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Functional Analysis of Human P5, a Protein Disulfide Isomerase Homologue

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Human P5 (hP5) was expressed in the *Escherichia coli* pET system and purified by sequential Ni^{2+}-chelating resin column chromatography. Characterization of purified hP5 indicated that it has both isomerase and chaperone activities, but both activities are lower than those of human protein disulfide isomerase (PDI). Moreover, hP5 was observed to have peptide-binding ability, and its chaperone activity was confirmed with rhodanese and citrate synthase as substrates, but not with D-glyceraldehyde-3-phosphate dehydrogenase, showing that hP5 has substrate specificity with respect to chaperone activity. Mutation of two thioredoxin-related motifs in hP5 revealed that the first motif is more important than the second for isomerase activity and that the first cysteine in each motif is necessary for isomerase activity. Since thioredoxin motif mutants lacking isomerase activity retain chaperone activity with the substrate citrate synthase, the isomerase and chaperone activities of hP5 are probably independent, as was shown for PDI.

Key words: human P5, molecular chaperone, protein disulfide isomerase, thioredoxin motif.

The isolation of a cDNA clone encoding human P5 (hP5) revealed that hP5 shares several structural similarities with protein disulfide isomerase (PDI, EC 5.3.4.1) (*1*). However, no function has yet been assigned to hP5. PDI is an abundant and important protein in the lumen of the endoplasmic reticulum (ER) (*2*), and its concentration in the ER of certain tissues reaches mM levels (*3*). PDI catalyzes the oxidation, reduction, and isomerization of disulfide bonds in proteins; it also catalyzes the disulfide-coupled folding of proteins in the ER (*4*). Many PDI homologues have been identified (*5–7*), and elucidation of their functions, most of which are unknown, may explain the observed diversity of these proteins. PDI has two distinct regions that contain the Cys-Gly-His-Cys (CGHC) sequence, which is similar to a motif found in thioredoxin, Cys-Gly-Pro-Cys (CGPC) (also called the TX motif or CXXC motif). The CGHC sequences in PDI are the active sites for thiol-disulfide bond exchange reactions (*8*). PDI and its homologues form a diverse protein superfamily whose members are characterized by two or three CXXC motifs (*5*). The deduced amino acid sequence of hP5 indicates that it has two CGHC sequences and the ER retention signal Lys-Asp-Glu-Leu, which are also found in PDI. Based on the number and the relative positions of CXXC motifs, we have classified PDI and its homologues into four groups: hPDI and hP5 are assigned to class 1 and class 2, respectively (*6*).

hPDI has been proposed to have a-b-b′-a′-c domain structure, and hP5 to have an a^0-a-b-c domain structure (*7*). The a^0, a, and a′ regions are redox-active thioredoxin domains, b and b′ are redox-inactive thioredoxin domains, and c is a highly acidic region. Two of the PDI CGHC sequences are located in the a and a′ domains, and the hP5 motifs are in the a^0 and a domains (*7*). A candidate peptide-binding site is located in each of the b′ and c domains of PDI (*7*). The c domains of both hP5 and PDI are rich in acidic amino acids, but little overall homology is observed between the two proteins.

Studies of the role of the mammalian PDI CXXC motifs in the refolding of bovine pancreatic ribonuclease A indicate that the N-terminal CXXC motif is more active than the C-terminal motif (*9*). However, recent studies of yeast PDI have demonstrated the opposite result: the N-terminal CXXC motif is not as effective as the C-terminal motif in the renaturation of lysozyme (*10*). The reason for this difference has not been determined. Mammalian and yeast PDIs have limited sequence similarity, and the de-gree of sequence similarity between hPDI and hP5 is also small. Recently, some functions of the rat hP5 homologue, CaBP1, which is the rat luminal ER calcium-binding protein, have been examined, and it was concluded that CaBP1 lacks chaperone activity (*11*). Here we report on the functions of

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Abbreviations: hPDI, human protein disulfide isomerase; hP5, human P5; ER, endoplasmic reticulum; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CBB-R250, Coomassie Brilliant Blue R250; RU, response units; GAPDH, D-glyceraldehyde-3-phosphate dehydrogenase.

hP5 including chaperone, isomerase and peptide-binding activities, and the roles of the CXXC motifs of hP5, as compared with those of hPDI.

MATERIALS AND METHODS

Materials—Rhodanese, citrate synthase, and D-glyceraldehyde-3-phosphate dehydrogenase (GADPH) were purchased from Sigma Chemical (St. Louis, USA). Other reagents were mostly from Wako Pure Chemical Industries (Osaka). All reagents were of reagent grade quality.

Strains and Plasmids—Escherichia coli AD494(DE3) [Δ*ara*$^-$, *leu7967*, Δ*lacX74*, Δ*phoA*, *Pvu II*, *phoR*, Δ*malF3*, *F'* [*lac*$^+$, (*lacI*^{q}), *pro*], *trxB*:: *kan*(*DE3*)] and pET-15b (Novagen, Madison, USA) were used for expression of hP5 and hPDI.

Expression and Purification of hP5 and hPDI—Two *Nde*I sites were created in the hP5 cDNA (*1*) and hPDI cDNA (cloned in our lab.) by site-directed mutagenesis (*12*) using PCR. One hP5 *Nde*I site was introduced at the end of the signal sequence coding region, and the other was introduced 10–15 nucleotides downstream from the stop codon using 5′-CTGGCAGTGCATATGCTGTATTCCTCT-3′ and 5′-GGTCTGAAGCATATGTTGTGGCTCTC-3′ as the upper and lower primers, respectively (mutated nucleotides are underlined). For the PDI cDNA, one *Nde*I site was created at the end of the signal sequence coding region, and the other was at 5–10 nucleotides downstream from the stop codon using 5′-CCCTGGTGCATATGGACGCCCCC-3′ and 5′-CGGGTCTGCATATGCGTATTACAGTTC-3′ as the upper and lower primers (mutated nucleotides are underlined). These cDNAs were inserted downstream of the His-tag coding region of pET-15b in the *Nde*I site. *E. coli* AD494 (DE3) harboring one of the expression plasmids was grown at 37°C to an optical density of 0.4–0.6 at 600 nm, then expression was induced by the addition of IPTG to a final concentration of 1 mM, and cultivation was continued at 30°C for 5 h. Cells were harvested by centrifugation, suspended in a 20 mM sodium phosphate buffer (pH 7.4), and disrupted with an ultrasonic cell disrupter. The supernatant was passed through a filter (0.45 μm) and applied onto a Ni^{2+}-chelating resin column for purification.

Assay of hP5 Activities—The isomerase activity of hP5 was determined according to the method of Lambert and Freedman (*13*), 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. To discriminate between isomerase and chaperone activities of hP5, rhodanese, citrate synthase, and GAPDH, which contain no disulfide bonds, were used as the substrate. The chaperone activity of preventing denatured rhodanese aggregation was defined by the methods of Martin *et al.* (*14*). Briefly, bovine rhodanese was denatured in buffer A (6 M guanidinium-HCl, 30 mM Tris-HCl, 1 mM dithiothreitol [pH 7.4]) at 25°C for 1 h, then diluted into buffer C (30 mM Tris-HCl, 50 mM KCl [pH 7.2]). The aggregation of denatured rhodanese was investigated by monitoring the increase in absorbance at 320 nm. The aggregation of citrate synthase upon thermal denaturation was assayed by the method of Shao *et al.* (*15*). Briefly, citrate synthase was diluted into prewarmed HEPES buffer (40 mM HEPES [pH 7.4]) at 43°C. Protein aggregation was monitored by measuring light scattering with a Hitachi Spectrofluorometer F4010. The excitation and emission wavelengths were set to 500 nm, and the excitation and emission slit widths were set to 3 nm. The denaturation and reactivation of GAPDH in the presence of hP5 were carried out according to the method of Cai *et al.* (*16*). In all experiments, purified hPDI was used as a control.

Interaction of hP5 and Mastoparan—Surface plasmon resonance experiments were performed with the BIACORE biosensor system 3000 (BIACORE, Uppsala, Sweden). hP5 was immobilized on the surface of the CM5 sensor chip as described previously (*17*). As a control of nonspecific binding, the unreacted carboxymethyl groups of a sensor chip lacking immobilized PDI were blocked with ethanolamine. As an analyte, mastoparan was injected over the flow-cell at a flow rate of 20 μl/min at 25°C. HBS buffer (10 mM HEPES, 150 mM NaCl, 0.005% Tween 20, 3 mM EDTA [pH 7.4]) was used as running buffer during the assay to prevent nonspecific binding. Data analysis was carried out using BIA evaluation software, version 3.1.

RESULTS

Expression and Purification of Wild Type and Mutant hP5—To understand the functional roles of the hP5 CXXC motifs, various CXXC motif mutations were introduced into the hP5 cDNA by site-directed mutagenesis, and all mutations were confirmed by nucleotide sequence determination (primers are not shown). Figure 1 shows the domain structure of hP5 and of the CXXC motif mutants, in which cysteine residues were replaced with serine. After growth as described in "MATERIALS AND METHODS," *E. coli* cells were harvested and disrupted. Native hP5 and its CXXC motif mu-tant proteins were soluble, and their expression levels reached 18.4–37.5% of total *E. coli* proteins. The supernatant was applied onto a Ni^{2+}-chelating resin column for purification. As shown in Fig. 2, all proteins were purified as a single band after a one-step purification.

Isomerase and Chaperone Activities of hP5—As shown in Fig. 3, hP5 has a concentration-dependent isomerase activity, which is about 45% of that of hPDI. The inhibition of the aggregation of rhodanese and citrate synthase, which lack disulfide bonds, is shown in Fig. 4, A and B, respectively, demonstrating that hP5 has about 50–60% of the chaperone activity of hPDI. To examine the peptide-binding activity of hP5, which is believed to be necessary for its chaperone activity, we studied the interaction between hP5

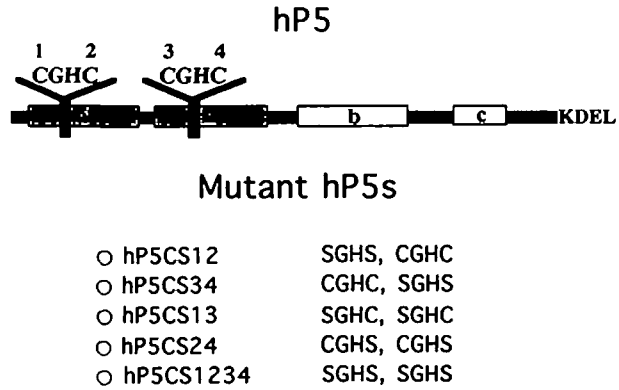

Fig. 1. **Domain structure of hP5 and alterations introduced into the thioredoxin motifs of mutant proteins.**

and mastoparan as described in "MATERIALS AND METHODS." As shown in Fig. 5, mastoparan binds hP5 in a concentration-dependent manner, with a K_D value of 5.66×10^{-5} M. Based on these observations, hP5 was confirmed to have a chaperone activity that acts independently of its isomerase activity. However, the reactivation of GAPDH by hP5 was not observed. These observations indicate that hP5 has both isomerase and chaperone activities, both of which are lower than those of hPDI.

Effect of CXXC Motif Mutations on the Isomerase Activity of hP5—Several CXXC motif mutations were introduced into the hP5 cDNA, and the isomerase activities of the mutant proteins were examined. As shown in Figs. 6 and 7, the activities of the CS12, CS34, and CS24 mutants are about 50, 75, and 10% of that of the wild-type protein, respectively, whereas the CS13 and CS1234 mutants have no isomerase activity. These results demonstrate that the contributions of the two CXXC motifs to isomerase activity are not equal and that a single CXXC motif is sufficient for isomerase activity. In addition, the N-terminal motif is more important for activity, and the presence of two cysteine residues in the same motif strengthens the isomerase activity. However, the contribution to isomerase activity of

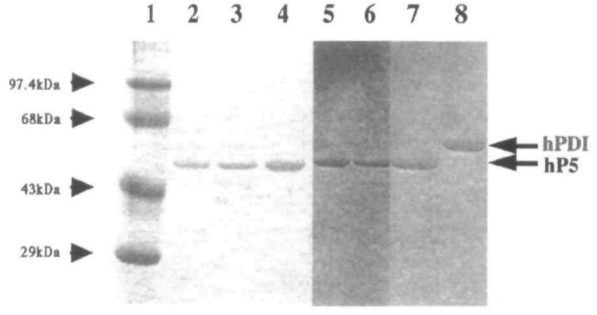

Fig. 2. **SDS-PAGE of wild type and mutant hP5 proteins purified by Ni^{2+}-chelating resin column chromatography.** Electrophoresis was carried out on a 12.5% gel as described in "MATERIALS AND METHODS." Lane 1: marker proteins; lane 2: hP5; lane 3: hP5CS12; lane 4: hP5CS34; lane 5: hP5CS13; lane 6: hP5CS24; lane 7: hP5CS1234; lane 8: hPDI. The arrows at left indicate the locations of the molecular weight markers. The arrows at right indicate the locations of hPDI and hP5.

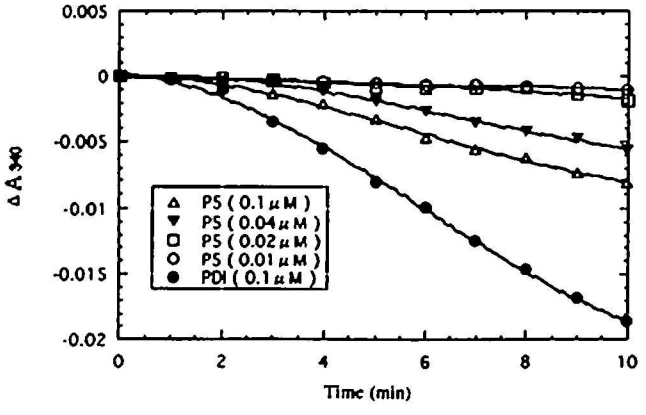

Fig. 3. **Isomerase activity of hP5, determined as described in "MATERIALS AND METHODS."** Inset indicates each protein and its concentration.

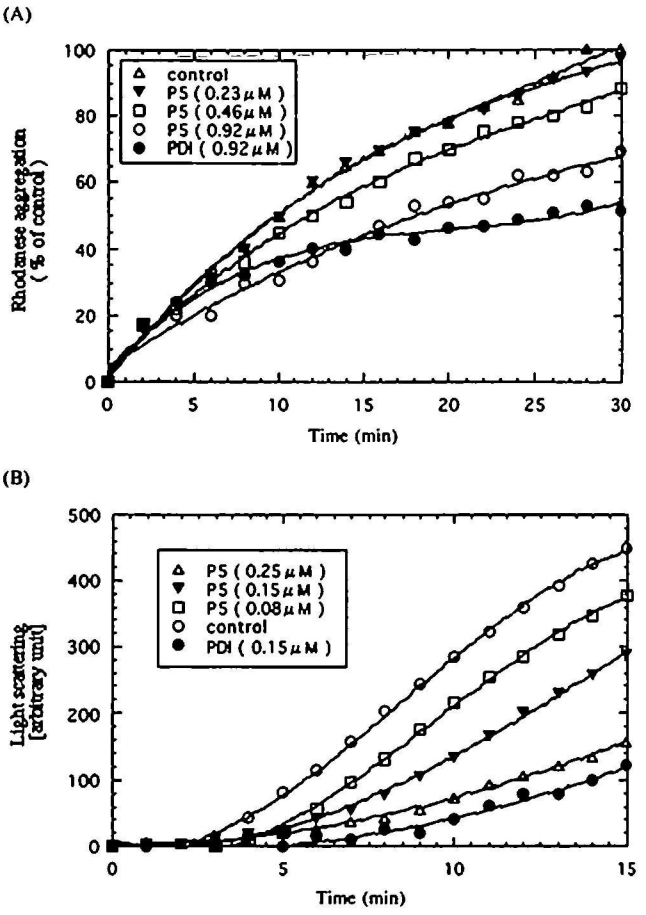

Fig. 4. **Chaperone activities of hP5 using rhodanese (A) and citrate synthase (B) as substrates.** (A) The suppression of denatured rhodanese aggregation by hP5 was investigated. Rhodanese was denatured and diluted (0.36 μM final concentration) in the absence (△) of hP5 as a control; this value was set to 100%. (B) The suppression of thermally denatured citrate synthase aggregation by hP5 was investigated. A 30 μM citrate synthase solution was diluted 200-fold into prewarmed Hepes buffer (0.15 μM final concentration) in the absence (○) of hP5 as a control.

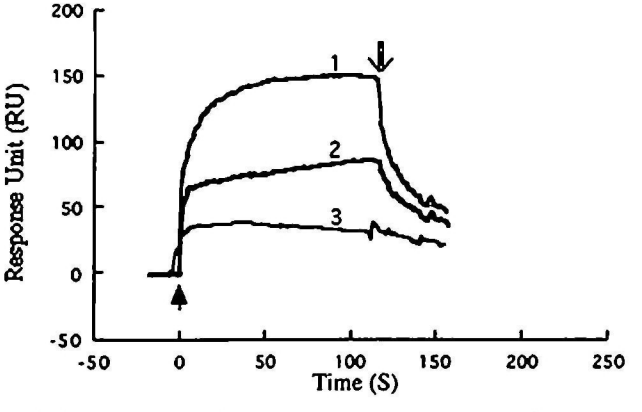

Fig. 5. **Sensorgrams of mastoparan bound to immobilized hP5, as determined with the BIACORE biosensor.** All analytes (1 [3.98 μM], 2 [1.98 μM], and 3 [0.995 μM]) were injected over hP5 immobilized on a sensor chip. The progress of binding to immobilized hP5 was monitored by following the increase in the signal [response units (RU)] induced by mastoparan. The arrow and double arrow indicate the beginning and end of the injection, respectively.

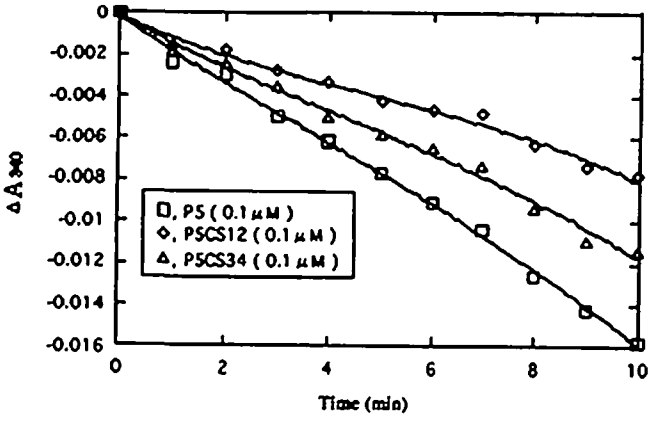

Fig. 6. **Isomerase activity of mutant hP5 proteins.** Inset indicates the concentrations of mutant of hP5 used.

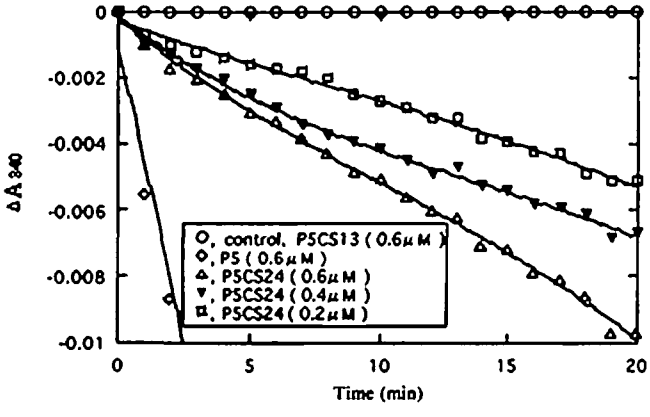

Fig. 7. **Isomerase activity of mutant hP5 proteins.** Inset indicates the concentrations of mutant of hP5 used.

the N-terminal cysteine residue in each motif is greater than that of the C-terminal residue.

Effect of CXXC Motif Mutations on the Chaperone Activity of hP5—The chaperone activities of the hP5 CXXC motif mutants were examined using citrate synthase as a substrate. As seen in Fig. 8, all of the motif mutants have almost the same activity as the wild-type hP5, indicating that CXXC motif mutations have no effect on chaperone activity. These mutations may produce free cysteine residues on the hP5 molecule, which might interfere with the inhibition of aggregation of citrate synthase. Thus we examined this activity in the presence of DTT (data not shown) and confirmed that the presence of DTT has little effect on the chaperone activities of CXXC motif mutants.

DISCUSSION

Our results show that hP5 has a lower isomerase activity than PDI, and that this activity is dependent on the two CXXC motifs of hP5. The CS12 mutant (50% activity), in which the N-terminal CXXC motif was destroyed by replacing both cysteine residues with serine, has a lower isomerase activity than the CS34 mutant (75% activity), in which the C-terminal CXXC motif (the second active site) was destroyed in the same manner. These results indicate

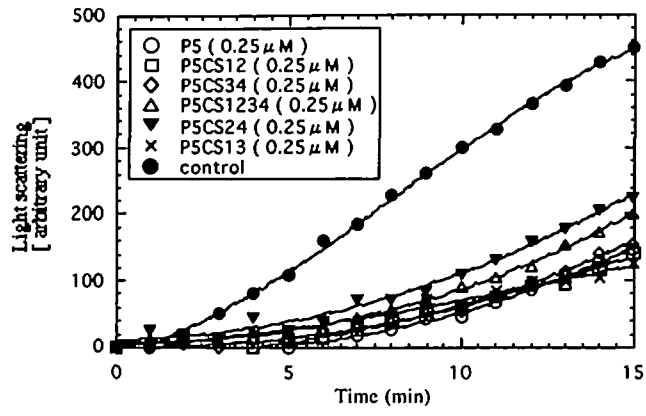

Fig. 8. **Chaperone activity of mutant hP5 proteins in the presence of citrate synthase.** Inset indicates the concentrations of mutant of hP5 used.

that the first hP5 CXXC motif is more important than the second for isomerase activity. Similar results were obtained for the calcium-binding protein CABP1 (*11*). In addition, our results show that two CXXC motifs are not necessarily required for isomerase activity. Yeast PDI homologues such as the MPDI (*18*) and the MPD2 gene products (*19*), which contain only one CXXC motif, can compensate for the loss of PDI, also suggesting that a single CXXC motif is sufficient for isomerase activity, although overall activity is low.

The CS24 mutant, in which the second cysteine residue in each of the two CXXC motifs was replaced with serine, had only 10% of wild-type hP5 isomerase activity, and the CS13 mutant, in which the first cysteine residue in each of the two CXXC motifs was replaced with serine, showed no isomerase activity. Thus the first cysteine in each hP5 motif is necessary for isomerase activity, and the second cysteine makes a lesser contribution. Moreover, effective isomerase activity is observed when two cysteine residues are present in the same CXXC motif. These results explain why the mutant CS1234 does not exhibit isomerase activity. Similar results were obtained for mammalian PDI (*8*) and CABP1 (*11*). Eug1p, a PDI-related yeast protein with one CXXS motif, can complement PDI deficiency (*20*), and mutation of CXXS to CXXC results in a dramatic increase in PDI activity (*21*). These observations show that the presence of two cysteine residues in one motif strengthens isomerase activity. It has been reported that the redox/isomerase ac-tivities of mammalian PDI and yeast PDI are due to the reactivity of the first cysteine residue in each CXXC motif (*10, 21*). Thus it seems likely that the two CXXC motifs and their constituent cysteine residues have same functional role in hP5 isomerase activity as do those of PDI.

Generally, protein aggregation is a non-productive and off-pathway reaction, and chaperones such as GroEL/GroES interact with folding intermediates and prevent or minimize aggregation, increasing the refolding yield (*22*). Many reports have stated that PDI influences the refolding of denatured and reduced proteins with multiple disulfide bonds as an isomerase and as a chaperone. PDI variants that are completely devoid of isomerase activity are not able to accelerate proinsulin folding (*22*). Based on these observations, rhodanese, citrate synthase, and GAPDH, which contain no disulfide bonds, were used as substrates

to discriminate between isomerase and chaperone activities of hP5. hP5 inhibits the aggregation of rhodanese and citrate synthase, which lack disulfide bonds, showing that hP5 has chaperone activity. However, its activity is also lower than that of hPDI, and the reactivation of GAPDH by hP5 is not observed. PDI inhibits the aggregation of rhodanese and citrate synthase, and it also reactivates GAPDH (*16*). Thus the absence of reactivation distinguishes hP5 from PDI. hPDI does not show chaperone activity when dihydrofolate reductase (DHFR) is used as a substrate (*23*), and hP5 does not reactivate GAPDH, suggesting that hPDI and hP5 exhibit substrate specificity with respect to chaperone activity. Kramer *et al.* examined the chaperone activity of CABP1 using only GADPH and concluded that CABP1 has no chaperone activity (*11*). Generally, PDI interacts with a variety of denatured proteins and peptides, and peptide binding is suggested to be necessary for chaperone activity. Thus the interaction between PDI and peptides has been examined by using mastoparan as a model peptide. Mastoparan and somatostatin have been used as model peptides to examine the peptide binding of PDI (*24*). By use of the BIACORE system, we have confirmed that hP5 binds mastoparan, which is composed of 14 amino acids and has been reported to bind to PDI (*24*). Based on these observations, we consider that hP5 has chaperone activity.

hP5 and hPDI share only 10.9% amino acid sequence homology, as determined with the DNASIS-Mac v3.7 program; nevertheless, both hP5 and hPDI have isomerase and chaperone activities. On the other hand, mutants lacking chaperone activity do not exist, and both chaperone and isomerase activities are involved in many refolding experiments. The first CXXC motif of mammalian PDI has been reported to be more active than the second CXXC motif in the refolding of bovine pancreatic ribonuclease A (*9*). However, the second CXXC motif of yeast PDI is more effective in the renaturation of lysozyme (*10*). Mammalian and yeast PDIs share only limited sequence similarity, and the reason for this difference has not yet been determined. These facts strongly suggest that local structure, and not amino acid sequence, is important for these activities, and that the CXXC motif is required for isomerase activity.

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