The b’x Region of Yeast Protein Disulfide Isomerase is Not Essential for Saccharomyces cerevisiae Viability at 30 °C

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Protein disulfide isomerase (PDI) catalyzes thiol oxidation, reduction and isomerization of disulphide bond of cell surface and secreted proteins. Yeast PDI1 consists of two catalytic domains (a and a’) which are separated by two non-catalytic domains (b and b’), and a x region linked the b’ and a domains. The b’ domain is important for the non-covalent binding of partially folded protein. To understand the contribution of b’ domain and x-linker of yeast PDI1 we have deleted the b’x and investigated its functional role in vitro and in vivo. Yeast PDI1 without b’x region retained only 50% activity and became more sensitive toward Proteinase K. Interestingly, yeasts containing full length PDI1 and pdi1∆b’x showed approximately the same growth rate. However, the yeast pdi1∆b’x mutant growth impaired severely at 37 °C compared to that of full length PDI1. Our results suggested that the a-b-a’-c domains of PDI seems to be sufficient to support the growth of yeast cells in normal condition, but the b’x region might be essential in assisting refolding of highly accumulated unfolded protein at high temperature (37 °C).

Key words: b’ domain, protein disulfide isomerase, Saccharomyces cerevisiae, x-linker

Formation of correct disulfide bonds is essential for proper folding of the majority of cell surface and secreted proteins in eukaryotic cells. Protein disulfide isomerase (PDI), which is a resident of the endoplasmic reticulum (ER), catalyzes the formation, reduction and isomerization of disulphide bond of proteins. It also has a chaperone role which mediates the folding and assembly of nascent peptides into mature proteins in the cells (Wang and Tsou 2003). The chaperone activity is independent of its catalytic activity as it has been demonstrated that PDI improves secretion of redox-inactive β-glucosidase (Powers and Robinson 2006). These hydrophobic residues are demonstrated that PDI1 improves secretion of redox-inactive β-glucosidase (Powers and Robinson 2007). In addition, PDI independent of its catalytic activity as it has been demonstrated that PDI improves secretion of redox-inactive β-glucosidase (Powers and Robinson 2007). In addition, PDI demonstrated that pdi1−/−Δ is essential for protein substrate interactions (Klappa et al. 2000; Denisov et al. 2009).

In Saccharomyces cerevisiae PDI is encoded by an essential gene PD1 (Farquhar et al. 1991). The three dimensional structure of yeast PD1 has a twisted U-shape in which the a and a’ domain facing each other on the end of U while the b and b’ domain forming the base (Tian et al. 2006). The b’ and a’ domains is connected by 17 residues which is referred as x-linker. The crystal structure shows that the b and b’ domain have hydrophobic patch which together with hydrophobic areas surrounding the active sites, form a continuous hydrophobic surfaces across the a, b’ and a’ domain (Tian et al. 2006). These hydrophobic residues are involved in the interaction of PDI with its substrates.

In this paper we described that yeast pdi1 without b’x region still maintains half of its insulin reductase activity, although it becomes more sensitive toward Proteinase K compared to that of full length PD1. Furthermore, we demonstrated that pdi1∆b’x can support yeast viability.

MATERIALS AND METHODS

Materials. Plasmid pRS314-PDI1 (a generous gift from Prof. W. J. Lennarz, Department of Biochemistry and Cell Biology, State University of New York at Stony Brook, USA) containing PDI1 was used as a template for amplification of a DNA fragment encoding for pdi1 without b’x. Escherichia coli DH5α was used for plasmid propagation and BL21 (DE3) (ara, leu7967, lacX74, phoA, PvuI, phoR, malF3, F’[lac, lacF], pro), trxB::kan(DE3) was used for expression host of PDI proteins. Yeast S. cerevisiae strain 2736 (MATa ade2-1 can1-100 ura3-1 leu2-3,112 trpl-1 his3-11,15 pdi1::HIS3) containing pCT37-PDI1 [URA3] (obtained from Prof. T. H. Steven, Institute of Molecular Biology, University of Oregon, USA) and pUKC639 (kindly provided by Prof. M. F. Tuite, School of Biosciences, University of Kent, United Kingdom) were used for plasmid shuffling in yeast cell. Plasmid pT7.7 (Novagen, Madison, WI) was used for expression of a pdi-Δb’x mutant in E. coli. Plasmid pGEM-®-T, Vent and Taq DNA polymerases were obtained from Promega. Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs, Inc. All syntetic DNA oligomers were synthesized by Pro-Oligo Singapore. Yeast cells were grown in minimal medium [0.7% (w/v) yeast nitrogen base (YNB) without amino acid, 2% (w/v) glucose or 2% (w/v) galactose] supplemented with appropriate amino acids and bases, and YEPD/Gal yeast extract ([1% (w/v), (w/v) bacto peptone 1%, (w/v) glucose 2%, (w/v) galactose 2%]) media. Escherichia coli was grown in Luria-Bertani (LB) (Sambrook et al. 1989).

Construction of pdi1∆b’x Mutant. A yeast PDI lacking b’x region coted by nucleotides at position 739-1149 in PD1 gene was constructed through 3 steps of PCR (Fig 1) using

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pRS314-PDII as a template. Oligonucleotide primers for PCR were designed based on published sequences of PDII gene (Accession number 850314). Primers FMP1(5'-ACT TTGGTGAAATCGCGTGAAGGCAACCATGACGAA-3') and RD2(5'-GAGGAGGATCCTTACAATTCATCGTGAATGG-3') were used to amplify a DNA fragment corresponding to the coding region 719-738 of PDII. The FMP1 contained 20 nucleotides corresponding to the coding region 719-738 of PDII, while RD2 primer had a NdeI restriction site. The resulted DNA fragment from the first round PCR as a mega primer together with primer FD1 (5'-GAG GAC ATC CCG TCA TTA -3') and a template of reverse mega primer together with primer FD1 (5'-GAG GAC ATC CCG TCA TTA -3') were used to amplify a DNA fragment corresponding to coding region 1150-1569 of PDII. The FMP1 contained 20 nucleotides corresponding to the coding region 719-738 of PDII, while RD2 primer had a BamHI restriction site.

The resulted DNA fragment from the first round PCR as a reverse mega primer together with primer FD1 (5'-GAG GAC ATA TGA AGT TTT CTG CTG GTG-3') and a template of pRS314-PDII were used in the second PCR. The third PCR was carried out using primers forward FD1 and reverse RD2 (5'-GAGGAGGATCCTAATTCATCGTGAATGG-3') and the second PCR results as a template. All PCR products were analyzed by agarose gel electrophoresis and purified by GFX purification kit.

The resulted pdi1Δb’x DNA fragment was ligated with pGem®-T vector and the ligation product was transformed into E. coli DH5α using CaCl, method (Sambrook et al. 1989). The pGem®-T-pdi1Δb’x fragment was digested with NdeI and BamHI restriction enzymes and the resulted 1400 bp pdi1Δb’x fragment was isolated and then ligated with an expression vector previously digested with NdeI and BamHI. The resulted pT7-7-pdi1Δb’x was confirmed by restriction analysis. The pT7-7-pdi1Δb’x was subjected to DNA sequence analysis.

**Construction of pdi1Δb’x in Yeast Expression Plasmid.** Plasmid pT7.7-pdi1Δb’x as a template, and primers PDIF1 (GCTAGCATGAAAGTTTTTCGCTGTTGC) and PDIR1 (GCTAGCTTACAATTCATCGTGAATGGC) were used to amplify pdi1Δb’x. The PCR program was 94 °C for 4 min, 25 cycles of 1 min at 94 °C, 1 min at 55 °C, 2 min at 72 °C and 5 min at 72 °C. NheI sites were included in both forward and reverse primers. The PCR products were gel purified, subcloned into pGem®-T vector. An NheI fragment of pdi1Δb’x taken out from pGem®-T-pdi1Δb’x/NheI was then inserted into pUKC639 previously digested with similar restriction enzyme. The resulted pUKC639-pdi1Δb’x was sequenced to confirm the presence of the deletion.

**Transformation of Yeast.** The resulted pUKC639-pdi1Δb’x was used to transform S. cerevisiae 2736 using a modified lithium acetate method (Ito et al. 1983). The transformed cells were plated onto minimal medium supplemented with 0.001% (w/v) adenine and 0.002% (w/v) tryptophan and grown at 30 °C for 4-7 d.

**Plasmid-Shuffling.** The yeast transformants carrying pUKC639-pdi1Δb’x were grown in solid minimal medium containing 2% (w/v) galactose, 1 mg mL⁻¹ 5’fluoroorotic acid (5’FOA), 0.002% (w/v) tryptophan, 0.001% (w/v) adenine and 0.001% (w/v) uracil at 30 °C for 4-7 d. The yeast cells designated as 2736ΔD were then transferred into a minimal medium supplemented with similar amino acid and bases.

**Protein Expressions.** Escherichia coli strain BL21 (DE3) carrying PDII or pdi1Δb’x genes on plasmid pT7-7 were grown in 10 mL LB media containing 100 g mL⁻¹ ampicillin at 37 °C for 16 h. One mL of overnight culture was transferred into a fresh 50 mL LB/ampicillin media and grown until the culture OD₆₀₀ reached 0.5. Isopropyl-α-thiogalactopyranoside (IPTG) was added to the culture to a final concentration of 0.5 mM and the culture was grown for further 2.5 h. Cell cultures were precipitated by centrifugation at 2100 x g for 30 min at 4 °C. The pellet cells were resuspended in lysis buffer (50 mM Tris-HCl pH 7.5, 0.2 mM NaCl and 10% glycerol) containing 1 mg mL⁻¹ lysozyme. Cells were lysed using
sonication with 10x 1 min burst at maximum frequency and cooled on ice for 1 min after each 1 min burst. The lysed cells was then centrifuged at 9500 x g for 30 min at 4 °C.

**Assay of PDI Protein Activities.** The ability of PDI1 to reduce disulphide bond was determined as described by Sutter et al. (1994). The assay mixture contained 1.67 mM insulin in 100 mM potassium phosphate buffer, pH 7.0, 20 mM ethylene diamine tetraacetic acid, crude bacterial cell extract PDI, 10 mM dithiothreitol. The increase of OD at 650 nm from precipitation of insulin B chain was measured. One unit activity was defined as an amount of PDI to reduce disulfide bonds of insulin in order to produce $\Delta A_{650}$ of 0.001 per min at experimental condition.

**Stability Test of Mutant pdi1∆b’x.** Stability of mutant PDI toward proteinase K was conducted based on method described by Klappa et al. (2000). PDI was mixed with various concentration of Proteinase K (1; 3; 10 mg mL$^{-1}$) in PBS buffer (NaCl 140 mM, KCl 2.7 mM, Na$_2$HPO$_4$ 10 mM, KH$_2$PO$_4$ 1.8 mM, pH 8) for 30 min at 4 °C. The reaction was stopped with 5 mM phenyl methyl sulphonyl fluride (PMSF) for 5 min.

**SDS-Polyacrylamide Gel Electrophoresis and Westernblotting.** Protein electrophoresis was performed as described by Laemmli (1970). Protein blotting was done by using semi-dry blotter (BioRad). Protein in the gel was transferred onto nitrocellulose membrane (Hybond ECL). The membrane was incubated with an antibody anti PDI and subsequently with the peroxidase-conjugated anti rabbit Ig antibody. The signal was developed using ECL detection reagent (Amersham).

**RESULTS**

Yeast pdi1∆b’x has been Generated by Three Steps PCR. Yeast pdi1∆b’x mutant was constructed by deletion of a 739-1146 DNA region of PDI1 corresponding to Ser247-Gly383 using three steps of PCR (Fig 2B). The first PCR was performed to amplify the DNA fragment at nucleotide position of 1150 to 1569 corresponding to the a’c domains. The resulted DNA fragment with the size of 447 bp also contained 20 oligonucleotides of b domain (Fig 3, lane 3). The oligonucleotide of a’c domain was used as a reverse mega primer in combination with FD1 primer to generate a low yield of 1190 bp DNA fragment corresponding to aba’c domain in the second PCR (Fig 3, lane 4). To increase the amount of the 1190 bp pdi1∆b’x DNA fragment, a third PCR using FD1 primer and RD2 primer was performed. The resulted DNA fragment (Fig 3, lane 5) was first ligated with pGem®-T plasmid and then subcloned into pT7.7 expression vector. The sequence of the 1190 bp was confirmed by dideoxynucleotide sequence analysis and found to be free of mutation. A full length PDI1 generated by PCR was also subcloned into pT7-7 plasmid.

Mutant pdi1∆b’x Produced as a Soluble Protein in E. coli. The full length PDI1/pdi1∆b’x gene is placed under the control of φ10 promoter which requires T7 RNA polymerase to activate the promoter. The host E. coli strain used had already been transfected with phage DE3 containing the T7 RNA polymerase gene. The T7 RNA polymerase was under the control of of inducible lacUV5 promoter and thus addition
of IPTG induced the lacUV5 promoter to produce T7 RNA polymerase thereby allowing transcription of the PDI1 gene. Proteins were isolated from the E. coli cultures as described in Material and Methods. The full length PDI1 appeared as a protein with molecular weight of approximately 60 kDa on SDS-PAGE while the molecular weight of pdi1Δb’x was 45 kDa (Fig 4). These are in agreement with the predicted molecular weight from deduced amino acids in which full length PDI1 consists of 522 amino acid residues while pdi1Δb’x contains 386 amino acid residues.

**Mutant pdi1Δb’x has Lower Reductase Activity and is Proteinase K Sensitive.** To determine whether deletion of b’x region affected PDI activity, the ability of both pdi1Δb’x mutant and full length PDI1 to catalyze the reductive cleavage of insulin was determined. The in vitro assay for PDI activity in the crude bacterial cell extract showed that specific activity of the pdi1Δb’x was 1.4 x 10^5 U mg^-1 which is approximately 50% of that of the full length PDI1 (2.8 x 10^5 U mg^-1).

To know the stability of the yeast PDI lacking of b’x towards Proteinase K, the pdi1Δb’x and full length PDI were treated with Proteinase K. Proteolysis of crude bacterial cell extracts was carried out using different concentration of proteinase K. Western blot analysis using anti yeast PDI antibody showed that a significant amount of pdi1Δb’x were digested upon the increase of Proteinase K concentration. There is hardly pdi1Δb’x left after treatment with 10 mg mL^-1 of proteinase K (Fig 5, lane 8). The full length PDI1 remains stable upon Proteinase K treatment (Fig 5). A lower band observed in the full length PDI1 could be the degradation product during protein preparation. This results suggest that pdi1Δb’x mutant is more susceptible to Proteinase K degradation.

**Mutant pdi1Δb’x can Support Yeast Viability.** Since PDI1 is an essential gene, a plasmid-shuffle procedure to observe the function of mutant pdi1Δb’x on supporting yeast viability was conducted. The recombinant plasmid pUKC639-GAL1-pdi1Δb’x containing a LEU2 selectable marker was introduced into a yeast strain 2736. The strain 2736 containing pdi1::HIS3 null mutation carried a plasmid-borne GAL1-PDI1 with a URA3 marker (Tachibana and Steven 1992). After transformation, the cells were grown in a medium containing 5 FOA which allow cells to lose its URA based plasmid-containing full length PDI1. Cells grown in the FOA medium indicated that the newly introduced pdi1Δb’x mutant is able to complement the chromosomal pdi1 null mutation.

**The Growth of pdi1Δb’x Yeast is Almost the Same as the Full Length PDI1 Yeast.** The effect of b’x deletion on the growth rate of yeast cell was investigated by growing the yeast 2736 carrying full length PDI1 and 2736Δ containing pdi1Δb’x in rich media containing galactose at 30 °C. The growth rate of yeast carrying full length PDI1 was 0.13 U OD₆⁶₀ per h, while 2736Δ had a growth rate of 0.12 U OD₆⁶₀ per h. The results show that pdi1Δb’x and full length PDI1 can support the yeast growth at nearly the same growth rate 30°C.

The pdi1Δb’x Yeast Impaired Severely at High Temperature. Protein disulphide isomerase is one of the main cellular chaperones in the ER. Under temperature stress, the amount of unfolded proteins accumulated in the ER will increase. We further investigated the effect of b’x deletion on the yeast growth at temperature stress (37 °C). As shown in Fig 6 both 2736 and 2736Δ grew well at 30 °C. However, reduced growth were observed for both yeast strains at 37 °C in which 2736Δ growth impaired severely.

**The pdi1Δb’x Yeast Shows No Cell Wall Defect.** To study whether the b’x region is responsible in the folding of cell wall forming protein, the pdi1Δb’x mutant was grown in a medium containing calcofluor white. Defect on the cell wall forming protein leads to calcofluor sensitive phenotype. The pdi1Δb’x mutant seems to grow in a similar manner as the full length PDI1 (Fig 7) which indicates that there is no defect on the synthesis of cell wall in pdi1Δb’x.

**DISCUSSION**

Protein disulphide isomerase as a key player in the formation of correct disulphide bond in protein was discovered 40 years ago by Anfinsen and co workers (Goldbeger et al. 1964). It is a member of thioredoxin superfamily which consists of five consecutive domains a, b, a’ and c (Fig 2A). There have been many studies reported on the contribution of each domain or domain combinations in PDI activities both as an enzyme or as a chaperone. We have constructed a yeast PDI without b’ domain and x-linker in which the N-terminals of 19 amino acid residues of the b’ domain and 7 amino acid residues of the a’ domain were still included (Fig 2B).

The pdi1Δb’x was expressed as a soluble protein in E. coli and retained only 50% of its activity in the reductive cleavage of insulin B chain. This represented that b’ domain is required to PDI activity. However, other domains is also capable of interacting with insulin. Other study has shown that deletion of a,b and b’ a’ c domains of bovine PDI resulted in a decrease of insulin reductase activity of 94% and 78%, respectively (Sun et al. 2000). Since pdi1Δb’x still has two a and a’ active site domains which are responsible in the reduction of disulphide bond of insulin, hence it possesses higher activity compared to the other two bovine PDI variants. Several researches have shown that multi domain fragments had enhanced catalytic activities compared with individual a or a’ domains, that the b’ domain had a particularly important role in this enhancement (Darby et al. 1998; Klappa et al. 1998; Tian et al. 2006).

To analyze whether there is a correlation between stability as defined by protease-resistance, crude bacterial cell extract containing pdi1Δb’x mutants was treated with Proteinase K. It appeared that pdi1Δb’x is protease sensitive while the full length PDI1 was only slightly affected at high Proteinase K concentration (Fig 4). The stability of full length human PDI toward Proteinase K had also been reported (Klappa et al. 2000). From the crystal structure of PDI1, it was found that the two flexible catalytic a and a’ domains are attached to more rigid b’ and b domains (Tian et al. 2006), hence deletion of b’x region will decrease conformational stability.

Furthermore, we had investigated how the function of mutant yeast PDI lacking of b’x to support yeast viability. Plasmid shuffling experiment showed that pdi1Δb’x can rescue the pdi1::HIS3 null mutation in 2736 yeast strain. LaMantia and Lennarz (1993) have demonstrated that pdi1 mutant containing a, b and b’ domains can support yeast.
growth, while the ab domain has lost its essential function in yeast viability. We speculate that in the pdi1Δb'x, the abac domains might adopt the modular U shape structure which presumably able to interact with polypeptide substrate to carry out its activity in vivo. Crystal structure of pdi1Δb'x would be able to reveal the three dimensional structure of the mutant.

Protein disulfide isomerase can act both as a catalyst and also as a chaperone. Deletion of the b' domain significantly slows down the refolding rate of misfolded RNase (Darby et al. 1998; Tian et al. 2006). Furthermore, it has also been demonstrated that the b' domain is generally important for refolding of proteins, whereas the b domain might contribute to the refolding rate in selected cases (Tian et al. 2006). Our data showed that the growth of pdi1Δb'x yeast strain producing the a,b,a',c protein was reduced dramatically at non permissive temperature. At high temperature the rate of protein unfolding increased, hence the chaperone role of PDI becomes predominant. Taken together, we propose that the presence of b' region together with b domain is required in assisting refolding of misfolded protein at non-permissive temperature.
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REFERENCES


Klappa P, Ruddock LW, Darby NJ, Freedman RB. 1998. The b' domain provides the principles peptides-binding sites of protein disulfide isomerase but all domains contribute to binding of misfolding protein. EMBO J 17:927-35.


