Purification and Characterization of the Pea Chloroplast Pyruvate Dehydrogenase Complex[1]

A SOURCE OF ACETYL-CoA AND NADH FOR FATTY ACID BIOSYNTHESIS

PAMELA J. CAMP[2] AND DOUGLAS D. RANDALL*
Department of Biochemistry, University of Missouri, Columbia, Missouri 65211

ABSTRACT

The pyruvate dehydrogenase complex has been purified 76-fold, to a specific activity of 0.6 μmoles per minute per milligram protein, beginning with isolated pea (*Pisum sativum* L. var Little Marvel) chloroplasts. Purification was accomplished by rate zonal sedimentation, polyethylene-glycol precipitation, and ethyl-agarose affinity chromatography. Characterization of the substrates as pyruvate, NAD^+, and coenzyme-A and the products as NADH, CO_2, and acetyl-CoA, in a 1:1:1 stoichiometry unequivocally established that activity was the result of the pyruvate dehydrogenase complex. Immunochemical analysis demonstrated significant differences in structure and organization between the chloroplast pyruvate dehydrogenase complex and the more thoroughly characterized mitochondrial complex. Chloroplast complex has a higher magnesium requirement and a more alkaline pH optimum than mitochondrial complex, and these properties are consistent with light-mediated regulation *in vivo*. The chloroplast pyruvate dehydrogenase complex is not, however, regulated by ATP-dependent inactivation. The properties and subcellular localization of the chloroplast pyruvate dehydrogenase complex are consistent with its role of providing acetyl-CoA and NADH for fatty acid synthesis.

The pyruvate dehydrogenase complex is a multicomponent system composed of the three enzymes, pyruvate dehydrogenase, dihydrolipoyl transacetylase, and dihydrolipoyl dehydrogenase. The irreversible reaction catalyzed by PDC[3] results in the oxidative decarboxylation of pyruvate according to the equation:

$$\text{Pyruvate} + \text{CoA} + NAD^+ \rightarrow \text{Acetyl-CoA} + CO_2 + \text{NADH} + H^+$$

Additionally, PDC activity is dependent on the coenzymes TPP, lipoic acid, flavin adenine dinucleotide, and divalent cations.

In animals and fungi PDC has been localized in the mitochondria (17). Plant cells, though, are typified by a greater degree of subcellular organization than other eukaryotes. All higher plants examined to date contain a typical PDC located within the mitochondrial compartment (15, 18, 19). Additionally, a second subcellular location of PDC, unique to plant cells, is within the plastids. Plastid PDC activity has been demonstrated in both green (6, 27) and nongreen (18) plant tissues.

Mitochondrial PDC catalyzes the entry of carbon into tricarboxylic acid cycle and related metabolism. Plastid PDC, on the other hand, provides acetyl-CoA for lipid biosynthesis. This role for plastid PDC has been well established in developing castor oil seeds, where large quantities of fatty acids are synthesized for storage (5). Similarly, chloroplast PDC has been postulated to supply acetyl-CoA for *de novo* synthesis of fatty acids in green tissues (27), a process demonstrated to occur exclusively in chloroplasts (14).

It has been widely demonstrated that PDC catalyzes a nonequilibrium reaction. One form of regulation generally observed is at the level of product inhibition (15, 20). There is also evidence for regulation of mitochondrial PDC by interconversion between an inactive and active form via a reversible covalent modification (11, 15, 16). Phosphorylation (inactivation) of pyruvate dehydrogenase is catalyzed by a tightly associated kinase and dephosphorylation (activation) by a more loosely associated phosphatase (10).

The pivotal position of PDC in metabolism has resulted in a vast amount of research on the enzyme from nonplant tissues. By comparison, relatively little is known about plant PDC, and most information that is available concerns the mitochondrial form of the enzyme. The limited knowledge of plastid PDC prompted the current investigation with the purpose of establishing the chloroplast enzyme as a true PDC with properties distinct from the mitochondrial enzyme and consistent with its postulated role in chloroplast fatty acid biosynthesis.
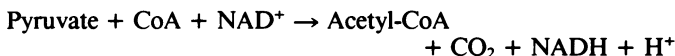
MATERIALS AND METHODS

Chemicals. Pyridine nucleotides, ATP, and CoA were obtained from P. L. Biochemicals. Nitrocellulose paper and electrophoretic materials were from Bio-Rad. Glycerol was obtained from J. T. Baker. Brays solution was from Research Products International and [1-^{14}C]pyruvate from ICN. Buffers were obtained from Research Organics, Inc. All other materials were from Sigma or Fischer Scientific. Purified *Escherichia coli* PDC was generously provided by Professor L. J. Reed.

Plant Material. Pea seeds (*Pisum sativum* L. var Little Marvel) were soaked overnight in running tap water and planted in vermiculite. Plants were grown for 10 to 12 d in a greenhouse under ambient light conditions and with a night-day temperature range of 21 to 32°C. Green shoots (2–4 cm) were harvested early in the morning prior to significant starch accumulation and immediately used for chloroplast isolation.

Isolation of Chloroplasts. Chloroplasts were isolated by the method of Williams and Randall (27). Pea shoots (75 gm) were

[2] Present address: Biology Department, University of Dayton, 300 College Park, Dayton, OH 45469.

[3] Abbreviations: PDC, pyruvate dehydrogenase complex; RuBPCase, ribulose-1,5-bisphosphate carboxylase; TPP, thiamine pyrophosphate.

homogenized in 150 ml of a partially frozen slush consisting of 0.33 M D-sorbitol, 50 mM Tricine (pH 7.8), 4.4 mM $Na_2P_4O_7$, 1 mM EDTA, and 3.5 mM $MgCl_2$, using a modified Braun homogenizer with 4-s high-speed bursts. Homogenates were filtered through two followed by eight layers of cheesecloth and then centrifuged at 3,020g for 90 s (start to stop). Chloroplast pellets were washed twice by resuspension in grinding buffer and repelleting. The final pellet was resuspended in 50 mM Hepes (pH 7.6), 1 mM $MgCl_2$, and 14 mM 2-mercaptoethanol. To facilitate breakage, chloroplasts were frozen and stored at −20°C until processed.

Preparation of Mitochondrial and Chloroplast PDC. A chloroplast-enriched preparation was used as the starting material for purification of plastid PDC. Mitochondrial contamination was assessed by measuring NAD^+-dependent isocitrate dehydrogenase (EC 1.1.1.41), and citrate synthetase (EC 4.1.3.7) activities and none were observed after the second wash. Frozen chloroplasts were thawed and diluted to 8.5 mg protein ml^{-1} resulting in a final solution containing 50 mM Hepes (7.6), 1 mM $MgCl_2$, 1 mM benzamidine, 1 mM ξ-aminocaproic acid, 5 μM leupeptin, 14 mM 2-mercaptoethanol, and 200 mM NaCl (buffer A). After stirring for 30 min the extract was centrifuged at 12,000g for 12 min. The resulting supernatant (stromal extract) was made 3% with respect to PEG by dropwise addition of a 50% (w/v) solution of PEG-8,000. The solution was allowed to equilibrate for 30 min on ice followed by centrifugation at 12,000g for 10 min. The green pellet was discarded and the supernatant brought to a final concentration of 7% PEG and allowed to equilibrate for 30 min. Following centrifugation at 12,000g for 12 min, the pellet was resuspended in buffer A plus 1 mM pyruvate, but only 100 mM NaCl (buffer B). To remove membranes, the preparation was centrifuged 15 min at 80,000g in a Ti 50 rotor using a Beckman L8-55 Ultracentrifuge. The resulting supernatant was then centrifuged for 4 h at 183,000g. The pellet was resuspended in a minimum volume of buffer B and applied to a 50-ml ethyl-agarose column equilibrated with the same buffer. Fractions containing PDC activity were pooled and the enzyme again centrifuged at 183,000g. The pellet was resuspended in a minimum volume of buffer B and either used immediately or frozen for later use. The entire purification was performed at 4°C.

An acetone powder of pea leaf mitochondria was prepared as described by Randall *et al.* (16) and stored at −20°C until used.

Immunochemical Analysis. Antibodies to broccoli (*Brassica oleracea* var *italica*) floral bud mitochondrial PDC were obtained as described by Randall *et al.* (16) and frozen at −20°C until used. Antibodies were prepared to apparently homogeneous complex as determined by analytical ultracentrifugation (19). The antibodies were purified by $(NH_4)_2SO_4$ fractionation and DEAE-Affigel-Blue according to the Bio-Rad technical bulletin. Antibodies to purified *E. Coli* PDC were similarly prepared. Chloroplast PDC was purified through the first ultracentrifugation step and the resulting pellet resuspended in 50 mM Hepes (pH 7.6) and 5 mM DTT. Pea mitochondrial PDC (prepared as described above) was resuspended using the same buffer. Equal activities of PDC from mitochondria, chloroplasts, and *E. coli* were incubated overnight on ice with varying concentrations of antibodies to either broccoli mitochondrial PDC or *E. coli* PDC. Aliquots of enzyme were also incubated with preimmune serum. After incubation all samples were centrifuged at 12,000g for 4 min and the supernatants assayed for PDC activity.

Partially purified chloroplast and mitochondrial PDC were analyzed by SDS-PAGE using the discontinuous buffer system of Laemmli (9). A linear 9 to 12.5% gradient acrylamide separating gel, with a 5% stacking gel, was electrophoresed in a Hoeffer slab gel apparatus at 30 mamps constant current for 4 h at 4°C. Prior to electrophoresis, samples were dissociated at 50°C for 30 min in 4 M urea, 2% 2-mercaptoethanol, and 2% SDS.

Proteins from the SDS gel were then electrophoretically transferred onto nitrocellulose in 12.4 mM Tris, 96 mM glycine (pH 8.3) containing 20% (v/v) methanol for 12 h at 200 mamps. Sigma high mol wt markers were also electroblotted. These markers and M_r values (in parenthesis) are carbonic anhydrase (29,000); egg albumin (45,000); bovine albumin (66,000); phosphorylase b (97,400); β-galactosidase (116,000); and myosin (205,000).

The standard lane was removed and stained for protein with 0.1% Amido Black in methanol:acetic acid:water (45:7:48, v/v) and destained in methanol:acetic acid:water (45:10:45, v/v).

The remaining nitrocellulose blot was incubated with antibodies to broccoli mitochondrial PDC, *E. coli* PDC, or preimmune serum. Antigens were detected with goat antirabbit antibody conjugated with alkaline phosphatase. A more detailed description of this procedure is in preparation.

Assay Procedures. PDC activity was determined spectrophotometrically according to Williams and Randall (27) with slight modifications. The final 1 ml assay mixture contained: 50 mM Tricine (pH 8.0), 0.2 mM TPP, 5 mM $MgCl_2$, 1.4 mM NAD^+, 0.12 mM CoA, 2.6 mM cysteine-HCl, and 1.5 mM pyruvate. The formation of NADH was monitored at 340 nm on a Gilford model 240 recording spectrophotometer. This procedure was used for substrate specificity and metal ion requirement studies with the appropriate substitutions made as indicated in "Results". One unit of PDC activity is defined as 1 μmol NADH formed per min.

PDC was also assayed radiochemically with $[1-^{14}C]$pyruvate (0.11 Ci/mol) using the same reaction mixture as used for the spectrophotometric procedure. Assays were performed in glass vials topped with inverted scintillation minivials containing a 2-cm^2 glass microfiber filter (Whatman GF/A) impregnated with 175 μl of 5% KOH. The two vials were connected with a rubber sleeve through which the reaction was stopped by injection of 100 μl of 1 N acetic acid (final pH = 3–4). After 1 h the amount of $^{14}CO_2$ released was determined by liquid scintillation spectroscopy in a Beckman LS-7000 scintillation counter.

PDC activity was additionally determined by measurement of acetyl-CoA production. The assay mixture was the same as for the spectrophotometric assay except 50 mM phosphate buffer was substituted for Tricine. The reaction was stopped by addition of 40 μl of 10% $HClO_4$ and subsequently neutralized with 25 μl of 1 M K_2CO_3. The sample was then centrifuged at 12,000g for 2 min and passed through a 0.45 μm filter. Acetyl-CoA was measured by the HPLC method of Ingebretson and Farstad (8) using a 250 × 4.6 mm column packed with 5 μm beads. The mobile phase consisted of 220 mM K-phosphate (pH 4) and 12% (v/v) methanol, at a flow rate of 1 ml·min^{-1} at 2500 p.s.i. The eluant was monitored at 250 nm using 0.32 mM thymidine as an internal standard. The retention times in min for substrates and reaction products are NAD^+, 3.3; TPP, 2.7; CoA, 12.4; NADH, 4.5; thymidine, 8.9; and acetyl CoA, 37.5. Other substrates and products showed no A at 250 nm.

Glycerol concentrations were monitored by refractometry. NAD^+-isocitrate dehydrogenase (4) and citrate synthase (12) were assayed by standard procedures. Dihydrolipoyl dehydrogenase was assayed by the method of Reid *et al.* (18). Protein was determined by the method of Bradford (2) using BSA as the standard.

Glycerol Gradients. A 0.3-ml sample from the first ultracentrifugation step was loaded onto a 17-ml, linear 10 to 50% (w/v) glycerol gradient. The gradient contained 50 mM Hepes (pH 7.6), 1 mM $MgCl_2$, 1 mM pyruvate, 14 mM 2-mercaptoethanol, 5 μM leupeptin, 1 mM benzamidine, 1 mM ξ-aminocaproic acid, and 100 mM NaCl. Bovine heart and *E. coli* PDCs were analyzed in an identical manner. Gradients were centrifuged for 16 h at 25,000 rpm in a Beckman L8-55 ultracentrifuge using an

SW-28.1 rotor.

Phosphorylation Experiments. Isolated chloroplasts were broken by resuspension in 0.02% Triton X-100, 50 mM Hepes (pH 7.6), and 5 mM DTT. After a 30-min incubation on ice, the reaction mixture was centrifuged for 10 min at 12,000g. Pea leaf mitochondrial PDC was prepared as described for immunochemistry. the chloroplast and mitochondrial complexes were incubated at room temperature for 10 min with 1 mM ATP and aliquots assayed for PDC activity (15, 16). In some experiments, mitochondrial and chloroplast PDCs were mixed both in the presence and absence of ATP. Similarly, chloroplast complex was incubated with beef heart protein kinase catalytic subunit or phosphorylase kinase and ATP.

RESULTS

Chloroplast PDC Preparation. A typical purification scheme for chloroplast PDC from pea leaves is summarized in Table I. Enzyme from stromal extracts of broken chloroplasts was pelleted with 7% PEG-8000 resulting in a 10- to 15-fold purification and recoveries of PDC activity in excess of 95%. PDC was then pelleted by ultracentrifugation with a 1.2- to 1.6-fold enrichment and recoveries about 85%. Application of the enzyme from the ultracentrifugation step to an ethyl-agarose column resulted in a 2- to 3-fold enrichment with a 40 to 50% recovery of activity. Ultracentrifugation of pooled PDC fractions from the column characteristically resulted in a doubling of specific activity and a recovery of approximately 70%. The final specific activity of PDC was around 0.6 units·mg^{-1} with a 23% recovery of activity from the original chloroplast extracts. The partially purified enzyme was stable at $-20°C$ for at least 2 weeks with only a 10 to 20% loss of activity. Repeated thawing and refreezing, however, resulted in considerable loss of PDC activity.

Substrate Specificity, Cofactor Requirements, and pH Optimum. The chloroplast PDC had an absolute requirement for NAD$^+$, CoA, and pyruvate (Table II). The partially purified complex utilized hydroxypyruvate and 2-ketobutyrate at 9% of the rate of pyruvate but showed no activity with glyoxylate, 2-ketoglutarate, 2-ketoisocaproate, or 2-ketoisovalerate. Omission of TPP from the reaction mixture resulted in only a 2% loss of activity when enzyme that had been chromatographed on Sephadex G-25 or dialyzed in the absence of TPP was used. NADP$^+$ could only slightly replace NAD$^+$, giving a rate of 4% of that with NAD$^+$.

The metal ion requirements of chloroplast PDC are shown in Figure 1. Mg^{2+} best fulfilled the requirement for a divalent cation with a K_m of 1.0 mM. Both Ca^{2+} and Mn^{2+} could partially replace Mg^{2+}, though neither was as effective.

The activity of partially purified chloroplast PDC was measured over the pH range of 6.4 to 8.8. Optimal activity was observed at 8.0 with 50% maximal activity at pH 7.1 and 8.8 (data not shown).

Products of Plastid PDC. The products of the PDC reaction are NADH, CO$_2$, and acetyl-CoA. These products were measured as described in "Materials and Methods" with each experiment repeated three times with at least three replications per point. Simultaneous determination of decarboxylation and NAD$^+$ reduction showed 0.11 ± 0.01 μmol·min^{-1}·ml^{-1} CO$_2$ released and 0.09 ± 0.006 μmol·min^{-1}·ml^{-1} NADH formed. Similarly, in separate experiments, the formation of 0.053 ± 0.004 μmol·min^{-1}·ml^{-1} acetyl-CoA and 0.055 ± 0.003 μmol·min^{-1}·ml^{-1} NADH was observed.

Immunochemical Analysis. Antibodies to PDC from mitochondria of broccoli floral buds were used for precipitation studies of pea mitochondrial and chloroplast enzyme complexes. Figure 2 shows the results of a representative experiment. At twice the concentration of antibody required to precipitate all of the mitochondrial PDC, only 40% of an equal activity of chloroplast enzyme had precipitated. Addition of preimmune serum had no effect. Activity lost from the supernatant could be measured quantitatively in the resuspended pellet; thus, loss of activity from the supernatant was a result of precipitation of PDC enzyme and not caused by inactivation. Antibodies to the *E. coli* complex did not result in any precipitation of chloroplast enzyme even at concentrations four times that required to totally precipitate an equal activity of *E. coli* PDC (data not shown).

A more detailed immunological analysis was performed with Western blots of the pea chloroplast and mitochondrial complexes. The banding patterns observed for the two complexes are different but do have 98,000 and 58,000 M_r bands in common (Fig. 3). A diffuse band was consistently observed with the chloroplast PDC at approximately 66,000 M_r that could not be removed or further resolved. The identity of observed bands is under investigation. Antibodies to *E. coli* PDC did not react with pea chloroplast enzyme but did show two strong bands with *E. coli* enzyme. No bands were observed with preimmune serum.

Mol Wt Estimation. Enzyme purified through the first ultracentrifugation was used for mol wt estimation by sedimentation on glycerol gradients (Fig. 4). Application of more highly purified enzyme resulted in 85% or greater losses of activity. Sucrose gradients resulted in greater losses of activity than glycerol gradients. An accurate estimation of the complex mol wt cannot be made as enzyme activity appeared as a broad peak (fractions 18–24) over one-fourth of the gradient as opposed to the relatively sharp peaks obtained with *E. coli* or bovine mitochondrial PDC (Fig. 4). Attempts to improve gradient resolution of the complex using detergent (Triton X-100), low or high ionic strengths (0–0.5 M NaCl), or inclusion of other reaction constituents (NAD$^+$ and TPP) were unsuccessful.

Phosphorylation Experiments. Table III shows the results of a representative attempt to phosphorylate chloroplast PDC. Addition of 1 mM ATP to a preparation of broken chloroplasts did not result in any loss of PDC activity, whereas similarly treated mitochondrial PDC was completely inactivated. Increasing ATP up to 5 mM or incubating with ATP for longer time periods also did not result in inactivation of the chloroplast PDC (data not shown). Mitochondrial PDC was incubated with the chloroplast enzyme and ATP to determine whether the mitochondrial PDC kinase could inactivate chloroplast PDC. As shown in Table III,

Table I. *Purification of the Pyruvate Dehydrogenase Complex from Pea Chloroplasts*

Fraction	Total Activity[a]	Total Protein	Specific Activity	Purification	Recovery
	units	*mg*	*units/mg*	*—fold*	*%*
Stromal extract	8.74	1083.0	0.008	1	100
PEG fraction (3–7%)	8.38	79.8	0.105	13	96
Ultracentrifugation	6.73	53.9	0.125	15	77
Pooled ethyl-agarose	2.84	4.2	0.340	42	32
Ultracentrifugation	2.05	3.4	0.610	76	23

[a] One unit of activity is defined as 1 μmol of NADH formed per min.

Table II. *Substrate and Cofactor Requirements*

Substrate and cofactor requirements of pea chloroplast PDC. Partially purified enzyme was chromatographed on a Sephadex G-25 column equilibrated with 50 mM Hepes (pH 7.6) and 14 mM 2-mercaptoethanol. The desalted enzyme was then used for spectrophotometric determination of substrate and cofactor requirements.

Assay Mixture	Relative Activity
	%
Complete	100
−TPP	98
−CoA	0
−NAD	0
+2.3 mM NADP	4
−Pyruvate	0
+1.5 mM hydroxypyruvate	9
+1.5 mM glyoxylate	0
+1.5 mM 2-ketobutyrate	9
+1.5 mM 2-ketoglutarate	0
+3.0 mM 2-ketoisocaproate	0
+3.0 mM 2-ketoisovalerate	0

FIG. 2. Immunoprecipitation of chloroplast and mitochondrial PDCs. Antibodies to broccoli mitochondrial PDC were used to attempt precipitation of equal units of PDC activity from pea chloroplasts or mitochondria.
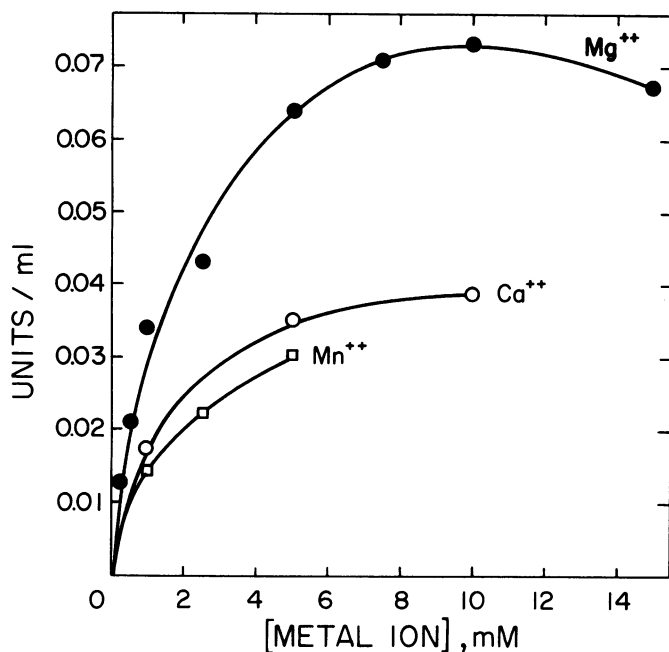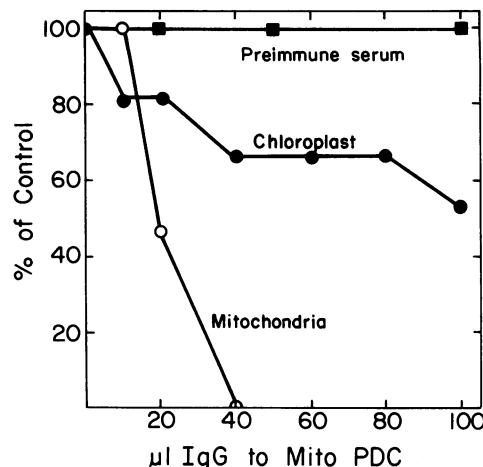

FIG. 1. Divalent cation specificity of pea chloroplast PDC. Partially purified enzyme was chromatographed on Sephadex G-25 equilibrated with 50 mM Hepes, 0.1 mM EDTA, and 14 mM 2-mercaptoethanol immediately prior to these experiments. For experiments with Mn^{2+}, cysteine was replaced with 2 mM DTT. PDC was assayed spectrophotometrically.

only the equivalent of mitochondrial enzyme activity was lost. Additionally, neither beef heart protein kinase catalytic subunit nor phosphorylase kinase plus ATP resulted in inactivation of chloroplast PDC.

DISCUSSION

Pea leaf chloroplast PDC has been partially purified using isolated chloroplasts as the starting material to avoid contamination by mitochondrial PDC. Yields of enzyme were improved when the broken chloroplast extracts were diluted upon thawing to reduce entrapment of the enzyme in the large membrane pellet resulting from the initial centrifugation. The 0.2-mM NaCl

was also added at this point to minimize charge interactions with chloroplast membranes. The supernatant of this low-speed centrifugation was still significantly green, but the addition of PEG-8000 to 3% helped to clarify the extract, without altering the concentration of protein. Fractionation with PEG has been frequently used in mitochondrial PDC purifications (15, 19) and again proved useful for the chloroplast complex. While mitochondrial PDC extracts are usually warmed to room temperature prior to PEG addition in order to improve the fractionation, the chloroplast enzyme was unstable to warming for any extended period of time. Therefore, it was necessary to do the PEG fractionation, as well as all other purification steps, at 4°C.

The large size of the complex allows sedimentation by ultracentrifugation and has previously resulted in significant purification of mitochondrial PDCs (10, 15, 18, 19). The chloroplast enzyme could also be sedimented (Table I); however, purification was minimal (1.5-fold), possibly because of removal of the majority of the lower mol wt proteins by PEG fractionation and the fact that the large quantity of RuBPCase present in chloroplasts also partially co-sediments with the complex. The presence of RuBPCase in this fraction was demonstrated by rocket immunoelectrophoresis (data not shown).

Ethanol-Sepharose has been successfully used as an affinity matrix for the purification of PDC from *E. coli* and *Bacillus* (25, 26), but in the present study a recovery of only 16% was obtained using this procedure. However, a similar matrix, ethyl-agarose, resulted in purifications with about 50% recoveries. Chloroplast PDC could be adsorbed to and eluted from the ethyl-agarose; however, again yields were low. If, on the other hand, the ethyl-agarose was equilibrated with 0.1 M NaCl the enzyme eluted from the column in greater yields. In the latter capacity, the column appears to retard the elution of other proteins either by size or some partial interaction with the matrix allowing separation from PDC.

The second ultracentrifugation was primarily used as a means of concentrating the enzyme but also resulted in 2-fold purifications. Further attempts at purification using several affinity matrices, numerous variations of ion-exchange chromatography, gel permeation chromatography, isoelectric precipitation, and other purification procedures resulted in almost complete losses of enzyme activity.

The oxidative decarboxylation of pyruvate could be the result of other complexes such as the 2-ketoglutarate or the branched chain ketoacid dehydrogenase complexes since they have some
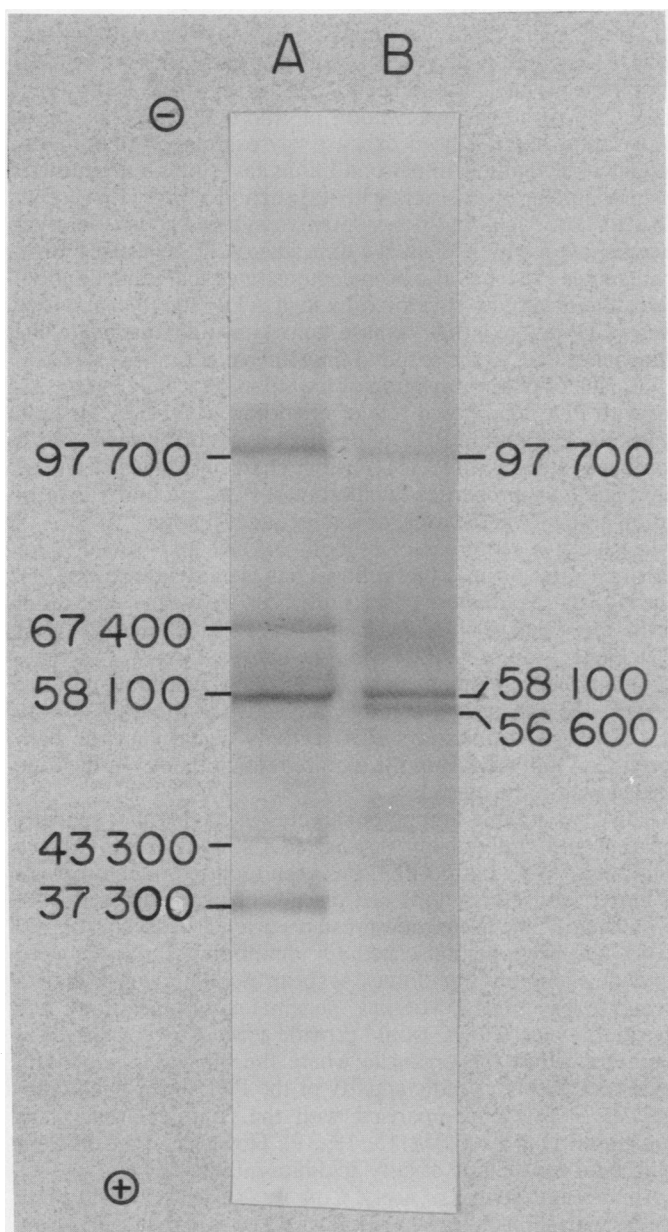
FIG. 3. Electrophoretic analysis of pea mitochondrial and chloroplast PDCs. Proteins were separated on a 9.5 to 12% acrylamide SDS gel and electrophoretically transferred to nitrocellulose. Antibodies to broccoli mitochondrial PDC were used to probe for PDC in the pea preparations. Lane A is a immuno-blot from pea mitochondria and lane B from pea chloroplasts. M_r values were determined using Sigma high mol wt standards.
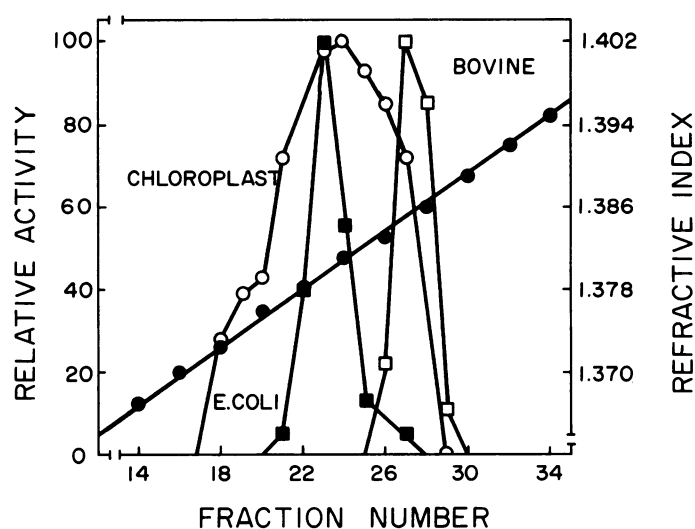

FIG. 4. Mol wt estimation by rate-zonal sedimentation on linear glycerol gradients. PDC from pea chloroplasts, *E. coli* and bovine heart were layered onto a 10 to 50% glycerol gradient as described in the text. The gradient was fractionated from the top in 0.5-ml fractions.

Table III. *ATP-Dependent Inactivation of Pea Leaf PDCs*

Chloroplast and mitochondrial PDCs were incubated together or separately, in the presence or absence of 1 mM ATP for 10 min at room temperature prior to assay. Purified chloroplasts from peas were ruptured by resuspension in 0.02% Triton X-100, 50 mM Hepes (pH 7.6), and 5 mM DTT for 30 min at 4°C and then centrifuged at 12,000g for 10 min to remove particulate material. Acetone powder of pea leaf mitochondria was resuspended in 25 mM Hepes (pH 7.6) and 5 mM DTT.

Enzyme Source	ATP	Relative Activity	
		Observed	Calculated
	mM		
Chloroplasts	0	37	
	1	37	
Mitochondria	0	48	
	1	0	
Chloroplast plus mitochondria	0	88	85
	1	38	37

substrates in common. It has also been proposed that the PDC activity in spinach chloroplast was attributable to acetolactate synthetase activity (13). To unequivocally establish PDC as the enzyme responsible for the observed activity, both substrate specificity and product analyses were performed. The pea chloroplast PDC showed an absolute requirement for pyruvate, NAD^+, and CoA and no activity was obtained with 2-ketoglutarate, 2-ketoisovalerate, or 2-ketoisocaproate (Table II). Thus, oxidative decarboxylation of pyruvate resulting from other keto acid dehydrogenase complexes is very unlikely. $NADP^+$ was also examined as a substrate (Table II) because of the large pool in chloroplasts; however, only minor $NADP^+$-dependent activity was observed.

Since the validity of chloroplast PDC has been questioned in the literature (13), it was essential to demonstrate the predicted products of the PDC reaction were indeed resultant. The chloroplast complex was therefore assayed by three techniques, each one quantitating a different product. The PDC reaction equation predicts a 1:1:1 stoichiometry of CO_2:acetyl-CoA:NADH. Our results are in agreement with this prediction and verify the chloroplast complex as a true PDC.

Mitochondrial PDCs from plant sources (19, 20) require the addition of TPP for maximal activity, indicating the ready dissociation of TPP from the enzyme complex. In plastids, however, a tighter association appears to exist. After extended dialysis, PDC was only stimulated 30% by exogenous TPP. Addition of TPP to the assay of pea chloroplast PDC from G-25 Sephadex treatment resulted in only a 2% increase in activity. Reid *et al.* (18) also observed only a minor stimulation of *Ricinus* endosperm plastid PDC with TPP. On the other hand, Rubin *et al.* (20) proposed possible regulation of mitochondrial PDC by TPP because of its very rapid dissociation. Regulation of this type seems improbable for the plastid complex.

Previously it has been possible to estimate the mol wt of PDC by rate-zonal sedimentation on linear glycerol gradients (18).

Although we could successfully perform these experiments with *E. coli* and bovine PDCs, attempts to obtain a sharp peak with chloroplast PDC were unsuccessful (Fig. 1). One possible reason for the broad profile of chloroplast complex activity could be the formation of aggregates of PDC; however, addition of Triton X-100 up to 0.2% or salt up to 0.5 M did nothing to sharpen the peak. Another possibility for the broad peak of activity could be the dissociation of one or more of the component enzymes. Dissociation of the dihydrolipoyl dehydrogenase component of PDC has been reported in mitochondrial PDC preparations (18), but in the present study the activity of this component was coincident with total complex activity (data not shown). Association of PDC with other stromal enzymes is a third possible explanation, and such associations have been reported between mitochondrial PDC and certain matrix enzymes (24). The size of the chloroplast complex does, however, appear to be comparable to reported values for other PDCs.

Although chloroplast and mitochondrial PDC have the same substrates, products, and cofactors, by immunological criteria there are structural differences between the two complexes. Precipitation of a portion of the chloroplast enzyme with antibodies to the broccoli mitochondrial complex indicate only a partial homology between the two enzymes. More detailed analysis using Western blotting techniques show differences exist in subunit mol wt and in number of subunits that react with antibody. The two complexes do have a $58,100$-M_r band in common on Western blots which is the dihydrolipoyl dehydrogenase subunit of PDC (J. A. Miernyk, unpublished data). Both complexes also have a $97,700$-M_r band but the identity of this subunit is currently unknown. Since the regulatory properties of chloroplast PDC appear to be more similar to the *E. coli* complex than to eukaryotic mitochondrial complexes, immunological characteristics were also compared between these two PDCs. Antibodies to *E. coli* complex did not, however, cross-react with pea chloroplast PDC. Homology has not been observed between *E. coli* PDC and from other sources (25). This does not exclude the possibility that antibodies made to specific components of the *E. coli* complex, rather than to the whole complex, might react.

Chloroplast PDC had an absolute requirement for divalent cations with a preference for Mg^{2+}. Optimal Mg^{2+} concentrations for the chloroplast complex were approximately 11-fold greater than required by plant mitochondrial PDCs (15, 19). The requirement for high Mg^{2+} may be of regulatory significance. Stromal Mg^{2+} concentrations have been shown to increase 3 to 5 mM in the light (3). If the concentration of free Mg^{2+} were to vary anywhere in the range of 0 to 6 mM regulation of PDC would be achieved in the light. Also consistent with postulated maximal activity during illumination was the alkaline pH optimum of chloroplast PDC. The chloroplast stromal pH shifts from seven in the dark to eight in the light (3). Such a rise in pH would result in approximately a 2-fold increase in PDC activity. Nongreen castor oil seed plastid PDC also has a high Mg^{2+} requirement and a sharp alkaline pH optimum (18). These characteristics may be typical of plastid PDCs in general. Considering both Mg^{2+} and pH effects, significant regulation of chloroplast PDC could be achieved by changes occurring in dark-light transitions. *De novo* fatty acid synthesis is reported to occur during illumination (14). Increased PDC activity in the light would provide acetyl-CoA for this process at the time when it is required.

Mitochondrial PDC is regulated by covalent modification (11, 15, 16); however, the chloroplast PDC does not appear to undergo typical ATP-dependent inactivation. The chloroplast complex was not inactivated in the presence of mitochondrial PDC kinase under conditions that did inactivate the mitochondrial complex, although this may be a steric problem if mitochondrial kinase cannot dissociate or reach the chloroplast PDC. Regula-

tion by covalent modification is also not observed with prokaryotic PDCs, but is achieved by other means such as product inhibition by NADH and acetyl-CoA, or by the GTP to GDP ratio (21). Although inactivation of chloroplast PDC by phosphorylation has not been exhaustively examined, an ATP-dependent inactivation under conditions favoring photosynthesis (light) would be inconsistent with its function of providing acetyl-CoA for fatty acid and polyisoprenoid synthesis. Lipid biosynthesis is favored in the light at a time when ATP levels are known to increase (23). It has also been demonstrated that mitochondrial PDC kinase activity is favored by high ATP (10, 15, 16). However, ATP-favored PDC kinase activity would inactivate the chloroplast PDC at the required time for synthesis of acetyl-CoA. Thus, lack of phosphorylation of the chloroplast PDC is consistent with PDC's proposed role of providing acetyl-CoA for lipid synthesis. The plastid PDC of developing castor oil seeds also is not inactivated by ATP (18). Further characterization of kinetic and regulatory properties of chloroplast PDC are under investigation and will be the topic of a subsequent report.

In plants, a requirement of both NADH and NADPH for maximal rates of lipid biosynthesis has been demonstrated (1). The NADH requirement has recently been shown to be the result of the specificity of chloroplast enoyl-ACP reductase for NADH (22). Generation of NADH could be achieved by PDC activity. Although there are other chloroplast enzymes that could produce NADH, the extent to which they are active at the time of fatty acid synthesis is unknown. PDC activity would generate both acetyl-CoA and NADH in the required stoichiometry at the time needed within the plastid.

Plants are unique among eukaryotes in having two spatially separate and functionally distinct PDCs. In addition to pea chloroplast PDC, plastid PDC has been reported in chloroplasts of butter lettuce and nongreen plastids of castor oil seeds (13, 18). Recently, the more widespread occurrence of specific plastid PDCs has been demonstrated for developing sunflower seed cotyledons, cauliflower florets, soybean nodule cytosol, and in green rye leaves (J. A. Miernyk, personal communication). It is logical that plastid PDC would provide acetyl-CoA for fatty acid synthesis within the organelle where the biosynthesis of fatty acids occurs. The specific activity of the PDC from lysed chloroplasts is low in comparison with the complex from plant mitochondria (0.02–0.05) (15, 18, 19). However, it is sufficient to account for the rate of fatty acid biosynthesis.

An alternate source of acetyl CoA was suggested based upon studies with spinach leaves (13). It was proposed that acetyl-CoA was formed first by mitochondrial PDC and then hydrolyzed to acetate via acetyl-CoA hydrolase. The acetate would then diffuse through the cytosol and into the chloroplast where it would be reesterified with CoA in an energy dependent reaction catalyzed by acetyl-CoA synthetase (13). This proposal was based upon an apparent lack of chloroplast PDC in spinach as well as the presence of the other enzymes. The general presence of acetyl-CoA hydrolase has recently been questioned by Givan and Hodgson (7) who were unable to detect this activity in pea mitochondria. It is possible that spinach chloroplast is an exception in not having a plastid PDC but a more detailed analysis of both pathways in a variety of species will be necessary before any generalizations can be made.

In summary, we have isolated and partially purified chloroplast PDC from green pea shoots. It was verified that the observed activity was PDC through substrate requirements and appropriate product formation. Comparison of the chloroplast and mitochondrial enzyme from peas show a number of differences in properties including a higher pH optimum for chloroplast PDC as well as a higher optimal Mg^{2+} requirement. Also, there is an apparent lack of ATP-dependent inactivation of the chloroplast complex. The two PDCs have distinct immunological character-

istics as well as different subunit structures.

LITERATURE CITED

1. AGRAWAL PK, DT CANVIN 1971 The pentose phosphate pathway in relation to fat synthesis in the developing castor oil seed. Plant Physiol 47: 672–675
2. BRADFORD MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254
3. BUCHANAN BB 1980 Role of light in the regulation of chloroplast enzymes. Annu Rev Plant Physiol 31: 341–374
4. COX GF 1969 Isocitrate dehydrogenase (NAD-specific) from pea mitochondria. Methods Enzymol 13: 47–51
5. DENNIS DT, JA MIERYNK 1982 Compartmentation of nonphotosynthetic carbohydrate metabolism. Annu Rev Plant Physiol 33: 27–50
6. ELIAS BA, CV GIVAN 1979 Localization of pyruvate dehydrogenase complex in *Pisum sativum* chloroplasts. Plant Sci Lett 17: 115–122
7. GIVAN CV, JM HODGSON 1983 Formation of coenzyme A from acetyl-coenzyme A in pea leaf mitochondria: A requirement for oxaloacetate and the absence of hydrolysis. Plant Sci Lett 32: 233–242
8. INGEBRETSEN OC, M FARSTAD 1980 Direct measurement of free coenzyme A in biological extracts by reversed-phase high-performance liquid chromatography. J Chromatogr 202: 439–445
9. LAEMMLI UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685
10. LINN TC, JW PELLEY, FH PETTIT, F HUCHO, DD RANDALL, LJ REED 1972 α-Keto acid dehydrogenase complexes XV. Purification and properties of the component enzymes of the pyruvate dehydrogenase complexes from bovine kidney heart. Arch Biochem Biophys 148: 327–342
11. LINN TC, FH PETTIT, LJ REED 1969 Keto acid dehydrogenase complexes, X. Regulation of the activity of the pyruvate dehydrogenase complex from beef kidney mitochondria by phosphorylation and dephosphorylation. Proc Natl Acad Sci USA 62: 234–241
12. MIERYNK JA, RN TRELEASE 1981 Role of malate synthase in citric acid synthesis by maturing cotton embryos: A proposal. Plant Physiol 67: 875–881
13. MURPHY DJ, PK STUMPF 1981 The origin of chloroplastic acetyl-CoA. Arch Biochem Biophys 212: 730–739
14. OHLROGGE JB, DN KUHN, PK STUMPF 1979 Subcellular localization of acyl carrier protein in leaf protoplasts of *Spinacia oleracea*. Proc Natl Acad Sci USA 77: 1194–1198
15. RANDALL DD, PM RUBIN, M FENKO 1977 Plant pyruvate dehydrogenase complex purification, characterization and regulation by metabolites and phosphorylation. Biochim Biophys Acta 485: 336–349
16. RANDALL DD, M WILLIAMS, BJ RAPP 1981 Phosphorylation-dephosphorylation of pyruvate dehydrogenase complex from pea leaf mitochondria. Arch Biochem Biophys 207: 437–444
17. REED LJ 1974 Multienzyme complexes. Acc Chem Res 7: 40–46
18. REID EE, P THOMPSON, CR LYTTLE, DT DENNIS 1977 Pyruvate dehydrogenase complex from higher plant mitochondria and proplastids. Plant Physiol 59: 842–848
19. RUBIN PM, DD RANDALL 1977 Purification and characterization of pyruvate dehydrogenase complex from broccoli floral buds. Arch Biochem Biophys 178: 342–349
20. RUBIN PM, WL ZAHLER, DD RANDALL 1978 Plant pyruvate dehydrogenase complex: Analysis of the kinetic properties and metabolite regulation. Arch Biochem Biophys 188: 70–77
21. SCHWARTZ ER, LJ REED 1970 Regulation of the activity of the pyruvate dehydrogenase complex of *Escherichia coli*. Biochemistry 9: 1434–1439
22. SHIMAKATA T, PK STUMPF 1982 Purification and characterization of α-keto-[acyl-carrier-protein] reductase, α-hydroxyacyl-[acyl-carrier-protein] dehydrase, and enoyl-[acyl-carrier-protein] reductase from *Spinacia oleracea* leaves. Arch Biochem Biophys 218: 77–91
23. STITT M, RMcC LILLEY, HW HELDT 1982 Adenine nucleotide levels in the cytosol, chloroplasts, and mitochondria of wheat leaf protoplasts. Plant Physiol 70: 971–977
24. SUMEGI B, I ALKONYI 1983 A study on the physical interaction between the pyruvate dehydrogenase complex and citrate synthase. Biochim Biophys Acta 749: 172–179
25. VISSER J, H KESTER, K JEYASEELAN, R TOPP 1982 Pyruvate dehydrogenase complex from *Bacillus*. Methods Enzymol 89: 399–408
26. VISSER J, M STRATING 1982 Pyruvate dehydrogenase complex from *Escherichia coli*. Methods Enzymol 89: 391–399
27. WILLIAMS M, DD RANDALL 1979 Pyruvate dehydrogenase complex from chloroplasts of *Pisum sativum* L. Plant Physiol 64: 1099–1103