Mutagenic studies on human protein disulfide isomerase by complementation of *Escherichia coli dsbA* and *dsbC* mutants

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Abstract Protein disulfide isomerase (PDI) exhibits both an oxido-reductase and an isomerase activity on proteins containing cysteine residues. These activities arise from two active sites, both of which contain pairs of redox active cysteines. We have developed two simple in vivo assays for these activities of PDI, based on the demonstration that PDI can complement both a *dsbA* mutation and a *dsbC* mutation when expressed to the periplasm of *Escherichia coli*. We constructed a variety of mutants in and around the active sites of PDI and analysed them using these complementation assays. Our analysis showed that the active site amino acid residues have a major role in determining the activities exhibited by PDI, particularly the N-terminal cysteine of the N-terminal active site. The roles of the histidine residue at position 38 and the glutamic acid residue at position 30 were also studied using these assays. The results show that these two in vivo assays should be useful for rapid screening of mutants in PDI prior to purification and detailed biochemical analysis.

Key words: Human protein disulfide isomerase; Zymogram; Urokinase; *dsbA*; *dsbC*; *Escherichia coli*

1. Introduction

Protein disulfide isomerase (PDI), a resident of the lumen of the endoplasmic reticulum of eukaryotic cells, catalyses the isomerisation and net formation of disulfide bonds in folding proteins (for reviews see [1–5]). The initial characterisation of PDI involved its ability to catalyse the oxidative refolding of reduced RNase in vitro [6]. Further demonstrations of PDI activity have included the restoration of defective co-translational disulfide bond formation in PDI-deficient microsomes of the endoplasmic reticulum [7], the complementation of a phenotype associated with defective disulfide bond formation in vivo in *Escherichia coli* [8,9], in vivo analysis in *Saccharomyces cerevisiae* [10], and the assisted folding of various model substrate proteins in vitro [11–17]. PDI has also been demonstrated to exhibit activities separate from dithiol:disulfide interchange. It has been identified as the β-subunits of both human prolyl 4-hydroxylase [18] and the microsomal triacylglycerol transfer protein [19] where the putative role of PDI is to maintain the α-subunits in an active conformation [20–22]. PDI also has chaperone activity, demonstrated by its ability to help the folding of non-disulfide-bonded substrate proteins [23,24]. This requires non-covalent binding of PDI to protein substrates, which has been experimentally demonstrated [25–30]. The ability of PDI to bind peptide and its dithiol:disulfide interchange activities appeared linked in that the full multi-domain structure of the PDI molecule is required for the efficient catalysis of most dithiol:disulfide reactions by PDI [31].

PDI has two conserved thioredoxin (Trx)-like active sites with the ubiquitous redox active CXXC motifs which are typical of this family of proteins. Early work on PDI demonstrated its cysteine residues to be essential for its enzymatic activity. Biochemical modification of the active site cysteine residues of PDI caused irreversible inactivation of the protein [32]. Subsequently, PDI molecules were produced with various active site cysteine to serine or alanine substitutions, some of which had a major influence on the dithiol:disulfide interchange activities of PDI in vivo and in vitro [10,33–35]. Detailed studies of PDI using site-directed mutagenesis have been hampered by the lack of a three-dimensional structure for the protein and the relatively complex in vitro assays. There is thus a need for a quick assay for rapid screening of mutant proteins in vivo.

The periplasm is the site of isomerisation and net formation of disulfide bonds in *E. coli*. The major catalyst of net formation of disulfide bonds in *E. coli* is DsbA [36–38]. Isomerisation is catalysed predominantly by the DsbC protein, the activity of which has been demonstrated both in vitro and in vivo [39–42]. We have previously demonstrated that human PDI expressed to the periplasm can complement a *dsbA* mutant in *E. coli*, using alkaline phosphatase, which requires oxidase but not isomerase activity for its efficient folding, as an in vivo substrate [8]. In this study, we show that human PDI can complement a *dsbC*-dependent phenotype on a substrate (mouse urokinase, mUK) that requires the action of an isomerase to fold efficiently. Thus, we can distinguish and quantify the oxidase and isomerase activities of PDI in two simple in vivo assays. We used these assays to analyse the effects of a series of site-directed mutants in and around the active sites of PDI. The various forms of PDI produced differed greatly in their ability to complement *dsbA* and *dsbC* phenotypes in vivo in *E. coli*. This enabled us to screen for amino acid residues critical to the ability of PDI to catalyse the isomerisation and net formation of disulfide bonds in folding protein.

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Abbreviations: PDI, human protein disulfide isomerase; mUK, mouse urokinase; Trx, thioredoxin; carb, carbenicillin; cm or cam, chloramphenicol; kan, kanamycin

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2. Materials and methods

2.1. Materials

Unless otherwise stated all growth media were obtained from BBL, and all chemicals from BDH or Fisher Scientific. All restriction enzymes and modifying enzymes were obtained from New England Biolabs. Mutagenic primers and sequencing primers were obtained from Alta Biosciences (University of Birmingham). VCMS13 Helper phage was obtained from Stratagene, kanamycin and chloramphenicol from Sigma, carbenicillin from Duchefa Biochemie BV (Netherlands), plasminogen from Calbiochem, rabbit polyclonal anti-bovine PDI antibody from BioQuote, secondary HRP-conjugated polyclonal anti-rabbit antibody from Sigma, Hybond C from Amersham and fat-free powdered milk from Marvel.

2.2. Bacterial strains and plasmids

Plasmids and bacterial strains used in this study are listed in Table 1.

2.3. General DNA manipulations, immunoblotting and detection

Standard methods were used [47,48].

2.4. Growth conditions

Cultures grown in Luria broth supplemented with antibiotics were shaken (200 rpm) at 37°C. Expression of PDI from pDPH5 [8] was from the uninduced *trp* promoter. Expression of mUK from pJRS7 was induced by inclusion of arabinose at a concentration of 0.2% (w/v).

2.5. Plasmid and strain construction

A *Bam*HI/*Hin*dIII fragment from pDPH5 containing the PDI gene was ligated into the pMac5.8 vectors producing plasmids pJRS1 and pJRS2 respectively. Oligonucleotide-directed mutagenesis of PDI was carried out in these plasmids by the gapped-duplex method [46]; the plasmids were designated pJRS2-*x* where *x* was the number of the particular mutation. Oligonucleotides used are shown in Table 2. *Bam*HI/*Hin*dIII fragments from pJRS2-*x* plasmids containing mutagenised forms of the PDI gene were ligated into pDPH5, producing the pJRS3-*x* series of plasmids. An *Eco*47III/*Bgl*I fragment of pBAD33mUK was blunt ended by treatment with T_4 DNA polymerase and then ligated into plasmid pDPH5 which had previously been digested with *Bam*HI and the ends filled in by treatment with Klenow. The resulting plasmid (pJRS7) expresses PDI from the *trp* promoter and mUK from the pBAD promoter. *Pvu*I/*Xba*I fragments of pJRS3-*x* plasmids containing mutagenised forms of PDI were ligated into pJRS7 to produce the pJRS7-*x* series of plasmids. As a control, a *Pvu*I/*Hpa*I fragment of pCT54 was ligated into pJRS7 producing pJRS8 which expresses mUK but not PDI. Plasmids in the pJRS2-*x*, pJRS3-*x* and pJRS7-*x* series were demonstrated to contain the desired mutational changes by restriction digestion and additionally by sequencing in the pJRS3-*x* plasmids (data not shown).

TG1 strains carrying mutations of the *dsbA* and *dsbC* genes were constructed using P1 transduction [49] from RI90 and RI179 respectively.

2.6. Assays of PDI dithiol:disulfide interchange activities

The alkaline phosphatase and urokinase (zymogram) assays were performed as described earlier [8,42]. Zymograms were approximately quantified by measuring the zones of clearing by optical densitometry using an IS-1000 Digital Imaging System (Alpha Innotech Corp.) using the software provided (AlphaImager 2000) and displaying them graphically. A calibration curve was constructed by making several dilutions of bacterial periplasmic extract from JRS3 containing pJRS7, and the activity exhibited by the PDI mutants when complementing the *dsbC* phenotype was estimated by comparison with this curve.

3. Results and discussion

3.1. PDI can complement a dsbC mutation in E. coli

We first studied the ability of periplasmic PDI to act as an isomerase in *E. coli*, using as a substrate the protein mUK (which contains 12 disulfide bonds) [42]. This protein was used by Rietsch et al. [42] to assay for the in vivo activity of the DsbC protein. When mUK is expressed to the *E. coli* periplasm, it requires the activity of the DsbC protein in order to reach its active state, which can then be detected on a zymogram using plasminogen as a substrate. The 12 disulfide bonds of the mUK protein must be correctly formed to produce functional protein [42]. We expressed mUK to the periplasm using plasmid pJRS7 which contains the mUK gene under the control of the arabinose-inducible *pBAD* promoter and PDI under the control of the *trp* promoter. As a control the plasmid pJRS8 was used which expresses mUK but not PDI. These plasmids were transformed into the *dsbC* strain JRS3 and production of active mUK was detected using zymograms as described in Section 2. The results are shown in Fig. 1.

As can be seen, expression of PDI restored mUK activity in the *dsbC* strain whereas in the absence of PDI, no mUK activity was detectable. We conclude from this that the PDI is acting as an isomerase in the *E. coli* periplasm.

3.2. Analysis of mutations in the redox active cysteines of PDI

Initially, we constructed single Cys to Ser changes in each of the four redox active cysteines of PDI. We then determined the oxidase activity of PDI by measurement of alkaline phosphatase activity in a *dsbA* background, and the isomerase

Table 1
Bacterial strains and plasmids used in this study

Strain/plasmid	Genotype/important features	Source or reference
RI89	MC1000 *phoR* Δara714	[42]
RI90	RI89 *dsbA::kan1*	[42]
RI179	RI89 Δ*dsbC::cam*	[42]
WK6	Δ(*lac-proAB*) *galE strA*/F′ *traD36 lacI*^{q} *lacZ*D*M15, proA*$^+$*B*$^+$	[43]
WK6*mutS*	WK6 *mutS*::Tn*10*	[43]
TG1	*supE hsdD5 thi* Δ(*lac-proAB*)/F′ *traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*	[44]
JRS1	TG1 *dsbA::kan1*	This study
JRS3	TG1 Δ*dsbC::cam*	This study
pCT54	Expression plasmid, *trp* promoter, ColE1 origin, Amp^R	[45]
pDPH5	pCT54 expressing PDI from *trp* promoter	[8]
pMa5.8	f1 origin, colE1 origin, Amp^R, Cm^S	[46]
pMc5.8	f1 origin, colE1 origin, Amp^S, Cm^R	[46]
pJRS3-x	pDPH5 with mutagenised PDI	This study
pBAD33mUK	mUK expression plasmid, *pBAD* promoter, pACYC184 origin, Cm^R	D. Belin
pJRS7-*x*	Plasmids expressing wild type or mutagenised PDIs and mUK from the *trp* and *pBAD* promoters respectively, colE1 origin, Amp^R	This study
pJRS8	Control plasmid expressing mUK from the *pBAD* promoter, colE1 origin, Amp^R	This study

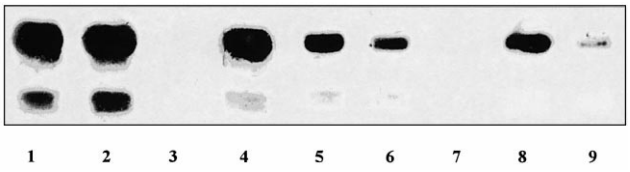

Fig. 1. Zymogram demonstrating the ability of PDI to complement the effect of *dsbC* mutation on urokinase activity. Lysates of cultures induced with 0.2% (w/v) arabinose and grown to an OD_{600} of approximately 1.0 were separated on non-reducing SDS-PAGE gels. Urokinase activity was shown by activation of plasminogen on plasminogen/casein agar. Zymograms of lysates are from strains TG1 containing pJRS8 and pJRS7 (lanes 1 and 2), JRS3 (TG1 ΔdsbC) containing pJRS8 and pJRS7 (lanes 3 and 4) and JRS3 containing pJRS7-*x* plasmids expressing PDI with the amino acid changes C383S, C380S, C36S, H38Y and H38P (lanes 5, 6, 7, 8 and 9 respectively). A typical zymogram of three independent experiments is shown.

activity by measurement of mUK activity in a *dsbC* background. The effects on oxidase and isomerase activity in our experiments were dramatically different (Table 3). None of the mutations had any significant effect on the in vivo oxidase activity of PDI. However, mutation of either of the two cysteines in active site one led to a complete loss of isomerase activity in vivo. Mutation of either of the two cysteines in active site two led to a severe reduction in isomerase activity, particularly for mutation of the first cysteine (Table 3, rows 2–5).

These results demonstrate two things. First, the requirements for redox active cysteines for isomerase activity are clearly more stringent than for oxidase activity. Second, with regard to isomerase activity there is evidence for some non-equivalence between the two sites, with site one being more important than site two. This has also been demonstrated by in vitro assays [50], although other investigations suggested that both active sites contribute equally to the isomerase activity of PDI and thus inactivation of either site led to a loss of 50% of the activity [33]. We considered the possibility that a difference in activity might be seen if assays were performed on cultures before they reached saturation, but experiments on cultures at different times in their growth failed to reveal any such difference (data not shown). The difference between different studies may arise from the use of different substrates or from the conditions under which the assays were carried out.

We next looked at the effects of mutating both of the active site cysteines in one or the other of the two active sites (Table 3, rows 6 and 7). Oxidase activity was unaffected by this; the PDI with either site one or site two inactivated by mutation of both cysteine residues to serine still gave 100% alkaline phosphatase activity in a *dsbA* background. Isomerase activity in contrast was completely lost if the first active site was mutated and largely though not completely lost if the second site was inactivated, further supporting the suggestion that the two sites are non-equivalent with regard to isomerase activity [50].

The fact that the oxidase activity of PDI was unaffected in our assays by inactivation of either one of the two active sites enabled us to look at the contribution of the different cysteine residues of each site to the activity, without any background activity from the other site (Table 3, rows 8–11). Mutation of the first cysteine to a serine in either site one or site two, with the other site inactivated, led to complete loss of oxidase activity. Mutation of the second cysteine to serine in site one (with no active site two) led to an approximately 50% loss of activity, whereas mutation of the second cysteine in site two (with no active site one) led to an approximately 70% loss of activity. These results were again consistent with a degree of non-equivalence between the two sites and also demonstrated that the first cysteine is the crucial one in the oxidase activity of PDI. We thus predicted that a double mutant where both the first cysteines in active sites one and two were changed to serine would be inactive, for both oxidase and isomerase activity, whereas a double mutant with both the second cysteines mutated would retain some oxidase activity but no isomerase activity. This prediction was confirmed (Table 3, rows 12 and 13). A mutant with all four cysteine residues converted to serines has no oxidase activity above background, as expected (Table 3, rows 14 and 15).

These results agree in part with previous studies [33,34], which identified the first cysteine of both active sites as essential for the oxidase and isomerase activities of PDI. However, our data identify the second cysteine of both active sites as being essential for isomerase activity only. This is discussed later.

3.3. Analysis of other active site mutations in PDI

The *E. coli* proteins DsbA, DsbC, and Trx, and PDI, all contain the same active site cysteine residues but show dramatically different in vivo and in vitro activities. DsbA is active primarily as an dithiol oxidase, DsbC as an isomerase, PDI as both, and Trx as a reductase. Alignments of the known polypeptide sequences of PDI, DsbA, DsbC and Trx shows the differences in and around the active site for each protein (see Fig. 2). The requirements for some of these amino acids in the bacterial proteins have already been analysed [51–58]. We therefore made changes in the first PDI active site

Table 2
Oligonucleotides used for construction of mutations in PDI

Oligonucleotide[a]	Restriction enzyme[b]	Amino acid change
5′-GGGGCATAGAAGACCACCAGCAGG-3′	*Bbs*I	E30V
5′-GCAGTGACCGCTCCATGGGGC-3′	*Bsr*BI	C36S/C380S
5′-GGCCAGAGCCTTGGAGTGGCC-3′	*Bst*XI	C39S
5′-GGGAGCCAACTGTTTGGAGTGACC-3′	*Bst*XI	C383S
5′-GCCTTGCAGTGCGGGCACCAAGGG-3′	*Ban*I	G37P
5′-GCCTTGCACGGACCACACCAAGGG-3′	*Ava*II	H38P
5′-GCCTTGCACTAGCCACACCAAGGG-3′	*Bfa*I	H38Y
5′-GCCTTGCAGAGGCCACACCAAGGG-3′	*Eco*NI	H38L
5′-GCCAGAGCCTTACAGCGGCCGCACCAAGGG-3′	*Eag*I	H38R

[a]Changes in the oligonucleotides from the wild type PDI DNA sequence are underlined.
[b]The restriction endonuclease used to initially check the successful mutagenesis of the PDI gene.

Redox Protein		Active Site Sequence																				Ref.s
human	PDI	A	**H**	K	Y	L	**L**	**V**	**E**	**F**	**Y**	**A**	**P**	**W**	**C**	**G**	**H**	**C**	**K**	**A**	**L**	**A**	[18]
human	Trx	G	D	K	L	**V**	V	**V**	**D**	**F**	S	**A**	T	**W**	**C**	**G**	**P**	**C**	**K**	**M**	**I**	K	[62]
E. coli	Trx	A	D	G	A	**I**	L	**V**	**D**	**F**	W	**A**	E	**W**	**C**	**G**	**P**	**C**	**K**	**M**	**I**	A	[63]
E. coli	DsbA	A	G	A	**P**	Q	**V**	L	**E**	**F**	F	**S**	**F**	F	**C**	**P**	**H**	**C**	**Y**	**Q**	**F**	**E**	[36, 37]
E. coli	DsbC	Q	**E**	**K**	**H**	**V**	**I**	**T**	**V**	**F**	**T**	**D**	**I**	**T**	**C**	**G**	**Y**	**C**	**H**	**K**	**L**	**H**	[39, 40]

Fig. 2. Comparison of the active site sequences of various eukaryotic and prokaryotic oxidoreductase proteins [18,36,37,39,40,62,63]. The PDI active site sequence is the N-terminal active site where the cysteine residues are at positions 36 and 39 of the PDI molecule. Multiple sequence alignments were carried out using the ClustalW algorithm [61]. For PDI 17 polypeptide sequences from the SWISS-PROT database were analysed. For DsbA, DsbC and Trx 8, 4 and 46 sequences were analysed respectively. Conserved amino acid residues within a redox protein subfamily are in bold.

that made it more closely resemble the active sites from the different bacterial proteins, and analysed their effect on both the oxidase and isomerase activities of PDI.

Mutation of the glycine residue at position 37 to a proline (found in the corresponding position in DsbA, which acts predominantly as an oxidase) did not reduce the oxidase activity of PDI but completely inactivated the isomerase activity irrespective of the presence or absence of a functional site two (Table 4, rows 3 and 4). Mutation of the histidine at position 38 to a proline as found in Trx, completely abolished the oxidase activity of PDI but intriguingly left a small residual isomerase activity (Table 4, rows 5 and 6). Mutation of the same histidine to a tyrosine (found in DsbC) led to a significant loss of oxidase activity, but although the isomerase activity was also reduced when combined with an unchanged site two, it was greater than that seen with a wild type site one and an inactivated site two (Table 4, rows 7 and 8). Thus in all cases, the changes in site one had the same effect on the particular activity being assayed irrespective of the nature of site two. The reason for this unexpected result is not clear, but it was highly reproducible.

The changes introduced above to mimic the active site sequences of other redox proteins pointed to the histidine residue at position 38 of PDI as having a key role in the dithiol:disulfide interchange activities of PDI. We investigated the effects of changing this residue to a more basic residue (arginine) or a neutral one (leucine). Both of these changes caused a complete loss of isomerase activity, irrespective of the presence or absence of an active site two (Table 4, rows 9–12). These mutations had no effect on the oxidase phenotype when an active site two was present, but in the absence of an active site two, mutation to leucine caused a very significant loss of oxidase activity.

Finally we looked at the possible role of the glutamate residue at position 30. This residue was chosen for study because the equivalent residue in Trx, aspartate 26, has been proposed to play an important role in the second stage of disulfide reduction as catalysed by Trx, acting as a proton acceptor to facilitate the ionisation of the second active site cysteine to the reactive thiolate form, which has a key catalytic role in the isomerase activity of PDI [54,55,59,60]. Our earlier results had already demonstrated that the second cysteine is not required for oxidase activity in our assay, thus mutation of glutamate 30 to a neutral residue such as valine was predicted to have little or no effect on the oxidase activity but to reduce or destroy the isomerase activity of PDI. The results obtained confirmed that this was indeed the case (Table 4, rows 13 and 14).

In this study we have demonstrated the ability of PDI to catalyse the isomerisation and net formation of disulfide bonds by complementation of *dsbA* and *dsbC* mutations in *E. coli*. This enabled us to quantify the importance of the active site residues to the dithiol:disulfide interchange activities of PDI. The first cysteine of both PDI active sites is essential for the ability of PDI to catalyse the isomerisation and net formation of disulfide bonds. We showed that the histidine residue at position 38 is critical for PDI function and that the glutamate residue at position 30 is required by

Table 3
Oxidase and isomerase activities of PDI and PDI mutants with Cys→Ser replacements in the active sites, determined in vivo

	Amino acids in PDI at active sites (mutagenised bases underlined)		Alkaline phosphatase activity[a]	Urokinase activity[b]
	N-terminal active site	C-terminal active site		
1	CGHC	CGHC	100	100
2	SGHC	CGHC	97	0
3	CGHS	CGHC	103	0
4	CGHC	SGHC	96	6.3
5	CGHC	CGHS	87	12.5
6	SGHS	CGHC	100	0
7	CGHC	SGHS	94	3.1
8	SGHC	SGHS	12	0
9	SGHS	SGHC	14	0
10	CGHS	SGHS	56	0
11	SGHS	CGHS	29	0
12	SGHC	SGHC	12	0
13	CGHS	CGHS	64	0
14	SGHS	SGHS	10	0
15	no PDI	no PDI	11	0

[a]Results are shown as percentage restoration of alkaline phosphatase activities compared to wild type PDI. The means of at least five independent experiments are shown, standardised against the activity of wild type PDI in RI90.
[b]Results are shown as percentage restoration of urokinase activities compared to wild type PDI. The means of at least three independent experiments were calculated, and standardised against the activity of wild type PDI in JRS3.

Table 4
Oxidase and isomerase activities of PDI and of mutagenised forms of PDI, determined in vivo

	Amino acids in PDI at active sites (mutagenised bases underlined)		Alkaline phosphatase activity[a]		Urokinase activity[b]	
	N-terminal active site	C-terminal active site		[c]		[c]
1	CGHC	CGHC	100	–	100	–
2	CGHC	SGHS	94	100	3.1	100
3	CPHC	CGHC	114	–	0	–
4	CPHC	SGHS	116	123	0	0
5	CGPC	CGHC	14	–	1.6	-
6	CGPC	SGHS	11	12	0.8	25
7	CGYC	CGHC	57	–	12.5	–
8	CGYC	SGHS	57	61	12.5	400
9	CGLC	CGHC	104	–	0	–
10	CGLC	SGHS	29	31	0	0
11	CGRC	CGHC	107	–	0	–
12	CGRC	SGHS	86	91	0	0
13	VxxxxxCGHC	CGHC	92	–	~0.1	–
14	VxxxxxCGHC	SGHS	92	98	~0.1	~3.2
15	no PDI	no PDI	11	–	0	0

[a]Results are shown as percentage restoration of alkaline phosphatase activities compared to wild type PDI. The means of at least five independent experiments were calculated.
[b]Results are shown as percentage restoration of urokinase activities compared to wild type PDI. The means of at least three independent experiments were calculated.
[c]The values in these columns are for mutant forms of PDI with an inactivated second active site, standardised against PDI with an intact first active site and inactivated second active site.

PDI to facilitate the isomerisation of disulfide bonds in folding protein.

In the absence of a three-dimensional structure for PDI, mutagenic studies have to be based on homologies with other proteins with related activities. The availability of these simple in vivo assays for rapid screening of site-directed mutants should considerably speed up the process of identifying mutant forms of PDI for further study by purification and detailed characterisation in vitro.

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