Biochem. J. (1996) 318, 997–1006 (Printed in Great Britain)

Kinetic studies on two isoforms of acetyl-CoA carboxylase from maize leaves

Derek HERBERT*, Lindsey J. PRICE*, Claude ALBAN†, Laure DEHAYE*, Dominique JOB†, David J. COLE‡, Kenneth E. PALLETT‡ and John L. HARWOOD*§

*School of Molecular and Medical Biosciences, University of Wales College of Cardiff, P.O. Box 911, Cardiff CF1 3US, Wales, U.K., †Unité Mixte C.N.R.S./Rhône-Poulenc (Unité associée au Centre National de la Recherche Scientifique, U.M.R. 41), Rhône-Poulenc Agrochimie, 14–20 rue Pierre Baizet, 69263 Lyon, France, and ‡Rhône-Poulenc Agriculture Ltd., Ongar, Essex CM5 OHW, U.K.

The steady-state kinetics of two multifunctional isoforms of acetyl-CoA carboxylase (ACCase) from maize leaves (a major isoform, ACCase1 and a minor isoform, ACCase2) have been investigated with respect to reaction mechanism, inhibition by two graminicides of the aryloxyphenoxypropionate class (quizalofop and fluazifop) and some cellular metabolites. Substrate interaction and product inhibition patterns indicated that ADP and P_i products from the first partial reaction were not released before acetyl-CoA bound to the enzymes. Product inhibition patterns did not match exactly those predicted for an ordered Ter Ter or a random Ter Ter mechanism, but were close to those postulated for an ordered mechanism. ACCase2 was about 1/2000 as sensitive as ACCase1 to quizalofop but only about 1/150 as sensitive to fluazifop. Fitting inhibition data to the Hill equation indicated that binding of quizalofop or fluazifop to ACCase1 was non-cooperative, as shown by the Hill constant (n_{app}) values of 0.86 and 1.16 for quizalofop and fluazifop respectively. Apparent inhibition constant values (K' from the Hill equation) for ACCase1 were 0.054 μM for quizalofop and 21.8 μM for fluazifop. On the other hand, binding of quizalofop or fluazifop to ACCase2 exhibited positive co-operativity, as shown by the n_{app} values of 1.85 and 1.59 for quizalofop and fluazifop respectively. K' values for ACCase2 were 1.7 mM for quizalofop and 140 mM for fluazifop. Kinetic parameters for the co-operative binding of quizalofop to maize ACCase2 were close to those of another multifunctional ACCase of limited sensitivity to graminicide, ACC220 from pea. Inhibition of ACCase1 by quizalofop was mixed-type with respect to acetyl-CoA or ATP, but the concentration of acetyl-CoA had the greater effect on the level of inhibition. Neither ACCase1 nor ACCase2 was appreciably sensitive to CoA esters of palmitic acid (16:0) or oleic acid (18:1). Approximate IC_{50} values were 10 μM (ACCase2) and 50 μM (ACCase1) for both CoA esters. Citrate concentrations up to 1 mM had no effect on ACCase1 activity. Above this concentration, citrate was inhibitory. ACCase2 activity was slightly stimulated by citrate over a broad concentration range (0.25–10 mM). The significance of possible effects of acyl-CoAs or citrate *in vivo* is discussed.

INTRODUCTION

Acetyl-CoA carboxylase (ACCase) is a biotinylated enzyme that catalyses the ATP-dependent carboxylation of acetyl-CoA to form malonyl-CoA, with bicarbonate as the source of carbon [1]. This is the first committed step in the biosynthesis of fatty acids [2]. The ACCase-catalysed reaction takes place at two catalytic sites via two partial reactions [1]. In the biotin carboxylase partial reaction, ATP and bicarbonate are used to carboxylate the biotin prosthetic group that is covalently bound to the biotin carboxyl carrier protein (BCCP). The second partial reaction is that catalysed by the carboxyltransferase, where the carboxy group attached to the biotin residue of the BCCP is transferred to acetyl-CoA to produce malonyl-CoA. The plant ACCases have attracted particular attention recently because they are the site of action of the grass-specific herbicides (graminicides) of the aryloxyphenoxypropionate class (e.g. quizalofop, fluazifop, diclofop) [3], although only the grass chloroplastic isoform of ACCase is appreciably sensitive [4,5].

Plastidic and extraplastidic isoforms of ACCase have recently been purified from leaves of several species. In grasses (the Graminaceae) both isoforms are nuclear-encoded and composed of high-molecular-mass multifunctional polypeptides of 219–240 kDa (4–6) where the three functional domains, biotin carboxylase, BCCP and carboxyltransferase, are present on a single polypeptide chain. In this respect they resemble the ACCases from animals and yeast [7,8]. Plant multifunctional ACCases seem to function as homodimers *in vivo* [4–6,9,10]. The major isoform in maize leaves, representing approx. 80 % of total leaf ACCase activity [4,5], is localized in mesophyll chloroplast stroma [4], where it supplies malonyl-CoA for the synthesis of fatty acids *de novo* [2]. The minor isoform is absent from mesophyll chloroplasts but has not so far been localized. It might be enriched in epidermal cells, as in pea [11], to supply malonyl-CoA for cuticular wax synthesis or the synthesis of flavonoids, both of which occur in the epidermal cytoplasm [12,13]. In dicotyledons, such as pea, the chloroplastic isoform is a multienzyme complex (of which one subunit is chloroplast-encoded) [15] of four functional proteins [11,14,16–18] and therefore resembles ACCase from micro-organisms [19]. Only the minor, extraplastidic, form in pea leaves and seeds, ACC220, is multifunctional [9,11].

Two general reaction mechanisms are possible for multifunctional ACCases [20,21]. In the Ping Pong system, intermediate products ADP and P_i from the biotin carboxylase partial reaction are released before acetyl-CoA binds to the carboxyltransferase site for the second partial reaction to take place. The other general system that is possible is a Ter Ter, where ADP and P_i are not released before acetyl-CoA binds, but instead a ternary complex is formed between the carboxybiotinyl-

Abbreviations used: ACCase, acetyl-CoA carboxylase (EC 6.4.1.2); BCCP, biotin carboxyl carrier protein; MCCase, methylcrotonoyl-CoA carboxylase (EC 6.4.1.4); DTT, dithiothreitol.

§ To whom correspondence should be addressed.

enzyme and ADP, P_i and acetyl-CoA. Kinetic data from maize [22] and castor bean [23] are in keeping with a Ping Pong mechanism. However, those experiments were performed with preparations containing more than one isoform of ACCase. In contrast, analysis of ACCase from chick liver indicated a Ter Ter mechanism [20].

Most characterizations of grass-leaf ACCases have been performed on a mixture of isoforms that were not separated during purification. In particular, this might lead to apparent negative co-operativity in graminicide binding [10]. In the present study we performed separate kinetic analyses on two multifunctional isoforms of ACCase from maize leaves and a multifunctional ACCase from pea seeds to determine whether co-operative behaviour was linked with insensitivity to graminicide.

It had been shown that aryloxyphenoxypropionate graminicides are reversible, non-competitive inhibitors of ACCase preparations containing unresolved isoforms from maize or wheat [22,24,25] and that the carboxyltransferase partial reaction is more sensitive to inhibition than the biotin carboxylase partial reaction [26,27]. We performed statistical analyses on kinetic data for the inhibition of the major maize isoform by quizalofop, with acetyl-CoA or ATP as varied substrates.

Although results from kinetic studies with graminicides are extremely important for the understanding of their herbicidal action and possible reasons for resistance at the target protein level, they do not have relevance to the regulation of acetyl-CoA carboxylase under normal conditions. Because ACCase has been shown to exert high flux control over lipid synthesis in leaves [28,29], an understanding of this regulation is obviously important. Moreover the different subcellular locations (and roles) for the two isoforms of maize ACCase lend particular interest in their control. Accordingly we have also studied the possible effects of physiologically relevant constituents (acyl-CoAs and citrate) on their activity.

MATERIALS AND METHODS

Materials

Maize (*Zea mays* cv. Champ) seeds were bought from Nickerson Seeds Ltd. (Lincoln, U.K.). Plants were grown in soil-less compost at 20 °C with 650 μE/s per m^2 illumination and a 12 h day/12 h night cycle. Plants were watered with tap water when required. Pea plants (*Pisum sativum*) were grown as described previously (9).

Quizalofop free acid {(*R*,*S*)-2-[4-(6-chloroquinoxalin-2-yloxy)-phenoxy]propionic acid} was provided by Rhône-Poulenc Agriculture Ltd. Fluazifop free acid {(*R*,*S*)-2-[4-(5-trifluoromethyl-2-pyridyloxy)phenoxy]propionic acid} was bought from Cluzeau Info. Laboratoire (Ste.-Foy-la Grande, France). Quizalofop and fluazifop free acids were racemic mixtures and were at least 98 % pure. Sodium [^{14}C]bicarbonate (3.7–37 GBq/mmol) was bought from Amersham International, Amersham, U.K. Other materials were bought from Sigma (Poole, Dorset, U.K.), Pharmacia Biosystems Ltd. (Central Milton Keynes, Bucks., U.K.) or Fisons Scientific Equipment Ltd. (Loughborough, U.K.) unless stated otherwise, and were the best available grades.

Purification and separation of two isoforms of ACCase from maize leaves

All steps were performed at 4 °C unless stated otherwise. Up to 100 g of freshly harvested leaves from 7-day-old maize plants were ground to a fine powder in liquid nitrogen with a pestle and mortar. The powder was transferred to another mortar to prevent the buffer from freezing and resuspended in two volumes of extraction buffer [0.1 M Hepes/KOH, pH 8.0, 1 mM NaEDTA, 10 % (v/v) glycerol, 5 mM dithiothreitol (DTT), 5 mM ϵ-aminohexanoic acid, 1 mM benzamidine/HCl and 1 mM PMSF, the last four ingredients being added immediately before use]. The homogenate was left for 5 min with occasional stirring, before filtering through four layers of Miracloth (Calbiochem, San Diego, CA, U.S.A.). The crude extract was centrifuged at 30000 ***g*** for 30 min to remove cell debris and then fractionated with ammonium sulphate. Material precipitating between 30 % and 55 % ammonium sulphate was collected by centrifugation at 30000 ***g*** for 30 min and dissolved in approx. 20 ml of resuspension buffer [20 mM Hepes/KOH, pH 8.0, 1 mM NaEDTA, 20 % (v/v) glycerol, 1 mM DTT, 5 mM ϵ-aminohexanoic acid, 1 mM benzamidine/HCl and 1 mM PMSF]. The solution was centrifuged at 105000 ***g*** for 30 min to remove cell membranes and transferred in column buffer (resuspension buffer without PMSF) onto a Sephadex G-25 column. The desalted fraction was loaded (flow rate 0.5 ml/min) on to a column of Red 120-agarose (2.6 cm × 15 cm, 80 ml) connected to a HiLoad (Pharmacia) automated gradient system and previously equilibrated with column buffer. The column was washed with this buffer (flow rate 1 ml/min) until the absorbance at 280 nm returned to the baseline, showing that all non-retained proteins had been removed. The bulk of the retained proteins that were not ACCase were then eluted with an 80 ml gradient of 0–0.2 M KCl followed by a 160 ml wash at 0.2 M KCl, both in column buffer. ACCase was then eluted with a 160 ml wash at 0.4 M KCl in column buffer. Fractions (8 ml) containing ACCase activity were pooled, made up to 50 mM in Hepes/KOH, pH 8.0, by the addition of 500 mM Hepes, pH 8.0, stock buffer, and brought to 70 % saturation by the addition of solid ammonium sulphate. Precipitated proteins (approx. 20 mg) were collected by centrifugation (30 000 ***g*** for 30 min), redissolved in resuspension buffer and desalted into column buffer before loading (flow rate 0.5 ml/min) on to a column of Q-Sepharose (1 cm × 12 cm, 9.4 ml) previously equilibrated with column buffer. The column was washed with this buffer (flow rate 1 ml/min) to remove non-retained proteins. Non-ACCase retained proteins were eluted with a 20 ml gradient of 0–0.15 M KCl followed by a 30 ml wash at 0.15 M KCl, both in column buffer. Two peaks of ACCase activity were then separated with an 80 ml linear gradient of 0.15–0.3 M KCl in column buffer. Fractions (1 ml) containing the two separated peaks of ACCase activity were pooled separately, concentrated to a final protein concentration of approx. 0.5 mg/ml with Macrosep-10 concentrators (Flowgen Instruments Ltd., Sittingbourne, Kent, U.K.) and stored at −80 °C until used.

Separation of the two peaks of activity was verified by assaying aliquots in the presence of 1 μM quizalofop, which almost totally inhibited ACCase activity in the major peak (more than 95 % inhibition) but had no effect on ACCase activity in the minor peak. Separation was also verified by performing Western blotting to detect biotinylated proteins (see the Results section).

Purification of a multifunctional ACCase from pea leaves or seeds

Purification of a multifunctional ACCase from pea was performed as described previously [9].

Assay for ACCase

All assays were optimized with respect to the concentration of each reaction component and to the pH of the reaction mixture. ACCase activity was measured under a fume hood as the incorporation of radioactivity from $NaH^{14}CO_3$ into an acid-

stable product, as described previously [9]. The acid-stable product was identified as malonyl-CoA by HPLC in preliminary experiments (results not shown). The standard assay mixture was 50 mM Hepes/KOH, pH 8.0 or 7.5, 2.5 mM $MgCl_2$, 1 mM ATP, 0.5 mM DTT, 10 mM $NaH^{14}CO_3$ (37 GBq/mmol), 1.5 mM acetyl-CoA and 2–5 μg of ACCase-containing protein, in a final volume of 200 μl in a 1.5 ml Eppendorf tube. Assays were preincubated for 10 min at 30 °C and then started by the addition of acetyl-CoA. Assays were terminated up to 20 min after the addition of acetyl-CoA, which was within the linear labelling period. Initial reaction velocities could then be calculated conveniently. Aliquots (150 μl) of the reaction mixtures were added to 20 ml scintillation vials containing 40 μl of 12 M HCl to stop the reaction and drive off unincorporated radiolabel. The vials were agitated gently by hand and the solutions were dried at room temperature with a stream of nitrogen. The dried residue was dissolved by adding 0.25 ml of distilled water, agitating gently by hand and leaving for 15 min. Acid-stable radioactivity was measured by adding 10 ml of scintillant (Optifluor; Canberra-Packard) and liquid-scintillation counting. Assays without acetyl-CoA were run to measure background activities; these values were subtracted from all readings. Background activities were less than 3 % of control activities in purified fractions. Assays were duplicated unless stated otherwise. ACCase activity was expressed as μmol of bicarbonate fixed per min per mg of enzyme protein. Quizalofop, fluazifop and other effectors were added to the assay mixtures before the 10 min preincubation. DTT was omitted from the assay mixture when the thiol-modifying reagent *N*-ethylmaleimide and the arginyl-modifying reagent phenylglyoxal were used. The omission of DTT did not affect ACCase activity significantly.

Tissue separation and subcellular fractionation

Leaf tissue from 7-day-old maize leaves was digested enzymically and subcellular fractionation performed as described by Baldet et al. [30]. Bundle-sheath strands were separated from mesophyll cells as described by Hawke et al. [31]. Proteins from the different preparations were analysed electrophoretically as described previously [11].

Preparation of quizalofop or fluazifop solutions

Stock solutions (30 mM) were made in 1.5 ml Eppendorf tubes by dissolving herbicide in acetone and then adding 0.5 M Hepes/KOH (pH 8.0) to give an acetone concentration of 9 % (v/v). Stock solutions were protected from light, stored at −20 °C and used within three weeks. After appropriate dilution in distilled water, solutions were added immediately to assay mixtures. Final acetone concentrations in the assays were less than 0.3 %, a concentration that did not affect ACCase activity.

Protein concentration measurement

Protein concentration was measured by the method of Bradford [32] by using Bio-Rad protein assay reagent with BSA as standard.

Determination of kinetic parameters

Parameter values ± S.D. were estimated by fitting duplicated initial reaction velocity values to different steady-state rate equations. All data shown were fitted by least-squares analysis with the SigmaPlot version 4.0 computer program (Jandel Scientific, Corte Madera, CA, U.S.A.) to the equation chosen by the variance ratio test [33].

Substrate interactions with enzyme were investigated by fitting data from each double-reciprocal plot to the reduced equation that applies to a parallel-line pattern (Ping Pong mechanism) [eqn. (1)] and to an equation for an intersecting-line pattern (Ter Ter mechanism) [eqn. (2)] [21]:

$$v = V_{max}/(1 + K_a/[a] + K_c/[c]) \quad (1)$$

or

$$v = V_{max}/(1 + K_b/[b] + K_c/[c])$$

$$v = V_{max}/\{1 + K_a/[a] + K_c/[c] + K_{ac}/([a][c])\} \quad (2)$$

or

$$v = V_{max}/\{1 + K_b/[b] + K_c/[c] + K_{bc}/([b][c])\}$$

where v is the experimentally determined initial velocity; V_{max} is the maximum velocity when the concentrations of substrates a (ATP) and c (acetyl-CoA), or b (bicarbonate) and c are saturating; K_a, K_b and K_c are the Michaelis constants for the varied substrates a, b and c respectively; and K_{ac} and K_{bc} are interaction terms.

Data from product inhibition studies were fitted to the rate equations for non-competitive inhibition [eqn. (3)] [mixed-type, with the slope and intercept inhibition constants (K_{ii} and K_{is} respectively) being different], and to equations for the limiting cases of competitive [eqn. (4)] or uncompetitive [eqn. (5)] inhibition when K_{ii} and K_{is} respectively tend to infinity:

$$v_i = \frac{V'}{(1+[I]/K_{is})K'_a + (1+[I]/K_{ii})[a]} \quad (3)$$

$$v_i = \frac{V'}{(1+[I]K_{is})K'_a + [a]} \quad (4)$$

$$v_i = \frac{V'}{K'_a + (1+[I]/K_{ii})[a]} \quad (5)$$

where v_i is initial enzyme velocity in the presence of different concentrations of inhibitor, a is the varied substrate, I is the product inhibitor and K'_a and V' are the Michaelis constant and maximum velocity respectively, in the presence of inhibitor. The ranges of concentrations for varied substrates and products were: ATP, 0.02–0.5 mM; $NaHCO_3^-$, 0.2–10 mM; AcCoA, 0.005–0.5 mM; P_i (Na_2HPO_4), 7.5–40 mM; MgADP, 0.4–3 mM; malonyl-CoA, 0.025–0.5 mM. Concentrations of the non-varied substrates were fixed at ATP, 1 mM; HCO_3^-, 10 mM; AcCoA, 0.8 mM.

Data for inhibition of the maize or pea ACCases by quizalofop or fluazifop were fitted to different steady-state rate equations [21], as follows.

First is an equation for simple hyperbolic inhibition:

$$v_i = \frac{V_0}{1 + ([I]/K_i)} \quad (6)$$

where V_0 is the control enzyme velocity (i.e. in the absence of inhibitor); v_i is the initial enzyme velocity in the presence of different concentrations of inhibitor, I; and K_i is the inhibition constant. Values of v_i were expressed as percentages of V_0 (100 %).

Next is the Hill equation:

$$v_i = \frac{V_0 K'}{K' + [I]^{n_{app}}} \quad (7)$$

An apparent Hill coefficient, n_{app}, or more than 1 indicates positive co-operativity; $n_{app} < 1$ indicates negative co-

operativity; $n_{app} = 1$ indicates that there is no co-operativity. K' is an inhibition constant.

Finally there is the equation for a two-site enzyme:

$$v_i = \frac{V_0}{1 + ([I]/K_{1i}) + ([I]^2/K_{1i}K_{2i})} \quad (8)$$

which assumes that the native enzyme has two quizalofop-binding sites, each with its own apparent inhibition constant, K_{1i} or K_{2i}.

RESULTS

Purification of two multifunctional ACCases from maize leaves

The three-step purification is summarized in Table 1. ACCase activity eluted from the Red 120-agarose column in the wash at 0.4 M KCl. Approx. 41 % of the applied ACCase activity was recovered from this column. Pigment–protein complexes mostly passed directly through the Red 120-agarose column and were therefore eliminated at that stage. The other major biotin-containing carboxylase, methylcrotonoyl-CoA carboxylase (MCCase), was not eluted from the column. In the young (7-day-old) leaves that were used for the purification the MCCase activity was small compared with ACCase (approximate specific activities in nmol/min per mg of soluble leaf proteins were 11 for ACCase and less than 0.25 for MCCase). Anion-exchange chromatography with Q-Sepharose separated two peaks of ACCase activity, which were eluted at approx. 210 mM KCl (ACCase2) and 250 mM KCl (ACCase1), as observed previously in analogous purifications of maize leaf ACCase with a similar anion-exchange material, Mono-Q [4,34]. It was necessary to use a shallow KCl concentration gradient (0.15–0.30 M in 8 column volumes) and to take many small fractions (80 × 1 ml) to achieve complete separation of the two peaks. ACCase1 represented 80–85 % of the total activity recovered from the column (23 % of the original crude extract activity) and had a specific activity of approx. 1.2 μmol/min per mg. The specific activity of ACCase2 was less than 20 % of that of ACCase1, in agreement with Egli et al. [4].

The above protocol allowed the rapid separation of maize ACCase isoforms in reasonable yield. Most importantly, the two isoforms were completely separated from each other. This procedure was therefore used to permit further study of these proteins.

Biochemical properties of ACCases 1 and 2

SDS/PAGE analysis and staining with Coomassie Brilliant Blue suggested that ACCases 1 and 2 were each approx. 30 % pure. Western blotting showed biotinylated subunit molecular masses of 230 and 220 kDa respectively, for the two isoforms. Gel-filtration with Sephacryl-S-300HR indicated that both isoforms were dimers. These results were in agreement with previous studies on multifunctional ACCases from maize and other plant species [4–6,9,10,35]. Only ACCase1 was appreciably sensitive to quizalofop or fluazifop, in agreement with previous studies [4,5].

Table 1 Purification of two isoforms of ACCase from maize leaves

A representative purification is shown beginning with 79 g fresh weight of 7-day-old leaves.

	Protein (mg)	Total activity (μmol/min)	Specific activity (μmol/min per mg)	Yield (%)	Purification (fold)
Crude extract	401	5.0	0.011	100	1
$(NH_4)_2SO_4$ (30–55 %)	217	5.7	0.026	114	2.4
Red 120-agarose	20	2.3	0.12	46	11
Q-Sepharose (ACCase1)	1	1.15	1.2	23	109
Q-Sepharose (ACCase2)	1.2	0.25	0.2	5	19

Table 2 Apparent K_m values of maize ACCases 1 and 2

Values were obtained by fitting data from three experiments using different enzyme preparations to the Michaelis–Menten equation. Assay details are given in the Materials and methods section. Results are means ± S.D.

	K_m (M)	
Substrate	ACCase1	ACCase2
Acetyl-CoA	$(49 \pm 17) \times 10^{-6}$	$(37 \pm 12) \times 10^{-6}$
ATP	$(27 \pm 14) \times 10^{-6}$	$(21 \pm 5) \times 10^{-6}$
Bicarbonate	$(1.8 \pm 0.3) \times 10^{-3}$	$(1.4 \pm 0.3) \times 10^{-3}$

Western blotting with streptavidin–peroxidase of a stromal extract from purified mesophyll cell chloroplasts showed a biotinylated polypeptide of approx. molecular mass 230 kDa but not of 220 kDa (results not shown). ACCase1 was therefore thought to be localized in the mesophyll chloroplast stroma as suggested previously [4]. Western blotting of total soluble protein extracts of mesophyll protoplasts and bundle sheath strands also detected only ACCase1. However, crude extracts from whole maize leaves showed biotin-containing proteins of both 230 and 220 kDa. Therefore it seems probable that the soluble ACCase2 is enriched in epidermal cells (the only tissue fraction that could not be isolated separately), as was demonstrated for the extra-chloroplastidic isoform, ACC220, in pea leaves [11].

Both ACCases 1 and 2 were active over a wide pH range, with optimal activity at approx. pH 8.0 for ACCase1 and at approx. pH 7.5 for ACCase2 (results not shown). All enzyme preparations required Mg^{2+}, ATP, bicarbonate and acetyl-CoA for activity. Velocity was maximal when 2.5 mM Mg^{2+} and 1 mM ATP were included in assays. K_m values (Michaelis constants) are shown in Table 2. These K_m values were similar to those measured for multifunctional ACCases from maize and other plant species [4,10,23,36–38].

Both ACCases 1 and 2 were inhibited by the thiol-modifying reagent *N*-ethylmaleimide and the arginyl-modifying reagent phenylglyoxal (results not shown). After 15 min of incubation of reagent with enzyme, ACCases 1 and 2 were inhibited approx. 25 % by 0.01 mM *N*-ethylmaleimide or 0.2 mM phenylglyoxal and 100 % by 0.1 mM *N*-ethylmaleimide or 5 mM phenylglyoxal. Similarly we detected little difference in sensitivity between the isoforms at other concentrations of inhibitor. Preincubation (10 min) of enzyme with substrate (ATP or acetyl-CoA) gave some protection against phenylglyoxal inactivation: 5 mM ATP decreased inhibition by 1 mM phenylglyoxal from 68 % to 43 %; 2 mM acetyl-CoA decreased inhibition by 1 mM phenylglyoxal from 66 % to 55 %. Compared with plant MCCase [39], ACCase1 and ACCase2 activities were about twice as sensitive to phenylglyoxal and five times more sensitive to *N*-ethylmaleimide. Furthermore, preincubation of MCCase with equivalent concentrations of substrates gave greater protection (70–100 %) than for ACCase1 or ACCase2.

Substrate interaction kinetics of maize ACCases 1 and 2

Steady-state kinetic experiments were performed on the separated maize isoforms, ACCases 1 and 2, to determine whether data

Table 3 Michaelis constants for ACCases 1 and 2

Values were obtained by fitting duplicated assay values to $v = V_{max}/\{1+K_a/[a]+K_c/[c]+K_{ac}/([a][c])\}$ or $v = V_{max}/\{1+K_b/[b]+K_c/[c]+K_{bc}/([b][c])\}$, as described in the Materials and methods section. Substance a is ATP; b is bicarbonate; c is acetyl-CoA. Concentrations of the non-varied substrate were fixed at: ATP, 1 mM; bicarbonate, 10 mM; AcCoA, 1 mM. Results are means ± S.D.

	Varied substrates*	Parameter	Parameter value (M)	Kinetic pattern
ACCase1				
ATP	Acetyl-CoA	K_a	$10.9 \pm 3.6 \times 10^{-6}$	Intersecting
		K_c	$28.8 \pm 8.6 \times 10^{-6}$	
		K_{ac}	$2.1 \pm 0.5 \times 10^{-3}$	
Bicarbonate	Acetyl-CoA	K_b	$1.3 \pm 0.4 \times 10^{-3}$	Intersecting
		K_c	$37.2 \pm 20.2 \times 10^{-6}$	
		K_{bc}	$82.5 \pm 21.0 \times 10^{-3}$	
ACCase2				
ATP	Acetyl-CoA	K_a	$6.6 \pm 2.1 \times 10^{-6}$	Intersecting
		K_c	$28.6 \pm 4.8 \times 10^{-6}$	
		K_{ac}	$1.1 \pm 0.3 \times 10^{-3}$	
Bicarbonate	Acetyl-CoA	K_b	$0.9 \pm 0.1 \times 10^{-3}$	Intersecting
		K_c	$58.7 \pm 10.9 \times 10^{-6}$	
		K_{bc}	$66.0 \pm 10.3 \times 10^{-3}$	

* The substrate in the second column was varied at different fixed concentrations of the substrate in the first column.

could be fitted best to an equation for a Ping Pong [eqn. (1)] or a Ter Ter [eqn (2)] mechanism. The concentrations of two substrates were varied while maintaining a fixed concentration of the third. The concentrations of acetyl-CoA (20–400 μM), ATP (30 μM to 1 mM) and bicarbonate (0.1–10 mM) were chosen to span their apparent K_m values. The fixed concentrations used were 1 mM ATP or 10 mM bicarbonate. Inhibition by substrate was not observed at any substrate concentration used.

Initial velocity data of ACCase1 or ACCase2 against acetyl-CoA concentration at different fixed levels of ATP or bicarbonate could not be fitted satisfactorily to a parallel-line equation for a Ping Pong mechanism. The data fitted better to an intersecting-line equation for a Ter Ter mechanism. Intersecting lines indicate that binding of the two varied substrates in each experiment is connected by reversible steps [21], implying that there was no release of ADP or P_i before acetyl-CoA bound to the enzyme. Michaelis constants, K_a, K_b, K_c, K_{ac} and K_{bc}, for interaction of the varied substrates with the two isoforms were obtained by fitting data to an equation for a Ter Ter mechanism [eqn (2)] (Table 3). These constants were similar for the two isoforms.

ACCase2 was about 10-fold more sensitive to inhibition by malonyl-CoA, a product of the carboxyltransferase partial reaction [1], than was ACCase1. However, ACCase2 was less sensitive than ACCase1 to graminicides that inhibit this partial reaction. Together these findings could indicate a higher specificity of the carboxyltransferase active site of ACCase2 for its substrates and products, compared with ACCase1.

Product inhibition kinetics of maize ACCases 1 and 2

The product inhibition patterns for ACCases 1 and 2 were similar. The observed patterns for acetyl-CoA are consistent with the existence of a two-site enzyme, as found previously for other biotin-dependent carboxylases [4,21]. The observed patterns are compatible with the idea that no product release is possible without prior fixation of all three substrates, in agreement with substrate interaction kinetic data. In particular, competitive

Table 4 Estimates of the product inhibition kinetic parameters for maize ACCases 1 and 2

Experimental details are as given in the Materials and methods section.

			Parameter value (mM)		
Substrate	Product	Term	Mean	S.D.	Kinetic pattern
ACCase1					
ATP	P_i	K'_a	0.05	0.003	Uncompetitive
		K_{ii}	4.30	1.53	
ATP	ADP	K'_a	0.04	0.01	Uncompetitive
		K_{ii}	0.24	0.04	
ATP	Malonyl-CoA	K'_a	0.04	0.01	Non-competitive
		K_{is}	0.22	0.06	
		K_{ii}	0.13	0.03	
HCO_3^-	P_i	K'_a	1.58	0.16	Non-competitive
		K_{is}	7.24	1.72	
		K_{ii}	21.40	3.92	
HCO_3^-	ADP	K'_a	1.56	0.15	Non-competitive
		K_{is}	2.08	0.76	
		K_{ii}	1.12	0.13	
HCO_3^-	Malonyl-CoA	K'_a	1.76	0.28	Non-competitive
		K_{is}	0.10	0.04	
		K_{ii}	0.23	0.06	
Acetyl-CoA	P_i	K'_a	0.03	0.003	Non-competitive
		K_{is}	7.60	2.40	
		K_{ii}	7.10	0.90	
Acetyl-CoA	ADP	K'_a	0.03	0.003	Uncompetitive
		K_{ii}	0.3	0.03	
Acetyl-CoA	Malonyl-CoA	K'_a	0.06	0.01	Non-competitive
		K_{is}	0.08	0.02	
		K_{ii}	0.13	0.02	
ACCase2					
ATP	P_i	K'_a	0.05	0.01	Uncompetitive
		K_{ii}	8.37	1.28	
ATP	ADP	K'_a	0.02	0.004	Uncompetitive
		K_{ii}	0.5	0.05	
ATP	Malonyl-CoA	K'_a	0.04	0.01	Non-competitive
		K_{is}	0.02	0.005	
		K_{ii}	0.012	0.003	
HCO_3^-	P_i	K'_a	1.53	0.21	Non-competitive
		K_{is}	6.68	1.52	
		K_{ii}	19.0	3.76	
HCO_3^-	ADP	K'_a	1.47	0.15	Uncompetitive
		K_{ii}	0.37	0.04	
HCO_3^-	Malonyl-CoA	K'_a	1.95	0.21	Non-competitive
		K_{is}	0.02	0.005	
		K_{ii}	0.03	0.004	
Acetyl-CoA	P_i	K'_a	0.030	0.005	Non-competitive
		K_{is}	0.60	0.05	
		K_{ii}	0.19	0.03	
Acetyl-CoA	ADP	K'_a	0.05	0.01	Uncompetitive
		K_{ii}	0.47	0.06	
Acetyl-CoA	Malonyl-Co A	K'_a	0.05	0.004	Non-competitive
		K_{is}	0.003	0.001	
		K_{ii}	0.008	0.005	

inhibition by either phosphate or ADP with respect to ATP would be predicted in a Ping Pong system [21], not the uncompetitive patterns that were observed. The product inhibition parameters obtained for maize ACCases 1 and 2 are listed in Table 4. The product inhibition patterns of ACCases 1 and 2 and some other Ter-reactant systems are summarized in Table 5. Like studies on the castor-bean ACCase [23] and the mammalian pyruvate carboxylase [40], the product inhibition patterns did not exactly match those theoretically predicted for an ordered or for a random Ter Ter mechanism [21]. However, they were close to those for an ordered mechanism (see Table 5)

Table 5 Product inhibition patterns for various Ter-reactant systems

Abbreviations: NC, non-competitive; U, uncompetitive, C, competitive; n.d., not determined.

System	Product inhibitor	Varied substrate		
		ATP	HCO_3^-	Acetyl-CoA
Predicted for Ter Ter				
Hybrid (two-site) ordered (ATP-HCO_3^--AcCoA, P_i-ADP-MalCoA)	P_i	NC	NC	NC
	ADP	U	U	U
	Malonyl-CoA	NC	NC	NC
Hybrid (two-site) random	P_i	C	NC	NC
	ADP	C	NC	NC
	Malonyl-CoA	NC	NC	C
Observed for Ping Pong				
Pyruvate carboxylase [40]	P_i	C	NC	U
	ADP	C	NC	U
	Malonyl-CoA	NC	NC	C
Castor oil seed ACCase [23]	P_i	NC	n.d.	n.d.
	ADP	C	NC	U
	Malonyl-CoA	NC	n.d.	NC
Observed for:				
maize ACCase 1	P_i	U	NC	NC
	ADP	U	NC	U
	Malonyl-CoA	NC	NC	NC
maize ACCase 2	P_i	U	NC	NC
	ADP	U	U	U
	Malonyl-CoA	NC	NC	NC

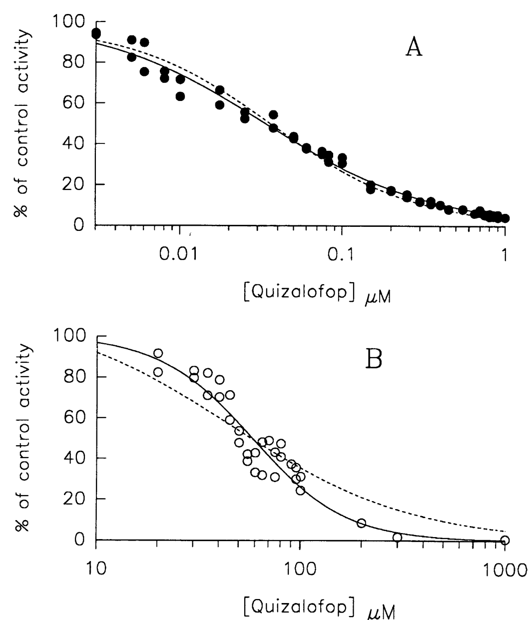

Figure 2 Curve fits for co-operative (solid line) or non-cooperative (broken line) binding of quizalofop to maize ACCase1 (A) or maize ACCase2 (B)

Assays were performed as described in the Materials and methods section. Curve fit parameters are listed in Table 6.

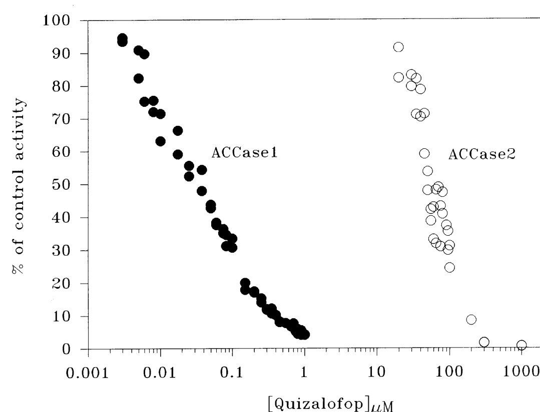

Figure 1 Inhibition of maize ACCases 1 and 2 by quizalofop

ACCase activity was measured as described in the Materials and methods section.

in which the order of substrate binding is ATP, bicarbonate and acetyl-CoA, with an ordered release of phosphate, ADP and malonyl-CoA.

Binding of quizalofop or fluazifop to maize ACCase1, maize ACCase2 or pea ACC220

Figure 1 shows the results obtained when the separated maize isoforms, ACCases 1 and 2, were assayed in the presence of different concentrations of quizalofop. Both isoforms were inhibited in a concentration-dependent manner but ACCase2 was approx. 1/2000 as sensitive as ACCase1. (Approximate IC_{50} values were 0.030 μM for ACCase1 and 60 μM for ACCase2.) Another multifunctional ACCase, pea ACC220, had a similar sensitivity to quizalofop as ACCase2 from maize (IC_{50} 57 μM) [9]. Another aryloxyphenoxypropionate graminicide, fluazifop, was tested against ACCases 1 and 2 (results not shown). ACCase2 was again less sensitive than ACCase1. However, ACCase2 was only approx. 1/150 as sensitive as ACCase1 to fluazifop. (Approximate IC_{50} values were 10 μM for ACCase1 and 1.5 mM for ACCase2.)

Inhibition data were fitted to different steady-state rate equations to investigate the kinetics of quizalofop or fluazifop binding to the different ACCases. Maize ACCase1 inhibition data could be fitted satisfactorily to the simple hyperbolic inhibition equation [eqn (6)]. In contrast with maize ACCase1, data for inhibition of maize ACCase2 or pea ACC220 by quizalofop or fluazifop could not be fitted satisfactorily to the simple hyperbolic equation.

Inhibition data were examined for co-operativity in quizalofop or fluazifop binding, by fitting to the Hill equation [eqn (7)]. Maize ACCase1 data fitted only slightly better to the Hill equation than to the simple hyperbolic equation, producing apparent Hill coefficients (n_{app}) close to 1 when quizalofop or fluazifop was the inhibitor [n_{app}(quizalofop) $= 0.86 \pm 0.03$; n_{app}(fluazifop) $= 1.16 \pm 0.09$]. This indicated only slight co-operativity in binding these graminicides [21], in agreement with results for ACCase from a graminicide-susceptible variety of *Lolium multiflorum*, which also showed only slight co-operativity in binding another graminicide of the aryloxyphenoxypropionate class, diclofop ($n_{app} = 1.2$) [10]. Although the latter result was not with a purified isoform, it would tend to reflect results for the chloroplastic isoform (i.e. ACCase1 in maize) because this represents approx. 80 % of the total leaf ACCase activity [4,5]. In contrast, maize ACCase2 and pea ACC220 data fitted better to the Hill equation than to the simple hyperbolic equation. Values of n_{app} for ACCase2 were 1.85 ± 0.19 (quizalofop) and 1.59 ± 0.09 (fluazifop), and for pea ACC220 1.70 ± 0.16 (quizalofop). A value of n_{app} greater than 1 indicates positive co-operativity [21].

Table 6 Summary of curve-fit parameters for inhibition of maize ACCases 1 and 2 and pea ACC220 by quizalofop or fluazifop

Parameter values (±S.D.) were estimated from eqns. (6) and (7) as described in the Materials and methods section.

	Hill			Simple hyperbolic	
	V_0 (%)	K' (M)	n_{app}	V_0 (%)	K_i (M)
Maize ACCase1					
Quizalofop	101±1.9	$54±4×10^{-9}$	0.86±0.03	98±1.5	$37±2×10^{-9}$
Fluazifop	102±2.2	$21.8±4.8×10^{-6}$	1.16±0.06	105±2.4	$13.0±0.9×10^{-6}$
Maize ACCase2					
Quizalofop	102±4.5	$1.77±0.15×10^{-3}$	1.85±0.19	112±6.7	$46.4±6.2×10^{-6}$
Fluazifop	98±1.7	$140±70×10^{-3}$	1.59±0.09	110±3.3	$1.26±0.11×10^{-3}$
Pea ACC220					
Quizalofop	101±2.4	$1.34±0.95×10^{-3}$	1.70±0.16	112±4.5	$56.8±8.1×10^{-6}$

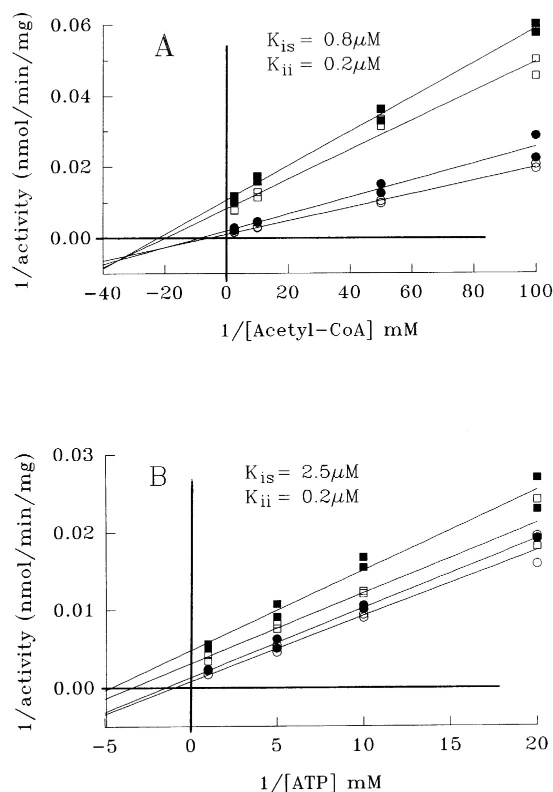

Figure 3 Double-reciprocal plots of the inhibition of maize ACCase1 by quizalofop at various concentrations of acetyl-CoA (A) or ATP (B)

Assays were performed as described in the Materials and methods section. (**A**) Concentrations of acetyl-CoA used were 10, 20, 100 and 400 μM. Concentrations of quizalofop used were 0 (○), 0.05 (●), 0.25 (□) and 0.5 μM (■). (**B**) Concentrations of ATP used were 50, 100, 200 and 1000 μM. Concentrations of quizalofop used were 0 (○), 0.05 (●), 0.1 (□) and 0.5 μM (■). A first-order regression was fitted to duplicate data points obtained at each fixed concentration of quizalofop in (**A**) or (**B**).

Positive co-operativity is also evident from the steepness of the slope in the ACCase2–quizalofop inhibition curve shown in Figure 1. The n_{app}(quizalofop) value of pea ACC220 was very close to that of ACCase2, as were the other kinetic parameters of ACC220. Figure 2 shows data for the inhibition of ACCase1 or ACCase2 by quizalofop fitted to the Hill equation or to the equation for simple hyperbolic inhibition. The smaller value of n_{app} obtained for binding of fluazifop to ACCase2 than for binding of quizalofop indicates a weaker co-operative effect. Curve-fit parameters for inhibition of the different ACCases by quizalofop or fluazifop are listed in Table 6.

Data were also examined by using the two-site equation [eqn (8)]. Maize ACCase1 data for quizalofop or fluazifop could not be fitted to this equation. In each case the value of K_{2i} obtained was over six orders of magnitude greater than K_{1i}, indicating that this parameter was virtually insignificant (results not shown). Data for maize ACCase2 and pea ACC220, in contrast, could be fitted to the two-site equation [maize ACCase2, K_{1i}(fluazifop) = 4.6 mM and K_{2i}(fluazifop) = 1.2 mM, K_{1i}(quizalofop) = 462 μM and K_{2i}(quizalofop) = 8 μM; pea ACC220, K_{1i}(quizalofop) = 256 μM, K_{2i}(quizalofop) = 25 μM] and these fits were very close to those of the co-operative model.

Kinetic analysis of the inhibition of maize ACCase 1 by quizalofop

Figure 3 shows double-reciprocal plots (Lineweaver–Burk plots) of the reaction velocity of maize ACCase 1 against varied concentrations of acetyl-CoA (Figure 3A) or ATP (Figure 3B) at different fixed concentrations of quizalofop. Inhibition was mixed-type with respect to both of these substrates. The concentration of acetyl-CoA had the greater effect on the level of inhibition and hence the value of the slope inhibition constant for acetyl-CoA [K_{is}(acetyl-CoA)] [21] was lower than K_{is}(ATP). This effect has previously been observed with maize and wheat [25–27]. The small uncompetitive element in the acetyl-CoA plot ($K_{is} > K_{ii}$) has not been observed previously. Instead, inhibition was virtually competitive ($K_{ii} \gg K_{is}$) [10,25–27] or virtually non-competitive ($K_{is} \approx K_{ii}$) [22] when unseparated ACCase isoforms from various grass species were analysed.

Because aryloxyphenoxypropionate graminicides are thought to inhibit only the transcarboxylation partial reaction [27], uncompetitive inhibition (a parallel-line pattern) would be expected when the level of ATP, a substrate of the biotin carboxylase partial reaction, is varied. However, a small slope effect was still observed [K_{is}(ATP) was an order of magnitude greater than K_{ii}(ATP), the *y*-axis intersect inhibition constant] (Figure 3B). Inhibition with respect to ATP (and bicarbonate) has been found to be mixed-type for maize and other plant ACCases [35–37]. Mixed-type inhibition would result if the graminicide interfered with the movement of biotin between the biotin carboxylase and carboxyltransferase sites on ACCase. As noted previously, this could happen if graminicide bound close to the biotin pocket or interfered indirectly with the binding of ATP by binding near to the acetyl-CoA site and causing a conformational change at the ATP-binding site [41].

Effect of fatty acyl-CoA esters on ACCases 1 and 2

In mammals, acyl-CoAs have been cited as important regulators of ACCase activity [42]. For plants, because of the extra-chloroplastic (cytosolic) location of fatty acyl-CoA esters [43], they might be expected to play a role in regulating the cytosolic but not the plastid isoform of ACCase (i.e. ACCase2 rather than ACCase1). The effects of different concentrations of palmitoyl-CoA or oleoyl-CoA on ACCases 1 or 2 are shown in Figure 4. Although neither isoform was appreciably sensitive to either fatty acyl-CoA, the putative cytosolic isoform, ACCase2, was indeed the more sensitive [approximate IC_{50} values were 10 μM (ACCase2) and 50 μM (ACCase1) for both fatty acyl-CoAs].

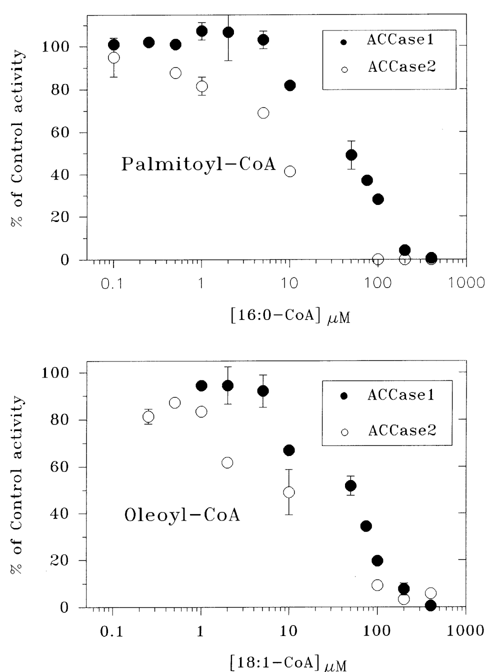

Figure 4 Effect of palmitoyl-CoA (upper panel) or oleoyl-CoA (lower panel) on the activities of maize ACCases 1 and 2

Assays were performed as described in the Materials and methods section. Enzyme (2 μg) was preincubated for 5 min in the presence of different concentrations of palmitoyl-CoA or oleoyl-CoA before assays were started by the addition of acetyl-CoA. Error bars are S.D. ($n = 3$) where larger than the symbols.

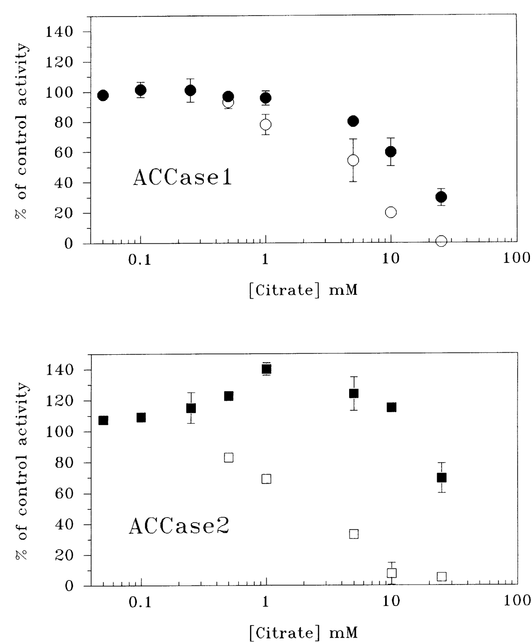

Figure 5 Effect of citrate on the activities of maize ACCase1 (upper panel) and ACCase2 (lower panel)

Assays were performed as described in the Materials and methods section. Enzyme (2 μg) was preincubated for 5 min with different concentrations of citrate in the presence (filled symbols) or absence (open symbols) of equimolar magnesium ions before assays were started by adding acetyl-CoA. Stock citrate solutions were made up in 0.5 M Hepes, pH 8.0, and an equal volume of buffer minus citrate was added to controls. Error bars are S.D. ($n = 3$) where these are larger than the symbols.

Octanoyl-CoA (8:0) and docosanoyl-CoA (22:0) did not inhibit either isoform at 10 or 100 μM. This is particularly interesting because the latter acyl-CoA is a typical substrate for fatty acid elongation [43], a reaction sequence that takes place in the cytosolic compartment on endoplasmic reticulum or Golgi membranes [44] and therefore would be dependent on ACCase2 activity for a supply of malonyl-CoA substrate.

Effect of citrate on ACCases 1 and 2

Another important effector of ACCase activity in mammals is citrate (and other tricarboxylic acids) [45]. This molecule has been widely tested with various plant ACCases. Figure 5 shows the effect of citrate on the activities of ACCases 1 and 2. When citrate concentration was increased in the assay medium of either isoform of maize ACCase, inhibition was observed above approx. 0.5 mM. However, when the chelatory effect of citrate on the magnesium ion ACCase cofactor [36] was compensated for (by preparing the citrate stock solution in an equimolar solution of magnesium chloride), the two isoforms showed different activity profiles. Citrate up to approx. 1 mM had no significant effect on the activity of ACCase1, but a gradually increasing inhibitory effect was apparent by 5 mM. A similar effect has previously been observed with ACCase purified from seeds of a variety of soya bean [36] and from castor bean [23]. ACCase2 activity, in contrast, was stimulated by citrate over a broad concentration range (0.25–10 mM) with maximal stimulation (40 %) at approx. 1 mM. A similar effect has previously been observed with ACCase purified from seeds of another variety of soya bean [36] and in that from spinach or avocado plastids [46].

DISCUSSION

Data from substrate interaction and product inhibition studies with ACCase1 or ACCase2 were in keeping with an ordered Ter Ter reaction mechanism for these two isoforms. This is the first report for purified plant ACCase isoforms and agrees with the results reported previously for an animal ACCase [20] that is also a multifunctional protein. Nakahira et al. [22] suggested a Ping Pong mechanism when they carried out experiments with a mixture of maize ACCase isoforms, although they did not show any data. Finlayson and Dennis [23] reported that the reaction mechanism of castor-bean seed ACCase was also Ping Pong. However, their experiments were with an undefined enzyme preparation that, in the light of recent work with another dicotyledonous species, pea [11], is likely to have contained a major part of its activity as a multienzyme complex form of ACCase. This makes direct comparison of the castor bean results with ours for resolved isoforms rather difficult.

An important feature of the results presented here for two multifunctional isoforms of maize ACCase and the multifunctional ACCase from pea is that positive co-operativity in graminicide binding was found in two minor, extrachloroplastidic, isoforms of moderate sensitivity to quizalofop or fluazifop. Thus maize ACCase2 and pea ACC220, minor extrachloroplastidic isoforms with similar moderate sensitivities to graminicide, both show strong positive co-operativity in binding quizalofop. Maize ACCase1 is highly sensitive to graminicides and exhibits only slight co-operativity. All these ACCases are homodimers [4–6,9,10] and, presumably, have two identical graminicide-binding regions, one on each subunit. According to

the co-operative model, binding of graminicide to the binding region of one subunit increases the affinity of the vacant binding region on the other subunit of maize ACCase2 and pea ACC220, but not that of maize ACCase1. Hence two apparent inhibition constant values ($K_{1i} > K_{2i}$) can be generated from inhibition curves of ACCase2 and ACC220, but not ACCase1, if data are fitted to an equation for a two-site enzyme. This was shown previously for ACC220 [9]. It was unlikely that the observed positive co-operativities of maize ACCase2 and pea ACC220 were caused by contamination with the major isoforms in these two species, because separation of isoforms was verified by their large differences in graminicide sensitivity and differences in subunit molecular mass (for details of pea, see [9]). It is also unlikely that ACCase2 was a degradation product *in vitro* of ACCase1. ACCase2 was present in similar proportions to the levels of graminicide-insensitive ACCase activity in crude leaf extracts, as reported by Ashton et al. [5]. Furthermore similar proportions of a graminicide-insensitive minor ACCase have been obtained by different laboratories [4,5,33]. The presence of two cDNAs for maize ACCase, only one of which codes for ACCase1 [5], also suggests that ACCase2 is not formed *in vivo* by post-translational modification of ACCase1.

The greater difference in co-operativity between maize isoforms in the binding of quizalofop than in the binding of fluazifop, together with the smaller difference in sensitivity between isoforms towards fluazifop (approx. 150-fold) than towards quizalofop (approx. 2000-fold) and the greater potency of quizalofop, indicate the highly specific nature of binding of individual graminicides.

Kinetic analysis suggested a higher affinity of ACCase1 for quizalofop near the acetyl-CoA-binding region than near the ATP-binding region, in keeping with results from previous studies. The small element of uncompetitive inhibition apparent in the acetyl-CoA plot has not been detected in mixtures of isoforms examined previously [10,22,25–27].

Because the putative cytosolic isoform, ACCase2, was appreciably more sensitive than the plastid isoform, ACCase1, to palmitoyl-CoA or oleoyl-CoA, and because acyl-CoAs are present in the cytosol rather than in the plastid, it is important to consider the relevance of the results to the situation *in vivo*. In this connection the inhibition by palmitoyl-CoA or oleoyl-CoA but not by docosanoyl-CoA is interesting because it might imply a physiological role for controlling carbon flux through different lipid pathways. Thus, in maize leaves, palmitoyl-CoA and oleoyl-CoA would be used for membrane fatty acid generation and could down-regulate fatty acid elongation by inhibiting the cytosolic ACCase, which provides malonyl-CoA for the latter. However, it would make good sense that docosanoyl-CoA, an intermediate in the production of very-long-chain fatty acids, did not affect elongation significantly. A key question is whether the concentration of acyl-CoAs ever reaches inhibitory levels in plants. Unfortunately, there are no data for leaf cytosol although essentially all of the CoA in chloroplasts (pea or spinach) was in the form of acetyl-CoA [28]. There seems no doubt that in animals ACCase is regulated by feedback inhibition by long-chain fatty acyl-CoA [42]. Inhibition of ACCase1 activity by citrate gradually began to become apparent above approx. 1 mM, whereas ACCase2 was stimulated. However, from current evidence in plants it does not seem that these changes have physiological relevance. The activity of ACCase1 was not altered by citrate concentrations below approx. 1 mM and the citrate concentration in chloroplast stroma (where ACCase1 is located) does not rise above 0.05 mM under different conditions of illumination [47], at least in oat. Most citrate in plant cells is in the cytosol/vacuole compartment, where its concentration is approx. 5 mM [47,48]. Although ACCase2 (which is believed to be cytoplasmic) was maximally stimulated by citrate concentrations around this value, citrate concentrations in the cytoplasm/vacuole are believed not to change much [47,48] and therefore would not have regulatory significance.

Conclusions

It is clear from our studies that the different multifunctional ACCase enzymes, although showing very similar molecular masses and binding constants for their substrates, have subtle differences in their structures. These differences not only contribute to their different sensitivities against graminicides but are also reflected in distinct enzyme kinetics and sensitivities to other possible effectors. It is obvious that we need to know far more about the detailed structure of plant ACCase isoforms before we can understand the factors that influence their inhibition by herbicides or, indeed, to rationally design new types of effector molecules. Moreover, elucidation of the regulation of ACCase (which is accepted as a key enzyme in lipid biosynthesis) in plants would also benefit from more work on the structure of these proteins.

We thank Dr. R. A. John (School of Molecular and Medical Biosciences, University of Wales College of Cardiff, Wales, U.K.) for help with the kinetic analyses. D.H., L.J.P. and J.L.H. thank Rhône-Poulenc Agriculture Ltd. for financial support.

REFERENCES

1 Knowles, J. R. (1989) Annu. Rev. Biochem. **58**, 195–221
2 Harwood, J. L. (1988) Annu. Rev. Plant Physiol. Plant Mol. Biol. **39**, 101–138
3 Harwood, J. L. (1991) in Target Sites for Herbicide Action (R. Kirkwood, ed.), pp. 57–94, Plenum Press, New York
4 Egli, M. A., Gengenbach, B. G., Gronwald, J. W., Somers, D. A. and Wyse, D. L. (1993) Plant Physiol. **101**, 499–506
5 Ashton, A. R., Jenkins, C. L. D. and Whitfield, P. R. (1994) Plant Mol. Biol. **24**, 35–49
6 Elborough, K. M., Simon, J. W., Swinhoe, R., Ashton, A. R. and Slabas, A. R. (1994) Plant Mol. Biol. **24**, 21–34
7 Iverson, A. J., Bianchi, A., Nordlund, A. C. and Witters, L. A. (1990) Biochem. J. **269**, 365–371
8 Witters, L. A. and Watts, T. D. (1990) Biochem. Biophys. Res. Commun. **169**, 369–376
9 Dehaye, L. Alban, C., Job, C., Douce, R. and Job, D. (1994) Eur. J. Biochem. **225**, 1113–1123
10 Evenson, K. J., Gronwald, J. W. and Wyse, D. L. (1994) Plant Physiol. **105**, 671–680
11 Alban, C., Baldet, P. and Douce, R. (1994) Biochem. J. **300**, 557–565
12 Hrazdina, G., Marx, F. A. and Hoch, H. C. (1982) Plant Physiol. **70**, 745–748
13 Kolattukudy, P. E., Croteau, R. and Buckner, J. S. (1976) in Chemistry and Biochemistry of Natural Waxes (Kolattukudy, P. E., ed.), pp. 289-347, Elsevier, New York
14 Konishi, T. and Sasaki, Y. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 3598–3601
15 Sasaki, Y., Hakamada, K., Suama, Y., Nagano, Y., Furusawa, I. and Matsuno, R. (1993) J. Biol. Chem. **268**, 25118–25123
16 Alban, C., Jullien, J., Job, D. and Douce, R. (1995) Plant Physiol. **109**, 927–935
17 Sasaki, Y., Konishi, T. and Nagano, Y. (1995) Plant Physiol. **108**, 445–449
18 Shorrosh, B. S., Roesler, K. R., Shintoni, D., van de Loo, F. J. and Ohlrogge, J. B. (1995) Plant Physiol. **108**, 805–812
19 Guchhait, R. B., Polakis, S. E., Dimroth, P., Stoll, E., Moss, J. and Lane, M. D. (1974) J. Biol. Chem. **249**, 6633–6645
20 Beaty, N. B. and Lane, M. D. (1982) J. Biol. Chem. **257**, 924–929
21 Segel, I. H. (1993) Enzyme Kinetics, John Wiley and Sons, New York
22 Nakahira, K., Hayashi, O., Uchiyama, M. and Suzuki, K. (1990) J. Pesticide Sci. **15**, 245–247
23 Finlayson, S. A. and Dennis, D. T. (1983) Arch. Biochem. Biophys. **225**, 586–595
24 Rendina, A. R., Felts, J. M., Beaudoin, J. D., Craig-Kennard, A. C., Look, L. L., Paraskos, S. L. and Hagenah, J. A. (1988) Arch. Biochem. Biophys. **265**, 219–225
25 Burton, J. D., Gronwald, J. W., Keith, R. A., Somers, D. A., Gengenbach, B. G. and Wyse, D. L. (1991) Pest. Biochem. Physiol. **39**, 100–109

26 Rendina, A. R., Beaudoin, J. D., Craig-Kennard, A. C. and Breen, M. K. (1989) in Proceedings, British Crop Protection Conference – Weeds, pp. 163–172, The British Crop Protection Council, Farnham
27 Rendina, A. R., Craig-Kennard, A. C., Beaudoin, J. D. and Breen, M. K. (1990) J. Agric. Food Chem. **38**, 1282–1287
28 Post-Beittenmiller, D., Roughan, G. and Ohlrogge, J. B. (1992) Plant Physiol. **100**, 923–930
29 Page, R. A., Okada, S. and Harwood, J. L. (1994) Biochim. Biophys. Acta **1210**, 369–372
30 Baldet, P., Alban, C., Axiotis, S. and Douce, R. (1993) Arch. Biochem. Biophys. **303**, 67–73
31 Hawke, J. C., Leese, B. M. and Leech, R. (1974) Phytochemistry **13**, 403–413
32 Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
33 Garland, W. J. and Dennis, D. T. (1977) Arch. Biochem. Biophys. **182**, 614–625
34 Howard, J. L. and Ridley, S. M. (1990) FEBS Lett. **261**, 261–264
35 Gornicki, P. and Haselkorn, R. (1993) Plant Mol. Biol. **22**, 547–552
36 Charles, D. J. and Cherry, J. H. (1986) Phytochemistry **25**, 1067–1071
37 Slabas, A. R. and Hellyer, A. (1985) Plant Sci. **39**, 177–182
38 Egin-Bühler, B. and Ebel, J. (1983) Eur. J. Biochem. **133**, 335–339
39 Alban, C., Baldet, P., Axiotis, S. and Douce, R. (1993) Plant Physiol. **102**, 957–965
40 Barden, R. E., Fung, C.-H., Utter, M. F. and Scrutton, M. C. (1972) J. Biol. Chem. **247**, 1323–1333
41 Taylor, W. S., Hixon, M., Chi, H., Marsilli, E. and Rendina, A. R. (1994) in Proceedings, Eighth IUAPC Congress on Pesticide Chemistry, Washington, DC
42 Numa, S. and Tanabe, T. (1984) in Fatty Acid Metabolism and its Regulation (Numa, S., ed.), pp. 1–23, Elsevier, Amsterdam
43 Browse, J. and Somerville, C. (1991) Annu. Rev. Plant Physiol. Plant Mol. Biol. **42**, 467–506
44 Cassagne, C., Lessire, R., Bessoule, J. J., Moreau, P., Créach, A., Schneider, F. and Sturbois, B. (1994) Prog. Lipid Res. **33**, 55–69
45 Lane, M. D., Moss, J. and Polakis, S. E. (1974) in Current Topics in Cell Regulation, vol. 8 (Horecker, B. L. and Stadtman, E. R., eds.), pp. 139–195, Academic Press, New York
46 Mohan, S. B. and Kekwick, G. O. (1980) Biochem. J. **187**, 667–676
47 Hampp, R., Goller, M., Füllgraf, H. and Eberle, I. (1985) Plant Cell Physiol. **26**, 99–108
48 Davies, D. D. (1987) in Biochemistry of Plants (Stumpf, P. K. and Conn, E. E., eds.), vol. 11, pp. 1–38, Academic Press, New York

Received 27 March 1996/28 May 1996; accepted 3 June 1996