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The Chaperone Activity of Protein Disulfide Isomerase Is Affected by Cyclophilin B and Cyclosporin A *In Vitro*

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To elucidate the function of protein disulfide isomerase (PDI), we screened for PDI-binding proteins in a bovine liver extract using affinity column chromatography. One of the binding proteins was identified by SDS-PAGE and N-terminal amino acid sequence analysis to be cyclophilin B (Cyp B). Use of the BIACORE system revealed that purified bovine Cyp B bound specifically to bovine PDI with a K_D value of 1.19×10^{-5} M. Interestingly, the binding affinity between PDI and Cyp B was strengthened by preincubation of the Cyp B with cyclosporin A (CsA), yielding a K_D value of 3.67×10^{-6} M. Although the interaction between PDI and Cyp B affected neither the isomerase activity of PDI nor the peptidyl-prolyl *cis-trans* isomerase activity of Cyp B, Cyp B increased the chaperone activity of PDI. However, the complex of Cyp B and CsA completely inhibited the chaperone activity of PDI. Thus, PDI and Cyp B appear to cooperate with each other to regulate the functional expression of proteins *in vivo*.

Key words: BIACORE system, cyclosporin A, molecular chaperone, peptidyl-prolyl *cis-trans* isomerase, protein disulfide isomerase.

It is generally accepted that secretory and membrane proteins, which are translocated as unstructured polypeptides into the luminal space of the endoplasmic reticulum (ER) on their way to synthesis, fold and in some cases assemble into oligomeric complexes before being transported to the *cis*-Golgi compartment. Some ER resident proteins, such as immunoglobulin heavy chain binding protein (BiP/GRP78) (*1, 2*), protein disulfide isomerase (PDI) (*3, 4*), peptidyl prolyl *cis-trans* isomerase (PPI) (*5, 6*), and calnexin (*7–9*), appear to assist the folding of these polypeptides.

PDI is known to catalyze the formation, reduction, and isomerization of disulfide bonds (*10*), and is essential for yeast viability (*11–13*). Recently, PDI was shown to have both chaperone and anti-chaperone activities (*14*), and was suggested to be involved in the quality control system, which serves to degrade misfolded proteins in the cell (*15, 16*). Another function of PDI is to bind other proteins, such as the prolyl 4-hydroxylase α subunit (*17*) and microsomal triglyceride transfer protein large subunit (*18*), and thereby stabilize them. These observations suggest that PDI is a multifunctional and indispensable protein in the cell. However, the physiological roles these multiple functions of PDI play in the cell remain unclear.

To examine the *in vivo* functions of PDI in more detail, we screened for PDI-binding proteins in a bovine liver extract using affinity column chromatography. Subsequent SDS-PAGE analysis showed that several proteins bound to PDI and the main binding protein was identified by N-terminal amino acid sequence analysis to be cyclophilin B (Cyp B). Cyp B is an ER protein belonging to the PPI family, which catalyze the *cis-trans* isomerization of Xaa-Pro peptide bonds (where Xaa is the preceding amino acid) in oligopeptides and accelerate the slow rate-limiting steps in the folding of several proteins *in vitro* (*19, 20*). The PPIs are divided into three structurally unrelated subfamilies on the basis of their ability to bind particular immunosuppressive drugs, namely, the cyclophilin family, the FK506-binding protein (FKBP) family, and the parvulin family (*21–23*). Cyp B, like its cytosolic homologue Cyp A, binds cyclosporin A (CsA) (*6, 24*), which inhibits its peptidyl prolyl *cis-trans* isomerase (PPIase) activity (*6, 25, 26*). Like PDI, Cyp B may be involved in protein folding *in vivo* since it is co-localized in the ER with calreticulin, calsequestrin, and Ca^{2+}-ATPase (*27*), and *in vivo* folding of collagen (*28*) and transferrin (*29*) appears to be slightly delayed after CsA treatment.

In this study we investigate the interaction between PDI and Cyp B in more detail. We find that the interaction of PDI with Cyp B increases the chaperone activity of PDI in a substrate-specific manner and that CsA completely blocks this.

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Abbreviations: BiP, immunoglobulin heavy chain binding protein; BSA, bovine serum albumin; CBB-R250, Coomassie brilliant blue R250; CsA, cyclosporin A; Cyp B, cyclophilin B; ER, endoplasmic reticulum; FKBP, FK506 binding protein; FPLC, fast protein liquid chromatography; GAPDH, D-glyceraldehyde-3-phosphate dehydrogenase; PDI, protein disulfide isomerase; PPI, peptidyl-prolyl *cis-trans* isomerase; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; RU, response units.

MATERIALS AND METHODS

Materials—CNBr-activated Sepharose 4B, Hitrap Q, and Hitrap SP were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Bovine liver was purchased from Nippon Ham (Osaka). Rhodanese, GAPDH, and *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide were purchased from Sigma Chemical (St. Louis, USA). PVDF membrane was purchased from Bio-Rad (California, USA). Cyclospolin A, chymotrypsin, and other reagents were mostly from Wako Pure Chemical Industries (Osaka). All reagents were of reagent grade quality.

Preparation of a PDI-Sepharose 4B Affinity Column—Bovine PDI was prepared from bovine liver according to the method of Lambert and Freedman (*30*). Briefly, after bovine liver (1 kg) had been homogenized and treated at 54°C, the 55–85% ammonium sulfate-saturated fraction was retained. After dialysis against 0.025 M sodium citrate buffer (0.025 M citric acid, 0.025 M sodium citrate [pH 5.3]), the extract was applied to a CM-Sephadex C-50 column and the pass fractions were pooled. After concentration of the pass fractions with 95% ammonium sulfate-saturation, the extract was dialyzed against 0.02 M sodium phosphate buffer (0.02 M NaH_2PO_4, 0.02 M Na_2HPO_4 [pH 6.3]), and then applied to a column of DEAE-Sephacel. PDI was eluted with a linear gradient of 0–0.7 M NaCl. Further purification was performed on a Hitrap Q FPLC column. A PDI-Sepharose 4B column was then prepared according to the method previously described (*31*).

Preparation of a Crude Bovine Liver Extract and Screening of Proteins Binding to Bovine PDI—Bovine liver was cut into small pieces, and then homogenized in homogenizing buffer (0.1 M sodium phosphate buffer [pH 7.5], 5 mM EDTA, 1% [v/v] Triton X-100) with an Excel Auto Homogenizer (NIPPON SEIKI), and then with a Teflon homogenizer (RIKAGAKU SYOUSI SEISAKUJYO). The homogenate was centrifuged at 18,000 ×*g* for 30 min at 4°C, and the pellet was discarded. Ammonium sulfate-saturated fractions 0–55% and 55–95% were separately precipitated, dissolved in 0.05 M sodium phosphate buffer, and then dialyzed against the same buffer before being applied to the PDI-Sepharose column. Unbound materials were thoroughly washed out with the same buffer and the bound proteins were eluted with the elution buffer (0.05 M NaH_2PO_4, 0.05 M Na_2HPO_4, 1 M NaCl [pH 7.4]). To identify the proteins binding to PDI, SDS-PAGE was carried out according to the method of Laemmli (*32*) using 12.5% (w/v) gels. The gels were stained with CBB-R 250. Analysis of the N-terminal amino acid sequences of the proteins was then performed by transferring the protein bands on the SDS-PAGE gel to a PVDF (polyvinyliden difluoride) membrane. Proteins were extracted from the paper and their N-terminal amino acid sequences were determined according to the method of Edman (*33*) using a peptide sequencer PPSQ-10 (SHIMADZU, Kyoto).

Preparation of Cyp B from Bovine Liver—Microsomes were prepared from 300 g of bovine liver by the differential centrifugation method of Tangen *et al.* (*34*) using homogenizing buffer (0.25 M sucrose, 5 mM EDTA, 0.05 M Tris-HCl [pH 7.5]). The microsomes were dialysed against 0.02 M Tris-HCl (pH 7.0) and then applied to the PDI-Sepharose affinity column. After washing out unbound proteins with the same buffer, bound proteins were eluted with the elution buffer (0.02 M Tris-HCl [pH 7.0], 1 M NaCl). The extract was dialysed against 0.02 M Tris-HCl (pH 7.0), applied to a Hitrap SP FPLC column, and then eluted with a linear gradient of 0–1 M NaCl.

Measurement of the Interaction of PDI with Cyp B—Surface plasmon resonance experiments were performed with a BIACORE biosensor system 3000 (BIACORE Inc., Uppsala, Sweden). Bovine PDI was immobilized on the surface of CM5 sensor chips *via* *N*-hydroxysuccinimide and *N*-ethyl-*N'*-(dimethylaminopropyl)carbodiimide activation chemistry according to the manufacturer's instructions. As a control for nonspecific binding, the unreacted carboxymethyl groups of a sensor chip lacking immobilized PDI were blocked with ethanolamine. As an analyte, Cyp B or a mixture of CsA and Cyp B was injected over the flow-cell at a flow rate of 20 μl/min at 25°C. HBS buffer (0.01 M HEPES, 0.15 M NaCl, 0.005% Tween 20, 3 mM EDTA [pH 7.4]) was used as the running buffer during the assay to prevent non-specific binding. Data analysis was performed with BIA evaluation ver.3.1 software.

Assaying of the Effect of Cyp B on PDI Activities—The isomerase activity of PDI was determined according to the method of Lambert and Freedman (*35*). Here, the reduction of the disulfide bonds in insulin catalyzed by GSH is linked to the reduction of GSSG to GSH, which is mediated by NADPH and glutathione reductase. The suppression of denatured rhodanese aggregation, which is mediated by PDI, was investigated by the methods of Martin *et al.* (*36*). 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 and then diluted with buffer C (30 mM Tris-HCl, 50 mM KCl [pH 7.2]) containing various concentrations of PDI. The aggregation of denatured rhodanese was investigated by monitoring the increase in absorbance at 320 nm. The suppression of denatured GAPDH aggregation mediated by PDI was investigated by the methods of Cai *et al.* (*37*). Protein aggregation was monitored by light scattering measurement with a Hitachi Spectrofluorometer F-4010. The excitation and emission wavelengths were both 488 nm. The excitation and emission slit widths were both 3 nm. The aggregation of citrate synthase upon thermal denaturation mediated by PDI was assayed by the method of Shao *et al.* (*38*).

Measurement of the PPIase Activity of Cyp B—The PPIase activity of Cyp B was determined by a coupled assay with chymotrypsin, using the synthetic short peptide *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide as described previously (*39*). Briefly, 50 μl of enzyme solution was preincubated with 50 μl of chymotrypsin (6 mg/ml) in 1,850 μl of 0.05 M HEPES (pH 8.0) for 5 min, and then 50 μl of the substrate (2 mg/ml) was added to the reaction system. The PPIase activity of Cyp B was assayed with time by measuring the absorbance at 390 nm with an U-3210 spectrophotometer (HITACHI). The temperature was kept constant at 8°C throughout the assay.

Measurement of Intrinsic Fluorescence Emission Spectra of Cyp B and CsA-Bound Cyp B—An excitation wavelength of 280 nm was used to measure the fluorescence intensity. Intrinsic fluorescence emission spectra were obtained with a Hitachi Spectrofluorometer F-4010. The reactions were carried out at 25°C.

RESULTS

Screening of Proteins Binding to PDI—A protein extract prepared from bovine liver was applied to a PDI-Sepharose column. Unbound materials were thoroughly washed out and the proteins eluted from the PDI-Sepharose column were analyzed by SDS-PAGE. Several bands were observed for the 55–95% ammonium sulfate-saturated fraction (Fig. 1A), but not for the 0–55% fraction (data not shown). The predominant eluted protein was 20 kDa (Fig. 1A, lane 2), and its N-terminal amino acid sequence was determined to be DEKKKGPKVTV, which is identical to that of bovine Cyp B (Fig. 1B). Other bands, I, II, and III, were identified as aspartate aminotransferase (42 kDa), malate dehydrogenase (33 kDa), and glutathione S-transferase (27 kDa) by N-terminal amino acid sequence analyses, respectively (data not shown). These observations suggest that the primary bovine liver protein that binds to PDI is Cyp B.

Preparation of Cyp B from Bovine Liver—Since Cyp B is a luminal protein, we prepared it by first fractionating cells by differential centrifugation to avoid contamination by cytosolic Cyp A. It was then passed into and eluted from a PDI-Sepharose column and purified on a Hitrap SP column (Fig. 2, lane 2). The N-terminal amino acid sequence of the purified protein was determined to be DEKKKGPKVT, which is identical to that of bovine Cyp B (data not shown). Moreover, the purified protein is a luminal protein of 20 kDa, and has peptidyl-prolyl *cis-trans* isomerase activity, which is inhibited by CsA (shown later). These characteristics show the protein is Cyp B. We succeeded in obtaining approximately 4.6 mg of homogeneous protein from 300 g of bovine liver (data not shown). This preparation was used in the following experiments.

Interaction of PDI with Cyp B or Cyp B Preincubated with CsA—To confirm that Cyp B and PDI interact, purified bovine PDI was immobilized on the surface of a CM5 sensor chip. The final immobilization level was approximately 3000RU (data not shown). Cyp B was then injected over the chip and its binding to the immobilized bovine PDI was monitored by following the increase in the signal measured with a BIACORE 3000. As shown in Fig. 3, the signal varied with the concentration of Cyp B, indicating that PDI interacts with Cyp B. The progress of the signal also showed that Cyp B easily associated with and dissociated from PDI. The resulting sensorgrams allowed the dissociation constant to be calculated with the BIA evaluation ver. 3.1. software. The K_D of PDI as to Cyp B was determined to be 1.19×10^{-6} M.

CsA is known to bind to Cyp B and to inhibit the PPIase activity of Cyp B (*6, 24–26*). Consequently, we examined the effect of CsA on the interaction between PDI and Cyp B. Interestingly, when Cyp B preincubated with CsA was used as an analyte, the interaction between PDI and Cyp B

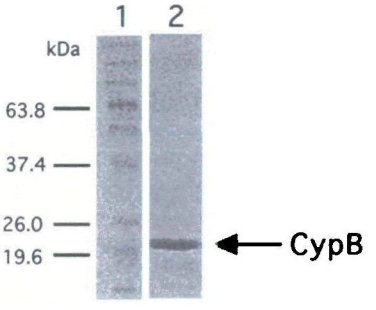

Fig. 2. **SDS-PAGE of Cyp B prepared from bovine liver microsomes.** Lane 1, molecular weight standards; lane 2, purified Cyp B (10 μg). The arrow at the right indicates the location of Cyp B (20.2 kDa).

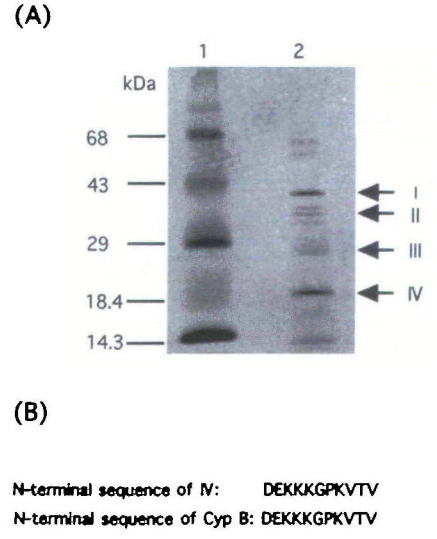

Fig. 1. **SDS-PAGE of PDI-binding proteins and N-terminal sequence of the band IV material.** (A) Electrophoresis of the ammonium sulfate-saturated fraction (55–95%) was carried out on a 12.5% gel. Lane 1, molecular weight standards; lane 2, fraction eluted from the PDI-Sepharose column. (B) N-terminal sequences of the band IV material in (A) and its alignment with Cyp B.

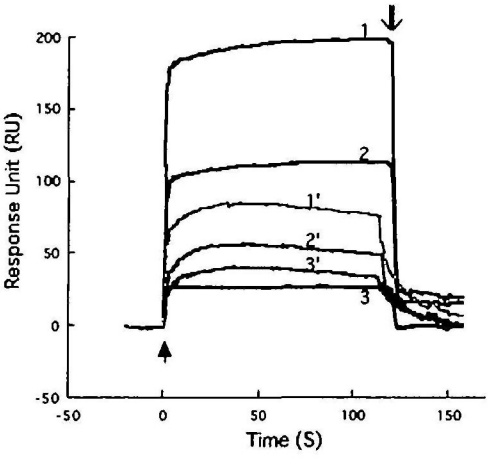

Fig. 3. **Sensorgrams of Cyp B and Cyp B preincubated with CsA injected over bovine PDI immobilized on a BIACORE biosensor chip.** The analytes injected over the sensor chip were: 1, Cyp B (4.48 μM); 2, Cyp B (2.24 μM); 3, Cyp B (0.56 μM); 1′, Cyp B (4.48 μM) preincubated with CsA (5 μM); 2′, Cyp B (2.24 μM) preincubated with CsA (5 μM); and 3′, Cyp B (0.56 μM) preincubated with CsA (5 μM). The progress of analyte binding to immobilized bovine PDI was monitored by following the increase in the signal [response units (RU)] induced by Cyp B or Cyp B with CsA. The arrow and double arrow indicate the beginning and end of the injection, respectively.

changed the progress of the signals, indicating that Cyp B now associated more slowly and dissociated more easily from PDI (Fig. 3, sensorgrams 1′–3′). A K_D value of 3.67×10^{-6} M was calculated. CsA itself appeared not to interact with PDI, and the addition of CsA after the injection of Cyp B did not have any effect on the interaction between PDI and Cyp B (data not shown).

Effect of Cyp B or Cyp B Preincubated with CsA on Various PDI Activities—As described above, Cyp B and Cyp B preincubated with CsA both bind to PDI, albeit with different kinetics. This finding led us to study the effects of Cyp B and Cyp B preincubated with CsA on various activities of PDI. As shown in Fig. 4, Cyp B did not have any effect on the isomerase activity of PDI. The addition of CsA after the incubation of PDI and Cyp B also had no effect on the isomerase activity. However, Cyp B preincubated with CsA slightly inhibited the isomerase activity of PDI (Fig. 4).

When the chaperone activity of PDI was investigated using rhodanese and GAPDH as substrates, Cyp B was found to increase the chaperone activity in a concentration-dependent manner (Figs. 5 and 6). However, when BSA was used instead of Cyp B, it had no effect on the chaperone activity of PDI (data not shown). On the other hand, when citrate synthase was used as a substrate, Cyp B did not increase the chaperone activity of PDI (data not shown). Interestingly, the addition of Cyp B preincubated with CsA completely inhibited the chaperone activity of PDI for both rhodanese and GAPDH, while the addition of CsA after the incubation of PDI and Cyp B had no effect (Figs. 5 and 6).

Effect of PDI on the PPIase Activity of Cyp B—As shown in Fig. 7, PDI did not have any effect on the PPIase activity of Cyp B. In contrast, CsA completely inhibited the PPIase activity of Cyp B (Fig. 7).

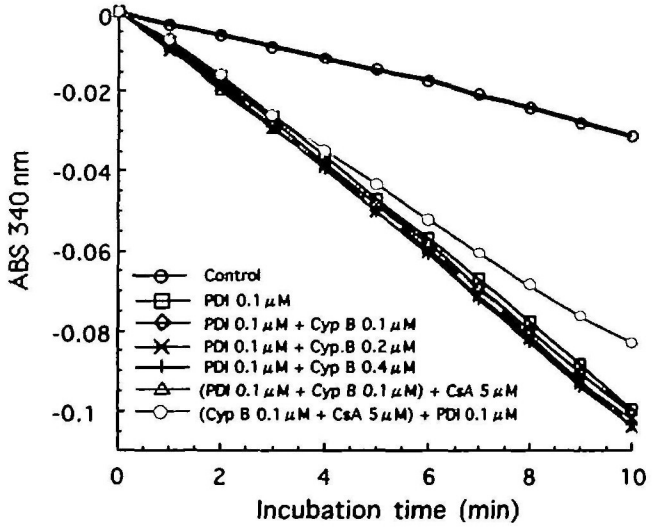

Fig. 4. **Effects of Cyp B and Cyp B preincubated with CsA on the isomerase activity of PDI.** The absence of both PDI and Cyp B from the reaction mixture was used as a negative control. The decrease in ABS 340 nm was measured to monitor the isomerase activity of PDI. The following combinations of agents were studied: PDI (0.1 μM), PDI (0.1 μM) in the presence of Cyp B (0.1 μM, 0.2 μM, and 0.4 μM), CsA (5 μM) in the presence of PDI (0.1 μM) preincubated with Cyp B (0.4 μM), and PDI (0.1 μM) in the presence of Cyp B (0.4 μM) preincubated with CsA (5 μM).

Intrinsic Fluorescence Emission Spectra of CsA-Bound Cyp B—Intrinsic fluorescence experiments on Cyp B and Cyp B preincubated with CsA were carried out. The spectrum of Cyp B alone had a sharp peak around 325–350 nm, while that of Cyp B preincubated with CsA showed a broad

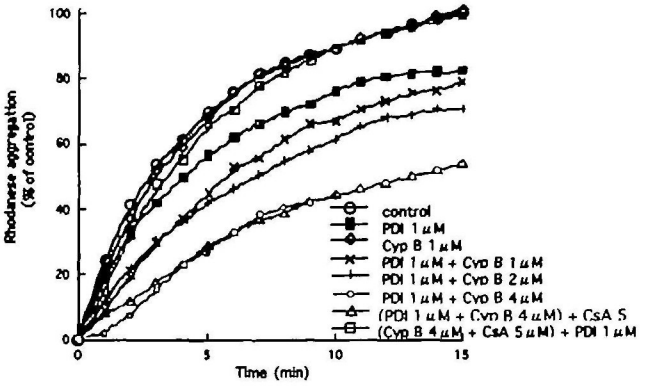

Fig. 5. **Effects of Cyp B and Cyp B preincubated with CsA on the chaperone activity of PDI with rhodanese.** The increase in ABS 320 nm was monitored to measure rhodanese aggregation. Bovine rhodanese was denatured and diluted (final concentration, 0.36 μM) in the absence of both PDI and Cyp B as a control, and this value was set as 100%. The following combinations of agents were studied: PDI (1 μM), Cyp B (4 μM), PDI (1 μM) in the presence of Cyp B (1 μM, 2 μM, and 4 μM), CsA (5 μM) in the presence of PDI (1 μM) preincubated with Cyp B (4 μM), and PDI (1 μM) in the presence of Cyp B (4 μM) preincubated with CsA (5 μM).

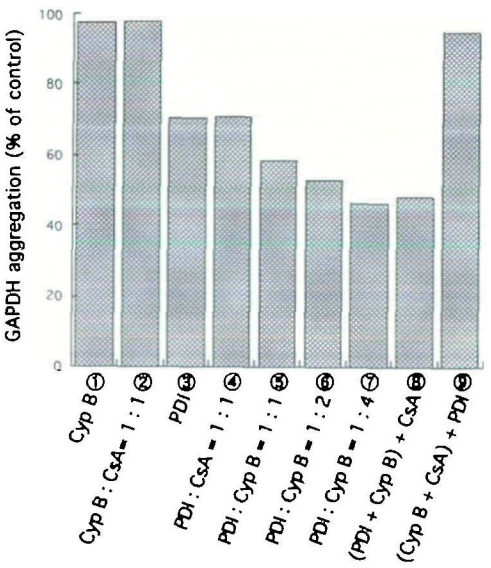

Fig. 6. **Effects of Cyp B and Cyp B preincubated with CsA on the chaperone activity of PDI with GAPDH.** The suppression of denatured GAPDH aggregation by bovine PDI was investigated by light scattering measurements as described under "MATERIALS AND METHODS." GAPDH was denatured and diluted in the absence of both PDI and Cyp B as a control, which was set as 100%. 1, Cyp B (8 μM); 2, Cyp B (8 μM) in the presence of CsA (8 μM); 3, PDI (2 μM); 4, PDI (2 μM) in the presence of CsA (2 μM); 5, 6, and 7, PDI (2 μM) in the presence of Cyp B (2 μM, 4 μM, and 8 μM); 8, CsA (8 μM) in the presence of PDI (2 μM) preincubated with Cyp B (8 μM); and 9, PDI (2 μM) in the presence of Cyp B (8 μM) preincubated with CsA (8 μM).

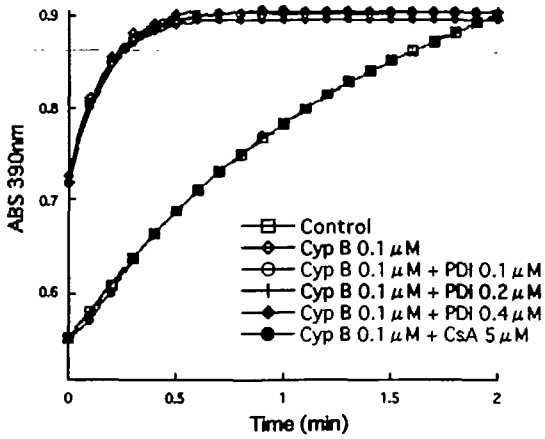

Fig. 7. **Effect of PDI on the PPIase activity of Cyp B. The absence of both PDI and Cyp B was used as a negative control.** The PPIase activity of Cyp B (0.1 μM), Cyp B (0.1 μM) in the presence of PDI (0.1 μM, 0.2 μM, and 0.4 μM), and Cyp B in the presence of CsA (5 μM) was assessed by measuring the increase in ABS 390 nm.

peak around 320–345 nm, indicating the tertiary structure of Cyp B changes when it has been preincubated with CsA (data not shown).

DISCUSSION

PDI is essential for cell viability and appears to possess a number of different functions. To assess the functions of PDI in more detail, PDI-binding proteins present in a bovine liver protein extract were screened using a PDI-Sepharose column. The predominant binding protein was identified as Cyp B by SDS-PAGE (Fig. 1A) and N-terminal amino acid sequence analysis (Fig. 1B). Surface plasmon resonance experiments using a BIACORE 3000 system confirmed that the interaction between PDI and Cyp B was specific (Fig. 3). Other PDI-binding proteins were identified as aspartate aminotransferase (42 kDa), malate dehydrogenase (33 kDa), and glutathione S-transferase (27 kDa), but the interaction between PDI and these PDI-binding proteins was found to be unspecific (data not shown).

The surface plasmon resonance experiments showed that the K_D of the interaction of Cyp B with PDI was 1.19×10^{-5} M. Cyp B is a cyclophilin-type PPI that is found in the ER, and like that of its cytosolic homologue Cyp A, its activity is inhibited by CsA (*6, 24–26*). We confirmed that the PPIase activity of Cyp B is inhibited by CsA (Fig. 7). We therefore examined the effect of CsA on the interaction of Cyp B with PDI and found that preincubation of Cyp B with CsA strengthened the binding affinity of PDI with Cyp B, as Cyp B preincubated with CsA associated more slowly with and dissociated more easily from PDI. The K_D value was 3.67×10^{-6} M. The sensorgrams of CsA-bound Cyp B with PDI are clearly different from those obtained for the interaction between Cyp B alone and PDI (Fig. 3). It has been reported that the tertiary structure of Cyp B changes when CsA binds to it (*40, 41*), and we confirmed this by intrinsic fluorescent experiments (data not shown). This structural change is likely to be responsible for the prolonged transient interaction between PDI and Cyp B. We then investigated the effects of Cyp B and CsA-bound Cyp B on various PDI activities. Cyp B did not have any effect on the isomerase activity of PDI but CsA-bound Cyp B slightly inhibited it (Fig. 4). This slight inhibition might be artificial, however, CsA-bound Cyp B completely inhibited the chaperone activity of PDI (Figs. 5 and 6), although Cyp B itself increased it in a concentration-dependent manner. In addition, it has been shown that the tertiary structure of Cyp B changes when CsA binds to it (*40, 41*). The binding affinity between PDI and Cyp B was strengthened by preincubation of Cyp B and CsA. These observations may suggest that the structural change of Cyp B had a negative effect on both the chaperone and isomerase activities of PDI, but the details are not clear.

The identification of the Cyp B binding site of PDI is helpful for understanding the PDI–Cyp B interaction. We have constructed several mutant PDIs to investigate the Cyp B binding site of PDI, however, we have not yet specified the region. It has been generally accepted that determination of the binding site of PDI is not easy. With regard to the substrate binding site of PDI, two reports (*42, 43*) have proposed that the different domains of PDI all contribute to the binding site. This means that they were not able to definitely determine the substrate binding site of PDI. We have continued to attempt to determine the Cyp B binding site of PDI. It is not clear why an increased ratio of Cyp B to PDI increases the chaperone activity of PDI. One possibility may be that the Cyp B protein loses its activity during purification. Thus we purified it again, but the results were the same. On the other hand, fast-folding species U_F of RNase T1 has correct prolyl isomers and refolds very rapidly (*44, 45*). Such structural intermediates with correct, native prolyl peptide bonds would be better substrates for the action of PDI. Rhodanese and GADPH contain 18 and 11 proline residues, respectively, and the binding of PDI and Cyp B is not so strong. Thus the other possibility is that unbound Cyp B would cause the substrate to form correct prolyl peptide bonds and assist refolding. Elucidation of the PDI–Cyp B interaction mechanism will settle these matters.

It has been reported that cytoplasmic PPI from *Escherichia coli*, whose PPIase activity is not inhibited by either CsA or FK 506 (*46, 47*), improves the ability of PDI to catalyze disulfide bond formation in the course of oxidative folding *in vitro* (*48*). In contrast, our studies revealed that Cyp B does not have any effect on the isomerase activity of PDI (and *vice versa*). Klappa *et al.* performed immunoprecipitation studies to show that PDI and Cyp B are in transient contact during a late stage of translocation (*49*). Our surface plasmon resonance experiment showed that the interaction between PDI and Cyp B is not very strong, and that the binding affinity of PDI and Cyp B is increased in the presence of CsA.

It appears that the interaction between PDI and Cyp B promotes the chaperone activity of PDI when rhodanese and GADPH are used as substrates. However, when the chaperone activity of PDI was analyzed using citrate synthase as a substrate, the activity was not increased by Cyp B (data not shown). Since the latter assay was performed at 43°C, the thermostability of Cyp B at 43°C was investigated by means of intrinsic fluorescent experiments. Cyp B was confirmed to be stable and did not change structurally at 43°C (data not shown). Thus, the mechanism by which

Cyp B promotes the chaperone activity of PDI might involve some substrate specificity. Such substrate specificity in chaperone functions may not be unusual. That the ER contains several resident proteins that assist the folding of newly synthesized polypeptides already suggests that there may be specificity in the proteins being chaperoned. Supporting this is that ERp57 has been found to interact exclusively with glycosylated secretory proteins after their translocation into the ER (*50*). No such interaction was observed with nonglycosylated proteins (*50*). Thus, it is conceivable that some proteins with chaperone activity may only act on specific substrates. Based on our observations described above, PDI and Cyp B appear to cooperate with each other to control the functional expression of proteins *in vivo*.

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