Localization and characterization of two structurally different forms of acetyl-CoA carboxylase in young pea leaves, of which one is sensitive to aryloxyphenoxypropionate herbicides

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Young pea leaves contain two structurally different forms of acetyl-CoA carboxylase (EC 6.4.1.2; ACCase). A minor form, which accounted for about 20% of the total ACCase activity in the whole leaf, was detected in the epidermal tissue. This enzyme was soluble and was purified to homogeneity from young pea leaf extracts. It consisted of a dimer of two identical biotinyl subunits of molecular mass 220 kDa. In this respect, this multifunctional enzyme was comparable with that described in other plants and in other eukaryotes. A predominant form was present in both the epidermal and mesophyll tissues. In mesophyll protoplasts, ACCase was detected exclusively in the soluble phase of chloroplasts. This enzyme was partially purified from pea chloroplasts and consisted of a freely dissociating complex, the activity of which may be restored by combination of its separated constituents. The partially purified enzyme was composed of several subunits of molecular masses ranging from 32 to 79 kDa, for a native molecular mass > 600 kDa. One of these subunits, of molecular mass 38 kDa, was biotinylated. This complex subunit structure was comparable with that of micro-organisms and was referred to as a 'prokaryotic' form of ACCase. Biochemical parameters were determined for both ACCase forms. Finally, both pea leaf ACCases exhibited different sensitivities towards the grass ACCase herbicide, diclofop. This compound had no effect on the 'prokaryotic' form of ACCase, while the 'eukaryotic' form was strongly inhibited.

INTRODUCTION

Acetyl-CoA carboxylase (ACCase; EC 6.4.1.2), a biotin-dependent enzyme, catalyses the ATP-dependent formation of malonyl-CoA from acetyl-CoA and bicarbonate (Knowles, 1989). This transcarboxylation reaction is the first committed step in the biosynthesis of fatty acids. In chloroplasts, ACCase plays a regulatory role in fatty acid biosynthesis (Post-Beittenmiller et al., 1992). Malonyl-CoA is also an intermediate in a number of other metabolic pathways, such as the biosynthesis of cuticular waxes and flavonoids (for a review see Harwood, 1989). Investigations of plant ACCase have increased over the last 5 years, following the demonstration that this enzyme represents the molecular target of two classes of powerful herbicides effective against grasses (the *Graminaceae*) including grass weeds, the cyclohexanediones and the aryloxyphenoxypropionates (Harwood, 1989). This enzyme has been studied and purified at least partially from different plant sources, essentially in seeds. ACCase purified from wheat germ, soyabean seed, parsley cells and developing pea embryos, is composed of high-molecular-mass polypeptides ranging from 210 to 240 kDa, depending on the plant source. In this respect, these plant ACCases resemble the yeast and animal ACCases, the subunit sizes of which are between 250 kDa and 280 kDa. On the other hand, the situation in green leaves is much more confusing. Thus SDS/PAGE analysis of ACCase purified from maize leaves revealed a single subunit of 60–61 kDa (Nikolau and Hawke, 1984). However, Egli et al. (1993) found that the enzyme purified from the same material was composed of high-molecular-mass polypeptides of 219–227 kDa. Furthermore, ACCases analysed in oil-seed rape leaves, soyabean leaves and in wheat leaves had molecular masses in the range 220–240 kDa (Hellyer et al., 1986; Charles and Cherry, 1986; Gornicki and Haselkorn, 1993). In contrast, ACCase partially purified from spinach chloroplasts would be composed of three or four different subunits ranging from 47 kDa to 120 kDa distributed between the soluble- and the membrane-phase of the chloroplast (Mohan and Kekwick, 1980). Such a structure would be comparable with that of ACCase from micro-organisms which contain three functional domains on four distinct separable polypeptides with molecular masses ranging from 17 kDa to 49 kDa (Guchhait et al., 1974).

Because ACCase in leaves is required in a number of different cell types (to supply malonyl-CoA for different biosynthetic pathways) and is present in different compartments of leaves from C_3 and C_4 plants, it is possible that these activities represent isoenzymes of ACCase (Nikolau et al., 1984). In support of this suggestion, two isoforms of ACCase were isolated from maize leaves, with molecular masses of 219 and 227 kDa (Egli et al., 1993). One of these isoforms was detected in mesophyll chloroplasts, while the other one seemed to be present in other cells or cell-type compartments. It is clear therefore that all of these results raise the question of whether isoenzymes of ACCase exist in plants.

In this paper, we present the characterization and the localization of a 'prokaryotic' form (a dissociable multienzyme complex) and an 'eukaryotic' form (a high-molecular-mass multifunctional protein) of ACCase in young pea leaves. These two forms were purified and their biochemical properties were compared.

Abbreviations used: ACCase, acetyl-CoA carboxylase; Chl, chlorophyll; DTT, dithiothreitol; PMSF, phenylmethanesulphonyl fluoride; PVP, polyvinylpyrrolidone.

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MATERIALS AND METHODS

Reagents

ATP, dithiothreitol (DTT), acetyl-CoA, avidin, D-biotin, and Red 120–agarose were obtained from Sigma Chimie SARL (La Verpillère, France). $NaH^{14}CO_3$ (53.1 mCi/mmol) was purchased from Amersham. Horseradish peroxidase colour development reagent and peroxidase-labelled streptavidin were purchased from Bio-Rad Laboratories (Munich, Germany). Superdex 200 prepacked column, Sepharose CL-4B, Sephadex G-25 and Sephacryl S-300 High Resolution were obtained from Pharmacia. QHyperD was obtained from Sepracor. All other chemicals were of analytical grade.

Plant material

Pea (*Pisum sativum L.*, var Douce provence) plants were grown from seeds in soil under a 12-h photoperiod of white light from fluorescent tubes (10–40 $\mu E/m^2$ per s) at 18 °C. The plants were watered every day with tap water.

Preparation of tissue and leaf extracts

Pea leaves (8 days after planting) were peeled immediately after harvesting to yield intact lower epidermal tissue. Upper epidermal tissue could not be routinely obtained because of a high level of cross-contamination by mesophyll cells.

Epidermal and whole-leaf tissues (approx. 0.2 g and 1 g respectively) were frozen in liquid N_2 in Eppendorf tubes and ground to a fine powder with a pestle. Upon N_2 evaporation, 100–500 μl of 50 mM Hepes (pH 8.0)/5 mM DTT/1 mM EDTA/10% (v/v) glycerol/1 mM phenylmethanesulphonyl fluoride (PMSF)/1 mM benzamidine hydrochloride/5 mM ϵ-aminohexanoic acid were added. The mixture was then further homogenized and centrifuged at 10000 *g* for 30 min. The supernatant was either assayed for enzyme activity immediately or treated for SDS/PAGE analysis.

Preparation of pea leaf mesophyll protoplasts

Freshly harvested pea leaves (3–5 g) were cut into fine strips (0.5–1 mm) under incubation medium: 10 mM Mes/NaOH (pH 5.5)/0.5 M sorbitol/1 mM $CaCl_2$/0.05% (w/v) polyvinylpyrrolidone (PVP)-25. The medium was removed and replaced with fresh incubation medium containing 2% (w/v) cellulase Onozuka R10, 0.5% (w/v) macerozyme R10 and 0.2% (w/v) pectolyase Y-23 (Yakult Honsha Co., Shingikancho, Nishinomiya, Japan). After vacuum infiltration the leaf strips were incubated at 25 °C for 2.5 h. All subsequent procedures were carried out at 4 °C. Protoplasts were released from the digested tissue by gentle shaking, and then separated from the leaf strips by filtering through a 100-μm nylon mesh (Züricher Beuteltuchfabrik AG, Rüschlikon, Switzerland). The filtrate was centrifuged at 100 *g* for 5 min (Beckman, JS 13 rotor) and the supernatant fluid was discarded. The pelleted protoplasts were resuspended in a medium containing 0.5 M sucrose, 1 mM $CaCl_2$ and 10 mM Mops, pH 7.0 (total volume 20 ml) and divided among four 15-ml glass centrifuge tubes. Into each tube was layered 2 ml of 0.4 M sucrose/0.1 M sorbitol/1 mM $CaCl_2$/10 mM Mops, pH 7.0, and 1 ml of 0.5 M sorbitol/1 mM $CaCl_2$/10 mM Mops, (pH 7.0). The resulting three-step gradients were centrifuged at 250 *g* (Beckman, JS 13 rotor, no brake) for 5 min. Intact mesophyll protoplasts sedimented at the interface of the top two layers and were collected using a Pasteur pipette and washed twice with washing medium: 0.5 M sorbitol/1 mM $CaCl_2$/10 mM Mops (pH 7.0). A careful examination of the final preparation by microscopy indicated that the mesophyll protoplasts containing chloroplasts were almost devoid of non-green protoplasts which derive from epidermal tissues.

Protoplasts, resuspended in the washing medium [at a chlorophyll concentration of 0.2 mg/ml] were gently ruptured by passing twice through a 10-μm nylon mesh attached to a 1-ml syringe. Chloroplasts were pelleted by centrifuging aliquots (1 ml) of the lysed protoplast suspension at 300 *g* for 5 min. The supernatant was decanted and centrifuged at 12000 *g* for 20 min to produce the mitochondrial fraction (pellet) and cytosolic fraction (supernatant). The chloroplast and mitochondrial pellets were resuspended in a minimal volume of the washing medium. In order to obtain a total extract, the pellet of washed protoplasts was resuspended in hypotonic medium (50 mM Hepes, pH 8.0/1 mM EDTA/5 mM DTT/1 mM PMSF/1 mM benzamidine hydrochloride/5 mM ϵ-aminohexanoic acid/0.5 μg/ml leupeptin/0.7 μg/ml pepstatin) and submitted to three cycles of freeze–thawing to ensure complete lysis. Under these conditions, all the organelles (chloroplasts, mitochondria, etc.) were lysed. The lysate suspension comprised the total protoplast extract and was used immediately for measurement of enzyme activity or treated for SDS/PAGE analysis.

Preparation and fractionation of purified chloroplasts

Young pea leaves (12 days; 2–3 kg) were homogenized in 330 mM sorbitol/50 mm Hepes, pH 8.0/1 mM EDTA/1 mM DTT/0.1% (w/v) BSA using a Waring blender for 3–5 s at low speed. Intact chloroplasts were rapidly prepared and purified as described by Douce and Joyard (1982) using discontinuous Percoll gradients. Intact chloroplasts (2–4 g of protein) were rapidly washed in the same buffer without BSA and then lysed with lysis buffer containing 50 mM Hepes, pH 8.0, 1 mM EDTA, 5 mM DTT, 1 mM PMSF, 5 mM ϵ-aminohexanoic acid and 1 mM benzamidine hydrochloride. After vortex mixing, chloroplasts were left on ice for 10 min to ensure complete lysis. The suspension of broken chloroplasts was centrifuged at 72000 *g* for 30 min on a 0.6 M sucrose layers (5 ml). The pellet and the supernatant comprised the chloroplast membranes (envelope membranes and thylakoids) and the soluble fraction (stroma) respectively. All procedures were carried out at 4 °C.

Purification of the high-molecular-mass form of ACCase

Up to 500 g of young pea leaves (7 days after planting) were harvested and finely ground in liquid N_2 using a pestle and mortar. The powder was then homogenized in 2 vol. of 0.1 M Hepes, pH 8.0/10% (v/v) glycerol/1 mM EDTA/10 mM 2-mercaptoethanol/1 mM PMSF/1 mM benzamidine hydrochloride/5 mM ϵ-aminohexanoic acid. The suspension was filtered through one layer of nylon mesh, and the filtrate centrifuged at 30000 *g* (Beckman, JA 14 rotor) for 30 min. The supernatant was brought to 20% satn. by addition of solid $(NH_4)_2SO_4$. After 15 min of constant stirring the mixture was centrifuged at 30000 *g* for 20 min and the supernatant recovered and brought to 45% satn. with solid $(NH_4)_2SO_4$. The precipitate was recovered by centrifugation and resuspended in a minimum volume of a buffer containing 20 mM Hepes, pH 8.0/10% (v/v) glycerol/1 mM EDTA/1 mM DTT/1 mM benzamidine hydrochloride and 5 mM ϵ-aminohexanoic acid (buffer A). After centrifugation at 30000 *g* (Beckman, JA 20 rotor) for 5 min to

remove insoluble material, the resulting supernatant was desalted on a Sephadex G-25 column (5 cm × 35 cm) and applied (flow rate 2 ml/min) to a Q-Sepharose column (1.6 cm × 20 cm) equilibrated with buffer A. The column was connected to a f.p.l.c. system (Pharmacia). The enzyme was eluted with a gradient of 0–0.5 M KCl in buffer A (170 ml). Fractions containing ACCase activity were pooled and brought to 70% satn. with solid $(NH_4)_2SO_4$. After centrifugation at 30000 *g* (Beckman, JA 20 rotor) for 30 min, the pellet was recovered and resuspended in a small vol. of buffer A. After desalting on a PD10 column, the fraction was loaded (0.5 ml/min) on a Red 120–agarose column (1.6 cm × 15 cm) previously equilibrated with buffer A. The column was extensively washed with buffer A to remove unbound proteins, then the enzyme was eluted with a 200-ml gradient of 0–1 M KCl in buffer A. Active fractions were pooled and applied to a monomeric avidin–Sepharose CL-4B column (1.6 cm × 15 cm) prepared as described previously (Kohansky and Lane, 1990; Alban et al., 1993), equilibrated with buffer A containing 0.5 M KCl (buffer B). After exposure to the protein fraction for 16 h, the column was washed with 100 ml of buffer B (flow rate 0.2 ml/min). The enzyme was finally eluted with 20 ml of the same buffer containing biotin (0.5 mg/ml). Active fractions were pooled, concentrated to a final concentration of about 0.5 mg/ml with Macrosep-10 tubes (Filtron) and stored at −80 °C until used. The entire purification procedure was carried out at 4 °C.

Assay for ACCase

All assays were optimized with respect to the concentration of each reaction component and to the pH of the reaction mixture. The activity of ACCase was measured as the incorporation of radioactivity from $NaH^{14}CO_3$ into an acid-stable product as described earlier (Baldet et al., 1993). The standard assay consisted of 50 mM Hepes, pH 8.0, 2.5 mM $MgCl_2$, 1 mM ATP, 0.5 mM DTT, 10 mM $NaH^{14}CO_3$ (1 mCi/mmol), 20 mM KCl, 0.8 mM acetyl-CoA and 1–200 μg of protein in a final volume of 200 μl. The assays were initiated by the addition of acetyl-CoA. After incubation for 5–20 min at 30 °C in a shaking water bath, aliquots of the reaction mixture (150 μl) were mixed vigorously with 40 μl of 12 M HCl to stop the reaction. The solution was then taken to dryness under N_2, and the acid-stable radioactivity was quantified in a liquid-scintillation counter. Duplicate assays without acetyl-CoA were run as controls.

Electrophoretic analyses of proteins

SDS/PAGE was performed at room temperature in SDS/polyacrylamide slab gels (15 cm × 15 cm) containing a 7.5–15% (w/v) acrylamide gradient. The experimental conditions for gel preparation, sample solubilization, electrophoresis and gel staining were as detailed by Chua (1980). In some experiments, polypeptides were also transferred electrophoretically to nitrocellulose sheets (Gelman Science), essentially according to Towbin et al. (1979). Biotin-containing polypeptides were detected with a system analogous to Western blotting using horseradish peroxidase-labelled streptavidin as specific reagent (Baldet et al., 1992).

Protein and chlorophyll content determination

Protein was measured by the method of Bradford (1976) using Bio-Rad protein assay reagent with γ-globulin as standard.

Chlorophyll content was measured by the method of Arnon (1949), modified according to Bruinsma (1961).

RESULTS

Localization of ACCase in pea leaf cells

In a previous publication, we showed the existence in cell-free extracts from whole pea leaves, of three different biotinyl polypeptides of 220, 76 and 38 kDa (Baldet et al., 1993). The 76 kDa polypeptide corresponded to the biotin-containing subunit of 3-methylcrotonoyl-CoA carboxylase (EC 6.4.1.4) localized in mitochondria (Baldet et al., 1992; Alban et al., 1993; Chen et al., 1993). The 38 kDa polypeptide, localized in mesophyll chloroplasts seemed to be associated with ACCase (Baldet et al., 1993). On the other hand, the 220 kDa biotinyl polypeptide, which may correspond to a high-molecular-mass form of ACCase, could not be localized in any of the different compartments of mesophyll cells. ACCase is known to be very sensitive to proteolytic degradation and it is possible that the 38 kDa biotinyl fragment present in the chloroplast was derived from proteolytic attack of a 220 kDa polypeptide during the course of chloroplast preparation. However, this hypothesis is most unlikely as incubation of purified 220 kDa biotinyl polypeptide (see below) with a total chloroplast extract did not modify the apparent molecular mass of the protein. This result strongly suggests therefore that the 220 kDa biotinyl polypeptide is localized elsewhere in a leaf territory distinct from the mesophyll cells, such as the epidermis.

To confirm this hypothesis, we have isolated epidermal tissue from young pea leaves. Pea plants were stored overnight at 4 °C before leaf peeling. This facilitated the removal of intact and

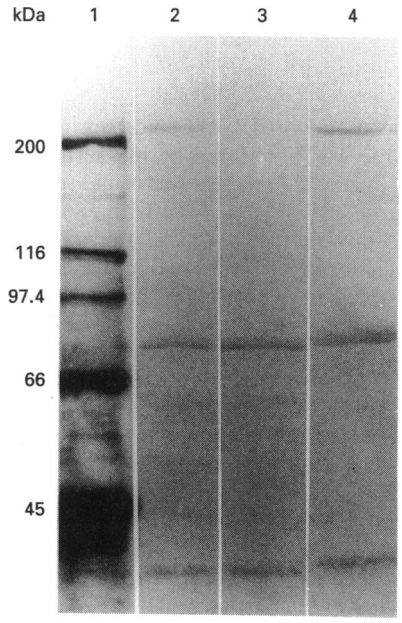

Figure 1 Analysis of biotinyl polypeptides from pea whole leaf crude extract, mesophyll protoplasts and lower epidermis lysates separated by SDS/PAGE

Preparation of protein extracts was carried out as described in the Materials and methods section. Proteins were analysed by Western blotting with peroxidase-labelled streptavidin. Lane 1, biotinylated molecular mass markers; lane 2, crude pea leaf extract (100 μg); lane 3, mesophyll protoplasts lysate (100 μg); lane 4, lower epidermis lysate (100 μg). Molecular masses on the left are given in kDa.

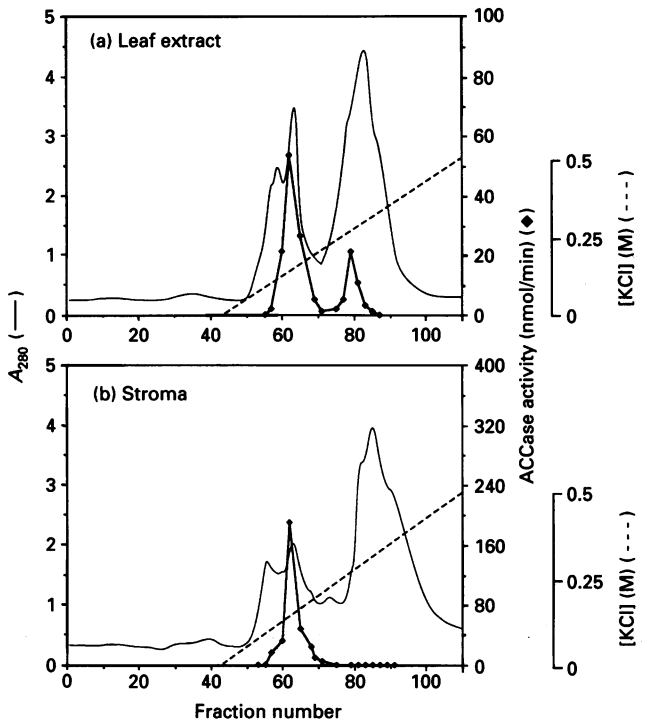

Figure 2 Separation of ACCase activity in leaf extract (a) and chloroplast extract (b) from pea leaves by ion-exchange chromatography

Extracts, equivalent to 200 mg of soluble protein were fractionated by anion-exchange chromatography on a Q-Sepharose column (1.6 cm × 20 cm) in buffer A. After sample loading, the column was washed with 50 ml of buffer A, then samples were eluted with a gradient of 0–0.5 M KCl (140 ml) in the same buffer at a flow rate of 2 ml/min. Fractions of 2 ml were collected and assayed for ACCase activity.

essentially uncontaminated lower epidermis. Contamination by mesophyll tissue was monitored by measuring the chlorophyll content of isolated epidermis and was found to be negligible (less than 2 μg/mg of protein compared with 180 μg/mg of protein for mesophyll protoplasts). ACCase activity was measured in crude homogenates of whole leaves, protoplasts and epidermal tissues, and was found to be 0.7, 0.6 and 0.9 nmol/min per mg of protein respectively. To investigate the distribution of protein-bound biotin in leaf, proteins from leaf extract, mesophyll protoplasts and epidermal tissue were analysed by Western blotting with peroxidase-labelled streptavidin. Figure 1 clearly shows that the 220 kDa biotinyl polypeptide, which was detected in the whole-leaf extract, was not present in mesophyll protoplasts and was enriched in the epidermis extract, indicating a specific localization of this polypeptide in this compartment. On the other hand, the 38 kDa biotinyl polypeptide was detected in the two leaf compartments, i.e. the epidermis and the mesophyll cells.

When a crude extract from pea leaves was fractionated by anion-exchange chromatography on a Q-Sepharose column, and the eluted fractions assayed for ACCase activity, two distinct peaks of activity were resolved. The major one, corresponding to about 80% of the total activity, was eluted at a concentration of 100 mM KCl. A minor peak was eluted at 200 mM KCl (Figure 2). Analysis of biotinyl polypeptides by SDS/PAGE and Western blot revealed that the major peak of activity was enriched in the 38 kDa biotinyl polypeptide, whereas the second peak was enriched in the 220 kDa polypeptide (results not shown). When an extract from purified pea chloroplasts was fractionated on the Q-Sepharose column, ACCase activity was eluted as a single peak at a concentration of 100 mM KCl. Under these conditions, active fractions contained only one biotinyl polypeptide of 38 kDa. These results, first confirmed that the 38 kDa and the 220 kDa biotinyl polypeptides are associated with ACCase activity. Secondly, they strongly suggest that young pea leaves contain two different forms of ACCase, a low-molecular-mass form according to the size of the biotinyl subunit, present in mesophyll chloroplasts and probably in the epidermis (see Figure 1), and a high-molecular-mass form, detectable only in the epidermal tissue, which accounts for a substantial part of the total ACCase activity detected in this leaf compartment.

Purification of the high-molecular-mass form of ACCase

The 220 kDa form of ACCase was purified from 7-day-old pea leaves. At this stage of development, this protein was found to be present in higher proportions compared with other biotinyl polypeptides, especially 3-methylcrotonoyl-CoA carboxylase (Figure 3). Table 1 shows a representative purification of the high-molecular-mass form of ACCase from a crude leaf extract. Separation of the two forms of ACCase was achieved by chromatography on the Q-Sepharose column. This can explain the apparent low recovery of ACCase activity after this chromatographic step. Indeed, the total enzyme activity reported in Table 1 only represents the activity of the high-molecular-mass form of ACCase eluted at a KCl concentration of about 200 mM. Chromatography on the Red 120–agarose column eliminated all the contaminating 3-methylcrotonoyl-CoA carboxylase activity. Thus, the only biotinyl polypeptide remaining in the extract at this stage of purification was the 220 kDa polypeptide. This protein was finally purified to apparent homogeneity on a monomeric avidin–Sepharose CL-4B column which is very specific for biotinyl proteins (Henrikson et al., 1979) (Figure 4). The procedure resulted in an overall 1774-fold purification. With respect to the high-molecular-mass form of ACCase, the purification factor may be estimated to be around 9000-fold. On the basis of SDS/PAGE, ACCase thus isolated gave a single band corresponding to a molecular mass of about 220 kDa for the subunit (Figure 4). The molecular mass of the native protein was estimated by gel-filtration chromatography on a prepacked Superdex 200 column (1.6 cm × 60 cm) equilibrated in buffer containing 20 mM Tris/HCl, pH 7.5, 1 mM EDTA and 200 mM KCl. Based on this technique, the molecular mass value of ACCase was estimated to be 450 kDa. We conclude therefore that the native enzyme is composed of two subunits, each with an apparent molecular mass of 220 kDa.

Partial purification of the low-molecular-mass form of ACCase

The enzyme was isolated from pea chloroplasts purified on Percoll gradients. The amount of extrachloroplastic contamination in the Percoll-purified chloroplast fraction was determined by measuring the activity of various marker enzymes of cytosol (pyrophosphate:fructose-6-phosphate-1-phosphotransferase; EC 2.7.1.90), mitochondria (fumarase; EC 4.2.1.2) and peroxisomes (catalase; EC 1.11.1.6) (not shown). This analysis showed that extrachloroplastic contaminations were negligible after purification through Percoll gradients. The isolated intact chloroplasts were lysed and soluble proteins (stroma) were separated from thylakoids and envelope membranes (membranes fraction) by centrifugation on a sucrose layer. Each fraction was assayed for ACCase activity. Over 95% of the activity was found in the soluble protein fraction (Table 2).

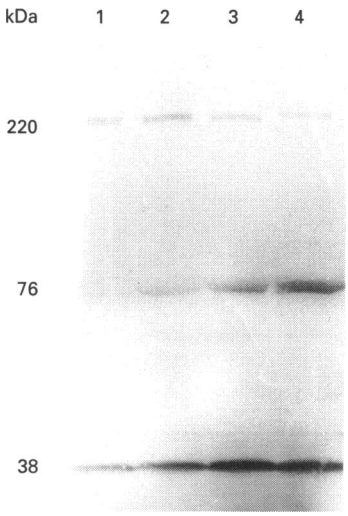

Figure 3 Evaluation of biotinyl polypeptides during development of pea leaves

Extracts from lane 1, 4-; lane 2, 7-; lane 3, 10- and lane 4, 16-day-old pea leaves were analysed by Western blotting with peroxidase-labelled streptavidin. The molecular masses (in kDa) of the biotinyl polypeptides are indicated on the left. Protein loaded on each lane was equivalent to 200 μg.

Table 1 Purification of the high-molecular-mass form of pea leaf ACCase

For this preparation 190 g (fresh weight) of young pea leaves were used.

	Protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min per mg)	Yield (%)	Purification (-fold)
Crude extract	2200	1375	0.62	100	1
$(NH_4)_2SO_4$ (20–45% satn.)	800	1127	1.4	82	2.2
Q-Sepharose	208	224	1.08	16	1.7
Red 120-agarose	2.18	120	55	8.7	88.7
Monomeric-avidin–Sepharose CL-4B	0.05	55	1100	4	1774

Stroma (up to 1 g of protein in a final volume of 60–80 ml) was supplemented with glycerol to a final concentration of 10% (v/v). Addition of glycerol was found to be essential for the preservation of the enzyme activity, especially for long periods of storage at −20 °C or −80 °C. The fraction was then concentrated using Macrosep-10 tubes (Filtron) to a final volume of 3–5 ml (protein concentration ranged from 180 to 250 mg/ml). Concentrated stroma was subjected to chromatography on a gel-permeation column (Sephacryl S-300; 2.6 cm × 35 cm) with buffer A and the ACCase activity was recovered just after the void volume (exclusion limit of about 600 kDa) with a recovery of about 80% and an enrichment in specific activity of 5- to 6-fold. Active fractions were pooled and subjected to $(NH_4)_2SO_4$ fractionation with solid $(NH_4)_2SO_4$. Preliminary experiments showed that with successive short 'cuts' of $(NH_4)_2SO_4$, that is 0–20%, 20–35%, 35–45%, 45–60% and 60–80%, almost all the initial ACCase activity was lost (Table 3). Different combinations of the $(NH_4)_2SO_4$ fractions were then assayed for ACCase

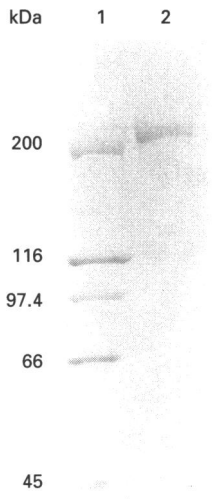

Figure 4 Analysis by SDS/PAGE of purified high-molecular-mass form of ACCase from pea leaves

Polypeptides were separated on a 7.5–15% (w/v) gradient polyacrylamide/SDS slab gel and stained with Coomassie Brilliant Blue R-250. Lane 1, molecular mass markers (10 μg each); lane 2, purified ACCase (15 μg). Molecular masses on the left are given in kDa.

Table 2 Distribution of ACCase activity in pea leaf chloroplastic subfractions

Preparation and fractionation of purified chloroplasts were carried out as described in the Materials and methods section. These data are from a representative experiment.

	Protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min per mg)
Lysed chloroplasts	2000	1800	0.9
Stroma	1000	1950	1.95
Membranes (thylakoids + envelope)	800	80*	0.1

* A careful washing of the membrane system removed all the remaining ACCase activity.

Table 3 $(NH_4)_2SO_4$ fractionation of ACCase activity from pea leaf chloroplasts

Active proteins equivalent to 45 mg, eluted from the Sephacryl S-300 column, were used. Total ACCase activity was 602 nmol/min. These results are from a representative experiment repeated three times.

$(NH_4)_2SO_4$ (% satn.)	Protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min per mg)	Yield (%)
0–20	0.65	0	0	0
20–35	17	23	1.35	3.8
35–45	8.8	0	0	0
45–60	9.2	0	0	0
60–80	8	0	0	0
20–35 + 35–45	25.8	652	25.2	108

Table 4 Purification of the low-molecular-mass form of ACCase from pea leaf chloroplasts

	Protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min per mg)	Yield (%)	Purification (-fold)
Stroma	1008	1965	1.95	100	1
Sephacryl S-300	144	1555	10.8	79.1	5.5
$(NH_4)_2SO_4$ (20–45% satn.)	82.6	1927	23.3	98	11.9
Monomeric-avidin–Sepharose CL-4B					
Void	78	0	0	0	–
Eluted	0.44	1.5	3.4	0.2	–
Void + eluted*	78.5	2120	27	108	13.8

* Optimum restored ACCase activity was obtained by combination of 150 μg of the void fraction and 8 μg of the affinity-eluted fractions from the monomeric-avidin–Sepharose CL-4B column.

activity. A total restoration of ACCase activity was achieved by combining the 20–35% and 35–45% $(NH_4)_2SO_4$ fractions (Table 3). All other mixtures not including these two fractions together were unsuccessful in restoring the full ACCase activity (not shown). These results strongly suggest that ACCase is dissociated during the course of $(NH_4)_2SO_4$ fractionation into at least two components separated in the 20–35% and 35–45% $(NH_4)_2SO_4$ cuts and that this dissociation is reversible by simple recombination of the two fractions. In an independent experiment, the Sephacryl S-300 active pool was chromatographed on a Red 120–agarose column. Proteins were eluted with fixed concentrations of KCl in buffer A (0.1, 0.2, 0.3, 0.5, 1 and 2 M KCl) and ACCase activity measured in all protein peaks. None of these fractions was found to contain detectable ACCase activity. However, combination experiments of the different protein peaks allowed an almost complete recovery of ACCase activity (up to 90% of the activity loaded on the column) upon mixing aliquots of the non-retained protein peak (wash fraction) and of the proteins eluted with concentrations of KCl of 0.2, 0.5 and 2 M. These results indicate a complex subunit structure for ACCase from pea chloroplasts, comparable with that of ACCase from micro-organisms, which readily dissociates into its different subunits under certain chromatographic conditions and whose activity may be restored by recombination of these separated constituents (Guchhait et al., 1974). To purify this ACCase further, the 20–45% $(NH_4)_2SO_4$ fraction was subjected to chromatography on a monomeric avidin–Sepharose CL-4B column under the same conditions described for the 220 kDa form of ACCase (see the Materials and methods section). Protein fractions eluted with free biotin exhibited only trace amounts of ACCase activity (Table 4) and no activity was found in the 'avidin-void' fraction, which is the protein fraction which was washed unbound from the affinity column. However, enzyme activity was recovered after mixing aliquots of these two fractions in the incubation medium (Table 4). The rate of acetyl-CoA carboxylation was dependent upon the protein concentration of both the affinity-eluted and the 'avidin-void' fractions (results not shown), and was found to be optimum with an approx. 20-fold protein excess of the last fraction over the former. Polypeptide analysis of the affinity-purified fraction by SDS/PAGE revealed the presence of six different polypeptides of 32, 35, 38, 47, 49 and 56 kDa, among which only one, of molecular mass of about 38 kDa, was biotinylated (Figure 5). No biotinyl protein was present in the 'avidin-void' fraction. The remaining component(s) of ACCase present in the 'avidin-void' fraction was purified further by chromatography on a QHyperD column (1 cm × 5 cm) and a Red 120–agarose column (1 cm × 5 cm) and the ACCase activity followed by combination of the various fractions eluted from these columns with aliquots of the affinity-eluted fraction from the avidin–Sepharose column (Table 5). Active fractions were eluted at concentrations of 100 mM KCl in buffer A from the QHyperD column and 300 mM from the Red 120–agarose column (see the legend of Table 5 for details). The purified fraction contained a major polypeptide of 64 kDa and two minor polypeptides of 79 and 56 kDa respectively (Figure 6).

Biochemical properties

All the biochemical parameters were determined on the pure high-molecular-mass form of ACCase and on a partially purified chloroplastic form of ACCase [i.e. as obtained after chromatography on Sephacryl S-300 column and fractionation with $(NH_4)_2SO_4$].

Both enzyme preparations were active over a wide pH range, with optimum activity at pH 8.0. The optimal temperature for activity of the enzyme *in vitro* was 33 °C in both cases. Purified high-molecular-mass form and partially purified low-molecular-mass form from pea leaves exhibited Michaelis–Menten kinetics with respect to ATP, acetyl-CoA and bicarbonate. The apparent K_m values for these substrates were determined, by using the double-reciprocal plot method (Lineweaver–Burk equation), to be 0.17, 0.015 and 2.5 mM respectively for the high-molecular-mass form, and 0.27, 0.25 and 0.87 mM respectively for the low-molecular-mass form of ACCase. Both enzyme preparations required Mg^{2+} for activity. Maximal velocity of ACCase activity was measured at 2.5 mM Mg^{2+} when 1 mM ATP was included in the reaction mixture. In these conditions, most of the total ATP concentration existed as $MgATP^{2-}$ complex (Storer and Cornish-Bowden, 1976).

Citrate, an allosteric activator of the animal ACCase (Lane et al., 1974), had no effect on the high-molecular-mass form of ACCase at concentrations up to 2 mM, but inhibited it above this concentration. In marked contrast, the low-molecular-mass form of ACCase was strongly inhibited even at physiological concentrations of citrate. Indeed, a 35% inhibition was observed at 2 mM and a 55% inhibition at 3 mM citrate. The same inhibitory effect was observed for the wheat germ and the soyabean seed enzymes (Finalyson and Dennis, 1983; Charles et al., 1986). However, the activity of ACCase isolated from spinach or avocado plastids was stimulated by 3 mM citrate (Mohan and Kekwick, 1980).

As already observed for other plant ACCases, both forms of ACCase from pea leaves were stimulated by the presence of KCl in the assay mixture. The high-molecular-mass form of ACCase

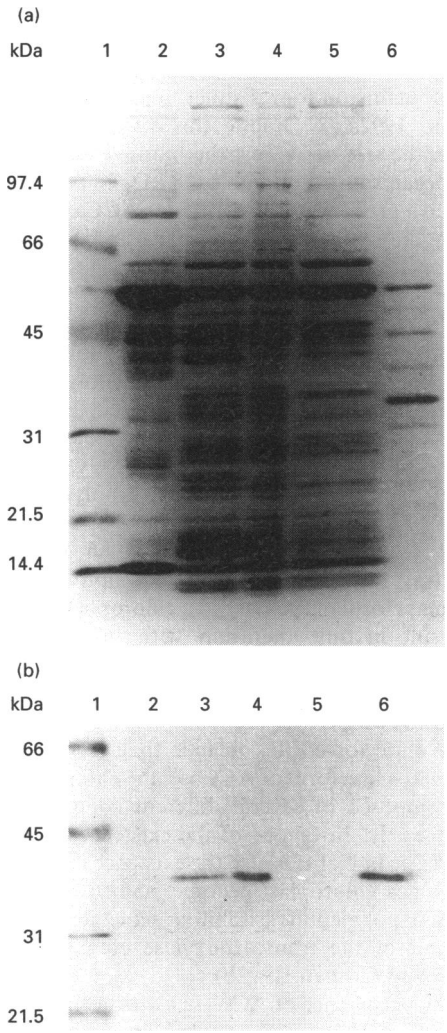

Figure 5 Analysis of purified samples of pea leaf low-molecular-mass form of ACCase by SDS/PAGE

Polypeptides were separated on a 7.5–15% (w/v) gradient polyacrylamide/SDS slab gel and stained with Coomassie Brilliant Blue R-250 (**a**) or analysed by Western blotting with peroxidase-labelled streptavidin (**b**). Lane 1, molecular mass markers (biotinylated in **b**) (5 μg each); lane 2, stroma (100 μg); lane 3, Sephacryl S-300 fraction (100 μg); lane 4, $(NH_4)_2SO_4$ 20–45% fraction (100 μg); lane 5, 'avidin-void' fraction (100 μg); lane 6, monomeric-avidin–Sepharose CL-4B-eluted fraction (8 μg). Molecular masses on the left are given in kDa.

Table 5 Purification of the remaining protein component(s) of pea leaf chloroplast ACCase from the 'avidin-void' fraction

ACCase from pea leaf chloroplast stroma was purified by chromatography on a Sephacryl S-300 column, $(NH_4)_2SO_4$ precipitation and monomeric-avidin–Sepharose CL-4B chromatography (see Table 4). After the last chromatographic step, monomeric-avidin–Sepharose CL-4B, the ACCase 'complex' was dissociated, one part ('avidin-void') being washed unbound from the column and the other part ('affinity eluted'), comprising the biotinyl subunit of ACCase, being retained on the affinity column and specifically eluted with free biotin. Proteins from the 'avidin-void' fraction were then chromatographed on a QHyperD column and eluted with a 50-ml KCl gradient (0–0.5 M) in buffer A. ACCase activity was assayed by combining each eluted fraction from the QHyperD column with aliquots of the protein fraction eluted from the monomeric-avidin–Sepharose CL-4B column. Active combinable fractions eluted from the QHyperD column were then pooled and chromatographed on a Red 120–agarose column. Fractions eluted from the Red 120–agarose column with a 50-ml KCl gradient (0–0.5 M) in buffer A were assayed for ACCase activity according to the same procedure. Incubation was at 30 °C for 20 min in the reaction mixture described in the Materials and methods section.

	Protein (mg)	Protein/assay (mg)	$H^{14}CO_3^-$ incorporated/assay (d.p.m.)
QHyperD	2.2	0.004	120
QHyperD + affinity-eluted fraction	2.2 + 0.44	0.04 + 0.008	7320
Red 120–agarose	0.3	0.006	132
Red 120–agarose + affinity-eluted fraction	0.3 + 0.44	0.006 + 0.008	11560

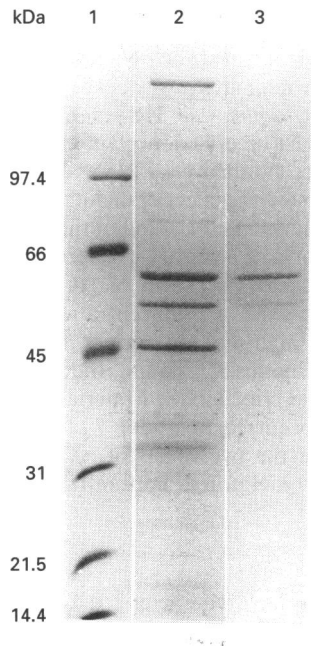

Figure 6 Analysis by SDS/PAGE of polypeptides from the 'avidin-void' fraction after chromatography on a QHyperD column and a Red 120–agarose column

Polypeptides were separated on a 7.5–15% (w/v) gradient polyacrylamide/SDS slab gel and stained with Coomassie Brilliant Blue R-250. Lane 1, molecular-mass markers (5 μg each); lane 2, QHyperD fraction (50 μg); lane 3, Red 120–agarose fraction (5 μg). Molecular masses on the left are given in kDa.

was stimulated approx. 2-fold with a concentration of 50 mM KCl, while the low-molecular-mass form of ACCase was stimulated about 1.5-fold with a concentration of 100 mM KCl.

Ethanol at a concentration of 15% (v/v) activates by 10-fold *Escherichia coli* ACCase activity (Guchhait et al., 1974). At this ethanol concentration, both pea leaf ACCase activities were completely inhibited. Lower ethanol concentrations also inhibited plant ACCases, although not always completely. For example, at concentrations of 5% and 10% ethanol, the chloroplastic ACCase was inhibited 65% and 95% respectively.

An interesting observation was made concerning the effect of protein concentration on the activity of both ACCase forms. For the pure high-molecular-mass form of ACCase, the assay was linear up to at least 100 μg/ml and for 20 min. On the other hand, the evolution of the activity of the low-molecular-mass form of ACCase with respect to protein concentration exhibited a sigmoidal response, suggesting a possible association–dissociation effect between the different proteins of a putative enzymic complex involved in ACCase activity (results not shown).

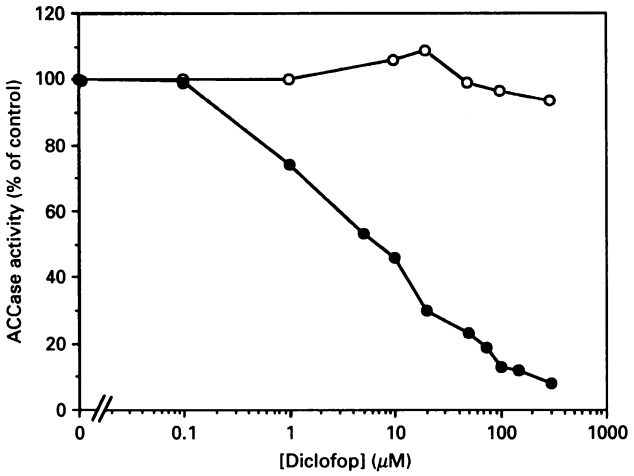

Figure 7 Effect of increasing concentrations of diclofop on the activity of the pure high-molecular-mass form (●) and partially purified low-molecular-mass form (○) of ACCase from pea leaves

ACCase activity was measured under the standard assay conditions of the Materials and methods section. Incubation was at 30 °C for 20 min.

This effect was independent of the presence of BSA in the reaction mixture.

ACCase is the established site of action for diclofop and other aryloxyphenoxypropionates, as well as for cyclohexanedione herbicides in the *Graminaceae*. However, ACCase activity from crude or chloroplast dicotyledon plant extracts is not inhibited by these herbicides (Walker et al., 1988a,b; Rendina and Felts, 1988). We have tested the effect of diclofop on the activity of the pure high-molecular-mass form and of the partially purified low-molecular-mass form of pea leaf ACCase. Diclofop had no effect on the low-molecular-mass form of ACCase. Less than 10% inhibition was observed at a concentration of 0.3 mM diclofop. In contrast, the high-molecular-mass form of ACCase was severely inhibited by this compound. Indeed, under the standard assay conditions described in the Materials and methods section, 50% inhibition occurred at about 7 μM diclofop (Figure 7). Finally, neither of the two forms of ACCase was found to be affected by methyl-diclofop at concentrations up to 0.1 mM.

DISCUSSION

In this report, we have characterized two different forms of ACCase in young green pea leaves. The minor one was detected specifically in the epidermal tissue. This enzyme was soluble and consisted of a dimer of two identical biotinyl subunits of molecular mass 220 kDa. This structure is very similar to that described for wheat germ, wheat leaves, soyabean seeds, parsley cells, maize leaves and developing pea embryos (Egin-Buhler and Ebel, 1983; Slabas and Hellyer, 1985; Bettey et al., 1992; Gornicki and Haselkorn, 1993; Egli et al., 1993) and for other eukaryotes such as yeast, mammals and chicken (Lopez-Casillas et al., 1988; Takai et al., 1988; Al-feel et al., 1992). In this respect, this pea leaf enzyme can be referred to as an eukaryotic form of ACCase. On the other hand, the predominant form of ACCase was present both in epidermal and mesophyll tissues. In mesophyll protoplasts, ACCase was detected exclusively in the soluble phase of chloroplast and consisted of a freely dissociating enzyme composed of at least four different subunits of molecular masses ranging from 32 to 79 kDa. Only one of these subunits, of molecular mass 38 kDa, was biotinylated. This suggests a prokaryotic structure, because *E. coli* ACCase contains three functional domains on four distinct, separable polypeptides (Li and Cronan, 1992a,c). Despite this structural similarity, the difference in the size of at least the biotinyl subunit of ACCase in the two organisms is noticeable (38 kDa in pea and 17 kDa in *E. coli*). Such a prokaryotic structure for ACCase was previously observed in the 1970s by Kannangara and Stumpf (1972) in spinach chloroplasts, Kannangara and Jensen (1975) and Reitzel and Nielsen (1976) in barley chloroplasts, and Mohan and Kekwick (1980) in spinach chloroplasts and avocado plastids, except that in all these cases the biotinyl subunit of ACCase was found to be associated with the membrane fraction of chloroplasts. However, it has been postulated, and then generally agreed, that the occurrence of the low-molecular-mass biotinyl polypeptide was essentially, if not exclusively, due to a severe degradation of the high-molecular-mass polypeptide form by endogenous proteinases during the course of ACCase isolation (Slabas and Hellyer, 1985; Hellyer et al., 1986; Harwood, 1988). On the contrary, in our experiments, addition of the pure high-molecular-mass form of ACCase to a chloroplast stroma fraction did not result in any alteration with time of the protein. Furthermore, the two ACCase activities described in this report differed in several aspects including biochemical parameters, anion-exchange properties, herbicide inhibition and tissue distribution. We are forced to conclude that, in contrast with the high-molecular-mass form of ACCase, the chloroplastic ACCase system is composed of several different components that dissociate very easily. In favour of the existence of a prokaryotic form of ACCase in plants is also the existence of an open reading frame in the pea chloroplast genome, named *accD*, and coding for a putative polypeptide exhibiting sequence similarities with the β subunit of the transcarboxylase component of *E. coli* ACCase (Li and Cronan, 1992b).

In fact, the occurrence of ACCase isoforms in leaves localized in different compartments is not surprising as malonyl-CoA, the product of the catalysed ACCase reaction, is required for multiple biosynthetic pathways specifically compartmentalized in the leaf. In mesophyll cells, *de novo* fatty acid synthesis occurs predominantly, if not exclusively, in chloroplasts (Ohlrogge et al., 1979) and the initial substrate for this pathway is malonyl-CoA. On the other hand, the biosynthesis of the very-long-chain fatty acids required for cuticular waxes occurs in the epidermal tissue (Kolattukudy et al., 1976). This process involves *de novo* C_{18} fatty acid synthesis in plastids and their elongation (that also requires malonyl-CoA as substrate) to C_{20}–C_{30} fatty acids in an extraplastidic compartment. Furthermore, chalcone synthase (EC 2.3.1.74), an enzyme requiring malonyl-CoA for the biosynthesis of flavonoids, occurs exclusively in the epidermis of pea leaves (Hrazdina et al., 1982).

Interestingly, maize leaves also contain two ACCase activities: a predominant herbicide-sensitive plastid-localized enzyme (ACCase I) and a minor enzyme (ACCase II), which differs from ACCase I in several properties including tissue distribution. Surprisingly, in this case both enzymes were of the 'eukaryotic' type, consisting primarily of a single (227 kDa, ACCase I; 219 kDa, ACCase II) biotinylated polypeptide (Egli et al., 1993). Thus, in the frame of the endosymbiont hypothesis of chloroplast origin, this would suggest different evolutionary pathways for ACCases of C_3- and C_4-metabolizing plants.

K_m values measured for the prokaryotic and eukaryotic forms of pea leaf ACCase are very similar, except for acetyl-CoA. Thus, a K_m value of 0.015 mM was obtained for the high-molecular-mass form of ACCase compared with 0.25 mM in the

case of the chloroplastic form of ACCase. This last value is high compared with the probable concentration of acetyl-CoA in chloroplasts. For example, this concentration was recently calculated to be 46–54 μM in pea chloroplasts (Post-Beittenmiller et al., 1992). Thus, these observations indicate that the availability of acetyl-CoA for the reaction catalysed by ACCase may be rate limiting in the fatty acid biosynthetic pathway within plastids.

Finally, the results presented in this article also demonstrate that the two pea leaf ACCases share different sensitivities towards the monocotyledon ACCase herbicide, diclofop. First, we observed that neither of the two enzyme forms was sensitive to methyl-diclofop. This is not surprising as it is known that the free acid is the active form of the herbicide (Walker et al., 1988b). Secondly, we showed that in contrast with the prokaryotic form, the high-molecular-mass form of pea leaf ACCase was inhibited by diclofop, with an IC_{50} value of about 7 μM. This is to our knowledge the first report of the occurrence in dicotyledons of an ACCase that shows 'sensitivity' to an aryloxyphenoxypropionate herbicide. However, this value is higher than those reported for monocotyledon ACCases. For example, for diclofop, IC_{50} values of 70 nM in the case of wheat ACCase (Rendina et al., 1990), 100 nM in the case of chloroplastic oat ACCase (Rendina et al., 1988) and 1 μM in the case of maize ACCase (Secor et al., 1989) were given. Furthermore, the chloroplastic form of pea leaf ACCase, which accounts for about 80 % of the total leaf ACCase activity, was found to be totally insensitive to diclofop. Taken together, these results would help to explain the tolerance of dicotyledon plants like pea to the aryloxyphenoxypropionate herbicides.

Further characterization of the proteins of the dissociable multienzyme complex may provide valuable molecular insights for understanding the respective roles and origins of both ACCases in dicotyledon plants.

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