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Suborganellar Localization and Molecular Characterization of Nonproteolytic Degraded Leukoplast Pyruvate Kinase from Developing Castor Oil Seeds[1]

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Plastid pyruvate kinase (PK_p) activity and anti-(castor oil seed [COS] PK_p) immunoglobulin G immunoreactive polypeptides were recovered in the stroma but not from envelope membranes of purified COS leukoplasts that had been subfractionated by sucrose density gradient centrifugation. The PK_p was highly purified from isolated leukoplasts using anion-exchange and ADP-agarose chromatographies. Proteolysis of PK_p was almost entirely eliminated by including 2,2'-dipyridyl disulfide in purification buffers. The final preparation contained 63.5-kD (α subunit) and 54-kD (β subunit) polypeptides that stained for protein and cross-reacted with anti-(COS PK_p) immunoglobulin G with similar intensities. These two polypeptides co-eluted following gel-filtration chromatography and co-migrated during nondenaturing isoelectric focusing-polyacrylamide gel electrophoresis. The enzyme's native M_r was estimated to be 334,000. This PK_p thus appears to exist as an $\alpha_3\beta_3$-heterohexamer. Comparison of the respective N-terminal sequences of the α and β subunits with the deduced amino acid sequences for several PK_p cDNAs indicated that (a) the α and β subunits are encoded by COS genes previously designated as PK_pA and PK_pG, respectively, and (b) respective transit peptides of 4.8- and 5.5-kD are cleaved from the α and β subunit preproteins following their translocation into the leukoplast.

PK (ATP:pyruvate phosphotransferase, EC 2.7.1.40) is an important regulatory enzyme of the glycolytic pathway that catalyzes the irreversible reaction:

$$PEP + ADP + H^+ \xrightarrow{Mg^{2+}, K^+} Pyruvate + ATP$$

Plant PK is known to exist as PK_c and PK_p that not only differ in their respective physical and kinetic characteristics but are also immunologically unrelated and genetically distinct (Ireland et al., 1980; Plaxton, 1989; Blakeley et al., 1991; Plaxton et al., 1990, 1993). The molecular, immunological, and kinetic characteristics of a variety of highly purified or homogeneous higher plant PK_c's have been studied in detail (Ireland et al., 1980; Plaxton, 1988, 1989; Podestá and Plaxton, 1991, 1992, 1993, 1994). Castor oil plant PK_c appears to exist as tissue-specific isozymes that exhibit marked differences in their respective physical and kinetic/regulatory properties (Hu et al., 1995; Podestá and Plaxton, 1991, 1992, 1994). The subunit structure of the homotetrameric COS cotyledon PK_c is comparable to that reported for the PK_c of developing COS endosperm but differs from that of the heterotetrameric PK_c of castor leaves and germinated endosperm (Plaxton 1988, 1989; Podestá and Plaxton 1993, 1994; Hu et al., 1995). In contrast, because of its extreme lability and proteolytic susceptibility, far less is known about plant PK_p relative to PK_c.

A PK_p having a native molecular mass of about 305 kD was purified to near homogeneity from developing COS endosperm (Plaxton et al., 1990). SDS-PAGE and immunoblot analysis of the final preparation revealed two immunologically related major protein-staining bands of 57.5 and 44 kD. That both polypeptides were associated with a single native form of the COS PK_p was suggested by the observations that the 57.5- and 44-kD protein-staining bands co-eluted following gel-filtration FPLC, and the single protein-staining band from the nondenaturing gel still produced the same two polypeptides upon SDS-PAGE. It was concluded that there was either partial degradation of the 57.5-kD polypeptide yielding the 44-kD polypeptide or that the enzyme might exist as a heteromer composed of two different types of related subunits (Plaxton et al., 1990).

In a subsequent study, Plaxton (1991) demonstrated that the novel polypeptide composition initially observed for the purified enzyme (Plaxton et al., 1990) artifactually arose after tissue homogenization via partial proteolysis of the enzyme's 63.5-kD (α) and 54-kD (β) subunits. The generation of a 57.5-kD degradation product from the 63.5-kD α subunit arises from the specific action of an endogenous asparaginyl endopeptidase, since the site of cleavage is on the carboxy-terminal side of a unique sequence of four consecutive Asn residues (Blakeley et al., 1991). The asparaginyl endopeptidase of developing COS was shown to be a Cys protease that displays characteristics consistent with its putative involvement in the turnover and/or elimination of PK_p during COS maturation (Plaxton, 1991; Cornel and Plaxton, 1994). The developmental period during which PK_p activity and concentration show maximal increases is coincident with the onset of the most active phase of storage-lipid accumulation by both developing COS endosperm and *Brassica napus* embryos (Plaxton, 1991; Sang-

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Abbreviations: COS, castor oil seed; DPDS, 2,2'-dipyridyl disulfide; FPLC, fast protein liquid chromatography; PK, pyruvate kinase; PK_c and PK_p, cytosolic and plastidic pyruvate kinases, respectively; PVDF, polyvinylidene difluoride.

wan et al., 1992). These data support earlier proposals (Dennis and Miernyk, 1982) that a fundamental role for PK_p in developing oil seeds is to generate precursors (i.e. pyruvate and ATP) required for long-chain fatty acid biosynthesis in leukoplasts.

Antibodies against proteolyzed COS PK_p (Plaxton et al., 1990) were used by Blakeley and co-workers (1991, 1992, 1995) to immunoselect several clones for PK_p (i.e. PK_pA, PK_pB, and PK_pG) from a developing COS cDNA library. The deduced sequence of PK_pA encodes eight amino acid residues that have been identified as the N terminus of the 57.5-kD α subunit of the proteolyzed COS PK_p (Blakeley et al., 1991). In vitro import assays utilizing isolated COS leukoplasts, radiolabeled translation products of the PK_pA and PK_pG cDNAs, and specific antibodies against the overexpressed C termini of PK_pA and PK_pG initially led to the hypothesis that leukoplasts of developing COS may contain distinct PK_p isozymes in the leukoplast envelope (i.e. PK_p A) and stroma (i.e. PK_pG) (Blakeley et al., 1992; Blakeley and Dennis, 1993; Dennis and Blakeley, 1993; Wan et al., 1993). However, subsequent work has revealed that, although PK_pA precursors appear to accumulate on the outer envelope membrane of developing COS leukoplasts both in vivo and in vitro, the 66-kD PK_pA preprotein can be processed to a mature 63-kD form following its in vitro import into isolated leukoplasts at relatively high (2 to 3 mM) levels of exogenous ATP (Wan et al., 1995). In contrast, the 61-kD preprotein encoded by PK_pG does not accumulate on the outer envelope membrane and is imported into isolated COS leukoplasts and processed to a 55-kD form at lower (1 mM) levels of exogenous ATP (Wan et al., 1995). The conservation of active site residues and differential in vitro import characteristics led to the proposal that the mature proteins encoded by COS PK_pA and PK_pG are not subunits of a single heteromeric PK_p but may represent distinct homomeric isoforms of PK_p (Blakeley et al., 1995; Wan et al., 1995).

The objective of the present work was to investigate further the suborganellar localization and molecular characteristics of nonproteolyzed COS PK_p. Furthermore, N-terminal sequencing of the purified enzyme's α and β subunits (a) demonstrated that they are encoded by the cDNAs previously designated as PK_pA and PK_pG, respectively (Blakeley et al., 1991, 1995), and (b) allowed identification of the processing sites for transit peptides of the respective PK_p preproteins.

MATERIALS AND METHODS

Chemicals and Plant Material

Biochemicals, rabbit muscle lactate dehydrogenase, alkaline phosphatase-conjugated goat anti-(rabbit IgG) IgG, SDS-PAGE M_r standards, ADP-agarose, and bisacrylamide were purchased from Sigma. DTT was purchased from Research Organics (Cleveland, OH). Tris and SDS were from Schwartz/Mann Biotech (Cambridge, MA). Protein assay reagent and ammonium persulfate were from Bio-Rad. PVDF membranes for immunoblotting were from Millipore, whereas those for N-terminal sequencing were from Bio-Rad. All other reagents were of analytical grade and were obtained from BDH Chemicals (Toronto, Ontario, Canada).

Castor oil plants (*Ricinus communis* L., var Baker 296) were grown in a greenhouse under natural light, supplemented with 16 h of fluorescent light.

Enzyme Assay

The PK reaction was coupled to the lactate dehydrogenase reaction and assayed at pH 8.0 and 30°C by monitoring NADH oxidation at 340 nm. Standard assay conditions for PK_p were as previously described (Plaxton et al., 1990). Assays were initiated by the addition of enzyme preparation. In all cases, the rate of reaction was linear with respect to time and concentration of enzyme assayed. One unit of enzyme activity is defined as the amount of enzyme catalyzing the oxidation of 1 μmol NADH/min at 30°C.

Buffers Used in Leukoplast Isolation and Fractionation

Buffer A contained 50 mM Hepes-KOH (pH 7.5), 0.4 M sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM DTT, 1% (w/v) Ficoll, 1% (w/v) BSA, 1 mM PMSF, 5 μg/mL leupeptin, 5 μg/mL chymostatin, 1 mM benzamidine-HCl, and 5 mM ϵ-aminocaproic acid; buffer B was the same as buffer A minus Ficoll and BSA. Buffer C contained 20 mM Hepes-KOH (pH 7.5), 1 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 1 mM PMSF, 5 μg/mL leupeptin, 5 μg/mL chymostatin, 1 mM benzamidine-HCl, and 5 mM ϵ-aminocaproic acid; buffer D was the same as buffer C plus 0.15% (v/v) Triton X-100 and 10% (v/v) glycerol.

Isolation and Lysis of COS Leukoplasts

All procedures were carried out at 0 to 4°C. Endosperm (25–30 g) was dissected from COS at the late cotyledonary stage of development (or developmental stages 5 to 7 according to Greenwood and Bewley, 1981) and ground for 2 min together with a scoop of sea sand in 2 volumes of buffer A. The homogenate was filtered through six layers of cheesecloth and centrifuged at 500*g* for 5 min. The supernatant was centrifuged at 6000*g* for 15 min, and the resulting pellet was resuspended in 5 mL of buffer A. Discontinuous Percoll gradient centrifugation of the resuspended pellet as described by Boyle et al. (1986) resulted in a band of leukoplasts sedimenting at the 22 to 35% Percoll interface. Collected leukoplasts were washed with 25 mL of buffer B and centrifuged at 6000*g* for 5 min. The pellet was re-washed with 25 mL of buffer B and centrifuged at 4354*g* for 5 min. The final pellet represents the purified leukoplasts, which were devoid of contamination by other organelles and cytosol as judged by the absence of marker enzyme activities. Marker enzymes were assayed as follows: catalase (Luck, 1965), fumarase (Hatch, 1978), acid phosphatase (Duff et al., 1991), isocitrate dehydrogenase (Smith et al., 1992), and alcohol dehydrogenase (Racker, 1955). The ratio of 6-P-gluconate dehydrogenase, $NADP^+$-dependent malic enzyme, and enolase activities before and after the addition of Triton X-100 was used to estimate leukoplast intactness (Alban et al., 1988; Smith et al., 1992).

The assay conditions for 6-P-gluconate dehydrogenase were as described by ap Rees et al. (1976), whereas those for $NADP^+$-malic enzyme and enolase were as described by Smith et al. (1992). All assays had a final volume of 1 mL and were initiated by the addition of an aliquot of the purified COS leukoplasts. After 3 min, 10 μL of 10% (v/v) Triton X-100 were added, and the three activities were monitored for a further 3 min.

The following treatments were examined with respect to their relative effectiveness for lysis of the isolated COS leukoplasts. The leukoplast fraction was resuspended in (a) 1 mL of buffer B, frozen at −20°C for 1 h, and thawed at room temperature; (b) 1 mL of buffer C; or (c) 1 mL of buffer B, frozen at −80°C for 1 h, thawed at room temperature, treated with 2 mL of buffer C, and homogenized (10 strokes) with a Potter-Elvehjem homogenizer equipped with a loose-fitting Teflon pestle (Alban et al., 1988). Each preparation was stored on ice for 1 h, and leukoplast intactness was assessed by determining enolase latency as described above.

Subfractionation of COS Leukoplasts into Stroma and Envelope Membranes

Ruptured leukoplasts were subfractionated by discontinuous Suc density gradient centrifugation in the presence of buffer C according to the method of Alban et al. (1988). Centrifugation for 15 h at 95,000*g* resulted in the separation of three fractions: a supernatant on top of the tube, representing the stromal material; a yellow band at the 0.6 M/0.93 M Suc interface representing the plastid envelopes; and a yellow pellet at the bottom of the tube representing unlysed leukoplasts (Alban et al., 1988). The supernatant and the yellow band were removed successively from the top of the tube using a Pasteur pipet. The envelope and pellet fractions were washed with buffer C. All fractions including the stroma were centrifuged at 93,000*g* for 30 min. Envelope and pellet fractions were solubilized in 1 mL of buffer D.

Triton X-114 Treatment

Phase partitioning with Triton X-114 was performed as described by Bordier (1981). Ruptured leukoplasts at a final protein concentration of 2 mg/mL were suspended in 25 mM Hepes-KOH (pH 7.5), 150 mM KCl, and 1% (v/v) Triton X-114. The suspension was mixed gently on ice for 1 h and centrifuged in an Eppendorf microcentrifuge at 16,000*g* at 4°C for 2 min. The supernatant was loaded onto 2 volumes of 10 mM Hepes-KOH (pH 7.5), containing 6% (w/v) Suc, 150 mM KCl, and 0.06% (v/v) Triton X-114. The solution was incubated at 30°C for 5 min, followed by centrifugation at 300*g* for 5 min at room temperature. The detergent and aqueous phases were assayed for PK_p activity and analyzed by immunoblotting using anti-(COS PK_p) IgG.

Enzyme Purification

For PK_p purification, leukoplasts were isolated as described above. The final pellet was resuspended in 2.5 mL of 20 mM Hepes-KOH (pH 7.5) containing 1 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 1 mM PMSF, 1.5 mM DPDS, and 20% (v/v) glycerol, quick frozen in liquid N_2, and stored at −80°C. Except where indicated, all procedures were carried out at 0 to 4°C.

Leukoplast Extract

Quick-frozen COS leukoplasts were thawed, ruptured with a Potter-Elvehjem homogenizer, and filtered through a 0.2-μm membrane.

Anion-Exchange Chromatography

The clear filtrate was absorbed at 0.75 mL/min onto a prepacked Waters Protein Pak-Q 8 HR anion-exchange column (1 × 10 cm), which had been connected to a FPLC system and pre-equilibrated with 20 mM Mes-NaOH (pH 6.4) containing 20% (v/v) glycerol, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT, and 0.5 mM DPDS. The column was washed with 60 mL of this buffer, and PK_p activity was eluted by the application of a linear 0 to 600 mM KCl gradient (200 mL) in buffer A. The pooled peak activity fractions were concentrated to 0.8 mL with an Amicon (Toronto, Ontario, Canada) YM-30 ultrafilter, quick-frozen in liquid N_2, and stored overnight at −80°C.

Dialysis

The pooled, concentrated fractions (0.8 mL) from the Waters Q column were thawed and dialyzed for 3 h against two 200-mL aliquots of 20 mM Hepes-KOH (pH 8.0) containing 20% (v/v) glycerol, 2 mM DTT, 5 mM $MgCl_2$, 1 mM EDTA, and 0.3 mM DPDS.

ADP-Agarose Chromatography

Affinity chromatography on ADP-agarose was conducted at room temperature, since the COS PK_p failed to bind to this medium at 4°C. The dialyzed pooled fractions from the Waters Q column were absorbed at 0.5 mL/min onto a column (0.5 × 2.0 cm) of ADP-agarose pre-equilibrated with the dialysis buffer. The column was washed with 16 mL of this buffer, and PK_p activity was eluted with the same buffer containing 2 mM ADP. The peak activity fractions were pooled, concentrated to 0.5 mL in an Amicon Centricon 30 ultrafilter, quick-frozen in liquid N_2, and then stored at −80°C. The activity of the purified enzyme was stable for at least 6 weeks when stored frozen.

SDS-PAGE and Immunoblot Analysis

SDS-PAGE was performed in a Bio-Rad minigel apparatus according to the method of Laemmli (1970) using 1-mm-thick slab gels and a 7.5% (w/v) monomer concentration for the separating gel. Immunoblotting was performed using rabbit anti-(total pea chloroplast envelope protein) immune serum (Ko et al., 1992) or affinity-purified rabbit anti-(COS PK_p) IgG as previously described (Plaxton et al., 1990). Immunological specificity was confirmed by performing immunoblots in which rabbit preimmune se-

rum was substituted for the various IgGs. Subunit molecular masses were estimated by comparing the mobilities of the PK_p subunits with those of the following standard proteins: myosin (205 kD), β-galactosidase (116 kD), phosphorylase *b* (97.4 kD), BSA (66 kD), ovalbumin (45 kD), and carbonic anhydrase (29 kD).

Gel-Filtration FPLC and Nondenaturing IEF-PAGE

For gel-filtration and IEF-PAGE analyses the COS PK_p was partially purified from isolated leukoplasts using anion-exchange FPLC as described above, except that a Pharmacia Mono-Q HR 5/5 column (0.5 × 5 cm) was substituted for the Waters Protein Pak-Q column, and the volume for the KCl gradient was decreased to 50 mL (fraction size, 1 mL). PK_p activity eluted as a single, sharp peak at approximately 0.17 M KCl. The fraction containing maximal PK_p activity (specific activity, 2.6 units mg^{-1}) was concentrated 5-fold using an Amicon YM-30 ultrafilter and applied at 0.2 mL min^{-1} onto a prepacked Superose 6 HR 10/30 column connected to a FPLC system and pre-equilibrated with 50 mM Hepes-KOH (pH 7.5) containing 1 mM EDTA, 5 mM $MgCl_2$, 2 mM DTT, 50 mM KCl, 20% (v/v) glycerol, and 0.04% (w/v) NaN_3 (fraction size, 0.2 mL). The native M_r of the PK_p was determined as described by Plaxton et al. (1990).

A 10-μL aliquot of the Mono-Q fraction containing maximal PK_p activity was subjected to nondenaturing IEF-PAGE over the pH range of 5 to 9 using 0.75-mm-thick minigels as described by Bollag and Edelstein (1991). Following IEF, a lane was sliced into 2.5-mm segments, and the relative PK activity of each segment was determined as previously described (Plaxton et al., 1990). The pH gradient was determined by incubating gel segments excised from an adjacent lane in 1 mL of 10 mM KCl for 1 h at room temperature and measuring the pH of the resultant solutions with a pH microelectrode. For second-dimension PAGE, the gel segment containing maximal PK_p activity was incubated for 2.5 h at 32°C in 100 μL of 62 mM Tris-HCl (pH 6.8) containing 10% (v/v) glycerol, 100 mM DTT, and 2% (w/v) SDS. After equilibration in SDS, the gel segment was subjected to SDS-PAGE and immunoblotting using anti-(COS PK_p) IgG as described above.

N-Terminal Microsequencing

Purified COS PK_p was subjected to SDS-PAGE, blotted onto a Bio-Rad PVDF membrane, stained overnight with Ponceau S (Sigma), and destained with 1% (v/v) acetic acid. Polypeptides corresponding to PK_p's nondegraded α and β subunits were individually excised, washed with water, air dried, and sent to the Harvard Microchemistry Facility (Cambridge, MA) for N-terminal microsequencing.

Protein Determination

Protein concentration was determined according to the method of Bradford (1976) using bovine γ-globulin as a standard.

RESULTS AND DISCUSSION

Preparation and Rupture of COS Leukoplasts

The purified COS leukoplasts were found to be highly intact, as judged by latencies of 100, 82, and 86% obtained for the stromal enzymes 6-P-gluconate dehydrogenase, $NADP^+$-malic enzyme, and enolase, respectively. A gentle osmotic shock or freeze-thawing in isotonic buffer has been reported to effectively rupture chloroplast envelope membranes (Douce et al., 1973). However, either lysis technique ruptured only 40% of the envelopes of the purified COS leukoplasts. Analogous results have been reported for the nonphotosynthetic amyloplasts of cauliflower buds (Alban et al., 1988). However, Alban and co-workers (1988) attained almost complete rupture of cauliflower amyloplasts when they homogenized previously frozen plastids in a hypotonic medium using a Potter-Elvehjem apparatus equipped with a Teflon pestle. Application of this protocol to purified COS plastids also proved to be highly satisfactory because it ruptured about 90% of the envelopes of the intact COS leukoplasts. All subsequent experiments were performed using plastids that had been lysed by this technique.

Localization of PK_p in COS Leukoplasts

The distribution of PK_p in the three fractions that were obtained following Suc density gradient fractionation of ruptured COS leukoplasts was assessed by measurements of PK activity (Table I) and immunoblotting with anti-(COS PK_p) IgG (Fig. 1). Table I shows that approximately 90% of the total PK activity was recovered in the stromal fraction, with the remaining 10% in the pellet (representing unbroken leukoplasts). Essentially all (i.e. 93%) of the total PK activity in the leukoplast preparation that was layered on the Suc gradient was subsequently recovered in the stromal and pellet fractions. PK activity was not detected in the envelope fraction. This experiment was repeated at least four times with essentially identical results. PEP phosphatase activity was undetectable in all leukoplast subfractions.

The stromal and envelope fractions contained a distinct polypeptide complement as indicated by Coomassie blue staining of SDS gels of the respective preparations (Fig. 1A). Immunoblots of the ruptured leukoplasts, stroma, and pellet fractions were probed with anti-(COS PK_p) IgG and

Table I. *Distribution of PK_p activity in various fractions obtained after subfractionation of purified castor seed leukoplasts*

These results are from a representative experiment. Ruptured leukoplasts corresponding to 10.1 units of PK activity and 20.2 mg of protein were fractionated by discontinuous Suc gradient as described in "Materials and Methods."

Fraction	Activity		Protein	
	units	%	*mg*	%
Stroma	8.5	90	10.0	59
Envelope	0	0	0.5	3
Pellet	0.9	10	6.4	38
Total	9.4	100	16.9	100

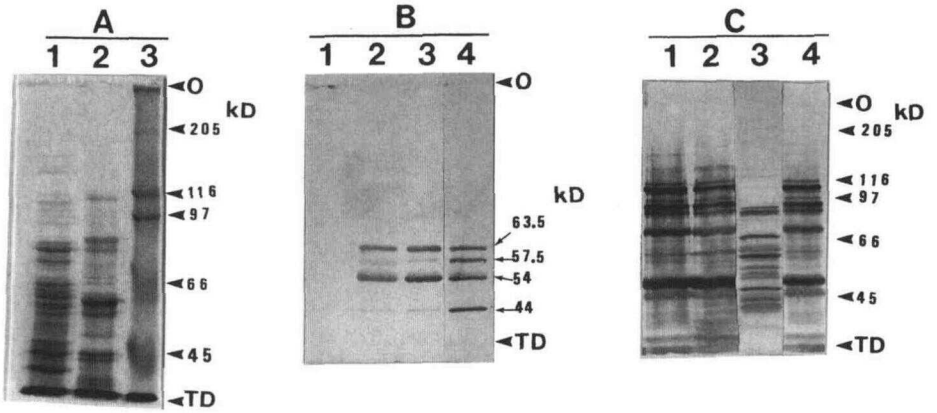

Figure 1. SDS-PAGE and immunoblot analysis of subfractions obtained following Suc gradient centrifugation of ruptured COS leukoplasts. A, Protein staining was performed with Coomassie blue R-250. Lanes 1 and 2 contained 20 μg of protein each of the stromal and envelope fractions, respectively. Lane 3 contained 3 μg of various molecular mass standards. B and C, Immunological detection of COS PK_p (B) and total envelope proteins (C). Samples (20 μg/lane) were subjected to SDS-PAGE and blot transferred to a PVDF membrane. Blots were probed with affinity-purified anti-(COS PK_p) IgG (B) (Plaxton et al., 1990) or anti-(total pea chloroplast envelope protein) immune serum (C) (Ko et al., 1992), and antigenic polypeptides were detected by using an alkaline phosphatase-conjugated secondary antibody. Lanes 1, Isolated envelopes; lanes 2, ruptured leukoplasts; lanes 3, stromal fraction; lanes 4, pellet. The molecular mass positions indicated were based on the mobility of standard molecular mass markers as described in "Materials and Methods." O, Origin; TD, tracking dye front.

revealed two major immunoreactive polypeptides of approximately 63.5 and 54 kD that corresponded to the α and β subunits, respectively, of nonproteolytic degraded COS PK_p (Fig. 1B, lanes 2–4) (Plaxton, 1991). The less-intense staining 57.5- and 44-kD antigenic polypeptides seen in Figure 1B (lanes 2–4) represent in vitro proteolytic degradation products of the α and β subunits, respectively (Plaxton, 1991). By contrast, no antigenic cross-reaction occurred when an immunoblot of an equivalent amount of protein from the leukoplast envelope fraction was probed with the anti-(COS PK_p) IgG (Fig. 1B, lane 1). Probing immunoblots of the same fractions with anti-(total pea chloroplast envelope protein) immune serum (Ko et al., 1992) demonstrated that the envelope fraction was enriched with plastid envelope proteins (Fig. 1C, lane 1).

To investigate further the localization of PK_p in isolated COS leukoplasts, we used Triton X-114 phase partitioning. When Triton X-114 is used to solubilize cellular proteins at low temperature and then is warmed to 30°C, the detergent phase contains the hydrophobic membrane proteins, whereas hydrophilic soluble proteins partition into the aqueous phase (Bordier, 1981). Following fractionation of ruptured COS leukoplasts with Triton X-114, all of the recovered PK activity was associated with the aqueous phase, with no activity detected in the detergent phase. Likewise, immunoblot analysis confirmed that anti-PK_p IgG immunoreactive polypeptides corresponding to the α and β subunits of PK_p were completely restricted to the aqueous phase (results not shown).

Our findings agree with those of Miernyk (1985), who reported the absence of a membrane association of the activities of PK and other glycolytic enzymes in COS leukoplasts. Our results are also similar to those of Wan and co-workers (1995), who observed immunoreactive polypeptides of 63 and 55 kD when immunoblots of COS leukoplast stromal fractions were probed with specific antibodies against overexpressed fusion proteins containing the carboxyl termini of the polypeptides encoded by PK_pA and PK_pG cDNAs, respectively. However, Wan et al. (1995) also observed an antigenic polypeptide of 66 kD (putatively representing the PK_pA preprotein) when immunoblots of developing COS leukoplast envelopes were probed with their anti-(COS PK_pA-fusion protein) IgG. By contrast, the results presented in Figure 1B (lane 1) demonstrate that this 66-kD envelope protein was not recognized by monospecific antibodies raised against purified COS PK_p that (a) effectively immunoprecipitate COS PK_p activity (Plaxton et al., 1990), (b) cross-react strongly with the enzyme's mature (63.5 kD) α subunit (Fig. 1B), and (c) were used to immunoselect the COS PK_pA cDNA used by Wan and co-workers (1995) to generate the the COS PK_pA fusion protein construct for overexpression in *Escherichia coli*. We argue that the results reported in Table I and Figure 1 and discussed above demonstrate that the COS PK_p is a soluble protein confined to the leukoplast stroma.

Purification of Nondegraded COS PK_p

A previous study demonstrated that several cysteinyl-modifying reagents such as iodoacetate and *p*-hydroxymercuribenzoate are potent inhibitors of the protease activity, which causes rapid, partial degradation of COS PK_p in vitro (Plaxton, 1991). However, these reagents either inactivate PK_p (W.C. Plaxton, unpublished data) or have the potential to covalently modify PK_p in a nonspecific manner and were therefore considered to be unsuitable for routinely inhibiting the degradation of PK_p during the enzyme's isolation. Since the endogenous Cys endopeptidase that causes partial proteolysis of COS PK_p in vitro is localized outside of the leukoplast (Plaxton, 1991; Cornel and

Plaxton, 1994), the strategy used to isolate nondegraded COS PK_p was to initiate the enzyme's purification with purified intact leukoplasts. The degradation of PK_p was substantially reduced in extracts prepared from purified leukoplasts, relative to extracts prepared from a COS endosperm homogenate (Fig. 2A, lanes 1 and 2). Negligible PK_p degradation was observed, however, when 1.5 mM DPDS was included in the leukoplast lysis buffer (Fig. 2A, lane 3). DPDS has been shown to partially alleviate PK_p's degradation during incubation of clarified COS endosperm homogenates at 4°C (Plaxton, 1991) and to act as a substrate analog of papain by specifically reacting with a Cys residue in the active site of the native, but not denatured, enzyme even in the presence of -SH reducing reagents such as DTT (Brocklehurst and Little, 1973). Because the experiment was started with isolated leukoplasts and included DPDS in purification buffers, the COS PK_p remained almost entirely intact during its purification, with only slight proteolysis of the enzyme's α subunit (Fig. 2A, lanes 4 and 5).

Table II shows a representative purification of PK_p from COS leukoplasts. When the leukoplast extract was subjected to anion-exchange chromatography on a Waters Protein Pak-Q column, a single, sharp peak of PK activity was resolved following application of a linear KCl gradient (Fig. 3A). ADP-agarose affinity chromatography of the pooled Waters Q peak fractions also resolved a single, sharp peak of PK activity (Fig. 3B). The PK_p purification resulted in a 68-fold purification with an overall yield of 13% (Table II). However, the preparation of the leukoplast fraction also represents an important purification of the enzyme. Thus, the actual purification of PK_p from a crude COS extract is more than 800-fold. The final specific activity of about 12 units mg^{-1} is significantly lower than the values of 41 and 200 units mg^{-1} obtained for the nearly homogeneous but proteolyzed COS PK_p (Plaxton et al., 1990) and homogeneous germinating COS endosperm PK_c (Plaxton, 1988), respectively. However, COS PK_p is known to be an extremely labile enzyme (Ireland et al., 1980; Plaxton et al., 1990). Plaxton and co-workers (1990) reported that in the absence of 50% (v/v) glycerol the activity of the purified degraded enzyme exhibited a half-life of less than 2 min. In the present study, activity losses during purification could be only partially alleviated by the inclusion of 20% (v/v) glycerol in all buffers. All attempts to further purify the enzyme resulted in enzyme inactivation or large reductions in specific activity.

Table II. *Purification of PK_p from leukoplasts isolated from endosperm of developing COS*

Fraction	Activity	Protein	Specific Activity	Purification	Yield
	units	*mg*	*units mg^{-1}*	*-fold*	*%*
Leukoplast extract	3.7	20.9	0.18	–	100
Waters-Q	2.2	0.67	3.3	18.3	60
ADP-agarose	0.46	0.038	12.3	68.3	13

SDS-PAGE of the final preparation resolved three major Coomassie blue staining bands of 69, 63.5, and 54 kD (Fig. 2B). Since only the 63.5- and 54-kD polypeptides cross-reacted with anti-(COS PK_p) IgG (Fig. 2A, lane 5), this indicates that the 69-kD protein-staining band is a contaminating polypeptide. The 63.5- and 54-kD PK_p polypeptides co-purified in an approximate 1:1 ratio during anion-exchange and ADP-agarose chromatographies (Fig. 2), as well as during hydrophobic interaction FPLC of a leukoplast extract on Phenyl Superose (results not shown).

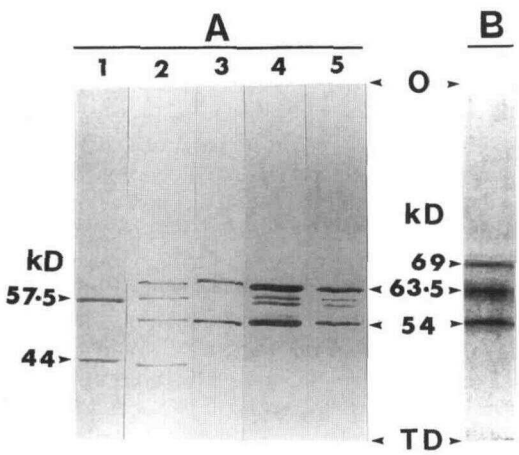

Figure 2. Immunoblot and SDS-PAGE analysis of PK_p from developing COS. A, Immunological detection of COS PK_p. Samples were subjected to SDS-PAGE and blot transferred to a PVDF membrane. Blots were probed with affinity-purified anti-(COS PK_p) IgG (Plaxton et al., 1990), and antigenic polypeptides were detected by using an alkaline phosphatase-conjugated secondary antibody. Lane 1 contained 20 μg of protein of a clarified extract prepared from whole, developing COS endosperm in the absence of any protease inhibitors and incubated for 21 h at 4°C. Lanes 2 and 3 contained 20 μg each of protein of a COS leukoplast extract prepared in the absence and presence of 1.5 mM DPDS, respectively. Lane 4 contained 4 μg of the concentrated Waters Q column pooled fractions. Lane 5 contained 0.1 μg of protein of the concentrated ADP-agarose pooled fractions. B, SDS-PAGE of 2 μg of protein of the concentrated ADP-agarose pooled fractions. The gel was stained with Coomassie blue R-250. The molecular mass positions indicated were based on the mobility of standard molecular mass markers as described in "Materials and Methods." O, origin; TD, tracking dye front.

Analysis of Nondegraded COS PK_p by Gel-Filtration FPLC and Nondenaturing IEF-PAGE

The peak PK_p activity fraction obtained following Mono-Q chromatography of a COS leukoplast extract was analyzed by Superose 6 FPLC and nondenaturing IEF-PAGE. In each instance a single, major peak of PK activity was resolved (Figs. 4 and 5). The native molecular mass of the enzyme as estimated by Superose 6 FPLC was 334 kD ($n = 2$). This is about 30 kD greater than the native molecular mass reported for the proteolytically degraded COS PK_p (Plaxton et al., 1990), and it is significantly greater than the values of 200 to 240 kD estimated for most other plant and nonplant PKs (Plaxton, 1988, 1989; Podestá and Plaxton, 1994, and refs. therein). SDS-PAGE of fractions 73 to 77 from the Superose 6 column demonstrated that the peak of PK_p activity co-eluted with 63.5- and 54-kD polypeptides that silver stained with similar intensities (Fig. 4, inset) and cross-reacted strongly with the anti-(COS PK_p) IgG (not

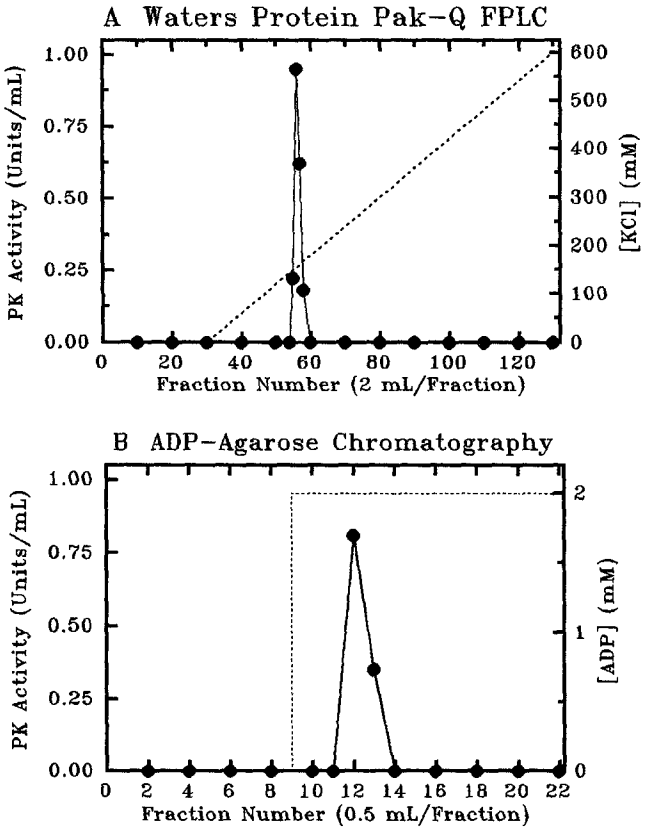

Figure 3. Purification of PK_p from leukoplasts isolated from endosperm of developing COS. A, Waters Protein Pak-Q anion-exchange FPLC. B, ADP-agarose affinity chromatography. Details of each procedure are described in the text. ●, PK activity; - - - - -, KCl concentration as estimated by the gradient programmer of the FPLC system (A) or ADP concentration (B). The relative A_{280} could not be determined owing to interference by the DPDS present in the column buffers.

shown). Similarly, when the gel segment containing maximal PK activity that was obtained following IEF-PAGE was equilibrated with SDS, subjected to SDS-PAGE, and immunoblotted using the anti-(COS PK_p) IgG, immunoreactive polypeptides of 63.5 and 54 kD were resolved that stained with similar intensities (Fig. 5, inset).

Overall, the data presented in Figures 2 to 5 are consistent with our previous studies (Plaxton et al., 1990; Plaxton, 1991) and indicate that nonproteolyzed native COS PK_p exists as a heterohexameric protein composed of equal proportions of α (63.5 kD) and β (54 kD) subunits.

N-Terminal Microsequencing

Polypeptides corresponding to the nonproteolyzed α and β subunits of COS PK_p (purified according to Table II) were separated by SDS-PAGE, electroblotted onto a PVDF membrane, and subjected to N-terminal microsequencing. A sequence of nine amino acid residues of the N terminus for the intact PK_p α subunit is identical with a portion of the deduced amino acid sequence for the COS PK_pA cDNA clone isolated by Blakeley and co-workers (1991) (Fig. 6A). This confirms that the PK_pA clone encodes the α subunit of COS PK_p. Furthermore, comparison of the N-terminal sequence of PK_p's α subunit with the cDNA-deduced PK_pA sequence indicates that a 44-amino acid (or 4.8 kD) transit peptide is cleaved from the PK_p-α preprotein following its import from the cytosol into the leukoplast (Fig. 6A). This is consistent with import studies of in vitro translated, ^{35}S-labeled COS PK_pA into COS leukoplasts, which estimated by SDS-PAGE that a 3-kD transit peptide was cleaved from the PK_pA preprotein during import (Wan et al., 1995). Computer analysis of the deduced primary structure of the processed (mature) COS PK_p α subunit indicates that this protein has an actual M_r of 59,330 and a pI of 4.88 (S.D. Blakeley, personal communication).

N-terminal analysis of the β subunit of COS PK_p yielded a sequence of 10 amino acid residues (Fig. 6B). Although a partial cDNA clone designated PK_pG has been isolated and sequenced from a developing COS cDNA library (Blakeley et al., 1995), this clone is missing a portion of its 5′ end. Hence, the deduced N-terminal sequence for the polypeptide encoded by this cDNA is unavailable. However, a full-length PK_pG cDNA, which shows 89% homology at the amino acid level with the COS PK_pG gene, has been isolated and sequenced from a *B. napus* developing embryo cDNA library (K.P. Cole, S.D. Blakeley, and D.T. Dennis, personal communication). Immunoblotting studies have revealed that, similar to the enzyme from developing COS, the PK_p from developing *B. napus* embryos appears to be composed of an equal ratio of α and β subunits having molecular masses of approximately 64 and 58 kD, respectively (Sangwan et al., 1992). As shown in Figure 6B, the N-terminal sequence for the nondegraded β subunit of COS PK_p is very similar to a portion of the deduced amino acid sequence for the *B. napus* PK_pG cDNA clone. This indicates that the PK_pG gene encodes the β subunit of PK_p. Furthermore, comparison of the N-terminal amino acid sequence of COS PK_p's β subunit with the deduced amino acid sequence for the *B. napus* PK_pG gene indicates the cleavage of a 50-amino acid (5.5 kD) transit peptide from the PK_pG preprotein (Fig. 6B). This is consistent with import studies of in vitro translated, ^{35}S-labeled PK_pG into COS leukoplasts, which estimated by SDS-PAGE that a 6-kD transit peptide was cleaved from the PK_pG preprotein during import (Wan et al., 1995). Computer analysis of the deduced primary structure of the processed (mature) *B. napus* PK_p β subunit indicates that this protein has an M_r of 57,450 and a pI of 5.87 (S.D. Blakeley, personal communication).

CONCLUDING REMARKS

Conditions that effectively rupture chloroplast envelope membranes (i.e. freeze-thaw in isotonic buffer) were completely unsuitable for rupturing COS leukoplasts. However, efficient lysis of COS leukoplasts was achieved by homogenizing previously frozen plastids in a hypotonic buffer.

That PK_p is confined to the stroma of COS leukoplasts was demonstrated by the sequestration of PK_p activity and anti-(COS PK_p) immunoreactive polypeptides into the (a)

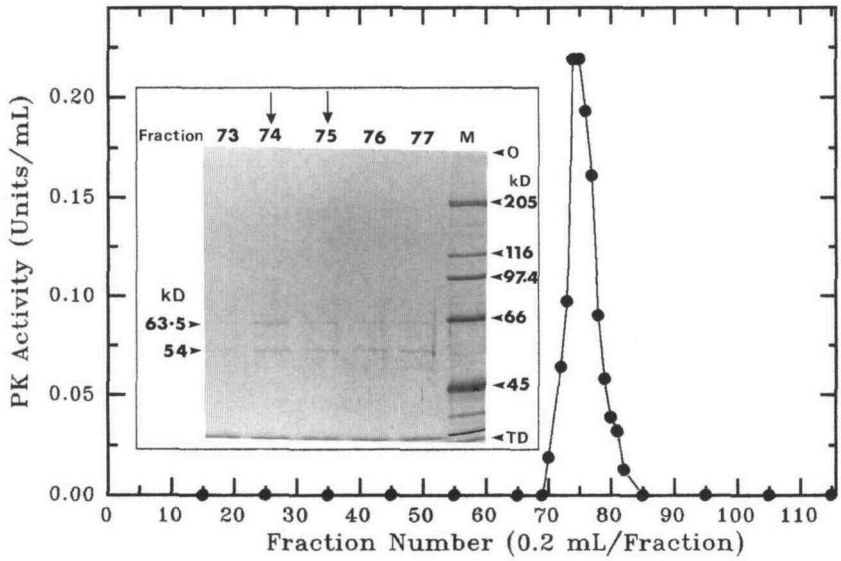

Figure 4. Superose 6 gel-filtration FPLC of partially purified PK_p from developing COS. Details of the procedure are described in the text. Inset, SDS-PAGE of 10-μL aliquots of the PK_p activity peak fractions (73–77) obtained following Superose 6 FPLC. The arrows indicate the peak PK_p activity fractions. The lane labeled M contained 2 μg of various molecular mass standards. The gel was stained with silver as described by Hochstrasser et al. (1988). O, Origin; TD, tracking dye front.

stroma, but not envelope membranes, of COS leukoplasts that had been subfractionated by Suc density gradient centrifugation (Table I; Fig. 1B) and (b) aqueous, but not detergent, phase of COS leukoplasts subjected to Triton X-114 phase partitioning.

The protein biochemical data of the present and earlier (Plaxton et al., 1990; Plaxton, 1991) studies consistently indicate that the nonproteolyzed native PK_p of developing COS exists as a heterohexameric protein composed of equivalent proportions of α and β subunits having molecular masses of about 63.5 and 54 kD, respectively. This conclusion is compatible with the observation that the 63.5- and 54-kD PK_p polypeptides are expressed in a constant 1:1 ratio throughout COS development and maturation (Plaxton, 1991). The kinetic and regulatory properties of purified nondegraded COS PK_p are currently being investigated.

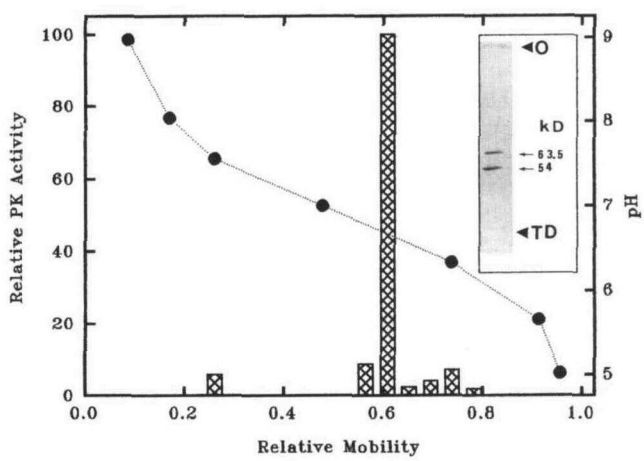

Figure 5. Nondenaturing IEF-PAGE of partially purified PK_p from developing COS. Details of the procedure are described in the text. ●, pH gradient. Inset, SDS-PAGE followed by immunoblotting with anti-(COS PK_p) IgG of the IEF-PAGE gel segment containing maximal PK_p activity that had been equilibrated with SDS as described in "Materials and Methods." O, Origin; TD, tracking dye front.

The ability to isolate nondegraded PK_p will also facilitate the analysis of the effect of proteolysis on the enzyme's catalytic properties.

Comparison of the respective N-terminal sequences of the nonproteolyzed COS PK_p subunits with the deduced amino acid sequences for several cDNAs revealed that the enzyme's α and β subunits are encoded by COS genes previously designated as PK_pA and PK_pG, respectively. Although the evidence presented here and elsewhere

A

NH_2-M S Q S L H F S P N L T F A K Q P F P K L P L P F P T S N S R Y P V N N Y K S L S I

K A ⇓ S T S P S S S S D P Q V L V A D N G T G N S G V L Y N N N N K S V T V S D P ---- COO-

S T S P S S S - D P *K S V T V S D P*

B

NH_2-M A Q V V A T R S I Q G S M L S P N G G S A S T R S D K F L K P A S F A V K V L G N E A

K K S G R V S V R G G R K V D T ⇓ T V R S A R V E T E V I P ----- COO-

A N R S T R V E T E

Figure 6. Alignment of the N-terminal amino acid sequences for the nonproteolyzed α and β subunits of COS PK_p with the N-terminal portions of the deduced amino acid sequences for COS PK_pA (Blakeley et al., 1995) (A) and *B. napus* PK_pG (K.P. Cole, S.D. Blakeley, and D.T. Dennis, personal communication) (B) cDNA clones. A, Bold and underlined letters represent the amino acids determined by N-terminal sequencing of the intact (63.5 kD) α subunit of COS PK_p. Italicized and underlined letters represent the N-terminal amino acid sequence previously determined by Blakeley et al. (1991) for the 57.5-kD COS PK_p α subunit that had been proteolyzed during the enzyme's purification from a developing COS endosperm homogenate (Plaxton et al., 1990). The arrow indicates the processing site for the 44-amino acid transit peptide that is cleaved during import of the α subunit preprotein into the leukoplast. The dashes represent the remainder of the cDNA deduced amino acid sequence. B, Bold and underlined letters represent the amino acids determined by N-terminal sequencing of the intact (54 kD) β subunit of COS PK_p. The arrow indicates the processing site of a putative 60-amino acid transit peptide that is cleaved during the import of the β subunit preprotein into the leukoplast. The dashes represent the remainder of the cDNA-deduced amino acid sequence.

(Plaxton et al., 1990; Plaxton, 1991) is inconsistent with the suggestions that the PK_pA and PK_pG genes encode distinct native isozymes of PK_p in developing COS (Blakeley et al., 1995; Wan et al., 1995), we cannot discount the possibility that PK_pA or PK_pG are independently expressed into active homomeric PK_p isoforms in other tissues of the castor oil plant.

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