Plant Organelles Contain Distinct Peptidylprolyl cis,trans-Isomerases*

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Peptidylprolyl cis,trans-isomerase (PPIase) activity was detected in the cytosol, mitochondria, and chloroplast of pea plants. Cyclosporin A inhibited the activity largely localized to the mitochondrial matrix while rapamycin inhibited the PPIase activity associated with the mitochondrial membranes. Differential inhibition by the two immunosuppressive drugs, the specific binding of these drugs to different mitochondrial fractions, and the immunological detection of a putative 25-kDa rapamycin-binding protein (RBP) in mitochondrial extracts attests to the presence in plant mitochondria of both cyclophilin and RBP classes of PPIases. Cyclosporin A-sensitive PPIase detected in the chloroplast was mostly localized to the thylakoids, which is suggestive of its function in the folding of membranal proteins. PPIase associated with the chloroplast stroma and the thylakoids was not inhibited by rapamycin nor was any cross-reactive RBP detected in chloroplast extracts. These results demonstrate the presence of distinct classes of PPIases in the mitochondria and the chloroplasts of plants.

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 (Schrieber, 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; Siekerka 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" (Schrieber, 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-HCl, pH 7.4, 20 mM MgCl2, 0.1 mM EDTA, 10% glycerol, 2 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 5 μM β-mercaptoethanol, and then vortexed. The broken chloroplasts were pelleted at 7,000 × 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-HCl, 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., 1988). 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 20,000 × 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 (Hack 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 PCMB [pH 7.2]) and sonicated 6 times for 5 s at 25-s intervals with a microprobe of a Vircsonic 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 15,600 × 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 Triton-HCl, pH 7.2, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1.8% N-octylglucoside 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 PPIase activity, the mitochondrial fraction was solubilized in 1.8% N-octylglucoside for 60 min on ice and centrifuged at 15,600 × g for 10 min to remove the insoluble material.

**Peptidylprolyl cis-trans-Isomerase Assay—**PPIase 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. (1988) 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, was multiplied by the amount of substrate (in each reaction). Under equilibrium conditions, with SP buffer, and the second membrane portion was resuspended following the addition of chymotrypsin the change in absorbance at 390 nm, was multiplied by the amount of substrate in each reaction. The interpretation that the putative 25-kDa RBP is peripheral to pea mitochondrial membranes and that its removal was 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 PPIase activity (Table I). In contrast, rapamycin inhibited PPIase 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. 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 membrane fractions of purified mitochondria was next determined. Results (Table I) showed a 13-fold higher specific activity of PPIase 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). PPIase activity was peripherally associated with washed mitochondrial membranes because it could be easily removed by treatment with 0.1 M sodium carbonate (Table I).

**RESULTS AND DISCUSSION**

**Pea Mitochondrial PPlase—**Solubilized pea and mitochondrial extracts were found to contain a bona fide PPIase activity; the enzyme activity exhibited first order kinetics (Fig. 1A), was linearly correlated to the amount of the mitochondrial 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 membrane fractions of purified mitochondria was next determined. Results (Table I) showed a 13-fold higher specific activity of PPIase 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). PPIase activity was peripherally associated with washed mitochondrial membranes because it could be easily removed by treatment with 0.1 M sodium carbonate (Table I).

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TABLE I
Characteristics of PPIase activity in pea mitochondrial fractions

| Fraction                      | Addition   | PPIase activity nmo1·s⁻¹ mg protein⁻¹ | Inhibition % | CsA binding cpm/mg protein | Cross-reactivity to anti-RBP |
|------------------------------|------------|--------------------------------------|--------------|---------------------------|-----------------------------|
| Total mitochondrial extract  | None       | 12.78 ± 1.62                          | 0            | 90,773                    | +                           |
|                              | 14 μM CsA  | 1.82 ± 0.55                           | 86           |                           |                             |
|                              | 24 μM rapamycin | 16⁴                                  |              |                           |                             |
| Mitochondrial matrix         | None       | 26.4 ± 3.97                           | 0            | 78,800                    | +                           |
|                              | 14 μM CsA  | 86⁴                                  |              |                           |                             |
| Mitochondrial membranes      | None       | 28.6 ± 0.68                           | 0            | 31,900                    | +                           |
|                              | 14 μM CsA  | 100                                  |              |                           |                             |
|                              | 30 μM rapamycin | 39                                   |              |                           |                             |

*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 nmo1·s⁻¹ mg⁻¹, which in the presence of 24 μM rapamycin was reduced to 5.85 ± 0.28 nmo1·s⁻¹ mg⁻¹.

TABLE II
Characteristics of PPIase activity in the chloroplast fractions

| Fraction                | Addition   | PPIase activity nmo1·s⁻¹ mg protein⁻¹ | Inhibition % | CsA binding cpm/mg protein | Cross-reactivity with anti-RBP |
|-------------------------|------------|--------------------------------------|--------------|---------------------------|-------------------------------|
| Chloroplast stroma      | None       | 9.8 ± 1.54                           | 0            | 85,917                    | -                             |
|                         | 14 μM CsA  | 100                                  |              |                           |                               |
| DEAE-fractionated thylakoids | None       | 5.88 ± 2.13                          | 100          | 533,140                   | -                             |
|                         | 14 μM CsA  | 1.96 ± 0.092                         | 66           |                           |                               |
| Total chloroplast membranes | ND        |                                      |              |                           |                               |
| Grana                   | ND        |                                      |              |                           |                               |
| Stroma lamellae         | ND        |                                      |              |                           |                               |
| Stroma lamellae, pH 9.0, washed | ND        |                                      |              |                           |                               |

The values represent means ± S.E.

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 SP buffer (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 foldases 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.
that our inability to detect a cross-reactive RBP in the chlo-
roplast fractions could be due to epitope differences and, the-
by, non-recognition of any chloroplast RBP by an anti-
body 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 CsA-
binding PPIases and that a putative RBP (rapamycin-sensi-
tive PPIase) 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 corre-
sponding 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 pro-
line-containing soluble and membrane proteins in plant or-
ganelles 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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FIG. 3. Elution profile of solubilized thylakoids on DEAE-
Toyopearl 650 S column. Thylakoids were solubilized in 2% Triton
X-100 as described under "Experimental Procedures.” Two-mg eq of
chlorophyll were applied to a 2-cm column previously equilibrated
with 50 mM Tris-NaOH, pH 7.2, containing 0.2% Triton X-100. The
column was washed with 10 ml of the equilibration buffer, and then
the bound proteins were eluted with 20 ml of 0-1 M NaCl gradient.
Fractions (0.5 ml) were collected and analyzed for chlorophyll content
(●) and CsA binding (△).

FIG. 4. A, kinetics of the hydrolysis of N-succinyl-Ala-Ala-Pro-
Phenyl-p-nitroanilidine were followed in the absence (0) or presence
(●) of partially purified thylakoids (50 μg of protein/assay) as de-
scribed under “Experimental Procedures.” Thylakoid proteins were
fractionated on a DEAE-Toyopearl 650 S column before determining
PPIase activity. The effect of 14 μM CsA is also shown (+). B, de-
pendence of the rate constant as a function of protein concentra-
tion. PPIase activity kinetics were followed as in A in triplicate each
at 0, 25, 50, and 75 μg of protein of DEAE-purified thylakoid fraction/
assay.

(Fig. 3) as described under “Experimental Procedures.” The
solubilized thylakoidal PPIase thus obtained exhibited first
order kinetics (Fig. 4A), showed a linear increase in activity
with increasing concentrations of the protein (Fig. 4B), and
was strongly inhibited by CsA (Table II).

CsA bound relatively stronger with the chloroplast fractions
than the mitochondrial ones (Tables I and II); this binding was to both the stroma and the thylakoids. However, in
contrast to the peripheral association of the PPIase with the
mitochondrial membranes, the binding associated with the
thylakoids could not be removed by washing at alkaline pH
(Table II).

Further, a clear distinction was apparent between the two
organelles in their abilities to bind rapamycin. While a cross-
reactive 25-kDa RBP was identified in the mitochondria and the
mitochondrial PPIase activity was partially inhibited by
rapamycin (Fig. 2 and Table I), such was not the case with
the chloroplast fractions. We were not able to detect any
cross-reactive chloroplast RBP nor was the chloroplast
PPIase inhibited by rapamycin (data not shown). It is possible