The Very-Long-Chain Fatty Acid Synthase Is Inhibited by Chloroacetamides

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The first elongation step to form very-long-chain fatty acids (VLCFAs) is catalyzed by the VLCFA-synthase. CoA-activated fatty acids react with malonyl-CoA to condense a C2-unit. As shown with recombinant enzyme this reaction is specifically inhibited by chloroacetamide herbicides. The inhibition is alleviated when the inhibitor (*e.g.* metazachlor) is incubated together with adequate concentrations of the substrate (*e.g.* oleoyl-CoA). Malonyl-CoA has no influence. However, once a chloroacetamide has been tightly bound to the synthase after an appropriate time it cannot be displaced anymore by the substrate. In contrast, oleoyl-CoA, is easily removed from the synthase by metazachlor. The irreversible binding of the chloroacetamides and their competition with the substrate explains the very low half-inhibition values of 10^{-8} M and below. Chiral chloroacetamides like metolachlor or dimethenamid give identical results. However, only the (*S*)-enantiomers are active.

Key words: Fatty Acid Elongation, Recombinant VLCFA-Synthase, Tight-Inhibitor Binding

Introduction

Since several decades chloroacetamides have been used for weed control in maize, soybean or rice. They are still going strong and are expected to contribute a significant share in future weed control. They are typical preemergent herbicides, active in the plant in very low concentrations, and weed resistance is very rare. Although these herbicides belong to the "classics" their specific target has only been found in recent years. The elongation of very-long-chain fatty acids (VLCFAs) is specifically inhibited (Matthes *et al.*, 1998). A majority of VLCFAs is located in the plasma membrane. When absent the membrane loses stability and becomes leaky leading to death of the herbicide-treated plant (Matthes and Böger, 2002). Fatty acids up to C18-chains are formed in the plastid while those with C > 18 are synthesized in the endoplasmic reticulum of the cytosol. CoA-activated C18- or C20-acyl substrates react with malonyl-CoA yielding elongation with a C2-unit. Four clustered enzymes are required for each elongation step as shown by Fig. 1. The first enzyme is the VLCFA-synthase, a condensing enzyme essentially depending on a reactive cysteinyl sulfur in its reaction center (Ghanevati and Jaworski, 2002) which should allow for a nucleophilic attack of the enzyme towards chloroacetamides (Böger *et al.*, 2000). Using yeast transformed with the FAE1-gene (from *Arabidopsis*) we got evidence that the VLCFA-synthase, catalyzing the reaction of malonyl-CoA with the activated substrate, is inhibited by chloroacetamides and functionally related compounds (Böger, 2003). This paper will illustrate this finding with a cell-free assay system in more detail.

Abbreviations: dimethenamid: 2-chloro-*N*-(2,4-dimethyl-3-thienyl)-*N*-(2-methoxy-1-methylethyl)acetamide; metazachlor: 2-chloro-*N*-(pyrazol-1-ylmethyl)aceto-2′,6′-xylidide; metolachlor: 2-chloro-6′-ethyl-*N*-(2-methoxy-1-methylethyl)aceto-2′-toluidide; for structures see Tomlin (2000).

Materials and Methods

Organism

Yeast (*Saccharomyces cerevisiae*) has been used after its transformation with the plasmid pYES2 (from Invitrogen, Karlsruhe) including the VLCFA-synthase gene FAE1 from *Arabidopsis thaliana* under control of a galactose-inducible promoter (Millar and Kunst, 1997). The transformed cells were selected on uracil-free medium.

Cultivation and homogenisation

A 20 ml-preculture (grown for 8 h at 28 °C) was added to FAE-medium adjusted to pH 6.0 (1.7 g/l yeast nitrogen base; 1.9 g/l yeast minimal dropout uracil; 5 g/l ammonium sulfate; 20 g/l galactose),

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and cultivated in 2 l-baffle flasks at 28 °C for about 40 h up to an optical density of 2 at 660 nm. The washed pellet was suspended in an appropriate homogenisation medium [80 mM Hepes (*N*-2-hydroxylethylpiperazine-*N'*-ethanesulfonic acid), pH 7.2, 320 mM sucrose, 5 mM EDTA, 1 mM dithiothreitol (DTT), 10 mM KCl]. Homogenisation was achieved by 0.5 mm glass beads in a rotary homogenizer under cooling with solid carbon dioxide (model Merkenschlager; Braun Comp., Melsungen, Germany). After removal of the glass beads the homogenate was centrifuged for 20 min at 10,000 × *g*, and the supernatant was centrifuged again at 100,000 × *g* for 1 h. The microsomal pellet thus obtained was suspended in a cold mixture of 80 mM Hepes, pH 7.2, 1 mM DTT, 1 mM $MgCl_2$ and 15% glycerol. For the FAE-assay aliquots of 10 μg microsomal protein/μl were stored and frozen in liquid nitrogen.

FAE-assay

5 μl 1 mM malonyl-CoA, 1 μl 127 μM ^{14}C-labeled C18:1-CoA (0.26 MBq/ml), 1 μl FAE-enzyme sample of above (= 10 μg protein) and 43 μl assay medium (80 mM Hepes, pH 7.2, 2 mM EDTA, 1 mM $MgCl_2$, 0.1% Triton X100) were mixed on ice. If not mentioned otherwise the assay mixture was incubated at 30 °C for 30 min. The amount of substrate of the control was converted within 5 min with a linear rate of about 3 μmol product per mg protein and min. Subsequently, free fatty acids were obtained after addition of 20 μl 50% KOH and heating at 60 °C for 30 min.

The acids were partitioned into a mixture of 350 μl 2 N HCl, 400 μl acetone, 300 μl hexane and 10 μl Ponceau Red. The hexane phase was evaporated under nitrogen. The acids were separated by TLC (Merck, RP-18 F_{254S}) with acetonitrile/acetic acid (99:1, v/v). Radioactivity in the spots was visualized by X-ray film using the Image-Quant program.

Results and Discussion

We did not succeed to purify the VLCFA-synthase from a plant source (like *Allium porrum*) without enzyme inactivation. Our previous reports on cell-free inhibition of VLCFA-formation related to the elongase activity that is the enzyme cluster including the four enzymes as shown in Fig. 1. Wild-type yeast is not able to synthesize C20–C22 or C24 VLCFAs, but the FAE1-trans-

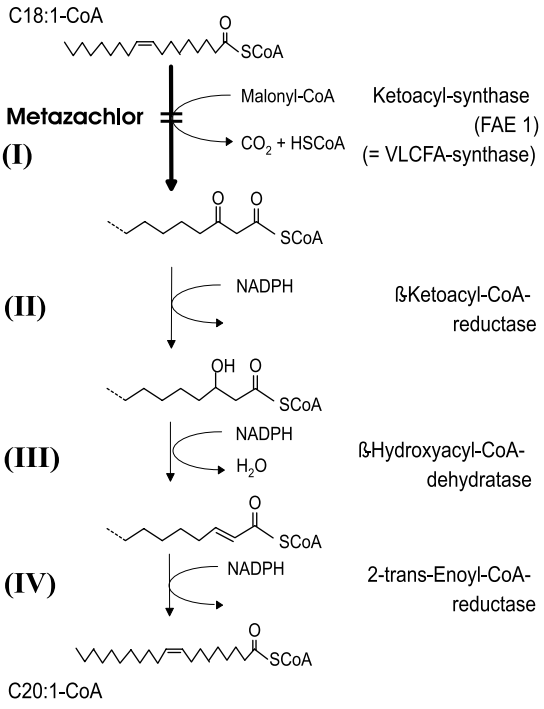

Fig. 1. A 4-step reaction sequence produces the elongated fatty acid. Acyl-CoA (here C18:1-CoA) reacts with malonyl-CoA (step I) with subsequent reduction, dehydratization and reduction (steps II, III, IV). The four clustered enzymes represent the elongase system. The VLCFA-synthase alone is inhibited by chloroacetamides and functionally related agrochemicals. The ketoacyl produced by step I is detected by the assay used in this study.

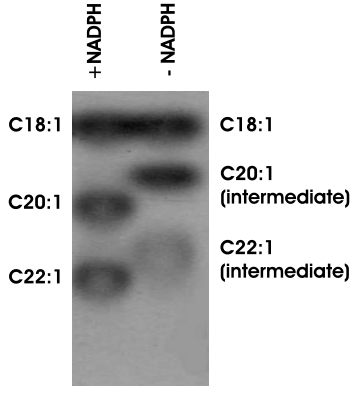

Fig. 2. Removal of NADPH from the assay mixture allows for the detection of the C20:1 and C22:1 ketoacyl products of step I (see Fig. 1) when oleoyl-CoA (= C18:1-CoA) is used as substrate. Due to their higher polarity the ketoacyl intermediates move faster on thin-layer than the reduced C20:1- and C22:1-fatty acids. The small formation of C24:1 fatty acid by FAE1-transformed *intact* yeast (Böger, 2003) is not observed with the cell-free system and the assay used here.

formant can do so (Böger, 2003). Obviously, the two reductases and the dehydratase (steps II, III, IV of Fig. 1) are present in the yeast for biosynthesis of "normal" < C18 fatty acids. In the transformant they are used to process the reaction products of the synthase (step I). With our TLC-detection system we could identify the cell-free formation of C20:1 and C22:1 keto intermediates when no NADPH was present in the assay (Fig. 2, right lane). NADPH (but not NADH) had to be included in the assay to produce the free C20- and C22-fatty acids (Fig. 2, left lane). The (oxidized) labeled condensation products were found stable and were detected in the subsequent inhibition assays. Their formation was inhibited by chloroacetamides. This finding excludes a putative inhibition of steps II–IV. Chloroacetamides like metazachlor or dimethenamid showed their inhibitory activity after a pre-incubation of 30 min or longer with the FAE1-synthase alone (Fig. 3, top). A similar lag phase of inhibition was also observed with the complete assay mixture (Fig. 3, bottom). Using moderate concentrations of metazachlor oleoyl-CoA could protect the enzyme against inhibition in a concentration-dependent manner (Fig. 4, top) while malonyl-CoA had no effect. Apparently, substrate and inhibitor compete for the same target domain. As shown in Table I inhibitor activity was reciprocal to the enzyme concentration.

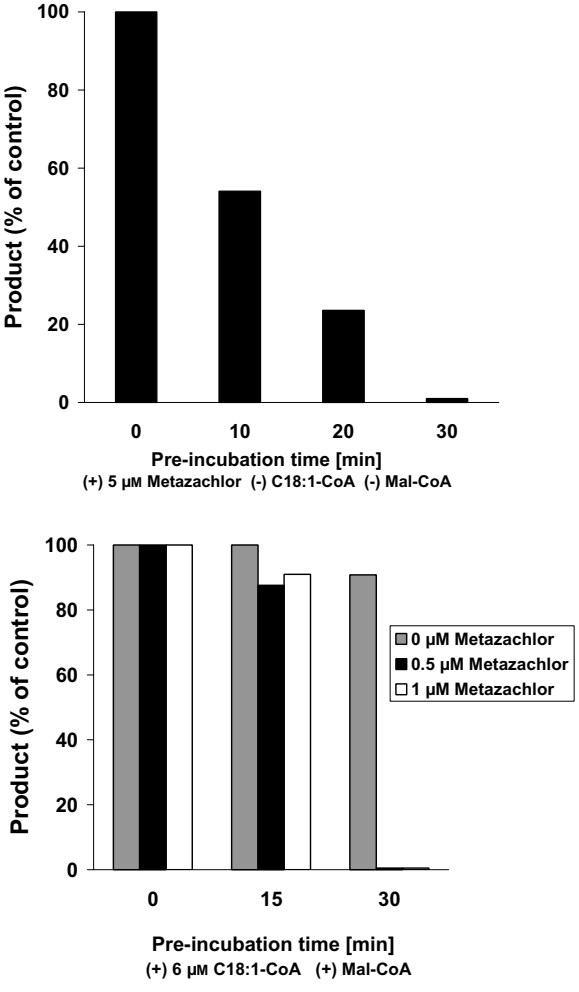

Fig. 3. Lag phase of inhibition. A 30-min pre-incubation of the VLCFA-synthase is required for complete irreversible inhibition. This is observed with the buffered enzyme alone (upper part) as well as with the complete assay mixture (lower part). Note that 0.5 or 1 μM metazachlor produce complete inhibition.

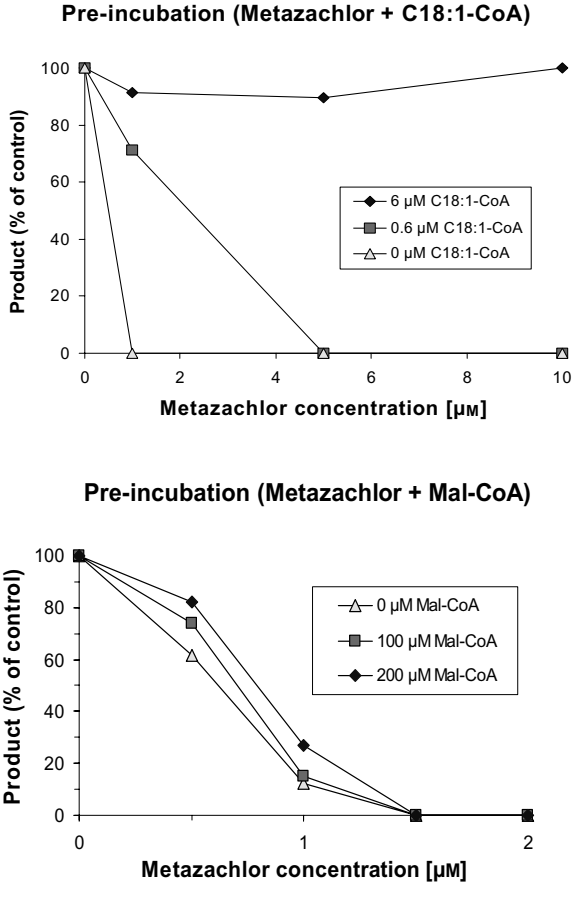

Fig. 4. Protection of the enzyme against metazachlor inhibition by higher concentrations of acyl-CoA substrates (upper part), while malonyl-CoA has no effect (lower part).

Table I. Inhibition by metazachlor relating to concentration of VLCFA-synthase (protein).

Metazachlor [μM]	5 μg Protein	10 μg Protein	20 μg Protein
0	88.6*	146.2	127.4
0.5	73	120.4	124.7
1	9.5	19.1	23.5
1.5	0	0	23.2
2	0	0	0

* Data in pmol product formed/30 min.

Table II. Part A: Increasing metazachlor concentrations outcompete the protection by substrates, like oleoyl-CoA. The enzyme was first pre-incubated for 30 min with oleoyl-CoA (1), then with metazachlor (2). Part B: Pre-incubation (1) with metazachlor and subsequent incubation with acyl-CoA (2) did not alleviate inhibition.

Pre-incubation 1	Pre-incubation 2	Product formation (% of control)
A	no metazachlor	100.0
	1 μM metazachlor	83.1
	2 μM metazachlor	68.6
6 μM C18:1-CoA	5 μM metazachlor	33.0
	10 μM metazachlor	31.1
	25 μM metazachlor	0
B	6 μMC18:1-CoA	0
2 μM metazachlor	32 μM C18:1-CoA	0
	6 μM C18:1-CoA	0
5 μM metazachlor	32 μM C18:1-CoA	0

The data allow the conclusion that the enzyme titrates off the inhibitor in a stoichiometric manner. The inhibition data of Table II demonstrate that the protection by *e.g.* oleoyl-CoA was impaired by increasing metazachlor concentrations (upper part). Once metazachlor has been tightly bound after a 30-min pre-incubation time, its inhibition could not be alleviated by oleoyl-CoA (Table II, lower part). Together with the observed lag phase of inhibition (Fig. 3) a tight irreversible binding between inhibitor and enzyme is evident as was already hypothesized previously (Böger *et al.*, 2000). This explains the very low I_{50}-values obtained with intact plants (see *e.g.* Fuerst *et al.*, 1991; Couderchet *et al.*, 1994, 1997). As known from previous studies with intact plants (Couderchet *et al.*, 1997) and from elongase assays (with *Allium porrum* homogenates; Schmalfuss *et al.*, 2000) only the (*S*)-enantiomers of chiral inhibitors were active. Only they show herbicidal activity in the field. Fig. 5 demonstrates the activity of both (*S*)-metolachlor and (*S*)-dimethenamid on the recombinant FAE1-synthase. A similar lag phase of inhibition was observed together with the findings demonstrated by Fig. 4 and Table I.

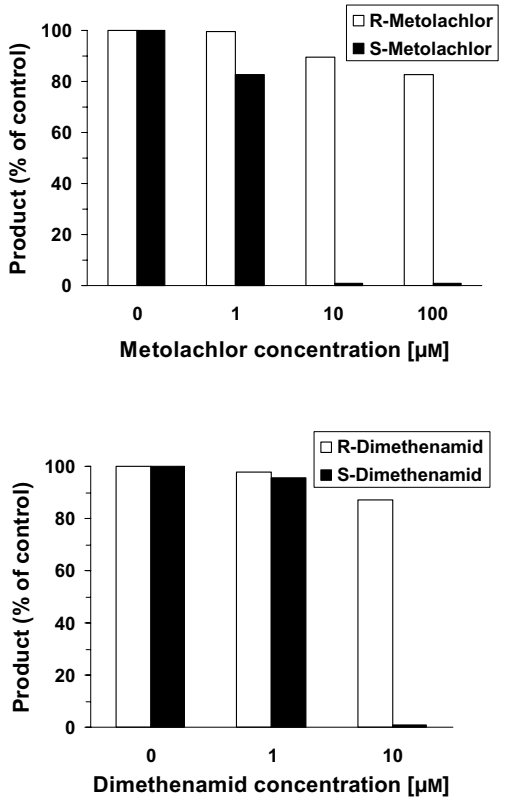

Fig. 5. Chirality and inhibition. Only the (*S*)-enantiomers of metolachlor or dimethenamid are inhibitors for the VLCFA-FAE1 synthase. The small inhibition observed with higher concentrations of the (*R*)-form is due to minor impurities with the (*S*)-enantiomer.

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