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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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 cyclophilin binding, it is now thought unlikely that this inhibition is responsible for the immunosuppressive effect (DeFranco, 1991; Schreiber, 1991). It is known that cyclophilin inhibits some part of the Ca2+-dependent signal-transduction pathway (Kay et al., 1989), and recent data suggests that the cyclosporin-cyclophilin complex inhibits a Ca2+-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 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 Ca2+ (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 Ca2+ (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 presquences 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

<table>
<thead>
<tr>
<th>Purification stage</th>
<th>Total protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg)</th>
<th>Purification (fold)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Matrix</td>
<td>863.00</td>
<td>156.7</td>
<td>0.18</td>
<td>1.0</td>
<td>100.0</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>357.00</td>
<td>91.4</td>
<td>0.26</td>
<td>1.4</td>
<td>58.3</td>
</tr>
<tr>
<td>Q- + S-Sepharose</td>
<td>18.20</td>
<td>59.0</td>
<td>3.24</td>
<td>18.0</td>
<td>37.0</td>
</tr>
<tr>
<td>Mono S</td>
<td>2.34</td>
<td>23.1</td>
<td>9.87</td>
<td>54.4</td>
<td>14.7</td>
</tr>
<tr>
<td>Superose 12</td>
<td>0.17</td>
<td>18.8</td>
<td>110.58</td>
<td>614.0</td>
<td>11.9</td>
</tr>
</tbody>
</table>

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. (NH₄)₂SO₄ cut (P₂) 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.

**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.
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. PPlase 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 PPlase was expressed in units of $\mu$mol of peptide converted/min calculated from the difference in $e_{490}$ (13400 litre·mol$^{-1}$·cm$^{-1}$) between the substrate and the released $\alpha$-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 PPlase

Table 1 summarizes the procedure used for the purification of the matrix PPlase. 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 (Handschemacher 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 PPlase 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 $\mu$M-cyclosporin A (results not shown). Thus it would seem that both proteins must be PPlases 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 PPlase 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 [PH]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 PPlase 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% of 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 ($\pm$ 95% confidence limits) were $0.90 \pm 0.12 \mu$M$^{-1}$·s$^{-1}$ and $3.10 \pm 0.70$ 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 PPlase, 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.

N-Terminal sequences of rat liver mitochondrial and cytosolic PPlases 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 PPlase. 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
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 properties 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 Ca²⁺ permeability of the mitochondrial inner membrane.

**REFERENCES**


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

<table>
<thead>
<tr>
<th>Cyclophilin or PPIase</th>
<th>Reference</th>
<th>Sequence</th>
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</thead>
<tbody>
<tr>
<td>Rat mitochondrial 17.5 kDa PPIase</td>
<td>The present work</td>
<td>S...E...Q...K...G...A...D...G...E...L</td>
</tr>
<tr>
<td>Rat mitochondrial 18.6 kDa PPIase</td>
<td>The present work</td>
<td>A...D...G...A...R...G...A...N...S...E...D...G...E...L</td>
</tr>
<tr>
<td>Rat liver cytosol PPIase</td>
<td>The present work</td>
<td>V...P...T...V...F...D...I...T...A...D...G...E...P...L</td>
</tr>
<tr>
<td>Human cyclophilin A</td>
<td>Harding et al. (1986)</td>
<td>S...T...P...T...A...D...G...E...L</td>
</tr>
<tr>
<td><em>Candida albicans</em> cyclophilin</td>
<td>Koser et al. (1990)</td>
<td>S...Q...T...V...D...A...D...G...E...P...L</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em> cyclophilin</td>
<td>Haendler et al. (1989)</td>
<td>S...C...P...F...D...V...A...D...G...E...P...L</td>
</tr>
<tr>
<td><em>Neurospora mitochondrial</em> PPIase</td>
<td>Tropschug et al. (1989)</td>
<td>V...T...V...F...P...Q...G...I...G...R...C</td>
</tr>
<tr>
<td>Rat cyclophilin B</td>
<td>Iwai &amp; Inagami (1990)</td>
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Purification of mitochondrial cyclophilin


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