

Graminicide insensitivity correlates with herbicide-binding co-operativity on acetyl-CoA carboxylase isoforms

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The sensitivity of grass species to important classes of graminicide herbicides inhibiting ACCase (acetyl-CoA carboxylase) is associated with a specific inhibition of the multifunctional ACCase located in the plastids of grasses. In contrast, the multisubunit form of ACCase found in the chloroplasts of dicotyledonous plants is insensitive and the minor cytosolic multifunctional isoforms of the enzyme in both types of plants are also less sensitive to inhibition. We have isolated, separated and characterized the multifunctional ACCase isoforms found in exceptional examples of grasses that are either inherently insensitive to these graminicides, or from biotypes showing acquired resistance to their use. Major and minor multifunctional enzymes were isolated from cell suspension cultures of *Festuca rubra* and the 'Notts A1'-resistant biotype of *Alopecurus myosuroides*, and their properties compared with those isolated from cells of wild-type sensitive *A. myosuroides* or from sensitive maize. Purifications of up to 300-fold were necessary to separate the two isoforms. The molecular

masses (200–230 kDa) and K_m values for all three substrates (ATP, bicarbonate and acetyl-CoA) were similar for the different ACCases, irrespective of their graminicide sensitivity. Moreover, we found no correlation between the ability of isoforms to carboxylate propionyl-CoA and their sensitivity to graminicides. However, insensitive purified forms of ACCase were characterized by herbicide-binding co-operativity, whereas, in contrast, sensitive forms of the enzymes were not. Our studies on isolated individual isoforms of ACCase from grasses support and extend previous indications that herbicide binding co-operativity is the only kinetic property that differentiates naturally or selected insensitive enzymes from the typical sensitive forms usually found in grasses.

Key words: acetyl-CoA carboxylase, co-operativity, graminicide, herbicide sensitivity, inhibitor binding.

INTRODUCTION

AOPPs (aryloxyphenoxypropionic acids) and CHDs (cyclohexanediones) are two successful and chemically distinct groups of herbicides that selectively control grasses (Gramineae). Together they account for approx. 10% of the current global herbicide market [1]. Although the mechanism of action of both AOPPs and CHDs was unclear at first, work in several laboratories pointed to ACCase (acetyl-CoA carboxylase) as the target site for both groups of herbicides [2–5] although much of the evidence was circumstantial owing to the problems of measuring acetyl-CoA and malonyl-CoA in intact chloroplasts [6]. Unequivocal evidence was provided by Walker et al. [7], and further detailed enzymology by Rendina et al. [8] showed that the carboxyl-transferase partial reaction was the specific site of action.

Development of both AOPPs and CHDs as successful selective herbicides was, in some ways, surprising because ACCase catalyses the first committed reaction in fatty acid biosynthesis [9], which is virtually ubiquitous in nature [10]. However, it turned out that these herbicides were selective against the plastid isoform of ACCase from grasses and did not affect the enzyme significantly from other monocotyledons, dicotyledons or from other species such as bacteria and animals. These observations helped to extend research into the nature of plant ACCases [11]. Thus it is now known that dicotyledons (with the exception of certain members of the *Geraniaceae*) [12] contain a multisubunit form of ACCase in their plastids [13–15], whereas the Gramineae contain a multifunctional protein. Both dicotyledons and Gramineae contain a second multifunctional form of ACCase outside the plastid

[11]. In addition, *Brassica napus* has been reported to contain a small proportion of the multifunctional form of ACCase in its plastids as well as in the usual multisubunit enzyme [16,17].

The completely different structure of the plastid ACCase in dicotyledons [18] (as well as some different properties such as its inability to carboxylate propionyl-CoA [19]) make it easy to surmise why such an enzyme may have different sensitivity towards AOPP and CHD herbicides. However, in Gramineae, both isoforms of ACCase are multifunctional proteins and yet they show very different sensitivities towards AOPP and CHD herbicides. Thus the plastid form in maize has an IC_{50} value of 0.03 μ M for quizalofop, whereas the non-plastid form has an IC_{50} value of 60 μ M [20]. Moreover, the non-plastid ACCase in dicotyledons, such as pea, is also relatively insensitive [13]. In an effort to pinpoint differences between the insensitive and sensitive multifunctional forms of ACCase, we examined the reaction mechanism for the two isoforms from maize in detail [20]. In both cases, the mechanism was the same and there was no obvious difference in the general properties of the isoforms except towards herbicide binding.

Both AOPP and CHD herbicides, despite their very different structures, show double inhibition on kinetic analysis. Thus binding of one herbicide class prevents binding of a herbicide from another class [8]. For the maize isoforms, the inhibition of activity of the sensitive ACCase by AOPPs could be fitted to a simple hyperbolic curve, whereas activity of the resistant isoform showed co-operativity. That is, binding of a herbicide molecule to one subunit of the native dimer of ACCase affected binding to the second subunit [20]. This agreed with the observation by Evenson et al.

Abbreviations used: ACCase, acetyl-CoA carboxylase; AOPP, aryloxyphenoxypropionic acid; CHD, cyclohexanedione; DTT, dithiothreitol.

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[21], who compared ACCase activity from resistant and sensitive biotypes of *Lolium multiflorum*. Although these workers did not separate the two isoforms from each biotype, which can confuse data interpretation, they noted that ACCase from the resistant biotype showed a different pI and, importantly, negative co-operativity of diclofop binding [21]. Similarly, a mixture of iso-enzymes was tested for the graminicide-resistant ACCase from *Poa annua* and, again, negative co-operativity for herbicide binding was noted [22]. In addition, the insensitivity of ACCase isoforms to graminicides seemed also to be correlated with a lesser ability to carboxylate propionyl-CoA [22].

The subtlety of the structural differences that determine whether a particular multifunctional ACCase isoform is sensitive or insensitive to graminicides is reinforced by our knowledge of resistance. Apart from a few species that can metabolize particular graminicides (e.g. wheat and diclofop) [23], resistance in the whole plant is almost invariably correlated with the presence of an insensitive plastid isoform of ACCase [24]. Even in cases where it appeared that there were no differences in the ACCase from resistant and sensitive populations [25], further work has revealed a target-site basis for resistance [26]. Some grasses, such as various species of *Festuca* and *P. annua*, show inherent resistance to at least some AOPPs and CHDs. In addition, acquired resistance is becoming an increasing problem in agriculture [1,24]. Examination of ACCase activity from a whole variety of resistant species has shown that acquired resistance is associated with the presence of insensitive forms of the enzyme [24]. Several of the herbicide-resistant biotypes have been characterized and reduced ACCase sensitivity has been shown to be an important resistance mechanism (see e.g. [27,28]). Inheritance studies have shown that ACCase-based resistance to both AOPP and CHD herbicides is governed by a single dominant or semi-dominant nuclear gene and that resistance to different herbicides is encoded at the same gene locus (see e.g. [29,30]).

Recently, work with chimaeric genes containing wheat plastid ACCase that complemented the yeast *ACC1* mutation identified a graminicide-sensitivity determinant to a 400-amino-acid fragment. By comparison with sequences for other ACCases, this 400-amino-acid stretch was suggested to be part of a carboxyl transferase domain [31]. Further work with these chimaeric isoforms, as well as with other ACCases exhibiting acquired or inherent resistance to graminicides, identified an isoleucine to leucine substitution as being critical for herbicide interaction [32]. However, as pointed out in the latter paper, other residues may play a role in defining binding specificity.

Both AOPP and CHD herbicides are thought to have overlapping binding sites on ACCase [8]. However, the development of resistance is often associated with distinct changes in the relative efficacy of different graminicides against the enzyme [24]. Indeed, similar phenomena were noted in the detailed studies on chimaeric proteins by Nikolskaya et al. [31]. All these facts point to subtle variations in the conformation of ACCases, which can influence graminicide sensitivity.

Although different multifunctional ACCases can vary considerably in their sensitivity to graminicides, they all seem to have similar enzymic properties (see e.g. [21]). Even the reaction mechanism of individual isoforms from the same plant seems to be identical [20]. However, the presence of co-operative binding properties for the graminicides seems to be associated with insensitivity [20–22]. To test this relationship, we have separated ACCase isoforms from grasses with sensitivity or with inherent or acquired resistance to graminicides. Detailed study of this range of enzymes has provided strong support for the hypothesis that the insensitivity is associated with co-operativity of herbicide binding.

MATERIALS AND METHODS

Plant material

Maize seeds (*Zea mays* cv Celebration) were from Nickerson Seeds (Lincoln, U.K.) and red fescue (*Festuca rubra*) seeds from Herbiseed (Wokingham, Berks., U.K.). Seeds of the wild-type (Rothamsted) and the graminicide-resistant (Notts A1) biotypes of *Alopecurus myosuroides* (black-grass) were collected by Dr Stephen Moss (Rothamsted Research, Rothamsted, Harpenden, Herts., U.K.). Seeds were grown in soil-less compost at 20 °C for a 14 h light period of 650 $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ and watered daily. They were harvested normally 5–7 days following germination through the compost surface.

Tissue cultures were established from seeds of *A. myosuroides* (both biotypes) and *F. rubra*. Seeds were sterilized in 20% (w/v) sodium hypochlorite for 20 min. After thorough washing with sterile distilled water, the seeds were aseptically placed on Murashige/Skoog-based salt medium (Sigma, Poole, Dorset, U.K.) containing 2 mg/l 2,4-dichlorophenoxyacetic acid, 20 g/l sucrose and 8 g/l agar. Calli were formed from the seeds at different rates, but once they were large enough to support their own growth they were removed from the seeds and placed on a fresh medium.

Maize cell cultures were from the Black Mexican Sweetcorn variety and from Bayer Crop Science (Ongar, Essex, U.K.) as was the *P. annua* culture, which was of root origin.

All cultures were maintained routinely on the supplemented Murashige/Skoog-based salt medium by subculturing every 3 weeks. Additionally, cells were maintained in suspension in the above medium without agar. A 15% inoculum was placed in 50 ml of the medium in 100 ml flasks, agitated at 200 rev./min on an orbital shaker at 27 °C under 650 $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ lighting. Cells were subcultured every 7 days.

Chemicals

Quizalofop-free acid (*R,S*)-2-[4-(6-chloroquinoxalin-2-yloxy)-phenoxy]propionic acid was from Bayer Crop Science and fluazifop-free acid (*R,S*)-2-[4-(5-trifluoromethyl-2-pyridyloxy)-phenoxy]propionic acid was from Cluzeau Info. Laboratoire (Ste-Foy-La Grande, France). Sethoxydim was obtained from Greyhound Chem-Services (Birkenhead, Merseyside, U.K.). Sodium [^{14}C]acetate (1.85–2.29 GBq/mmol) and $\text{NaH}^{14}\text{CO}_3^-$ (1.85–2.29 GBq/mmol) were from Nycomed-Amersham (Bucks, U.K.).

All other chemicals and materials were from Sigma, Fisher Scientific (Loughborough, Leics., U.K.), Pharmacia Biosystems (Milton Keynes, U.K.) or BDH–Merck Ltd. (Poole, Dorset, U.K.) and were of the best available grades.

Assay of carboxylase activity

ACCase was assayed by measuring the incorporation of radioactivity from $\text{NaH}^{14}\text{CO}_3^-$ into malonyl-CoA by the method of Burton et al. [2]. The enzyme solution was mixed in the ratio 1:1 (v/v) with the assay buffer [100 mM HEPES/KOH (pH 8.0)/5 mM $\text{MgCl}_2/2$ mM ATP/20 mM $\text{NaH}^{14}\text{CO}_3^-$ (37 MBq/mmol)/1.0 mM acetyl-CoA in a final volume of 100 μl]. The above assay concentrations were found to be optimal for all the ACCases assayed (results not shown). Samples were pre-incubated for 10 min and the assay started by the addition of acetyl-CoA and continued at 30 °C for 10 min. Acid-stable radioactivity [2] was counted in Optifluor scintillation fluid (Packard Bioscience, Groningen, The Netherlands) and quench corrections were made by the external standard channels ratio method.

Duplicated assays without acetyl-CoA were used as controls, and background readings subtracted in all the cases. The identity of the malonyl-CoA product was confirmed by TLC [33].

Propionyl-CoA carboxylase activity was assayed as above, with the replacement of the acetyl-CoA by propionyl-CoA. The assay conditions and identity of the product had been demonstrated previously [34].

Purification of ACCase

Maize leaf isoforms

The method was described by Herbert et al. [20] and used ammonium sulphate precipitation followed by chromatography on Sephacryl S-400, agarose-linked Reactive Red 120 (Red Agarose; Sigma), Sepharose-linked Cibacron Blue and Q-Sepharose. The two maize isoforms were resolved on the latter using a 15–30 % gradient of 1 M KCl in 20 mM HEPES/KOH (pH 8)/1 mM Na-EDTA/20 % (w/v) glycerol/5 mM DTT (dithiothreitol)/5 mM ϵ -aminocaproic acid/1 mM benzamidine/HCl/1 mM PMSF (buffer A). De-salting was with Sephadex G-25 and fractions were concentrated with Macrosep 50 concentrators (Pall Filtron, Northborough, MA, U.S.A.). Separation of the two peaks was confirmed by assaying fractions in the presence and absence of 1 mM quizalofop which inhibited the sensitive isoform > 95 % but had no effect on the activity of the minor isoform. Samples were stored at -70°C until required. Freezing and thawing were always performed as rapidly as possible to minimize enzyme degradation [35].

Extraction of ACCase from cell cultures

For enzyme extraction, cell suspension cultures were grown in 250 ml of medium in 500 ml flasks. Cells were harvested 4 days after subculturing when ACCase activity was optimal. Cells were filtered under vacuum through Whatman no. 1 filter paper, frozen rapidly in liquid nitrogen and then grounded to a fine powder using a pestle and mortar. The powder was then suspended in buffer A.

Maize cell suspension isoforms

The procedure similar to the maize leaf ACCase was followed, except that the Blue-Sepharose step was omitted. The latter proved unnecessary and achieved no significant purification.

Isoforms from *A. myosuroides*

After preparation of powder from cell suspensions, ammonium sulphate fractionation was performed as for maize. It was followed by purification using Sephacryl S-400. For the Rothamsted biotype, activity eluted in one peak shortly after the void volume. Active fractions were collected, concentrated in Macrosep 50 concentrators before purifying on Q-Sepharose columns. Two peaks were separated with the minor one eluting at approx. 210 mM KCl and the major at approx. 350 mM KCl in a 200–500 mM KCl gradient in buffer A. For the Notts A1 biotype, ACCase activity eluted earlier from the Sephacryl S-400 column with separation of the minor peak from the latter major peak. Usually the separation was adequate, but in cases where the peaks overlapped or where more purification was needed, a Q-Sepharose column was used. The minor peak eluted off the latter column in the void volume and the major peak at approx. 350 mM KCl in a 0–500 mM KCl gradient in buffer A.

F. rubra isoforms

The ACCases from *F. rubra* were partially purified to the salt precipitation stage by following the procedure used for maize

cells. Two columns were then used. The Sephacryl S-450 column gave good purification but no resolution of isoforms. The latter was achieved using a column of Fractogel EMD TMAE 650 (Merck, Frankfurt, Germany) equilibrated with buffer A. A 150–500 mM KCl gradient in buffer A was then applied and the major fraction eluted first at approx. 230 mM KCl with the minor isoform at approx. 360 mM KCl.

Protein analysis

SDS/PAGE was used with 7.5 or 5 % (w/v) running gels and 5 % (w/v) stacking gels to separate reduced protein samples. The gels were stained with Coomassie Blue. Electroblooming was performed using the wet-blot system of Bio-Rad (Munich, Germany) at 4°C on nitrocellulose membranes (Hybond-C-Super; Nycomed-Amersham). Transfer of the large ACCase proteins required up to 24 h. Protein markers used were from Sigma or Bio-Rad including pre-stained (Rainbow) sets.

Immunodetection of isoenzymes from maize was performed using antibodies raised against the multifunctional isoform of ACCase from pea. This was a gift from Dr C. Alban (Centre National de la Recherche Scientifique, Bayer Crop Science LaboMixte, Lyon, France). Alternatively, biotin-containing proteins were detected using horseradish peroxidase-labelled streptavidin (Sigma). Bands were detected with the developing reagent 4-chloronaphthol (Sigma).

Protein was quantified by the Coomassie Blue dye-binding method [36] using the Bio-Rad protein assay reagent. BSA was used as the protein standard.

Inhibition of cell suspension growth

Inhibition of growth of cell suspension cultures by different graminicides was measured in terms of reduction (on a dry weight basis) of cells after 7 days growth compared with control. Susceptibility of the cultures was expressed in terms of an IC_{50} value. The herbicides were made up as stock solutions in acetone and diluted so that the final concentration of acetone in the growth medium was 0.1 % (v/v). Such acetone concentrations had no effect on the growth of any of the cell cultures used. Control flasks had 0.1 % acetone but no herbicide.

Enzyme kinetic studies

For determination of the K_m values for each substrate, the other two substrates were present at saturating concentrations. These were checked for each of the purified isoforms and were 2 mM ATP, 1 mM acetyl-CoA and 20 mM bicarbonate. For each enzyme–substrate pair, data (in triplicate) were fitted to the Michaelis–Menten equation by least-squares analysis using MicroMath Scientist to obtain apparent K_m values.

Herbicide binding to ACCase

Data for inhibition of isoforms by different concentrations of herbicides were fitted to different steady-state rate equations [37] to determine which mechanism of inhibition was more probable. Data were first fitted to the following equation, which describes simple hyperbolic inhibition:

$$v = \frac{v_0}{1 + (i/K_i)}$$

where v_0 is the control enzyme velocity (i.e. in the absence of inhibitor), v is the initial enzyme velocity at different concentrations of the inhibitor i and K_i is the inhibition constant.

Table 1 Purification of ACCase isoforms from different cell cultures

For details of the purification procedures see the Materials and methods section.

Isoform	Total protein (mg)	Total activity ($\mu\text{mol} \cdot \text{min}^{-1}$)	Yield (% recovery)	Specific activity ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg of protein}^{-1}$)	Purification (fold)
<i>Z. mays</i>					
Homogenate	517.0	628.0	100.0	1.2	1.0
(NH ₄) ₂ SO ₄ cut	129.5	178.2	28.3	1.3	1.1
Sephacryl	60.2	138.1	22.0	2.2	1.9
Red agarose	3.2	109.0	17.4	34.1	28.2
Q-Sepharose					
ACCCase 1	0.4	93.5	14.8	217.0	224.2*
ACCCase 2	0.2	7.4	0.68	34.9	144.2*
<i>A. myosuroides</i> Rothamsted					
Homogenate	132.0	145.2	100.0	1.1	1.0
(NH ₄) ₂ SO ₄ cut	56.0	95.2	63.0	1.7	1.5
Sephacryl	15.5	69.8	48.1	4.5	4.1
Q-Sepharose					
ACCCase 1	0.4	54.2	15.0	154.9	176.0*
ACCCase 2	0.4	13.2	3.7	41.3	187.7*
<i>A. myosuroides</i> Notts A1					
Homogenate	575.0	248.8	100.0	0.9	1.0
(NH ₄) ₂ SO ₄ cut	138.8	203.8	58.4	1.4	1.8
Sephacryl					
ACCCase 1	21.9	149.0	42.7	6.8	9.1
ACCCase 2	59.0	21.4	6.1	0.4	1.9
Q-Sepharose					
ACCCase 1	0.33	17.3	4.9	52.4	72.8*
ACCCase 2	1.5	5.2	1.5	3.5	19.4*
<i>F. rubra</i>					
Homogenate	125.0	95.9	100.0	0.8	1.0
(NH ₄) ₂ SO ₄ cut	24.3	66.0	68.8	2.7	3.5
Sephacryl	4.7	34.3	35.7	7.3	9.5
TMAE					
ACCCase 1	0.1	22.1	23.1	184.2	299.0*
ACCCase 2	0.2	4.8	5.0	24.9	162.0*

* Calculated from the original homogenate activity assuming that the minor isoform accounted for 20% of the total activity.

Secondly, data were fitted to the following equation:

$$v = \frac{v_0}{[1 + (i/K_i)h_{\text{app}}]}$$

where h_{app} is the apparent Hill coefficient.

The second equation is of the same form as the Hill equation and takes into account the co-operativity involved in inhibitor binding. h_{app} is a measure of the co-operativity between enzyme subunits for the binding of the inhibitor. If $h_{\text{app}} > 1$, then there is positive co-operativity between the inhibitor binding sites, whereas $h_{\text{app}} < 1$ indicates negative co-operativity. In addition, where there is positive co-operativity, the next highest integer above the value of h_{app} represents the number of inhibitor binding sites. The Hill equation is identical with the equation for simple hyperbolic inhibition if $h_{\text{app}} = 1$, i.e. if there is no co-operativity.

RESULTS

Separation of different isoforms of ACCase

In general, plants contain two isoforms of ACCase, one plastid-localized (also known as ACCase 1 or the major isoform) and the second outside the plastid [15], presumed to be cytosolic (also known as ACCase 2 or the minor isoform). For the Gramineae, both isoforms are high-molecular-mass (200–240 kDa) multi-functional proteins which function as native dimers [11]. Fol-

lowing our successful purification of the two ACCase isoforms from maize leaves [20], we used the same method as a basis of the purification method for other plant species. The final method adopted is described in the Materials and methods section and is summarized in Table 1. For maize cell cultures, we were able to omit the Blue-Sepharose step used for the leaf enzymes but otherwise the method was virtually unchanged. The Blue-Sepharose step gave hardly any purification for the suspension cells which was surprising since, with preparations from maize leaves, it was quite effective, giving a purification of approx. 8-fold (results not shown). Presumably, this contrast reflected the different protein mixtures obtained from the two sources. Nevertheless, the final separation of the two isoforms was achieved on a Q-Sepharose column with similar ionic strengths needed to elute each of the isoforms (Figure 1). The final purification of the ACCase was 224-fold for ACCase 1 and 144-fold for ACCase 2 from the maize cell suspensions (Table 1). This compared with approx. 300- and 100-fold respectively for the same isoforms from maize leaves (cf. [20]).

Two biotypes of *A. myosuroides* (black-grass) were used. Cell cultures were established from an AOPP- and CHD-sensitive Rothamsted (wild-type) population and a population with acquired resistance, Notts A1 [38,39]. For *A. myosuroides* Rothamsted, we were able to omit the Red Agarose column and separated the two isoforms successfully (Figure 1) and with good purification (Table 1) in only three steps. For *A. myosuroides* Notts A1, the two isoforms were resolved from each other on the first, Sephacryl S-400, column (Figure 1). However, this gave only a

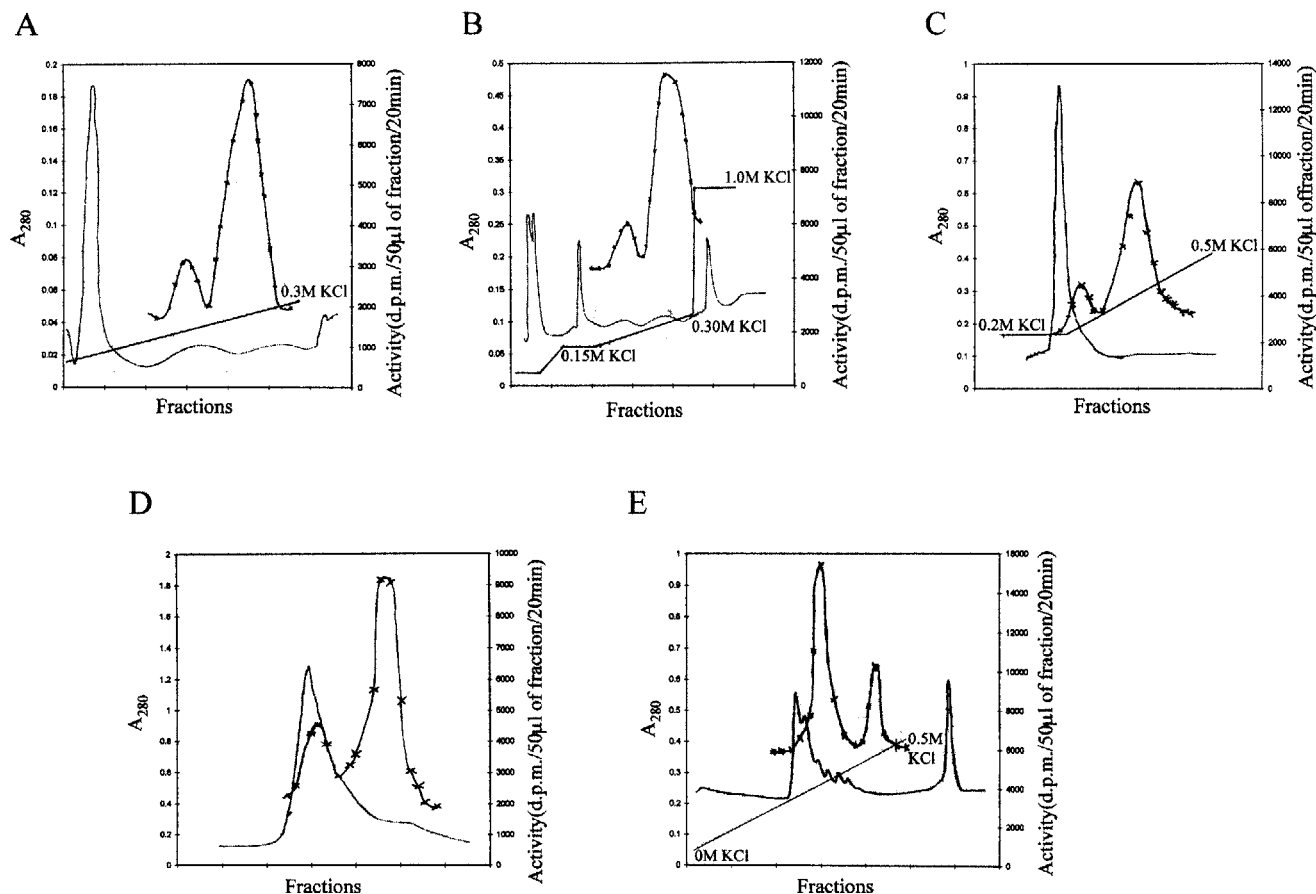


Figure 1 Separation of ACCase isoforms from various graminaceous species

The panels show separations on columns where both ACCase 1 and ACCase 2 isoforms were fully resolved. For details of the purification procedures see the Materials and methods section. Activity of individual fractions is indicated with x. The continuous traces show the absorbance at 280 nm. Separation of (A) maize leaf, (B) maize cell culture isoforms, (C) *A. myosuroides* biotype Rothamsted isoforms on Q-sepharose, (D) *A. myosuroides* biotype Notts A1 isoforms on Sephacryl S-400 and (E) *F. rubra* isoforms on TMAE anion exchange.

slight purification of ACCase 1 and virtually none for ACCase 2 (Table 1). Therefore for enzyme studies, we used a Q-Sepharose step as well, although the purification was still noticeably worse than for preparations from the Rothamsted population (Table 1).

For cell suspensions of *F. rubra*, purification proceeded similar to the maize and *A. myosuroides* Rothamsted cells to the Sephacryl S-400 step, where one peak of activity was obtained. However, subsequent attempts to resolve ACCase isoforms using Q-Sepharose anion exchange or Red Agarose affinity chromatography were unsuccessful. Eventually, success was achieved using anion exchange on Fractogel EMD TMAE 650 (Figure 1), where ACCase 1 eluted first. A very high purification was obtained using the TMAE column (Table 1).

The purified ACCase isoforms were analysed by SDS/PAGE followed by Western blotting. As expected, the maize leaf and maize cell suspensions gave identical patterns with the major ACCase 1 isoform giving cross-reactivity to the pea multifunctional ACCase antibody for a protein estimated to be 227 kDa. The minor ACCase 2 isoform had an estimated molecular mass of 219 kDa (Figure 2). The separated ACCase from both biotypes of *A. myosuroides* gave bands of approx. 230 kDa. In contrast with maize, the isoforms from *A. myosuroides* had molecular masses that were too close to distinguish confidently (Figure 2). For ACCases from *F. rubra*, estimated molecular masses were approx. 240 kDa for both isoforms.

Enzymic properties of the isoforms

Michaelis constants were calculated for the three substrates used by the ACCase isoforms. All curves corresponded to Michaelis-Menten kinetics, but there were differences in the properties of each pair of isoforms (Table 2); this provided additional proof of the successful resolution of the two isoforms for the various tissues. (This was also confirmed by the different sensitivities or binding characteristics towards graminicides for the isoform pairs, which are shown later.)

With the exception of the minor isoform from *A. myosuroides* Rothamsted, all ACCases had apparent K_m values for acetyl-CoA in the range 0.11–0.20 μM . For ATP, the K_m values were in a similar range (0.08–0.18 μM), with the exception of the two isoforms obtained from *A. myosuroides* Notts A1 where the value was significantly higher. For bicarbonate, the K_m values of all isoforms were considerably greater than for the other substrates.

Since the sensitivity of a limited number of ACCase isoenzymes towards graminicides had been correlated with their ability to carboxylate propionyl-CoA [19,34], we also tested the latter substrate. All the isoforms tested were able to use propionyl-CoA as an alternative substrate (Table 2). For the two graminicide-sensitive ACCases (ACCase 1 from *A. myosuroides* Rothamsted and maize), the K_m values for acetyl-CoA and propionyl-CoA were rather similar. However, for the graminicide-insensitive isoforms, the ratio of K_m values for the two acetyl-CoAs varied from

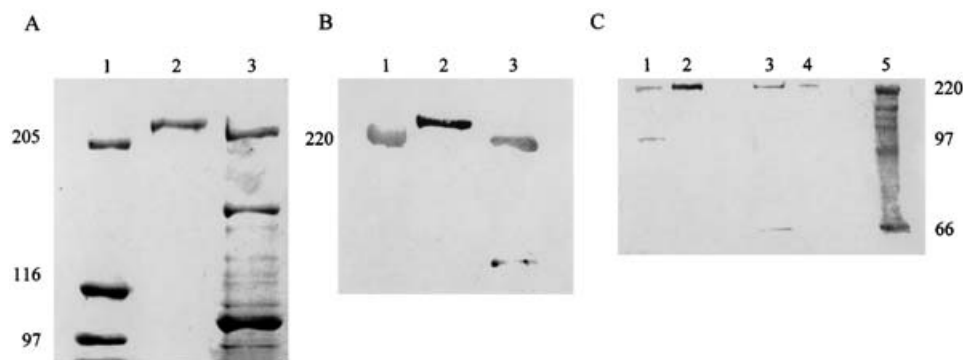


Figure 2 Electrophoretic separation of ACCase isoforms

Separated isoforms (see the Materials and methods section and Table 1) from maize leaf, stained with Coomassie Blue (**A**) (lane 1, marker proteins; lane 2, 227 kDa ACCase 1; and lane 3, 219 kDa ACCase 2), from maize cell cultures (Western blots) (**B**) (lane 1, 220 kDa marker; lane 2, 227 kDa ACCase 1; and lane 3, 219 kDa ACCase 2) and from two populations of *A. myosuroides* (Western blots) (**C**) (lane 1, ACCase 2 from Rothamsted; lane 2, ACCase 1 from Rothamsted; lane 3, ACCase 1 from Notts A1; lane 4, ACCase 2 from Notts A1; and lane 5, pre-stained marker proteins run simultaneously).

Table 2 Apparent K_m values (mM) for resolved ACCase isoforms from different sources

For separation of isoforms and assay details see the Materials and methods section. For determination of K_m of each substrate, the other two substrates were present at saturating concentrations. The latter concentrations were 2 mM ATP, 2 mM acetyl-CoA and 10 mM bicarbonate. For each substrate pair, data (in triplicate) were fitted to the Michaelis–Menten equation by least-squares analysis using MicroMath Scientist to obtain the apparent K_m values shown. For propionyl-CoA carboxylase assay, acetyl-CoA substrate was replaced with propionyl-CoA. Data are means \pm S.D. ($n = 3$). tr, < 0.005 ; n.m., not measured.

Substrate	Organism ... ACCase ...	Apparent K_m (mM)							
		<i>A. myosuroides</i> Rothamsted		<i>A. myosuroides</i> Notts A1		<i>F. rubra</i>		<i>Z. mays</i>	
		1	2	1	2	1	2	1	2
Acetyl-CoA		0.14 \pm 0.01	0.01 \pm tr	0.16 \pm 0.02	0.11 \pm 0.01	0.20 \pm 0.02	0.11 \pm 0.01	0.12 \pm 0.01	0.11 \pm 0.01
Propionyl-CoA		0.10 \pm 0.01	n.m.	0.27 \pm 0.03	0.08 \pm 0.01	0.07 \pm tr	n.m.	0.10 \pm 0.01	0.04 \pm tr
ATP		0.08 \pm 0.01	0.18 \pm 0.01	0.47 \pm 0.06	0.50 \pm 0.06	0.15 \pm 0.01	0.12 \pm tr	0.08 \pm 0.01	0.08 \pm tr
Bicarbonate		1.38 \pm 0.10	1.75 \pm 0.24	1.64 \pm 0.16	1.25 \pm 0.06	1.15 \pm 0.10	1.38 \pm 0.09	1.09 \pm 0.07	0.39 \pm 0.04

0.59 for *A. myosuroides* Notts A1 ACCase 1 to 2.86 for ACCase 1 from *F. rubra* (Table 2).

Models for herbicide binding

A graminicide-insensitive multifunctional ACCase 2 from pea leaves was shown to have two quizalofop binding sites, which had different affinities for the herbicide. Steady-state kinetic analysis showed that there was positive co-operativity of herbicide binding between the two sites [19]. Positive co-operativity has also been demonstrated for the insensitive maize ACCase 2, whereas the sensitive ACCase 1 did not show co-operativity [20]. Negative co-operativity has been observed for mixtures of insensitive isoforms from resistant biotypes of *L. multiflorum* [21] and from the inherently tolerant *P. annua* [22]. Since a correlation of co-operativity in herbicide binding with insensitivity is possible, we examined the phenomenon in some detail.

Curve fitting was performed as described in the Materials and methods section, and the fit to a simple hyperbolic equation versus the Hill equation (indicating co-operativity) was assessed. We tested both isoforms from each of the four tissue cultures against representatives of the AOPP herbicides. Some examples of the curve fitting of data are shown in Figure 3 and a summary of the results is given in Table 3. Using the sum of the squared deviations as a measure of fit, there were only two isoforms that gave data that could be fitted to the simple hyperbolic equation nearly as well as to the Hill equation. Further examination of the

data by estimating h_{app} revealed that these two values were close to unity, i.e. showing little, if any, co-operativity. These were for the ACCase 1 from *A. myosuroides* Rothamsted and maize, which are the only graminicide-sensitive enzymes and which also showed a better fit to the simple hyperbolic equation (see Figure 3A). For the herbicide-insensitive ACCases, h_{app} values showed positive co-operativity except for ACCase 2 from *A. myosuroides* Rothamsted (Table 3), which showed strong negative co-operativity.

DISCUSSION

Purification and properties of ACCase isoforms

By using a similar purification procedure as for maize leaf ACCases [20], we succeeded (with some modifications) in separating two active fractions for each tissue used. For maize, the two purified fractions clearly contained different proteins, which could be separated by mass (Figure 2). However, for the proteins from *A. myosuroides* or *F. rubra*, the molecular masses appeared very similar and the isoforms could not be distinguished by this technique. However, in all cases, the Michaelis constants and/or herbicide sensitivity were sufficiently distinct (Tables 2 and 3) to reassure us that the two separated peaks represented the major ACCase 1 and the minor ACCase 2 isoforms respectively.

Overall, the Michaelis constants obtained for the different isoforms were rather similar and consistent with those obtained

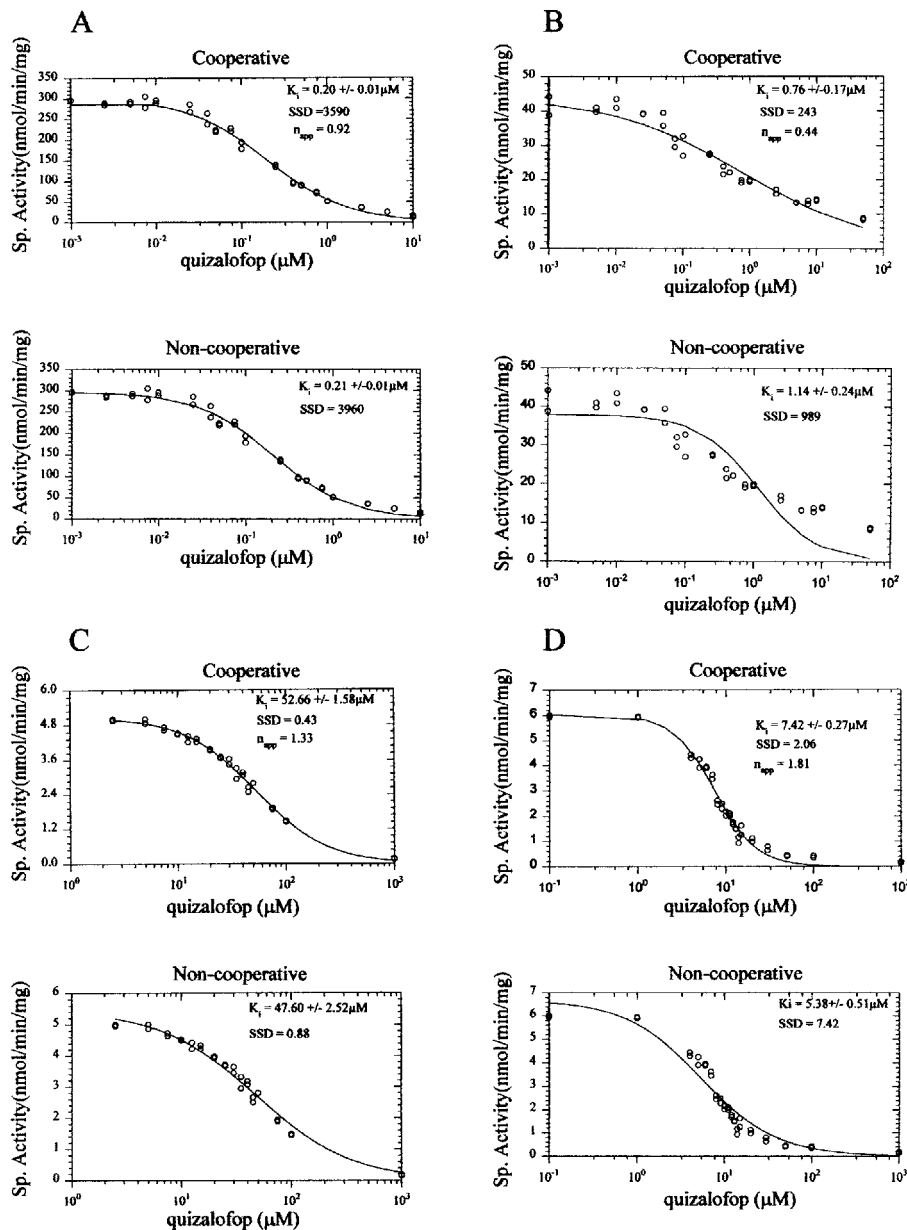


Figure 3 Curve fits for the co-operative and non-co-operative models for binding of quizalofop to ACCase isoforms of *A. myosuroides*

Binding to (A) the susceptible Rothamsted population ACCase 1, (B) Rothamsted ACCase 2, (C) the resistant Notts A1 population ACCase 1 and (D) Notts A1 ACCase 2. Data points were plotted and curve-fitting performed by least-squares analysis using MicroMath Scientist software. Measure of fit is expressed as the sum of the squared deviations (SSD).

for other ACCases (see e.g. [13,20]). In fact, the significantly higher K_m value for bicarbonate compared with those for ATP or acetyl-CoA is a consistent finding for ACCases in plants [40]. The minor, non-plastid, isoform from *A. myosuroides* Rothamsted had a particularly low K_m value for acetyl-CoA (Table 2) when compared with the other purified isoforms. However, its value of $10 \mu\text{M}$ was similar to that reported for the non-plastid multifunctional isoform from pea which was $15 \mu\text{M}$ [13].

The possible role of substrate concentrations in the regulation of plant ACCases has been discussed [41–43]. On the basis of the ATP concentrations estimated for maize, this substrate should not be limiting for the plastid ACCase isoforms even in the dark. An exception is the major isoform of *A. myosuroides* Notts A1, which has a K_m of 0.47 mM for ATP (Table 2). However, the ATP levels in chloroplasts from this species are unknown. The concentrations

of acetyl-CoA in spinach or pea chloroplasts have been reported to be maintained at $31\text{--}54 \mu\text{M}$. Thus comparing this range with the K_m values for plastid isoforms (Table 2) suggests that acetyl-CoA levels may exert some control on the enzyme activity. For each of the grasses studied, the ACCase 2 had a lower K_m value for acetyl-CoA when compared with ACCase 1. In spinach leaves, it is thought that 77% of the total acetyl-CoA is chloroplastic [43] so that even the activity of the minor (non-plastid) ACCase 2 isoform could be limited by substrate availability.

In discussing the substrate K_m values relative to possible substrate levels and, hence, control from this source *in vivo*, it must be pointed out that recent evidence suggests that oxidation/reduction of enzyme protein is important for regulation. Experiments so far have concentrated on the multisubunit form of ACCase 1, where the redox status of the carboxyl transferase component seems to be

Table 3 Comparison of models for graminicide binding in different acetyl-CoA carboxylases

ACCcase isoforms were assayed in the presence of increasing concentrations of graminicides. Duplicate data points were fitted to the Hill equation or the equation which describes simple hyperbolic inhibition (see the Materials and methods section) by least-squares analysis using MicroMath Scientist. Column 3 represents the relative measure of fits to the two equations. The higher this value from 1, the better fit to the Hill equation, suggesting co-operative binding of the herbicide. SSD, sum of the squared deviations; Notts A1, *A. myosuroides* Notts A1; Rothamsted, *A. myosuroides* Rothamsted.

	SSD non-co-operative/SSD co-operative	h_{app}	IC ₅₀ (μ M)
Quizalofop			
<i>F. rubra</i>			
ACCcase 1	2.3	1.33	25.3
ACCcase 2	1.8	1.21	18.6
Notts A1			
ACCcase 1	2.0	1.33	52.6
ACCcase 2	3.6	1.81	7.4
Rothamsted			
ACCcase 1	1.1	0.92	0.2
ACCcase 2	4.1	-0.44	0.8
Fluazifop			
Notts A1			
ACCcase 1	1.8	1.23	2540
ACCcase 2	2.2	1.49	2980
<i>Z. mays</i>			
ACCcase 1	1.2	0.86	0.04
ACCcase 2	3.3	1.85	57.0

responsible for regulation [44,45]. All of our isoforms were purified and stored in the presence of DTT, which has been shown to activate the pea ACCase effectively [44,46]. Thus we are confident that interpretation of the results in Table 2 is not complicated by a variable amount of protein oxidation.

Previous studies by Dehaye et al. [19] had shown a linear relationship between the V_{max} values of propionyl-CoA/acetyl-CoA and the IC₅₀ values for quizalofop inhibition of pea ACCase activity. Similar observations were made by Herbert [34]. However, in the present studies, all the isoforms tested showed V_{max} values for acetyl-CoA which were 2–2.5 times higher than those for propionyl-CoA (results not shown), regardless of whether the ACCase was sensitive or insensitive to quizalofop. Moreover, the K_m values determined for the two substrates usually showed a lower value for propionyl-CoA, regardless of whether the enzyme was sensitive or not (Table 2). In addition, the herbicide-insensitive ACCase 1 of *A. myosuroides* Notts A1 actually had a higher K_m value for propionyl-CoA when compared with acetyl-CoA. Thus although all the multifunctional ACCase proteins examined were able to carboxylate both acetyl-CoA and propionyl-CoA, their activity with these two substrates did not correlate well with graminicide sensitivity.

Co-operativity of herbicide binding

It is clear from the above discussion that the different multifunctional ACCases share very similar properties despite a wide variation in their sensitivity to graminicides. The possible correlation of the latter property with co-operativity of herbicide binding was examined with our purified isoforms. The concept of co-operativity of herbicide binding in ACCases from herbicide-insensitive grasses was first observed for an insensitive biotype of *L. multiflorum* [21]. It was also detected in the inherently resistant *P. annua* [22]. As noted before, however, in both cases a mixture of isoforms was assayed which could complicate the interpretation

of results. Nevertheless, examination of separated isoforms of ACCase also confirmed that an insensitive pea enzyme exhibited herbicide-binding co-operativity [19] whereas the sensitive maize ACCase 1 did not [20].

Results of the present study strongly support the above hypothesis. Thus for *A. myosuroides* Rothamsted, the sensitive ACCase 1 showed no co-operativity towards quizalofop, whereas the insensitive ACCase 1 isoforms from *A. myosuroides* Notts A1 or *F. rubrum* did. Moreover, the sensitive ACCase 1 from maize cells showed no co-operativity towards a second AOPP, fluazifop, whereas the insensitive ACCase 1 from *A. myosuroides* Notts A1 showed positive co-operativity. All the insensitive ACCase 2 isoforms showed co-operativity against the AOPPs (Table 3).

Generally, it is accepted that acquired resistance towards both AOPP and CHD herbicides in grasses is mainly through point mutations to the target protein ACCase. Since such mutations can lead to different alterations in sensitivity to AOPPs as opposed to CHDs (despite both these graminicide classes binding to the same site on the enzyme) [8] and, indeed, to changes in the relative sensitivity to herbicides within one chemical class [24], there must be a series of possible mutations which can occur. Studies on the molecular basis of resistance conferred by an insensitive ACCase in the Notts A1 population [47] confirmed that sethoxydim resistance in *A. myosuroides* is associated with the leucine substitution for isoleucine also found in resistant *Setaria viridis* [48], *Avena fatua* [49] and *Lolium rigidum* [32]. This identical substitution adds to the evidence of an important function for this particular mutation. In view of the clear evidence that isoleucine substitution by leucine converts a sensitive ACCase into an insensitive enzyme [32], it would be interesting to study the enzyme properties in such cases.

Substitution of isoleucine by leucine was also the first point mutation identified which confers resistance to ACCase inhibitors in *A. myosuroides* [47]. In the latter study, only a small region of the *A. myosuroides* ACCase gene was examined, and it is possible that other mutations are involved in the production of the insensitive enzyme. In fact, it is, at first, surprising that a conservative amino acid substitution has such a large effect on ACCase activity. Nevertheless, similar changes in enzyme activity have been noted, especially when active-site residues (such as valine to leucine) are involved [50]. However, given the different cross-resistance patterns seen in resistant populations of many grassweeds, it is probable that other mutations exist which are yet to be identified [51]. Moreover, because detected mutations have altered herbicide sensitivity they must all be affected at the herbicide binding site. Therefore it makes sense that biotypes with acquired insensitivity also show changes in herbicide binding in the form of co-operativity, similar to ACCase isoforms with inherent insensitivity.

Thus our results confirm the proposal that graminicide insensitivity is associated with co-operativity of herbicide binding to the ACCase dimer. So far, this is the only associated property that seems to distinguish graminicide-sensitive from -insensitive isoforms of ACCase.

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