Peptidyl prolyl *cis*–*trans*-isomerase activity associated with the lumen of the endoplasmic reticulum

Suchira BOSE* and Robert B. FREEDMAN

Research School of Biosciences, Biological Laboratory, University of Kent, Canterbury CT2 7NJ, Kent, U.K.

Peptidyl prolyl *cis*–*trans*-isomerase (PPI) activity was detected in microsomal fractions from bovine and rat liver. Extensive washing, proteinase and sonication treatments indicated that although some of this activity was due to adsorbed cytosolic enzymes, there was also an active but latent microsomal PPI activity. Density-gradient subfractionation indicated that activity

was associated with vesicles derived from both the rough and the smooth endoplasmic reticulum (ER), suggesting that the activity was located within the ER lumen. The luminal PPI activity was inhibited by cyclosporin A and was active towards an unfolded protein substrate as well as towards the standard peptide substrate.

INTRODUCTION

The *cis*–*trans* isomerization of prolyl–peptide bonds is a rate-determining step in the *in vitro* refolding of some proteins (Fischer et al., 1989). A 17 kDa cytosolic protein which accelerates this process has been detected in many sources and purified from porcine kidney (Fischer et al., 1984). It is possible that this enzyme acts during initial protein folding in the cell, either to introduce native *cis* prolyl–peptide bonds, or to reconvert into *trans* any non-native *cis* prolyl–peptides which accumulate at random in the period between translation and folding. Such a role would imply the classification of PPI (peptidyl prolyl *cis*–*trans*-isomerase; EC 5.2.1.8) with PDI (protein disulphide-isomerase; EC 5.3.4.1) as a catalyst of protein folding in the cell, related in function to the molecular chaperones.

However, this cellular role is not finally established. Sequencing of porcine kidney cytosolic PPI has revealed it to be identical with cyclophilin, originally identified as the major high-affinity binding protein for the immunosuppressant cyclosporin A (Takahashi et al., 1989). While cyclosporin A has now been found to inhibit the PPI activity of cyclophilin/PPI, this is not thought to be the route by which it acts (Bierer et al., 1990). It appears to act on the immune system by inhibiting the initial steps of T-lymphocyte activation (Schreiber, 1991). The cyclophilin–cyclosporin A complex binds and modulates the activity of the protein phosphatase calcineurin (Friedman and Weissman, 1991; Liu et al., 1991). This would imply that the physiological role of cyclophilin/PPI is as a component of a major cellular signalling pathway.

Thus the question of the role of PPI is unresolved. If its major function is in initial protein folding, it would be expected that PPI activity would be found not only in the cytosol, but in other compartments where such folding occurs. This would be consistent with the situation in bacteria, where two forms of PPI have been detected: one in the cytoplasmic and one in the periplasmic compartment (Liu and Walsh, 1990; Hayano et al., 1991). Evidence from mammalian cDNA sequences indicates the existence of homologues of cyclophilin/PPI with classical N-terminal secretory signal sequences and lacking any identifiable retention sequences (Hasel et al., 1991; Price et al., 1991; Spik et

al., 1991). Such sequences could encode secretory proteins or proteins targeted to any location in the secretory pathway.

The lumen of the ER is an organelle with a specialist function of protein folding and assembly. Nascent proteins enter this compartment co-translationally in an unfolded form, but their exit to distal compartments of the secretory pathway requires the protein to have completed native folding and assembly (Hurtley and Helenius, 1989; Freedman, 1992). The co- and post-translational events which secretory proteins undergo in the ER compartment are mediated by proteins of the ER membrane or resident proteins of the ER lumen (Bulleid and Freedman, 1988). The lumen contains enzymes (PDI) and molecular chaperones (IgG-heavy-chain-binding protein, BiP) which participate in this folding function (Gething and Sambrook, 1992), in addition to other enzymes which catalyse early post-translational covalent modifications (Rowling and Freedman, 1993). If the cellular function of PPI were to act as a catalyst of initial protein folding, it would be expected that PPI activity would also be found in the ER lumen; many secretory proteins, especially collagens, are very rich in proline residues.

The aim of the present study was to establish biochemically whether a PPI activity was located within the ER lumen of secretory cells. We present data from subcellular-fractionation and latency studies indicating the existence of such an activity in rat and bovine liver; an account of some of these studies has appeared in preliminary form (Bose and Freedman, 1992).

MATERIALS AND METHODS

Materials

All chemicals were obtained from Sigma Chemical Co. unless otherwise stated. All assays were performed in a Beckman DU 70 spectrophotometer unless otherwise stated.

Preparation of microsomes

Rat and bovine liver microsomes were prepared by differential centrifugation, a method based on that of Tangen et al. (1973) being used. A cocktail of proteinase inhibitors was added to the buffer used (5 mM EDTA, 1 mM PMSF and 1 µg/ml aprotinin).

Abbreviations used: PPI, peptidyl prolyl *cis*–*trans*-isomerase; ER, endoplasmic reticulum; PDI, protein disulphide-isomerase; Suc, succinyl; DTT, dithiothreitol; sRNAase, scrambled RNAase.

* To whom correspondence should be addressed.

The procedure of Lambert and Freedman (1985) was used for washing and proteinase treatments of microsomes.

Subfractionation of microsomes

Microsomes were subfractionated into smooth and rough vesicles by sucrose-gradient centrifugation, using the method of Hawkins (1976). Approx. 10 ml of microsomal suspension was gently layered over 6 ml of 1.35 M sucrose/TKM (50 mM Tris/25 mM KCl/5 mM MgCl₂, pH 7.5), which in turn was layered over 4 ml of 2.0 M sucrose/TKM in a polystyrene tube and these were centrifuged in a swing-out rotor at 105000 *g* for 3.5 h at 4 °C. After this time, two bands were apparent, that of rough vesicles at the lower interface and smooth vesicles at the upper interface. These bands were harvested with a pasteur pipette and hand-homogenized in TKM buffer. These were centrifuged in a fixed-angle rotor in order to remove the excess sucrose; samples were stored at -20 °C for further analysis.

Sonication

Subcellular fractions were sonicated at maximum power for 1 min in a Jencons Soniclean sonicator which was continuously cooled with ice.

Protein determination

The Bradford method, as altered by Stoscheck (1990), was used to evaluate protein concentrations, BSA standards were used.

PPI activity

PPI activity was measured in a coupled assay with chymotrypsin, employing the synthetic peptide *N*-Suc-Ala-Ala-Pro-Phe *p*-nitroanilide as described by Fischer et al. (1984). The assay relies on the fact that the *p*-nitroanilide can only be cleaved by chymotrypsin when the preceding Ala-Pro bond is in the *trans* configuration. About 10 % of the peptide has a *cis* Ala-Pro bond at equilibrium. In the presence of high chymotrypsin concentrations, hydrolysis of the *trans* peptide occurs within seconds, but cleavage of the *cis* peptide is rate-limited by the *cis-trans* isomerization of the Ala-Pro bond. This reaction is catalysed by PPI. Reactions were carried out at pH 7.8 in buffered solutions containing 35 mM Hepes/5 mM β -mercaptoethanol. Chymotrypsin at 7 μ M and peptide at 40 μ M were used. The temperature was kept constant at 10 °C throughout the assay. Assays were carried out in duplicate or triplicate, and the data collected were fitted to a first-order rate equation using the Enzfitter package. Units of PPI activity are expressed in s⁻¹ and specific activity is expressed as units/mg of protein.

Refolding of RNAase T1

PPI activity was also monitored by its ability to increase the rate of refolding of RNAase T1. The method used was essentially that of Kiefhaber et al. (1990). Measurements were carried out on a Perkin-Elmer LS-5B spectrofluorimeter. Unfolded RNAase T1 was produced by a 2 h incubation of 100 μ M RNAase T1 in 8.0 M urea/1 M Tris/HCl, pH 8.0, at 10 °C. Refolding was initiated by a 40-fold dilution into 0.1 M Tris/HCl, pH 8.0.

Refolding conditions were 2.5 μ M RNAase T1 in 0.2 M urea/0.1 M Tris/HCl, pH 8.0. The temperature in the cell was maintained at 10 °C. Refolding was monitored by the increase in fluorescence at 320 nm after excitation at 268 nm.

Inhibition by cyclosporin A

The dependence of residual sonicated microsomal PPI activity on cyclosporin A concentrations was measured by incubating 100 μ l of sonicated washed microsomes with various concentrations of cyclosporin A (range 0–60 nM final concn.) for 10 min at 10 °C. The activity of PPI was determined by measuring the catalysis of the *cis-trans* interconversion of *N*-Suc-Ala-Ala-Pro-Phe *p*-nitroanilide, as described previously. PPI activity in the absence of cyclosporin A was taken as 100 %. The experiment was repeated using 100 μ l of sonicated trypsin-treated washed microsomes.

PDI activity

PDI activity was measured by the method of Hawkins et al. (1991). 'Scrambled ribonuclease' (sRNAase) is fully re-oxidized, but its disulphide bonds are non-native and hence it is inactive. PDI activity is measured by its ability to re-activate sRNAase by catalysing the interchange of the disulphide bonds to native bonds. Essentially, 160 μ l of sample was preincubated at 30 °C with 20 μ l dithiothreitol (DTT) for 2 min. Then 20 μ l of sRNAase was added to give a final volume of 200 μ l. From this PDI incubation, 10 μ l samples were taken at five time points between 0.5 and 12 min and added to a cuvette containing 250 μ g of highly polymerized RNA in 3.0 ml of TKM buffer. RNAase activity was assayed by the increase in A_{260} on a Perkin-Elmer 356 spectrophotometer in dual-wavelength mode with a slit width of 2.5 nm, recording A_{260} relative to A_{280} .

NADPH:cytochrome *c* reductase activity

NADPH:cytochrome *c* reductase activity was assayed spectrophotometrically by monitoring the reduction of cytochrome *c* at 550 nm using the method of Philips and Langdon (1962).

NDPase activity

The hydrolysis of UDP to UMP and P_i by NDPase was detected using the stopped assay method of Ishibashi et al. (1978).

Lactate dehydrogenase (LDH) activity

LDH activity was measured by the method of Bergmeyer and Bernt (1974), where the reduction of pyruvate to lactate by NADH is monitored spectrophotometrically at 340 nm.

RESULTS

Subcellular fractionation

The classical PPI is an abundant cytosolic protein, so that any attempt to detect a specific PPI in the secretory pathway requires controls to exclude the participation of cytosolic activity. Initial subcellular fractionation experiments on bovine liver showed that most PPI activity was found in the soluble (cytosolic) fraction, but significant activity was also noted in the microsomal fraction (Figure 1). Comparison with the distribution of the marker cytosolic enzyme, LDH (results not shown) indicated that the bulk of this PPI activity in the microsomal fraction could be accounted for by 'artefactual' adsorption or entrapment of

Figure 1 Subcellular distribution of PPI activity

Subcellular fractions were obtained as described in the Materials and methods section, using 50 g of bovine liver. PPI activity was assayed in all subcellular fractions (H, homogenate; NP, nuclear pellet; MP, mitochondrial pellet; C, cytosol; CM, crude microsomes).

Figure 2 Enhancement of microsomal PPI activity following sonication

Bovine liver was subfractionated as described in the Materials and methods section. PPI activity was assayed in subcellular fractions before and after sonication in order to measure latency of PPI activity [crude-microsome (CM) latency, 31%]. For other abbreviations, see Figure 1.

cytosolic protein. However, sonication to release the luminal content from microsomal vesicles enhanced PPI activity considerably (Figure 2), implying that these preparations contain a latent activity, presumably located within the interior of the vesicles; time-course experiments revealed that 1 min of sonication was sufficient time to release maximal activity. No other subcellular fraction had a significant latent PPI activity. Experiments with rat liver microsomes showed comparable results.

Washing treatments

In order to minimize contamination of the microsomal fraction with cytosolic PPI, crude liver microsomes were subjected to a series of washing treatments; gentle treatments (150 mM Tris, pH 8.0; 50 mM Tris + 500 mM KCl, pH 7.5; 50 mM Tris + 500 mM KCl + 1 mM puromycin, pH 7.5; 250 mM sucrose + 50 mM Tris + 5 mM EDTA, pH 7.5) were chosen to ensure retention of microsomal vesicle integrity. These treatments removed from 17.1% to 38% of microsomal protein and up to 42% of the PPI activity detectable in unsonicated crude microsomal preparations. Washed microsomes were sonicated for

Figure 3 Latency of PPI activity in crude (CM) and washed (WM) microsomal preparations

Washing and sonication conditions were as described in the text (CM latency, 31%; WM latency, 50%).

various lengths of time in order to find the optimum conditions required for disrupting microsomal vesicles and releasing active soluble components; 50 mM Tris/500 mM KCl, pH 7.5, was used in all subsequent washing experiments as these gave maximum release of cytosolic contaminants, with a sonication time of 1 min, as this was the optimum condition for disrupting microsomal vesicles. Sonication of washed preparations again enhanced the PPI activity, and the overall latency of PPI activity [defined as (activity after sonication – activity before sonication)/activity after sonication] was markedly greater than in the crude preparations (50% latent activity associated with the washed microsomes as compared with 31% associated with the crude microsomes; see Figure 3), indicating that the wash treatments had selectively removed external adsorbed PPI.

Figure 4 illustrates the distribution of marker enzymes during subcellular fractionation. PDI is a well-characterized ER luminal enzyme. Here PDI is shown to have high latency in both the crude and the washed microsomal preparations, indicating that the microsomes were intact. NDPase, a second ER luminal marker, also shows a high latency in the washed microsomes. NADPH:cytochrome *c* reductase activity was the marker for the outer surface of the ER; it appears almost exclusively in the microsomal fraction and is not affected significantly by washing or sonication, as expected of an integral membrane protein. The cytosolic marker, LDH, mainly distributes with the cytosolic fraction, although some activity was noted in other fractions, owing to adsorption.

Proteinase treatments

To provide a more stringent test for the presence of PPI located within the luminal compartment of microsomal vesicles, washed microsomes were treated with various amounts of proteinases (trypsin/proteinase K) for 10 or 20 min, and the proteinases were subsequently inactivated with appropriate inhibitors (Table 1). PPI activity in the proteinase-treated vesicles was assayed before and after sonication. In some cases all directly detectable PPI had been degraded, but a latent activity inaccessible to proteinase could be detected (trypsin, 100 µg/mg for 10 min or 50 µg/ml for 20 min; proteinase K, 25 or 50 µg/mg; 10 or 20 min). Figure 5 illustrates the results using 50 µg/mg trypsin for 20 min; it shows that very little PPI activity was detectable in proteinase-treated washed microsomes in the absence of sonication, indicating that all contaminating adsorbed cytosolic PPI had been removed or inactivated. However, sonication of the preparation released significant PPI activity (2.5 units/mg). Prolonged proteinase treatments were necessary to ensure that all cytosolic PPI

Figure 4 Activities of marker enzymes during subfractionation

Bovine liver homogenate was subfractionated as described in the Materials and methods section, and crude microsomes were washed as in Figure 3. Marker enzymes (a, PDI; b, NDPase; c, LDH; d, NADPH:cytochrome *c* reductase) were assayed in all fractions, before and after sonication. Abbreviations are as in Figures 1 and 3.

Table 1 Treatment of microsomes with proteinases

Microsomes were incubated with various amounts of proteases (trypsin/proteinase K) for 10 or 20 min and subsequently inactivated with appropriate inhibitors. PPI activity was measured before and after sonication (+, activity detected; −, activity not detected). In some cases a latent activity inaccessible to proteinase could be detected.

Incubation time (min)	Proteinase	Sonication	Amount of proteinase (μ g/mg of protein)		
			25	50	100
10	Trypsin	No	+	+	−
		Yes	+	+	+
	Proteinase K	No	−	−	?
		Yes	+	+	?
20	Trypsin	No	+	−	−
		Yes	+	+	−
	Proteinase K	No	−	−	?
		Yes	+	+	?

Figure 5 Latency of PPI activity in washed or proteinase-treated microsomes

Washed microsomes were treated with trypsin as described in the text. Various treated microsome preparations were sonicated as above (crude-microsome latency, 31%; washed-microsome latency, 49%; proteinase-treated-microsome latency, 95%).

adsorbed to the microsomal membrane had been degraded and this would have damaged some of the microsomal membranes and resulted in some proteolysis of luminal content. This explains

the lower latent specific activity after proteinase treatment as compared with that observed in the crude and washed preparations.

Figure 6 PPI activity located in the ER subfractions

PPI activity was assayed in smooth-ER (sER) and rough-ER (rER) fractions before and after sonication. Smooth and rough vesicles were obtained by subfractionation of bovine liver microsomes, as described in the Materials and methods section [washed-microsome (WM) latency, 50%; sER latency, 60%; rER latency, 66%].

Figure 7 Properties of luminal PPI activity: (a) catalysis of refolding of RNAase T1 by luminal content and (b) inhibition by cyclosporin A of luminal PPI activity

(a) Refolding of RNAase T1 was followed by monitoring the increase in tryptophan fluorescence in the absence (+) and presence (Δ, ○) of washed microsomes, native (Δ) and sonicated (○). The fluorescence at 120 min was taken as 100% relative fluorescence. The data were fitted to a first-order rate equation. (b) The coupled assay with chymotrypsin was used to monitor the effect of cyclosporin A on PPI activity.

Sucrose-gradient subfractionation

Washed microsomes were subfractionated over a discontinuous sucrose gradient to obtain rough vesicles and smooth vesicles.

Each fraction was found to have latent PPI activity, higher activity being located within the rough vesicles (18.75 units/mg specific activity present in the rough vesicles as compared with 12.5 units/mg specific activity in the smooth vesicles; see Figure 6).

Refolding of RNAase T1

Native washed microsomes were able to catalyse *cis-trans* isomerization of prolyl-peptide bonds due to the presence of adsorbed cytosolic enzymes. The luminal component of microsomes, released after sonication of washed microsomes, catalysed peptidyl-prolyl isomerization-dependent folding of RNAase T1 (Figure 7a) more effectively than native microsomes. Thus this luminal activity is able to catalyse prolyl isomerization of an unfolded protein in addition to the standard PPI activity employing the synthetic peptide.

Effect of cyclosporin A

Cyclosporin A is a potent inhibitor of the major cytosolic PPI (cyclophilin). At present it is unknown whether this inhibition of PPI activity is on the pathway mediating immunosuppressive action of cyclosporin A. Figure 7(b) illustrates that the luminal activity, detected after sonication of washed or washed/proteinase-treated microsomes, is also inhibited by cyclosporin A, and activity was found to decrease with increasing concentration of cyclosporin A. In these crude preparations, 50% inhibition was found at 10 nM cyclosporin A, whereas for purified cytosolic PPI, IC_{50} is at 4 nM cyclosporin A (Schönbrunner et al., 1991).

DISCUSSION

The physiological role of the PPI/cyclophilins is presently unclear. Its ability to catalyse the prolyl-peptide *cis-trans* isomerization steps in the folding of small proteins has led to the proposal that it may promote protein folding (Fischer and Schmid, 1990). Our study has been concerned with detecting PPI activity within the secretory pathway, where it may be involved in the folding of newly synthesized secretory proteins.

A number of cDNA clones have been isolated from human, mouse, chick etc. which show significant (~60%) sequence identity with the vertebrate cytosolic cyclophilin/PPI (Caroni et al., 1991; Hasel et al., 1991; Price et al., 1991; Spik et al., 1991), but also have a distinct N-terminal signal sequence which suggests that they are directed to the secretory pathway. They have been termed S-cyclophilins or cyclophilin B. These sequences lack any identifiable retention motifs and hence could encode secretory proteins or proteins targeted to undefined compartments of the secretory pathway. When the cloned genes are expressed, the products are found partly secreted and partly within the secretory pathway (Caroni et al., 1991; Spik et al., 1991). More specific localization by immunocytochemistry (Arber et al., 1992) or Western blotting of subcellular fractions (Hasel et al., 1991) suggested a location within the ER or within an ER subdomain specialized for Ca^{2+} storage.

However, none of these reports indicated that these gene products show PPI enzymic activity in vertebrate cells or suggested any role for the gene products in protein folding. Nevertheless, two reports from studies in intact cells suggest that there is a cyclosporin-sensitive step in the folding of secreted proteins. Steinmann et al. (1991) monitored the synthesis, folding and assembly of procollagen in chick embryo tendon fibroblasts and demonstrated a delay in the incorporation of procollagen

chains into folded triple helices. Subsequently, Lodish and Kong (1991) showed that cyclosporin delayed the exit of newly synthesized transferrin from the ER of Hep G2 cells. In the case of procollagen, *cis-trans*-isomerization of prolyl-peptide bonds is established as being rate-determining for the folding of trimers into the triple-helical conformation. The two findings together imply, but do not directly demonstrate, the presence of a cyclosporin-sensitive PPI active towards newly synthesized secretory proteins within the lumen of the ER.

A more direct demonstration of the importance of the cyclophilin/PPI family in events associated with protein exit from the ER is provided by studies on the *Drosophila ninaA* gene product (Stamnes and Zuker, 1990). *NinaA* was first defined functionally; mutations in this gene led to reduction in the level of expression of Rh1 rhodopsin in *Drosophila* R1-6 photoreceptor cells; the gene was cloned and shown to be a homologue of cyclophilin/PPI with a secretory signal sequence and a C-terminal hydrophobic sequence (Schneuwly et al., 1989; Shieh et al., 1989). Subsequent work has shown that the *ninaA* gene product is an integral membrane protein in the *Drosophila* retina (Stamnes et al., 1991) and that it functions in the ER, in that mutations in *ninaA* inhibit transport of newly synthesized opsin from the ER (Colley et al., 1991).

The data on the sequences, expression and subcellular locations of the S-cyclophilin/cyclophilin B gene products suggest that these homologues of the cytosolic PPI/cyclophilin are expressed in the secretory pathway, but do not indicate the nature of their activity. The studies of cyclosporin effects on protein secretion imply that a cyclosporin-sensitive folding step occurs within the ER, but do not identify the target of cyclosporin action. The *ninaA* data suggest that PPI/cyclophilin homologues are active in protein folding in the ER, but this gene is expressed only in specialized retinal cells.

We have sought to test directly whether a PPI activity is present within the secretory compartment of vertebrate cells, and to localize this activity. Our data show that there is PPI activity associated with the microsomal fraction of mammalian liver and that a latent activity is released after sonication of extensively washed and proteinase-treated microsomes, indicating the presence of a luminal PPI distinct from adsorbed cytosolic PPI. Furthermore, subfractionation of washed microsomes by sucrose-density centrifugation to form smooth- and rough-vesicle fractions indicated that the latent activity associated with the rough vesicles was greater than that of the smooth vesicles. Since the smooth-vesicle fraction will contain fragments of various membranes in addition to those from the smooth ER, while the rough vesicles will be predominantly derived from the rough ER, the evidence strongly indicates that the latent microsomal PPI is located within the ER lumen.

This result is consistent with the earlier immunochemical data on the location of the S-cyclophilin/cyclophilin B gene products. The finding that the latent microsomal PPI activity is sensitive to inhibition by cyclosporin A and is active towards protein substrates suggests that it is this activity which is responsible for catalysing the folding events in procollagen and transferrin biosynthesis which earlier data have shown to be cyclosporin-sensitive.

In the following paper (Bose et al., 1994) we describe the

purification of the ER luminal PPI, its N-terminal sequence and its kinetic properties; this work confirms that the latent microsomal PPI enzyme which we have described in the present paper is identical with the product of the S-cyclophilin/cyclophilin B gene.

S.B. was supported by a studentship from the Science and Engineering Research Council (U.K.).

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