Fatty Acid and Lipoic Acid Biosynthesis in Higher Plant Mitochondria*

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Fatty acid and lipoic acid biosynthesis were investigated in plant mitochondria. Although the mitochondrial lack acetyl-CoA carboxylase, our experiments reveal that they contain the enzymatic equipment necessary to transform malonate into the two main building units for fatty acid synthesis: malonyl- and acetyl-acyl carrier protein (ACP). We demonstrated, by a new method based on a complementary use of high performance liquid chromatography and mass spectrometry, that the soluble mitochondrial fatty-acid synthase produces mainly three predominant acyl-ACPs as follows: octanoyl(C8)-, hexadecanoyl(C16)-, and octadecanoyl (C18)-ACP. Octanoate production is of primary interest since it has been postulated long ago to be a precursor of lipoic acid. By using a recombinant H apoprotein mutant as a potential acceptor for newly synthesized lipoic acid, we were able to detect limited amounts of lipoylated H protein in the presence of malonate, several sulfur donors, and cofactors. Finally, we present a scheme outlining the new biochemical pathway of fatty acid and lipoic acid synthesis in plant mitochondria.

Lipoic acid (6,8-thioctic acid or 1,2-dithiolane-3-pentanoic acid) is a sulfur-containing cofactor involved in several multienzyme complexes such as pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, branched-chain keto acid dehydrogenase, and glycine decarboxylase complex. The carboxyl group of lipoic acid is attached to the dihydrolipoamide acyltransferase subunits (E2) of the keto acid dehydrogenase complexes and to the H protein of the glycine decarboxylase complex, by an amide linkage to the e-amino group of a specific lysine (1–4).

Recent studies have highlighted the potential of free lipoic acid and dihydrolipoic acid as powerful metabolic antioxidants that are able to scavenge the reactive oxygen species, to recycle other antioxidants (vitamin C, glutathione, and vitamin E), and even to intervene in redox regulation of gene transcription (5, 6). Consequently, lipoic acid is now increasingly used as a therapeutic agent in pathologies associated with oxidative stress (for a review see Packer et al. (5)).

In prokaryotic cells, Parry (7) and White (8) showed by labeling experiments that octanoic acid was a direct precursor of lipoic acid, 6-thiooctanoate and 8-thiooctanoate being possible intermediates in lipoic acid biosynthesis (9–11). Mutant strains of Escherichia coli defective in lipoic acid biosynthesis have allowed the isolation of several genes involved in lipoic acid biosynthesis (12–14). The characterization of the lip locus revealed that it contained the lipA gene encoding for a 36-kDa protein (14–16). Despite the fact that LipA activity has never been measured in vitro, the protein is expected to be related to a lipoate synthase. Sequence similarity to biotin synthase strongly suggests that lipA encodes a sulfur insertion enzyme analogous to biotin synthase and, consequently, that the sulfur insertion mechanisms of the two systems could be related (15, 16). Moreover, biotin synthase is known to contain a $[4Fe-4S]^{2+}$ iron-sulfur cluster, and recent works (17, 18) have demonstrated that LipA is also an iron-sulfur protein. Strains of E. coli with mutations in lipA were shown to grow only in the presence of 8-thiooctanoate (16, 19) or 6-thiooctanoate (16) implicating the involvement of LipA in the insertion of the first sulfur into octanoate. At last, two other genes of E. coli were shown to intervene in the metabolism of lipoic acid, lipA and lipB encoding, respectively, for proteins of 38 and 25 kDa (16, 20). These proteins were shown to be involved in the attachment of lipoic acid to the apoprotein form of the lipoyl-containing protein. In the presence of free exogenous lipoic acid, LpA utilizes ATP to generate the activated lipoyl-AMP species and then transfers the lipoyl group to the acceptor protein (20–22), whereas lipB is required for the attachment of lipoyl groups, linked to ACP, produced via endogenous biosynthesis (21, 23).

In eukaryotic cells, most of the lipoate-containing enzymes are located in the mitochondria and have been studied intensively. However, the mechanisms underlying the biosynthesis of lipoic acid and its incorporation into apoproteins have been less investigated. Despite the existence of four independent complementation groups (lip1–4) defective in lipoic acid metabolism in Saccharomyces cerevisiae (24), the exact functions of the corresponding genes remain unknown. Lip5 from S. cerevisiae (25) and Lip1 from Arabidopsis thaliana (26) have been isolated and found to be homologous to the E. coli lipA. In addition, an analogue of E. coli lipB in the yeast Kluyveromyces lactis has also been isolated (27). In contrast with the situation observed in E. coli, in mammals, the activation of free exogenous lipoic acid in lipoyl-AMP and its coeffector

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1 The abbreviations used are: ACP, acyl carrier protein; DTT, dithiothreitol; MOPS, 4-morpholinoalpropanesulfonic acid; MALDI-MS, matrix-assisted laser desorption ionization-mass spectrometry; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; FAS, fatty-acid synthase.
attachment to the lipoyl-accepting enzyme is catalyzed by two distinct mitochondrial enzymes, the lipote-activating enzyme and the lipoyl-AMP-N'-lysine lipoyltransferase (28). The latter enzyme, which appeared as two isoforms, has been isolated (28) and cloned (29, 30).

Production of fatty acids up to 14 carbons from malonate was detected in *Neurospora crassa* mitochondria (31) and is related to the presence of ACP within the organelle (32). Recent work suggests that the mitochondrial fatty acid biosynthesis pathway was involved in lipic acid synthesis (23, 33, 34). Jordan and Cronan (23) also demonstrated that plant mitochondria contain a lipote transferase that used lipoyl-ACP as the lipote donor to attach lipote to the dehydrogenase complexes.

By taking advantage of an available system for producing recombinant H apoprotein, a lipote-accepting protein (35), we sought to investigate the lipic acid biosynthesis in plant mitochondria, using malonate as the precursor. We have thus characterized the different biochemical steps involved in the transformation of malonate into lipic acid by a soluble protein extract (matrix extract) isolated from pea leaf mitochondria. We have also devised a new method using mass spectrometry to monitor all the acyl-ACP and H protein intermediates formed during the functioning of the enzymes involved in the mitochondrial fatty acid and lipic acid biosynthesis.

**MATERIALS AND METHODS**

Isolation of Mitochondria and Preparation of a Soluble Mitochondrial Protein Extract (Matrix Extract)—Mitochondria were isolated and purified from pea leaves (washed with 5% sodium hypochlorite to eliminate contaminating bacteria) as described by Douc et al. (36). The purified mitochondria (about 200 mg of proteins) were diluted in 40 ml of buffer containing 40 mM MOPS (pH 7.4) and 1 mM DTT. Total release of the matrix protein was achieved by three cycles of freezing and thawing. The suspension of broken mitochondria was then centrifuged on a 3-kDa Diaflo membrane using a stirred ultrafiltration cell (Amicon) on a magnetic stirring table to a concentration of about 50 mg/ml.

Purification of the Proteins—Recombinant H apoproteins (wild type and HE14A mutant) were produced and purified as described by Gueguen et al. (37). P protein of the glycine decarboxylase complex was purified from pea leaf mitochondria as described by Bourguignon et al. (38). *E. coli* ACP (5 mg) from Sigma was purified by fast performance liquid chromatography using a Mono Q-HR5/5 column (Amersham Pharmacia Biotech) previously equilibrated with 50 mM Tris-HCl (pH 7.4) and 2 mM DTT. Proteins were eluted with a continuously increasing NaCl gradient (0–1 M) (flow rate, 0.5 ml/min; fraction size, 1 ml). The fractions containing ACP (analyzed by SDS-PAGE) were pooled and dialyzed extensively at 4 °C against 4 liters of 50 mM Tris-HCl (pH 7.4) and 2 mM DTT. The proteins were then concentrated using a 3K-Centric (Filtron) by centrifugation to a final concentration of about 2 mg/ml. The mass of the matrix proteins in a final volume of 0.5 ml was determined with a Uvicon 810 spectrophotometer.

**Biochemical Assays**

Malonyl-Coenzyme A Synthetase—Malonyl-CoA synthetase activity was measured as described by Kim and Bang (39) using the malonyloxydrazate assay. This assay is based on the determination of maloneydrazate formed by the reaction of hydroxylamine with malonyl-CoA produced by the enzyme. This assay was conducted at 30 °C in 40 mM Tris (pH 7.4) containing 40 mM sodium malonate, 200 mM NH4OH, 10 mM MgCl2, 10 mM ATP, 500 mM CoASH, and 200 μg of matrix extract proteins in a final volume of 0.5 ml. After 2 h, the reaction was stopped by the addition of 0.25 ml of 10% trichloroacetic acid. Then 0.5 ml of 15% FeCl3 solution in 0.66 N HCl was added to the reaction mixture. After elimination of the precipitated proteins by centrifugation, the intensity of the red-brown color developed by the formation of Fe3+ complex of malonylhydroxamate was monitored at 540 nm (ε = 842 M–1 cm–1) using an Uvicon 810 spectrophotometer.

Malonyl-CoA:Acyl Carrier Protein Transacylase—Malonyl-CoA:Acyl carrier protein transacylase activity was measured as described by Guerra and Ohlrogge (40).
Fatty Acid and Lipoic Acid Biosynthesis

In order to verify the presence of lipoylated HE14A protein in the incubation medium and to discriminate the lipoylated form from the octanoylated form, aliquots of the purified H proteins were incubated at 30 °C during 30 min in presence of 0.2 μM of the P protein of the glycine decarboxylase complex, 20 μM pyridoxal phosphate, and 20 mM glycine. In these conditions, the oxidative decarboxylation of glycine occurs, and the active lipoylated H protein becomes loaded with methylamine. The result of the acylation of the H protein by the methylamine group (+32 Da) was followed by MALDI-MS. On the other hand, the octanoylated H protein remains unaltered under the same conditions.

High Performance Liquid Chromatography—The ACP mixtures (see under “Fatty-acid Synthase Assay”) were first dried and then dissolved in water containing 1% trifluoroacetic acid (v/v). Separations of the different acyl-ACPs were performed with an Applied Biosystems A 130 (Perkin-Elmer) HPLC apparatus using a reversed-phase column (Brownlee, C8, 5 μm, 1 × 100 mm). Elution of polypeptides, monitored at 214 nm, was performed by an isocratic gradient during 5 min at 100% solvent A (water containing 0.1% trifluoroacetic acid), and then a linear gradient was run from 0 to 50% solvent B (acetonitrile/water, 90/10, containing 0.08% trifluoroacetic acid) with a flow rate of 50 μl/min and then from 50 to 100% solvent B in 5 min. Peaks were collected and analyzed by MALDI-MS.

Mass Spectrometry—Matrix-assisted Laser Desorption Ionization (MALDI-Mass Spectrometry Analysis)—Mass spectra of modified ACPs were obtained with a Time of Flight Mass Spectrometer Voyager Elite XI (Perseptive Biosystems, Framingham, MA) equipped with a 337-nm nitrogen laser. A mass spectrum of each sample was recorded by averaging approximately 128 laser shots at various locations across the spot. All spectra were acquired in the positive ion mode with delayed extraction. After a 200-ns delay time, the first acceleration grid was set to 25,000 V and the second acceleration grid to 93.7% of this value or 23.425 V. The guide wire in the flight tube was held at 0.075% of the pulse acceleration value (18.75 V). Aliquots of the protein and the matrix solutions (0.5 μl each) were mixed on the stainless steel sample plate and dried in the air prior to mass spectrometry analysis. External calibration was performed with porcine insulin (m/z 5835) obtained from Sigma. All experiments were performed using saturated solution of 2,5-dihydroxybenzoic acid prepared in a 50% (v/v) solution of acetonitrile/water containing 0.1% trifluoroacetic acid.

Electrospray Ionization Mass Spectrometry Analysis—Electrospray ionization mass spectrometry was performed using a SCIEX API III+ triple quadrupole mass spectrometer (Perkin-Elmer) equipped with a nebulizer-assisted electrospray source. Calibration was performed in positive mode using poly(propylene glycol) ions. The H protein spectra were acquired in multichannel acquisition mode on a m/z range from 600 to 1600, with a scan of 0.4 m/z and a dwell time of 2 ms.

Electrophoretic Analyses of Proteins—Protein samples were analyzed by SDS-PAGE (15% acrylamide, 0.2% bisacrylamide, 0.1% SDS as stacking gel and 0.1% SDS as stacking gel) as described by Sambrook et al. (41). Gels were stained with R-250 Coomassie Brilliant Blue. After 30 min of incubation in a solution containing 3% acetic acid and 7% glycerol, gels were dried onto Whatman No. 3MM paper. Protein concentrations were estimated by the method of Lowry et al. (42) with bovine serum albumin as standard.

RESULTS

Plant Mitochondria Contain a Malonyl-CoA Synthetase and Malonyl-CoA:Acyl Carrier Protein Transacylase That Allow the Conversion of Malonate into Malonyl-ACP—Plant mitochondria are devoid of acetyl-CoA carboxylase (43) that prohibits them from synthesizing malonyl-CoA from acetyl-CoA. Malonate entering into the mitochondria must be activated before its incorporation into fatty acids. We therefore postulated that malonate could be activated into malonyl-CoA, and we investigated the presence of a malonate-CoA synthetase in the soluble fraction of the plant mitochondria. This enzyme has been purified and characterized from Pseudomonas fluorescens that was grown on malonate as a sole source of carbon (44) and from Rhizobium japonicum (45). By using the malonohydroxamate assay described by Kim and Bang (39), we showed that plant mitochondria contain a malonate-CoA synthetase with a V\textsubscript{m} value of 1.9 nmol of malonyl-CoA formed per min and per mg of matrix extract proteins (Table I, part A). The conversion of malonate into malonyl-CoA shows an absolute requirement for ATP, CoASH, and a divalent cation (Mg\textsuperscript{2+}). The reaction of the malonyl-CoA synthetase is linear from 0 to 120 min, and there is a linear relationship between malonyl-CoA formation rates and the amount of matrix enzymes. The activity of malonyl-CoA synthetase was assayed as a function of pH by buffering the reaction medium with either MOPS or Tris from pH 6.5 to 10. At 30 °C, the enzyme showed a broad pH optimum (with maximum ranging from pH 7.3 to 9). The malonyl-CoA synthetase displays a rather low affinity for Mg\textsuperscript{2+} with an apparent K\textsubscript{m} value of 1 mM. The kinetics experiments were carried out under steady-state conditions at saturated Mg\textsuperscript{2+} concentration (10 mM) with various concentrations of substrates. Malonyl-CoA synthetase from pea mitochondria exhibited Michaelis-Menten kinetics with respect to malonate, CoASH, and ATP. The apparent K\textsubscript{m} values for these substrates, determined by using double reciprocal plot method (Lineweaver-Burk equation), were 5.2 mM, 215 μM, and 384 μM, respectively.

The presence of a malonyl-CoA:ACP transacylase in the matrix extract isolated from pea leaf mitochondria was investigated. Detection was performed according to the method described by Guerra and Ohrrogge (40), and we could show that plant mitochondria contain a powerful malonyl-CoA:ACP transacylase (V\textsubscript{m} value of 52 nmol of malonyl-ACP formed per min and per mg of soluble mitochondrial proteins).

Plant Mitochondria Contain a Malonyl-ACP Synthetase That Allows the Direct Conversion of Malonate into Malonyl-ACP—We tried to detect the presence of a “malonyl-ACP synthetase” in the matrix extract. This enzymatic activity (V\textsubscript{m} value of 0.2 nmol of malonyl-ACP formed per min and per mg of matrix extract proteins) was characterized in a medium devoid of CoASH by following the production of acid-insoluble radiolabeled malonyl-ACP in the presence of [2-14C]malonic acid (see “Material and Methods”). The conversion of malonate to malonyl-ACP shows an absolute requirement for ATP and Mg\textsuperscript{2+} (Table I, part B), and a 50% stimulation was observed with 1 mM DTT (Table I, part B). Malonyl-ACP synthetase from pea mitochondria exhibited Michaelis-Menten kinetics with respect to malonate. The apparent K\textsubscript{m} value for malonate was determined to be 53 μM.

Matrix-assisted laser desorption ionization (MALDI)-mass spectrometry (MS) was also used to monitor the disappearance of free ACP concomitant with the production of malonyl-ACP. The reaction that was performed using cold malonate and ACP as substrates was stopped by addition of an equal volume of

<table>
<thead>
<tr>
<th>Table I</th>
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<tr>
<td>Malonyl-CoA synthetase (A) and Malonyl-ACP synthetase (B) activities in a matrix extract of mitochondria isolated from pea leaves</td>
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<tr>
<td>Reaction mixture</td>
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<td></td>
</tr>
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<td>A. Complete system</td>
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<td>- ATP</td>
</tr>
<tr>
<td>- Malonate</td>
</tr>
<tr>
<td>- CoASH</td>
</tr>
<tr>
<td>- MgCl\textsubscript{2}</td>
</tr>
<tr>
<td>- MgCl\textsubscript{2} + EDTA</td>
</tr>
<tr>
<td>B. Complete system</td>
</tr>
<tr>
<td>- ATP</td>
</tr>
<tr>
<td>- Malonate</td>
</tr>
<tr>
<td>- ACP</td>
</tr>
<tr>
<td>- MgCl\textsubscript{2}</td>
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<td>- DTT</td>
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</table>

During the absence of either malonate, ATP, or MgCl₂ (spectra not shown) and acetyl-ACP (m/z 8886). The reaction was also performed by taking advantage of its high solubility in 2-propanol (Ref. 46, materials). Kinetic study was performed at 30 °C as described under “Materials and Methods.”

Malonyl-ACP synthesis by a soluble matrix extract of pea leaf mitochondria. ACP modification in the presence of malonate, catalyzed by a pea matrix extract, was followed by MALDI mass spectrometry after partial purification of ACPs by 2-propanol precipitation method. All the acyl intermediates occurring as thioesters of ACP during the kinetics of the mitochondrial fatty acid biosynthesis reaction were separated by HPLC using a reverse phase column (see “Material and Methods”). The different elution profiles obtained at each time are presented in Table II. At time 0, the HPLC elution profile of the partially purified ACP shows a double peak called peak I (Fig. 2), presents only the spectra corresponding to the mass spectrometry analysis of the major peaks (I to V) present at t = 6 h (Fig. 2E). Indeed, when the same analysis was carried at 0, 0.5, 2, and 4 h, we found the same qualitative content in each peak (results not shown). For a better comprehension of the mass spectra, a list of molecular masses of the acyl-ACP intermediates that could be synthesized during the fatty acid synthesis reaction is presented in Table II. At time 0, the HPLC elution profile of the partially purified ACP shows a double peak called peak I (Fig. 2A). The mass spectrometry analysis of peak I (Fig. 2F) shows that only free ACP is present (m/z = 8843), the other peak (m/z = 8974) corresponding to the matrix adduct of the peak at 8843. The appearance of a double peak, which occurs at this elution time, is attributable to the H protein. Indeed, the protein present at high concentration (3 mM) in the matrix extract is not entirely precipitated by 2-propanol and almost co-eluted with ACP during the HPLC. After 30 min of incubation, two new major peaks (peaks II and III) appeared distinctly (Fig. 2F). Indeed, when the same analysis was carried at 0, 0.5, 2, and 4 h, we found the same qualitative content in each peak (results not shown).

This experiment demonstrated therefore that the major compounds synthesized by the plant mitochondrial fatty-acid syn-
FIG. 2. Purification by HPLC and MALDI mass spectrometry analysis of the acyl-ACP intermediates formed during the functioning of the mitochondrial fatty-acid synthase. The different ACP intermediates formed during the incubation of a pea matrix extract in the
that these are the octanoyl(C8)-ACP, hexadecanoyl(C16)-ACP, and octadecanoyl(C18)-ACP, the octanoyl-ACP being probably the precursor of lipoate.

\[ \text{[2-14C]Malonate Incorporation into Lipoate-accepting H Apoprotein by Soluble Mitochondrial Extract—} \]

To proceed further toward the biosynthesis of lipoic acid, we investigated whether the mitochondrial fatty acid pathway could be diverted by the addition of a recombinant lipoate acceptor protein (H apoprotein). The reaction was performed with [2-14C]malonate acid as a primary carbon donor. Proteins were then analyzed by SDS-PAGE, and the dried gel was autoradiographed. The autoradiography presented in Fig. 3 shows that, during the incubation with the matrix extract, the H protein (molecular mass estimated at 15.5 kDa by SDS-PAGE) is labeled (Fig. 3, lanes 2–3) and that the reaction is dependent on the addition of the H apoprotein (lane 9). ATP as expected was required for the labeling of the H protein (Fig. 3, lane 4). In addition, we observed a labeling of a compound (molecular mass estimated at 10 kDa) that was strongly dependent on the presence of \( E. \) \textit{coli} ACP in the incubation medium (lane 8). It appears likely that this labeled band corresponds to one of the acyl-ACP intermediates characterized in the previous experiment. Nevertheless, in the absence of \( E. \) \textit{coli} ACP, the H protein is still labeled because of the presence of the mitochondrial ACP in the matrix extract. The presence of a reduced pyridine nucleotide in the incubation medium was required (lane 7); however, NADH or NADPH were equally effective (lanes 5–6).

Fig. 4 shows the kinetics of protein labeling by [2-14C]malonic acid in the absence (A and C) or in the presence (B and D) of H apoprotein (2 nmol/200 \( \mu \)l) with a matrix extract protein concentration of 2 mg/ml (A and B) and 20 mg/ml (C and D). The time course of protein labeling analyzed with a Phosphorimager presented in Fig. 3 shows that, during the incubation medium was required (lane 8) and that the reaction is dependent on the addition of the H apoprotein (lane 9). ATP as expected was required for the labeling of the H protein (Fig. 3, lane 4). In addition, we observed a labeling of a compound (molecular mass estimated at 10 kDa) that was strongly dependent on the presence of \( E. \) \textit{coli} ACP in the incubation medium (lane 8). It appears likely that this labeled band corresponds to one of the acyl-ACP intermediates characterized in the previous experiment. Nevertheless, in the absence of \( E. \) \textit{coli} ACP, the H protein is still labeled because of the presence of the mitochondrial ACP in the matrix extract. The presence of a reduced pyridine nucleotide in the incubation medium was required (lane 7); however, NADH or NADPH were equally effective (lanes 5–6).

The mass graphs of the peak III (H) shows the unique presence of the monocharged octanoyl-ACP (m/z = 8970 Da), whereas the mass spectrometry analysis of peak IV (I) and peak V (J) shows the unique presence of the hexadecanoyl-ACP (m/z = 9082) and the octadecanoyl-ACP (m/z = 9110), respectively.

\[ \text{Table II} \]

The different acyl-ACP intermediates in the fatty acid synthesis

<table>
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<th>Group name chemical formula</th>
<th>Group name ACP (8842 Da) + group name (Da)</th>
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<th>Group name ACP (8842 Da) + group name (Da)</th>
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The presence of malonate was partially purified by 2-propanol precipitation, separated on C8 Brownlee column by HPLC, and analyzed by MALDI mass spectrometry (see “Materials and Methods”). The different elution profiles obtained by HPLC are presented as follows: A, \( t = 0 \); B, \( t = 0.5 \) h; C, \( t = 2 \) h; D, \( t = 4 \) h; and E, \( t = 6 \) h. All the fractions of the five chromatographies were analyzed by mass spectrometry. In \( F \)–\( J \) are presented only the mass spectra corresponding to the analysis of peak I, II, III, IV, and V, respectively, obtained during the elution profile presented in \( E \) (at \( t = 6 \) h). The peak at \( m/z = 8843 \) corresponds to the monocharged unacylated form of ACP and that at \( m/z = 8974 \) to its matrix adduct (\( F \)). In \( G \), the peaks at \( m/z = 8843 \), at \( m/z = 8886 \), and \( m/z = 8928 \) correspond, respectively, to the monocharged unacylated form of ACP, acetyl-ACP, and malonyl-ACP or ketobutyryl-ACP (see Table II). The mass spectra of the peak III (\( H \)) shows the unique presence of the monocharged octanoyl-ACP (\( m/z = 8970 \) Da), whereas the mass spectrometry analysis of peak IV (\( I \)) and peak V (\( J \)) shows the unique presence of the hexadecanoyl-ACP (\( m/z = 9082 \)) and the octadecanoyl-ACP (\( m/z = 9110 \)).
phosphorImager (Molecular Dynamics)(Fig. 4E) indicates that the rate of the reaction is proportional to the amount of the matrix protein. Vm values were approximately 2 nmol of H protein labeled per mg of matrix proteins and per h. Fig. 4E also indicates that the enzymatic systems exhibited an extremely high affinity for H apoprotein in the overall reaction carried out in the presence of malonate as substrate.

As a whole, all these results suggest first that [2-14C]malonic acid is utilized for [14C]octanoyl-ACP synthesis via fatty acid biosynthetic pathway and second that a lipoate protein ligase catalyzed the attachment of the octanoyl or lipoyl moiety to the specific Lys-63 residue of H apoprotein (1, 23).

Evidence of Lipoate Biosynthesis by a Matrix Extract of Mitochondria Isolated from Pea Leaves—Since it is impossible to discriminate by mass spectrometry newly formed lipoylated H apoprotein from endogenous H protein (14,135 Da), we decided to use as a lipoate (or octanoyl)-accepting H apoprotein a mutant of H protein called HE14A (with Glu-14 replaced by Ala) (37). This mutant, which is correctly folded (37), should be an ideal final substrate to study the in vitro biosynthesis of lipoate since its slightly lower mass (13,889 Da) allows us to monitor the appearance of octanoylated or lipoylated form outside the peak of endogenous H protein. The matrix extract was, therefore, incubated with the HE14A apoprotein mutant in the presence of malonic acid as a primary carbon donor. The reaction medium contained some potential sulfur donors (Na2S, cysteine) and AdoMet as a possible source of deoxyadenosyl radical (47). After incubation, the various forms of H protein were partially purified from the reaction medium by ammonium sulfate fractionation (see “Materials and Methods”) and analyzed by MALDI mass spectrometry. Spectra obtained by mass spectrometry analysis of H protein mutant (HE14A) modified with a pea matrix extract in presence of malonate, the H proteins were partly purified by ammonium sulfate precipitation and analyzed by MALDI mass spectrometry (see “Materials and Methods”). A, spectra obtained at the initial time (t = 0). Two forms of H protein are present as follows: the peak at m/z = 13,889 corresponds to monocharged recombinant HE14A apoprotein, and the peak at m/z = 14 137 corresponds to the endogenous lipoylated H protein. B, purified sample analysis after 6 h of incubation at 30 °C. The peak at m/z = 14,016 corresponds to the octanoylated HE14A protein and the peak at m/z = 14,078 to the lipoylated HE14A protein. C, the sample analyzed in B was incubated for 30 min at 30 °C with P protein of the glycine decarboxylase in presence of glycine and pyridoxal phosphate prior to analysis by mass spectrometry. The peaks at m/z = 14,110 and 14,169 correspond to the methylamine-loaded form of HE14A and H protein, respectively.

![Fig. 5. MALDI mass spectrometry analysis of H protein mutant (HE14A) modified with a pea matrix extract in presence of malonate. The H proteins were partly purified by ammonium sulfate fractionation and analyzed by MALDI mass spectrometry (see “Materials and Methods”). A, spectra obtained at the initial time (t = 0). Two forms of H protein are present as follows: the peak at m/z = 13,889 corresponds to monocharged recombinant HE14A apoprotein, and the peak at m/z = 14 137 corresponds to the endogenous lipoylated H protein. B, purified sample analysis after 6 h of incubation at 30 °C. The peak at m/z = 14,016 corresponds to the octanoylated HE14A protein and the peak at m/z = 14,078 to the lipoylated HE14A protein. C, the sample analyzed in B was incubated for 30 min at 30 °C with P protein of the glycine decarboxylase in presence of glycine and pyridoxal phosphate prior to analysis by mass spectrometry. The peaks at m/z = 14,110 and 14,169 correspond to the methylamine-loaded form of HE14A and H protein, respectively.](image-url)
alyzed by the P protein of the glycine decarboxylase complex. Sample containing the different H protein forms was incubated in presence of P protein and glycine. Under these conditions, the lipoylated forms of H protein became loaded with methylamine. Proteins present in the reaction medium were then analyzed immediately by mass spectrometry. Fig. 5C shows that the intensity of the peak corresponding to the lipoylated HE14A protein (m/z = 14,078) has decreased, whereas a peak at m/z = 14,110 corresponding to the mass of the methylamine-loaded HE14A protein appeared symmetrically. In the same proportion, the peak corresponding to the wild type H protein present in the matrix extract (m/z = 14,137) declined and was replaced by a peak at m/z = 14,169 corresponding to the mass of the methylamine-loaded wild type H protein. Other peaks were not modified during the reaction with P protein.

In conclusion, these results demonstrated that the soluble compartment of the plant mitochondria contains all the enzymes required to synthesize octanoic acid from malonate and subsequently to carry out the post-translational modification of H apoprotein to yield octanoylated or lipoylated protein.

**DISCUSSION**

Mitochondria are semi-autonomous organelles whose universally recognized function is to provide cellular ATP by the process of oxidative phosphorylation. In plants, mitochondria have recently been shown to be the site of synthesis of essential cofactors such as biotin (49), folate (50), and lipoate (23, 33). Thus, beyond their role in cell bioenergetics, it is evident that plant mitochondria carry other primordial biosynthetic functions reflecting the autotrophic status of plants. With regard to lipid metabolism, the discovery of a mitochondrial ACP in yeast and plant mitochondria (31, 51) supports the existence of a FAS in this organelle. However, the complete enzyme pathway has not been elucidated so far. By using malonate as a precursor, we could demonstrate, by an original method based on HPLC separation coupled to mass spectrometry analysis of acyl-ACP intermediates, that a mitochondrial soluble protein extract was capable to synthesize fatty acids. The major fatty acids synthesized were octanoic acid (C8), hexadecanoic acid (C16), and octadecanoic acid (C18).

The usual pathway of fatty acid synthesis (FAS I or FAS II) is initiated with acetyl-CoA that is carboxylated by acetyl-CoA carboxylase into malonyl-CoA. The latter compound is then transformed into malonyl-ACP (C3 unit) and condensed with acetyl-CoA or acetyl-ACP (C2 unit) by β-ketoadyl synthase (52, 53). Plant mitochondria cannot use acetyl-CoA or acetyl-ACP as the sole precursor for fatty acid synthesis (33), essentially because they lack acetyl-CoA carboxylase (43). However, the results presented here show that malonate alone is able to provide both C2 and C3 units for fatty acid synthesis by matrix extracts from plant mitochondria. Therefore, we decided to investigate the mechanism of activation of malonate into malonyl-ACP. We demonstrated for the first time that plant mitochondria contain a malonyl-CoA synthetase and a malonyl-CoA:ACP transacylase (Table I) which are able to activate malonate into malonyl-ACP (see Fig. 6). Upon determination of kinetic parameters of malonyl-CoA synthetase activity, we found a high apparent Km value of around 5 mM for malonate. As this compound is a well-known competitive inhibitor of succinate dehydrogenase (54), this implies that high metabolic fluxes through the fatty acid synthesis pathway would be paralleled by a transient decrease of the flux through the tricarboxylic acid cycle. Furthermore, it has been shown that soybean leaf tissue contains two isoforms of malonyl-CoA:acyl carrier protein transacylase (40). One of these enzymes could be the mitochondrial malonyl-CoA:acyl carrier protein transacylase detected in the present work. Besides, we found that malonate could be directly activated into malonyl-ACP in the presence of ATP (Table I and Figs. 1 and 6). This activity could be either attributed to a novel enzyme that we call malonyl-ACP synthetase or to a side activity of malonyl-CoA synthetase. The latter hypothesis appears unlikely since the Km of the malonyl-CoA synthetase for malonate is much higher (5 m M) than that of malonyl-ACP synthetase (around 50 μM). Nevertheless, to confirm the presence of these two enzymes in the matrix space of plant mitochondria, the purification of these enzymes is under progress in our laboratory. It should be notice that the rate of activation of malonate by the couple malonyl-CoA synthetase/malonyl-CoA:acyl carrier protein transacylase is 10-fold higher than the direct activation by malonyl-ACP synthetase (see Table I) but with a lower affinity (2 orders of magnitude less). Ours results also indicate that acetyl-ACP is an intermediary product of malonate-dependent fatty acid synthesis (Figs. 1 and 2). Although we cannot provide experimental evidence for the origin of acetyl-ACP, we hypothesize that this reaction could be performed by β-ketoadyl synthase as it was described in E. coli (53, 55).

Ours results raise the problem of the origin of malonate for fatty acid synthesis in plant mitochondria. In fact, the biosynthesis of this dicarboxylic acid is poorly understood. The study of Riley et al. (56) on the origin of free brain malonate suggests that it could be the result of the following sequential reactions: acetyl-CoA → malonyl-CoA → malonate. The first reaction...
could be catalyzed by the cytosolic acetyl-CoA carboxylase, and the latter step could occur by transfer of the CoA group from malonyl-CoA to succinate and/or acetoacetate (56). Malonate could enter into mitochondria via the dicarboxylic acid transporter characterized by Vivekananda et al. (57). In some plants, malonate can be present at high concentrations. For example, in soybean, this metabolite is the predominant organic acid in leaf and root tissues where its concentration reaches 2–5 μmol/g of fresh weight (58). As acetyl-CoA supply in mitochondria should not be limiting in vivo, we expected to find an acetyl-CoA:ACP transacylase activity catalyzing the transfer of acetyl moiety from CoASH to ACP. We were surprised to be unable to detect this activity in vivo because we did not yet observe any lipoyl-ACP intermediates and also because some transfer of lipoyl moiety seems to occurs between endogenous H protein and mutant H apoprotein lowering therefore the real amount of de novo biosynthesis. Although we find this unlikely, we cannot exclude that sulfur insertion may occur directly on the octanoylated protein (Fig. 6).

In conclusion, this work is summarized in Fig. 6, which shows the biochemical pathway of fatty acid and lipic acid synthesis in plant mitochondria as deduced from our present knowledge.

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REFERENCES

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