Identification and Molecular Characterization of the β-Ketoacyl-[Acyl Carrier Protein] Synthase Component of the Arabidopsis Mitochondrial Fatty Acid Synthase*†

Rie Yasunod, Penny von Wettstein-Knowles§, and Hajime Wada¶

From the ‡Department of Life Sciences, Graduate School of Arts and Sciences, University of Tokyo, Komaba 3-8-1, Meguro-ku, Tokyo 153-8902, Japan and the §Department of Genetics, Molecular Biology Institute, University of Copenhagen, Oester Farimagsgade 2A, DK-1353 Copenhagen K, Denmark

Substrate specificity of condensing enzymes is a predominant factor determining the nature of fatty acyl chains synthesized by type II fatty acid synthase (FAS) enzyme complexes composed of discrete enzymes. The gene (mtKAS) encoding the condensing enzyme, β-ketoacyl-[acyl carrier protein] (ACP) synthase (KAS), constituent of the mitochondrial FAS was cloned from Arabidopsis thaliana, and its product was purified and characterized. The mtKAS cDNA complemented the KAS II defect in the E. coli CY244 strain mutated in both fabB and fabF encoding KAS I and KAS II, respectively, demonstrating its ability to catalyze the condensation reaction in fatty acid synthesis. In vitro assays using extracts of CY244 containing all E. coli FAS components, except that KAS I and II were replaced by mtKAS, gave C4–C18 fatty acids exhibiting a bimodal distribution with peaks at C8 and C14–C16. Previously observed bimodal distributions obtained using mitochondrial extracts appear attributable to the mtKAS enzyme in the extracts. Although the mtKAS sequence is most similar to that of bacterial KAS IIIs, sensitivity of mtKAS to the antibiotic cerulenol that of E. coli KAS I. In the first or priming condensation reaction of de novo fatty acid synthesis, purified His-tagged mtKAS efficiently utilized malonyl-ACP, but not acetyl-CoA as primer substrate. Intracellular targeting using green fluorescent protein, Western blot, and deletion analyses identified an N-terminal signal conveying mtKAS into mitochondria. Thus, mtKAS with its deletion analyses identified an N-terminal signal conveying using green fluorescent protein, Western blot, and deletion analyses identified an N-terminal signal conveying mtKAS into mitochondria. This is accomplished by FAS start with condensation of the acyl primer substrate with the C2-unit to give a β-ketoacyl-ACP. The introduced β-keto group is then removed by three reactions, a β-keto reduction, a β-dehydration, and an enoyl reduction. The resulting saturated acyl-ACP serves as a substrate for the next extension. Most frequently seven or eight cycles yield palmitoyl (C16)-ACP and stearoyl (C18)-ACP, respectively. Additional FAS activities transfer the acyl chains to other compounds depending upon their final destination; for example, acyltransferases and thioesterases channel C16 and C18 fatty acids to membrane lipids.

The β-ketoacyl-ACP synthase (KAS) components of the type II FAS enzyme complexes present in bacteria and plant plastids carry out the condensation steps in fatty acid synthesis using a Claisen reaction consisting of three parts: (i) transfer of acyl bound acyl primer substrate to the active site cysteine of KAS, (ii) decarboxylation of malonyl-ACP to form the acetyl-ACP carbamation, and (iii) condensation of the carbamation with the carboxyl carbon of the acyl primer substrate. As shown in Fig. 1 (A and B), three KAS enzymes contribute to construction of fatty acyl chains. KAS III is singular in using a CoA-activated primer substrate, acetyl-CoA, for the initial condensation with a C2-unit (5, 6). All the subsequent extensions are with ACP-activated acyl chains. They are carried out by KAS I and II, which differ in their substrate specificities. In plastids (Fig. IA) KAS I utilizes butyryl (C4)- to myristoyl (C14)-ACP’s as substrates, and KAS II executes the last step to yield C15-ACP (7). KAS IV enzymes in some seeds, such as those from Cuphea sp., show a marked preference for medium (C6–C14) acyl chains (8).

Fatty acids are synthesized by the enzymatic reactions of acetyl-CoA carboxylase (ACCase)3 and fatty acid synthase (FAS). FAS enzyme complexes are classified into two groups based on their structural forms and organization. Complexes consisting of multifunctional polypeptides encoded by one or two genes (type I) are present in the cytoplasm of animals and fungi (1, 2), whereas those composed of monofunctional enzymes (type II) are present in most bacteria and plant plastids (3, 4) as well as in mitochondria, as will be detailed below. The incipient reaction of fatty acid synthesis is catalyzed by ACCase to form malonyl-CoA from acetyl-CoA, the initial carbon source. The first FAS activity, malonyl-CoA:ACP transacylase (MCAT), transfers the malonyl group from CoA to acyl carrier protein (ACP) to form the donor substrate malonyl-ACP that provides the C2-units for elongation. Repetitive elongation cycles accomplished by FAS start with condensation of the acyl primer substrate with the C2-unit to give a β-ketoacyl-ACP. The introduced β-keto group is then removed by three reactions, a β-keto reduction, a β-dehydration, and an enoyl reduction. The resulting saturated acyl-ACP serves as a substrate for the next extension. Most frequently seven or eight cycles yield palmitoyl (C16)-ACP and stearoyl (C18)-ACP, respectively. Additional FAS activities transfer the acyl chains to other compounds depending upon their final destination; for example, acyltransferases and thioesterases channel C16 and C18 fatty acids to membrane lipids.

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4 The online version of this article (available at http://www.jbc.org) contains Supplemental Fig. 1.

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In *Escherichia coli* KAS I and II carry out all the elongations starting with C_4-ACP, with the exceptions that KAS I is unique for the initial step and KAS II for the final step in the pathway for unsaturated fatty acids (Fig. 1B) (9). Although double bonds are inserted into the growing acyl chain in *E. coli*, they are inserted by desaturases into the synthesized acyl chains in plastids. Mutants of *fabF* encoding KAS II have no phenotype as the absence of cis-vaccenoyl (C_{18:1} cis Δ11) acyl chains is compensated for by increased amounts of palmitoleoyl (C_{16:1} cis Δ9) acyl chains (Fig. 1B) (10). In combination with mutants of *fabB* encoding KAS I, KAS II defects are revealed by the failure of the double mutant to grow in the presence of oleic acid (C_{18:1} cis Δ9) (9).

ACP, a protein cofactor, is one of the best characterized of the FAS components, and carries the acyl chains through the FAS reactions. That ACP functions in additional pathways has been recognized for some time, for example, in polyketide synthesis reactions in mitochondrial fatty acid synthesis. The mtKAS component of a type II FAS, namely the condensing enzyme that catalyzes all the condensation reactions in mitochondrial fatty acid synthesis. The mtKAS had an N-terminal mitochondrial target sequence and its cDNA complemented the *fabF* mutation in the *E. coli* strain CY244. Assays of purified mtKAS revealed that malonyl-ACP reductase (KR), respectively, but definitive proof of their role in mitochondrial fatty acid synthesis has not been obtained. mtACP-1 is imported into mitochondria and can function as a cofactor in fatty acid synthesis (16). No candidate genes for the β-hydroxyacyl-ACP dehydrase have yet been isolated. During the final stages of preparing this report, a publication appeared characterizing human ACP and MCAT proteins that were targeted to mitochondria and had the requisite activities to function in fatty acid synthesis (24).

In the present study, we have purified and characterized a mitochondrial FAS constitutent, namely the condensing enzyme (mtKAS) of *A. thaliana* that catalyzes all the condensation reactions in mitochondrial fatty acid synthesis. The mtKAS had an N-terminal mitochondrial target sequence and its cDNA complemented the *fabF* mutation in the *E. coli* strain CY244. Assays of purified mtKAS revealed that malonyl-ACP served as both the primer and donor substrates for de novo fatty acid synthesis, explaining the absence of KAS III in mitochondria. Moreover, the fatty acids synthesized in a heterologous system exhibited an unusual bimodal distribution that was attributable to the mtKAS component of a type II FAS.

**EXPERIMENTAL PROCEDURES**

*Plant Material—* *A. thaliana* (Columbia ecotype) was grown on vermiculite under continuous light at a photon flux density of 40 μmol m^{-2}s^{-1} at 25 °C. Bright yellow-2 (BY-2) cells of tobacco (*Nicotiana tabacum*) were obtained from Dr. T. Nagata (University of Tokyo, Tokyo, Japan). The cells were grown in modified Linsmaier and Skoog medium as described by Nagata et al. (25).

Construction of a cDNA Library and Cloning of the mtKAS cDNA

Using Polymerase Chain Reaction (PCR)—Poly(A)^+ RNAs were isolated from leaves and roots of 3-week-old *A. thaliana* with an mRNA purification kit (Amersham Biosciences). The cDNA library was constructed with cDNAs, which were prepared from the poly(A)^+ RNAs with a
cDNA synthesis kit (Amersham Biosciences), and a phage vector bgt11 (Stratagene) according to the protocols from the manufacturer. A phage DNAs prepared from this library served as templates for PCR. The primers, 5'-TGGCCGTTAAGCGCTTCATCT-3' and 5'-TGCTGCTTAACTACATCT-3', were designed to amplify the central region of a predicted mtKAS cDNA derived from T103.5. The 5'-terminal region of mtKAS cDNA was amplified using the primers, 5'-TTGACACCAGCACACGGAGGAAGGTCCTT-3' and 5'-CCGTGTTG-3', that anneal to the cloning site of bgt11 and to an internal region of the deduced mtKAS cDNA, respectively. The 5'-terminal region of mtKAS cDNA was amplified by the 5'-rapid amplification of cDNA ends method (5'-Full RACE Core Set from Takara). The phosphorylated primer, 5'-GACAACCTGCTTATAGGAGC-3', was used for synthesis of cDNA using total RNA isolated from A. thaliana grown on Murashige and Skoog (MS) media with a polyvinylpyrrolidone (RNeasy plant mini kit from Qiangen). The following protocols were used for the first and second PCR in the 5'-rapid amplification of cDNA ends, respectively: (i) 5'-TTTGGGGCTTTATGGAATCAACTTCTC-3' and 5'-GCACGAGTCTTAAAGAAAGTGA-3', (ii) 5'-GGTTAAGGAATAGTGAACCCCT-3' and 5'-AGAGTCAATCTCTTATCATTCC-5'. Each of the three amplified fragments was subcloned into pCRII (Orig. TA cloning kit; Invitrogen), and its nucleotide sequence was determined. Combining the nucleotide sequences of the 5'-, internal, and 3'-regions gave a full-length cDNA of the predicted A. thaliana mtKAS of 1,566 bp. Homology Search and Phylogenetic Analysis—Sequences homologous to the predicted sequence of mtKAS were retrieved using a BLAST search at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). These sequences from bacteria, plant plastids, yeast, fungi, and animals were aligned with that of mtKAS using CLUSTAL X version 1.8 (26), followed by adjustment with the naked eye. The known or deduced N-terminal regions corresponding to transit peptides and internal gaps were removed prior to analysis. Distance matrices were generated with the PROTDIST program in PHYLIP version 3.57c (27) using the PAM matrix of Dayhoff. Sequences homologous to mtKAS were obtained from 1,566 bp of 1,566 bp. The reactions were stopped by addition of 1 ml of cold 5% trichloroacetic acid. The precipitated acyl-ACP's were collected by centrifugation, resolved by 1,3, or 4 M urea-PAGE on the basis of the chain length and presence of double bonds, electroblotted to a PVDF membrane, and subjected to autoradiography using a Phosphorimager as described (30). 3'-C-Labeled standards included C12-ACP (30), acetyl- and malonyl-ACPs prepared as described below, plus C12-ACP prepared using a labeled C12 fatty acid (Amersham Biosciences) and acyl-acetyl synthase purified as described (36) according to Rock and Cronan (37). Identity of additional acyl-ACPs was deduced based on previous work (31). The expression of the sensitive ESK enzymes to cerulenin was investigated as previously described (31) with modifications. Cerulenin (Sigma) (0–10 nmol) dissolved in ethanol was transferred to Eppendorf tubes and dried under vacuum. Soluble protein extracts (10 μl) were added to the tubes. After standing on ice for 15 min, 90 μl of the reaction mixture was added and the assay carried out as described above. Characterization of the First Condensation Reaction—The mtKAS Δ20 cDNA was re-cloned into pQE-30 (Qiagen). This resulted in a N-terminal sequence in which Met-Gly was replaced with Arg-Gly-Ser plus a His tag of six residues followed by Gly-Ser-Met (38). Expression and protein purification using Ni-NTA technology were carried out as detailed previously (39, 38) with overnight induction and using only one step of detergent depletion. The protein extract was divided into two C units, each 30% of the total yield. The ability of the Ni-NTA-purified mtKAS enzyme to initiate fatty acid synthesis by joining two C units together in the first condensation reaction, an assay was developed. The assay also reveals whether the primer substrate is acetyl-CoA or acetyl-ACP. ACP was pre-incubated (30) in the presence of DTT and then mixed with the other components of the assay mixture. The 100-μl assay mixture consisting of 10 μM ACP, 0–120 μM acetyl-CoA ÷ 22–123 kdpm [1-14C]acetyl-CoA (Amersham Biosciences, specific activity 2.18 GBq/ mmol), 10.1 μM malonyl-CoA ÷ 10 kdpm [2-14C]malonyl-CoA (PerkinElmer Life Sciences, specific activity 2.2 GBq/mmol), 1 mM DTT, 50 mM potassium phosphate buffer (pH 6.8), as well as 1.2 μg of the Ni-NTA-purified mtKAS, or 0.8 μg of the enzyme, with or without 2.5 μg of the protein (KR) (38) and MCAT was incubated for 15 min at 37 °C. Acyl-ACP products were analyzed as described above. Radio-labeled acetyl- and malonyl-ACP standards were prepared by carrying out decarboxylase assays in the presence and absence of E. coli KAS I mutant protein that has a substitution of amino acid Cys-183 with Ala and a functionally decarboxylase-deficient mtKAS or KAS II of E. coli. Northern and Reverse Transcription (RT)-PCR Analyses—Poly(A) + RNAs used for Northern blot analysis were extracted from 2-week-old A. thaliana leaves using an mRNA purification kit (Amersham Biosciences). Approximately 3 μg of poly(A)+ RNAs were separated by electrophoresis in a 1% (w/v) agarose gel, and transferred to a nylon membrane. cDNA probes were labeled by nick-translation, nick-translation, and hybridization detection were performed using a DNA labeling and detection system (ECL kit; Amersham Biosciences). Total RNAs used for RT-PCR were extracted from leaves, roots, and flowers of 4-week-old A. thaliana with an RNA extraction kit (RNAeasy Plant Mini Kit; Qiagen). The primer...
set used for amplifying and cloning of the mtKAS-Δ20 sequence was used for RT-PCR as described before (39). As a control the expression of the gene for α-tubulin of A. thaliana (40) was also checked with the same RNA preparations using the primer set 5′-CTACTGAGAGAGATGGCAG-3′ and 5′-CAACATCTCTCCTGTTACTCATC-3′.

Expression of mtKAS-Δ20 in E. coli and Antibody Production—The mtKAS-Δ20 sequence was amplified using the primer set 5′-GCCCATGGCATCATCTCCATGG-3′ and 5′-GCCCATGGATAGATAAGGCAAAAGA-3′. In constructing these primers, the sequence 5′-GCCCATGG-3′ including an Ncol site was added to the 5′ end of each primer. The obtained PCR products were digested with Ncol and ligated into the same site of the expression vector pET-30a (+) (Novagen). The obtained plasmid was used to transform E. coli BL21(DE3) (41).

Construction and Visualization of mtKAS-Green Fluorescent Protein (GFP) Fusion Protein—The mtKAS cDNA sequence encoding the 5′ N-terminal residues was amplified by PCR with the primer set 5′-GCG-TGTGACATGGCAGATATACCTGCTGTA-3′ and 5′-CATGGCATGTGGTTTCCACGCTCTACCAAG-3′. The sequences 5′-GCCCATG-3′ or 5′-CATGGCATG-3′, including a SalI site and an Ncol site, respectively, were added to the 5′ end of each primer. The PCR product was digested with SalI and Ncol, and ligated into the SalI-Ncol site of the CaMV35S-eGFP (S65T)-nos3′ plasmid, designated pGFP (42). The obtained recombinant plasmid pmtKASpGFP and pGFP were introduced into tobacco BY-2 cells prepared from a 5-day-old culture, with a particle bombardment device (PDS-1000/He Biolistics® Particle Delivery System; Bio-Rad) according to instructions from the manufacturer. The applied conditions were 1,100 p.s.i. of helium gas pressure, a distance of 9 cm from macrocarrier to cell suspension, and a decompression vacuum of 28 inches Hg. Tungsten particles (1.1 μm) were used as a carrier of plasmid DNAs. The bombarded cells were incubated at room temperature under dark overnight and stained with a mitochondrion-selective probe (Mito Tracker® Red CM-H_2Xros; Molecular Probes) following the recommendations from the manufacturer. Cells were examined with a confocal laser scan microscope (LSM410; Carl Zeiss). Excitation wavelengths were set at 488 nm for GFP and at 543 nm for Mito Tracker Red. All obtained images were processed by Adobe Photoshop software.

RESULTS

Isolation of a cDNA Encoding mtKAS—The T1O3.5 sequence in GenBankTM was previously suggested to code for a KAS participating in mitochondrial fatty acid synthesis in A. thaliana (43). To confirm this hypothesis, we cloned and characterized a cDNA, designated mtKAS, corresponding to T1O3.5. The mtKAS cDNA contained an open reading frame of 1,383 bp encoding a polypeptide of 461 amino acids (molecular mass 49,379 Da). Comparison of the sequence of the cDNA and T1O3.5 revealed that mtKAS is composed of 14 exons and 13 introns. Southern blot analysis confirmed that mtKAS is a single-copy gene in the A. thaliana genome (data not shown), as predicted from the genome sequence. The deduced amino acid sequence of mtKAS is compared with those of KAS I and KAS II of E. coli and A. thaliana plasids in Fig. 2. Residues 28–461 of mtKAS share 36 and 49% identities with E. coli KAS I and II, and 39 and 40% with A. thaliana KAS I and II, respectively. Comparison with other KAS I and II isozymes of bacteria and plant plastids disclosed greater identities with bacterial KAS II enzymes (~50%) than with bacterial KAS I and plant plastidial KAS I and II enzymes (~40%). Potential active site residues, Cys-209 and two histidines, His-350 and His-389, requisite for catalysis are present in the mtKAS sequence, placing it in the CHH group of KAS enzymes to which KAS I and II belong (46).

To examine the effect of the N-terminal extension on the activity of mtKAS, complementation tests with CY244 were carried out with mtKAS cDNAs encoding mtKAS having a full-length N-terminal extension and four N-terminal deletions. Comparative growth of transformants is shown in Fig. 3B. Transformants with pmtKAS-Δ0 (full-length), pmtKAS-Δ20, and pmtKAS-Δ27 grew well, whereas those with pmtKAS-Δ30 and pmtKAS-Δ34 showed at best only the same faint growth exhibited by the empty vector pKK233-2 on oleic acid containing medium at 42 °C. Western blot analysis of soluble extracts of all the transformants readily detected mtKAS proteins corresponding in size to Δ0, Δ20, and Δ27, but none in the vector Δ30 and Δ34 extracts (data not shown). These results demonstrate that the 27 N-terminal residues of mtKAS are not necessary for the function of mtKAS, and that when 30 or more residues are deleted the translated protein is unstable. Thus, for a functional mtKAS, at least three residues need to precede Val-33, which corresponds to the first residue of the first β-strand in the α-β-α-β-α core structure of the E. coli KAS I and
FIG. 2. Comparison of the amino acid sequences of KAS enzymes. The alignment shows the deduced amino acid sequences of *A. thaliana* mtKAS, KAS I (44), and KAS II (45), and those of *E. coli* KAS I (33) and II (31). The amino acid residues conserved in all sequences are indicated by asterisks. The Cys, His, Lys, and His residues participating in catalysis in the CHH group of KAS enzyme are highlighted with gray. Hyphens represent gaps in the alignment of sequences. The arrows labeled with Δ plus numbers represent number of N-terminal residues removed from mtKAS to delineate the primary structure of mature mtKAS.
KAS II enzymes (47, 49). Our experimental results reveal that the PSORT prediction of a cleavage site between Gly-47 and Val-48 for the Arabidopsis mtKAS target sequence, which is just after the loop following the first β-sheet in the core structure, is incorrect. Presumably the analogous PSORT predictions for the deduced sequences of human and mouse mtKAS are also incorrect.

**Synthesis of Acyl-ACPs by mtKAS in Crude Extracts of E. coli CY244**—To investigate the ability of the mtKAS to carry out the condensation reactions, crude soluble protein extracts prepared from the CY244 transformants grown at 42 °C in the presence of oleic acid were used for elongation assays. The control transformant with the vector pKK233-2 was grown at 30 °C. Acetyl-CoA and [2-14C]malonyl-CoA were incubated as potential substrates with each extract. Labeled acyl-ACPs produced during a 30-min incubation at 42 °C were separated by electrophoresis with a polyacrylamide gel containing 2 M urea, electroblotted to PVDF membrane, and visualized by autoradiography. Only acetyl- and malonyl-ACPs were detected in the extract of the vector control (Fig. 4A, lane 1). Malonyl-ACP was generated by transfer of the malonyl group from malonyl-CoA to ACP by MCAT, and acetyl-ACP by decarboxylation of malonyl-ACP by KAS III. Both MCAT and KAS III are present in E. coli soluble protein extracts. The pmtKAS-S20 transformant extract, by contrast, synthesized a series of saturated acyl-ACPs with up to 18 carbons (Fig. 4B, lane 2). Of these, medium chain C8- and C14-ACPs plus the long chain C16-ACP were detected as major bands, resulting in a bimodal distribution. With the pFabB and pFabF transformant extracts, as expected, predominantly long chain acyl-ACPs, both saturated and unsaturated, were produced (Fig. 4B, lanes 3 and 4). These results demonstrate that mtKAS can catalyze many of the condensation reactions in fatty acid synthesis, and that it is compatible with all relative components of the E. coli FAS. Whether mtKAS is able to carry out the first condensation step to yield C2-ACP was unresolved, however, because of the presence of E. coli KAS III in the extracts.

Cerulenic, known as an irreversible inhibitor of type II FAS, binds to the active site cysteine residue of KAS I and II enzymes, and blocks their condensation activities (33, 50). Although plastidial and bacterial KAS I enzymes are more sensitive to cerulenic than KAS IIs, the structural basis for this difference is not yet known (46). To check the effect of cerulenic on the activity of mtKAS, extracts of CY244 transformants were incubated in the presence of 0–10 nmol of cerulenic before adding the substrates. As shown in Fig. 4C (lane 2 versus lane 3), mtKAS activity was almost completely inhibited by the addition of 1 nmol of cerulenic. Thus, it is at least as sensitive to cerulenic as KAS I of E. coli (lanes 5–7 versus lanes 2–4). Treating E. coli KAS II with 5 nmol of cerulenic (lane 9) had a little effect on saturated fatty acid synthesis compared with that on mtKAS and E. coli KAS I (lanes 4 and 7) as expected. Even with 10 nmol of cerulenic, some elongation still occurred (lane 10). The present results disclose that mtKAS is very sensitive to cerulenic and imply that Cys-209 is essential for catalysis.

The mtKAS and E. coli KAS I and II proteins expressed in CY244 were unstable in the crude extracts during storage at 0 °C in break buffer, which lacks glycerol. This was most marked for mtKAS, which initially synthesized prominent amounts of C14 and C16 acyl chains (Fig. 4B, lane 2). After being stored for a week at −20 °C, however, mtKAS synthesized predominantly C8 acyl chains (Fig. 4C, lane 2), an ability that was maintained for at least 8 weeks of storage (data not shown).

The First Condensation Reaction Carried out by mtKAS—To examine the ability of mtKAS to carry out the first condensation reaction, the mtKAS-S20 protein was purified with aid of a His tag. This is the first report to our knowledge of a plant CHH type KAS being readily purified as an active enzyme. As shown in Fig. 5A, the activity assay was designed to reveal whether mtKAS could condense two C2-units to give a C4 acyl chain, and if so whether the primer substrate was activated by CoA as characteristic for KAS III or by ACP as characteristic for KAS I and II. In the course of the assay, malonyl-ACP is generated from malonyl-CoA by MCAT. Decarboxylation of malonyl-ACP to acetyl-ACP, the donor substrate, is the second part of the tripartite KAS reaction. Decarboxylation assays using similar concentrations of mtKAS as in the present assay revealed an activity similar to that exhibited by E. coli KAS I (data not shown and Ref. 48). Acetyl- and malonyl-ACP standards are shown in lanes 1, 2, 10, and 11. In the final step of the KAS reaction, β-ketobutyryl (β-keto C4)-ACP is synthesized if labeled malonyl-CoA is present in the reaction mixture as illustrated for mtKAS (lanes 3–7). The addition of KR and NADPH to the assay resulted in the reduction of the β-keto C2-ACP to β-hydroxybutyryl (β-OH C4)-ACP (lanes 3–5). The latter was not formed if either KR or the cofactor NADPH upon which it is dependent was omitted (lanes 6 and 7). Why the reduction is not more efficient is unknown. A 4-fold increase in KR had no effect on the reduction of β-OH C4-ACP (data not shown). The presence or absence of unlabeled acetyl-CoA had no noticeable effect on the production of β-OH C4-ACP (lanes 3–5), and incorporation of radioactivity into β-OH C4-ACP from [2-14C]acetyl-CoA was not detected under the tested conditions (lanes 8 and 9). These results demonstrate that (i) mtKAS possesses the ability to carry out the first condensation reaction and (ii) malonyl-ACP is a sole carbon source for the first condensation reaction catalyzed by mtKAS, i.e. it provides both the primer and donor substrates.

The same series of assays was carried out using a purified His-tagged E. coli KAS II (45) in place of mtKAS (Fig. 5B). Two interesting differences emerged. First, only traces of β-ketoacyl C2-ACP were detectable as predicted from its known instability (lanes 3–5). Second, acetyl-CoA can serve as the primer substrate for KAS II albeit inefficiently, as witnessed by a minor amount of β-OH C4-ACP (lanes 8 and 9). This band was absent.
if either KR or NADPH was omitted from the assay mixture (lanes 6 and 7).

Expression of mtKAS in A. thaliana—The size of the transcript of the mtKAS gene was checked by Northern blot analysis using poly(A)⁺ RNA prepared from 2-week-old A. thaliana leaves. A single hybridizing signal was detected at the position...
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Fig. 6. RT-PCR analysis of the A. thaliana mtKAS gene. RT-PCR analysis monitors the expression of mtKAS (upper panel) compared with that of the housekeeping gene for α-tubulin (lower panel) used as a control. Total RNAs extracted from leaves (lane 1), roots (lane 2), and flowers (lane 3) served as templates for RT-PCR.

of 1,600 nucleotides, which was close to the size of the 1,566 nucleotides of the cloned mtKAS cDNA (data not shown). This discrepancy in size presumably arises from the absence of the 3′ downstream region and poly(A)′ tail in the cDNA.

To estimate the expression level of the mtKAS gene in organs, RT-PCR analysis was carried out using total RNAs prepared from leaves, roots, and flowers. In all tested organs, a DNA fragment corresponding to mtKAS cDNA was detected at relatively similar levels (Fig. 6, upper panel) as was the control gene for α-tubulin (Fig. 6, lower panel). These results suggest that the mtKAS gene is expressed at the same level in these three organs.

Intracellular Localization of mtKAS in A. thaliana—As mentioned above, mtKAS has a putative mitochondrial transit peptide on its N terminus, implying its presence in this organelle. To explore this possibility, we prepared cytosolic (Fig. 7, lane 1), microsomal (lane 2), chloroplast (lane 3), and mitochondrial (lane 4) fractions from A. thaliana leaves and investigated the intracellular localization by Western blot analysis using an antibody against mtKAS. A single band at ~47 kDa corresponding in size to the mtKAS lacking ~20 residues was detected only in the mitochondrial fraction (Fig. 7, upper panel). To check the purity of the mitochondrial proteins, the same fractions were analyzed with an antibody against pea H-protein, a subunit of the glycine decarboxylase complex (glycine cleavage system) present in mitochondrial matrix (51). The lower panel in Fig. 7 shows that H-protein was primarily in the mitochondrial fraction. These results support the contention that mtKAS is located in mitochondria.

To provide further evidence of the mtKAS target site, we prepared a recombinant plasmid that encodes the 50 N-terminal residues of mtKAS fused to GFP. This recombinant plasmid, pmmtKAS-GFP, was introduced into BY-2 tobacco cells by particle bombardment. As a control, the same construct lacking the mtKAS sequence (pgFP) was also introduced. The transformed BY-2 cells were examined with a laser scanning microscope. As shown in Fig. 8, the green fluorescence (panels A and B) represents the site where GFP is present, and the red fluorescence (panel C) from Mito Tracker Red identifies the mitochondria. In the cells in which pmmtKAS-GFP was introduced, the green fluorescence from GFP overlapped extensively with the red fluorescence from mitochondria (panel D), indicating that the mtKAS-GFP fusion protein was targeted into mitochondria in BY-2 cells. By contrast, in the cells in which pgFP was introduced, the green fluorescence from GFP was observed in the cytoplasm and nucleus (Fig. 8A). These results demonstrate that mtKAS is located in mitochondria and suggest that the N-terminal region of mtKAS functions as a mitochondrial transit peptide.

DISCUSSION

Although there is no longer any doubt that de novo fatty acid synthesis takes place in mitochondria of eukaryotic cells, as well as in plant plastids and the cytoplasm of animal cells, numerous questions remain about the functions of the synthetized fatty acids and about the enzymes of the FAS complex and their genes. Least is known about the condensing, ketoacyl reductase and dehydrase enzymes. As a step toward remedying these deficiencies, we have cloned and carried out the initial characterization of the mitochondrial condensing enzyme component, mtKAS, from A. thaliana. In vivo the Arabidopsis mtKAS gene complemented the fabF defect in E. coli CY244 that bears mutations in both fabB and fabF, demonstrating a role of mtKAS in fatty acid synthesis as a condensing enzyme. mtKAS mRNA was present in leaves, roots, and flowers. The biosynthesis of fatty acids in mitochondria would occur in these organs if the fatty acids were vital for a mitochondria to function in respiration, as suggested by the respiration-deficient phenotypes of knockout mutants (19–21, 23, 24). Western blot and intracellular targeting analyses with the mtKAS-GFP fusion protein revealed that the amino terminus of mtKAS serves as a signal to target protein into mitochondria. Deletion analyses demonstrated that the maximum length of the presequence was 29 residues. Thus, mtKAS falls into the most common class of mitochondrial precursor proteins destined for the matrix, having a 20–50-residue N-terminal transit peptide that forms an amphipathic helix (52). Phylogenetic analysis disclosed that the mitochondrial KAS enzyme from A. thaliana and its homologues from M. musculus, H. sapiens, N. crassa, and S. pombe are more closely related to bacterial KAS II enzymes than to those from plastids. This is in accord with the hypothesis that mitochondria and plastids each originated from a different bacterial endosymbiont. With respect to cerulenin sensitivity, however, mtKAS is similar to E. coli KAS I and not to KAS II.

Although acetyl-CoA was shown in 1962 (53) to participate in fatty acid synthesis, it was only recently identified as the

Fig. 7. Intracellular localization of mtKAS in A. thaliana. Proteins of cytosolic (lane 1), microsomal (lane 2), chloroplast (lane 3), and mitochondrial (lane 4) fractions prepared from A. thaliana leaves were subjected to SDS-PAGE, blotted to a nitrocellulose membrane, and used for Western blot analysis with antibodies against mtKAS (upper panel) and pea mitochondrial H-protein of glycine decarboxylase (lower panel). One hundred micrograms of protein were applied to each lane. Arrows indicate the positions of mtKAS and H-protein.

Fig. 8. Targeting of mtKAS-GFP fusion protein into mitochondria in tobacco BY-2 cells. The control plasmid pgFP (A) and pmmtKAS-GFP encoding the 50 N-terminal residues of mtKAS fused to GFP (B–D) were transformed into BY-2 cells by particle bombardment. Green fluorescence from GFP (A and B), red fluorescence from mitochondria stained with Mitotracker red (C), and a combined image of both green and red fluorences (D) were detected by confocal microscopy.
primer substrate for KAS III in E. coli (5) and spinach (6). KAS III has the same α-β-α-β-α fold as KAS I and II, and also carries out a Claisen condensation, although its sequence has little homology to KAS I and II (46). Because the second substrate utilized by KAS III is malonyl-ACP, it is not surprising that both a CoA and an ACP docking site in KAS III for delivering the substrates to the active site have been identified in crystallographic studies (54, 55), whereas a single ACP docking site is predicted for KAS I and II. The presence of only one KAS enzyme in A. thaliana mitochondria infers an ability to carry out both the priming and the elongating reactions. Our results demonstrate that mtKAS has this capacity. Specifically, (i) the purified His-tagged mtKAS synthesized C4-ACP using malonyl-ACP as the primer substrate and (ii) in vitro and in the in vitro elongation assays, mtKAS complemented the KAS II defect in the E. coli strain CY244 extending C4-ACP in iterative reactions to yield C18-ACP. This broad substrate specificity of mtKAS (Fig. 1C) is similar to that of the KAS domain of the cytosolic mammalian type I FAS (1), for example, and dissimilar to the restricted substrate specificities characterizing most plastid and bacterial KAS enzymes in type II FAS systems.

The seminal characterization of E. coli KAS II and its comparison to KAS I revealed that both enzymes could use acetyl-ACP as the priming substrate (56). Subsequently, the same was shown for spinach KAS I (7). Thus, mtKAS is not unique in being able to use acetyl-ACP as well as longer acyl-ACPs in fatty acid synthesis, but its efficiency in using acetyl-ACP is presumably improved compared with that of the mentioned E. coli and plastidial enzymes. To address the question of the specificity of mtKAS for its primer substrate, additional assays employing the purified His-tagged mtKAS were carried out. The results disclosed that labeled acetyl-CoA in the presence of malonyl-ACP failed to serve as a primer substrate under the experimental conditions used, inferring that mtKAS lacks acetyl-CoA transacylase activity. By comparison, in analogous assays, the purified His-tagged E. coli KAS II incorporated minor amounts of label into C4-ACP from acetyl-CoA, revealing that, in contrast to KAS I (57), it has acetyl-CoA-ACP transacylase activity. The apparent inability to use acetyl-CoA as a primer substrate exhibited by the purified A. thaliana mtKAS in vitro fits with the observations that mitochondrial extracts from pea leaves incorporated only insignificant amounts of label from acetate into fatty acids, which could have been caused by traces of plastid contamination (17), and that the extracts lack detectable acetyl-CoA-ACP transacylase activity (58). We are unable to explain why, in the latter study, the label from acetyl-CoA was “readily incorporated” into fatty acids in the presence of malonyl-ACP (58). Given that mitochondria, in contrast to plastids, lack ACCase activity (17, 59), it is not surprising that acetyl-CoA is an unlikely carbon source for mitochondrial fatty acid synthesis and that malonate assumes this role. Although the source of the malonate is still a question, the enzyme activities to produce malonyl-ACP from malonate are present in mitochondria (58).

Our results lead to the conclusion that malonyl-ACP provides both the primer and donor substrate in the first condensation reaction carried out by A. thaliana mtKAS (Fig. 1C). If the active site structure of mtKAS resembles that of the E. coli KAS I and II enzymes (46), the following scenario can be envisaged. The first malonyl-ACP enters the decarboxylating pocket resulting in a C2 primer substrate, acetyl-ACP, that is transferred to the active site cysteine of the acyl binding pocket, and the concomitant release of the ACP from the enzyme. Decarboxylation of a second malonyl-ACP yields the extender C2-unit, which is also transferred to the active site cysteine effecting synthesis of a C4 acyl chain. The latter is released from the cysteine and linked to the phosphopantetheine arm of an ACP molecule in the docking site, enabling its exit from the active site. Thus, one compound provides both primer and extender units also occurs during synthesis of the derailment product triacetic acid lactone in E. coli (60, 61). In this case the initial C4 acyl chain serves as a substrate for an additional extension, giving a C6 acyl chain that upon release from the enzyme cyclizes, yielding triacetic acid lactone.

As a step toward explaining the function of FAS in mitochondria, the nature of the fatty acyl chains synthesized in this organelle has been the subject of several earlier investigations. Studies using Neurospora mitochondria and soluble extracts revealed major amounts of C8 and C14 acyl chains (18), whereas those with pea mitochondria disclosed C10 to C14 acyl chains (17). Subsequently, using a more sensitive detection system, C8, C12, and C14 were identified as the predominant acyl chains synthesized by FAS in soluble mitochondrial pea extracts (58). Our results with the A. thaliana mtKAS expressed in a heterologous system also reveal a bimodal distribution, in this case C4 versus C14 and C16. Such a distribution was only obtained when the KAS components of the E. coli FAS complex were replaced by mtKAS. This infers that the bimodality, noted in earlier work with mitochondrial extracts (17, 18), may well reflect activity of the mtKAS component in the extracts. Even though the FAS complex in E. coli produces C4 acyl chains for lipid A, C14 acyl chains for lipid A, and C16 and C18 acyl chains for membrane phospholipids, we have never detected in our assay systems using KAS I and II any suggestion for a phenomenon similar to the bimodality characterizing mtKAS. Our combined results infer that, although the KAS component of the mitochondrial FAS plays a major role in determining the observed fatty acid distributions in this organelle, acyltransferases, such as PlsB and LpxA, plus lipoic acid synthase (LipA) are the predominant factors in E. coli (62–64).

An intriguing question for the future will be to probe the structural basis of the observed bimodality. Interestingly, the capacity of mtKAS to synthesize longer acyl chains is more readily lost than that to synthesize the shorter ones as the time of storage before assay increases. Although today it is accepted that the C8 acyl chains are precursors of lipoic acid in vivo, the function(s) of the longer ones remain to be clarified. The longer acyl chains are deduced to play a vital role, as C14 acyl chains are found in Neurospora on subunits of the mitochondrial cytochrome c oxidase (65) and NADH dehydrogenase (66) and because mutants of BPL1 (biotin ligase protein) in S. cerevisiae with normal levels of lipoic acid are respiration-deficient as the result of a failure in mitochondrial fatty acid synthesis (67).

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