Protein disulfide isomerase mutant lacking its isomerase activity accelerates protein folding in the cell

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Abstract **We investigated the effect of protein disulfide isomerase (PDI) on in vivo protein folding of human lysozyme (h-LZM) in a specially constructed yeast coexpression system. Coexpression with PDI increased the amounts of intracellular h-LZM with the native conformation, leading to an increase in h-LZM secretion. The results indicated that PDI is a real catalyst of protein folding in the cell. The secretion of h-LZM increased even when both active sites of PDI were disrupted, suggesting that the effect of PDI resulted from a function other than the formation of disulfide bonds. This is the first finding that PDI without isomerase activity accelerates protein folding in vivo.**

Key words: Protein disulfide isomerase; Gene expression; Human lysozyme; Molecular chaperone; Protein folding; Secretion

1. Introduction

Protein disulfide isomerase (PDI) activity was first discovered by Anfinsen's group in rat liver microsome as a catalyst of reduced ribonuclease reactivation [1] and PDI catalyzes the formation, reduction, and isomerization of disulfide bonds in vitro [2]. Two sequences consisting of -Cys-Gly-His-Cys- are responsible for the oxidoreductase activity of PDI [3]. Glutathione is involved in PDI-mediated disulfide bond formation of a protein in the cell [4]. The genetic substitution of wild type PDI with a mutant lacking its activity leads to a delay in disulfide bond formation of carboxypeptidase Y in yeast cells, suggesting that PDI assists folding of disulfide-bonded proteins in vivo [5]. On the other hand, PDI exhibits chaperone-like activity in the in vitro refolding of proteins [6–8]. Furthermore, PDI is suggested to be involved in the quality control system, in which misfolded proteins are destined to degradation in the cell [9,10]. The physiological role of these multiple function of PDI during protein folding in the cell remains obscure.

As PDI promotes the in vitro folding of various proteins, the mechanism of the in vivo protein folding catalyzed by PDI can be used to improve expression systems for foreign genes. In this study, we constructed a coexpression system of PDI and human lysozyme (h-LZM) as the substrate in a single yeast cell and we investigated the effect of PDI on the in vivo folding of the disulfide-bonded protein. We describe the potential function of PDI other than the formation of disulfide bonds during the in vivo protein folding and the use of PDI in developing more efficient expression systems for foreign genes.

2. Experimental

2.1. Strains and vectors

The *Saccharomyces cerevisiae* haploid strain YPH499 (*MAT***a**, *ura3-52*, *lys801*^{amber}, *ade-101*^{ochre}, *trp1-Δ63*, *his3-Δ200*, *leu2-Δ1*) [11] was used as the host for integrating expression units of h-lysozyme and PDI genes. Plasmids pRS303, pRS304, pRS305, and pRS306 [11] were used to construct integration vectors.

2.2. Materials

The h-lysozyme gene was from Takeda Chemical Industries Ltd. The yeast glyceraldehyde 3-phosphate dehydrogenase (GAP) promoter and the yeast alcohol dehydrogenase (ADH) terminator were from Tonen K.K. Restriction enzymes, pUC119, the DNA ligation kit, the site-directed mutagenesis kit (Mutan-K) were obtained from Takara Shuzo. The GeneAmp PCR reagent kit was from Perkin-Elmer. Centricon10 was from Amicon. DEAE Sephacel, Con A Sepharose, and Sephacryl S-200 were from Pharmacia. Oligonucleotides used for PCR and site-directed mutagenesis were synthesized by an automated DNA synthesizer (380A, Applied Biosystems). Dihydrofolate reductase (DHFR) and rhodanese were from Sigma. Chemicals of reagent quality were from Sigma, Wako Pure Chemical Industries, and Nacalai Tesque Inc.

2.3. General methods

Yeast cells were grown in YPD medium or SD medium with auxotrophic requirements [12]. Yeast transformations were carried out as described [13]. Routine recombinant DNA manipulations were performed as described [14].

2.4. Amplification and cloning of genes

Polymerase chain reaction [15] was performed, using a Perkin-Elmer Cetus DNA Thermal Cycler, in 10 mM Tris-HCl, pH 8.3, containing 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin, 200 μM dNTPs, 2.5 units of AmpliTaq DNA polymerase, 2 μmol each of sense and antisense primers, and 200 ng of yeast high-molecular weight DNA as a template. Amplification was performed for thirty cycles of 1 min at 95°C, 2 min at 37°C, and 3 min at 72°C, followed by an incubation at 7 min at 72°C. Primers for amplification of the yeast PDI (y-PDI) gene [16,17] were: 5′-AAAACTCGAGTTATGAAGTTTTCTGCT-3′ (sense) and 5′-TTTTTGGATCCGTTCGTGCAGTGTGA-3′ (anti-sense). Full-length human PDI (h-PDI) cDNA was cloned by screening a human liver λgt11 cDNA library (Clontech) using the oligonucleotide, 5′-TGGCGTCCACCTTGGCCAACCTGATCTCGGAACCTTCTGC-3′ [18], as a probe.

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Abbreviations: ADH, alcohol dehydrogenase; DHFR, dihydrofolate reductase; ER, endoplasmic reticulum; GAP, glyceraldehyde 3-phosphate dehydrogenase; GSH, glutathione (reduced form), GSSG, glutathione (oxidized form); h-LZM, human lysozyme; h-PDI, human protein disulfide isomerase; HSA, human serum albumin; NADPH, nicotinamide adenine dinucleotide phosphate (reduced form); ribonuclease, RNase; TPOR, thiol:protein disulfide oxidoreductase; PDI, protein disulfide isomerase; y-PDI, yeast protein disulfide isomerase; PPI, peptidyl-prolyl *cis–trans* isomerase.

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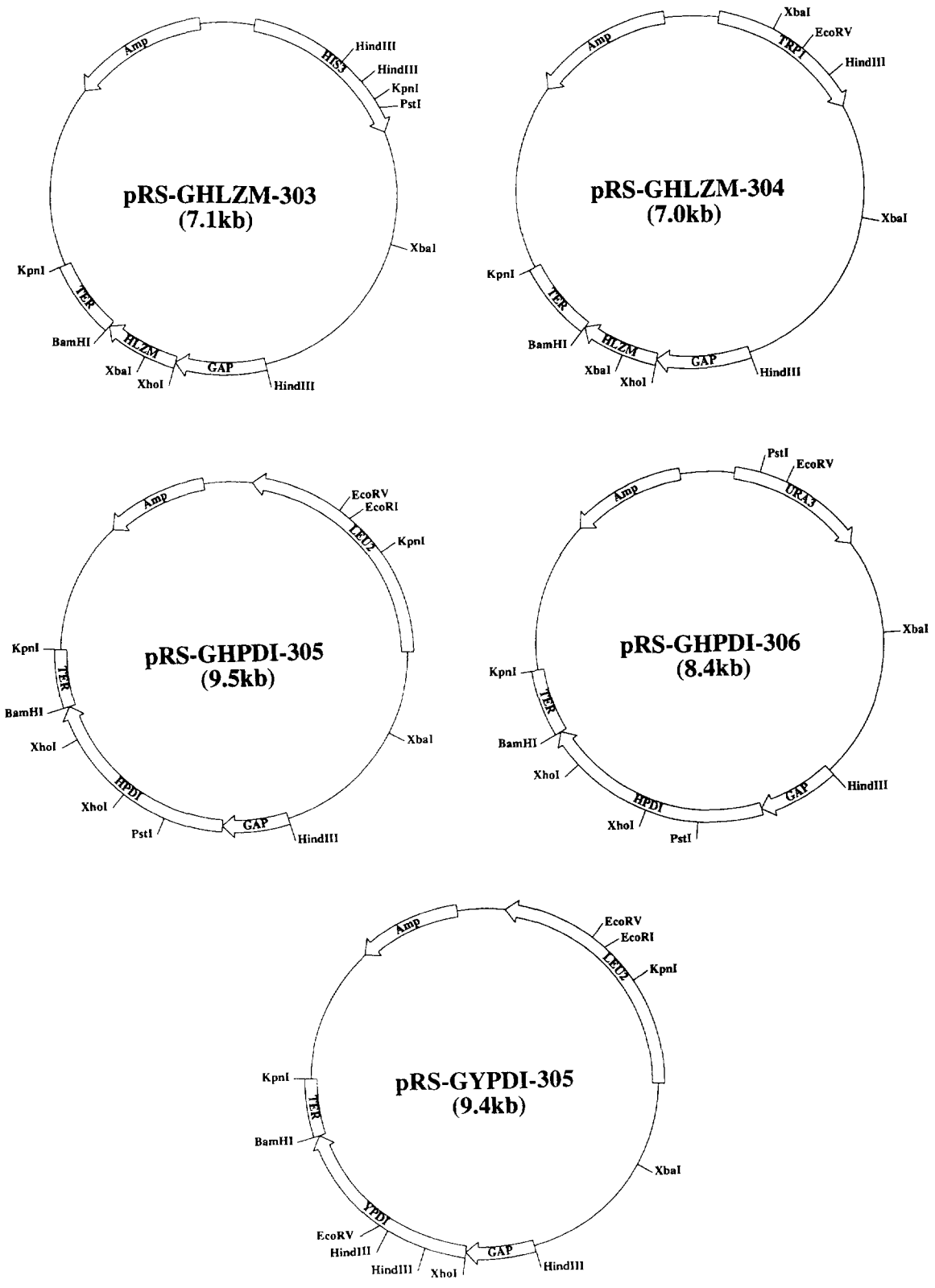


Fig. 1. Expression vectors of h-LZM, h-PDI, and y-PDI. HLZM, HPDI, and YPDI represent coding regions of h-LZM, h-PDI, and y-PDI, respectively. GAP and TER show the coding sequences of the yeast GAP promoter and the yeast ADH terminator, respectively. Four selective marker genes (HIS3, TRP1, LEU2, and URA3) are shown in each vector. Physical maps of the expression vectors of mutant h-PDI (pRS-GHPDIM-305 and pRS-GHPDIM-306) are the same as those of wild type h-PDI (pRS-GHPDI-305 and pRS-GHPDI-306), respectively, except that the coding sequence of mutant h-PDI carries the mutations in its catalytic sites.

2.5. Site-directed mutagenesis

Site-directed mutagenesis, as described by Kunkel [19] was used to introduce a mutation at the junction of the signal sequence and the N-terminus of mature h-PDI to generate a *Nae*I restriction site. The mutagenic primer was: 5′-CGGGGGCGCCGGCGCGC-3′. Mutations in the active sites of h-PDI were introduced as described above. The mutagenic primers used for generating mutations in the first and the second active sites of h-PDI were 5′-CTTGCAGTGGCCAGACCAAGGGGCATA-3′ and 5′-AGTGACCAGACCATGGG-3′, respectively.

2.6. Construction of integration vectors for expression of h-LZM, h-PDI, and y-PDI

The *Hin*dIII/*Xho*I fragment encoding GAP promoter and the *Bam*HI/*Kpn*I fragment encoding ADH terminator were introduced to the multi-cloning sites of the integration vectors: pRS303, pRS304, pRS305, and pRS306 [11]. The resulting plasmids were designated pRS303E, pRS304E, pRS305E, and pRS306E, respectively. Two integration vectors for the expression of h-LZM, designated pRS-GHLZM-303 and pRS-GHLZM-304, were constructed by introducing an *Xho*I/*Bam*HI fragment encoding h-LZM between the GAP promoter and the ADH terminator in pRS303E and pRS304E, respectively. An expression vector for y-PDI, designated pRS-GYPDI-305, was constructed by introducing the *Xho*I/*Bam*HI fragment encoding y-PDI into the *Xho*I/*Bam*HI site in pRS305E. Integration vectors for expression of h-PDI were constructed as follows. A DNA fragment encoding the signal sequence of human serum albumin (HSA) was generated by the annealing and subsequent ligation of the phosphorylated oligonucleotides (5′-TCGAGAATTCATGAAGTGGGTTACCTTCATCTCTTTGTTGTT-3′, 5′-AACAAGAACAACAAAGAGATGAAGGTAACCCACTTCATGAATTC-3′, 5′-CTTGTTCTCTTCTGCTTACTCTAGAGGTGTTTTCAGAAGGCCTG-3′, and 5′-GATCCAGGCCTTCTGAAAACACCTCTAGAGTAAGCAGAAGAG-3′), and subcloned into the *Xho*I site of pUC119X, which was produced by introducing a *Xho*I linker into the *Eco*RI site of pUC119. The plasmid was designated pUC119sig. The *Eco*RI/*Bam*HI fragment encoding h-PDI was subcloned into the *Eco*RI/*Bam*HI site of pUC119 and a *Nae*I restriction site was generated at the junction between the signal sequence and the N-terminus of mature h-PDI by site-directed mutagenesis as described above. The plasmid was digested with *Nae*I and *Bam*HI, then the mature h-PDI coding sequence was excised and cloned into the *Stu*I/*Bam*HI site of pUC119sig. The resulting plasmid, designated pHPDI, was digested with *Eco*RI, followed by fill-in with the Klenow fragment, and the DNA fragment containing h-PDI with the signal sequence of HSA was excised from the vector by *Bam*HI digestion and cloned into the *Xho*I/*Bam*HI site, in which the former protruding end had been filled-in with Klenow fragment, just downstream of the GAP promoter in pRS305E and pRS306E, respectively. These integration vectors were designated pRS-GHPDI-305 and pRS-GHPDI-306, respectively. Expression vectors of mutant h-PDI (pRS-GHPDIM-305 and pRS-GHPDIM-306) was constructed in the same manner as described for wild type h-PDI. The control integration vectors, designated pRSΔ305 and pRSΔ306, were produced by *Xho*I/*Bam*HI digestion, filled-in with the Klenow fragment, followed by the self-ligation of pRS305E and pRS306E, respectively. All of the vectors were linearized at unique restriction sites in selective marker genes (*HIS4*, *TRP1*, *LEU2*, and *URA3*) before transformation.

2.7. Purification of recombinant h-PDI

An yeast strain expressing recombinant wild type h-PDI (HP1) was grown in selective SD medium at 30°C for 48 h and further grown in 12 liters of fresh YPD medium, of which the pH was adjusted to 7.0 by 50 mM sodium phosphate, at 30°C for 48 h. After centrifugation, the supernatant was applied directly on a DEAE-Sephacel column (5.0 × 30 cm) equilibrated with 20 mM sodium phosphate buffer (pH 6.3) and eluted at a flow rate of 25 ml/h with a linear gradient of NaCl (0–1.0 M). Recombinant PDI was detected in the eluate with about 0.6 M NaCl. The fractions containing PDI were collected, concentrated with a Centricon10, applied to a Con A Sepharose column (1.5 × 15 cm) equilibrated with 0.5 M NaCl/20 mM Tris-HCl buffer (pH 7.4) and eluted with the same buffer. The flow through fractions were pooled, applied to a Sephacryl S-200 column (1.5 × 170 cm) equilibrated with 0.5 M NaCl/100 mM sodium phosphate buffer (pH 7.0), and eluted at a flow rate of 0.1 ml/min with the same buffer. PDI was eluted at around 120 ml. Mutant h-PDI was purified in the same manner as described above except that the yeast strain secreting the mutant protein (HP2) was used. Protein concentrations were measured using the protein assay kit (Bio-Rad) with bovine γ-globulin as standard.

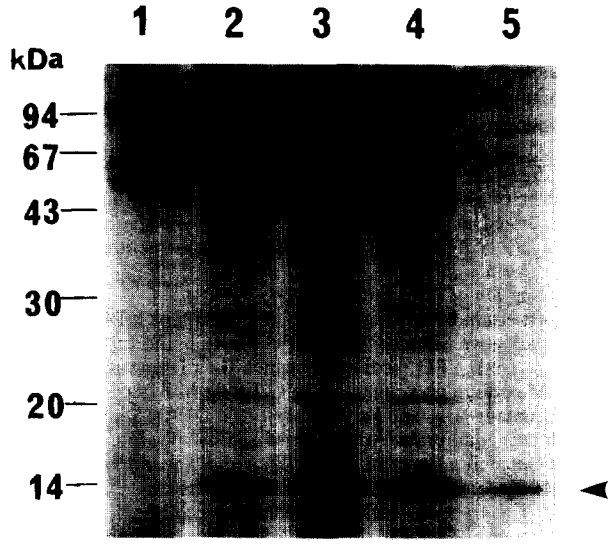
2.8. Preparation of crude yeast extracts

Yeast crude cell extracts were prepared basically as described [20]. Stationary cultures of yeast were inoculated into 250 ml of fresh YPD medium and grown at 30°C to mid-logarithmic phase. The cells were harvested, suspended in 50 ml of buffer A (50 mM sodium phosphate, 20 mM EDTA, 50 mM NaCl, pH 7.5), and ruptured at 9,000 psi for five times with a French pressure cell press (SLM Instruments Inc.). The resulting suspension was adjusted to pH 7.0 with 1 M Tris, then phenylmethylsulfonyl fluoride was added (2 mM). After centrifugation at 15,000 × *g* for 30 min, the supernatant was concentrated with ammonium sulfate (30–85% saturation) and dialyzed against buffer B (50 mM sodium phosphate, 10 mM EDTA, 50 mM NaCl, pH 7.0). The dialyzate was used for assay of intracellular PDI activity.

Table 1
Strains used in this study

Strain	Genotype
YPH499	*MATa, ura3, lys2, ade2, trp1, his3, leu2*
HL1	*MATa, ura3, lys2, ade2, trp1, his3::(HIS3, GAP-HLZM), leu2*
HL2	*MATa, ura3, lys2, ade2, trp1::(TRP1, GAP-HLZM), his3::(HIS3, GAP-HLZM), leu2*
HL10	*MATa, ura3, lys2, ade2, trp1::(TRP1, GAP-HLZM), his3::(HIS3 GAP-HLZM), leu2::(LEU2, GAP-Δ)*
HLP11	*MATa, ura3, lys2, ade2, trp1::(TRP1, GAP-HLZM), his3::(HIS3, GAP-HLZM), leu2::(LEU2, GAP-HPDI)*
HLP12	*MATa, ura3, lys2, ade2, trp1::(TRP1, GAP-HLZM), his3::(HIS3, GAP-HLZM), leu2::(LEU2, GAP-HPDIM)*
HLP21	*MATa, ura3, lys2, ade2, trp1::(TRP1, GAP-HLZM), his3::(HIS3, GAP-HLZM), leu2::(LEU2, GAP-YPDI)*
HLP30	*MATa, ura3::(URA3, GAP-Δ), lys2, ade2, trp1::(TRP1, GAP-HLZM), his3::(HIS3, GAP-HLZM), leu2::(LEU2, GAP-HPDI)*
HLP31	*MATa, ura3::(URA3, GAP-HPDI), lys2, ade2, trp1::(TRP1, GAP-HLZM), his3::(HIS3, GAP-HLZM), leu2::(LEU2, GAP-HPDI)*
HP1	*MATa, ura3::(URA3, GAP-HPDI), lys2, ade2, trp1, his3, leu2::(LEU2, GAP-HPDI)*
HP2	*MATa, ura3::(URA3, GAP-HPDIM), lys2, ade2, trp1, his3, leu2::(LEU2, GAP-HPDIM)*

HLZM, HPDI, YPDI and HPDIM represent human lysozyme, human PDI, yeast PDI and mutant human PDI, respectively. Δ represents the integration of control plasmid pRSΔ305 and pRSΔ306.



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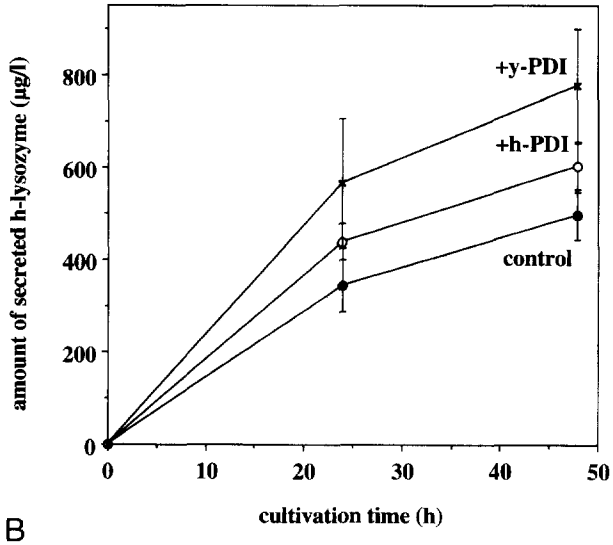

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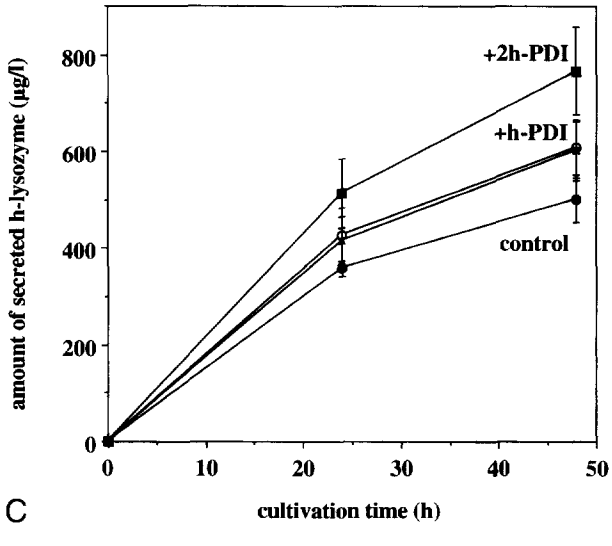

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2.9. Assay of enzyme activity

PDI activity was assayed by measuring the rate of reactivation of scrambled ribonuclease A (RNase A) [21]. Thiol: protein disulfide oxidoreductase (TPOR) activity was assayed by the method, in which the enzyme-catalyzed reduction of disulfide bonds of insulin by GSH is linked to the reduction of GSSG to GSH, by NADPH and glutathione reductase [22]. The lytic activity of h-lysozyme was measured using *Micrococcus lysodeikticus* cells as a substrate [23].

2.10. Assay of chaperone activity

The effect of purified recombinant h-PDI on the reactivation of unfolded DHFR and the aggregation of unfolded rhodanese was investigated as described [24].

3. Results and discussion

To elucidate the possible involvement of PDI in the step of protein folding in the cell, the coexpression system of PDI and h-LZM as a substrate was constructed using a set of yeast integrating plasmid vectors (YIp) with multi-cloning sites. In this study, h-LZM was selected as the substrate for PDI because it is a small and simple protein, consisting of 130 amino acid residues, with four disulfide bonds and because the formation of its disulfide bonds is crucial for in vivo folding [25,26]. In the integration vectors, h-LZM, h-PDI, and y-PDI were constitutively expressed under the control of the yeast GAP promoter with transcriptional termination by the yeast ADH terminator (Fig. 1). In h-PDI expression, it should be noted that the signal sequence of h-PDI was substituted with that of HSA because h-PDI was degraded in yeast, when expressed with its authentic signal sequence, possibly due to a failure in its processing. This construction actually allowed us to express functional h-PDI, while the amino-terminal residue (Asp) of native mature h-PDI was changed to Gly. The expression units of h-LZM, h-PDI, and y-PDI in the vectors were integrated by homologous recombination to the sites of the selective marker genes (*ura3*, *trp1*, *his3*, and *leu2*) on yeast chromosomes of the host strain (YPH499), which contain non-revertible auxotrophic mutations. The strains constructed and used in this study are described in Table 1.

As shown in Fig. 2A and B, h-PDI facilitated the secretion of h-LZM to an extent of 128% and 122% of the control after 24 and 48 h in culture, respectively. The effect of y-PDI on h-LZM secretion was greater than that of h-PDI (148% and 157% of the control at 24 and 48 h of culture, respectively). A dose effect of h-PDI on the h-LZM secretion was also evident (Fig. 2C), in which the increase was about 43% and 53% of the

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Fig. 2. The effect of h-PDI and y-PDI on h-LZM secretion. (A) Yeast strains were grown in the YPD medium [12] for 24 h at 30°C and 750 μl of cultures were concentrated by ethanol precipitation after removing the cells by centrifugation. The resulting samples were resolved by 13.5% SDS-polyacrylamide gel electrophoresis (SDS/PAGE) and the gel was stained with Coomassie Brilliant Blue R-250. The results of SDS/PAGE of typical clones are shown. Molecular weight standards are indicated on the left. The bands of h-LZM are denoted by an arrowhead. Lane 1 = YPH499; lane 2 = HL10; lane 3 = HLP11; lane 4 = HLP21; lane 5 = 0.2 μg of purified h-LZM. (B) Time course of the secretion of h-LZM from strains. HL10 (●: control), HLP11 (○: +h-PDI), and HLP21 (×: +y-PDI). The amount of secreted h-LZM was estimated for cultures at 24 and 48 h by measuring the lytic activity of h-LZM. Ten independent clones were analyzed for each strain. (C) The dose effect of h-PDI on the secretion of h-LZM was investigated as described above. HL10 (●: control). HLP11 (○: +h-PDI). HLP30 (×). HLP31 (■: +2h-PDI).

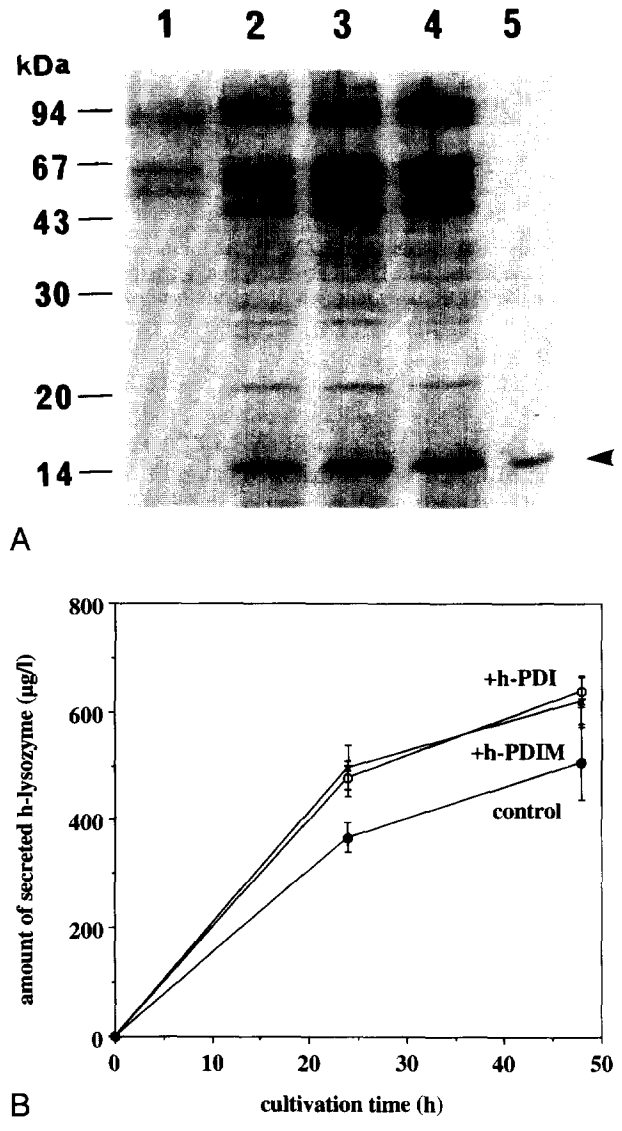

Fig. 3. The effect of mutant h-PDI on h-LZM secretion. The effect of mutant h-PDI, lacking isomerase activity, on the secretion of h-LZM was investigated as described in the legend to Fig. 2. (A) Lane 1 = YPH499; lane 2 = HL10; lane 3 = HLP11; lane 4 = HLP12; lane 5 = 0.2 μg of purified h-LZM. (B) HL10 (●: control). HLP11 (○: + h-PDI). HLP12 (×: + h-PDIM).

control at 24 and 48 h of culture, respectively. These results indicated that the effect was closely related to the amount of intracellular h-PDI. In these experiments, strains (HL10 and HL30), in which control vectors lacking only the coding sequences of h-PDI or y-PDI were integrated, were used as controls, showing that the effect resulted from the coexpression of PDI and not from the rescue of auxotrophic mutations by the integration. There were no apparent differences in the growth rates among strains (data not shown), indicating that the increase in the secretion did not result from the effect of PDI on the cell growth. Thus, functionally expressed h- and y-PDIs facilitate the secretion of the substrate in vivo.

The substitution of both catalytic site sequences of PDI with -Ser-Gly-His-Cys- leads to the complete loss of isomerase activity in vitro [3]. To determine whether the increase of intracellular isomerase activity was responsible for facilitating h-LZM secretion, the effect of mutant h-PDI on h-LZM secretion was examined. The mutant h-PDI accelerated the secretion of h-LZM to almost the same extent as wild type h-PDI (Fig. 3A and B). Whereas PDI is an endoplasmic reticulum (ER)-resident protein, recombinant PDI was secreted into the culture medium in small amounts in our expression system, probably due to overflow. Thus the wild type and the mutant h-PDIs secreted from yeast strains HP1 and HP2 respectively, were purified to homogeneity (Fig. 4). Purified proteins were assayed for the isomerase activity using scrambled RNase A and the TPOR activity using insulin as a substrate. We confirmed that the mutant h-PDI lost its activity completely (data not shown).

Next, we focused on the intracellular changes induced by the coexpression of PDI. Crude cell extracts were prepared from the yeast strains (HL10, HLP11, HLP12, and HLP21) and the amounts of intracellular h-LZM with the native conformation were estimated by measuring the lytic activity. As shown in Table 2, the increase of intracellular, folded h-LZM paralleled that of secreted h-LZM, indicating that the effect of PDI is exerted in the pre-secretory pathway. Together with the observation that the coexpression of PDI did not affected the transcriptional level of the h-LZM gene (data not shown), it was strongly suggested that PDI accelerates h-LZM folding in vivo. The effect of PDI on the increase of intracellular h-LZM was more remarkable than that of secreted h-LZM, possibly due to some limitations in the secretory steps, such as a deficiency of the secretory machinery. The intracellular activities of PDI were also measured by the scrambled RNase and TPOR assays (Table 2). In the former, the activity was increased 121% in the extracts of HLP11 and 243% in those of HLP21, compared with that of the control (HL10), respectively. In the latter, those were 112% for HLP11 and 248% for HLP21, respectively. Supposing that the specific activities of mammalian PDI and y-PDI are

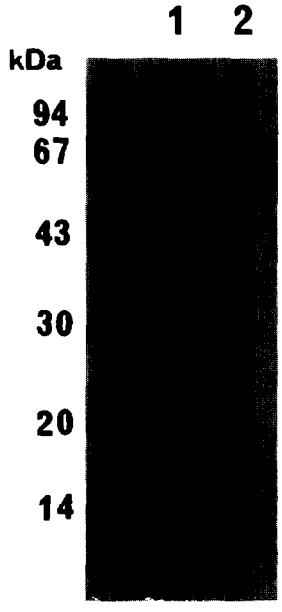

Fig. 4. SDS/PAGE of recombinant wild type and mutant h-PDIs. 3 μg of purified recombinant wild type (lane 1) and mutant (lane 2) h-PDIs were resolved by 13.5% SDS/PAGE, respectively, followed by staining with Coomassie Brilliant Blue. Molecular weight standards are shown on the left.

Table 2
Correlation between the coexpression of PDI and in vivo folding of h-LZM

Strain	Secreted h-LZM	Intracellular h-LZM	Intracellular PDI activity	
			RNase assay	TPOR assay
HL10	100	100	100	100
HLP11	128	250	121	112
HLP12	135	200	101	101
HLP21	149	550	243	248

Average values obtained from the experiments described in Figs. 2 and 3 were used for the amount of secreted h-LZM. Values for intracellular h-LZM were estimated as the amount of h-LZM per total proteins in the crude cell extracts. For the RNase and TPOR assays, crude cell extracts containing 760 μg of total proteins were used. Values for intracellular h-LZM, the RNase assay, and the TPOR assay represent the mean from two independent experiments. In all columns, values for HL10 are set to 100%.

similar [20], the intracellular amount of y-PDI was estimated to be 7–12 fold greater than that of h-PDI. Together with the findings that the amount of folded h-LZM from HLP11 and HLP21 increased 250% and 550%, the effect of h-PDI on intracellular h-LZM folding is greater than that of y-PDI. In this experiment, the complete loss of PDI activities by the mutation and the effect of the mutant h-PDI, namely an increase in the secreted h-LZM level, were also confirmed (Table 2). Thus mutant h-PDI lacking its isomerase activity accelerated h-LZM folding to the same extent as wild type h-PDI, suggesting that a function of h-PDI besides oxidoreduction of proteins is responsible for the effect. Disruption of the PDI gene in yeast results in a recessive lethal mutation [16,17]. However, the loss of PDI activity by site-specific disruption in both catalytic sites does not affect the viability of yeast cells [5]. This indicates that the essential function of PDI does not reside in its isomerase activity but in its unidentified activity. On the other hand, the loss of PDI activity leads to a delay in the folding of carboxypeptidase Y [5]. In this light, our study describes a new finding that the mutant h-PDI, lacking isomerase activity, accelerates the in vivo folding of the disulfide-bonded protein.

Recently it was reported that PDI exhibits chaperone-like activity in the refolding of some proteins [6–8]. Chaperone-like activity of purified recombinant h-PDI was investigated. In the DHFR assay, no significant chaperone-like activity of h-PDI was evident even when PDI was present at a large molar excess (10-fold against DHFR at a molar ratio) (data not shown). On the other hand, the chaperone-like activity of recombinant h-PDI was observed in the rhodanese assay (Fig. 5A). Unlike GroEL, addition of Mg-ATP had no effect on the PDI-mediated suppression of rhodanese aggregation (Fig. 5A). The mutant h-PDI also had chaperone-like activity (Fig. 5B), indicating that PDI activity is not responsible for chaperone-like activity. The suppression of aggregation of denatured rhodanese by the mutant was more efficient than that by the wild type h-PDI. This could be explained by its anti-chaperone activity [6]. Wild type h-PDI partly prevented aggregation with its chaperone- like activity and partly promoted aggregation

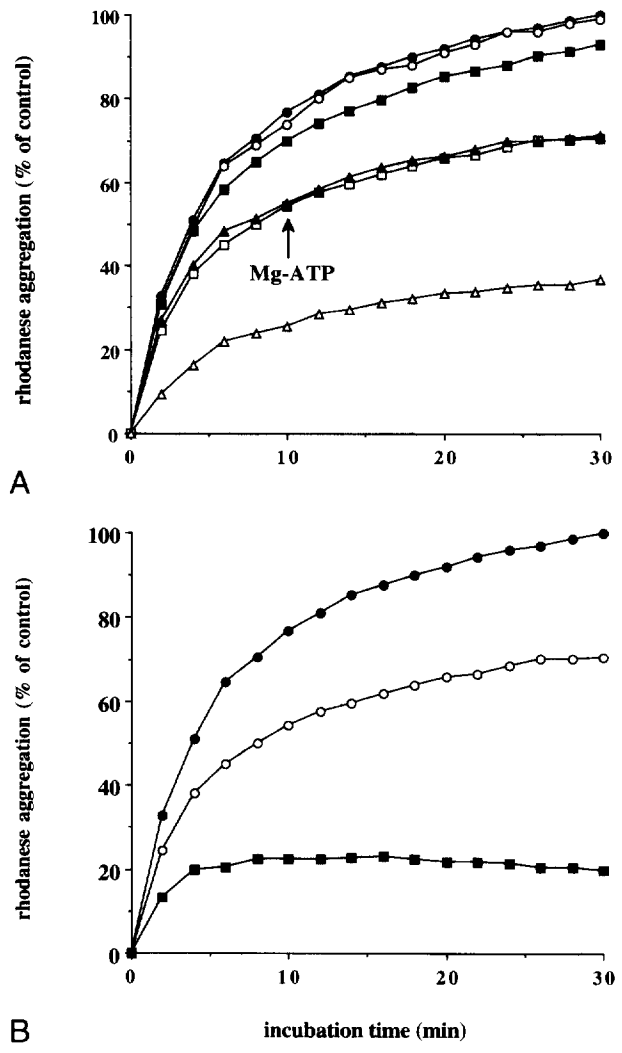

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Fig. 5. Chaperone-like activity of h-PDI. (A) The suppression of denatured rhodanese aggregation by h-PDI was investigated [24]. Bovine rhodanese (800 μg) was denatured in 1 ml of buffer A (6 M guanidinium-HCl, 30 mM Tris-HCl, pH 7.4, 1 mM dithiothreitol) at 25°C for 1 h and diluted (0.46 μM final concentration) 100-fold into buffer C (30 mM Tris-HCl, pH 7.2, 50 mM KCl) containing 0 (●), 0.23 (■), 0.46 (□), and 1.38 μM (△) recombinant h-PDI, and 1.38 μM bovine serum albumin (○) as a control. The aggregation of denatured rhodanese was investigated by monitoring the increase in absorbance at 320 nm. The effect of Mg-ATP was also investigated. Ten minutes after the dilution of denatured rhodanese (0.46 μM) in buffer C containing 0.46 μM h-PDI, 1 mM ATP and 5 mM magnesium acetate at final concentration were added (▲). Aggregation in the absence of h-PDI was set to 100%. (B) The effect of the mutant h-PDI on the suppression of denatured rhodanese aggregation was investigated. As described above, rhodanese was denatured and diluted in buffer C in the presence of 0.46 μM wild type h-PDI (○) and 0.46 μM mutant h-PDI (■). Aggregation in the absence of h-PDI (●) was set to 100%.

with its oxidation activity, of the intermolecular disulfide bonds of denatured and reduced rhodanese. Coupled with our finding that the mutant h-PDI accelerates the folding of h-LZM in vivo, the chaperone- like activity of PDI could be another mode of function, other than its isomerase activity, involved in the step of protein folding in the cell.

It should be noted that recombinant h-PDI has chaperone-like activity in the rhodanese, but not in the DHFR assay, suggesting that it has substrate-specificity in its chaperone activity. We suggest that proteins such as rhodanese, which are liable to aggregate and unable to refold spontaneously, are preferable substrates for PDI, whereas proteins such as DHFR or RNase A, which spontaneously refold, are not. From this perspective, h-LZM would not be a preferable substrate, which would explain why the effect of PDI described here was not so great as that described by Robinson et al. [27], in which the overexpression of PDI resulted in ten-fold higher levels of secretion of human platelet derived growth factor B and a four-fold increase in that of acid phosphatase. To apply PDI to an expression system for foreign genes, the substrate specificity of PDI needs to be understood. In addition, the factors that regulate the dissociation of the bound polypeptides from PDI remain to be elucidated. Peptidyl-prolyl *cis–trans* isomerase (PPI), another folding-assisting enzyme, has been also shown to possess chaperone activity in the folding of carbonic anhydrase [28]. Whether or not such folding-assisting enzymes would actually function as molecular chaperones in the cell remains an issue to be resolved.

Application of PDI to the expression of foreign genes was first challenged in the *E. coli* system, in which *E. coli* PDI, designated DsbA [29,30], was coexpressed with various antibody fragments including the single F_v fragment [31]. Coexpression did not significantly change the folding limit of the functionally folded antibody fragments. DsbA is required for the in vivo folding of alkaline-phosphatase, β-lactamase, and OmpA [29,30]. However, its in vivo substrates might be limited to the resident proteins in the periplasmic space of *E. coli*, because DsbA is smaller than eukaryotic PDI, and lacks the domain in PDI that is responsible for the nonspecific peptide binding activity [32]. Another explanation for the quite low effect of DsbA, is that aggregation steps in the periplasmic space, in which no molecular chaperone was found, would compete with protein folding and they would occur prior to disulfide bond formation [31]. In this context, our application of PDI to the expression system of foreign genes suggests that the ER-resident chaperones and/or PDI itself would promote the folding pathway, competing for the aggregation pathway of overexpressed proteins in yeast cells. Our system will be helpful for studying the cooperativity among PDI, PPI, and chaperones during protein folding in the cell and will be improved by the coexpression of some *sec* genes.

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