Pergamon

Phytochemistry, Vol. 44, No. 3, pp. 399–405, 1997
Copyright © 1997 Elsevier Science Ltd
Printed in Great Britain. All rights reserved
0031–9422/97 $17.00+0.00

PII: S0031–9422(96)00580–8

GRAMINICIDE-BINDING BY ACETYL-CoA CARBOXYLASE FROM *POA ANNUA* LEAVES

Derek Herbert, 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, U.K.; *Rhône-Poulenc Agriculture Ltd, Ongar, Essex CM5 0HW, U.K.

(*Received* 19 *June* 1996)

Key Word Index—*Poa annua*; Gramineae; leaves; acetyl CoA carboxylase; herbicide; kinetics.

Abstract—A partially purified acetyl-CoA carboxylase (ACCase) preparation from leaves of a graminicide-resistant grass, *Poa annua* (annual meadow grass), was weakly inhibited by an aryloxyphenoxypropionate graminicide, quizalofop. The I_{50} value of 10 μM was *ca* 300-fold higher than that previously determined for an ACCase from a susceptible grass species, maize. *Poa annua* ACCase exhibited negative cooperativity in binding quizalofop (Hill coefficient = 0.54 ± 0.02). Inhibition was approximately equal in sensitivity to the concentrations of acetyl-CoA or ATP in the assay medium and appeared close to being non-competitive for these substrates. A double-reciprocal plot of initial velocity with the substrate pair acetyl-CoA plus ATP produced a parallel line pattern which was the expected pattern for a Ping-Pong reaction mechanism. The results from these kinetic measurements are discussed in relation to possible reasons for the insensitivity of *P. annua* ACCase to graminicides. It is concluded that cooperativity of herbicide-binding may be a possible indicator of such insensitivity. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

Poa annua (annual meadow grass) is common in the U.K. and other temperate regions of Europe. It is a problem weed in horticulture, in grass seed crops and in turf grass [1]. *Poa annua* is resistant to certain aryloxyphenoxypropionate and cyclohexanedione graminicides [2], apparently because of the insensitivity of its acetyl-coenzyme A carboxylase (ACCase) enzyme [3, 4; K. A. Walker and J. L. Harwood, unpublished results].

ACCase (E.C. 6.4.1.2) is a biotinylated enzyme that catalyses the ATP-dependent carboxylation of acetyl-CoA to form malonyl-CoA, with bicarbonate as the source of carbon [5]. It catalyses the first committed step in lipid biosynthesis [6]. Interest in ACCase has increased in recent years with the discovery that it exerts strong flux control over fatty acid (and lipid) synthesis in leaves [7, 8], as well as being the target of two classes of important grass-specific herbicides (graminicides), the aryloxyphenoxypropionates and the cyclohexanediones [2, 9–12]. These graminicides specifically inhibit ACCase in susceptible grass species; dicotyledonous species and monocotyledonous species other than grasses are resistant to these compounds because of the insensitivity of their ACCases [2, 9, 10, 12–14]. It is the unique sensitivity of grass ACCases that results in the specific graminicidal action [15, 16], since uptake, translocation and metabolism of these compounds does not usually differ between susceptible and resistant species [17, 18].

However, there are exceptions to the susceptibility of grasses. Certain grass species, including *Poa annua*, are resistant to examples of the aryloxyphenoxypropionate or cyclohexanedione graminicides because their ACCases are inherently insensitive [4, 19, 20]. Resistant biotypes of other grass species have been selected for as a result of graminicide usage [e.g. 21]. Some other grass species are resistant to certain graminicides because they metabolize the herbicides, the most notable example being the resistance of wheat to diclofop methyl [22]. Graminicide resistance has been reviewed by Holt *et al.* [23].

The ACCase reaction takes place at two catalytic sites via two partial reactions [5]. In the biotin carboxylase partial reaction, ATP and bicarbonate are used to carboxylate the biotin prosthetic group, biotin carboxyl carrier protein (BCCP). The second partial reaction is that of carboxyl-transfer, where the carboxyl group attached to the biotin residue of the BCCP is transferred to acetyl-CoA to produce malonyl-CoA. In multifunctional ACCases, such as those found in animals, the three functional domains, biotin carboxylase, BCCP and carboxyltransferase, are present on a single polypeptide chain [24]. Grasses, such

---

† Author to whom correspondence should be addressed.

as maize, have two multifunctional ACCase isoforms, each being dimeric with a subunit M_r of *ca* 220 000 [15, 25]. The major isoform, representing *ca* 80% of total leaf ACCase activity, is localized in mesophyll chloroplast stroma, where it supplies malonyl-CoA for *de novo* fatty acid synthesis [6, 15]. The minor isoform is absent from mesophyll chloroplasts but so far has not been localized. It may be enriched in epidermal cells, as is the situation in pea [26], to supply malonyl-CoA for synthesis of cuticular wax or flavonoids, both of which are synthesized in the epidermal cytoplasm [27]. Only multifunctional plant ACCases are inhibited by graminicides [15, 16, 26] and, of these, only the grass chloroplastic isoform is appreciably sensitive [15, 28]. In dicotyledonous plant species, only the extraplastidic isoform is multifunctional [26]. In contrast, the chloroplastic isoform resembles the ACCase of bacteria, such as *Escherichia coli*, that is, it is a multienzyme complex of the three dissociable functional domains [29].

ACCase has been purified, at least partially, from a number of plant sources [e.g. 6, 15, 26, 28, 30], including leaves from graminicide-resistant grasses [20, 21, 31]. Because the multifunctional enzyme is susceptible to proteolysis during purification, the latter is carried out rapidly, and at low temperature, with proteinase inhibitors added to the buffers in order to obtain the high M_r form [6]. Separation of multifunctional isoforms of ACCase from maize have been achieved on anion-exchange columns [15, 25, 28].

In order to determine whether the insensitivity of *P. annua* ACCase to inhibition by graminicides was associated with and, indeed, could be explained by a different enzyme mechanism we have characterized the kinetics of the reaction using a partly purified preparation. These studies include an examination of the binding of an aryloxyphenoxypropionate herbicide, quizalofop, with respect to cooperativity. The sensitivity of the inhibition with respect to the substrates of each of the partial reactions was also measured.

RESULTS AND DISCUSSION

The three-step purification summarized in Table 1 (ammonium sulphate precipitation, Red 120-agarose dye-ligand chromatography and Q-Sepharose anion-exchange chromatography) typically yielded *ca* 2 mg of partially purified ACCase from 60 g fresh weight of 7-day-old *P. annua* seedling leaves. Because of the instability of the purified ACCase [see 6], the apparent purification by this procedure was not very high. However, from SDS/PAGE (sodium dodecylsulphate polyacrylamide gel electrophoresis) and Coomassie blue staining this ACCase was judged to be *ca* 20% pure (data not shown). The native M_r of ACCase in the Q-Sepharose fraction was estimated as *ca* 460 000 using gel-filtration with Sephacryl-S300-HR (data not shown). SDS/PAGE followed by Western blotting with streptavidin peroxidase revealed that this fraction contained two biotinylated polypeptides, of *ca* M_r 220 000 (major form) and 210 000 (minor form), as did the initial leaf homogenate (data not shown). By anology with all other grass species so far examined in this way (maize, wheat and rice), these were probably subunits of two multifunctional ACCase isoforms [15, 16, 25]. Since only the M_r 220 000 biotinylated polypeptide was detected in isolated mesophyll protoplasts, this was probably that of the chloroplast stroma isoform, by analogy with other species [15, 26]. It was thought likely that a minor multifunctional isoform of *P. annua* ACCase was localized mostly in epidermal cells (which we were not able to isolate and analyse separately), as had been demonstrated in pea leaves [26]. These data were very similar to those obtained for multifunctional ACCases from other plant species [15, 25, 26, 31], indicating that the ACCase fraction under investigation contained two isoforms of dimeric multifunctional ACCase.

Attempts to separate two peaks of ACCase activity during the Q-Sepharose step were unsuccessful, even though this column and other anion-exchange materials had previously separated two peaks (corresponding to two isoforms) of ACCase activity from leaves of another grass species, maize [15, 25, 28, 36]. Only a single peak of activity eluted (at *ca* 0.3 M KCl) when different extended KCl gradients were tried, in common with purification of another insensitive ACCase, that from *Lolium multiflorum* [31].

Fitting data to the Michaelis–Menten equation gave the K_m values shown in Table 2. These values were

Table 1. Purification of ACCase from *P. annua* leaves

	Protein (mg)	Total activity*	Specific activity†	Yield (%)	Purification (fold)
Homogenate	313	1.61	0.005	100	1
$(NH_4)_2SO_4$ (0–65% cut)	150	1.69	0.011	105	2.2
Red 120-agarose	12.4	0.57	0.05	35	9
Q-Sepharose	2.1	0.25	0.12	16	24

A representative purification is shown starting with 58 g fresh weight of 7-day-old leaves.

* Total activity, μmol bicarbonate fixed min^{-1}.

† Specific activity, mmol bicarbonate fixed min^{-1} mg^{-1} protein.

Table 2. Apparent K_m values of *P. annua* ACCase

Substrate	K_m (μM)
Acetyl-CoA	66 (±12)
	74 (±15)
ATP	109 (±29)
	94 (±22)
Bicarbonate	1137 (±304)
	1350 (±270)

Values (means (±s.d.)) were obtained by fitting duplicated assay data to the Michaelis–Menten equation (see Experimental). Data from two enzyme preparations are shown.

also similar to those measured for other multifunctional plant ACCases [e.g. 15, 25, 37]. A noticeable point is that the K_m for bicarbonate is always an order of magnitude higher than that for the other substrates (see ref. [6]). Once assay conditions had been optimized, graminicide-binding studies were carried out on Q-Sepharose-purified ACCase.

A graminicide of the aryloxyphenoxypropionate class, quizalofop, was tested against *P. annua* ACCase (Fig. 1). The level of inhibition was concentration-dependent and the I_{50} value (the concentration of inhibitor causing a 50% reduction in enzyme activity) was *ca* 10 μM, a value *ca* 300-fold higher than that obtained by us for ACCase from a graminicide-sensitive species, maize [28]. The inhibition curve for ACCase in a desalted leaf homogenate was very similar to that of the Q-Sepharose-purified fraction (data not shown), indicating that insensitivity was not caused by modification of the ACCase during purification. We had previously obtained a value of 0.1 μM for a desalted *P. annua* leaf homogenate [3]. It was possible that this difference resulted from biotypes of *P. annua* which differed in the sensitivity of their ACCases. However, this was not investigated further.

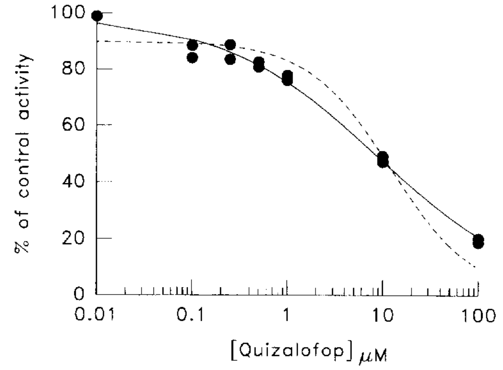

Fig. 1. Inhibition of *P. annua* ACCase by quizalofop. Duplicated assay values are shown. Lines are best fits of the data to the Hill equation (solid line) or to an equation for simple hyperbolic inhibition (dashed line). The kinetic parameters derived from these fits are listed in Table 3. Further details are given in the Experimental.

By fitting the data shown in Fig. 1 to different steady-state rate equations (see Experimental), it was found that *P. annua* ACCase exhibited negative cooperativity in binding quizalofop. Data fitted better to the Hill equation (an equation that takes account of the cooperativity involved in inhibitor binding) than to the equation for simple hyperbolic inhibition. This was indicated by the lower sum of residual squares (SRS) value from the Hill equation fit (Table 3). Moreover, the value of V_0 (control reaction velocity) was also estimated more accurately by use of the Hill equation (i.e. it was closer to 100%) and standard deviations of this and the inhibition constant values (K' or K_i) were also lower when data were fitted to the Hill equation (Table 3). The apparent Hill coefficient or n_{app} value, (0.54±0.02), was less than 1 which therefore indicated negative cooperativity in binding of quizalofop to *P. annua* ACCase [34]. It is also possible to estimate the value of n_{app} by using Hill Plots [35] (Fig. 2). This was carried out to check the curve-fitting estimate and to provide a more direct comparison with a study by Evenson *et al.* [31] on an insensitive ACCase preparation from a resistant biotype of *L. multiflorum*. The resulting Hill plot estimate of n_{app} (0.57±0.01) was very similar to our curve fit estimate and to the value of 0.62 that had been obtained with *L. multiflorum* ACCase. Therefore, insensitivity to graminicide in these two resistant species appears to be associated with negative cooperativity in graminicide-binding. significantly, sensitive ACCase (from a susceptible *L. multiflorum* biotype) showed only weak cooperativity (n_{app} = 1.2) [31]. As pointed out by Evenson *et al.* [31], interpretation of inhibition data and of the apparent negative cooperativity is not straightforward when examining a mixture of isoforms. When similar kinetic analyses to those described here were carried out on separated multifunctional ACCase isoforms from maize or pea it was found that the minor isoform from each species, which was only slightly sensitive to quizalofop and another aryloxyphenoxypropionate graminicide, fluazifop, also exhibited cooperativity (positive in this case) in binding each of these compounds [28]. In contrast, the same investigation showed that the major ACCase isoform in maize leaves, which is extremely sensitivity to quizalofop (I_{50} *ca* 0.03 μM), exhibited no significant cooperativity in binding these compounds (n_{app} quizalofop = 0.86±0.03), n_{app} fluazifop = 1.16±0.06).

In order to check the separate partial reactions of ACCase for sensitivity to quizalofop (see ref. [38]), Michaelis–Menten plots (enzyme velocity *vs* substrate concentration) were made of ACCase activity *vs* different concentrations of acetyl-CoA, a substrate of the carboxyltransferase partial reaction, or ATP, a substrate of the biotin carboxylase partial reaction (see ref. [5]), in the presence and absence of a fixed concentration of quizalofop (Fig. 3). In this way it was possible to compare inhibition with respect to the concentrations of acetyl-CoA or ATP in the assay medium. Inhibition appeared to be close to non-competitive with respect to each of these substrates,

Table 3. Kinetic parameters (means ($\pm$s.d.)) obtained from curve-fits of inhibition data for *P. annua* ACCase *vs* quizalofop

Hill equation $v_i = V_oK'/(K'+[I]^{n_{app}})$				Simple hyperbolic equation $v_i = V_o/(1+([I]/K_i))$		
V_o(%)	K' (μM)	n_{app}	SRS	V_o(%)	K_i (μM)	SRS
98.8$\pm$1.6	3.2$\pm$0.4	0.54$\pm$0.02	106	89.9$\pm$2.4	12.1$\pm$2.7	671

v_i = percent of control enzyme velocity in the presence of inhibitor. V_o(%) = control enzyme velocity (i.e. in the absence of inhibition); a value close to 100% equates with good agreement with the equation used. SRS = sum of residual squares; a low value equates with good agreement with the equation used. K' and K_i are inhibition constants; n_{app} is an apparent Hill constant. Parameters are discussed in the Experimental.

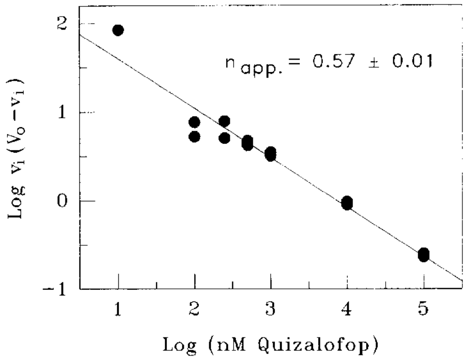

Fig. 2. Hill plot for the inhibition of *P. annua* ACCase by quizalofop. Each assay was carried out in duplicate and these values are plotted. The apparent Hill constant (n_{app}) was calculated as the slope of the first order regression fitted to the data. V_o = control enzyme velocity; v_i = enzyme velocity in the presence of quizalofop. These data are also shown in Fig. 1.

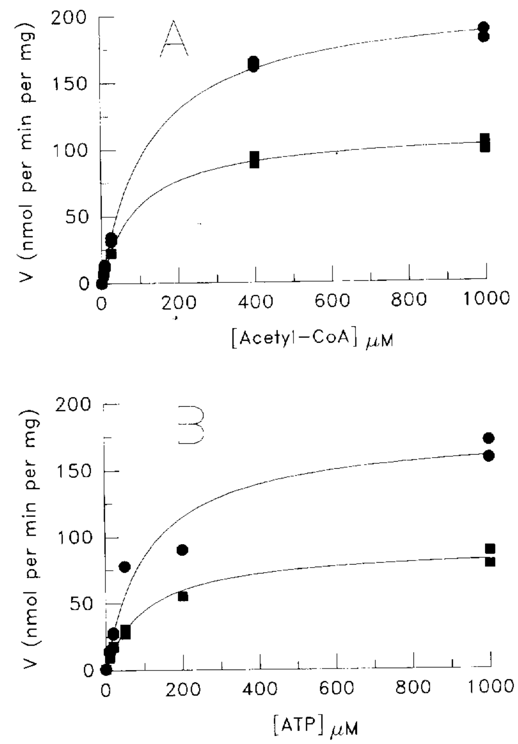

Fig. 3. Michaelis–Menten plots of *P. annua* acetyl-CoA carboxylase in the presence (■) or absence (●) of 10 μM quizalofop at different concentrations of acetyl-CoA (A) or ATP (B). Lines were best-fitted to the data points using SigmaPlot 4.0. V_{max} and K_m values obtained from these curves in the absence (first value) or presence (second value) of quizalofop, respectively, were: A, V_{max} = 210 ($\pm$6) and 112 ($\pm$3) nmol min^{-1} mg^{-1}, respectively. K_m = 120 ($\pm$15) and 94 ($\pm$9) μM, respectively. B. V_{max} = 177 ($\pm$8) and 91 ($\pm$4) nmol min^{-1} mg^{-1}, respectively. K_m = 109 ($\pm$30) and 103 ($\pm$13) μM, respectively.

because K_m was reduced to a much lesser extent than was V_{max} (maximal velocity). Furthermore, inhibition was nearly equal in sensitivity to the amounts of these two substrates in the assay mixture, as observed from similar intercept inhibition constant, K_{ii}, values [35] of *ca* 10 μM for both acetyl-CoA and ATP (estimated from secondary plots, not shown). In marked contrast, inhibition of ACCase from graminicide-sensitive species is more dependent on the concentration of acetyl-CoA than on ATP (or HCO_3^-) concentration [2, 13]. K_{ii} values for *P. annua* ACCase were *ca* 15 times greater than those measured for a graminicide-sensitive ACCase from maize [28], in keeping with the lower sensitivity of *P. annua* ACCase.

A steady-state kinetic experiment was carried out in order to determine whether a Ping-Pong or a Ter-Ter reaction mechanism [35] was the more likely for *P. annua* ACCase. In the Ping-Pong mechanism the products of the first partial reaction, ADP and Pi, are released before acetyl-CoA binds to enzyme; in a Ter-Ter mechanism there is no release of ADP and Pi before acetyl-CoA binds. Initial velocities were measured at varying concentrations of acetyl-CoA and ATP whilst maintaining a saturating concentration of the third substrate of the ACCase reaction, bicarbonate (10 mM). In this way, the kinetic analysis could be simplified by treating the system as two-substrate. The concentrations of acetyl-CoA (20 to 400 μM) and ATP (30 μM to 1 mM) were chosen to span their apparent K_m values. A double-reciprocal plot of initial velocity *vs* acetyl-CoA concentration at different fixed concentrations of ATP is shown in Fig. 4. These data could not be fitted accurately to an equation for a Ter-Ter reaction mechanism, an equation for an intersecting line pattern. However, they could be fitted to

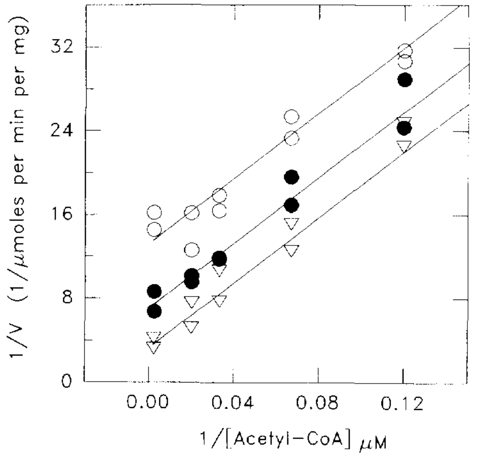

Fig. 4. Double-reciprocal plot of the initial velocity of *P. annua* ACCase *versus* acetyl-CoA concentration at different fixed concentrations of ATP. Concentrations of acetyl-CoA used were 20 μM, 30 μM, 50 μM and 400 μM. Concentrations of ATP used were 30 μM (○), 75 μM (●) and 1 mM (▽). Bicarbonate was at a saturating concentration (10 mM) in all assays. Data are shown fitted to $v = V_m/(1 + K_a/[a] + K_b/[b])$, where [a] is ATP concentration, [b] is acetyl-CoA concentration, K_a and K_b are the Michaelis constants for ATP and acetyl-CoA, respectively, v is the experimentally determined initial enzyme velocity, and V_m is the theoretical maximum velocity when the concentrations of ATP and acetyl-CoA are saturating (see Experimental). The kinetic parameters derived from this fit are discussed in the text.

an equation for a Ping-Pong reaction mechanism, an equation for a parallel line pattern (see Experimental). Parallel lines indicate that binding of the two varied substrates, acetyl-CoA and ATP, is connected by irreversible steps [35]. Because the two partial reactions of ACCase are freely reversible [38], and because the ATP-dependent carboxylation of biotin must take place before the transcarboxylation of acetyl-CoA, the simplest explanation for these data was that ADP and Pi were released from the enzyme before acetyl-CoA bound. A parallel line pattern has also been observed for ACCase purified from plastids of castor bean (*Ricinus communis*) [37]. By fitting data to a steady-state rate equation that applies to a Ping-Pong mechanism the apparent Michaelis constants, K_a ((0.054±0.006) mM ATP) and K_b ((0.106±0.013) mM acetyl-CoA), representing the apparent affinity of enzyme for these two substrates [35], were estimated. These values were very close to those obtained for castor bean ACCase [37].

Thus, in conclusion, we have found that the ACCase preparation from *Poa annua* showed Ping-Pong kinetics which contrasted with the sensitive as well as with the insensitive isoform from maize, a sensitive grass [28]. Perhaps of significance was the cooperativity of herbicide-binding observed. All examples of insensitive ACCase from resistant biotypes which have been studied so far (see also ref. [31]) show cooperativity, in contrast to the sensitive chloroplast isoform from maize [28]. It may be that this property is connected with the particular protein structures that confer sensitivity to ACCase.

EXPERIMENTAL

General. Poa annua seeds were bought from Herbiseed Ltd, Wokingham, U.K. Plants were grown in soil-less compost at 20° with 650 μE s^{-1} m^{-2} illumination and a 12 hr light/12 hr dark cycle and watered with tap water when required. Quizalofop, free acid ((*RS*)-2-[4-(6-chloroquinoxalin-2-yloxy)phenoxy] propionic acid), was provided by Rhône-Poulenc Agriculture Ltd; it was a racemic mixt. and at least 98% pure. Sodium [^{14}C]bicarbonate (3.7–37 GBq $mmol^{-1}$) was obtained from Amersham International, Amersham, U.K.

Purification of ACCase. Young leaves were used because these contained the highest sp. act. of ACCase per g fr. wt, in common with leaves of other species (e.g. 3, 4, 32). All steps were carried out at 4°. About 60 g freshly-harvested leaves from 7-day-old plants were ground to a fine powder in liquid N_2 with a pestle and mortar. The powder was transferred to another mortar to prevent the buffer from freezing and resuspended in 4 vols of extraction buffer (0.1 M Hepes–KOH (pH 8.0), 1 mM Na_4 EDTA, 10% (v/v) glycerol, 5 mM DTT (dithiothreitol), 5 mM ε-aminocaproic acid, 1 mM benzamidine-HCl and 1 mM PMSF (phenylmethylsulphonylfluoride), the last four ingredients being added immediately before use). The homogenate was filtered through four layers of Miracloth. The filtered homogenate was centrifuged at 30 000 *g* for 30 min to remove cell debris and then fractionated with $(NH_4)_2SO_4$. Material precipitating between 0% and 65% $(NH_4)_2SO_4$ was collected by centrifuging at 30 000 *g* for 30 min and dissolved in *ca* 20 ml resuspension buffer (20 mM Hepes–KOH (pH 8.0), 1 mM NaEDTA, 20% (v/v) glycerol, 1 mM DTT, 5 mM ε-aminocaproic acid, 1 mM benzamidine-HCl and 1 mM PMSF). The soln was centrifuged at 105 000 *g* for 30 min to remove cell membranes and desalted into column buffer (resuspension buffer minus PMSF) on Sephadex G-25. The desalted fr. was loaded (flow rate 0.5 ml min^{-1}) onto a column of Red 120-agarose (2.6 cm × 15 cm, 80 ml) that was connected to an automated gradient system and had been previously equilibrated with column buffer. The column was washed with this buffer (flow rate 1 ml min^{-1}) until the *A* at 280 nm returned to baseline, showing that all non-retained proteins had been removed. The bulk of the non-ACCase retained proteins were then eluted with a 80 ml gradient of 0–0.2 M KCl followed by a 160 ml wash at 0.2 M KCl, in column buffer. ACCase was then eluted with a 160 ml wash at 0.4 M KCl in column buffer. Frs (8 ml) containing ACCase activity were pooled, made up to 50 mM Hepes–KOH (pH 8.0) by the addition of 500 mM Hepes (pH 8.0) stock buffer and brought to 70% satn by the addition of solid $(NH_4)_2SO_4$. Precipitated proteins (*ca* 12 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^{-1}) onto a column of Q-Sepharose (1 cm × 12 cm, 9.4 ml) previously equilibrated with column buffer. The column was washed with 30 ml of this buffer (flow rate 1 ml min^{-1}) 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, in column buffer. ACCase-containing proteins were then eluted with a 140 ml linear gradient of 0.15–0.55 M KCl (flow rate 1 ml min^{-1}). Frs (1 ml) containing ACCase activity were pooled, concd to a final protein concn of *ca* 0.5 mg ml^{-1} with Macrosep-10 concentrators (Flowgen Instruments Ltd) and stored at −80°C until used.

Assay for ACCase. ACCase activity was measured as the incorporation of radioactivity from $NaH^{14}CO_3$ into an acid-stable product, essentially according to ref. [13]. All assays were optimized with respect to the concn of each reaction component and to the pH of the reaction mixt. The standard assay mixt. was 50 mM Hepes–KOH (pH 8.0), 2.5 mM $MgCl_2$, 1 mM ATP, 0.5 mM DTT, 10 mM $NaH^{14}CO_3$ (37 GBq mmol^{-1}), 0.8 mM acetyl-CoA and 2–10 μg ACCase-containing protein, in a final vol. of 200 μl in a 1.5 ml Eppendorf tube. Assays were preincubated for 10 min at 30° and then started by adding acetyl-CoA. Assays were terminated up to 20 min after adding acetyl-CoA, which was within the linear labelling period. Initial reaction velocities could then be calculated conveniently. Reaction mixtures (150 μl portions) were added to scintillation vials (20 ml) containing 40 μl 12 M HCl to stop the reaction and drive off unincorporated radiolabel. Vials were agitated gently by hand and the solns dried with a stream of N_2. The dried residue was solubilised by adding 0.25 ml dist. H_2O, agitating gently by hand and leaving for 15 min. Scintillant (10 ml; Optifluor; Canberra-Packard) was added and acid-stable radioactivity was measured in a liquid scintillation counter. The acid-stable product was identified as malonyl-CoA by HPLC in preliminary expts (data not show). Assays without acetyl-CoA were run to measure background activity and the latter was subtracted from all readings. Background activities in the Q-Sepharose frs, used for kinetic analyses, were less than 3% of the control velocities. Assays were duplicated. ACCase activity was expressed as μmoles bicarbonate fixed min^{-1} mg^{-1} enzyme protein. Where appropriate, quizalofop was added to the assay mixt before the 10 min preincubation.

Preparation of quizalofop solutions. Stock solutions (30 mM) were made in 1.5 ml Eppendorf tubes by dissolving herbicide in Me_2CO and then adding 0.5 M Hepes–KOH (pH 8.0) to give an Me_2CO concn of 9% (v/v). Stock solns were protected from light and stored at −20° between expts and used within 3 weeks. After appropriate diln in dist. H_2O, solns were added immediately to assay mixts. Final Me_2CO concns in assays were always less than 0.3%, a concn that had no effect on ACCase activity. Concns recorded refer to the active *R* enantiomer (see ref. [9]).

Tissue preparation and subcellular fractionation. Leaf tissue from 7-day-old plants was digested enzymically and mesophyll cells subjected to subcellular fractionation [33].

Estimation of protein M_r *and Western blotting.* SDS/PAGE, streptavidin-peroxidase Western blotting and estimation of native M_r by gel filtration were carried out as described in ref. [26].

Protein concentration. Protein concn was measured by the method of Bradford [34] using Bio-Rad protein assay reagent with BSA as standard.

Determination of kinetic parameters. Parameter values (±standard deviations) (s.d.) were estimated by fitting duplicated initial reaction velocity values to different steady-state rate equations. Fitting was carried out by linear least squares analysis with the SigmaPlot version 4.0 computer programme (Jandel Scientific, Corte Madera, California). All assays were carried out on Q-Sepharose-purified frs (see Table 1).

In order to examine data for evidence of cooperativity in the binding of quizalofop to *P. annua* ACCase, they were fitted to the Hill equation [35].

$$v_i = \frac{V_o K'}{K' + [\mathrm{I}]^{n_{app}}}. \tag{1}$$

which takes account of the cooperativity involved in inhibitor binding. Where V_o is control enzyme velocity (i.e. in the absence of inhibitor) and v_i is enzyme velocity in the presence of different concns of inhibitor, I. v_i values were expressed as percentages of V_o (100%). The apparent Hill coefficient, n_{app}, is a measure of the cooperativity between enzyme subunits for the binding of inhibitor. A n_{app} value >1 indicates positive cooperativity, a n_{app} value <1 indicates negative cooperativity. K' is an inhibition constant.

And an equation for simple hyperbolic inhibition,

$$v_i = \frac{V_o}{1 + ([\mathrm{I}]/K_i)}. \tag{2}$$

Substrate interactions with enzyme were investigated by fitting initial velocity data to the reduced equation that applies to a parallel line pattern (Ping-Pong mechanism) (eqn 3) and to an equation for an intersecting line pattern (Ter-Ter mechanism) (eqn 4) [35]:

$$v = V_m/\{1 + K_a/[\mathrm{a}] + K_b/[\mathrm{b}] + K_{ab}/([\mathrm{a}][\mathrm{b}])\} \tag{3}$$

$$v = V_m/(1 + K_a/[\mathrm{a}] + K_b/[\mathrm{b}]) \tag{4}$$

where, v is the experimentally determined initial velocity, V_m is the maximum velocity when the substrate concns [a] (ATP) and [b] (acetyl-CoA) are saturating. K_a and K_b are the Michaelis constants for the varied substrates a and b, respectively. K_{ab} is an interaction term. A saturating concn of bicarbonate (10 mM) was used in all assays.

Acknowledgement—D.H. and J.L.H. are grateful to Rhône-Poulenc Agriculture Ltd for financial support.

REFERENCES

1. Shildrick, J. P., Bowen, P. and Soper, D., in *Weed Control Handbook—Principles*, ed. H. A. Roberts. The British Crop Protection Council/Blackwell Scientific Publications, Oxford, 1982, p. 369.
2. Harwood, J. L., in *Target Sites for Herbicide Action*, ed. R. Kirkwood. Plenum Press, New York, 1991, p. 57.
3. Herbert, D., Cole, D. J., Pallett, K. E. and Harwood, J. L., *Pesticide Biochemistry and Physiology* (in press).
4. Lichtenthaler, H. K., Focke, M., Golz, A., Goffman, S., Kobek, K. and Motel, A., in *Proceedings of 10th International Symposium on Plant Lipids, Jerba*, ed. A. Cherif *et al.* Centre National Pédogogique, Tunis, 1992, p. 103.
5. Knowles, J. R., *Annual Review of Biochemistry*, 1989, **58**, 195.
6. Harwood, J. L., *Annual Review of Plant Physiology and Plant Molecular Biology*, 1988, **39**, 101.
7. Page, R. A., Okada, S. and Harwood, J. L., *Biochimica et Biophysica Acta*, 1994, **1210**, 369.
8. Post-Beittenmiller, D., Roughan, G. and Ohlrogge, J. B., *Plant Physiology*, 1992, **100**, 923.
9. Walker, K. A., Ridley, S. M., Lewis, T. and Harwood, J. L., *Biochemical Journal*, 1988, **254**, 307.
10. Walker, K. A., Ridley, S. M. and Harwood, J. L., *Biochemical Journal*, 1988, **254**, 811.
11. Burton, J. D., Gronwald, J. W., Somers, D. A., Connelly, J. A., Gengenbach, B. G. and Wyse, D. L., *Biochemical and Biophysical Research Communications*, 1987, **148**, 1039.
12. Secor, J., Cséke, C. and Owen, W. J., *Proceedings of British Crop Production Conference—Weeds*, 1989, **1**, 145.
13. Burton, J. D., Gronwald, J. W., Keith, R. A., Somers, D. A., Gengenbach, B. G. and Wyse, D. L., *Pesticide Biochemistry and Physiology*, 1991, **39**, 100.
14. Rendina, A. R., Beaudoin, J. D., Graig-Kennard, A. C. and Breen, M. K., *Proceedings of British Crop Protection Conference—Weeds*, 1989, **1**, 163.
15. Egli, M. A., Gengenbach, B. G., Gronwald, J. W., Somers, D. A. and Wyse, D. L., *Plant Physiology*, 1993, **101**, 499–506.
16. Konishi, T. and Sasaki, Y., *Proceedings of the National Academy of Sciences of the U.S.A.*, 1994, **91**, 3598.
17. Walker, K. A., Ridley, S. M., Lewis, T. and Harwood, J. L., *Review of Weed Science*, 1989, **4**, 71.
18. Owen, W. J., in *Herbicide Resistance in Weeds and Crops*, eds J. C. Caseley, G. W. Cussans and R. K. Atkin. Butterworth, Oxford, 1991, p. 199.
19. Stoltenberg, D. E., Gronwald, J. W., Wyse, D. L., Burton, J. D., Somers, D. A. and Gengenbach, B. G., *Weed Science*, 1989, **37**, 512.
20. Catanzaro, C. J., Burton, J. D. and Skrotch, W. A., *Pesticide Biochemistry and Physiology*, 1993, **45**, 147.
21. Gronwald, J. W., Eberlein, C. V., Betts, K. J., Baerg, R. J., Ehlke, N. J. and Wyse, D. L., *Pesticide Biochemistry and Physiology*, 1992, **44**, 126.
22. Shimabukuro, R. H., Walsh, W. C. and Hoerauf, R. A., *Journal of Agricultural Food Chemistry*, 1979, **27**, 615.
23. Holt, J. S., Powles, S. B. and Holtum, J. A. M., *Annual Review of Plant Physiology and Plant Molecular Biology*, 1993, **44**, 203.
24. Iverson, A. J., Bianchi, A., Nordlund, A. C. and Witters, L. A., *Biochemical Journal*, 1990, **269**, 365.
25. Ashton, A. R., Jenkins, C. L. D. and Whitfield, P. R., *Plant Molecular Biology*, 1994, **24**, 35.
26. Alban, C., Baldet, P. and Douce, R., *Biochemical Journal*, 1994, **300**, 557.
27. Hrazdina, G., Marx, F. A. and Hoch, H. C., *Plant Physiology*, 1982, **70**, 745.
28. Herbert, D., Price, L. J., Alban, C., Dehaye, L., Job, D., Gole, D. J., Pallett, K. E. and Harwood, J. L., *Biochemical Journal*, 1996, **318**, 997.
29. Guchait, R. B., Polakis, S. E., Dimroth, P., Stoll, E., Moss, J. and Lane, M. D., *Journal of Biological Chemistry*, 1974, **249**, 6633.
30. Hellyer, A., Bambridge, H. E. and Slabas, A. R., *Biochemical Society Transactions*, 1986, **14**, 565.
31. Evenson, K. J., Gronwald, J. W. and Wyse, D. L., *Plant Physiology*, 1994, **105**, 671.
32. Dehaye, L., Alban, C., Job, C., Douce, R. and Job, D., *European Journal of Biochemistry*, 1994, **225**, 1113.
33. Baldet, P., Alban, C., Axiotis, S. and Douce, R., *Archives of Biochemistry and Biophysics*, 1993, **303**, 67.
34. Bradford, M. M., *Analytical Biochemistry*, 1976, **72**, 248.
35. Segel, I. H., *Enzyme Kinetics*, 2nd edn. John Wiley and Sons Inc., New York, 1993.
36. Howard, J. L. and Ridley, S. M., *FEBS Letters*, 1990, **261**, 261.
37. Finlayson, S. A. and Dennis, D. T., *Archives of Biochemistry and Biophysics*, 1983, **225**, 586.
38. Rendina, A. R., Graig-Kennard, A. C., Beaudoin, J. D. and Breen, M. K., *Journal of Agricultural Food Chemistry*, 1990, **38**, 1282.
39. Rendina, A. R., Felts, J. M., Beaudoin, J. D., Craig-Kennard, A. C., Look, L. L., Pradkos, S. L. and Hagenah, J. A., *Archives of Biochemistry and Biophysics*, 1988, **265**, 219.
40. Alban, C., Baldet, P., Axiotis, S. and Douce, R., *Plant Physiology*, 1993, **102**, 957.