Studies on the Inhibition of Biotin-Containing Carboxylases by Acetyl-CoA Carboxylase Inhibitors

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In higher plants the biosynthetic machinery of *de novo* fatty acid biosynthesis, measured as [^{14}C]acetate incorporation into fatty acids, is predominantly located in plastids. A key enzyme in this pathway is the biotin-containing acetyl-CoA carboxylase (ACC, EC 6.4.1.2) which catalyzes the ATP-dependent carboxylation of acetyl-CoA to malonyl-CoA. The ACC from *Poaceae* is very efficiently blocked by two herbicide classes, the cyclohexane-1,3-diones (*e.g.* sethoxydim, cycloxydim) and the aryloxyphenoxy-propionic acids (*e.g.* diclofop, fluazifop). It is shown that within the *Poaceae* not only different species but also different varieties exist which exhibit an altered sensitivity and tolerance towards both herbicide classes, which points to a mutation of the target enzyme ACC. In purifying the ACC we extended our research to the possible presence of other biotin-containing plant enzymes. In protein preparations from maize, oat, barley, pea and lentil we were able to demonstrate the carboxylation of acetyl-CoA, propionyl-CoA and methylcrotonyl-CoA. The two herbicide classes not only block the ACC, but also the activity of the propionyl-CoA carboxylase (PCC), whereas the methylcrotonyl-CoA carboxylase (MCC), a distinct biotin-containing enzyme from mitochondria, is not affected. MCC may play a role in isoprenoid catabolism. Whether PCC is a separate plastid enzyme or only a side activity of ACC is under current investigation. The efficiency of the graminicides in sensitive *Poaceae* is then not only determined by the inhibition of ACC, malonyl-CoA and fatty acid biosynthesis, but also by the exclusion of the PCC-catalyzed metabolic pathways of the plant cell.

Introduction

The enzyme acetyl-CoA carboxylase ACC is the key enzyme of the *de novo* fatty acid biosynthesis in plant plastids and also supplies various other biosynthetic pathways with malonyl-CoA (such as flavonoid and polyketide biosynthesis). It is the major biotin-containing enzyme of plants. Until 1990 it was assumed that ACC was the only biotin-containing carboxylase known in plants [1]. In the green algae *Chlorella* additionally a urea carboxylase (UC) was detected [2].

More recently the activity of other biotin-containing carboxylases, *e.g.* propionyl-CoA carboxylase (PCC), methylcrotonyl-CoA carboxylase (MCC) and pyruvate carboxylase (PC), has been detected in non-purified preparations of sunflower, carrot, rape and maize [3] and the MCC activity also in pea, maize and oat [4, 5]. The enzyme PCC may function as in animals in the degradation of the amino acids valine, isoleucine, methionine and odd-numbered fatty acids, whereas MCC may play a role in the degradation of leucine and β-methyl-branched keto acids (isoprenoid residues).

All biotin-dependent carboxylases consist of three domains: a) the biotin carboxylase (BC), which catalyzes the ATP-dependent carboxylation of biotin with hydrogencarbonate; b) the carboxyl transferase (CT), which transfers the activated carbon dioxide from the biotin to an acceptor molecule (*e.g.* to acetyl-CoA in the case of ACC); c) the biotin carboxyl carrier protein (BCCP), which is covalently linked to the prosthetic biotin group by a mobile spacer, which allows the biotin to move between the two catalytic centers. The coupling site of biotin to BCCP is the ε-amino group of a lysyl side chain [6]. This modified amino acid residue is also called biocytin. The biotin-containing carboxylases work in a two-step mechanism, as is shown below for the ACC.

In the first step the biotin carboxylase catalyzes the carboxylation of the biotinylated prosthetic group at the biotin carboxyl carrier protein (reac-

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tion center I). After translocation of this group by a 1.4 mm long swivel arm to reaction center II, the carboxyl transferase catalyzes the carboxyl transfer from biotin to acetyl-CoA forming malonyl-CoA at the second step. The mechanism of this enzyme reaction can be described as a two-site ping-pong bi-bi uni-uni reaction [7]. The ACC reaction is ATP- and Mg^{2+}-dependent:

$$\begin{array}{lcl} \text{ACC-biotin} + \text{ATP} + HCO_3^- & \xrightarrow{Mg^{2+}} & \text{ACC-biotin}-COO^- + \text{ADP} + P_i \\ \text{ACC-biotin}-COO^- + \text{acetyl-CoA} & \longrightarrow & \text{ACC-biotin} + \text{malonyl-CoA} \\ \hline \text{Acetyl-CoA} + \text{ATP} + HCO_3^- & \longrightarrow & \text{ADP} + P_i + \text{malonyl-CoA}. \end{array}$$

It had been shown before that two groups of chemically different herbicides specifically inhibit the plant acetyl-CoA carboxylase ACC [8–13]. These are derivatives of either aryloxyphenoxypropionic acids (*e.g.* diclofop, fluazifop, haloxyfop) or cyclohexane-1,3-diones (*e.g.* sethoxydim, cycloxydim). Both herbicide groups are very effective in many grasses, whereas monocotyledonous plants outside the *Poaceae* and dicotyledonous plants appear to be resistant. Different species of the same genus exhibit different sensitivity towards both herbicide groups, whereby the one is sensitive *(e.g. Poa pratensis)* and the other tolerant *(e.g. Poa annua)* [14]. Certain *Poaceae* are able to develop resistance against diclofop by an increased metabolization of the herbicide in the cytoplasm, whereas the isolated ACC enzyme is sensitive. In oat the hydroxylation product is 5-OH-diclofop, which in barley, however, is found only in small amounts [15]. The ring hydroxylation is catalyzed by a NADPH-cytochrome *c* (P450) reductase [16]. 5-OH-diclofop is much less phytotoxic than diclofop itself [10].

In this paper we have tried to separate the three biotin-containing carboxylases ACC, PCC and MCC of plants and to test whether all of them are sensitive to the ACC inhibitors such as cycloxydim, sethoxydim and the aryloxyphenoxypropionic acid derivative diclofop. Furthermore we determined the I_{50}-values for cycloxydim and diclofop in chloroplasts of different varieties of the tolerant plant *Festuca rubra* in order to see whether the ACC tolerance was variable or constant within one plant species.

Materials and Methods

Plant growth

Barley (*Hordeum vulgare* L.) was grown on a mineral-containing peat (TKS II) in the light for 7 days and then kept for 8 days in the dark for the assay of MCC activity. For ACC purification barley was grown for 5 days in the dark. Maize (*Zea mays* L. var. Mona), lentil (*Lens culinaris* L.) and pea (*Pisum sativum* L. var. Lisa) were grown for about 10–12 days in the light.

For enzyme isolation and developmental studies the seedlings were cut above the ground and either analyzed directly or the different leaves were carefully separated including the leaf base and separately analyzed.

Purification of the ACC

The purification of ACC from barley leaf homogenates [20] was performed using different HPLC techniques (Table I). An essential step in this chromatographic purification was an anionic ion change chromatography of protein fractions precipitated by ammonium sulfate (0–40%) using a special chromatographic material (Fractogel, Merck, Germany). A second chromatographic step was dye ligand HPLC chromatography (Fractogel TSK AF-Red A, Merck, Germany) [17]. Thereafter the ACC enzyme was further purified using a self-fabricated affinity column with the immobilized herbicide diclofop bound to an agarose matrix, which is proved to be very specific for the binding of the ACC. The elution was performed using buffer containing 200–250 mM KCl.

Assay for carboxylase activity

Assays of the different carboxylase activities were performed by measuring the heat and acid-stable incorporation of [^{14}C]hydrogen carbonate into one of the offered thioester (acetyl-CoA, propionyl-CoA, methylcrotonyl-CoA). Enzyme fractions from leaf material of young lentil, pea, barley and maize seedlings were prepared by ammonium sulfate fractioning (0–40% precipitation) and

partly chromatographic separation on an anionic exchange column (Fractogel, Merck) as described [10].

Determination of different kinetic constants and inhibition studies for ACC and PCC was performed with a post anion exchange chromatographic fraction, which represents an acceptable combination of ACC purity and stability. The applied herbicides were gifts from different firms: diclofop from Hoechst GmbH, Germany, cycloxydim from BASF AG, Germany, and sethoxydim from Nippon Soda AG, Japan.

Results and Discussion

Studies on the enzyme activities of ACC, PCC and MCC in plants

In the course of our investigations on the starting enzymes of *de novo* fatty acid biosynthesis we purified ACC from barley to near homogeneity. The enzyme was very unstable and lost its activity very quickly during purification.

During the isolation procedure the crude leaf homogenate was precipitated by $(NH_4)_2SO_4$ addition. The 0–40% fraction contained the ACC and was desalted before loading the fractogel column (= load fractogel) (Table I). The fraction "post fractogel" was concentrated before loading the red dye ligand affinity column (= load red) and the fraction "post red" was desalted and concentrated before loading the diclofop column (= load diclofop). This last HPLC affinity chromatography step with the immobilized diclofop bound to an agarose matrix gave an extremely pure ACC preparation. The ACC from barley leaves was purified 342-fold with a specific activity of 1508 nmol/mg protein · min and a recovery of about 10%.

The purity of this preparation was proven with SDS-PAGE and showed a band at 220 kDa and two smaller bands at about 40 kDa and 50 kDa. Its biotin content was made visible after a Western blot with an extravidin/alkaline phosphatase. Addition of avidin to the enzymic assay inhibited the ACC activity as expected. For the purified ACC preparation we determined the K_m-values as 55 μM (acetyl-CoA), 3.3 mM (HCO_3^-) and 192 μM (ATP/Mg^{2+}). This preparation also exhibited PCC activity, but with different K_m-values as 32 μM (propionyl-CoA), 4.5 mM (HCO_3^-) and 90 μM (ATP/Mg^{2+}). This purified ACC/PCC enzyme preparation did not show any MCC activity.

In attempts to purify the ACC we dried to separate the ACC from the PCC activity which carboxylates propionyl-CoA. This is essential with respect to the question whether the carboxylation of propionyl-CoA is catalyzed by the ACC as a side activity or whether there exists a distinct propionyl-CoA carboxylase (PCC), which may exhibit similar isolation characteristics to the ACC enzyme. PCC activity was always found besides ACC activity in protein fractions from barley, maize, oat and pea. In all chromatographic HPLC systems with barley ACC preparations we could not, however, clearly separate the two carboxylating activities for acetyl-CoA and propionyl-CoA, but found quite different values for the ratios of the ACC/PCC activities (from 1:1 to 3:1) in the different enzyme fractions eluted *e.g.* from anion exchange or dye ligand columns. This variation could be taken as a strong indication of two separate enzymes ACC and PCC, though this is no proof.

In SDS-PAGE gels of the most purified fractions of ACC we still obtained besides the major

Table I. Purification of the ACC enzyme from 5 d old etiolated barley seedlings using HPLC with special anion exchange and affinity chromatography columns.

Fractions	Specific activity [nmol/mg and min]	Purification factor	Total activity [nmol/min]	% Recovery	Total protein [mg]
Homogenate	4.44	1	6566	100	1490
Load fractogel*	16.8	3.8	3119	47.5	186
Load red**	60	13.7	1393	21.2	23
Load diclofop***	690	156	1289	19.6	1.9
Active fraction	1508	342	600	10.2	0.45

* Anion exchange chromatography column.
** Dye ligand affinity chromatography column.
*** A special self-fabricated herbicide-containing affinity chromatography column.

high molecular band (*ca.* 220 kDa), two further and minor biotin-containing protein bands at about 40 and 50 kDa, which might originate from PCC. These two minor bands appear not to be identical to endogenous or trypsin-induced breakdown products of ACC, which showed up near 60 kDa in senescent plants or after a trypsin treatment of ACC. The PCC activity in the purified ACC preparations could thus be either a side activity of ACC or derive from an additional single PCC enzyme. A possible explanation for the difficulties in separating the two enzyme activities might be that a possible separate PCC enzyme might have a very similar chromatographic behaviour to the ACC, due to a parallel evolutionary development of both enzymes according to the theory of Lynen [18]. An enzyme preparation from parsley, in which ACC was purified to apparent homogeneity, showed propionyl-CoA carboxylating activity at 60% and butyryl-CoA carboxylating activity at 15% of the ACC activity [19]. The different developmental activity pattern for ACC and PCC [20] and the varying ratio of the ACC/PCC activities found in our investigation during development would support the theory of two separate enzymes.

In contrast, a third carboxylase activity, the methylcrotonyl-CoA carboxylase (MCC), is a clearly distinguishable separate biotin enzyme [21]. It had no activity for carboxylating either acetyl-CoA or propionyl-CoA, as was tested here. On the other hand, the purified ACC preparations (Table I) with PCC activity did not possess any activity for the carboxylation of methylcrotonyl-CoA. MCC activity does not coincide with the ACC activity on anion exchange and dye ligand columns and can clearly be separated from the ACC/PCC activities since it is eluted earlier. In preparations with ACC and PCC activity from parsley no methylcrotonyl-CoA carboxylation capacity was detected either [19]. MCC activity seems to exhibit a broad distribution in plants. In the shoots of seedlings of maize, oat, pea and *Poa annua* all three carboxylase activities ACC, PCC and MCC were present, as well as in roots of maize. Only in young etiolated barley seedlings could we not find any MCC activity, but in light-grown barley placed into a longer dark phase of several days it was induced (Fig. 1).

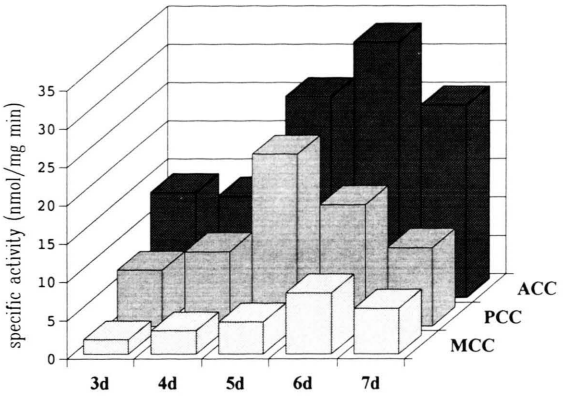

Fig. 1. Development of the activities of the biotin carboxylases ACC, PCC and MCC in the shoots of 5 d old light-grown barley seedlings during a subsequent dark period of several days.

The MCC activity in barley exhibited a quite different developmental pattern than the ACC activity. The latter is predominantly present in very young leaf tissue and then declines (Fig. 2), whereas the MCC exhibited its highest activity in older leaf tissue (Fig. 3). The PCC activity followed the activity pattern of the ACC, but was always lower than that of ACC. In contrast to ACC and PCC, the subcellular localization of MCC seems to be different. In isolated chloroplasts from barley plants no MCC activity was detectable, whereas ACC as well as PCC activity were measured. Further differential centrifugation steps at higher *g*-values gave evidence to the mitochondrial locali-

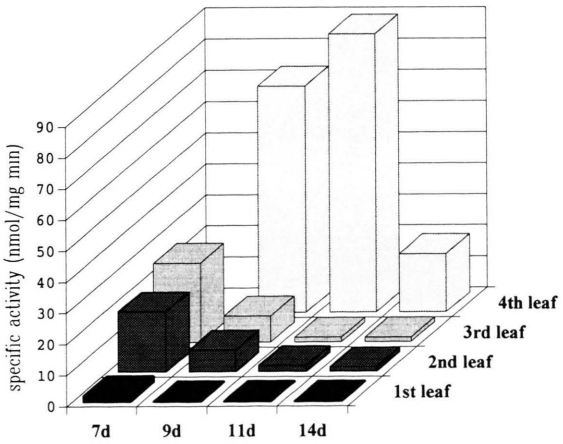

Fig. 2. Activity of the acetyl-CoA carboxylase ACC in different leaves of maize seedlings with increasing age.

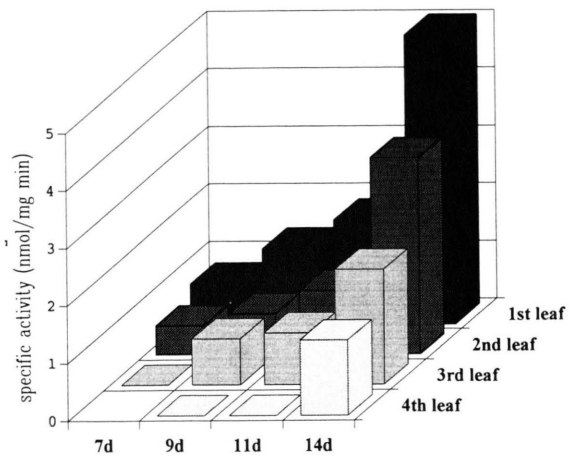

Fig. 3. Activity of the methylcrotonyl-CoA carboxylase MCC in different leaves of maize seedlings with increasing age.

zation of the plant MCC. The mitochondrial localization of the barley MCC observed by us is now confirmed for the MCC of pea [5] in an independent work. In animal tissue (bovine kidney) the MCC is also bound to mitochondria [22].

Studies on the inhibition of biotin carboxylases by the ACC inhibitors

The herbicides diclofop, cycloxydim and sethoxydim inhibit not only the ACC but also the PCC from barley in a dose-dependent manner with I_{50}-values for diclofop of 0.64 μM (ACC) and 0.63 μM (PCC activity), for cycloxydim of 4.8 μM (ACC) and 2.3 μM (PCC activity) and for sethoxydim of 5.6 μM (ACC) and 3.5 μM (PCC). In any case, the results indicate that with the aryloxyphenoxypropionic acid type herbicides (diclofop) and the cyclohexane-1,3-dione derivatives cycloxydim and sethoxydim both the ACC and PCC activities are inhibited. This demonstrates that these graminicides block not only *de novo* fatty acid biosynthesis in the sensitive plants, but also the PCC function. The latter may lie – as in animals – in the degradation of amino acids (valine, isoleucine, methionine) and odd-numbered fatty acids. The block of such PCC activities could contribute to the efficiency of the graminicides as herbicides in the sensitive plants.

The ACC from barley is equally sensitive to both kinds of herbicides, but there are also *Poaceae* that show medium or total tolerance to one or both herbicides. So is the ACC enzyme from the sensitive species *Festuca arundinacea* and *Poa pratensis* inhibited by cycloxydim with I_{50}-values of 1.7 μM and 2.6 μM, respectively, whereas *Festuca rubra* and *Poa annua,* which are tolerant species, have I_{50}-values of 300 μM and 2200 μM (Table II). These results on the ACC enzyme level also explain the tolerance of the intact plants towards cycloxydim.

In contrast, in the case of diclofop the differences on the ACC enzyme level between sensitive and tolerant species are not as large as for cycloxydim (Table II). The diclofop resistance, however, is primarily based on other mechanisms than the mutation of the target enzyme ACC. In tolerant *Poaceae* the herbicide diclofop is metabolized by mechanisms in the cytoplasm, such as hydroxylation and glycosylation [23, 24], whereas the isolated enzyme is still sensitive against diclofop. Differences in sensitivity towards herbicides exist not only in different species, but can also be observed in different varieties of the same species. There are varieties of *Festuca rubra* that show significant differences in their I_{50}-values for *de novo* fatty acid biosynthesis of isolated chloroplasts against both, cycloxydim and diclofop (Table III). The I_{50}-values for cycloxydim were much higher than those for diclofop. This was to be expected since the tolerance towards cycloxydim appears to be a mutation of the target enzyme only, whereas the tolerance of whole *Poaceae* plants towards diclofop primarily lies in the metabolization of diclofop in the cytoplasm. Those *Festuca rubra* varieties, which possessed the higher I_{50}-values for cycloxydim, simultaneously exhibited also higher I_{50}-values for diclofop. This indicates 1) a certain cross tolerance between cycloxydim and diclofop, though the major tolerance mechanisms are differ-

Table II. I_{50}-Values (μM) of herbicides for the inhibition of the ACC enzyme from different sensitive and tolerant plants.

Species	Cycloxydim	Diclofop
Festuca arundinacea (sensitive)	1.7	1.2
Festuca rubra (tolerant)	300	2.2
Factor	176 ×	1.8 ×
Poa pratensis (sensitive)	2.6	1.2
Poa annua (tolerant)	2200	6.9
Factor	846 ×	5.8 ×
Barley	4.8	0.64

Table III. I_{50}-Values of inhibition of *de novo* fatty acid biosynthesis in chloroplasts of several *Festuca rubra* varieties by the herbicides cycloxydim and diclofop.

Species, varieties	Incorporation rate* (Controls)	I_{50}-Values (μM)** Cycloxydim	Diclofop
Festuca rubra rubra (with long runners)			
var. Roland 21	109 ± 29	130	0.4
var. Robin	115 ± 34	360	0.7
var. Condor	71 ± 15	210	0.2
var. Pennlawn	16 ± 3	140	0.2
Festuca rubra trichophylla (short runners)			
var. Noro	41 ± 7	1000	2.1
Festuca rubra commutata (nest forming)			
var. Milan	92 ± 23	2400	3.3
var. W 539	119 ± 10	500	0.7
var. Rasengold	72 ± 23	290	0.6

* The [^{14}C]acetate incorporation rates in total fatty acids is given in nmol · mg chlorophylls $(a + b)^{-1} \cdot h^{-1}$.

** Mean of 6 determinations from 2 isolations with standard deviation of 10% or less.

ent, and 2) that the modification of the target enzyme ACC for higher tolerance against cycloxydim also causes a lower sensitivity towards diclofop.

These differences in sensitivity of particular grass genera, species and varieties towards cycloxydim appears to be due to a modification of the binding domain in the ACC peptide in medium and highly tolerant plants in such a way that the herbicide can no longer bind. It was proposed that there are two or more common binding points in the binding niche on the ACC molecule for the two herbicide groups cycloxydim and diclofop [13], whereby at least two lie in a direct neighbourhood [25] and at least one is shared by both herbicide groups [13, 26]. In view of the results of Table III, the question arises whether the large range of tolerance with I_{50}-values of cycloxydim from 140 to 2400 for the *Festuca rubra* varieties may all be due to differential mutations and submutations of the ACC target enzyme or whether other mechanisms, at least in part, may be responsible for these differences. Though the idea of submutations within the varieties of a medium-tolerant *F. rubra* species appears to be quite possible, another mechanism such as rate of herbicide uptake through the chloroplast envelope might also be a cause for these differences in I_{50}-values of isolated chloroplasts. In our previous work with many different *Poaceae,* the differences in the I_{50}-values found for cycloxydim or sethoxydim on the level of isolated chloroplasts, were, however, always confirmed in similar ways on the level of the isolated ACC enzyme.

In newer publications [27, 28] the sensitivity of membrane depolarization for diclofop lies within the same rate as the I_{50}-values of ACC from various sources (Table II), whereas in the original papers of Shimabukuro on diclofop [29, 30] and for sethoxydim [31] much higher concentrations were required. It is possible to correlate the depolarization and repolarization phenomenon with the sensitivity and resistance of plants towards diclofop and sethoxydim. The altered membrane potential is characterized by a raised K^+/Na^+ ratio in resistant plants as compared with sensitive plants. The authors assume that the ability of membrane repolarization in tolerant species can but must not necessarily be linked with a herbicide insensitive ACC [27, 28]. It is matter of future research whether changes of a membrane potential can occur at the chloroplast envelope or whether such changes are only restricted to the plasma

membrane. In any case, the analysis of the ACC genes of sensitive and tolerant plants, at present in progress in several laboratories, will answer in future many of the open questions on the resistance mechanisms towards the graminicides.

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