Reaction mechanism of recombinant 3-oxoacyl-(acyl-carrier-protein) synthase III from *Cuphea wrightii* embryo, a fatty acid synthase type II condensing enzyme

Amine ABBADI*, Monika BRUMMEL*, Burkhardt S. SCHÜTT*, Mary B. SLABAUGH†, Ricardo SCHUCH‡ and Friedrich SPENER*¹

*Institut für Biochemie, Universität Münster, Wilhelm-Klemm-Strasse 2, D-48149 Münster, Germany, †Department of Crop and Soil Science, Oregon State University, Corvallis, OR 97331, U.S.A., and ‡Institut für Chemo- und Biosensorik, Mendelstrasse 7, D-48149 Münster, Germany

A unique feature of fatty acid synthase (FAS) type II of higher plants and bacteria is 3-oxoacyl-[acyl-carrier-protein (ACP)] synthase III (KAS III), which catalyses the committing condensing reaction. Working with KAS IIIs from *Cuphea* seeds we obtained kinetic evidence that KAS III catalysis follows a Ping-Pong mechanism and that these enzymes have substrate-binding sites for acetyl-CoA and malonyl-ACP. It was the aim of the present study to identify these binding sites and to elucidate the catalytic mechanism of recombinant *Cuphea wrightii* KAS III, which we expressed in *Escherichia coli*. We engineered mutants, which allowed us to dissect the condensing reaction into three stages, i.e. formation of acyl-enzyme, decarboxylation of malonyl-ACP, and final Claisen condensation. Incubation of recombinant enzyme with [1-¹⁴C]acetyl-CoA-labelled Cys¹¹¹, and the replacement of this residue by Ala and Ser resulted in loss of overall condensing activity. The Cys¹¹¹Ser mutant, however, still

was able to bind acetyl-CoA and to catalyse subsequent binding and decarboxylation of malonyl-ACP to acetyl-ACP. We replaced His²⁶¹ with Ala and Arg and found that the former lost activity, whereas the latter retained overall condensing activity, which indicated a general-base action of His²⁶¹. Double mutants Cys¹¹¹Ser/His²⁶¹Ala and Cys¹¹¹Ser/His²⁶¹Arg were not able to catalyse overall condensation, but the double mutant containing Arg induced decarboxylation of [2-¹⁴C]malonyl-ACP, a reaction indicating the role of His²⁶¹ in general-acid catalysis. Finally, alanine scanning revealed the involvement of Arg¹⁵⁰ and Arg³⁰⁶ in KAS III catalysis. The results offer for the first time a detailed mechanism for a condensing reaction catalysed by a FAS type II condensing enzyme.

Key words: ACP, active-site residues, fatty acid synthesis, protein engineering.

INTRODUCTION

3-Oxoacyl-[acyl-carrier-protein (ACP)] synthase III (KAS III, EC 2.3.1.41) initiates the chain of reactions of the fatty acid synthase in plants and bacteria (FAS, type II) by catalysing the condensation of acetyl-CoA with malonyl-ACP to produce 3-oxobutyl-ACP, which is reduced in the FAS reaction to butyryl-ACP using NADPH and NADH as reducing equivalents. KAS III was originally detected in *Escherichia coli* [1] and then found to be present in various higher plant species [2–9] and in the red alga *Porphyra umbilicalis* [10], thus indicating the widespread occurrence of this condensing enzyme in organisms having type II FAS. In all these cases KAS III can be clearly distinguished from acyl-ACP-specific condensing enzymes (KAS I and KAS II) by its insensitivity towards the antibiotic cerulenin, a well-known inhibitor of FAS. Yet based on experiments with seed extracts from higher plants, it remained unclear whether this enzyme can also promote the elongation of short-chain acyl-ACPs [4,11]. With respect to the kinetics of the condensing reaction, we previously demonstrated that catalysis by native KAS III in *Cuphea lanceolata* seed extracts follows Ping-Pong kinetics [11], a result that we recently reproduced with purified recombinant KAS III from *Cuphea wrightii* (results not shown). Consequently, different attachment sites for acetyl-CoA and malonyl-ACP were postulated.

It is well accepted that the first step of the condensing reaction comprises the formation of an acyl-enzyme intermediate by

transfer of the acyl group from the pantetheine moiety of an acyl donor to the substrate-binding site on the condensing enzyme. In the condensing domain of the yeast multifunctional-fatty-acid synthase (FAS, type I), the residue responsible for accepting the acyl group was identified to be a cysteine [12,13] and the involvement of a cysteine in the condensing mechanism was also demonstrated for KAS I from *E. coli* [14], a FAS type II enzyme. Moreover, experiments with modifying reagents demonstrated the presence of basic residues, such as lysine and histidine, in or near the active sites of chicken liver FAS [15] and *Zoogloea ramigera* biosynthetic thiolase [16], respectively, which could perturb the pK_a of the cysteine nucleophile enabling it to attack the acyl substrate. Based on an amino-acid-sequence alignment of condensing enzymes, chalcone synthases and thiolases from different organisms, Siggaard-Anderson [17] identified conserved residues and proposed a model for the condensing mechanism in which a cysteine and five basic residues were involved. Very recent data on the crystal structure of KAS II from *E. coli* supported the involvement of a nucleophile cysteine and two histidine residues in the active site [18]. However, the amino-acid-sequence similarity between KAS IIIs and the acyl-ACP-specific condensing enzymes is poor, with the exception of a highly conserved cysteine [5]. This cysteine was assumed to be part of the putative active site of KAS III [17,18], but experimental proof has yet to be provided. Due to the lack of crystal-structure data on KAS III, the identification of the active-site residues of this enzyme is a basic requirement for an improved understanding

Abbreviations used: KAS, 3-oxoacyl-(acyl-carrier-protein) synthase; ACP, acyl carrier protein; FAS, fatty acid synthase.

¹ To whom correspondence should be addressed (e-mail spener@uni-muenster.de).

of the mechanism of the condensing reaction and of the substrate specificity of KAS III and may contribute to a more general understanding of condensing enzyme action as a whole.

In the present study we investigated the interaction of purified recombinant KAS III, originating from *C. wrightii*, with its substrates. Taking cues from sequence alignments of KAS IIIs, conserved amino acids were singled out as candidates for substrate labelling experiments and for site-directed mutagenesis with the objective of identifying the active-site residues and dissecting the overall condensing reaction into three steps, (i) acetyl-CoA binding, (ii) malonyl-ACP binding and decarboxylation, and (iii) the final Claisen condensation. This enabled us to explain KAS III catalysis on the basis of the Ping-Pong reaction mechanism.

MATERIALS AND METHODS

Materials

The host *E. coli* strain BL21(DE3)pLysS was purchased from Novagen (Madison, WI, U.S.A.), restriction endonucleases *Nde*I and *Xho*I from New England Biolabs (Schwalbach, Germany) and isopropyl β -D-thiogalactoside from MBI Fermentas (St. Leon-Rot, Germany). [14 C]acetyl-CoA (1.85 GBq·mmol⁻¹) and [14 C]malonyl-CoA (2.07 GBq·mmol⁻¹) were from Amersham (Braunschweig, Germany). Protease (LysC, sequencing grade) was from Promega (Heidelberg, Germany). Acyl-CoAs, fatty acids and NADPH were purchased from Sigma (Deisenhofen, Germany), and electrophoresis-grade reagents for peptide sequencing were from Bio-Rad (Munich, Germany). All other reagents were of analytical grade or better.

ACP and acyl-ACPs

ACP from *E. coli* was purchased from Sigma and purified by anion-exchange fast-performance liquid chromatography on Mono Q, as described previously [19]. Acyl-ACPs were synthesized chemically from ACP and fatty acids according to Cronan and Klages [20]. The purity and concentration of the acyl-ACP stock solutions were determined by conformationally sensitive gel electrophoresis in 20% (w/v) polyacrylamide gels containing 2.5 M urea (urea/PAGE), followed by visualization with Coomassie Blue and quantification by densitometry using purified ACP as a standard [21].

Malonyl-ACP

Malonyl-ACP was synthesised enzymically from ACP and malonyl-CoA using a partially purified malonyl-CoA:ACP S-malonyltransferase (EC 2.3.1.39) preparation from *C. lanceolata* seeds [22]. The reaction mixture (1 ml) contained 100 mM sodium phosphate, pH 7.6, 100 μ M purified ACP, 300 μ M malonyl-CoA, 100 μ l malonyl-CoA:ACP S-malonyltransferase preparation (corresponding to 0.22 nkat) and 2 mM dithiothreitol. For complete reduction ACP was preincubated with dithiothreitol (15 min, 37 °C). The reaction was carried out for 15 min at 37 °C and stopped by the addition of 112 μ l of 100% (w/v) trichloroacetic acid and transfer to ice for at least 10 min. The mixture was centrifuged (16000 g, 5 min, 4 °C) and the supernatant containing unreacted malonyl-CoA was discarded. The precipitate was washed with 800 μ l of 10% (w/v) trichloroacetic acid, centrifuged as above, dissolved in 50 mM Mes, pH 6.8, apportioned, and stored at -70 °C. The purity and concentration of the malonyl-ACP stock solution was determined as described for acyl-ACPs, except that a 1.0 M urea/PAGE was employed.

Table 1 Primer sequences used for PCR

The mutagenized codons are underlined.

Primer	Sequence
5'-flanking primer	5'-CATATGGGTTCTGACAGGCAG-3'
3'-flanking primer	5'-CTCGAGTTATCCCCACCTGAT-3'
Cys ¹¹¹ Ser 5'-mutagenic primer	5'-ACCGCTGCATCCAGTGGATT-3'
Cys ¹¹¹ Ala 5'-mutagenic primer	5'-ACCGCTGCAGCCAGTGGAT-3'
His ²⁶¹ Ala 5'-mutagenic primer	5'-CTGCTTCGTCAGAAATCAG-3'
His ²⁶¹ Arg 5'-mutagenic primer	5'-CTGCTTCGTCAGGCAAATCAG-3'
Arg ¹⁵⁰ Ala 5'-mutagenic primer	5'-ACTGATGCCGGAACCTGTATT-3'
Arg ²⁶⁶ Ala 5'-mutagenic primer	5'-AATCAGGCCATCATTGATGCA-3'
Arg ³⁰⁶ Ala 5'-mutagenic primer	5'-GCTGTGGCCAGTGAAATGTG-3'

For the synthesis of radiolabelled malonyl-ACP, [14 C]malonyl-CoA (0.37 GBq·mmol⁻¹) was used, and the final preparation was apportioned, and stored at -20 °C. The concentration of the [14 C]malonyl-ACP preparation was calculated from liquid scintillation spectrometry data.

Cloning of KAS III from *C. wrightii* embryo

Clone cwKAS IIIa, encoding KAS III from *C. wrightii*, was isolated from a developing embryo cDNA library as previously described [7].

Site-directed mutagenesis

Applying symmetric PCR with a single-mutant primer [23], Cys¹¹¹ of the wild-type KAS III was substituted with Ser or Ala, His²⁶¹ was substituted with Ala or Arg, whereas Arg at positions 150, 266 and 306 were subjected to alanine scanning. Primer sequences are shown in Table 1. The amplification of the mutant KAS III DNA fragments was achieved with 50 pmol of 3'-flanking primer, 50 pmol of 5'-mutagenic primer, 1.3 units of proof-reading *Pfu* polymerase (Stratagene, Heidelberg, Germany), 0.46 fmol of pET15b-cwKASIIIa plasmid as template and 200 μ M dNTPs in a total volume of 50 μ l. Conditions for PCR were as follows: denaturation at 93 °C for 1 min, followed by 30 cycles of 93 °C for 30 s, 55 °C for 45 s and 72 °C for 1 min and a final extension step at 72 °C for 10 min. To generate the full-length mutant cDNA, 2.5 pmol of the gel-purified DNA fragments were used as 3'-'megaprimers' in a second reaction with 2.5 pmol of both flanking primers, 5 $\times$ 10⁻⁶ fmol of wild-type DNA, 200 μ M dNTPs and 1.3 units *Pfu* polymerase in a final volume of 50 μ l. The amplification was performed as follows: denaturation for 1 min at 93 °C, followed by 5 cycles of 1 min at 93 °C, 1 min at 48 °C and 1 min at 72 °C, and 20 cycles using the same temperature program except that the renaturation temperature was raised to 55 °C. The final extension was carried out for 10 min at 72 °C. The resulting 1048 base pair DNA fragments were ligated into the pGEM-T Easy sequencing vector (Promega) according to the manufacturer's protocol. The entire encoding sequence of the mutant constructs was confirmed by DNA sequencing. For protein expression the mutant KAS III cDNAs were subcloned into the *Nde*I and *Xho*I restriction sites of pET15b plasmid (Novagen) following the supplier's instructions.

Construction of the double-mutant clones

The plasmids containing the mutations His²⁶¹Ala and His²⁶¹Arg were digested with *Fse*I and *Xho*I, the resulting DNA fragments

containing the mutations were gel-purified and each ligated into the *FseI/XhoI* digested plasmid containing the Cys¹¹¹Ser mutation.

Expression and isolation of recombinant wild-type and mutant KAS IIIs

E. coli strain BL21(DE3)pLysS was transformed with the recombinant plasmid and grown at 30 °C in 2 l of Terrific Broth ('TB') containing 50 µg·ml⁻¹ ampicillin (Serva, Heidelberg, Germany) and 25 µg·ml⁻¹ chloramphenicol (Roth, Karlsruhe, Germany). At a cell density corresponding to an attenuation of 0.7, the expression of KAS III was induced by adding isopropyl β-D-thiogalactoside at a final concentration of 0.4 mM, and incubation was continued for additional 3 h. Bacteria were harvested by centrifugation and stored overnight at -20 °C. Cell lysis was performed by resuspending the frozen bacterial mass in 20 ml lysis buffer [50 mM sodium phosphate, pH 8.0, 2 mM 2-mercaptoethanol, 10 % (w/v) glycerol, 0.45 % Triton X-100, 20 mM imidazole] in the presence of RNase and DNase (final concentrations of 5 µg·ml⁻¹ and 10 µg·ml⁻¹, respectively) and incubation for 30 min on ice. Cells were disrupted by sonication (3 × 10 s) and the cell debris was removed by centrifugation (20 000 g, 30 min, 4 °C). Soluble His-tagged KAS III was purified by immobilized-metal affinity chromatography by applying the supernatant to a Ni-nitrilotriacetic acid Superflow column (10 ml, Qiagen, Hilden, Germany) pre-equilibrated with 50 mM sodium phosphate, pH 8.0, containing 2 mM 2-mercaptoethanol, 10 % (w/v) glycerol, 0.01 % Triton X-100 and 20 mM imidazole, washing with 20 ml 50 mM imidazole and finally eluting the recombinant KAS III with 0.2 M imidazole. Pure KAS III fractions were pooled and concentrated using a Centrprep 10 concentration unit (Amicon, Beverly, MA, U.S.A.). The final preparations were apportioned, and stored at -70 °C. Purity of KAS III preparations was judged by SDS/PAGE as described by Laemmli [24]. KAS III concentration was determined by the method of Bradford [25], which was modified for a microplate reader [26], using ovalbumin as standard.

Enzyme assays and substrate specificity

KAS III activity was assayed by monitoring the incorporation of radioactive acetate from [1-¹⁴C]acetyl-CoA into acetoacetyl-ACP, as described previously [11]. The reaction mixture (50 µl) contained 100 mM sodium phosphate, pH 7.6, 10 µM [1-¹⁴C]-acetyl-CoA, 20 µM malonyl-ACP and 2.5 ng of recombinant KAS III. The reaction was started by the addition of the enzyme and carried out for 5 min at 30 °C. To investigate the reaction mechanism of KAS III the concentration of one substrate (acetyl-CoA or malonyl-ACP) was varied, whereas the concentration of the other substrate (malonyl-ACP or acetyl-CoA, respectively) was kept at saturation level. The data obtained were analysed by the method of Lineweaver-Burk [27] and are the result of numerous test series.

Substrate specificity of KAS III was monitored by the incorporation of malonate from [2-¹⁴C]malonyl-ACP into the condensation products. The assay (50 µl) contained 100 mM sodium phosphate, pH 7.6, 10 µM acyl-ACP or acetyl-CoA, 20 µM [2-¹⁴C]malonyl-ACP, 2 mM NADPH, 2.5 ng KAS III and 0.6 µkat of an affinity-purified recombinant glutathione-S-transferase-3-oxoacyl-ACP reductase fusion protein (EC 1.1.1.100) from *C. lanceolata* [28]. The 3-hydroxyacyl-ACPs formed were precipitated, washed and dissolved as described for malonyl-ACP and subsequently separated by 2.5 M urea/PAGE. Following transfer to the Immobilon P membrane (Millipore,

Eschwege, Germany) by electroblot at 0.8 mA/cm² for 1 h, the reaction products were visualized by autoradiography after 5 days exposure to an X-ray film (Hyperfilm MP, Amersham).

Decarboxylase activity of mutant KAS III was determined in a reaction mixture containing 100 mM sodium phosphate, pH 7.6, 10 µM acetyl-CoA, 20 µM [2-¹⁴C]malonyl-ACP and 20 ng of mutant KAS III. The reaction was carried out for 10 min at 30 °C. The reaction products were precipitated, washed, dissolved and separated by 2.5 M urea/PAGE, followed by transfer to an Immobilon P membrane and visualization by autoradiography, as described above.

Identification of the substrate attachment site of KAS III

Purified recombinant KAS III was incubated with a 3.5 M excess of [1-¹⁴C]acetyl-CoA (0.74 GBq·mmol⁻¹) at a final concentration of 2.24 mg KAS III·ml⁻¹ for 4 min on ice. The resulting [¹⁴C]acyl-enzyme complex was denatured by precipitation with trichloroacetic acid at a final concentration of 10 % (w/v) followed by incubation for 5 min on ice. After centrifugation (10 000 g, 5 min, 4 °C), the supernatant was discarded and the precipitate was washed three times with 400 µl of ice-cold 0.1 M acetic acid and finally resuspended in an appropriate volume of proteolysis buffer (25 mM Tris/HCl, pH 8.4, 1 mM EDTA). Proteolytic digestion of the [¹⁴C]acyl-enzyme complex was carried out at a final concentration of 1.5 mg·ml⁻¹ of the ¹⁴C-labelled KAS III by treatment with LysC protease (20 µg of LysC protease·mg⁻¹ KAS III) for 2 h at 37 °C. The resulting peptides were separated by SDS/Tricine/PAGE [1 cm spacer gel, 10 % (w/v) total acrylamide ('T'), which includes 3 % bisacrylamide ('C'); 5 cm separating gel, 16.5 % total acrylamide ('T'), which includes 3 % bisacrylamide ('C')] according to Schagger and von Jagow [29] under slightly reducing conditions (7 mM dithiothreitol) and subsequently transferred to an Immobilon P membrane by electroblot (0.8 mA/cm² for 1 h) using 50 mM Tris, pH 8.3, 50 mM boric acid, 20 % (v/v) methanol as transfer buffer. Peptides were then visualized by Coomassie Blue staining and by autoradiography after 5 days exposure to Hyperfilm MP. The radiolabelled 9.5 kDa peptide was excised from the membrane and directly subjected to Edman degradation in a liquid phase sequenator (Applied Biosystems, Weiterstadt, Germany). The radioactivity associated with each amino acid was measured by liquid scintillation spectrometry using Aquasafe 500 (Zinser, Frankfurt, Germany).

Determination of CD spectra

All CD spectra were collected with a Jobin Yvon CD6 dichrograph in a 0.01 cm cell at 25 °C. Scans were recorded with a bandwidth of 0.5 nm and a time response of 1 s. For each enzyme preparation the final spectrum is the result of five accumulated scans. Samples (0.5–1 mg·ml⁻¹) were desalted using a NAP-10 column (Pharmacia Biotech, Freiburg, Germany) and eluted in 10 mM sodium phosphate buffer, pH 8.0, containing 0.1 % Tween 20. Samples were centrifuged at 16 000 g for 15 min to remove any precipitated protein. The A₂₈₀ was measured to scale the CD data to the same protein concentration. Secondary structure estimations of wild-type and mutant KAS III were done with the CONTIN program [30].

RESULTS

The rationale of the present study required purified wild-type and mutant enzymes, which were achieved by employing a His-tagged KAS III expression vector and purification of the

Figure 1 CD spectra of wild-type and mutant KAS IIIs

Mean residue ellipticity $[\theta]$ is plotted as a function of wavelength for the wild-type (●), the mutants Cys¹¹¹Ser (■), His²⁶¹Ala (▲), Arg²⁶⁶Ala (○) and the double-mutant Cys¹¹¹Ser/His²⁶¹Ala (▼). The error in the CD measurements was approx. $\pm 5\%$.

functionally expressed enzymes to homogeneity with the aid of immobilized-metal affinity chromatography (results not shown). In order to investigate the structural integrity of wild-type and mutant KAS IIIs, their secondary structure composition was investigated by CD spectroscopy. An overlay of the far-UV CD spectra of the Cys¹¹¹Ser, His²⁶¹Ala, Cys¹¹¹Ser/His²⁶¹Ala mutants, and wild-type KAS III demonstrated that they were identical (Figure 1), indicative of structural preservation of the engineered KAS III mutants. Furthermore the CD spectra of the Arg¹⁵⁰Ala and Arg³⁰⁶Ala mutants were also indistinguishable from that of wild-type KAS III (spectra not shown for brevity). In contrast the Arg²⁶⁶Ala mutant differed significantly from the wild-type in its CD spectrum (Figure 1), indicating structural perturbation.

Substrate specificity of wild-type recombinant KAS III

The purified His-tagged KAS III showed a specific activity of $7.0 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$, when assayed in the presence of saturating concentrations of acetyl-CoA and malonyl-ACP. The K_m values for acetyl-CoA and malonyl-ACP were $1.4 \pm 0.1 \mu\text{M}$ and $4.1 \pm 0.3 \mu\text{M}$, respectively, and were comparable to those of the native enzyme from *C. lanceolata* [11]. The substrate specificity of purified recombinant KAS III was examined by monitoring the incorporation of [2-¹⁴C]malonyl-ACP into the condensation products. Since the unstable 3-oxoacyl-ACPs gave rise to rather diffuse bands in urea/polyacrylamide gels, they were converted, prior to analysis, to the corresponding stable 3-hydroxyacyl-ACPs by addition of 3-oxoacyl-ACP reductase and NADPH to the reaction mixture. The reaction products were then separated by conformationally sensitive electrophoresis, transferred to Immobilon P and visualized by autoradiography. As shown in Figure 2, recombinant KAS III was able to use propionyl-CoA (C_3) and butyryl-CoA (C_4) besides acetyl-CoA for condensation with malonyl-ACP. Surprisingly, short-chain acyl-ACPs (C_2 and C_3) also served as substrates, although at very low levels (Figure 2).

Figure 2 Substrate specificity of KAS III

Reaction mixtures containing 100 mM sodium phosphate (pH 7.6), $20 \mu\text{M}$ [2-¹⁴C]malonyl-ACP, $10 \mu\text{M}$ primer as indicated, 2 mM NADPH, 2.5 ng KAS III and $0.6 \mu\text{kat}$ 3-oxoacyl-ACP reductase, were incubated as described in the Materials and methods section. The products, converted into 3-hydroxyacyl-ACPs, were separated by 2.5 M urea/PAGE and visualized by autoradiography. The first number preceding the CoA or ACP of the primers indicates the carbon chain length of the acyl moieties; the second number refers to the number of carbon-carbon double bonds. Other abbreviations: Mal-ACP, malonyl-ACP; 3-OH-4:0-ACP, 3-hydroxybutyryl-ACP; 3-OH-5:0-ACP, 3-hydroxypentanoyl-ACP; 3-OH-6:0-ACP, 3-hydroxyhexanoyl-ACP.

Identification of active-site residues

To identify the amino acid residue responsible for covalent acetate binding in the acetyl-CoA attachment site of KAS III, we incubated the recombinant enzyme with [1-¹⁴C]acetyl-CoA at a 3.5 molar excess. The formation of the acetyl-enzyme intermediate, after acid denaturation was then investigated by separation on SDS/Tricine/PAGE, followed by transfer of the labelled intermediate on to an Immobilon P membrane and autoradiographic detection. As shown in the inset of Figure 3, a radioactive protein band was detected in the autoradiograph (lane 2) at a position corresponding to the band representing KAS III, which was visualized by staining the same membrane with Coomassie Blue (lane 1). The stability of the [¹⁴C]acetyl-enzyme complex, even under the conditions of proteolytic digestion and electrophoresis, strongly indicated covalent binding of the substrate. To identify the amino acid engaged in [1-¹⁴C]acetate binding we digested the complex with LysC protease. This resulted in several peptides (inset in Figure 3, lane 3), but the radioactivity was concentrated in a single peptide of 9.5 kDa (lane 4). The amino acid sequence of this peptide matched, after alignment, positions 101–113 of the sequence of recombinant KAS III. The determination of the radioactivity associated with each amino acid showed that labelling was localized at position 111 of the 9.5 kDa peptide, corresponding to Cys¹¹¹ of KAS III (Figure 3). To further characterize Cys¹¹¹ as the catalytically active residue and to gain more information on how a thioester effects catalysis relative to an oxoester in a Claisen condensation, we examined acetate binding and condensation activity with the aid of mutants Cys¹¹¹Ser and Cys¹¹¹Ala. The cDNAs encoding for both KAS III mutants were sequenced in order to confirm the desired mutation, the corresponding plasmids were expressed in *E. coli*, and all mutant proteins were isolated to the same level of purity as wild-type recombinant KAS III. In both cases, substitution of cysteine¹¹¹ led to complete loss of condensing activity (Table 2). Interestingly, incubation of the mutants with [1-¹⁴C]acetyl-CoA afforded the Cys¹¹¹Ser mutant in labelled form (Figure 4A), although at levels approximately 3-fold lower than that of the wild-type enzyme.

Amino acid sequence alignment of all available plant KAS IIIs and that of *E. coli* revealed the existence of one highly conserved His, corresponding to His²⁶¹ in the recombinant KAS III from *C. wrightii*. As this residue could be involved as a general base to

Figure 3 Identification of the acetyl-binding site

The radioactively labelled 9.5 kDa peptide was N-terminally sequenced and radioactivity corresponding to each amino acid position was determined by liquid scintillation spectrometry. Inset: Purified recombinant KAS III (200 μ g) was incubated with [14 C]acetyl-CoA and the [14 C]acetyl-enzyme complex was denatured by acid precipitation as described in the Materials and methods section. (A) Analysis of undigested labelled KAS III (5 μ g) by SDS/Tricine/PAGE and electrotransfer to Immobilon P membrane. (B) Digestion of labelled KAS III (29 μ g) with LysC protease prior to electrophoresis. Digested KAS III (9 μ g) was subjected to electrophoretic analysis. Peptides were visualized by Coomassie Blue stain (lanes 1 and 3) and autoradiography (lanes 2 and 4).

Table 2 Condensing activity of recombinant wild-type and mutant KAS IIIs

Reaction mixtures containing 100 mM sodium phosphate pH 7.6, 10 μ M [14 C]acetyl-CoA, 20 μ M malonyl-ACP and 2.5 ng of the enzymes indicated were incubated for 5 min at 30 $^{\circ}$ C. Condensing activity was determined as described in the Materials and methods section. Each value represents mean $\pm$ S.D. ($n = 4$). Abbreviation: nd, not detected.

Enzyme form	Specific activity (μ mol $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$)
Wild-type	7.0 $\pm$ 0.1
Cys ¹¹¹ Ser	nd
Cys ¹¹¹ Ala	nd
His ²⁶¹ Ala	nd
His ²⁶¹ Arg	0.8 $\pm$ 0.1
Arg ¹⁵⁰ Ala	1.9 $\pm$ 0.2
Arg ²⁶⁶ Ala	3.5 $\pm$ 0.1
Arg ³⁰⁶ Ala	0.2 $\pm$ 0.0

lower the pK_a of the active-site Cys we converted it to Ala and Arg by site-directed mutagenesis. The mutants were expressed in *E. coli*, purified and assayed for enzyme activity. Mutation to Ala caused a complete loss of KAS III condensing activity, whereas the His²⁶¹Arg mutant was still able to promote the condensation reaction, but at substantially decreased activity when compared to the wild-type (Table 2). When testing the ability of the His²⁶¹Ala and His²⁶¹Arg mutants to bind [14 C]acetyl-CoA at pH 7.6, only the latter became covalently labelled with [14 C]acetate at a level comparable to that of the wild-type enzyme (Figure 4A). These results demonstrated the importance of general-base catalysis to promote nucleophilicity of Cys¹¹¹ in the condensation reaction. Additional evidence was obtained from

Figure 4 Binding of labelled acetate by wild-type and mutant KAS IIIs

Labelling experiments were carried out with [14 C]acetyl-CoA, as described in the Materials and methods section. An aliquot of 5 μ g of each labelled enzyme was analysed by SDS/Tricine/PAGE, electrotransferred on to an Immobilon P membrane and visualized by autoradiography. (A) Labelling of wild-type and mutant KAS IIIs at pH 7.6. (B) pH-dependent labelling of the wild-type KAS III and His²⁶¹Ala mutant. For the pH range from 8.0 to 8.6 100 mM sodium phosphate buffers were applied. Lane 1, wild-type; lane 2, His²⁶¹Ala mutant. (a) pH 8.0; (b) pH 8.3; (c) pH 8.6.

the pH-dependency of this reaction. Whereas wild-type KAS III exhibited an activity optimum between pH 6.8 and 8.6, the activity of the His²⁶¹Arg mutant increased with a shallow slope

Figure 5 pH optima of the wild-type KAS III and the His²⁶¹Arg KAS III mutant

Wild-type (■) and mutant (□) enzymes were assayed for condensing activity in a reaction mixture containing 10 μM [¹⁴C]acetyl-CoA, 20 μM malonyl-ACP and 2.5 ng of wild-type or mutant KAS III in 100 mM buffer (sodium phosphate, Tris/HCl or glycine, for the pH ranges, 5.8–7.6, 7.6–9.4 and 9.4–12.0, respectively). Each value represents the mean of duplicate determinations.

Figure 6 Decarboxylase activity of mutant KAS IIIs

Decarboxylase activity was assayed in a reaction mixture containing 100 mM sodium phosphate, pH 7.6, 10 μM acetyl-CoA, 20 μM [¹⁴C]malonyl-ACP and 20 ng of mutant KAS III (see Materials and methods section). The substrate (malonyl-ACP) and the product (acetyl-ACP) of the reaction were separated by 2.5 M urea/PAGE, transferred to an Immobilon P membrane and visualized by autoradiography. Abbreviations: Mal-ACP, malonyl-ACP; Ac-ACP, acetyl-ACP.

up to pH 9.6, and then steeply up to pH 12 (Figure 5). The His²⁶¹Ala mutant was inactive at all pH values tested. Whether this lack of activity was due to the failure of active site cysteine¹¹¹ to attack the starter substrate, or to catalytic inactivity upon binding the elongation unit still was an open question. Thus wild-type KAS III and the His²⁶¹Ala mutant were assayed for labelling by [¹⁴C]acetyl-CoA at pH values around the pK_a of the Cys¹¹¹-SH group, according to the methods described above. As can be seen in Figure 4(B) the His²⁶¹Ala mutant became labelled and stayed labelled even after SDS/Tricine/PAGE and electroblotting. The incorporation of [¹⁴C]acetate from [¹⁴C]acetyl-CoA into this mutant was 5-fold lower than into wild-type KAS III, but interestingly, the rate of labelling of both enzymes increased as the pH increased from 8.0 to 8.6. The fact that the His²⁶¹Ala mutant lacked overall condensing activity indicated that His²⁶¹ was not only involved in the deprotonation of Cys¹¹¹, but that it also played a role in subsequent catalytic steps.

On the basis of Siggaard-Anderson's mechanistic model for FAS type II condensing enzymes, which involved a total of five

basic residues [17] and the two histidines postulated on the basis of the crystal structure of *E. coli* KAS II [18], we probed for further basic residues that could be involved in the reaction mechanism of KAS III, beyond His²⁶¹ which has already been identified. Thus we observed loss of activity upon treatment of the enzyme with arginine-specific phenylglyoxal (results not shown). A comparison with published KAS III sequences indicated Arg¹⁵⁰, Arg²⁶⁶ and Arg³⁰⁶ to be conserved residues. These residues were then subjected to alanine scanning, resulting in a substantial decrease of KAS III condensing activity in the case of Arg¹⁵⁰Ala and Arg³⁰⁶Ala (27 % and 3 % residual activity, respectively, Table 2), despite the fact that both mutants had fully conserved secondary structures. In contrast the 50 % residual activity observed with the Arg²⁶⁶Ala mutant correlated with the perturbation of the secondary structure (Figure 1). Thus only Arg¹⁵⁰ and Arg³⁰⁶ might be active in KAS III catalysis.

Induction of decarboxylase activity

While investigating the reaction products of the attempted condensation reactions catalysed by the Cys¹¹¹Ser and Cys¹¹¹Ala mutants, we observed decarboxylation of malonyl-ACP to acetyl-ACP only in the case of the Cys¹¹¹Ser mutant (Figure 6). This prompted us to take a close look into the role of His²⁶¹ in the decarboxylation reaction, since we knew already of the importance of this residue in KAS III catalysis. Two double mutants, Cys¹¹¹Ser/His²⁶¹Ala and Cys¹¹¹Ser/His²⁶¹Arg, incapable of catalysing the condensation reaction, were generated and incubated with [2-¹⁴C]malonyl-ACP in the presence of acetyl-CoA. As shown in Figure 6 decarboxylation of malonyl-ACP could be attained with the double mutant Cys¹¹¹Ser/His²⁶¹Arg only.

DISCUSSION

We could show here *in vitro* that recombinant *C. wrightii* KAS III not only accepted acetyl-CoA, but also propionyl- and butyryl-CoA as primers in the first condensing step of *de novo* fatty acid synthesis; this offers a tool for engineering odd-numbered fatty acids in plants. Yet recombinant *C. wrightii* KAS III was also able to promote the elongation of short-chain acetyl- and butyryl-ACPs, although with low activities; the respective products were not products of an acyl-CoA:ACP transacylase activity, since none of acetyl-CoA, CoA-SH, or ACP was present in the reaction mixture. Recent findings that cell-free seed extracts from *C. lanceolata* [11] and *C. wrightii* [31] could promote the elongation of butyryl-ACP in the presence of high concentrations of cerulenin can now be attributed in part to this extraordinary elongation. It is interesting to note that *E. coli* KAS III, also a FAS type II enzyme, also accepts short-chain acyl-CoAs, but not acyl-ACPs [32–34].

The mechanism of catalysis used by the condensing enzymes in general is thought to involve the action of an active site cysteine [5,12,13,17,18], which would correspond to Cys¹¹¹ in recombinant KAS III from *C. wrightii* embryo. Exactly this Cys became labelled upon incubation with [1-¹⁴C]acetyl-CoA in our experiments, and its substitution by Ser and Ala resulted in a complete loss of condensing activity of the enzyme. However, the replacement of the Cys¹¹¹ sulphur nucleophile by the Ser oxygen nucleophile did not affect the ability of this KAS III mutant to covalently bind [1-¹⁴C]acetate. A precedent for this phenomenon was reported for biosynthetic thiolase from *Zoogloea ramigera* [35], where a mutant thiolase, having the active site cysteine replaced by serine, lacked condensing activity but retained self-acetylation activity with acetyl-CoA as substrate. This phenom-

Scheme 1 Schematic presentation of the KAS III reaction

Cys¹¹¹ and His²⁶¹ are part of the active site. Two amino acid residues, B1 and B2, could also be needed for acid–base catalysis. According to the proposed model, the active cysteine thiol group, assisted by general-base catalysis of the active site His²⁶¹, makes an attack on the substrate acetyl-CoA, yielding an acyl-enzyme intermediate (**A**). The imidazolium ion generated during the formation of the acyl-enzyme intermediate is stabilized by the carboxylic acid group of malonyl-ACP, which is then positioned and decarboxylated by the same His²⁶¹, now acting as a general-acid catalyst (**B**). The condensing reaction occurs by a nucleophilic attack of the carbanion on the enzyme-bound carbonyl ester. The bond to the cysteine residue is broken to yield the product and the regenerated enzyme (**C**).

enon, observed in the two examples discussed here, can be explained by the overall rate of acyl-enzyme complex formation, which would be expected to be faster for the thiol enzyme due to the enhanced nucleophilicity of sulphur as compared with oxygen. This is consistent with model studies, which indicate that thiol groups are better nucleophiles than alcohols in displacement reactions at carbonyl carbons with good leaving groups [35–37].

Our kinetic data indicated that KAS III catalysed reactions follow a Ping Pong mechanism (results not shown), in which after binding the first substrate (acetyl-CoA) a first product (reduced CoA) is released before binding the second substrate. This was borne out by our observation that the acetylated Cys¹¹¹Ser was able to decarboxylate malonyl-ACP, i.e., after formation of the acetyl-enzyme complex and release of reduced CoA. This agreed also with the induction of decarboxylase activity in the case of yeast FAS with its active-site cysteine occupied by substrate or alkylated with iodoacetamide [12]. Decarboxylase activity was also observed with KAS I and KAS II from *C. lanceolata*, but in this case decarboxylation of malonyl-ACP was a consequence of 3-oxoacyl-ACP accumulation, the direct product of the condensing reaction [38].

The nucleophilic attack of Cys¹¹¹ on acetyl-CoA should be facilitated, or even made possible, when a general base lowers the pK_a of the cysteine residue's thiol group. Such a residue was found in His²⁶¹. Indeed, the requirement for general base action was demonstrated by the fact that, at pH 7.6, the His²⁶¹Ala substitution in KAS III completely abolished the binding of the first substrate and thus the overall condensing activity, whereas the His²⁶¹Arg mutant did bind acetate and was enzymically active. The results in Figure 4(B) indicated that, by virtue of being a potential nucleophile, the active-site Cys¹¹¹ of the His²⁶¹Ala mutant was capable of retaining enough reactivity at pH values ≥ 8.0 to attack the substrate for the formation of an acyl-enzyme intermediate even in the absence of His²⁶¹, due to pH-dependent deprotonation of Cys¹¹¹ (pK_a 8.3). The lack of condensing activity in the His²⁶¹Ala mutant, despite its ability to bind covalently acetate, even at higher pH, revealed the involvement of His²⁶¹ not only in proton abstraction from the Cys¹¹¹-thiol, but also in subsequent steps in the overall condensing reaction (see below).

The Cys¹¹¹Ser mutant, which was catalytically active in the decarboxylation of malonyl-ACP to acetyl-ACP, provided an ideal educt to produce the Cys¹¹¹Ser/His²⁶¹Ala and Cys¹¹¹Ser/His²⁶¹Arg double-mutants to study the first two steps in the overall condensing reaction (acetyl-enzyme formation and binding plus decarboxylation of malonyl-ACP) independently. The fact that the former double-mutant did not and the latter did promote decarboxylation of malonyl-ACP clearly established His²⁶¹ as part of the catalytic machinery for the liberation of CO₂, in this case functioning as a general acid.

Taken together, our approach allowed us to dissect the condensing reaction of KAS III from *C. wrightii* embryo into the formation of a covalent acetyl-enzyme complex, the decarboxylation of malonyl-ACP and the subsequent carbon–carbon ligation to complete the overall condensation. A mechanism of catalysis can now be envisaged (Scheme 1), whereby the formation of the acetyl-enzyme intermediate occurs through nucleophilic attack of the Cys¹¹¹ on acetyl-CoA assisted by general-base catalysis of His²⁶¹ with the release of reduced CoA (Panel A). The imidazolium ion generated during the formation of the acetyl-enzyme intermediate serves as a recognition center for malonyl-ACP and is stabilized by the carboxylate of malonyl-ACP, which is then decarboxylated to the carbanion of acetyl-ACP by His²⁶¹, now acting as a general-acid catalyst (Panel B). Then the new carbon–carbon bond is created by nucleophilic attack of the carbanion on the enzyme-bound carbonyl thioester function of the acetyl group. The bond to the cysteine residue is then broken to yield the 3-oxobutryl-ACP product and regenerated enzyme (Panel C). The proposed mechanism involves two further amino acids for acid–base catalysis, i.e. B1, which stabilizes the carbanion intermediate generated by decarboxylation of malonyl-ACP and B2, which enhances the electrophilicity of the enzyme-bound carbonyl ester function prior to condensation. Our inhibition studies with phenylglyoxal and alanine scanning indicated Arg¹⁵⁰ and Arg³⁰⁶ as candidates for these roles. Alternatively, they may take part in the recognition of the protein part of malonyl-ACP.

The recent data on the crystal structure of *E. coli* KAS II revealed the involvement of an active-site cysteine residue (Cys¹⁶³ corresponding to Cys¹¹¹ in KAS III) and two histidine residues

(His³⁰³ and His³⁴⁰) in the catalytic machinery of this FAS type II enzyme [18]. One of these histidine residues, His³⁴⁰ (corresponding to His²⁶¹ in KAS III; alignment by the HUSAR program, Heidelberg, Germany), is conserved and is supposed to have a general-base catalytic function similar to that found by us for KAS III, indicating that the Cys–His pair action could be a general catalytic diad of condensing enzymes as a whole. His³⁰³ in *E. coli* KAS II was supposed to play a role in malonyl-ACP decarboxylation [18], however, a corresponding residue in *C. wrightii* KAS III could not be identified. Since the primary structures of the two enzymes share only 17% identity, a reliable model of KAS III tertiary structure on the basis of *E. coli* KAS II crystal data could not be constructed. However, our mechanistic model ascribes to His²⁶¹ of *C. wrightii* KAS III general base (for acetate binding) and general acid (for malonyl-ACP decarboxylation) properties, a double function that is based on experimental evidence. Absence of this histidine residue led to a shift in the pH optimum for acetate binding, and condensing activity was to be expected in more alkaline conditions. This was not observed, however, due to the lack of general acid function exerted by His²⁶¹ necessary for decarboxylation of malonyl-ACP.

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