The characterization of a cyclophilin-type peptidyl prolyl *cis–trans*-isomerase from the endoplasmic-reticulum lumen

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A luminally located peptidyl prolyl *cis–trans*-isomerase (PPI) has been purified from bovine liver microsomes. It has a molecular mass of 20.6 kDa, and N-terminal sequencing demonstrates strong sequence similarity to the sequences of the cyclophilin B family. The enzyme catalyses the isomerization of the standard proline-containing peptide *N*-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide, as well as the refolding of RNAase T1. Kinetic properties, substrate-specificity data and inhibition by cyclosporin A indicate that it is a cyclophilin-type PPI, consistent with the amino-acid-sequence results.

INTRODUCTION

Prolyl *cis–trans* isomerization is a rate-determining step in the folding of some proteins *in vitro* (Fischer and Schmid, 1990). A 17 kDa cytosolic enzyme which catalyses this process has been purified from porcine kidney (Fischer et al., 1984). Sequencing of this enzyme revealed it to be identical with cyclophilin (Fischer et al., 1989; Takahashi et al., 1989), the major high-affinity binding protein for the immunosuppressive drug cyclosporin A (CsA). Another class of immunophilins that exhibit peptidyl prolyl *cis–trans*-isomerase (PPI) activity are the FKBPs (FK506-binding proteins), and they can be distinguished from the cyclophilins by their ability to bind the immunosuppressant FK506 (Siekierka et al., 1989).

CsA is a cyclic undecapeptide and FK506 is a member of the macrolide antibiotic family. The two are structurally unrelated, and cross-inhibition between the two is not observed. N.m.r. and X-ray crystal structures of cyclophilin, cyclophilin–CsA complex, FKBP, and FKBP–FK506 complex have been determined (Fesik et al., 1990; Kallen et al., 1991; Michnick et al., 1991; Ke et al., 1991; Van Duyne et al., 1991). These studies indicate that although both proteins are able to catalyse the process of prolyl isomerization, cyclophilin and FKBP share no primary sequence similarity, nor are there any similarities in their tertiary structures.

The functions of these immunophilins *in vivo* are unclear. The binding of immunophilin to its respective immunosuppressant (cyclophilin–CsA and FKBP–FK506) results in a number of responses which include the prevention of T-cell response to antigen, binding and regulating the activity of calcineurin, and preventing nuclear import of a T-cell activation transcription factor [for a review, see Schreiber and Crabtree (1992)]. Members of each family (cyclophilin and FKBP) are ubiquitous, show high sequence similarity and are able to catalyse the process of prolyl isomerization *in vitro*, and so they may play a role in protein folding *in vivo*.

The physiological role of these abundant cytosolic proteins remains unresolved. In another study [the preceding paper (Bose and Freedman (1994)] we examined the subcellular distribution of PPI activity. We demonstrated the existence of a latent PPI activity in washed and proteinase-treated microsomes. We proposed that this activity is due to an endoplasmic-reticulum (ER)-located PPI which may promote folding of secretory proteins at biosynthesis. Here, we have purified the ER-located PPI from bovine liver microsomes. Kinetic characterization of this PPI shows close similarity with the cytosolic (cyclophilin) form of the enzyme. N-terminal sequence, substrate-specificity data and inhibition by CsA indicate that the ER luminal PPI is a cyclophilin-type PPI.

MATERIALS AND METHODS

Materials

All chemicals were obtained from Sigma Chemical Co., Poole, Dorset, U.K., and were of analytical grade. CsA was generously given by Sandoz. Bovine liver was obtained from Anglo–Dutch Meat, Charing, Ashford, Kent. Recombinant RNAase T1 was generously given by Dr. Stefan Walter, Laboratorium für Biochemie, Universität Bayreuth, Bayreuth, Germany. Chromatography materials were obtained from Pharmacia, Uppsala, Sweden. Centrifugation and ultracentrifugation were carried out on Beckman centrifuges. Assays were performed on Beckman DU70 spectrophotometers unless otherwise stated. Problot was supplied by the AFRC Microchemical Facility, Babraham, Cambridge, U.K., where N-terminal sequencing was performed. Monoclonal anti-KDEL antibody (MAC 256) was generously given by Dr. Richard Napier, Horticultural Research International, West Malling, Maidstone, Kent, U.K.

Preparation of washed microsomes

Crude microsomal vesicles were prepared by differential centrifugation of fresh bovine liver as described by Tangen et al. (1973). Crude microsomal pellets were resuspended in minimum volume of 500 mM KCl/50 mM Tris/HCl, pH 7.5, and washed to minimize contamination with bound cytosolic PPI [the accompanying paper (Bose and Freedman (1994)]. The resuspended pellets were centrifuged at 100000 ***g*** (35500 rev./min) for 90 min at 4 °C using a fixed-angle rotor. The resultant pellet was resuspended in minimum volume of S-TKM (0.25 M sucrose/50 mM Tris/25 mM KCl/5 mM $MgCl_2$, pH 7.5), and this frac-

Abbreviations used: CsA, cyclosporin A; FKBP, immunosuppressant FK506-binding protein; PPI, peptidyl prolyl *cis–trans*-isomerase; ER, endoplasmic reticulum; PDI, protein disulphide-isomerase; ECL, enhanced chemiluminescence.

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tion is termed 'washed microsomes'. These washed microsomes formed the starting material for the purification of ER-specific PPI.

Protein characterization and immunoblotting

Protein concentrations were determined by the Bradford method, as modified by Stoscheck (1990), with BSA as standard. The purity of the PPI sample was analysed by SDS/15%-(w/v)-PAGE (Laemmli, 1970). Gels were stained with Coomassie Blue G. Proteins were transferred to nitrocellulose (Towbin et al., 1979) and probed with antibodies (Treharne et al., 1988). The affinity-purified rabbit anti-[bovine protein disulphide-isomerase (PDI)] was used at 1:250 dilution, and the rat monoclonal anti-KDEL (MAC 256) at 1:100, and the blots were developed with appropriate second antibodies (from DAKO-immunoglobulins, Glostrup, Denmark) using the ECL (enhanced chemiluminescence) method of detection.

PPI activity

PPI activity was measured using the standard coupled chymotrypsin assay (Fischer et al., 1984) employing the oligopeptide *N*-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide. For the sequence-specificity studies, the assay was carried out with the standard peptide (as above) as well as *N*-succinyl-Ala-Leu-Pro-Phe *p*-nitroanilide and *N*-succinyl-Ala-Glu-Pro-Phe *p*-nitroanilide. The assays were carried out at 10 °C and the data collected were fitted to a first-order rate equation and activity was calculated as described in the accompanying paper (Bose and Freedman 1994).

Purification of ER-specific PPI

Washed microsomes were permeabilized using 0.5% Triton X-100 at 4 °C for 20 min, and then microsomes were centrifuged at 100000 *g* (35500 rev./min) for 90 min at 4 °C using a fixed-angle rotor. The resultant supernatant was dialysed against 20 mM Tris/HCl, pH 8.0. This was loaded on to an HR 10/10 Mono Q column equilibrated with the same buffer. PPI activity was detected in the unbound protein peak which was dialysed against 20 mM Mes, pH 6.2. The dialysed sample was applied to a HR 5/5 Mono S column equilibrated with the same buffer. Bound protein was eluted at a flow rate of 0.5 ml/min with a linear 0–1 M NaCl gradient over 20 column volumes. Both columns were run using a Pharmacia f.p.l.c. apparatus.

Preparation of PPI for *N*-terminal sequencing

Purified ER-specific PPI was separated by SDS/15%-PAGE and electroblotted on to Problot membranes at 50 mA for 4 h. The problot was previously rinsed in 100% methanol before soaking in blotting buffer (25 mM Tris/192 mM glycine/20% methanol). The problot was stained with Coomassie Blue G for 30 s and then destained using acetic acid/methanol/double-distilled water (1:1:18, v/v) overnight. The membrane was allowed to air-dry overnight. The PPI band was cut out of the membrane for N-terminal sequencing.

Refolding of RNAase T1

Refolding of RNAase T1 in the presence and absence of ER-specific PPI was measured using the method of Kiefhaber et al. (1990c). Unfolded RNAase T1 was produced by a 2 h incubation, at room temperature, of 100 μM RNAase T1 in 8.0 M urea/0.1 M Tris/HCl, pH 8.0. Refolding was initiated by a 40-fold dilution of unfolded RNAase T1 with refolding buffer (0.1 M Tris/HCl, pH 8.0) in the cell of a Perkin-Elmer LS-5B fluorescence spectrophotometer equilibrated at 10 °C. Refolding conditions were 2.5 μM RNAase T1 in 0.2 M urea/0.1 M Tris/HCl, pH 8.0. Refolding was monitored by the increase in tryptophan fluorescence at 320 nm (band-pass 10 nm) after excitation at 268 nm (band-pass 2.5 nm). The fluorescence at 120 min was taken as 100% relative fluorescence. In those experiments where ER PPI was present, 24 nM enzyme was used.

Inhibition by CsA

The coupled chymotrypsin assay (see above) was used to monitor the effect of cyclosporin A on ER-specific PPI. Activity in the presence of 24 nM ER PPI and the absence of CsA was taken as 100% activity. The effect of various concentrations of CsA on this activity was then determined.

RESULTS AND DISCUSSION

Purification and N-terminal sequencing of an ER-specific PPI

Previous experiments [see Bose and Freedman (1992) and the accompanying paper (Bose and Freedman, 1994)] had shown that the luminal content of washed microsomes has a high specific PPI activity which is inhibited by CsA and is therefore presumably of the cyclophilin type. The purification method for this ER-specific PPI was developed using the high pI values of known cyclophilins (Handschumacher et al., 1984; Harding et al., 1986). Purification of an ER-specific PPI was achieved by solubilization of washed microsomes, followed by anion- and cation-exchange steps (Table 1). The active soluble component was applied to a Mono Q column (anion-exchanger) and, at the pH used, the active protein fractions were associated with the unbound-protein peak. This then bound to the Mono S column (cation-exchanger) at the pH used. Elution of this column with a linear salt gradient resulted in the release of proteins in two major peaks (Figure 1). Active fractions were associated with the second major peak (Figure 1), which was eluted at 0.35 M NaCl. Analysis of SDS/PAGE revealed this peak to contain a homogeneous 20.6 kDa protein (Figure 2). This 20.6 kDa PPI species was found to be 1% of the total soluble component of washed microsomes, and a 14-fold purification was achieved.

N-terminal sequencing of this active 20.6 kDa protein showed that this protein had high sequence similarity to the deduced N-termini of the cyclophilin B family of PPI that have been identified by cDNA sequencing (Figure 3). These N-terminal sequences are distinctive. Analysis of cDNA sequences of the cyclophilin B family shows that they have an N-terminal signal sequence, which directs them to the ER, followed by a distinct N-

Table 1 Purification procedure for bovine liver microsomal PPI

Purification stage	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)
Solubilized microsomes	200	972	4.86	1.0
Mono Q void	99	673	6.80	1.4
Mono S	2.1	141	67.30	14.0

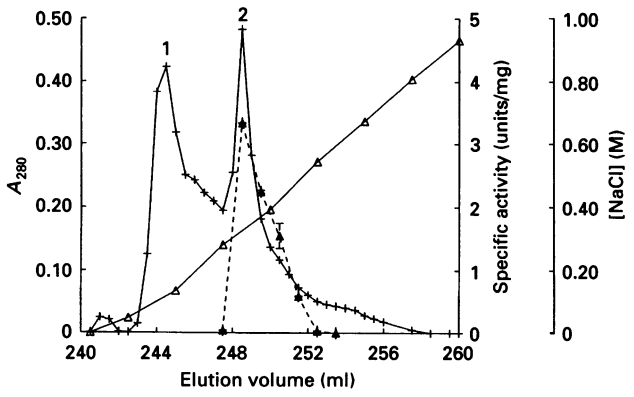

Figure 1 Elution profile of microsomal PPI from a Mono S column

Protein was eluted from the column using a linear salt gradient from 0 to 1 M (△) and detected by absorbance at 280 nm (+) (see the Materials and methods section for details). Fractions were of volume 0.5 ml. Fractions were assayed for PPI activity (▲).

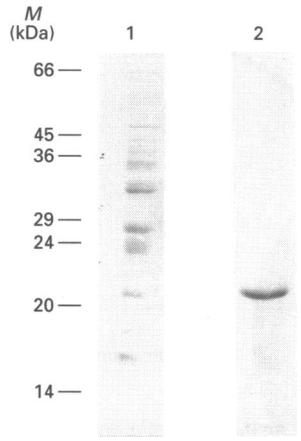

Figure 2 SDS/PAGE of purified microsomal PPI

The 15%-polyacrylamide gels were loaded with molecular-mass (*M*) standards, peak 1 fraction and peak 2 fraction (see Figure 1). Peak 2 consisted of a 20.6 kDa protein. Staining was with Coomassie Blue.

DEKKGGPKVTVKVYFDLRIG ER PPI (bovine liver)
DEKKKGPKVTVKVYFDLRIG CYPB H (cDNA)
NDKKKGPKVTVKVYFDLQIG CYPB M (cDNA)

Figure 3 N-terminal sequence comparisons of several PPIs

Data were taken from Hasel et al. (1991). The shaded area indicates regions of sequence identity. Abbreviations: CYPB H, human cyclophilin B; CYPB M, mouse cyclophilin B.

terminal extension, which is lysine-rich and is different from those of the cytosolic and mitochondrial cyclophilins. The cytosolic form (cyclophilin A) has no N- and C-terminal extensions, and the mitochondrial form (cyclophilin 3) has been shown to have a serine-rich N-terminal extension (Connern and Halestrap, 1992). In addition, the PPIs of the cyclophilin B

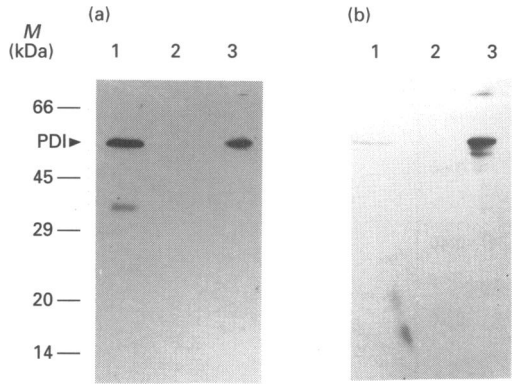

Figure 4 Western-blotting analysis

Duplicate blots of (1) washed microsomes, (2) pure microsomal PPI and (3) pure PDI were probed with (**a**) anti-KDEL and (**b**) anti-PDI antibodies. Positions of molecular-mass (*M*) standards are also shown.

family have been shown to have a C-terminal decapeptide extension which again is distinct from the other forms of PPI discussed (Arber et al., 1992). No direct evidence exists which confirms whether this family of PPIs are resident proteins of the ER. Further investigation using a monoclonal anti-KDEL antibody (Figure 4a) indicated that the isolated microsomal PPI does not cross-react with this antibody; however, PDI, a resident ER protein, does cross-react with the anti-KDEL antibody. This correlates with the published sequence data (Hasel et al., 1991; Price et al., 1991; Spik et al., 1991) on the cyclophilin B family, where no recognizable retention motif is present.

Arber et al. (1992) have shown that the expressed human s-cyclophilin (cyclophilin B) is not secreted out of the cell but co-localizes with the Ca^{2+}-binding protein calreticulin. But this does not reveal the mechanism by which the ER-associated PPI/cyclophilin remains in the cell. The α-subunit of prolyl-4-hydroxylase is required for the correct assembly of collagen within the lumen of the ER, yet it does not have any identifiable retention motif; the β-subunit of this enzyme is PDI (Pihlajaniemi et al., 1987), which does have the classic retention motif, KDEL. Perhaps ER-associated PPI is also bound to a resident ER protein. Further studies involving immunoprecipitation and immunofluoresence experiments may shed light on the matter.

Characterization of ER-specific PPI

Cytosolic PPI is a well-characterized protein. Its kinetics have been determined using the coupled assay based on peptide substrate for chymotrypsin, and it has also been shown to refold small proline-containing proteins. RNAase T1 has been used as a model system to demonstrate the latter function. In our study we examined the kinetics of the purified microsomal PPI and compared it with the well-characterized cytosolic PPI.

The purified protein was found to be active as a PPI when assayed with the synthetic peptide *N*-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide, using the well-characterized coupled assay with chymotrypsin (Fischer et al., 1984). The reaction obeyed first-order kinetics, and the rate constant of the catalysed reaction increased linearly with the ER PPI concentration (Figure 5). The $k_{cat.}/K_m$ ratio was determined to be 3.0×10^6 $M^{-1} \cdot s^{-1}$ from the gradient of this plot. This value compares well with those found by Schönbrunner et al. (1991), who compared the kinetics of cytosolic PPIs from different species and found that the catalytic

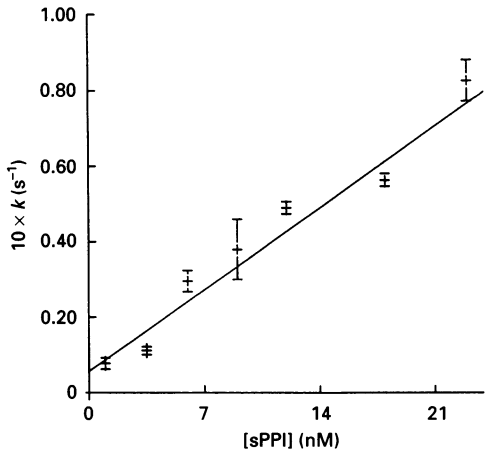

Figure 5 Prolyl isomerase activity of microsomal PPI

The rate constant of isomerization of the standard assay peptide *N*-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide is shown as a function of microsomal PPI concentration. Measurements were carried out in 0.035 M Hepes, pH 7.8, at 10 °C in the presence of 40 μM assay peptide and 7 μM chymotrypsin. Isomerization was monitored by the increase in absorbance at 390 nm. The slope of the line is equivalent to $k_{cat.}/K_m$.

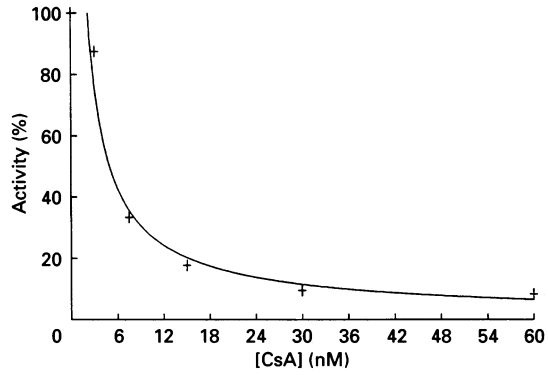

Figure 6 Inhibition by CsA of the prolyl isomerase activity of the microsomal PPI

The decrease in activity with increasing CsA concentration is expressed relative to the activity in the absence of the inhibitor; conditions were as described in the Materials and methods section.

Table 2 Substrate specificity of microsomal PPI compared with porcine cytosolic PPI (pCYP) and bovine FKBP (bFKBP)

Data for pCYP are from the work of R. Schönbrunner, F. X. Schmid and G. Fischer (F. X. Schmid, personal communication). Data for bFKBP are from Harrison and Stein, 1990. τ_0 refers to the initial time constant in the absence of PPI (−PPI) and τ refers to the time constant in the presence of PPI (+PPI). τ is the reciprocal of the first-order rate constant and will therefore have units of s.

Identity of Xaa in the substrate *N*-Suc-Ala-Xaa-Pro-Phe *p*-nitroanilide	−PPI (τ_0)	+PPI (τ)	τ_0/τ	τ_0/τ relative to Ala		
				ER PPI	pCYP	bFKBP
Ala	137	53	2.58	1.00	1.00	1.00
Leu	160	72	2.22	0.86	0.94	12.1
Glu	285	100	2.85	1.10	1.08	0.01

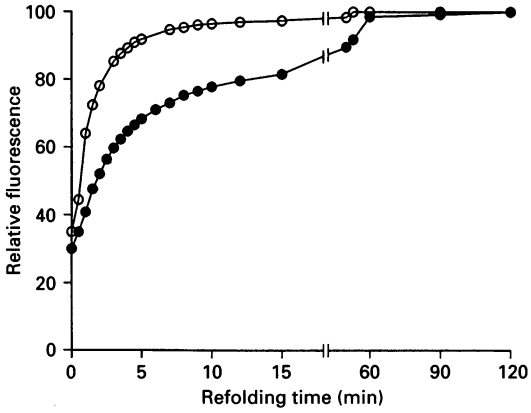

Figure 7 Catalysis of the slow refolding reactions of RNAase T1 by microsomal PPI

The increase in tryptophan fluorescence was monitored as a function of time. ●, Refolding of RNAase T1 in the absence of microsomal PPI; ○, refolding in the presence of 24 nM microsomal PPI. The conditions were as described in the Materials and methods section.

efficiencies of the various PPIs are high and the difference between species is small. Connern and Halestrap (1992) have reported that mitochondrial PPI is also kinetically similar to the cytosolic form. So it seems that all cyclophilins identified to date are kinetically similar, regardless of cellular localization and species.

CsA the immunosuppressive drug that binds only to the cyclophilin-type PPIs, was found to inhibit the activity of the purified protein (Figure 6); although data at low CsA concentrations are unreliable, owing to the hydrophobicity of CsA and its adsorption to glass, CsA is clearly powerfully inhibitory, and 50% inhibition occurred at 5 nM CsA concentration. This value corresponds well to that found for the cytosolic PPI (Schönbrunner et al., 1991). This supported the conclusion from sequencing that the purified PPI was a cyclophilin-type enzyme. To further verify this, the substrate specificity of the purified material was studied. Harrison and Stein (1990) have shown that cyclophilins do not discriminate among the amino acids preceding proline in substrate peptides, whereas the FKBPs are sensitive to this; from the amino acids studied, glutamate preceding proline was found to be most unfavourable by FKBPs, but not cyclophilins. In our study, we examined the specificity of three peptides (*N*-succinyl-Ala-Xaa-Pro-Phe *p*-nitroanilide where Xaa = Ala, Leu and Glu) and compared the kinetics of these with those of the cytosolic cyclophilin and FKBP. Table 2 clearly indicates that the purified material shows no discrimination among the peptides studied and confirms that it is a cyclophilin-type PPI.

To test the ability of ER PPI to catalyse slow protein-folding reactions, RNAase T1 was used as a model system. Its slow folding kinetics (Kiefhaber et al., 1990a,b,c) are determined by the isomerization of two proline residues, Pro-39 and Pro-55, both of which are in the *cis* conformation in the native protein (Heinemann and Saenger, 1982). The refolding of RNAase T1 in the presence or absence of ER PPI is shown in Figure 7. The catalytic property of this enzyme is clearly demonstrated. Both refolding steps are well catalysed by ER PPI. It resembles other

PPIs of the cyclophilin type in its efficiency as a catalyst of RNAase T1 folding. The intermediate phase of refolding is due to the isomerization of Ser-54–Pro-55, and the very slow phase is due to the isomerization of Tyr-38–Pro-39. The two refolding phases differ in rate and in catalysis by PPIs because they differ in the accessibility of the prolyl–peptide bond to the enzyme (Schönbrunner et al., 1991).

Conclusion

We have demonstrated the existence of a novel cyclophilin-type PPI that is associated with the luminal content of the ER. This PPI is abundant, highly active and is able to catalyse slow protein-folding reactions, indicating that it may play a role in protein-folding processes in this compartment. The specific activity with the standard peptide substrate, the substrate specificity, the K_i for CsA and the catalytic refolding of RNAase T1 all indicate a very clear identity in catalytic properties between this luminal PPI and the well-characterized cytosolic PPI/cyclophilin. This is consistent with the N-terminal sequence, which shows a high level of identity with those of the cyclophilin B family. The purification of an ER-specific PPI means that the role of this enzyme in the folding of nascent polypeptide chains can be examined.

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