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Purification and *N*-terminal sequencing of peptidyl-prolyl *cis–trans*-isomerase from rat liver mitochondrial matrix reveals the existence of a distinct mitochondrial cyclophilin

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1. Rat liver mitochondrial matrix peptidyl-prolyl *cis–trans*-isomerase (PPIase) has been purified. The major form of the enzyme has a molecular mass of 18.6 kDa, with a minor active component of 17.6 kDa. 2. The second-order rate constant for cyclosporin A binding to the enzyme was determined from the time-dependence of the inhibition of PPIase by low concentrations of cyclosporin A and found to be 0.9 $\mu M^{-1} \cdot s^{-1}$ at 10 °C. 3. The K_i for cyclosporin A inhibition of the enzyme was 3.6 nM, and the half-life for dissociation of the enzyme–inhibitor complex was 3.6 min. 4. From the specific activity of the pure enzyme it can be calculated that isolated liver mitochondria contain approx. 45 pmol of enzyme per mg of total mitochondrial protein. Higher values estimated previously [Halestrap & Davidson (1990) Biochem. J. **268**, 153–160] are explained by the use of a short (30 s) preincubation period of the enzyme with cyclosporin, which is insufficient to allow full equilibration of the binding of the inhibitor to the PPIase. 5. *N*-Terminal sequencing of the 18.6 and 17.5 kDa forms of PPIase show the presence of mitochondrial presequences of 13 and three amino acids respectively, with the remaining sequence having a strong sequence similarity to other cyclophilins. 6. Parallel purification and *N*-terminal sequencing of rat cytosolic PPIase showed the two proteins to have significant differences, implying that they are probably products of separate genes.

INTRODUCTION

The immunosuppressive drug cyclosporin A is known to exert its inhibitory effects on the activation of T-lymphocytes through binding to cyclophilin, a 17 kDa protein which is identical with peptidylprolyl *cis–trans*-isomerase (Fischer *et al.*, 1989; Ryffel, 1989; Takahashi *et al.*, 1989; Schreiber, 1991; DeFranco, 1991). Although inhibition of the isomerase activity accompanies cyclosporin binding, it is now thought unlikely that this inhibition is responsible for the immunosuppressive effect (DeFranco, 1991; Schreiber, 1991). It is known that cyclosporin inhibits some part of the Ca^{2+}-dependent signal-transduction pathway (Kay *et al.*, 1989), and recent data suggests that the cyclosporin–cyclophilin complex inhibits a Ca^{2+}-activated protein phosphatase, calcineurin. Regulation of this phosphatase may be involved in the translocation into the nucleus of the cytosolic component of a factor involved in activating T-lymphocyte interleukin-2 gene transcription (Flanagan *et al.*, 1991; Friedman & Weissman, 1991; Liu *et al.*, 1991).

Several closely related forms of cyclophilin have been characterized in mammalian cells, including secreted forms and those whose sequence implies an endoplasmic-reticulum location (Caroni *et al.*, 1991; Friedman & Weissman, 1991; Price *et al.*, 1991; Spik *et al.*, 1991). Related proteins have also been described in bacteria (Liu & Walsh, 1990; Hayano *et al.*, 1991; Schönbrunner *et al.*, 1991), plants (Gasser *et al.*, 1990) and fungi (Tropschug *et al.*, 1988, 1989; Haendler, 1989). In *Neurospora crassa* a mitochondrial form has been characterized and sequenced (Tropschug *et al.*, 1988). Work in this laboratory has demonstrated the presence of a peptidylprolyl *cis–trans*-isomerase (PPIase) activity in rat heart and liver mitochondria that is inhibited by cyclosporin A with a K_i similar to that for inhibition of the cytosolic enzyme (Halestrap & Davidson, 1990; Griffiths & Halestrap, 1991). This mitochondrial PPIase has been implicated in the damage that occurs to mitochondria that are overloaded with Ca^{2+} (Halestrap & Davidson, 1990; McGuinness *et al.*, 1990). Such mitochondria become permeable to low-molecular-mass molecules, a process that is inhibited by cyclosporin analogues with a similar activity profile to their inhibition of PPIase (Crompton *et al.*, 1988; Broekemeier *et al.*, 1989; Halestrap & Davidson, 1990; Griffiths & Halestrap, 1991). We and others have proposed that the increase in permeability occurs as the result of the mitochondrial PPIase interacting with a proline on a mitochondrial membrane protein that also binds Ca^{2+} (Halestrap & Davidson, 1990; McGuinness *et al.*, 1990; Griffiths & Halestrap, 1991). We have suggested that this protein is the adenine nucleotide translocase, with which PPIase may interact to cause a conformational change sufficient to form a pore in the membrane (Halestrap & Davidson, 1990; Griffiths & Halestrap, 1991). In order to test this hypothesis, we have now purified the PPIase from the matrix of rat liver mitochondria.

In the present paper we show that the mitochondrial matrix PPIase has a molecular mass of 18.6 kDa, with a minor component of 17.6 kDa. *N*-Terminal sequencing shows the presence of mitochondrial presequences of 13 and three amino acids respectively, with the remaining sequence having a strong sequence similarity to other cyclophilins. Parallel purification and *N*-terminal sequencing of rat cytosolic PPIase showed that the two proteins have significant differences, implying that they are probably products of separate genes.

EXPERIMENTAL

Materials

Antipain, leupeptin and pepstatin A were obtained from Cambridge Research Biochemicals, Northwich, Cheshire, U.K., and phenylmethanesulphonyl fluoride (PMSF) was from Sigma Chemical Co., Poole, Dorset, U.K. The sources of all the other

Abbreviations used: DTT, dithiothreitol; PPIase, peptidyl-prolyl *cis–trans*-isomerase; PMSF, phenylmethanesulphonyl fluoride.
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Table 1. Purification procedure for rat liver mitochondrial PPIase

Purification stage	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Recovery (%)
Matrix	863.00	156.7	0.18	1.0	100.0
$(NH_4)_2SO_4$	357.00	91.4	0.26	1.4	58.3
Q-+S-Sepharose	18.20	59.0	3.24	18.0	37.0
Mono S	2.34	23.1	9.87	54.4	14.7
Superose 12	0.17	18.8	110.58	614.0	11.9

chemicals and biochemicals were as given by Halestrap & Davidson (1990).

Methods

Preparation of mitochondrial matrix and cytosolic fractions. Matrix fractions were prepared from rat liver mitochondria by digitonin treatment and sonication as described previously (Davidson & Halestrap, 1989). PMSF, antipain, leupeptin and pepstatin were added at 1 μg/ml, and the matrix fraction was stored at −70 °C until required. The postmitochondrial supernatant was centrifuged at 100000 *g* for 30 min, and the resulting supernatant used as the starting material for the purification of the cytosolic PPIase.

Protein characterization. Protein was determined by the method of Bradford (1976), with BSA as the standard. The purity of PPIase samples was analysed by SDS/15%-(w/v)-PAGE (Laemmli, 1970). Where necessary, gels were stained for protein with either silver or Coomassie Blue G. PPIase was assayed by the method of Fischer *et al.* (1989), as described by Halestrap & Davidson (1990).

Purification of mitochondrial matrix PPIase. The purification procedure used was a substantial modification of that described by Fischer *et al.* (1984) and is summarized in Table 1. The 40–60%-satd. $(NH_4)_2SO_4$ cut (P_3) was dialysed against 10 mM-Tris, pH 8.2, containing 2 mM-EDTA, 0.5 mM-dithiothreitol (DTT) and applied to a Q-Sepharose column (2.5 cm × 22 cm). PPIase activity was detected in the unbound protein peak, which was transferred directly on to an S-Sepharose column (1.6 cm × 4.5 cm) equilibrated with 10 mM-Tris, pH 8.2, containing 2 mM-EDTA and 0.5 mM-DTT. The bound protein was eluted with a linear 0–200 mM-NaCl gradient. The active PPIase was eluted between 80 and 100 mM-NaCl, and this fraction was dialysed against 50 mM-Mes, pH 6.0, containing 2 mM-EDTA and 0.5 mM-DTT, before applying it to an HR 5/5 Mono S column. Bound protein was eluted with a linear 0–100 mM-NaCl gradient. The active PPIase fraction was concentrated by using an Amicon Centricon 10 (molecular-mass cut-off 10 kDa) and applied to a Superose 12 HR 10/30 column equilibrated with 10 mM-Tris, pH 8.2, containing 2 mM-EDTA and 0.5 mM-DTT. The profile of PPIase activity in eluted fractions is shown in Fig. 1, together with an analysis of their composition determined by silver staining of the proteins separated by SDS/15%-PAGE. Two peaks of activity were eluted: a larger peak containing a major protein of molecular mass 18.6 kDa and a smaller peak containing both this protein and a component of molecular mass 17.5 kDa.

Purification of cytosolic PPIase. Cytosolic PPIase was purified by using exactly the same protocol as described for matrix PPIase. A single protein of molecular mass 17 kDa was detected when the material was analysed by SDS/15%-PAGE, as shown in Fig. 2.

Preparation of PPIase for *N*-terminal sequencing. Purified PPIase was separated by SDS/15%-PAGE and electroblotted on to Problott membranes (Applied Biosystems) according to the method of Towbin *et al.* (1979). The membrane was washed for 1 min with distilled water and stained with Problott staining

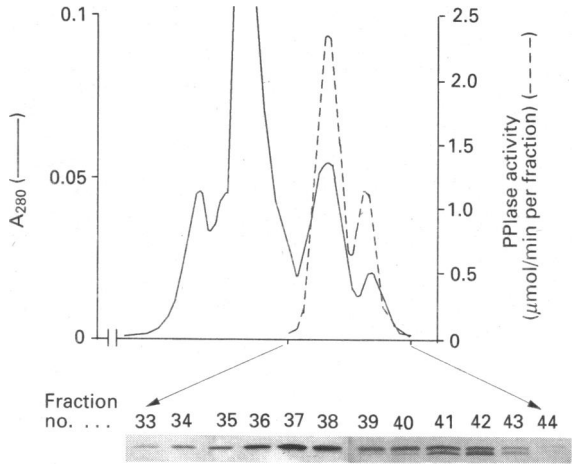

Fig. 1. Activity profile and polypeptide composition of mitochondrial matrix PPIase eluted from a Superose 12 column

Experimental details are given in the Experimental section. Fraction volumes were 0.5 ml (0–30) or 0.25 ml (31–45), and samples were both assayed for PPIase activity and subjected to SDS/PAGE, followed by silver staining to reveal the protein bands. Only the relevant area of the gel containing the two isoforms of PPIase is shown, there being no other proteins present in these fractions, as shown in Fig. 2.

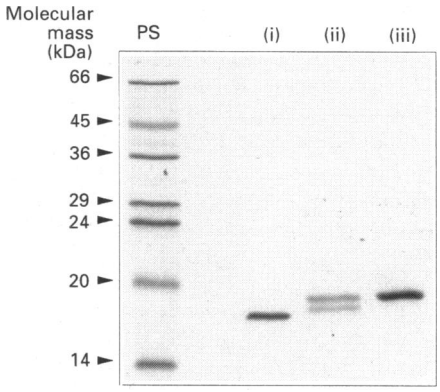

Fig. 2. SDS/PAGE of purified cytosolic and mitochondrial isoforms of PPIase

Tracks of a 15%-polyacrylamide gel were loaded with 925 ng of cytosolic PPIase (i), 1270 ng of mitochondrial PPIase containing both 18.6 and 17.5 kDa isoforms (ii) and 810 ng of the 18.6 kDa mitochondrial PPIase (iii). Protein standards (PS) were also run as indicated. Staining was with Coomassie Blue.

solution [45% (v/v) methanol/5% (v/v) acetic acid/0.1% (w/v) Coomassie Blue R250] for 30 s before destaining with Problott destaining solution (45% methanol/5% acetic acid). The membrane was washed with distilled water and allowed to dry overnight. PPIase was excised from the membrane, and sequencing was carried out using an Applied Biosystems 477A pulsed liquid–protein sequencer fitted with a Blott Cartridge. Approx. 80 pmol of protein was usually used for sequencing. In the case of the cytosolic enzyme, recoveries of the amino acid phenylthiohydantoin derivatives over the first few cycles were about 30% of the loaded protein. This is within the range normally recovered. However, when sequencing the mitochondrial enzyme, the yield of amino acids was only about 10% of the loaded protein, suggesting that a substantial portion of the enzyme might be *N*-terminally blocked.

Expression of enzyme activity. The activity of PPIase was expressed in units of μmol of peptide converted/min calculated from the difference in ϵ_{390} (13400 litre·mol^{-1}·cm^{-1}) between the substrate and the released *p*-nitroaniline (Harrison & Stein, 1990). Since it is not possible to saturate the enzyme with its artificial substrate under the conditions of the assay (Harrison & Stein, 1990; Kofron *et al.*, 1991), the specific activities quoted here and in other papers are critically dependent on the assay conditions used.

RESULTS AND DISCUSSION

Purification and characterization of mitochondrial matrix PPIase

Table 1 summarizes the procedure used for the purification of the matrix PPIase. The method used was developed from that of Fischer *et al.* (1989) and utilizes the high isoelectric points and low molecular masses of known cyclophilins (Handschumacher *et al.*, 1984; Harding *et al.*, 1986). Thus the protein passes straight through the Q-Sepharose column (anion-exchanger) at pH 8.2, but binds to the cation-exchanger S-Sepharose at this pH. Elution from S-Sepharose with a salt gradient and subsequent re-chromatography on a Mono S column gave a fraction contaminated with higher-molecular-mass material that could be separated using a sizing column (Superose 12). The two peaks of activity that were eluted contained a major component of molecular mass 18.6 kDa and a minor component of molecular mass 17.5 kDa. A fraction containing only the 18.6 kDa protein had a PPIase specific activity of 110.6 units/mg of protein, which was the same as that of a fraction containing approximately equal amounts of both proteins. Both fractions were also totally inhibited by 1 μM-cyclosporin A (results not shown). Thus it would seem that both proteins must be PPIases with very similar properties. We were unable to obtain a fraction that contained exclusively the 17.5 kDa protein, except by electroblotting after SDS/PAGE. Parallel purification of the cytosolic enzyme gave a single protein of molecular mass 17 kDa and a specific activity of 115.8 units/mg of protein.

From the specific activity and molecular mass of the pure mitochondrial PPIase and the enzyme activity and specific activity of a crude matrix fraction, it can be calculated that the concentration of the enzyme in the matrix is about 90 pmol per mg of protein. This compares with a value of 60 pmol per mg of total mitochondrial protein estimated by analysis of high-affinity binding of [^{3}H]cyclosporin to liver mitochondria (McGuinness *et al.*, 1990). The two values are in quite a good agreement, especially when it is recognized that only about 50% of total mitochondrial protein is matrix-derived (E. J. Griffiths, unpublished work). By contrast, the value of 300–400 pmol/mg of protein estimated from cyclosporin-inhibition studies of the enzyme activity in the crude matrix fraction (Halestrap & Davidson, 1990; Griffiths & Halestrap, 1991) appears to be a substantial overestimate. Such a result would be predicted if there were incomplete equilibration of cyclosporin with PPIase in the 30 s preincubation at 10 °C used for the kinetic experiments. The binding studies by McGuinness *et al.* (1990) used 8 min at 25 °C to obtain complete equilibration. The data shown in Fig. 3 confirm that inhibition of the pure matrix enzyme by low concentrations of cyclosporin is time-dependent, and at 30 s the inhibition observed will be less than 30% the equilibrium value. The time course of inhibition shown in Fig. 3 allows calculation of the second-order rate constant and the K_i for binding of cyclosporin A. The values obtained (±95% confidence limits) were 0.90±0.12 μM^{-1}·s^{-1} and 3.10±0.70 nM respectively. In a separate experiment (not shown) where inhibition by increasing cyclosporin concentrations was measured after equilibration for 6 min, a K_i value of 3.64±0.56 nM was obtained by using the procedure described previously (Halestrap & Davidson, 1990; Griffiths & Halestrap, 1991). From the measured K_i value and second-order rate constant for inhibitor binding to PPIase, the half-life for the dissociation of the inhibitor–enzyme complex was calculated to be 3.56 min. Data obtained with the pure cytosolic enzyme gave similar results (not shown) to those obtained for the mitochondrial enzyme.

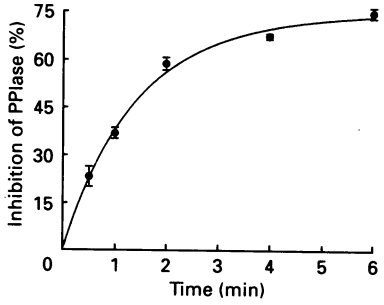

Fig. 3. Time course of the inhibition of mitochondrial PPIase by cyclosporin A

The 18.6 kDa isoform of mitochondrial PPIase (2 nM) was incubated at 10 °C with 10 nM-cyclosporin A for the time shown before initiation of the assay by addition of the substrate peptide. Each point is shown as the mean±S.E.M. (error bars) for three separate observations and the data was fitted by least-squares-regression analysis to the integrated rate equation for a second-order reaction reaching equilibrium using suitable software (FigP; Biosoft, Cambridge, U.K.). For the reaction:

$$E + I \rightleftharpoons EI$$

let the initial concentrations of E and I be a and b respectively and the concentration of EI at time t and equilibrium be p and q. Then:

$$p = abq(e^{\{[(ab/q)-q]kt\}} - 1)/[ab(e^{\{[(ab/q)-q]kt\}}) - q^2]$$

where k is the second order rate constant. To fit the inhibition data to this equation, the value for p is calculated from the percentage inhibition of PPIase (y) using $y = 100\,p/a$. For the fit shown, the derived values (±95% confidence limits) for k and q were 0.90±0.12 μM^{-1}·s^{-1} and 1.47±0.08 nM respectively. From the latter value the K_i was calculated to be 3.10±0.70 nM.

N-Terminal sequences of rat liver mitochondrial and cytosolic PPIases shows the enzymes to be distinct gene products

Table 2 shows the *N*-terminal sequences of the rat liver cytosolic and two mitochondrial isoenzymes of PPIase. The sequence of the first 28 residues of the cytosolic enzyme was identical with that obtained for rat brain cyclophilin obtained from the cDNA sequence (data only shown up to residue 16). However, the mitochondrial enzymes are quite distinct from the

Table 2. *N*-Terminal sequence comparisons of several PPIases and cyclophorins

Data were taken from the references cited and unless otherwise stated are for the cytosolic cyclophilin A. Where sequences have been derived from cDNA sequences, the *N*-terminal methionine residue has been omitted for consistency. The boxed area indicates sequence identity with rat brain cyclophilin, whereas underlined sequences show additional identities between the rat mitochondrial PPIase and the yeast and *Neurospora crassa* cyclophilins. the two mitochondrial sequences represent data from two identical analyses of the 18.6 kDa protein and one analysis of the 17.5 kDa protein. Ambiguity in the signal was consistently observed at the point shown. The putative second cleavage site of the 18.6 kDa protein is indicated with an arrow.

Cyclophilin or PPIase	Reference	Sequence
Rat mitochondrial 17.5 kDa PPIase	The present work	S S S Q N P L V Y L D V G A D G Q P L
Rat mitochondrial 18.6 kDa PPIase	The present work	A R D G G A R G A N S S S Q N P L V Y L D V G A D G Q P L
		S ↑
Rat liver cytosol PPIase	The present work	V N P T V F F D I T A D G E P L
Rat brain cyclophilin	Danielson *et al.* (1988); Lad *et al.* (1991)	V N P T V F F D I T A D G E P L
Human cyclophilin A	Harding *et al.* (1986)	V N P T V F F D I A V D G E P L
Candida albicans cyclophilin	Koser *et al.* (1990)	S T V Y F D V S A D G Q K L
Saccharomyces cerevisiae cyclophilin	Haendler *et al.* (1989)	S Q V Y F D V E A D G Q P I
Schizosaccharomyces pombe cyclophilin	De Martin & Philipson (1990)	S N C F F D V I A N G Q P L
Neurospora mitochondrial PPIase	Tropschug *et al.* (1989)	T Y S C A R A F S N T S S I M S K V F F D L E W E G P V L
Rat cyclophilin B	Iwai & Inagami (1990)	V T V K V Y F D F Q I G G R T C

cytosolic enzyme. The 18.6 kDa and 17.5 kDa forms show an *N*-terminal presequence of 13 and three amino acids respectively before a sequence similar to that of the cytosolic enzyme is encountered. Thereafter the two mitochondrial forms have sequences identical with one another, but show significant differences from the cytosolic enzyme. These differences all represent conservative changes and would not be expected to lead to major changes in the properties of the protein. The presequences of the two mitochondrial forms show properties consistent with there being a precursor protein with a mitochondrial targeting sequence that is differentially cleaved within the matrix to give the two forms. Such presequences are extremely variable, but usually show a high content of serine, alanine and arginine residues and are often cleaved at more than one site (Brandriss, 1988). The 13-amino-acid presequence of the 18.6 kDa form contains three alanine, three or four serine and one or two arginine residues, which conforms with this pattern. In the 17.5 kDa form there appears to have been additional cleavage to leave only three serine residues of the presequence. The mitochondrial PPIase from the fungus *Neurospora crassa* has a presequence with many similarities to that of the liver mitochondrial enzyme, and it is also cleaved in a two-step process. The remaining sequence of the liver mitochondrial enzyme appears to be more closely related to the cytosolic enzyme from the yeasts *Saccharomyces cerevisiae* and *Candida albicans* than to the liver cytosolic enzyme. Indeed we have prepared polyclonal antibodies to the cytosolic enzyme and these did not cross-react with the mitochondrial enzyme on Western blots (results not shown), nor was the sequence of the mitochondrial enzyme closely related to that of rat cyclophilin B, a membrane-associated form of cyclophilin (Iwai & Inagami, 1990; Price *et al.*, 1991).

Conclusions

Our data suggest that the mitochondrial and cytosolic PPIases are probably encoded for by distinct nuclear genes, although the resulting proteins appear to have almost identical properties. This contrasts with the situation in *Neurospora crassa*, where it has been shown that the mitochondrial and cytosolic enzymes are derived from the same gene by different processing of the mRNA (Tropschug *et al.*, 1988). The availability of the pure enzyme should enable us to elucidate more fully its role in mediating the increase in Ca^{2+} permeability of the mitochondrial inner membrane.

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