 min at 0°C. Samples that contained radiolabelled Δ -somatostatin only (lanes 3 and 6) or a mixture of radiolabelled and unlabelled Δ -somatostatin (lanes 2 and 5) were incubated for 10 min at 0°C and cross-linked, and served as controls. (B) Cross-linking of 10 μ M radiolabelled Δ -somatostatin to purified bovine liver PDI (2 μ M) and a cell lysate expressing the b' domain (2 μ M), respectively, was performed in the absence or presence of 0.3% Triton X-100 (TX-100).

observed cross-linking of peptides to purified fragments of cytosolic Hsc82 (kind gift of Dr J.Buchner, Regensburg). This was only observed in the presence of high concentrations of these fragments. Further, in the presence of small concentrations of purified PDI, these interactions between peptides and the fragments of cytosolic Hsc82 were abolished, and only cross-linking products comprising peptides and PDI could be detected (data not shown).

The interaction between PDI and radiolabelled Δ -somatostatin is reversible

When an excess of unlabelled Δ -somatostatin and [125 I]Bolton–Hunter-labelled Δ -somatostatin were added simultaneously to a cell extract expressing the b' domain (Figure 4A, lane 2), a decrease in the intensities of the cross-linking product was observed compared with that in the absence of the unlabelled peptide (lane 3); the same decrease was noted when unlabelled Δ -somatostatin was added after pre-incubation of the extract with radiolabelled Δ -somatostatin (lane 1). A similar result was obtained when Δ -somatostatin was cross-linked to purified bovine PDI (lanes 4–6). These results are in agreement with our previous observations that the interactions between PDI and peptides are reversible (Klappa *et al.*, 1997).

We have shown recently that hydrophobic interactions are essential for peptide binding. As shown in Figure 4B, the presence of Triton X-100 decreased cross-linking of radiolabelled Δ -somatostatin to purified PDI or to the b' domain in a crude bacterial lysate to the same extent.

These results demonstrate that the b' domain on its own has peptide-binding properties comparable with those of full-length PDI.

Binding of a fragment of BPTI requires additional domains of human PDI

BPTI 4–31 is a synthetic peptide containing two cysteine residues which has been used widely to study the action of PDI in catalysing disulfide bond formation and reduction in a large unstructured fragment of natural BPTI protein

(Darby *et al.*, 1994; Darby and Creighton 1995a). When this [125 I]Bolton–Hunter-labelled fragment was cross-linked to various fragments of recombinant human PDI, we observed cross-linking products with the a–b–b', a–b–b'–a', b'–a'–c or the b–b'–a'–c domains (Figure 5A). The b' domain is the only domain present in all these constructs but, in contrast to the results obtained with the smaller peptides, we did not observe any interactions with the b' domain alone. This result was confirmed by a competition experiment (Figure 5B). When a cell extract expressing the b' domain was subjected to cross-linking with radiolabelled Δ -somatostatin, an excess of the unlabelled BPTI fragment did not compete for the binding of radiolabelled Δ -somatostatin to the b' domain (lane 2). However, the unlabelled BPTI fragment showed competition for the binding of radiolabelled Δ -somatostatin to full-length recombinant PDI (lane 5). The presence of unlabelled Δ -somatostatin, however, strongly reduced binding of radiolabelled Δ -somatostatin to both PDI (lane 6) and the b' domain (lane 3).

In a similar competition experiment, the radiolabelled BPTI fragment was cross-linked to the b'–a'–c domain in the presence or absence of unlabelled Δ -somatostatin (Figure 5C). An excess of unlabelled Δ -somatostatin significantly inhibited the interaction of the radiolabelled BPTI fragment both with full-length recombinant PDI (lane 3) and with the b'–a'–c construct (lane 4), indicating that the BPTI fragment and Δ -somatostatin interacted with the same binding site.

From this experiment, we conclude that the b' domain on its own, although essential for the binding of peptides and the BPTI fragment, was not sufficient for efficient binding of the BPTI fragment.

Biotinylated 'scrambled' RNase interacts specifically with recombinant fragments containing the b'–a' domains of human PDI

When 'scrambled' RNase, another well-characterized substrate of PDI, was biotinylated and then cross-linked

Fig. 5. Interaction of various recombinant PDI fragments with a fragment of the bovine pancreatic trypsin inhibitor (BPTI 4–31). **(A)** Equal amounts of total cellular proteins (15 µg/µl) were incubated with 15 µM radiolabelled fragment of the BPTI with subsequent cross-linking. **(B)** Cell lysates expressing full-length PDI (2 µM) and the b' domain (2 µM), respectively, were incubated with 10 µM radiolabelled Δ-somatostatin in the presence of a 10-fold excess of unlabelled Δ-somatostatin or BPTI fragment with subsequent cross-linking. Samples without unlabelled competitor peptides served as controls. **(C)** Cell lysates expressing full-length PDI (2 µM) and the b'-a'-c domain (2 µM), respectively, were incubated with 10 µM radiolabelled BPTI fragment in the presence of a 10-fold excess of unlabelled Δ-somatostatin or BPTI fragment with subsequent cross-linking. Samples without unlabelled competitor peptides served as controls.

Fig. 6. Interaction of various recombinant PDI fragments with biotinylated 'scrambled' RNase. **(A)** Equal amounts of total cellular proteins (15 µg/µl) were incubated with 3 µM biotinylated 'scrambled' RNase with subsequent cross-linking. Unbound biotinylated 'scrambled' RNase is indicated by an arrow. A cross-linking product comprising biotinylated 'scrambled' RNase and an unidentified bacterial protein is indicated (*). **(B)** Cell lysates expressing full-length PDI (2 µM) and the b' domain (2 µM), respectively, were incubated with 10 µM radiolabelled Δ-somatostatin in the presence of a 10-fold excess of unlabelled Δ-somatostatin or 'scrambled' RNase with subsequent cross-linking. Samples without unlabelled competitor peptides served as controls. **(C)** Cell lysates expressing full-length PDI (2 µM) and the b'-a'-c domain (2 µM), respectively, were incubated with 3 µM biotinylated 'scrambled' RNase in the presence of a 10-fold excess of unlabelled Δ-somatostatin (Δ-S), 'scrambled' RNase (scR) or native RNase (nR) with subsequent cross-linking. Samples without unlabelled competitor peptides served as controls. Unbound biotinylated 'scrambled' RNase is indicated by an arrow. Relevant cross-linking products are indicated with a diamond.

to crude cell extracts expressing various recombinant fragments of human PDI, cross-linking products were detected with cell extracts expressing the a-b-b'-a', b'-a'-c or b-b'-a'-c domains, respectively (Figure 6A). In contrast to the experiments using short radiolabelled peptides, no cross-linking products could be observed when cell extracts expressing the b' domain were subjected to cross-linking, nor were such products observed with the a-b-b' construct, in contrast to the results obtained with the BPTI fragment. From this result, we conclude that

'scrambled' RNase interacted only with those fragments containing at least the b'-a' domains.

Various ligands were compared for their ability to compete with each other for binding to recombinant PDI fragments. An excess of unlabelled 'scrambled' RNase, like unlabelled Δ-somatostatin, could compete with radiolabelled Δ-somatostatin for the binding to recombinant human PDI (Figure 6B, lane 5 versus 6). However, when a cell extract expressing the b' domain was subjected to cross-linking with radiolabelled Δ-somatostatin, an excess of

Fig. 7. Interaction between various fragments and active site mutants of PDI and Δ-somatostatin in the presence of 'scrambled' RNase and BPTI fragment, respectively. **(A)** Radiolabelled Δ-somatostatin (10 μM) was cross-linked to various fragments of recombinant PDI in the presence of a 10-fold excess of unlabelled Δ-somatostatin, 'scrambled' RNase or the BPTI fragment. Samples without unlabelled competitor peptides served as controls. Quantification was performed by PhosphorImager analysis. **(B)** Radiolabelled Δ-somatostatin (10 μM) was cross-linked to 2 μM of recombinant PDI, PDIDS1 (C36S C39A) and PDIDS2 (C380S C383A), respectively, in the presence of a 10-fold excess of unlabelled Δ-somatostatin, 'scrambled' RNase or the BPTI fragment. Samples without unlabelled competitor peptides served as controls. The position of relevant cross-linking products is marked with a diamond. A cross-linking product comprising radiolabelled peptides and an unidentified bacterial protein is indicated (*). **(C)** Quantification of **(B)** performed by PhosphorImager analysis.

'scrambled' RNase did not compete for the binding (lane 2 versus 3). In the presence of unlabelled Δ-somatostatin, however, binding of radiolabelled Δ-somatostatin to PDI (lane 4) and to the b' domain (lane 1) was strongly reduced. Native RNase did not interfere with the binding of radiolabelled Δ-somatostatin to the recombinant b' domain or full-length recombinant PDI (data not shown).

The binding of biotinylated 'scrambled' RNase to recombinant human PDI or the b'-a'-c domain in a cell extract was inhibited by an excess of unlabelled Δ-somatostatin (Figure 6C, lanes 3 and 4) or of unlabelled 'scrambled' RNase (lanes 5 and 6). However, no inhibition could be observed in the presence of native RNase (lanes 7 and 8). These experiments clearly demonstrate that the b'-a'-c region is necessary and sufficient for the recognition of misfolded proteins.

The binding of larger peptides requires fragments in addition to the b' domain

From these competition experiments, we concluded that the b' domain is essential for the binding of peptides and misfolded proteins. However, in the case of 'scrambled' RNase and the BPTI fragment, the additional presence of other domains seemed to be essential. This was confirmed by cross-linking [¹²⁵I]Bolton-Hunter-labelled Δ-somatostatin to various fragments of recombinant human PDI in the presence of an excess of unlabelled 'scrambled' RNase or the unlabelled BPTI fragment. Competition by unlabelled Δ-somatostatin served as a control. As demonstrated in Figure 7A, the interaction between radiolabelled Δ-somatostatin and PDI was strongly inhibited in

the presence of unlabelled 'scrambled' RNase and the BPTI fragment, respectively, indicating that 'scrambled' RNase and the BPTI fragment showed efficient binding to full-length recombinant PDI with efficient competition for the binding site. A similar result was observed in the case of the a-b-b'-a' domains, demonstrating that the c domain was not involved significantly in peptide binding. However, in the presence of shorter PDI fragments, this inhibition of the binding of Δ-somatostatin to those fragments was successively reduced, indicating that the binding affinities for 'scrambled' RNase and the BPTI fragment depended on the composition of the fragments. This is in line with our observation that the b' domain on its own was not sufficient to bind 'scrambled' RNase and the BPTI fragment efficiently (Figures 5B and 6B). By contrast, unlabelled Δ-somatostatin efficiently inhibited cross-linking of labelled Δ-somatostatin to all PDI constructs. We noted that the b'-a'-c domain showed a higher affinity for the binding of 'scrambled' RNase and the BPTI fragment than the a-b-b' domains, which indicates that the a'-c domains are more important than the a-b domains with respect to substrate binding.

This result was confirmed by using mutants of full-length human PDI with the cysteine residues of the active sites replaced; [¹²⁵I]Bolton-Hunter-labelled Δ-somatostatin was cross-linked to human PDI, PDIDS1 (C36S C39A) and PDIDS2 (C380S C383A), respectively, in the presence of an excess of unlabelled 'scrambled' RNase or the unlabelled BPTI fragment. Competition by unlabelled Δ-somatostatin served as a control. As shown in Figure 7B and C, the interaction between PDI and radiolabelled

Δ -somatostatin was strongly inhibited in the presence of unlabelled 'scrambled' RNase and the BPTI fragment, respectively, indicating that 'scrambled' RNase and the BPTI fragment showed efficient binding to full-length recombinant PDI with efficient competition for the binding site. A similar result was observed in the case of PDIDS1, demonstrating that the replacement of the cysteine residues in the active site of the a domain had little effect on peptide binding. However, in the presence PDIDS2, the inhibition of the binding of Δ -somatostatin was reduced, indicating that the binding affinities for 'scrambled' RNase and the BPTI fragment were decreased by the replacement of the cysteine residues in the active site of the a' domain.

Discussion

It has been shown that PDI interacts with a variety of different molecules comprising denatured proteins and peptides, although the nature of these interactions is poorly understood. In this study, we investigated the interaction between various fragments of recombinant human PDI, expressed in *E.coli*, and different model peptides by chemical cross-linking. Chemical cross-linkers have been shown to be a powerful tool to study such interactions; recently we demonstrated that purified bovine liver PDI can be cross-linked specifically to peptides and non-native proteins in a manner that is saturable, reversible and independent of the presence of cysteine residues in the bound peptide (Klappa *et al.*, 1997). Subsequently, we reported the results of our studies using chemical cross-linking of model peptide substrates to PDI and related proteins in the presence of crude cell extracts (P.Klappa, T.Stromer, R.Zimmermann, L.W.Ruddock and R.B.Freedman, submitted). Clearly this technique can be employed to study specific interactions between proteins available in small amounts without time-consuming purification steps.

The homobifunctional cross-linker used in this study reacted with either the amino-terminus or the ϵ -amino group of lysine residues of the peptides and the fragments of PDI. Although labelling with [125 I]Bolton–Hunter also modified either the amino-terminus or the ϵ -amino group of lysine residues of the peptides, cross-linking could be carried out. This can be explained by the fact that all peptides contain at least two lysine residues and that, due to the conditions of radiolabelling, it is very unlikely that more than one of the reactive groups (either the amino-terminus or the ϵ -amino group of lysine residues) was blocked by the [125 I]Bolton–Hunter labelling reagent. The lysine residues are located at different positions in the various peptides so that the results do not arise from particular favourable locations of cross-linking groups on ligands and proteins.

Small peptides interact with the b' domain of recombinant human PDI

We found that small peptides like somatostatin, mastoparan and a fragment derived from the glucocorticoid receptor, after [125 I]Bolton–Hunter radiolabelling and precipitation with trichloroacetic acid (TCA), can be chemically cross-linked to those linear combinations of various PDI domains which include the b' domain. Fragments without the b' domain did not show any peptide binding. The b' domain

alone exhibited efficient binding of the various peptides. The peptide-binding properties of the b' domain were identical to the properties observed for purified bovine PDI (Klappa *et al.*, 1997), i.e. the binding was reversible and sensitive to detergents. The fact that the interactions between peptide and the b' domain are identical to those between peptide and purified PDI, combined with the observation that the b' construct is folded (unpublished observation), indicates that the b' domain contributes most of the peptide-binding domain of PDI.

From the observation that there was an interaction between the b' domain and several different peptides, we conclude that no specific sequence motif in the peptide is required for binding, which is in agreement with the findings of Morjana and Gilbert (1991). Peptide binding to the b' domain was observed whether or not the probed peptide contained a disulfide bond, as noted for purified bovine PDI (Klappa *et al.*, 1997). Mutation of the cysteine residues within the b' domain of full-length PDI at positions 295 and 326, respectively, did not show any effect with respect to peptide binding.

From these results, we conclude that the b' domain is essential and sufficient for the binding of small (10–15 residues) peptides. This does not define the peptide-binding site of PDI fully in spatial terms, and other domains of the enzyme may be in close proximity and may make some contribution to binding.

Interestingly, we found in competition experiments with mastoparan, somatostatin and Δ -somatostatin that the interactions between PDI and peptide were stronger if the peptide contained cysteine residues (Klappa *et al.*, 1997), most likely stabilized by the formation of mixed disulfide bonds. This suggests that domains involved in the enzymatic functions of PDI, i.e. the a and a' domains, also can contribute to the binding of peptides.

Earlier work of Noiva *et al.* showed that a radiolabelled tripeptide could be cross-linked specifically to residues within the C-terminal 50 amino acid residues of rat liver PDI (Noiva *et al.*, 1991, 1993). However, it was not demonstrated that the binding of this species was saturable, nor that its binding was competitive with binding of the unlabelled peptide or of standard PDI substrates. Indeed, Morjana and Gilbert (1991) showed that PDI exhibits only very weak affinity for tripeptides.

Larger peptides require domains in addition to the b' domain

Cross-linking experiments with a radiolabelled 28 amino acid fragment of BPTI or biotinylated 'scrambled' RNase A demonstrated that the b' domain alone was not sufficient for efficient binding of these well characterized substrates of PDI. In the case of 'scrambled' RNase, the additional presence of the a'–c domain was essential while in the case of the BPTI fragment the additional presence of either the a–b domain or the a'–c domain were sufficient. However, we did not observe binding to these domains in isolation; binding was only observed to constructs which contained the b' domain, indicating that the b' domain is also essential for the binding of 'scrambled' RNase and the BPTI fragment. This was confirmed by the observation that Δ -somatostatin, which binds to the b' domain, could compete for the binding of 'scrambled' RNase and the BPTI fragment to various fragments of human PDI.

From these experiments, we conclude that the b' domain is essential but not sufficient for the binding of 'scrambled' RNase and the BPTI fragment.

This idea was supported by the findings that the affinity of the various constructs of human PDI for the binding of 'scrambled' RNase and the BPTI fragment, as measured by competition experiments, increased with incremental length of the probed constructs. A special role might be assigned to the a' domain, since the b-b'-a'-c construct showed a 6-fold higher affinity for 'scrambled' RNase than the corresponding a-b-b' construct in competition experiments. The importance of the a' domain was emphasized further by the observation that the b'-a'-c construct was the smallest fragment that was able to bind a large misfolded protein as well as small peptides. From this result, we conclude that the a' domain contributes more to the binding of substrates to full-length PDI than does the a domain. This was confirmed by using active site mutants of full-length PDI; we observed that the affinity for the binding of 'scrambled' RNase was lower in the mutant lacking the active site cysteine residues in the a' domain than for either the wild-type PDI or for a mutant lacking the cysteine residues in the active site of the a domain. From this experiment, we conclude that the cysteine residues in the active site of the a' domain increase the affinity for the binding of misfolded proteins containing cysteine residues. This result is in agreement with our previous findings that the binding of ligands to PDI, although independent of cysteine residues within the ligand, is enhanced by the formation of mixed disulfide bonds between the ligand and PDI (Klappa *et al.*, 1997).

The pattern of results presented here is fully consistent with recent data on the various catalytic activities of the same set of PDI fragments (Darby *et al.*, 1998). It was observed that multidomain fragments had enhanced catalytic activities compared with individual a or a' domains, that the b' domain had a particularly important role in this enhancement and that the enhancement was more marked for the catalysis of disulfide bond rearrangements than for catalysis of simple thiol:disulfide oxidoreductions in BPTI folding intermediates. As per peptide binding, the smallest fragment that showed efficient catalysis of disulfide bond rearrangement was the b'-a'-c construct. These data indicate that the multidomain nature of PDI is crucial for its ability to act on complex protein substrates.

Since PDI is now believed to be constructed of four domains each with the thioredoxin fold, it now appears that it shows some parallels with other molecular chaperones such as secB (Smith *et al.*, 1996), the hsp60 family (Fenton and Horwich, 1997), etc. These all comprise multimeric proteins, and while some contain identical subunits (secB, GroEL), others, including the chloroplast chaperonins (Baneyx *et al.*, 1995) and mammalian CCT complex (Lin and Sherman, 1997), contain homologous but distinct subunits, comparable with the homologous but distinct domains of PDI.

A further interesting parallel of a different kind is to trigger factor, a peptidyl prolyl *cis-trans* isomerase from *E.coli* and other prokaryotes, associated with protein folding. As for PDI, trigger factor has a modular structure and shows competitive inhibition of protein folding by an unfolded protein. Furthermore, it has been demonstrated

that there are distinct and separate sites for protein binding and prolyl isomerization (Scholz *et al.*, 1997) located in different structural domains (Zarnt *et al.*, 1997). Hence, both in trigger factor and in PDI, domains specialized for a specific chemical isomerization activity are linked in sequence to domains specialized for binding of peptides or unfolded proteins; the combination provides for efficient catalysis of protein folding linked to disulfide isomerization and peptidyl prolyl *cis-trans* isomerization, respectively.

The substrate-binding site of PDI might be composed of different domains

From the results presented in this study, we propose a model in which various domains of PDI contribute to the substrate binding. The b' domain forms the essential core of the binding site and is sufficient for the binding of small peptides. The addition of further domains, i.e. the a and a' domains, provides additional binding interactions and thus is essential for the binding of more complex substrates. Finally, all the domains might be required for a binding site that is large enough to interact extensively with an unfolded protein. We speculate that the substrate binding extends over the various domains. What is not yet clear is whether the contributions made by each of the domains to substrate binding all involve structurally homologous regions within each domain, and whether the binding sites on each domain are structurally homologous to the peptide substrate-binding sites identified on DsbA (Guddat *et al.*, 1997) and thioredoxin (Qin *et al.*, 1996). However, without a three-dimensional structure of PDI, it remains impossible to assess to what extent the effects attributed to the individual domains are unique to that domain, or are a consequence of their position in the structure of PDI.

Materials and methods

Materials

Somatostatin, mastoparan (*Vespula lewisii*), 'scrambled' RNase A, native RNase A and the homobifunctional cross-linking reagent DSG were obtained from Sigma. [¹²⁵I]Bolton-Hunter labelling reagent and X-ray films were purchased from Amersham. *N*-Hydroxysuccinimidyl-biotin and streptavidin-horseradish peroxidase conjugates were from Pierce. The somatostatin derivative without cysteine residues (Δ -somatostatin), the glucocorticoid receptor fragment and the BPTI fragment were synthesized as described previously for other peptides (Klappa *et al.*, 1991). The amino acid sequences of each polypeptide construct used in this study are defined in Figure 1B. The polyclonal antibody raised against bovine liver PDI was from Stressgen. Bovine liver PDI was purified as described previously (Freedman *et al.*, 1995).

Methods

[¹²⁵I]Bolton-Hunter labelling of the peptides somatostatin, Δ -somatostatin, the glucocorticoid receptor fragment, the BPTI fragment and mastoparan was performed as recommended by the manufacturer. 'Scrambled' RNase A was biotinylated with *N*-hydroxysuccinimidyl-biotin as recommended by the manufacturer.

The construction of the vectors based on pET12 for the expression of PDI, PDI mutants and PDI domains was described previously (Darby *et al.*, 1998). Protein production was carried out in *E.coli* strain BL21 (DE3), which also contained the pLysS plasmid to control leakthrough expression. Cell extracts from *E.coli* were prepared by repeated freezing and thawing.

Binding of peptides and 'scrambled' RNase

After precipitation with TCA, the radiolabelled peptides were dissolved in distilled water. Labelled peptides or biotinylated 'scrambled' RNase

were added to buffer A (100 mM NaCl, 25 mM KCl, 25 mM triethanolamine pH 7.5, 2.5 mM CaCl₂), containing purified PDI or the crude cell extracts. The samples (10 µl) were incubated for 10 min on ice before cross-linking.

Cross-linking

Cross-linking was performed using the homobifunctional cross-linking reagent DSG as described for other cross-linkers (Klappa *et al.*, 1994). The samples were supplied with 1/10 volume of cross-linking solution (10 mM DSG in buffer A). The reaction was carried out for 60 min at 0°C. For competition experiments, the labelled probe was mixed with a 10-fold excess of unlabelled probe prior to the addition of the PDI fragments. Cross-linking was stopped by the addition of SDS-PAGE sample buffer (Klappa *et al.*, 1995, 1997).

The samples were subjected to electrophoresis in 17.5% SDS-polyacrylamide gels with subsequent autoradiography. Quantification was performed using a BioRad phosphorimager. Biotinylated products were detected using streptavidin-horseradish peroxidase conjugates.

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References

- Baneyx,F., Bertsch,U., Kalbach,C.E., van der Vies,S.M., Soll,J. and Gatenby,A.A. (1995) Spinach chloroplast cpn21 co-chaperonin possesses two functional domains fused together in a toroidal structure and exhibits nucleotide-dependent binding to plastid chaperonin 60. *J. Biol. Chem.*, **270**, 10695–10702.
- Creighton,T.E., Hillson,D.A. and Freedman,R.B. (1980) Catalysis by protein-disulphide isomerase of the unfolding and refolding of proteins with disulphide bonds. *J. Mol. Biol.*, **142**, 43–62.
- Darby,N.J. and Creighton,T.E. (1995a) Characterization of the active-site cysteine residues of the thioredoxin-like domains of protein disulfide-isomerase. *Biochemistry*, **34**, 16770–16780.
- Darby,N.J. and Creighton,T.E. (1995b) Functional properties of the individual thioredoxin-like domains of protein disulfide-isomerase. *Biochemistry*, **34**, 11725–11735.
- Darby,N.J., Freedman,R.B. and Creighton,T.E. (1994) Dissecting the mechanism of protein disulfide-isomerase—catalysis of disulfide bond formation in a model peptide. *Biochemistry*, **33**, 7937–7947.
- Darby,N.J., Penka,E. and Vincentelli,R. (1998) The multi-domain structure of protein disulfide isomerase is essential for high catalytic efficiency. *J. Mol. Biol.*, in press.
- DeLorenzo,F., Goldberger,R.F., Steers,E., Jr, Givol,D. and Anfinsen,C.B. (1966) Purification and properties of an enzyme from beef liver which catalyses sulfhydryl-disulfide interchange in proteins. *J. Biol. Chem.*, **241**, 1562–1567.
- Edman,J.C., Ellis,L., Blacher,R.W., Roth,R.A. and Rutter,W.J. (1985) Sequence of protein disulfide isomerase and implications of its relationship to thioredoxin. *Nature*, **317**, 267–270.
- Fenton,W.A. and Horwich,A.L. (1997) GroEL-mediated protein folding. *Protein Sci.*, **6**, 743–760.
- Freedman,R.B. (1984) Native disulfide bond formation in protein-biosynthesis—evidence for the role of protein disulfide isomerase. *Trends Biochem. Sci.*, **9**, 438–441.
- Freedman,R.B., Hirst,T.R. and Tuite,M.F. (1994) Protein disulfide-isomerase—building bridges in protein-folding. *Trends Biochem. Sci.*, **19**, 331–336.
- Freedman,R.B., Hawkins,H.C. and McLaughlin,S.H. (1995) Protein disulfide-isomerase. *Methods Enzymol.*, **251**, 397–406.
- Guddat,L.W., Bardwell,J.C., Zander,T. and Martin,J.L. (1997) The uncharged surface features surrounding the active site of *Escherichia coli* DsbA are conserved and are implicated in peptide binding. *Protein Sci.*, **6**, 1148–1156.
- Kemmink,J., Darby,N.J., Dijkstra,K., Scheek,R.M. and Creighton,T.E. (1995) Nuclear-magnetic-resonance characterization of the N-terminal thioredoxin-like domain of protein disulfide-isomerase. *Protein Sci.*, **4**, 2587–2593.
- Kemmink,J., Darby,N.J., Dijkstra,K., Nilges,M. and Creighton,T.E. (1997) The folding catalyst protein disulfide isomerase is constructed of active and inactive thioredoxin modules. *Curr. Biol.*, **7**, 239–245.
- Klappa,P., Mayinger,P., Pipkorn,R., Zimmermann,M. and Zimmermann,R. (1991) A microsomal protein is involved in ATP-dependent transport of presecretory proteins into mammalian microsomes. *EMBO J.*, **10**, 2795–2803.
- Klappa,P., Zimmermann,M. and Zimmermann,R. (1994) The membrane proteins TRAMP and sec61 alpha p may be involved in post-translational transport of presecretory proteins into mammalian microsomes. *FEBS Lett.*, **341**, 281–287.
- Klappa,P., Freedman,R.B. and Zimmermann,R. (1995) Protein disulphide isomerase and a luminal cyclophilin-type peptidyl prolyl *cis-trans* isomerase are in transient contact with secretory proteins during late stages of translocation. *Eur. J. Biochem.*, **232**, 755–764.
- Klappa,P., Hawkins,H.C. and Freedman,R. (1997) Interactions between protein disulphide isomerase and peptides. *Eur. J. Biochem.*, **248**, 37–42.
- Lin,P. and Sherman,F. (1997) The unique hetero-oligomeric nature of the subunits in the catalytic cooperativity of the yeast Cct chaperonin complex. *Proc. Natl Acad. Sci. USA*, **94**, 10780–10785.
- Morjana,N.A. and Gilbert,H.F. (1991) Effect of protein and peptide inhibitors on the activity of protein disulfide isomerase. *Biochemistry*, **30**, 4985–4990.
- Noiva,R., Kimura,H., Roos,J. and Lennarz,W.J. (1991) Peptide binding by protein disulfide isomerase, a resident protein of the endoplasmic-reticulum lumen. *J. Biol. Chem.*, **266**, 19645–19649.
- Noiva,R., Freedman,R.B. and Lennarz,W.J. (1993) Peptide binding to protein disulfide-isomerase occurs at a site distinct from the active-sites. *J. Biol. Chem.*, **268**, 19210–19217.
- Pihlajaniemi,T., Helaakoski,T., Tasanen,K., Myllyla,R., Huhtala,M.L., Koivu,J. and Kivirikko,K.I. (1987) Molecular cloning of the beta-subunit of human prolyl 4-hydroxylase—this subunit and protein disulfide isomerase are products of the same gene. *EMBO J.*, **6**, 643–649.
- Qin,J., Clore,G.M., Kennedy,W.P., Kuszewski,J. and Gronenborn,A.M. (1996) The solution structure of human thioredoxin complexed with its target from Ref-1 reveals peptide chain reversal. *Structure*, **4**, 613–620.
- Roth,R.A. and Pierce,S.B. (1987) *In vivo* cross-linking of protein disulfide isomerase to immunoglobulins. *Biochemistry*, **26**, 4179–4182.
- Scholz,C., Stoller,G., Zarnt,T., Fischer,G. and Schmid,F.X. (1997) Cooperation of enzymatic and chaperone functions of trigger factor in the catalysis of protein folding. *EMBO J.*, **16**, 54–58.
- Smith,V.F., Schwartz,B.L., Randall,L.L. and Smith,R.D. (1996) Electrospray mass spectrometric investigation of the chaperone SecB. *Protein Sci.*, **5**, 488–494.
- Wetterau,J.R. and Combs,K.A. (1989) Identification of the 58 kd subunit of the microsomal triglyceride transfer protein as protein disulfide isomerase. *Arteriosclerosis*, **9**, A691–A691.
- Wetterau,J.R., Combs,K.A., Spinner,S.N. and Joiner,B.J. (1990) Protein disulfide isomerase as a component of the microsomal triglyceride transfer protein complex. *J. Biol. Chem.*, **265**, 9800–9807.
- Zarnt,T., Tradler,T., Stoller,G., Scholz,C., Schmid,F.X. and Fischer,G. (1997) Modular structure of the trigger factor required for high activity in protein folding. *J. Mol. Biol.*, **271**, 827–837.

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