Plant Organelles Contain Distinct Peptidylprolyl cis,trans-Isomerases*

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The conceptual thinking about protein folding in living cells has undergone a major revision since the discovery of "protein-folding" enzymes and protein chaperones, classes of proteins implicated in regulating the maturation and assembly of a variety of proteins (Gething and Sambrook, 1992). Included in the family of enzymes that catalyze or facilitate protein folding are protein disulfide isomerase, which promotes correct disulfide pairings in proteins (Freedman, 1989), and peptidylprolyl cis,trans-isomerase (PPIase),† which catalyzes slow isomerization of peptide bonds in oligopeptides as well as cellular proteins involving the amino acid proline (Gething and Sambrook, 1992; Freedman, 1989). Protein disulfide isomerase activity has generally been found associated specifically with the endoplasmic reticulum of lower eukaryotes and mammals while proteins with PPIase activity have a wider distribution and are shown to be present in bacteria to mammals (Freedman, 1989; Fischer and Schmid, 1990; Liu and Walsh, 1990; Schonbrunner et al., 1991).

In plants, relatively more information is available on the involvement of chaperones in the biosynthesis, oligomerization, and maturation of proteins than on the occurrence and/or role of the protein-folding enzymes. Protein disulfide isomerase activity has been detected in plant tissues, but very little is known about PPIase in plants (Holmgren, 1985; Gasser et al., 1990). PPIases in other tissues have been characterized and found to be easily distinguishable from one another by selective inhibitory effects of the immunosuppressive agents, cyclosporin A (CsA) and FK506 (Schreiber, 1991). Proteins that bind CsA are called cyclophilins while structurally distinct drugs such as FK506 and rapamycin bind to proteins called FKBP (FK506-binding proteins) or rapamycin-binding proteins (RBP) (Handschumacher et al., 1984; Harding et al., 1989; Siekiera et al., 1989; Schonbrunner et al., 1991). These proteins show a wider distribution, being present in the cytosol (Koletsky et al., 1986), periplasmic space (Hultsch et al., 1991), and the mitochondria (Tropschug et al., 1988) of different organisms. Although their role(s) and physiological substrates remain to be determined, PPIases have been termed "conformases" (Fischer and Schmid, 1990) or "rotamases" (Schreiber, 1991) because they catalyze slow steps in the initial folding/rearrangement of proteins. The mechanism of this catalysis around specific peptide bonds is also unknown.

We have undertaken an investigation toward finding PPIases in pea plant organelles, the mitochondria and the chloroplasts. We show here for the first time that both the chloroplast and the mitochondria contain CsA-sensitive PPIases while a putative rapamycin-binding protein is found exclusively associated with pea mitochondria.

EXPERIMENTAL PROCEDURES

Plant Material—Pea seedlings (cv. Alaska) were grown for 10 days under white light in a growth chamber at 25 °C, and the leaves were harvested for chloroplast preparation. For mitochondrial preparations etiolated pea seedlings grown for 6 days at 25 °C were harvested.

Pea Chloroplast Stroma and Membrane Isolation—Intact pea chloroplasts were isolated by the method of Bartlett et al. (1982), resuspended in a lysis buffer containing 50 mM NaCl, 50 mM Tris-Cl, pH 7.4, 20 mM MgCl2, 10% glycerol, 2 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 5 mM β-mercaptoethanol, and then vortexed. The broken chloroplasts were pelleted at 7,000 x g for 10 min and the supernatant used as stroma. The chloroplast membranes were washed twice in the lysis buffer supplemented with 300 mM NaCl. Finally the membranes were washed twice in 10 mM Tris-Cl, pH 8.0.

Localization and Partial Purification of Cyclosporin A Binding Activity from Thylakoids—Thylakoids were isolated according to Marder et al. (1982) and fractionated into grana and stroma lamellae as previously described (Mattoo and Edelman, 1987; Callahan et al., 1989). For studying CsA binding, whole thylakoids (1 mg of chlorophyll/ml) were solubilized in 2% Triton X-100 for 30 min at 4 °C. The insoluble material was removed by centrifugation at 2,000 x g
for 10 min and the supernatant applied to a DEAE-Toyopearl 650 S column equilibrated with 50 mM Tris- NaOH, pH 7.2, and 0.2% Triton X-100. The column was washed with 10 ml of the same buffer and the bound material eluted with 20 ml of 0.5 M NaCl. Fractions (0.5 ml) were collected and analyzed for chlorophyll content and CsA binding.

Determination of Chlorophyll and Protein Concentrations—Chlorophyll was determined in leaf extracts prepared in 80% acetone (Arnon, 1949). Protein content was determined by the Bradford method (Bradford, 1976).

Mitochondria, Matrix, and Membrane Isolations—Mitochondria were isolated as previously described (Breiman, 1987) and fractionated into the matrix and membranal fractions (Hackett et al., 1991). The mitochondria were resuspended in sucrose phosphate buffer (SP) (0.3 M sucrose, 20 mM sodium phosphate, 0.25 mM EDTA, and 0.25 mM PVP-40 in pH 7.2) and sonicated 6 times for 5 s at 25-s intervals with a microprobe of a Virsonic 300 (Virtis) at 60% of full power and the tubes kept in a mixture of methanol/ice. The unbroken mitochondria and aggregated material were pelleted by centrifugation at 230,000 × g for 10 min. The supernatant was then centrifuged at 230,000 × g for 70 min to obtain the matrix (soluble) fraction. The pellet was resuspended in SP buffer, divided into two portions, and centrifuged at 230,000 × g for 70 min. One portion was washed twice with SP buffer, and the second membrane portion was resuspended in 0.1 M sodium carbonate (pH 11) to remove peripheral proteins (Fujiki et al., 1982). The tubes were incubated for 40 min on ice and then centrifuged at 230,000 × g for 70 min. The membrane pellets were resuspended in 10 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1.8% N-octyl glucoside and incubated on ice for 60 min. The solubilized mixture was centrifuged at 15,600 × g for 5 min to remove the insoluble material. For CsA binding assay and PPlase activity, the mitochondrial fraction was solubilized in 1.8% N-octyl glucoside for 60 min on ice and centrifuged at 15,600 × g for 10 min to remove the insoluble material.

Peptidylprolyl cis, trans-Isomerase Assay—PPlase activity was measured in a coupled assay with chymotrypsin (Boehringer Mannheim) using a Shimadzu UV-160 spectrophotometer essentially by the method of Fischer et al. (1989) with the following exceptions. The test peptide N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (Sigma) at 60 μM final concentration was added to a solution of the assay buffer (40 mM Hepes pH 8.0, 0.015% Triton X-100) and the plant extract in a final volume of 1.5 ml. The reaction was initiated by the addition of chymotrypsin to a final concentration of 20 μM. Immediately following the addition of chymotrypsin the change in absorbance at 390 nm (A390) was monitored for 100 s. First order rate constants were calculated from the absorbance change at 390 nm, was multiplied by the amount of substrate (in each reaction. Under equilibrium conditions, the amount of each plant extract tested is indicated in the appropriate columns. The concentration of each plant extract was studied using Sephadex LH-20 columns as previously described (Handschumacher et al., 1984). The reactions were carried out in a 100-μl final volume in a containing 10 mM Hepes pH 8, 100 mM NaCl, 0.015% Triton X-100, 5 mM β-mercaptoethanol, 0.5 μM CsA, [3H]CsA (106 cpm), and plant extract. In some reactions, different concentrations of nonradioactive CsA were added to study specific binding. The matrix and membranal fractions (not shown) were isolated as previously described (Breiman, 1987) and fractionated into the matrix and membranal fractions. The matrix extract (Fig. 1B), and was inhibited close to 90% in the presence of CsA (14 μM) or to 16% in the presence of rapamycin (24 μM) (Fig. 1A and Table I). The distribution of this activity between the soluble (matrix) and membranal fractions of purified mitochondria was next determined. Results (Table I) showed a 13-fold higher specific activity of PPlase in the matrix as compared with the mitochondrial membranes, suggestive of an enrichment of this activity in the soluble fraction. Of the total enzyme units present in the two mitochondrial fractions, 78% were recovered in the matrix fraction (not shown). PPlase activity was peripherally associated with washed mitochondrial membranes because it could be easily removed by treatment with 0.1 M sodium carbonate (Table I).

Both mitochondrial fractions strongly bound CsA, which resulted in the near total inhibition of their PPlase activity (Table I). In contrast, rapamycin inhibited PPlase activity of only the mitochondrial membranes. However, when an antibody against yeast RBP was used to detect cross-reactive protein bands in different mitochondrial fractions, a 25-kDa protein was detected in both matrix and membranes (Fig. 2). In washed membranes treated with 0.1 M sodium carbonate, which resulted in the removal of PPlase activity from the membranes (see Table I), this cross-reactive protein band was not detected (Fig. 2, lane 4). These data are consistent with the interpretation that the putative 25-kDa RBPs are peripheral to pea mitochondrial membranes and that its removal was possibly the cause for our inability to detect PPlase activity in mitochondria treated with sodium carbonate (Table I). The homologous 25-kDa RBPs have been recently identified and cloned from animal species (Galat et al., 1992; Wier...
Rotamases in Plants

TABLE I

Characteristics of PPIase activity in pea mitochondrial fractions

The values represent means ± S.E.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Addition</th>
<th>PPIase activity</th>
<th>Inhibition</th>
<th>CsA binding*</th>
<th>Cross-reactivity to anti-RBP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total mitochondrial extract</td>
<td>None</td>
<td>12.78 ± 1.62</td>
<td>0</td>
<td>90,773</td>
<td>+</td>
</tr>
<tr>
<td>Mitochondrial matrix</td>
<td>14 μM CsA, 24 μM rapamycin</td>
<td>1.82 ± 0.55</td>
<td>86</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitochondrial membranes</td>
<td>None</td>
<td>26.4 ± 3.97</td>
<td>0</td>
<td>78,800</td>
<td>+</td>
</tr>
<tr>
<td>Mitochondrial membranes washed</td>
<td>14 μM CsA, 30 μM rapamycin</td>
<td>28.6 ± 0.68</td>
<td>0</td>
<td>31,900</td>
<td>+</td>
</tr>
</tbody>
</table>

* The specific binding of CsA is given as a difference in the binding at 500 nM and that at 5 nM.
* This value is from another experiment where PPIase activity in the total extract was 7.02 ± 0.97 nmol·s⁻¹·mg⁻¹, which in the presence of 24 μM rapamycin was reduced to 5.85 ± 0.28 nmol·s⁻¹·mg⁻¹.
* This value is from another experiment where PPIase activity in the absence of CsA was 4.0 ± 0.54 nmol·s⁻¹·mg⁻¹.

FIG. 2. Identification of a cross-reactive rapamycin-binding protein in mitochondrial fractions. Solubilized total mitochondria (lane 1), mitochondrial matrix (lane 2), mitochondrial membranes washed with 0.1 M Na₂CO₃, pH 11 (lane 3), and mitochondrial membranes washed with 0.1 M Na₂CO₃, pH 11 (lane 4) were fractionated on SDS-polyacrylamide gel electrophoresis, then either stained with Coomassie Blue (A) or immunoblotted and incubated with an antibody against yeast RBP (B). Each lane was loaded with 20 μg of protein. The positions of prestained molecular mass markers is shown in kDa.

derrecht et al., 1992). The results presented here indicate the presence in pea mitochondria of two distinct activities of PPIase. The association of the majority of the CsA binding activity with the pea mitochondrial matrix is in agreement with previous observations made with Neurospora and rat liver mitochondria (Tropschug et al., 1988; Halestrap and Davidson, 1990).

Chloroplast PPIases—Chloroplast is a special organelle in green plants which houses most of the biosynthetic reactions and a distinct, highly organized and regulated genome. The photosynthetic membranes (thylakoids) of chloroplasts are comprised of stacked (granal) membranes, enriched in photosystem II components, interconnected with non-appressed (stromal) membranes, enriched in photosystem I components (see Callahan et al., 1989). Thylakoid proteins are classifiable into two types. One class represents those proteins that translocate within the thylakoids and thus are located on both membrane types. The second class constitutes those proteins that do not translocate and are exclusive to one of the membrane types (Callahan et al., 1989). Correct protein folding is therefore highly desirable in this photosynthetic organelle, as is apparent from the discovery and involvement of protein chaperones in the assembly of some of the multisubunit complexes in this organelle (Goloubinoff et al., 1989; Ellis, 1991). We sought to look for the presence of other classes of protein folds in the chloroplasts.

An active PPIase was present in the soluble (stromal) fraction of the purified chloroplasts, specific activity of which was lower than that in the mitochondrial matrix (compare Tables I and II). The chloroplast stroma PPIase was inhibited completely at 14 μM CsA (Table II). Relative to the ease with which PPIase activity could be determined with both of the mitochondrial fractions as well as the chloroplast stroma, chloroplast membranes (thylakoids) proved initially to be intractable. This inability to detect PPIase in the thylakoids was found linked to the presence of chlorophyll in these membranes, which interfered with the PPIase activity assay. To circumvent this problem, we solubilized the thylakoids and fractionated the proteins on the DEAE-Toyopearl column.

TABLE II

Characteristics of PPIase activity in the chloroplast fractions

Units and other descriptions are the same as given in Table I. ND, not detectable.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Addition</th>
<th>PPIase activity</th>
<th>Inhibition</th>
<th>CsA binding*</th>
<th>Cross-reactivity with anti-RBP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroplast stroma</td>
<td>None</td>
<td>9.8 ± 1.54</td>
<td>0</td>
<td>85,917</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>14 μM CsA</td>
<td>10.0 ± 2.04</td>
<td>0</td>
<td>533,140</td>
<td>-</td>
</tr>
<tr>
<td>DEAE-fractionated thylakoids</td>
<td>None</td>
<td>5.88 ± 2.13</td>
<td>0</td>
<td>533,140</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>14 μM CsA</td>
<td>1.96 ± 0.092</td>
<td>66</td>
<td>533,140</td>
<td>-</td>
</tr>
<tr>
<td>Total chloroplast membranes</td>
<td>ND</td>
<td>122,920</td>
<td>-</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Grana</td>
<td>ND</td>
<td>184,000</td>
<td>-</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Stroma lamellae</td>
<td>ND</td>
<td>81,352</td>
<td>-</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Stroma lamellae, pH 9.0, washed</td>
<td>ND</td>
<td>91,667</td>
<td>-</td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>
that our inability to detect a cross-reactive RBP in the chloroplast fractions could be due to epitope differences and, thereby, non-recognition of any chloroplast RBP by an antibody against a heterologous, yeast RBP. Our data are consistent with the absence of a chloroplast RBP activity, but more direct results are awaited to prove this unequivocally.

In conclusion, these results demonstrate for the first time that both plant organelles share similar but distinct Ca-binding PPIases and that a putative RBP (rapamycin-sensitive PPlase) is selectively localized to the mitochondria. These data further add to the diversity of PPIases in nature and should lead to the isolation and identification of the corresponding genes encoding the organellar PPIases in plants. In this regard, the cloning of the plant cytosolic cyclophilin is encouraging (Gasser et al., 1990). We speculate that PPIases are involved in the correct folding of newly synthesized proline-containing soluble and membrane proteins in plant organelles in a fashion similar to that demonstrated with the protein chaperone, the rubisco-binding protein (Ellis, 1990), for the assembly of the rubisco protein complex.

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