Identifying and characterizing a second structural domain of protein disulfide isomerase

Nigel J. Darby[a], Monique van Straaten[a,b], Elke Penka[a], Renaud Vincentelli[a], Johan Kemmink[a,b,*]

[a] *European Molecular Biology Laboratory, Meyerhofstrasse 1, D-69012 Heidelberg, Germany*
[b] *Biocenter and Department of Biochemistry, University of Oulu, P.O. Box 333, FIN-90570 Oulu, Finland*

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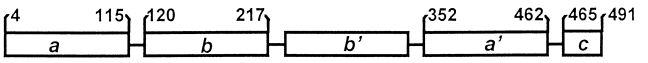
Abstract Recent protein engineering studies have confirmed the multidomain nature of protein disulfide isomerase previously suggested on the basis of analysis of its amino acid sequence. The boundaries of three domains, denoted *a*, *a′* and *b*, have been determined, and each domain has been expressed as an individual soluble folded protein. In this report, the boundaries of the final structural domain, *b′*, are defined by a combination of restricted proteolysis and protein engineering approaches to complete our understanding of the domain organization of PDI. Using these data an optimized polypeptide construct has been prepared and characterized with a view to further structural and functional studies.

Key words: Disulfide bond; Protein disulfide isomerase; Protein domain; Protein folding; Thioredoxin

1. Introduction

Protein disulfide isomerase (PDI) is a 55 kDa multifunctional protein, which catalyzes the formation of disulfide bonds in secreted proteins [1,2]. In addition, it is also a subunit of two multimeric enzymes, prolyl-4-hydroxylase [3] and the microsomal triglyceride transfer protein [4], in which it appears to stabilize the folded state of the catalytic subunits [5,6]. The ability of PDI to bind unfolded or partly folded proteins and prevent their aggregation also suggests a possible function as a chaperone, although the physiological significance of this is not yet known [7,8].

The multidomain nature of PDI first became apparent from analysis of its amino acid sequence, in which two domains, *a* and *a′*, were identified as being homologous to thioredoxin, each domain containing a CGHC active site [9]. Further sequence analysis suggested two other potential domains, *b* and *b′*, as being internal sequence repeats [9]. This resulted in the following model of the PDI domain structure:



The small stretch of sequence at the C-terminus, designated *c*, consists of primarily acidic residues, and is involved in calcium binding [10]. It probably does not constitute a folded domain.

To date, the available evidence supports the domain model of Eq. (1). The individual *a*, *b* and *a′* domains have been prepared in folded forms [11,12], albeit with some modifications to the original boundaries proposed by Edman et al. [9]. Studies on the isolated domains have provided the first detailed information about the structure of PDI and insight into its active site chemistry and functions [11–16]. The anticipated thioredoxin-like structure of the *a* domain has been confirmed directly [14], but, surprisingly, it was also found that the *b* domain has a thioredoxin-like fold, although it lacks the characteristic active site and has a very low sequence identity to any thioredoxin [15].

The existence of the *b′* domain has not yet been demonstrated experimentally and its boundaries are unknown. The sequence homology that exists between the *b* domain and the putative *b′* domain has raised the possibility that the *b′* domain also has a thioredoxin-like structure, so PDI would essentially consist of four linked thioredoxin modules, two with active sites and two without [15]. Recent evidence also points to an important role for the *b′* domain in the function of PDI, possibly as a major site of substrate interaction with the catalyst [16]. To address these questions, we now describe the elucidation of the boundaries of the *b′* domain of PDI, which has allowed the preparation and characterization of an isolated *b′* domain polypeptide construct that can be used in further structural and functional studies.

2. Materials and methods

2.1. Materials

The cloned gene for human PDI [3] was kindly provided as cDNA clone S-138 by K. Kivirikko (University of Oulu, Finland). PDI_{1-257} and PDI_{1-370} were prepared as previously described [16].

2.2. Construction of vectors for the expression of b′

Two polypeptide constructs were prepared that contained the *b′* domain, $PDI_{204-370}$ and $PDI_{213-351}$. The numbering scheme is based on the sequence of mature PDI. Vectors were constructed as described previously, to express the polypeptide constructs under the control of the T7 promoter of pET12a, with an additional initiating methionine [11,16].

*Corresponding author. Fax: +358 (8) 553 1141.
E-mail: johan.kemmink@oulu.fi

Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DTT, 1,4-dithiothreitol; HPLC, high pressure liquid chromatography; IPTG, isopropyl-β-D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; PDI, protein disulfide isomerase; PDO, protein disulfide oxidoreductase; PMSF, phenylmethylsulfonylfluoride; TLCK, N_α-*p*-tosyl-L-lysine chloromethylketone; TFA, trifluoroacetic acid; PDI_{i-j}, polypeptide construct corresponding to residues *i* to *j* using the residue numbering of mature human PDI

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2.3. Gene expression

Protein production was carried out in *Escherichia coli* strain BL21(DE3), which also contained the pLysS plasmid to control leak-through expression [17]. Cells were grown in LB medium or minimal medium at 37°C to an A_{600} of 0.4 and IPTG added to a concentration of 1 mM. After only 1–2 h further growth the cells were harvested by centrifugation and frozen at −20°C. Cell lysis occurred upon thawing [17]. After treatment with DNAse the PDI polypeptide constructs were purified from cell lysates as described below.

2.4. Purification of $PDI_{204-370}$

Ammonium sulfate was added to the lysate at 4°C to 40% saturation and the precipitated protein removed by centrifugation. The supernatant was loaded onto a 1.6×10 cm column of Octyl Sepharose equilibrated with 50 mM sodium phosphate (pH 7.4) containing ammonium sulfate to 80% saturation. The protein was eluted using a gradient from 80% to 0% ammonium sulfate saturation in the same buffer at a flow rate of 0.5 ml/min. Fractions containing $PDI_{204-370}$ were pooled and dialyzed against 50 mM Tris-HCl (pH 7.4) and further purified on a MonoQ HR5/5 column. Protein was eluted with a 0–120 mM NaCl gradient in the same buffer.

2.5. Purification of $PDI_{213-351}$

The lysate was dialyzed against 20 mM Tris (pH 8) and loaded onto a 1.6×15 cm column of Q-Sepharose equilibrated with 20 mM Tris-HCl (pH 8). The protein was eluted with a 700 ml gradient from 0 to 400 mM NaCl in the same buffer at a flow rate of 1 ml/min. When very high sample purity was required the sample was rechromatographed under the same conditions on a MonoQ column.

2.6. Limited proteolysis of PDI constructs

Limited proteolysis was carried out in 50 mM Tris (pH 8) at 37°C using trypsin or thermolysin at the protease concentrations described in the text. Proteolysis was stopped by addition of up to 5 mM of either PMSF or TLCK (for trypsin) or EDTA (for thermolysin), followed after a 2 min incubation by either acidification to pH 2 (for analysis by HPLC) or addition of electrophoresis sample buffer and boiling (for SDS-PAGE analysis). Samples were analyzed by SDS-PAGE on 15% (w/v) polyacrylamide gels [18] or by reverse phase HPLC on a Vydac C-18 column using gradients of acetonitrile in 0.1% (v/v) TFA. Proteolytic fragments isolated by HPLC were lyophilized and resuspended in 0.1% (v/v) TFA for mass spectrometry.

2.7. Electrophoresis

Urea gradient gel electrophoresis was carried out as described by Creighton and Shortle [19].

2.8. Gel filtration

Gel filtration analysis was carried out on a TSK 2000SWXL column with 50 mM sodium phosphate (pH 7.4), 0.1 M KCl as eluent.

2.9. Spectral analysis

Circular dichroism spectra were measured with a Jasco J710 spectrometer in a 1 mm pathlength cell at 20°C; spectra were averages of five scans. The protein was dissolved in 20 mM Tris (pH 7.4), also containing 0.2 mM DTT when cysteine residues were present in the protein. Samples of $PDI_{213-351}$ used for analysis by NMR spectroscopy were dialyzed against 10 mM sodium phosphate (pH 6.8) and concentrated to a volume of 1 ml using a Centricon-3 concentrator (Amicon). NMR spectra were recorded on a Bruker DRX-500 spectrometer operating at 500.132 MHz.

3. Results

3.1. The occurrence of a second structural domain in PDI

Initial evidence supporting the existence of the *b′* domain came from the observation that the construct PDI_{1-370} had markedly enhanced catalytic properties compared to PDI_{1-257} especially in isomerizing disulfide bonds in highly structured folding intermediates. This suggested that the additional sequence imparted some extra functionality [16]. The CD spectra of PDI_{1-370} confirmed that it contained additional regular

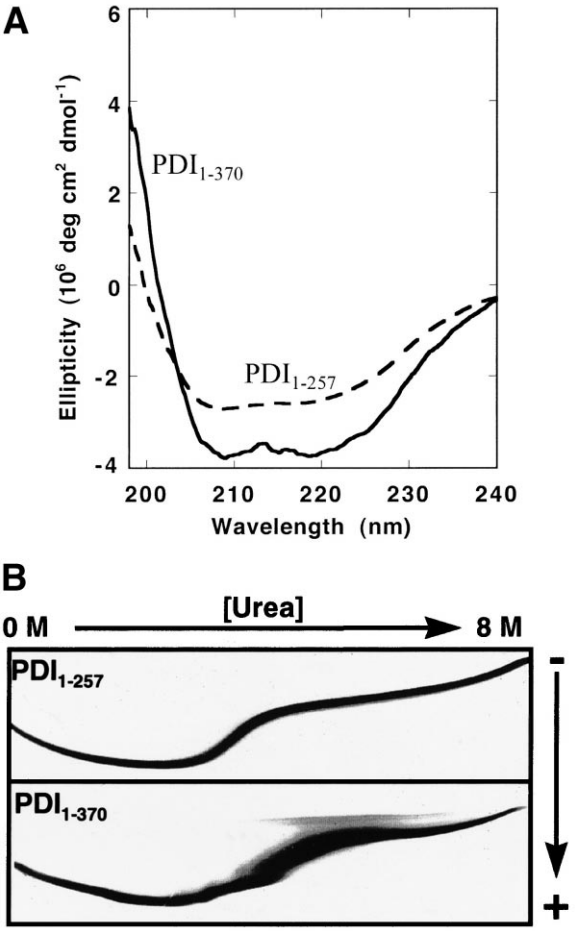

Fig. 1. Far UV CD spectra (A) and urea gradient electrophoresis (B) of the PDI_{1-257} and PDI_{1-370} constructs. The slight perturbations in mobility at very low and high urea concentrations were due to imperfections in the gel and are not indicative of further unfolding transitions.

structure compared to PDI_{1-257} (Fig. 1A), as might be expected from the presence of at least one additional domain.

Further analysis of the conformational properties of the PDI_{1-257} and PDI_{1-370} came from urea gel gradient electrophoresis (Fig. 1B), which monitors the hydrodynamic volume of a protein by its mobility during native gel electrophoresis through a transverse linear gradient of urea. At the extremes of the gradient, the fully folded protein (0 M urea) will have a higher mobility compared to the fully unfolded protein (8 M urea), because of its smaller hydrodynamic volume. The nature of the transition between these two states on the gel is characteristic of the conformational transitions that occur on the folding-unfolding pathway. Previously, it was demonstrated that PDI_{1-257} construct underwent a single unfolding transition, which was similar to that of the isolated *a* and *b* domains. This suggests that these two domains have similar stabilities and that they do not significantly affect each other's stability [12]. In contrast, PDI_{1-370} exhibits a more complex multistate transition, as might be expected from a protein that contains domains of different stability. The initial sharp transition is similar to that observed in PDI_{1-257}, but this is nearly obliterated by a second much broader transition that probably results from unfolding of the *b′* domain. The very broad nature of this transition probably reflects a slow event on the

electrophoretic timescale on the folding-unfolding pathway of b' [20]. A similar broad transition is seen on electrophoresis of intact PDI (unpublished observations).

3.2. Identifying the b′ domain

Initial attempts to prepare the b' domain used a construct consisting of residues 252 to 351 in line with previously suggested domain boundaries [9], but this did not result in the production of folded soluble protein (data not shown). As this was probably the result of using incorrect domain boundaries, a new construct was prepared, $PDI_{204-370}$, which contains polypeptide sequence at the N- and C-termini that can be assigned with certainty to the b and a' domains, respectively. This was then trimmed by restricted proteolysis, which results in preferential cleavage of flexible non-structured residues to leave the stable folded core intact.

Upon expression, $PDI_{204-370}$ was found in both the soluble and insoluble fractions of the cell. Time course studies showed that a short period of expression favored the soluble form.

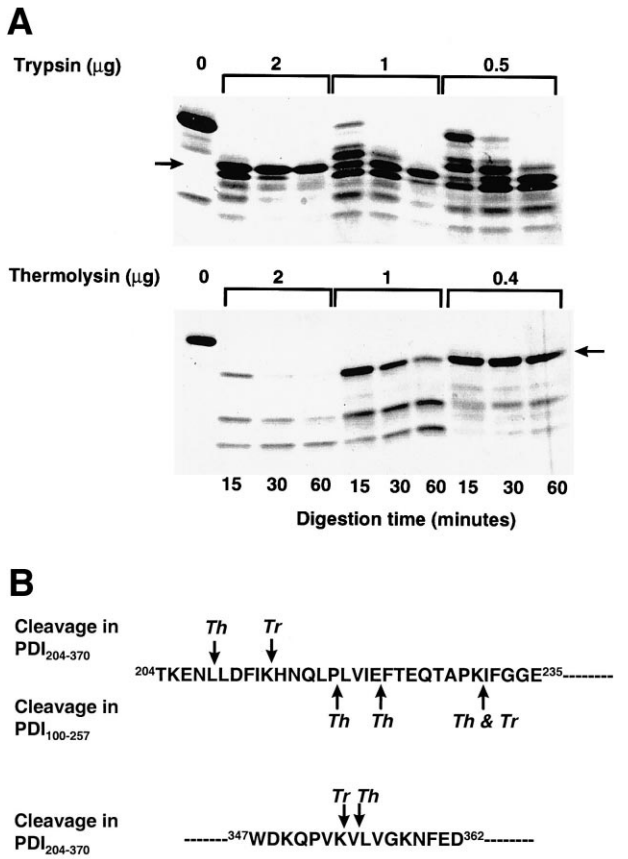

Fig. 2. A: Proteolytic trimming of polypeptide $PDI_{204-370}$ using trypsin and thermolysin. Digestions were carried out as described in Section 2 using the amounts of proteases and digestion times shown. The bands indicated by arrows correspond to the fragments isolated by HPLC, which were identified by mass spectrometry. B: Thermolysin (*Th*) and trypsin (*Tr*) cleavage sites in polypeptide constructs $PDI_{204-370}$ (arrows above line) and $PDI_{100-257}$ (arrows below line) [12]. Cleavage sites in $PDI_{204-370}$ were identified on the basis of three proteolytic fragments. The major product of trypsin digestion was a fragment of mass 16295.8, which was identified as H^{214} to K^{353} (expected mass 16296.6). Two fragments were isolated by HPLC from thermolysin digests that corresponded to the single fragment observed by SDS-PAGE. These had masses 17597.3 and 17012.47 which correspond to fragments T^{204} to V^{354} (expected mass 17598.1) and L^{209} to V^{354} (expected mass 17012.67).

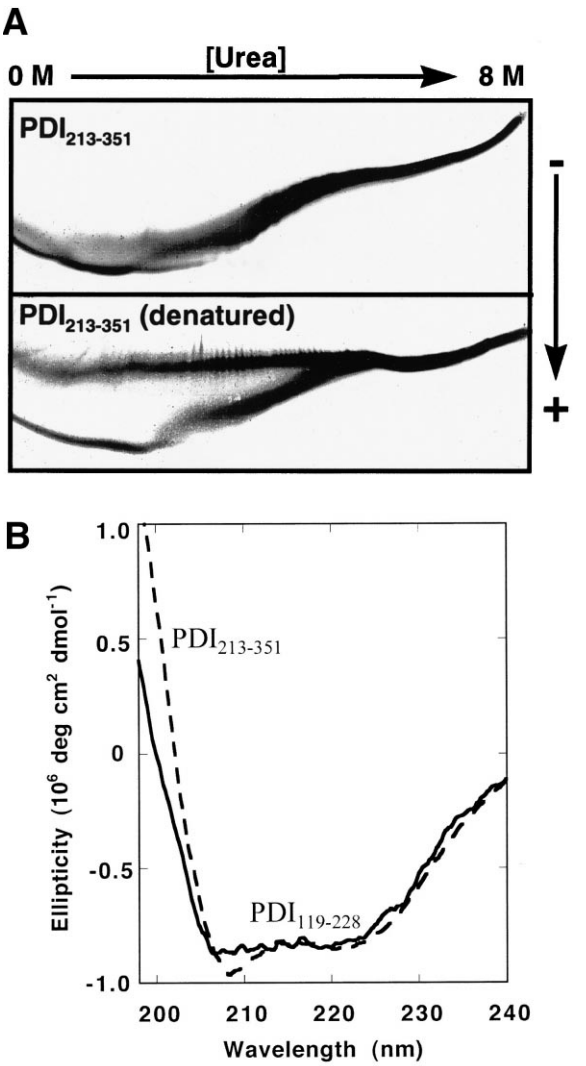

Fig. 3. A: Urea gradient gel electrophoresis of monomeric $PDI_{213-351}$ isolated by ion exchange chromatography. In the lower panel, the protein was denatured before electrophoresis by treatment with 8 M urea. The slight perturbations in mobility at very low and high urea concentrations were due to imperfections in the gel and are not indicative of further unfolding transitions. B: Far UV CD spectra of $PDI_{213-351}$ and $PDI_{119-228}$.

Mass spectroscopy indicated that the preparation was predominantly the form of the protein without the N-terminal methionine (measured mass 19432.5; expected mass 19433.2), and a small amount of the unprocessed form.

To define the domain boundaries, this polypeptide construct was subject to limited proteolysis using trypsin and thermolysin (Fig. 2). The conditions of proteolysis were optimized with respect to time and protease concentration using SDS-PAGE separations of digestions (Fig. 2A). The most stable large fragments, indicated by arrows in Fig. 2A, were isolated by reverse phase HPLC and identified by mass spectrometry.

The major points of proteolysis in $PDI_{204-370}$ are shown in Fig. 2B. Points of cleavage in a complementary construct consisting of residues 100 to 257 ($PDI_{100-257}$) are also shown. This construct contains the b domain and some flanking sequence at the C-terminus, which overlaps with that of

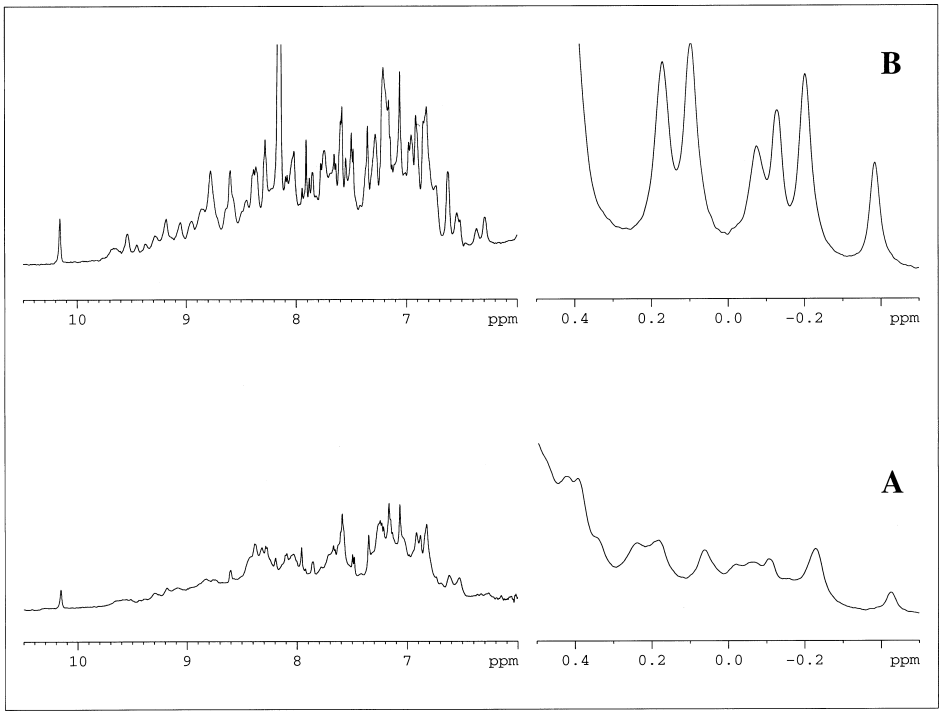

Fig. 4. A: The amide (left) and aliphatic (right) region of the 500 MHz ^{1}H-NMR spectrum of $PDI_{213-351}$ recorded in 10 mM phosphate buffer at pH 6.8. The spectrum was recorded at 300K using a spectral width of 6000.9 Hz and averaged over 512 scans. B: The spectrum of $PDI_{213-351}$ under the same conditions as described above with 10 mM CHAPS present.

$PDI_{204-370}$. $PDI_{100-257}$ has previously been defined as $EB_{100-257}$ [12]. NMR analysis of the *b* domain indicated that the last residue of this domain included in its folded structure was L^{217} [21]. This was consistent with the proteolysis studies on $PDI_{100-257}$, which showed thermolysin cutting shortly after this point in the sequence before residues L^{219} and F^{223}. In contrast, neither site was cut in the construct $PDI_{204-370}$ to generate the major stable fragment, which might be indicative that these residues are both located in folded structure. Instead, $PDI_{204-370}$ was cut before L^{209} by thermolysin and after K^{213} by trypsin; both residues are clearly part of the folded structure of the *b* domain [21]. These data would suggest that the domain next in sequence to the *b* domain starts probably after P218.

At the C-terminus of the molecule, two points of cleavage were observed, after residue K^{353} (trypsin) and before L^{355} (thermolysin). The close proximity of these residues and the apparent resistance to cleavage after the potential site K^{349} (trypsin) and before W347 (thermolysin), suggest that the cleavage is occurring near the end of the folded sequence.

3.3. Preparation of $PDI_{213-351}$

This construct contains the residues defined by proteolysis as belonging to the *b′* domain and, in addition, a few polar residues at the N-terminus. Like the $PDI_{204-370}$ construct, production of the soluble form was favored by limiting the period of protein expression to only 1 h. The protein could be readily purified by anion exchange chromatography to give a single band on SDS-PAGE. Analysis by anion exchange chromatography on MonoQ media, however, indicated that the preparation contained two components, which could also be separated by gel filtration and native gel electrophoresis. These had almost identical masses of 16328.9 and 16328.3 by mass spectrometry, which correspond to the expected mass of 16328.4. This suggested that the two forms are likely to be different aggregation states of the protein, which are probably a monomer and a dimer.

That $PDI_{213-351}$ formed a stable, folded structure could be demonstrated by urea gradient gel electrophoresis (Fig. 3A) of the monomeric form, freshly isolated by ion exchange chromatography. As might be expected $PDI_{213-351}$ exhibits the same broad transition, indicative of a slow conformational change on the folding-unfolding pathway that was also observed for PDI_{1-370}. $PDI_{213-351}$ was also analyzed after denaturation in 8 M urea. Two populations of molecules can be observed in this case: One readily refolds on the timescale of electrophoresis to give rise to a characteristic folding curve; the other population does not refold and runs as a continuous horizontal band of protein with a mobility corresponding to that of the denatured state. This pattern is characteristic of a situation where a slow transition between two unfolded forms blocks refolding of a portion of the molecule (e.g. cis-trans isomerization of peptide bonds preceding proline residues) [20].

CD spectra of $PDI_{213-351}$ also indicated that it adopts a folded conformation (Fig. 3B), but there was essentially no difference between the spectra of the monomeric or dimeric forms of the protein isolated by ion exchange chromatography (data not shown). For comparison, the CD spectra of a polypeptide construct that contains the *b* domain plus 10 extra residues at the C-terminus ($PDI_{119-228}$) [12] is also shown.

3.4. A non-aggregated protein preparation

Progress towards determining the structure of the *b′* domain requires that the protein is prepared in a stable and non-aggregated form at sufficiently high concentration. Pre-

liminary NMR spectra indicated that at a concentration of 0.5 mM, $PDI_{213-351}$ gave poor quality spectra, presumably because it was in a multimeric state. Various reagents, which potentially could affect the aggregation behavior of $PDI_{213-351}$, were rapidly screened using ion exchange chromatography. The cause of aggregation is still unclear, but one possibility is that it occurred because of the formation of disulfide bonds between two polypeptide chains via either of the cysteine residues present (C295 and C326). Addition of DTT, however, had no effect. No effect was also observed with various combinations of salt and pH. Efficient conversion to the lower molecular weight form could be achieved by the addition of either CHAPS (≥ 1 mM) or octyl-β-D-glucopyranoside (≥ 5 mM). The major point of interest was the effect of these detergents on the NMR spectra. In the absence of detergent, the amide and aliphatic regions of the $PDI_{213-351}$ ^1H spectrum (Fig. 4A) are not as expected for a protein of this size. The observed dispersion in chemical shifts, however, seems to be typical for a folded protein. Addition of 10 mM CHAPS improved the quality of the spectrum significantly indicating the protein is predominantly present as a monomeric, folded species (Fig. 4B).

4. Discussion

Although known for more than 30 years PDI has proved to be a difficult protein to understand at the molecular level, because of its size and complexity. Many of these problems have been overcome by dissecting the protein into its constituent domains and studying their structural and catalytic properties both in isolation and in combination. Key points that emerge are that PDI is likely to consist of four linked thioredoxin domains, although only two contain redox active sites, and that this multidomain architecture is required for the full range of PDI functions [15,16].

The preparation of a stable, folded form of the *b′* domain now allows a number of issues to be examined in more detail. Although the homology between the *b* domain of PDI and *b′* suggests that the *b′* domain should have a thioredoxin-like structure, this still needs to be confirmed experimentally. This should now be possible, using the preparations of $PDI_{213-351}$ in the presence of CHAPS for structure determination by NMR, and the quality of the spectra obtained appear sufficient for this process. Several successful examples of the application of CHAPS as an additive to NMR samples to manipulate the aggregation state of proteins have been reported previously [22–25].

The *b′* domain as defined by these studies is certainly confined within residues 213 to 351 of PDI, but in the absence of a three-dimensional structure for either the *b′* domain or PDI itself, the exact boundaries are unclear. Comparable studies involving proteolysis of intact PDI [26] generate fragments with an N-terminus at residues 222 and 230, which is quite similar to the present study. At the C-terminus there is a more substantial difference with several cleavages occurring between residues 328 and 349, which may suggest the region 328 to 349 is a linker between the *b′* and *a′* domains. Similar cleavages were not observed in the present study, but the conditions of proteolysis were not identical. Taken together, the two studies suggest the *b′* domain may be as short as 102 residues (230 to 328) or as long as 139 residues (213 to 351). There is some evidence from the CD spectrum of $PDI_{213-351}$ that there is at least some unstructured polypeptide in this construct, especially when compared to the *b* domain construct $PDI_{119-228}$, as evidenced by the more intense negative signal at 209 nm (Fig. 3B). Although we propose that the *b* and *b′* domains are homologous to each other and thioredoxin, this does not help define the length of this module. Thioredoxin has been adapted considerably throughout evolution, so the basic fold has been found with lengths of 90 to 120 amino acids and with insertions of up to 65 residues [27].

The differences observed between the *b′* domain construct and the *a* and *b* domain constructs may have functional significance, particularly in the light of studies showing that the *b′* domain may have some special role in the reactions catalyzed by PDI [16]. This has been enlarged upon in a parallel study on the ability of $PDI_{213-351}$ to bind peptides and proteins. Although other domains are also involved in substrate binding, it is clear that the *b′* domain plays an especially important role [28]. The ability of the isolated *b′* domain to form discrete oligomeric forms at higher protein concentrations may also reflect its ability to bind protein substrates. On the other hand, it may be due to some separate tendency to oligomerize, which may be related to the known ability of PDI to self-associate [29], or to interactions via uncovered domain-domain interfaces. The key to answering a number of these questions lies in the determination of the three-dimensional structure of the *b′* domain, which will then allow domain-domain and substrate-domain interactions to be mapped by NMR.

The experimental determination of the boundaries of the *b′* domain described here completes our experimental analysis of the domain organization of PDI. Although the original model proposed on the basis of sequence analysis [9] proved correct in that it identified four major domains in PDI, experimental studies have been required to identify the domain boundaries unambiguously. Attempts to identify the domain boundaries of PDI using sequence comparison and analysis alone have generally proved inaccurate. For example, even an integral part of the thioredoxin fold of the PDI *a* domain, the α4 helix (residues 101–116), has proved to be difficult to identify because of the sequence diversity found in this secondary structure element in the different members of the thioredoxin family. In turn, this led to speculation that a fifth domain might occur, designated *e*, which contained these residues at its N-terminus [30]. Only by direct experimental analysis could it be shown that the residues proposed to constitute the *e* domain really form integral parts of the *a* and *b* domains [31].

More recently, it was concluded on the basis of sequence analysis that the boundaries of the *b* and *b′* domains were residues 145–213 and 253–323, respectively [32]. This is clearly incorrect, however, as no account is taken of the experimental determination of the structure of the *b* domain showing that residues 120–217 are required for its folded structure [21]. On the basis of the work reported here the boundaries of the *b′* domain also appear to be incorrect: indeed, a construct containing residues 252–351 behaves as a heterogeneous, aggregated unfolded protein (unpublished data), whereas $PDI_{213-351}$ has been shown here to be a soluble folded protein.

The finding that PDI and its related proteins such as ERp72 and ERp61 [1] are likely to consist of linked thioredoxin-like domains [15], is interesting in the light of a number of studies showing this structural unit, with or without its active site, occurs in a wide variety of proteins. Recently identified exam-

ples include the calcium storage protein calsequestrin from rabbit skeletal muscle sarcoplasmic reticulum [33] and PDO from *Pyrococcus furiosus* [34], which consist of, respectively, three and two linked thioredoxin-like domains. In addition, ERp28, an endoplasmic reticulum protein of unknown function, is also thought to contain the thioredoxin module without its redox active site [32]. Clearly, the thioredoxin module has been used to fulfil a number of roles in proteins, though it is often not apparent what these roles are or how the structure of thioredoxin has been adapted to fulfil them. The ongoing structural and functional characterization of the b' domain of PDI made possible by the work described here should go some way to answering these questions.

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References

[1] Freedman, R.B., Hirst, T.R. and Tuite, M.F. (1994) Trends Biochem. Sci. 19, 331–336.
[2] Creighton, T.E., Zapun, A. and Darby, N.J. (1995) Trends Biotechnol. 13, 18–22.
[3] Pihlajaniemi, T., Helaakoski, T., Tasanen, K., Myllyla, R., Huhtala, M.-L., Koivu, J. and Kivirriko, K.I. (1987) EMBO J. 6, 643–649.
[4] Gordon, D.A., Wetterau, J.R. and Gregg, R.E. (1995) Trends Cell Biol. 5, 317–321.
[5] Vuori, K., Pihlajaniemi, T., Myllylä, R. and Kivirikko, K.I. (1992) EMBO J. 11, 4213–4217.
[6] Lamberg, A., Jauhiainen, M., Metso, J., Ehnholm, C., Shoulders, C., Scott, J., Pihlajaniemi, T. and Kivirriko, K.I. (1996) Biochem. J. 315, 533–536.
[7] Hayano, T., Hirose, M. and Kikuchi, M. (1995) FEBS Lett. 377, 505–511.
[8] Yao, Y., Zhou, Y.-C. and Wang, C.-C. (1997) EMBO J. 11, 4213–4217.
[9] Edman, J.C., Ellis, L., Blacher, R.W., Roth, R.A. and Rutter, W.J. (1985) Nature 317, 267–270.
[10] Lucero, H.A., Lebeche, D. and Kaminer, B. (1994) J. Biol. Chem. 269, 23112–23119.
[11] Darby, N.J. and Creighton, T.E. (1995) Biochemistry 34, 11725–11735.
[12] Darby, N.J., Kemmink, J. and Creighton, T.E. (1996) Biochemistry 35, 10517–10528.
[13] Darby, N.J. and Creighton, T.E. (1995) Biochemistry 34, 16770–16780.
[14] Kemmink, J., Darby, N.J., Dijkstra, K., Nilges, M. and Creighton, T.E. (1996) Biochemistry 35, 7684–7691.
[15] Kemmink, J., Darby, N.J., Dijkstra, K., Nilges, M. and Creighton, T.E. (1997) Curr. Biol. 7, 239–245.
[16] Darby, N.J., Penka, E. and Vincentelli, R. (1998) J. Mol. Biol. 276, 239–247.
[17] Studier, F.W., Rosenberg, A.H. and Dunn, J.W. (1990) Methods Enzymol. 185, 60–89.
[18] Laemmli, U.K. (1970) Nature 27, 680–685.
[19] Creighton, T.E. and Shortle, D. (1994) J. Mol. Biol. 242, 670–682.
[20] Creighton, T.E. (1980) J. Mol. Biol. 137, 61–80.
[21] Kemmink, J., Dijkstra, K., Mariani, M., Scheek, R.M., Penka, E., Nilges, M. and Darby, N.J. (1999) J. Biomol. NMR, in press.
[22] Davies, J. and Reichman, L. (1995) FEBS Lett. 339, 285–290.
[23] Kortt, A.A., Guthrie, R.E., Hinds, M.G., Power, B.E., Ivancic, N., Caldwell, J.B., Gruen, L.C., Norton, R.S. and Hudson, P.J. (1995) J. Protein Chem. 14, 167–178.
[24] Dingley, A.J., Mackay, J.P., Chapman, B.E., Morris, M.B., Kuchel, P.W., Hambly, B.D. and King, G.F. (1995) J. Biomol. NMR 6, 321–328.
[25] Anglister, J., Grzesiek, S., Wang, A.C., Ren, H., Klee, C.B. and Bax, A. (1994) Biochemistry 33, 3540–3547.
[26] Freedman, R.B., Gane, P.J., Hawkins, H.C., Hlodan, R., McLaughlin, S.H. and Parry, J.W. (1998) Biol. Chem. 379, 321–328.
[27] Martin, J.L. (1995) Structure 3, 245–250.
[28] Klappa, P., Ruddock, L.W., Darby, N.J. and Freedman, R.B. (1998) EMBO J. 17, 927–935.
[29] Lambert, N. and Freedman, R.B. (1981) Biochem. J. 213, 225–234.
[30] Tsibris, J.C.M., Hunt, L.T., Ballejo, G., Barker, W.C., Toney, L.J. and Spellacy, W.N. (1989) J. Biol. Chem. 264, 13967–13970.
[31] Kemmink, J., Darby, N.J., Dijkstra, K., Scheek, R.M. and Creighton, T.E. (1995) Protein Sci. 4, 2587–2593.
[32] Ferrari, D.M., Nguyen Van, P., Kratzin, H.D. and Söling, H.D. (1998) Eur. J. Biochem. 255, 570–579.
[33] Wang, S., Trumble, W.R., Liao, H., Wesson, C.R., Dunker, A.K. and Kang, C. (1998) Nat. Struct. Biol. 5, 476–483.
[34] Ren, B., Tibbelin, G., de Pascale, D., Rossi, M., Bartolucci, S. and Ladenstein, R. (1998) Nat. Struct. Biol. 5, 602–611.