

Decarboxylation of malonyl-(acyl carrier protein) by 3-oxoacyl-(acyl carrier protein) synthases in plant fatty acid biosynthesis

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In order to identify regulatory steps in fatty acid biosynthesis, the influence of intermediate 3-oxoacyl-(acyl carrier proteins) (3-oxoacyl-ACPs) and end-product acyl-ACPs of the fatty acid synthase reaction on the condensation reaction was investigated *in vitro*, using total fatty acid synthase preparations and purified 3-oxoacyl-ACP synthases (KASs; EC 2.3.1.41) from *Cuphea lanceolata* seeds. KAS I and II in the fatty acid synthase preparations were assayed for the elongation of octanoyl- and hexadecanoyl-ACP respectively, and the accumulation of the corresponding condensation product 3-oxoacyl-ACP was studied by modulating the content of the reducing equivalents NADH and NADPH. Complete omission of reducing equivalents resulted with either KAS in the abnormal synthesis of acetyl-ACP

from malonyl-ACP by a decarboxylation reaction. Supplementation with NADPH or NADH, separately or in combination with recombinant 3-oxoacyl-ACP reductase (EC 1.1.1.100), led to a decrease in the amount of acetyl-ACP and a simultaneous increase in elongation products. This demonstrates that the accumulation of 3-oxoacyl-ACP inhibits the condensation reaction on the one hand, and induces the decarboxylation of malonyl-ACP on the other. By carrying out similar experiments with purified enzymes, this decarboxylation was attributed to the action of KAS. Our data point to a regulatory mechanism for the degradation of malonyl-ACP in plants which is activated by the accumulation of the fatty acid synthase intermediate 3-oxoacyl-ACP.

INTRODUCTION

Plant fatty acid synthase (FAS II) has been described as a plastidic multienzyme complex composed of dissociable enzymes and a central acyl carrier protein (ACP). Starting from acetyl-CoA and malonyl-ACP, an acyl chain is progressively extended by two carbons. This pathway involves a condensation reaction producing a 3-oxoacyl-ACP, catalysed by 3-oxoacyl-ACP synthases (KASs; EC 2.3.1.41; also known as condensing enzymes), and the subsequent removal (reduction) of the 3-oxo group by the sequential action of a 3-oxoacyl-ACP reductase (KR; EC 1.1.1.100), a 3-hydroxyacyl-ACP dehydratase (EC 4.2.1.58–61) and an enoyl-ACP reductase (EC 1.3.1.9–10). This reduction process requires NADH and NADPH as reducing equivalents. The product of each extension cycle (FAS cycle) is a saturated acyl-ACP, which is the substrate for condensation with malonyl-ACP in the next cycle. For the synthesis of storage triacylglycerols in oil seeds, acyl-ACPs (mostly C₁₆ and C₁₈) are finally hydrolysed by acyl-ACP thioesterase (EC 3.1.2.14) and the resulting non-esterified fatty acids are exported into the cytoplasm.

Condensing enzymes are pivotal in this pathway, since they catalyse the reaction that is actually responsible for chain extension. KAS enzymes are distinguished by their acyl-chain specificity and their sensitivity to inhibitors. It is accepted that KAS I is highly cerulenin-sensitive (IC₅₀ 2 µM) and elongates acyl-ACPs ranging from C₂ to C₁₄, whereas KAS II is less sensitive (IC₅₀ 50 µM) and catalyses the elongation of C₁₆ to C₁₈ [1–3]. KAS III is cerulenin-insensitive and catalyses the condensation reaction of acetyl-CoA and malonyl-ACP [4–6], thus initiating fatty acid synthesis. Although there has been some controversy as to the substrate specificity of KAS III, the purified enzyme can extend only acetyl-CoA [4]. We showed recently [7] that KAS III uses exclusively acyl-CoAs as substrate and that the extension of short-chain acyl-ACPs, observed previously in

extracts treated with cerulenin [4,6], is the consequence of the action of a new condensing enzyme (KAS IV).

Although our knowledge of the enzymes and reactions involved in plastidic fatty acid synthesis has grown in the last few years, no data concerning the organization of the FAS multienzyme complex are available, and little is known about the mechanisms regulating this biosynthetic pathway. The role of end-products in this regulation has been studied recently in some laboratories. By investigating fatty acid biosynthesis in tobacco suspension cells [8], it has been verified that exogenously added oleic acid, in the form of oleoyl-Tween, causes a decrease in the synthesis of long-chain acyl-ACPs, which was suggested to be a consequence of a feedback control on the acetyl-CoA carboxylase (EC 6.4.1.2) reaction. By studying the synthesis of medium-chain fatty acids in *Cuphea lanceolata* seeds (which accumulate up to 90% decanoic acid in storage triacylglycerols), we demonstrated that decanoyl-ACP strongly inhibits KAS III, indicating that the first condensation step in the FAS reaction is feedback-regulated by the end-product [7]. Finally, Heath and Rock [9], working with *Escherichia coli* mutants also characterized by a FAS II complex, proposed a feedback regulatory loop that recycles malonyl-ACP to acetyl-CoA involving the malonyl-ACP decarboxylase reaction of KAS I and II. This loop is activated by inhibition of phospholipid synthesis, with concomitant down-regulation of fatty acid biosynthesis, and is dependent on the resulting accumulation of the long-chain acyl-ACP.

Our studies on the synthesis of fatty acids in *C. lanceolata* are aimed at the identification of regulatory mechanisms at the level of condensation reactions. In the present paper we elaborate *in vitro* conditions for investigation of the reactions catalysed by KAS I and II and the subsequent reduction of the 3-oxo group catalysed by KR, in order to verify the influence of the intermediate 3-oxoacyl-ACP as well as the product acyl-ACP on the condensation reaction.

Abbreviations used: ACP, acyl carrier protein; ecACP, *Escherichia coli* ACP; FAS, fatty acid synthase; KR, 3-oxoacyl-ACP reductase; KAS, 3-oxoacyl-ACP synthase; MAT, malonyl-CoA:ACP transacylase; NEM-extract, *N*-ethylmaleimide-treated FAS preparation from *Cuphea lanceolata* seeds.

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MATERIALS AND METHODS

Plant material

Seed material of *Cuphea lanceolata* was obtained from the experimental fields of the Department of Agronomy and Plant Breeding, University of Göttingen, Germany. Developing seeds were harvested 8–24 days post-pollination, frozen in liquid nitrogen and stored at -70°C until use.

Chemicals

[2- ^{14}C]Malonyl-CoA (1.85 GBq/mmol) and [1- ^{14}C]octanoic acid (0.16 GBq/mmol) were obtained from Amersham (Braunschweig, Germany). Enzymes were assayed by using a solution of 72 kBq of [2- ^{14}C]malonyl-CoA in 200 μM malonyl-CoA. Insoluble polyvinylpyrrolidone (Polyclar AT) was from Serva (Heidelberg, Germany). Cerulenin (stored as a 2 mM aqueous stock solution, pH 4, at 4°C), malonyl-CoA, NADH and NADPH were from Sigma (Deisenhofen, Germany). All other reagents were of analytical grade or better.

ACP and acyl-ACPs

Escherichia coli ACP (ecACP) was purchased from Sigma and further purified by anion-exchange chromatography on Mono Q, as described [10]. Acyl-ACPs were prepared chemically from ecACP and fatty acids by the method of Cronan and Klages [11]. The concentration and purity of the acyl-ACP stock solutions were determined by 2.5 M urea/PAGE, as described by Brück et al. [12].

Malonyl-CoA:ACP transacylase (MAT) and recombinant KR

MAT (EC 2.3.1.39) from *C. lanceolata* seeds was purified as described by Brück et al. [12]. The preparation obtained contained 0.1 mg/ml protein with an activity of 2.22 nkat/mg. An affinity-purified glutathione S-transferase fusion protein of KR from *C. lanceolata* [13] was used. The recombinant KR solution contained 0.1 mg/ml protein with an activity of 3.0 mkat/mg.

Extraction of FAS enzymes

A crude extract was prepared from 150 mg of frozen seeds of *C. lanceolata* using insoluble polyvinylpyrrolidone (1.5 g/g of seed), as described by Kopka et al. [10]. This crude extract was adjusted to 40% saturation with solid ammonium sulphate, stirred for 1 h at 4°C and centrifuged at 18000 g for 30 min. The supernatant was then adjusted to 65% saturation with solid ammonium sulphate, stirred and centrifuged as above. The resulting 40–65% ammonium sulphate precipitate containing FAS enzymes was stored at -70°C . For enzyme assays, an aliquot of this precipitate was dissolved in 100 mM potassium phosphate (pH 7.6), providing a FAS preparation containing 3.9 mg/ml protein, as determined by the method of Bradford [14] with ovalbumin as standard but using an adaptation for a microplate reader [15].

Purification of KAS enzymes

The 40–65% ammonium sulphate precipitate was taken up in 50 mM potassium phosphate (pH 7.6), 2 mM dithiothreitol and 10% (w/v) glycerol (buffer A) and loaded on to a 2.6 cm \times 90 cm column of Sephacryl S-200 HR (Pharmacia, Freiburg, Germany) equilibrated with the same buffer. The column was operated at 4°C with a flow rate of 2 ml/min. Fractions containing KAS

activity were pooled and fractionated by anion-exchange chromatography on a 1.5 cm \times 10 cm column of Q-Sepharose Fast Flow (Pharmacia) equilibrated with 20 mM potassium phosphate (pH 7.6), 2 mM dithiothreitol and 10% (w/v) glycerol (buffer B). Elution was performed at room temperature by running a linear salt gradient (0–1 M NaCl). Active fractions were adjusted to 1.7 M ammonium sulphate and then subjected to hydrophobic interaction chromatography on a phenyl-Superose HR 5/5 column (Pharmacia) equilibrated with buffer A containing 1.7 M ammonium sulphate. Proteins were eluted at room temperature in a linear gradient from 1.7 to 0 M ammonium sulphate. Fractions containing KAS activity were pooled and loaded on to a 2.6 cm \times 90 cm column of Sephacryl S-100 (Pharmacia) equilibrated with buffer A. Protein elution was performed at 4°C with a flow rate of 2 ml/min. The active fractions were diluted to 20 mM potassium phosphate and then subjected to anion-exchange chromatography on a Mono Q HR 5/5 (Pharmacia) column equilibrated with buffer B. Proteins were eluted at room temperature with a linear salt gradient from 0 to 0.2 M NaCl. These fractions (2 ml) contained acyl-ACP-specific condensing enzymes (KAS III activity was eliminated in earlier steps), and were stored at -70°C . The method of Bradford was not sensitive enough for quantification of the protein content in these fractions.

Enzyme assays

Condensation reactions were monitored by the incorporation of radioactive malonate from [2- ^{14}C]malonyl-ACP into acyl-ACPs of different chain lengths, using a two-step assay [4]. Whereas KAS I activity was measured with octanoyl-ACP as substrate [16], KAS II activity was detected by the elongation of hexadecanoyl-ACP in the presence of 100 μM cerulenin, since the latter enzyme is only partially inhibited at this cerulenin concentration. In the first assay step, the reaction mixture (50 μl) contained 100 mM potassium phosphate (pH 7.6), 2 mM dithiothreitol, 10 μM ecACP, 5 μM acyl-ACP, 2 μl of MAT preparation, 10 μM [2- ^{14}C]malonyl-CoA and 20% (v/v) FAS preparation or KAS preparation. ecACP was preincubated with dithiothreitol for 15 min at 30°C . The reaction was started by the addition of [2- ^{14}C]malonyl-CoA, which is converted into malonyl-ACP *in situ* by the action of MAT. After an incubation period of 5 min at 30°C , the reaction was stopped by the addition of 5.5 μl of 100% (w/v) trichloroacetic acid while keeping the samples on ice for at least 10 min. Subsequently, the mixture was centrifuged for 5 min at 16000 g, 4°C , and the supernatant was removed. The protein precipitate was washed with 100 μl of 1% (w/v) trichloroacetic acid and centrifuged as above.

In the second step the products of the condensation reaction (3-oxoacyl-ACPs) were enzymically reduced to saturated acyl-ACPs by the method of Brück et al. [7], by adding NEM-extract (a FAS preparation from *C. lanceolata* seeds treated with *N*-ethylmaleimide), 1 mM NADH and 2 mM NADPH. To investigate earlier steps of condensation reactions, NADH or NADPH was added to the first assay step, separately or in combination with 2 μl of recombinant KR solution. For product analysis, radioactive acyl-ACPs were precipitated as above and dissolved in 50 mM Mes, pH 6.8. Aliquots of this solution were subjected either to liquid scintillation spectrometry using Aquasafe 500 (Amersham-Buchler, Braunschweig, Germany) or to electrophoretic separation by 2.5 or 5.0 M urea/PAGE [17], with subsequent electrotransfer on to Immobilon-P (Millipore, Eschborn, Germany) and visualization by autoradiography.

For monitoring KAS activity during purification, this assay was modified to a single-step assay by supplying NEM-extract

and reducing equivalents directly to the whole reaction mixture. Product acyl-ACPs were precipitated as described, resuspended in 10 M NaOH and hydrolysed at 80 °C for 10 min. Non-esterified fatty acids were acidified with conc. HCl, extracted once with light petroleum (boiling range 60–80 °C; Merck, Darmstadt, Germany) and subjected to liquid scintillation spectrometry using Lipoluma (Baker, Deventer, The Netherlands).

RESULTS

In our investigation of the influence of FAS intermediates and end-products on the condensation reaction, we first used a FAS preparation from *C. lanceolata* seeds to perform a two-step assay in which the condensation of malonyl-ACP with octanoyl-ACP was monitored. In the first step of this assay, reduction equivalents (NADH and NADPH) were omitted. This promotes the accumulation of the condensation product, since under these conditions 3-oxoacyl-ACP could not be reduced to the corresponding acyl-ACP by the reduction enzymes present in the FAS preparation. As 3-oxoacyl-ACPs are highly unstable, resulting in rather diffuse bands on electrophoretic fractionation, a second assay step was performed to reduce these intermediates to stable products. Using the NEM-extract, which contains all enzymes necessary for reduction of the 3-oxo group but no condensing activity, saturated acyl-ACPs extended by two carbons compared with the primer are obtained as stable end-products and can be analysed by urea/PAGE (Figure 1). In the reaction involving [2-¹⁴C]malonyl-ACP and octanoyl-ACP, two other products, identified as acetyl- and butyryl-ACP, were synthesized in parallel to decanoyl-ACP (Figure 1A, lane 1). The

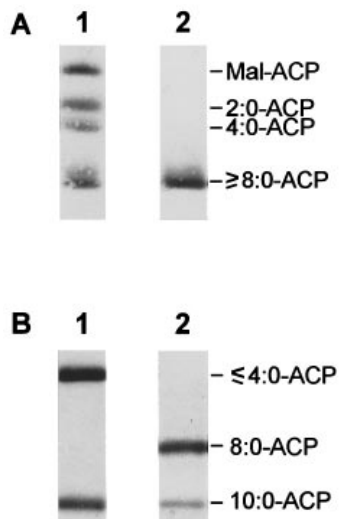


Figure 1 Products of the condensation reactions catalysed by FAS preparations from *C. lanceolata* seeds in the absence of reducing equivalents

The condensation reactions of [2-¹⁴C]malonyl-ACP and octanoyl-ACP (lanes 1) or malonyl-ACP and [1-¹⁴C]octanoyl-ACP (lanes 2) were performed in a first assay step (50 μ l reaction volume) containing 100 mM potassium phosphate, pH 7.6, 2 mM dithiothreitol, 10 μ M malonyl-CoA, 5 μ M octanoyl-ACP, 10 μ M ecACP, 4 μ l of MAT preparation and 10 μ l of FAS preparation. Reaction mixtures were incubated for 5 min at 30 °C and reactions were stopped by precipitation with 10% (w/v) trichloroacetic acid. For analysis, reaction products were reduced to acyl-ACPs in a second assay step (50 μ l) using 100 mM Tris/HCl, pH 8.0, 1 mM NADH, 2 mM NADPH and 3 μ l of NEM-extract. Acyl-ACPs were separated by 2.5 M (A) and 5.0 M (B) urea/PAGE, and radioactive products were visualized by autoradiography. The migration positions of standards are indicated. Acyl moieties are defined as (number of carbon atoms):(number of double bonds). Mal-ACP, malonyl-ACP.

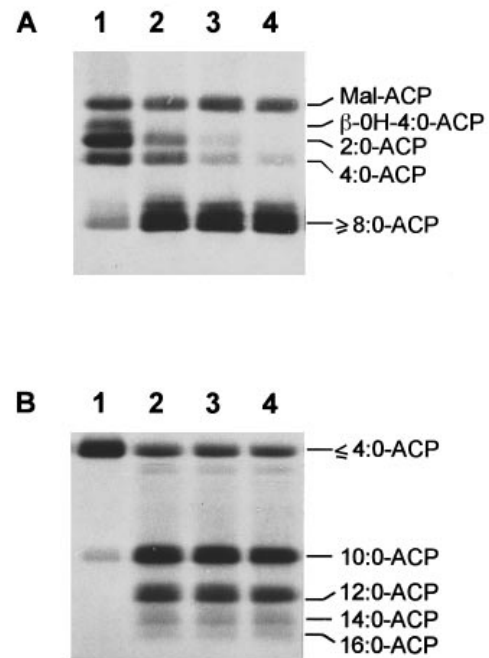


Figure 2 Products of the condensation reactions catalysed by the FAS preparation from *C. lanceolata* seeds in the presence of reducing equivalents

Products were analysed using the two-step assay as described in the legend to Figure 1, except that the condensation reaction of [2-¹⁴C]malonyl-ACP and octanoyl-ACP was supplied in addition with 1 mM NADH (lanes 1), 1 mM NADH plus 2 μ l of KR solution (lanes 2), 2 mM NADPH (lanes 3) or 2 mM NADPH plus 2 μ l of KR solution (lanes 4). Product acyl-ACPs were separated by 2.5 M (A) or 5.0 M (B) urea/PAGE and visualized by autoradiography. The migration positions of standards are indicated. Acyl moieties are defined as (number of carbon atoms):(number of double bonds). Mal-ACP, malonyl-ACP; β -OH-4:0-ACP, β -hydroxybutyryl-ACP.

presence of decanoyl-ACP as a reaction product could be more clearly identified using 5.0 M urea gels (Figure 1B, lane 1). In contrast, only one product could be identified in the reaction involving non-labelled malonyl-ACP and [1-¹⁴C]octanoyl-ACP (Figures 1A and 1B, lane 2), namely decanoyl-ACP resulting from the elongation of octanoyl-ACP. This indicates that the short-chain acyl-ACPs observed in Figure 1(A), lane 1, originated from malonyl-ACP as a result of decarboxylation.

To investigate the formation of acetyl- and butyryl-ACP in more detail, we performed a series of experiments in which the first assay step was supplemented with NADH or NADPH, separately or in combination with purified recombinant KR, the first reducing enzyme in the FAS cycle. Figure 2 clearly shows that, as in the reaction without reducing equivalents (Figure 1), the presence of NADH in the first assay step led to the formation almost exclusively of acetyl- and butyryl-ACP (Figures 2A and 2B, lane 1). On addition of both KR and NADH the elongation of octanoyl-ACP was clearly enhanced, although acetyl- and butyryl-ACP were still produced (Figures 2A and 2B, lane 2). On adding NADPH to the first assay step, decanoyl-ACP and acyl-ACPs of longer chain length were produced almost exclusively (Figures 2A and 2B, lane 3). Short-chain acyl-ACPs were present only in very low amounts. The addition of KR in this case resulted in only small changes in the production of elongated acyl-ACP, whereas acetyl-ACP was practically undetectable (Figures 2A and 2B, lane 4). These data indicate that, during the FAS reaction, NADPH is more efficient as a reducing equivalent

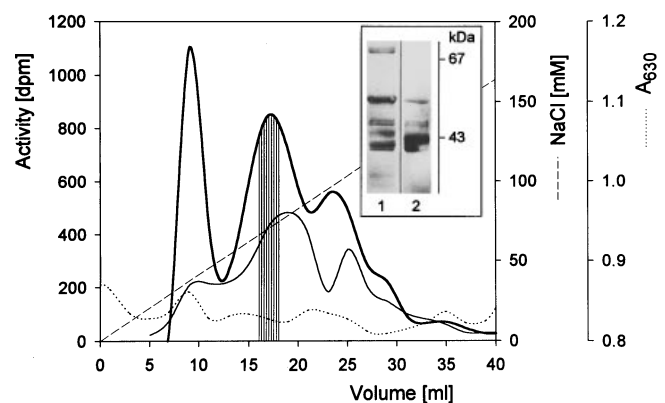


Figure 3 Fractionation of KAS enzymes from *C. lanceolata* seeds by anion-exchange chromatography on Mono Q HR 5/5

The column was equilibrated with 20 mM potassium phosphate (pH 7.6), 2 mM dithiothreitol and 10% (w/v) glycerol, and fractions were eluted at a flow rate of 1 ml/min. Enzymes were eluted at room temperature by applying a linear gradient of 0–1 M NaCl (dashed line). The protein profile (A_{630} ; dotted line) was determined according to Bradford [14]. The fraction indicated by the shaded region containing KAS I (upper trace) and KAS II (lower trace) activity was used as the KAS preparation in further experiments. The inset shows the protein pattern of the KAS preparation after fractionation by SDS/PAGE (13%) and visualization by Coomassie Blue staining (lane 1) and immunoblotting using anti-KAS antibody (lane 2).

than NADH. On the other hand, enhancement of the activity of KR, an NADPH-dependent enzyme, results in a greater degree of elongation and a concomitant decrease in decarboxylation activity, even in the presence of NADH.

To demonstrate that the decarboxylation activity observed is a consequence of KAS action, we performed experiments using a purified enzyme preparation obtained after fractionation of *C. lanceolata* seed extract. Figure 3 shows the KAS I and II activities present in fractions obtained after final anion-exchange chromatography on Mono Q. The fraction eluted at 68 mM NaCl was used as the KAS preparation in these experiments. KAS III, acetyl-CoA:ACP transacylase and reducing enzymes had already been eliminated in the earlier purification steps.

Using the purified KAS preparation we performed two-step assays monitoring the condensation reaction of $[2-^{14}\text{C}]$ malonyl-ACP with non-labelled octanoyl-ACP (KAS I activity) or hexadecanoyl-ACP (KAS II activity) in the absence and presence of cerulenin. The reaction products were analysed by 2.5 M urea/PAGE. Regardless of the primer applied, in the absence of cerulenin decarboxylation of malonyl-ACP was observed, as verified by the presence of acetyl-ACP among the reaction products (Figure 4, lanes 1 and 2). This result is identical with that obtained using the FAS preparation (containing all FAS enzymes). Cerulenin inhibited almost completely the elongation of octanoyl-ACP and the decarboxylation of malonyl-ACP (Figure 4, lanes 3 and 5), a result that can be explained by the high sensitivity of KAS I to the antibiotic. In contrast, the elongation of hexadecanoyl-ACP and the decarboxylation activity related to this reaction (detected by the presence of acetyl-ACP in Figure 4, lanes 4 and 6) were only partially inhibited, in agreement with the greater resistance of KAS II to cerulenin.

Finally, the purified KAS preparation was used to monitor the activity of KAS I in the presence of NADPH and purified recombinant KR by performing a single-step assay. In this assay, 3-oxoacyl-ACP formed by the condensation reaction is immediately reduced to β -hydroxyacyl-ACP, which gives a well defined band in urea/PAGE and can be analysed directly without

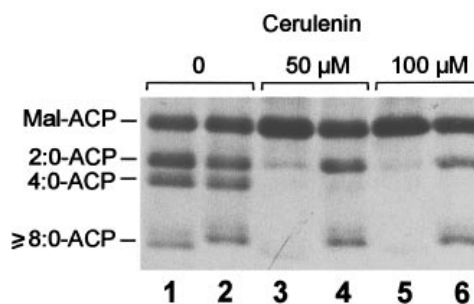


Figure 4 Products of the condensation reactions catalysed by KAS I and KAS II

Reaction products were analysed by the two-step assay as described in the legend to Figure 1, but using the purified KAS preparation from *C. lanceolata* seeds preincubated in the absence or the presence of cerulenin (50 μM or 100 μM) for 10 min at 30 °C. KAS I activity was monitored by the elongation of octanoyl-ACP (lanes 1, 3 and 5), and KAS II activity by the elongation of hexadecanoyl-ACP (lanes 2, 4 and 6). Acyl-ACPs were separated by 2.5 M urea/PAGE and visualized by autoradiography. The migration positions of standards are indicated. Acyl moieties are defined as (number of carbon atoms):(number of double bonds). Mal-ACP, malonyl-ACP.

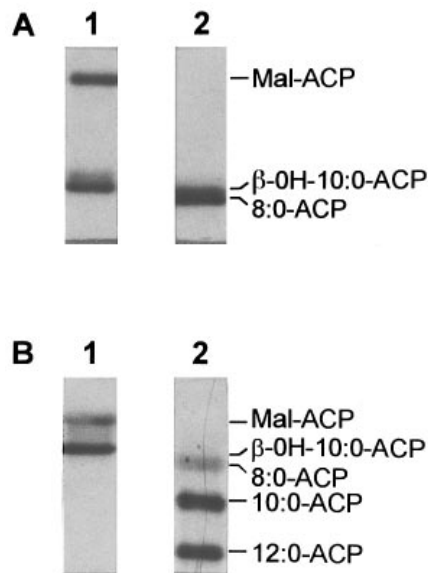


Figure 5 Products of the condensation reactions catalysed by KAS I in the presence of NADPH and recombinant KR

The condensation reaction of $[2-^{14}\text{C}]$ malonyl-ACP and octanoyl-ACP (lanes 1) was performed in a single-step assay as described for the first assay step in the legend to Figure 1, but using the purified KAS preparation and supplying 2 mM NADPH and 2 μl of KR solution. Reaction products were separated by 2.5 M (A) or 5.0 M (B) urea/PAGE and visualized by autoradiography. Lanes 2, migration positions of authentic acyl-ACP standards. Acyl moieties are defined as (number of carbon atoms):(number of double bonds). Mal-ACP, malonyl-ACP; β -OH-10:0-ACP, β -hydroxydecanoyl-ACP.

further reduction to acyl-ACP. As shown in Figure 5, no decarboxylation of malonyl-ACP was observed under these conditions, and only one reaction product, β -hydroxydecanoyl-ACP, was obtained.

These results, i.e. (i) decarboxylation related to elongation, (ii) modulation of decarboxylation by removal of the 3-oxo group,

and (iii) cerulenin sensitivity of the reaction, show clearly that the decarboxylation of malonyl-ACP observed in our studies is the consequence of KAS action. It should be noted that an enzyme with malonyl-ACP decarboxylation activity has not been described in plants so far.

DISCUSSION

In the *in vitro* studies presented here, we first used a preparation containing all the FAS enzymes and subsequently verified the results obtained by using a purified KAS preparation. We observed the unexpected synthesis of acetyl- and butyryl-ACP during the condensation reaction of malonyl-ACP with octanoyl-ACP in the absence of reducing equivalents (NADH and NADPH). Acetyl-ACP, however, can be synthesized in the absence of NADH and NADPH from acetyl-CoA and ACP, a reaction that has been attributed either to acetyl-CoA:ACP transacylase (EC 2.3.1.38) [4,18–20] or to KAS III [4,20,21]. Although both enzymes are present in our FAS preparation, acetyl-CoA was not supplied as a substrate in the experiments, and it seems unlikely that the concentration of any endogenous acetyl-CoA in this preparation would be sufficiently high to account for the extent of acetyl-ACP synthesis observed. On the other hand, acetyl-CoA might originate from malonyl-CoA, supplied for the *in situ* synthesis of malonyl-ACP, by the reverse reaction of the acetyl-CoA carboxylase. The low activity of the subsequent transacylase reaction [6,22], however, would be likely to result in low production of acetyl-ACP, not the large amounts observed. In support of this hypothesis, we showed that acetyl-ACP was also synthesized in the experiments using the purified KAS preparation, which was free from acetyl-CoA, acetyl-CoA:ACP transacylase and KAS III. By carrying out complementary experiments in which the radiolabelled substrates in the reaction were alternated, we could demonstrate that acetyl-ACP originates from malonyl-ACP by a decarboxylation reaction. Since this decarboxylation was also observed in the experiments using purified KAS preparations, containing KAS I and II but no KAS III, we attribute this reaction to the activity of the former enzymes. Moreover, we demonstrated previously that KAS III is unable to catalyse decarboxylation of malonyl-ACP [7].

Malonyl-ACP decarboxylation by KAS has been observed previously in various studies of fatty acid biosynthesis in *E. coli* [23,24], which also has a type II organization of the FAS. Recently, Heath and Rock [9], performing *in vivo* studies with *E. coli* mutants, suggested that this decarboxylation activity of KAS enzymes is feedback-induced by the accumulation of the end-products, long-chain acyl-ACPs, in a situation of inhibited phospholipid synthesis and concomitant down-regulated fatty acid biosynthesis. Our *in vitro* results show that, during the elongation of octanoyl-ACP, the decarboxylation of malonyl-ACP takes place even in the presence of low levels of the resulting elongation product decanoyl-ACP (Figure 2, lanes 1), the end-product of fatty acid biosynthesis in *C. lanceolata* seeds. The decarboxylation reaction is very active in the absence of reducing equivalents, and can be suppressed by supplying either NADPH or NADH plus KR. Moreover, in the presence of NADPH almost no acetyl-ACP is produced, although acyl-ACPs accumulate in large amounts. In contrast to the feedback mechanism proposed by Heath and Rock [9], our data strongly indicate that an intermediate of the FAS cycle, and not the final product acyl-ACP, is responsible for the induction of malonyl-ACP decarboxylation by KAS. The obvious candidate is 3-oxoacyl-ACP, since enhancement of KR activity, either by

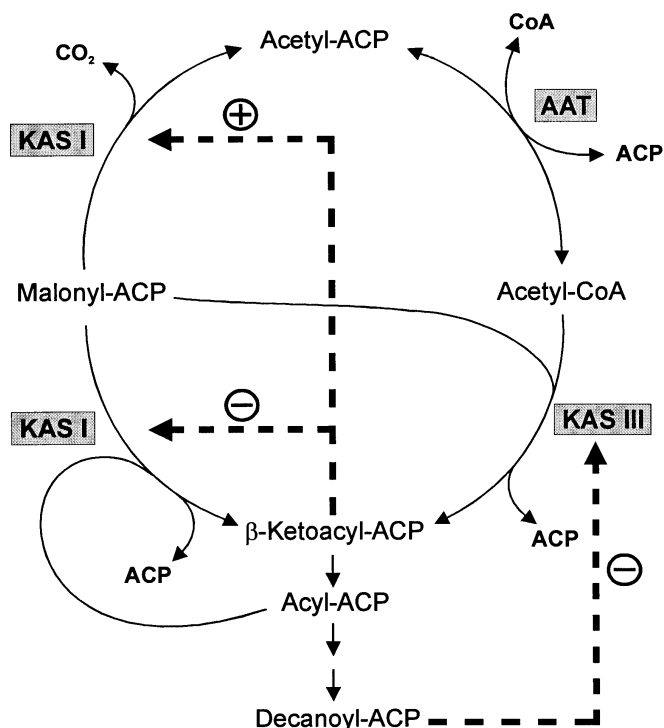


Figure 6 Hypothetical scheme for the regulation of fatty acid biosynthesis in *C. lanceolata* seeds

Fatty acid biosynthesis is initiated by the condensation reaction of acetyl-CoA with malonyl-ACP, catalysed by KAS III, producing 3-oxobutyryl-ACP. The 3-oxo group is removed during the course of the FAS reaction to produce saturated butyryl-ACP by the sequential action of KR, 3-hydroxyacyl-ACP dehydratase and enoyl-ACP reductase. Further elongation steps are catalysed by acyl-ACP-specific condensing enzymes, such as KAS I and KAS II. The latter catalyses the elongation of palmitoyl-ACP to stearoyl-ACP, which is a less important reaction in the biosynthesis of storage triacylglycerols in *C. lanceolata* seeds. Accumulation of the end-product of medium-chain fatty acid biosynthesis, decanoyl-ACP, results in direct and indirect methods of regulation. Directly, decanoyl-ACP causes feedback inhibition of KAS III, with the consequent accumulation of the starter substrates acetyl-CoA and malonyl-ACP. Indirectly, the accumulation of decanoyl-ACP leads to accumulation of the intermediate 3-oxodecanoyl-ACP, which inhibits the condensing activity and induces the decarboxylation activity of KAS I. Malonyl-ACP is then degraded to acetyl-ACP. The latter might be either (i) stored as a reserve substrate for restarting fatty acid biosynthesis, or (ii) recycled to regenerate acetyl-CoA, e.g. by the reverse action of acetyl-CoA:ACP transacylase (AAT), which is known for its very low activity for the synthesis of acetyl-ACP. β -Ketoacyl-ACP = 3-oxoacyl-ACP

supplying the enzyme exogenously or by supplying NADPH and/or NADH, resulted in a decrease in decarboxylation activity. We suggest, therefore, that the accumulation of the condensation product 3-oxoacyl-ACP inhibits the condensing activity by product inhibition and, concomitantly, induces the decarboxylation activity of acyl-ACP-specific KAS enzymes. This is conceptually feasible, and does not disagree with the observations made with the *E. coli* system of Heath and Rock [9], since our approach allowed us to investigate the condensation step of one FAS cycle directly. Indeed, it is conceivable that accumulation of the end-product of the reaction chain, as proposed by Heath and Rock, would lead to accumulation of all intermediates in this reaction chain.

The presence of butyryl-ACP as a reaction product in our condensation experiments in the absence of reducing equivalents is a puzzling result. Butyryl-ACP is formed, in this case, from the condensation reaction of acetyl-ACP and malonyl-ACP, and is thus an indirect consequence of the decarboxylation of malonyl-

ACP. The obvious question here is: to which enzyme can this activity be attributed? A first candidate would be KAS I, since our results show that the elongation of acetyl-ACP to butyryl-ACP is highly cerulenin-sensitive. However, we assume that the decarboxylation activity is induced by product inhibition, and it is difficult to envisage how KAS I could be inhibited for the elongation of octanoyl-ACP and yet could still elongate acetyl-ACP. The existence of a regulatory loop to recycle malonyl-ACP to acetyl-CoA via decarboxylation, as suggested for *E. coli* [9], might shed some light on this matter. In this case, acetyl-CoA would serve as a substrate for KAS III to produce butyryl-ACP. Our investigations using purified KAS preparations disagree with this hypothesis, however, since KAS III was absent from this preparation; furthermore, the synthesis of butyryl-ACP is cerulenin-sensitive, whereas KAS III is resistant to this antibiotic. As a third hypothesis, our experiments cannot exclude the existence of an as yet unknown, cerulenin-sensitive, condensing enzyme that elongates acetyl-ACP to butyryl-ACP.

In conclusion, our results demonstrate that plant condensing enzymes are able to promote the decarboxylation of malonyl-ACP. This reaction is a consequence of the accumulation of the direct product of the condensation reaction, i.e. 3-oxoacyl-ACP. Under these conditions, the condensing activity of the enzyme is inhibited and the decarboxylation activity is induced (Figure 6). We demonstrated recently that decanoyl-ACP is involved in the mechanism of regulation of fatty acid biosynthesis in *C. lanceolata* seeds, via feedback inhibition of KAS III [7]. Under such regulation, inhibition of the KAS III reaction would result in the accumulation of both substrates, i.e. acetyl-CoA and malonyl-ACP. Thus the existence of a mechanism regulating the malonyl-ACP concentration in the plastid is very attractive. The *in vitro* data described here suggest that decarboxylation of malonyl-ACP to acetyl-ACP might be activated concomitant with the inhibition of the condensing activity of KAS I or KAS II by 3-oxoacyl-ACP, thus modulating the levels of that substrate when necessary.

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REFERENCES

- 1 Shimakata, T. and Stumpf, P. K. (1982) Proc. Natl. Acad. Sci. U.S.A. **91**, 11027–11031
- 2 MacKintosh, R. W., Hardie, D. G. and Slabas, A. R. (1989) Biochim. Biophys. Acta **1002**, 114–124
- 3 Siggaard-Andersen, M., Kauppinen, S. and von Wettstein-Knowles, P. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 4114–4118
- 4 Clough, R. C., Matthis, A. L., Barnum, S. R. and Jaworski, J. G. (1992) J. Biol. Chem. **267**, 20992–20998
- 5 Tai, H. and Jaworski, J. G. (1993) Plant Physiol. **106**, 801–802
- 6 Schuch, R., Brummel, M. and Spener, F. (1994) J. Plant Physiol. **143**, 556–560
- 7 Brück, F. M., Brummel, M., Schuch, R. and Spener, F. (1996) Planta **198**, 271–278
- 8 Shintani, D. K. and Ohlrogge, J. B. (1995) Plant J. **7**, 577–587
- 9 Heath, R. J. and Rock, C. O. (1995) J. Biol. Chem. **270**, 15531–15538
- 10 Kopka, J., Robers, M., Schuch, R. and Spener, F. (1993) Planta **191**, 102–111
- 11 Cronan, Jr., J. E. and Klages, A. L. (1981) Proc. Natl. Acad. Sci. U.S.A. **78**, 5440–5444
- 12 Brück, F. M., Schuch, R. and Spener, F. (1994) J. Plant Physiol. **143**, 550–555
- 13 Klein, B., Pawlowski, K., Horicke-Grandpierre, C., Schell, J. and Töpfer, R. (1992) Mol. Gen. Genet. **233**, 122–128
- 14 Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
- 15 Simpson, J. A. and Sonne, O. (1982) Anal. Biochem. **119**, 424–427
- 16 Schuch, R., Brummel, M. and Spener, F. (1993) Grasas Aceites (Seville) **44**, 126–128
- 17 Post-Beittemiller, D., Roughan, G. and Ohlrogge, J. B. (1992) Plant Physiol. **100**, 923–930
- 18 Shimakata, T. and Stumpf, P. K. (1983) J. Biol. Chem. **258**, 3592–3598
- 19 Lowe, P. N. and Rhodes, S. (1988) Biochem. J. **250**, 789–796
- 20 Gulliver, B. S. and Slabas, A. R. (1994) Plant Mol. Biol. **25**, 179–191
- 21 Tsay, J.-T., Oh, W., Larson, T. J., Jackowski, S. and Rock, C. O. (1992) J. Biol. Chem. **267**, 6807–6814
- 22 Jaworski, J. G., Clough, R. C. and Barnum, S. R. (1989) Plant Physiol. **90**, 41–44
- 23 Alberts, A. W., Bell, R. M. and Vagelos, P. R. (1972) J. Biol. Chem. **247**, 3190–3198
- 24 Magnuson, K., Jackowski, S., Rock, C. O. and Cronan, Jr., J. E. (1993) Microbiol. Rev. **57**, 522–542