The objective of this study was to identify the products and possible role of a putative pathway for de novo fatty acid synthesis in mammalian mitochondria. Bovine heart mitochondrial matrix preparations were prepared free from contamination by proteins from other subcellular components and, using a combination of radioisotopic labeling and mass spectrometry, were shown to contain all of the enzymes required for the extension of a 2-carbon precursor by malonyl moieties to saturated acyl-ACP thioesters containing up to 14 carbon atoms. A major product was octanoyl-ACP and, in the presence of the apo-H-protein of the glycine cleavage complex, the newly synthesized octanoyl moieties were translocated to the lipoylation site on the acceptor protein. These studies demonstrate that one of the functions of the de novo fatty acid biosynthetic pathway in mammalian mitochondria is to provide the octanoyl precursor required for the essential protein lipoylation pathway.

Lipoic acid was discovered in the early 1950s, through a collaboration between Lester Reed and Eli Lilly and Company (1), and initially was thought to represent a new member of the B vitamin family. Thirty years elapsed before evidence was obtained indicating that lipoic acid was not a vitamin for animals and that radiolabel from acetate and octanoate could be incorporated into lipoic acid, most likely in the liver (2). However, the mechanism by which lipoic acid is synthesized in animals has yet to be elucidated. In addition to performing an important role as an antioxidant in the free acid form (3), lipooyl moieties are essential cofactors for several mitochondrial multienzyme complexes that play critical roles in energy metabolism, including the α-ketoacid dehydrogenases and the glycine cleavage system. In each of these enzyme complexes, lipooyl moieties are covalently attached via an amide bond to the ε-amino group of specific lysine residues (4). Studies in prokaryotes have established that lipooyl moieties can be derived either from endogenous lipoic acid, via a lipoyl-AMP intermediate formed by the lipoil ligase, LpLA (5), or from octanoyl-ACP, 2 an intermediate in fatty acid synthesis, by the insertion of sulfur atoms at C-6 and C-8 in a reaction catalyzed by lipoic acid synthase, LpA (6–8); insertion of the sulfur atoms can occur prior to, or following, transfer from ACP thioester linkage to the lysine residue on the acceptor protein in a reaction catalyzed by lipoyl transferase, LpB. Homologs of LpLA, LpA, and LpB have been identified in eukaryotes (9–11), suggesting that the same two pathways for production of lipooyl moieties may be operative in mitochondria. Recently, strong evidence has been obtained for the existence of a mitochondrial pathway for de novo fatty acid biosynthesis in both fungi (12) and plants (13, 14) and several of the enzymes required for the de novo synthesis of fatty acids by a putative type II “ACP track” pathway have been identified and characterized in humans and shown to be nuclear-encoded, mitochondrially targeted proteins (15–17). These freestanding, type II enzymes are quite distinct from the type I multifunctional polypeptide fatty acid synthase system found in the cytosol. However, direct evidence for the functioning of a mitochondrial pathway for fatty acid synthesis in animals is lacking. The objectives of this study were to determine whether the entire pathway could be reconstituted in mitochondrial extracts, to identify the products of the pathway, and to ascertain whether the pathway can provide the octanoyl precursor required for the de novo synthesis of lipooyl moieties. We chose to use bovine heart for these experiments because this organ is readily obtainable, it contains little or no cytosolic fatty acid synthase and is rich in mitochondria.

**EXPERIMENTAL PROCEDURES**

Isolation, Purification, and Subfractionation of Bovine Heart Mitochondria—Crude mitochondria were prepared from ~0.5 kg of beef heart essentially as described earlier (18). The crude mitochondrial pellet was resuspended in 0.25 M sucrose, 10 mM Tris-HCl, 1 mM Tris succinate (pH 7.8), 0.2 mM EDTA containing protease inhibitors, washed twice by centrifugation at 10,000 x g for 10 min, resuspended in the same medium without protease inhibitors, and purified by centrifugation for 3 h at 4 °C on an OptiPrep density gradient (19–27%). The layer containing purified mitochondria was diluted with 0.25 M sucrose, 10 mM Tris-HCl, 1 mM Tris succinate (pH 7.8) and centrifuged at 30,000 x g for 10 min. The mitochondrial pellet was washed by resuspension and centrifugation and finally resuspended in 0.25 M sucrose, 10 mM Tris-HCl, 1 mM Tris succinate (pH 7.8), then flash frozen in liquid nitrogen and stored at ~80 °C. As required, mitochondria were thawed, diluted with 40 mM MOPS (pH 7.4), 1 mM dithiothreitol to a protein concentration of ~5 mg/ml and lysed by subjecting to 5 freeze-thaw cycles;
conditions were optimized by monitoring the release of citrate synthase. The extract was centrifuged at 230,000 g for 28 min to pellet mitochondrial membranes and the supernatant containing matrix proteins was concentrated on a Vivaspin 5,000 Mₜ, cut-off device to ~27 mg/ml. Protein concentration was estimated by the BCA™ Protein Assay (Pierce) with bovine serum albumin as a standard.

Characterization of Mitochondrial Preparations Using Marker Proteins—1/170,000, 1/103,000, and 1/26,000 of final volumes of nuclei-free homogenate, gradient purified mitochondria, and mitochondrial matrix, respectively, were separated by SDS-PAGE on 8% (larger molecular mass subcellular markers) or 12% (smaller molecular mass subcellular markers) gels and transferred to nitrocellulose membranes. Membrane blocking, washing, and exposure to primary and secondary antibodies was performed by standard procedures. The primary antibodies used were: mouse anti-pyruvate dehydrogenase E1β subunit (Molecular Probes), rabbit anti-human mitochondrial β-ketoacyl synthase serum raised by Antibodies Inc., Davis, CA, from our purified protein (17), rabbit anti-prohibitin (BioLegend), rabbit anti-prostaglandin E synthase-2 (Cayman Chemical Co.), rabbit-anti-PMP70 (U.S. Biological), and goat anti-lactate dehydrogenase (U.S. Biological). The secondary antibodies used were: horseradish peroxidase-conjugated anti-rabbit IgG (H + L) (Bio-Rad), horseradish peroxidase-conjugated anti-mouse IgG (H + L) (Pierce), and horseradish peroxidase-conjugated anti-goat IgG (Santa Cruz Technology). Staining was performed with either ECL or SuperSignalWest Pico Chemiluminescent Substrate (Pierce).

Expression and Purification of Human Mitochondrial ACP, Synthesis of Holo-ACP and Acetyl-ACP—Human mitochondrial ACP was expressed in the insect Sf9 cell/baculoviral host/vector system and the apo-form purified as described previously (16). Holo-ACP, acetyl-ACP, and d15-labeled octanoyl-ACP were synthesized using the human phosphopantetheinyl transferase with CoA, acetyl-CoA, and d15-octanoyl-CoA, respectively, as donor substrate, and purified by anion exchange chromatography (17).

Expression and Purification of Bovine H-protein—Bovine H-protein was expressed in Escherichia coli and purified as the apo-form, essentially as described earlier (19) except that DEAE-Sepharose CL-6B and Sephadex G-100 were replaced with TSK gel DEAE-5PW (13 μ, 21.5 × 150 mm, Tosohaas) and HiPrep™ Sephacyr S-100 HR (16/60, Amersham Biosciences), respectively, and the hydroxylapatite step was omitted. The protein was at least 86% pure (SDS-PAGE). The m/z of the purified protein determined by MALDI-TOF MS was 13,847.4 ± 0.5, in good agreement with the estimated m/z for the apo-H-protein from which the N-terminal methionine had been cleaved (13,847.5). Full-length protein was not found either in the purified preparation or in the bacterial homogenate. A calculated absorbance coefficient for 280 nm 18,450 m⁻¹ cm⁻¹ (ProtParam, ExPASy server) was utilized to determine protein concentration.

Synthesis of d15-Labeled Octanoyl-CoA—d15-labeled octanoyl-CoA was synthesized from d15-octanoic acid (CDN Isotopes) and CoASH via thiophenol thioesters (20) and purified on a Waters C18 SepPak cartridge (21). Identity and purity was confirmed by MALDI-TOF MS and reversed-phase HPLC.

Analysis of 14C-Fatty Acyl-ACP Acyl Moieties Formed by Mitochondrial Matrix Preparations—The reaction mixture composition was based on earlier experiments using plant mitochondrial preparations (13) and consisted of 0.1 M MOPS buffer (pH 7.1), 2 mM dithiothreitol, 1 mM S-adenosylmethionine, 4 mM sodium sulfide, 1.72 mg/ml bovine mitochondrial matrix extract, 8.5 μM holo-ACP, 26.5 μM S-acetyl-ACP, 4.5 mM NADPH, and 4.5 mM NADH and either 0.1 mM [2-14C]malonyl-CoA, initial concentration (25.7 kdpm/nmol), or 3.9 mM [2-14C]malonate (21.1 kdpm/nmol). When [2-14C]malonyl-CoA was used as the substrate, additional [2-13C]malonyl-CoA (85 μM) was added every hour. Reaction mixtures containing malonate were supplemented with 5 mM ATP, 2 mM MgCl₂, and 4 mM CoA. Additional 5 mM ATP was added every 2 h. Incubations were continued at 37 °C for 2 or 6 h. Before incubation, all reaction mixtures were degassed under house vacuum for 40 s and vacuum was replaced with nitrogen. The procedure was repeated twice. Reactions were stopped by the addition of 1 M HCl to a final pH ~2 and ACPs were precipitated by treatment with ammonium sulfate, final concentration 2.4 M, for 1 h on ice. The precipitate was centrifuged and washed extensively with cold 2.4 M ammonium sulfate to remove free radioactivity. Subsequently, the S-acetyl-ACP thioesters were hydrolyzed by treatment of the precipitates with 1 M KOH for 1 h at 20 °C. The hydrolysate was acidified, fatty acid standards (10 nmol each) were added, and the contents was extracted with hexane/isopropyl alcohol. To avoid loss of short fatty acids during evaporation, fatty acids were converted to the potassium salts, solvent was removed by evaporation with nitrogen and the fatty acid salts were derivatized with phenacyl-h (Pierce). Phenacyl esters were analyzed by reversed-phase HPLC (22). To improve separation of phenacyl esters of short chain fatty acids and lipoic acid, the gradient was modified as follow: 45% solvent B (acetonitrile) for 5 min, then linear gradient to 56% B over 13 min, linear gradient to 90% B over 12 min, and finally linear gradient to 98% B over 9 min. Solvent A was water and the flow rate was 1 ml/min. Overall recoveries for the extraction and derivatization process were >90% for acyl chains longer than 4 C atoms, 80% for butyryl, 30% for acetyl, and 8% for malonyl standards.

MALDI-TOF MS Analysis of Fatty Acyl-ACP and Fatty Acyl-H-protein Species Formed by Mitochondrial Matrix Preparation—Large scale (440 μl) de novo synthesis of acyl-ACPs was carried out as described above for [2-14C]malonyl-CoA except that non-radioactive malonyl-CoA was used as a substrate. When products of transfer of acyl moieties from ACP to the apo-H-protein were analyzed, 20 μM apo-H-protein was present and the reaction volume was reduced to 100 μl. Assay mixtures were incubated for 6 h, then 2-propanol was added to 40%, samples were kept on ice for 20 min and then centrifuged (12,900 × g for 30 min). The supernatant, enriched in ACPs, was collected, concentrated to 40 μl in a VivaSpin 5-kDa molecular mass cut-off device (VivaScience), diluted with 0.1% trifluoroacetic acid to 145 μl, and the acyl-ACP species were separated on a C18 300 Å, 5 μm, 2.1 × 250-mm HPLC column (Vydac). The column was developed at 0.2 ml/min in 0.1% tri-
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fluorooacetic acid using a 30–45% acetonitrile gradient over 5 min, 45% acetonitrile for 20 min, and two-step acetonitrile gradient 45–90% over 10 min and 90–95% over 2 min; elution was continued for additional 10 min. The HPLC fractions were lyophilized or directly used in MALDI-TOF MS analysis. Transfer of \textsuperscript{14}C-octanoyl from ACP to H-protein was assayed in a 100-\mu l reaction where malonyl-CoA, NADPH, NADH, holocytochrome c, and acetyl-ACP were replaced with 20 \mu M \textsuperscript{15}N-labeled octanoyl-ACP and the matrix extract concentration was increased to 5.8 mg of protein/ml. The reaction was stopped after 2 h by addition of 2-propanol to 40% and the products analyzed as described above.

Identification of \textsuperscript{14}C-Fatty Acyl Moieties Covalently Linked to H-protein—Radiolabeled fatty acids were synthesized de novo by mitochondrial extract in a slightly modified procedure. To increase the amount of \textsuperscript{14}C label on the H-protein, the matrix protein concentration was increased to 7.7 mg/ml and the initial concentration of acetyl-ACP primer was 17.5 \mu M. Every 2 h, acetyl-ACP was replenished (11 \mu M each addition). Reactions were stopped by addition of ammonium sulfate (2.4 \mu M final concentration) that precipitated the acyl-ACPs. The supernatant containing H-protein was adjusted to pH 3.5 with 10% trifluoroacetic acid and injected onto an reversed-phase HPLC column, C18 300 Å, 5 \mu m, 2.1 \times 250 mm (Vydac). The column was eluted with a three-step gradient 5–40% over 15 min, 40–50% over 30 min, and 50–95% acetonitrile, 0.1% trifluoroacetic acid over 10 min and the elution was continued for another 5 min. Radioactive fractions eluting immediately following the unlabeled H-protein were collected, lyophilized, and H-protein radiopurity confirmed by SDS-PAGE, transferred to nitrocellulose membranes, probed with antibodies, and stained using the ECL system. Antibodies used were: PDH, anti-pyruvate dehydrogenase E1\beta subunit (mitochondrial matrix, 35.8 kDa); KS, anti-mitochondrial human \beta-ke-toacyl synthase (45.2 kDa); Proh, anti-prohibitin (inner mitochondrial membrane, 27.4 kDa); PSyn-2, prostaglandin E synthase-2 (microsomal membrane, 31.0–33 kDa); PMP70 (peroxisomal membrane, 68–76 kDa); LDH, lactate dehydrogenase (cytosol, 34.2 kDa). Estimated masses, calculated from the mobilities of molecular mass standards are shown on the right.

Results

Characterization of the Mitochondrial Matrix Preparation—Conditions for homogenization were optimized to provide efficient cell lysis with minimal damage to mitochondria by monitoring the release of lactate dehydrogenase and citrate synthase (markers for cytosol and mitochondrial matrix, respectively) into the high speed supernatant. The purity of the mitochondrial preparation and the matrix fraction derived therefrom was assessed using various marker proteins (Fig. 1). The purified mitochondrial fraction was free of contamination by the cytosolic marker but contained small amounts of both peroxisomal and microsomal membrane markers. The mitochondrial matrix fraction was essentially free of contamination by peroxisomal, microsomal, and mitochondrial membrane markers. The mitochondrial \beta-ke-toacyl synthase was recovered in the matrix fraction together with the matrix marker protein and this fraction was examined for its ability to synthesize fatty acyl chains.

Fatty Acid Synthesis by Mitochondrial Matrix Preparation—The system used to assess the lipogenic activity of mitochondrial matrix preparations included acetyl-ACP and [\textsuperscript{2,14}C]malonyl-CoA as substrates and both NADPH and NADH as a potential source of reducing equivalents. Preliminary experiments revealed that no radiolabel was incorporated into fatty acyl chains unless free ACP was included in the incubation system, indicating that malonyl-ACP, formed from malonyl-CoA by endogenous malonyl transacylase was the actual source of chain extender units for lipogenesis and confirming the earlier finding that mammalian mitochondrial \beta-ke-toacyl synthase uses malonyl-ACP but not malonyl-CoA as substrate (17). Control experiments also revealed that 90% of [\textsuperscript{2,14}C]malonyl-CoA was destroyed after 2 h incubation with mitochondrial matrix extract, being converted mainly to malonic, acetic acid, and traces of acetyl-CoA. Previous studies have identified acyl-CoA thioesterase activity in mitochondria (24) and we confirmed that our matrix preparations contained thioesterase activity toward malonyl- and C2-C10 acyl-CoA thioesters; in contrast, the corresponding ACP thioesters were stable in the presence of matrix extract (details not shown). Thus, in subsequent experiments [\textsuperscript{2,14}C]malonyl-CoA was routinely replenished every hour throughout the incubation period. Acyl-ACPs were precipitated with ammonium sulfate, the acyl chains were released by mild alkaline hydrolysis and derivatized with phenacyl-8. The major products formed after a 2-h incubation were C4- and...
C8-ACP with smaller amounts of C6-ACP and traces of C10-ACP (Fig. 2a). After 6 h of incubation, C8-ACP was the dominant product but significant amounts of C10, C12-, and C14-ACP were also formed (Fig. 2b). Essentially the same product profile was observed when the acyl-ACPs were collected by precipitation with trichloroacetic acid (data not shown). The only radiolabeled compounds detected in the supernatants remaining after precipitation of the acyl-ACPs were malonate and acetate, the latter presumably formed by decarboxylation of malonyl moieties. Thus, the acyl-ACP products appeared to be stable and the acyl moieties did not undergo significant hydrolysis. The average condensation rate in these reactions was 0.08 and 0.06 nmol h\(^{-1}\) mg of matrix protein per h for the 2- and 6-h incubations, respectively. The rate of uncoupled malonyl decarboxylation, as measured by the formation of radiolabeled acetyl moieties, was 0.08 and 0.06 nmol h\(^{-1}\) mg\(^{-1}\), respectively. No radiolabeled acyl-ACPs were synthesized in the presence of cerulenin (data not shown), an inhibitor of \(\beta\)-ketoacyl synthase involved in de novo fatty acid synthesis in prokaryotes, plant chloroplasts, and eukaryotic cytosol (25). Recombinant human mitochondrial \(\beta\)-ketoacyl synthase is also inhibited by cerulenin (17).

Utilization of the recombinant human mitochondrial His\(_6\)-tagged ACP as the acyl carrier for fatty acid synthesis in the bovine mitochondrial matrix preparations also allowed unambiguous identification of newly synthesized acyl-ACPs by mass spectrometry, because the human ACP (11,445.0 Da, for N-acetylated, His\(_6\), apo form) and endogenous bovine ACP (10,109.6 Da, for apo form) differ significantly in molecular mass. The acyl-ACP species formed during a 6-h incubation were fractionated directly by reversed-phase HPLC and analyzed by MALDI-TOF mass spectrometry (Fig. 3a). The recombinant acyl-ACP species eluted from the HPLC column in zones broader than anticipated, based on the elution profiles of purified ACP that had been incubated with the mitochondrial matrix extract, in the absence of any other substrates or cofactors, confirmed the presence of protease activity (Fig. 3b and c, and Table 1). For example, C8-ACP was detected in zone C of the chromatogram as both the His\(_6\)-tagged and His\(_6\)-tagged ACP species (Fig. 3b) and C14-ACP was detected in zone H as the His\(_6\)-tagged, His\(_6\)-tagged, and untagged ACP species (Fig. 3c). Mass spectrometric analysis of purified ACP that had been incubated with the mitochondrial matrix extract, in the absence of any other substrates or cofactors, revealed the presence of protease activity responsible for the removal of the C-terminal His tag (details not shown). Nevertheless, the data fully supported the results
obtained by analysis of the radiolabeled acyl moieties and confirmed that the matrix preparation was capable of synthesizing saturated acyl chains containing up to 14 C atoms.

The source of malonyl moieties for mitochondrial fatty acid synthesis is unclear because the two isoforms of acetyl-CoA carboxylase are located outside this organelle. One potential source could be free malonate and this possibility was evaluated experimentally using the bovine mitochondrial matrix preparation (Fig. 2c). When [2-14C]malonyl-CoA was replaced by [2-14C]malonate plus ATP, CoA, and MgCl2, and the fatty acids released from the acyl-ACP species by mild basic hydrolysis were analyzed as phenacyl esters, again significant amounts of de novo synthesized fatty acids were detected, predominantly octanoate, although the rate of formation was lower than when malonyl-CoA was added directly to the incubation system. No products were formed in the absence of ATP and MgCl2 (Fig. 2d), confirming that an ATP-dependent ligase present in the matrix preparation is able to convert malonate to malonyl-CoA that can be subsequently utilized for fatty acid synthesis.

**Coupling of Lipogenesis and Lipoylation Pathways in Mitochondrial Matrix Preparations**—One of the roles proposed for the mitochondrial fatty acid biosynthetic pathway to provide octanoyl moieties that can be utilized as a substrate for the lipoylation of mitochondrial proteins is the ability to synthesize fatty acids that can subsequently be utilized for fatty acid synthesis. The absence of radiolabel corresponding to residual [2-14C]malonyl moieties in the phosphorimages apparently is due to lability of the malonyl-CoA thioester during sample preparation and SDS-PAGE (Fig. 4, a and b). In the absence of apo-H-protein, the only radiolabeled protein detected was ACP, but inclusion of the apo-H-protein in the incubation system resulted in formation of a radiolabeled protein corresponding approximately to the apo-H-protein. The H-protein was radiolabeled when cerulenin was included in the incubation confirming that the radiolabel was associated with a protein fatty acid synthesis. The formation of octanoyl-H-protein was directly confirmed in a similar experiment where [2-14C]malonyl-CoA was replaced with non-radioactive substrate (Fig. 5a). The presence in the mitochondrial matrix extract of a enzyme capable of translocating octanoyl moieties from ACP thioester linkage to H-protein N-lysyl amide linkage was confirmed by incubating highly purified d15-labeled octanoyl-ACP with mitochondrial matrix extract and apo-H-protein. The H-proteins were puri-
fied and examined by MALDI-TOF mass spectrometry (Fig. 5b). Two species were identified with molecular masses cor-
responding to unreacted apo-H-protein and d\textsuperscript{15}-octanoyl-H protein.

DISCUSSION

The existence of a mitochondrial pathway for de novo fatty acid synthesis was first reported 40 years ago, when it was gen-
erally assumed that fatty acid synthesis proceeded by reversal of the mitochondrial pathway for fatty acid β-oxidation (26, 27).

Thus, the study showed unequivocally that the mitochondria possibly contribute enzymes capable of fatty acid elongation.

To eliminate the possibility that the mitochondrial matrix preparations used in our study might be contaminated with enzymes from other subcellular compartments, we utilized a rigorous purification protocol and monitored for the presence of contamination using a panel of marker enzymes. Using this approach, we determined that the beef heart mitochondrial matrix preparations were essentially free from contamination with microsomal and mitochondrial membranes that could possibly contribute enzymes capable of fatty acid elongation.

Thus, the study showed unequivocally that the mitochondria do indeed contain a malonyl-CoA-dependent, ACP-track sys-
tem for de novo fatty acid synthesis: ACP and malonyl transferase (16), enoyl reductase (15), and β-ketoacyl synthase (17).

To determine the catalytic efficiencies for the isolated β-ketoacyl synthase, we measured the enzyme activity using synthetic malonyl-ACP as substrate and apo-H-protein as acceptor. Although we routinely included in our preparations an octanoyl-ACP:protein N-oc-
tanoyltransferase transferase was ascertained by mass spectrometry using deuterated octanoyl-ACP as substrate and apo-H-protein as acceptor. Although we routinely included in our lipogenic assay system the S-adenosylmethionine and sodium sulfide required for insertion of the S atoms into C6 and C8 of the octanoyl moiety, we did not detect any significant amount of lipoyl-ACP among the products. In the procedure used to identify radiolabeled moieties formed on ACP, these moieties were identified as phenacyl esters (Fig. 2). The phenacyl ester of an authentic lipoic acid standard eluted at 18.4 min in a zone that did not contain radioactivity. In addition, when synthetic [\textsuperscript{1}\textsuperscript{14}C]octanoyl-ACP was incubated with matrix extract in the presence of cofactors for lipoic acid synthase, no significant amount of lipoyl-ACP was detected. Although we have been successful in synthesizing lipoyl-ACP from lipoyl-CoA, using the phosphopantetheinyl transferase at pH 7, in the presence of 1 mM diithiothreitol, we found the product to be remarkably

| Acyl chain | Synthesized by matrix preparation | β-Ketoacyl synthase catalytic efficiency (kcat/Km) |
|------------|----------------------------------|---------------------------------------------|
| C4         | 28.6                             | 24.0                                        |
| C6         | 3.0                              | 3.0                                         |
| C8         | 11.2                             | 49.1                                        |
| C10        | 0.3                              | 6.7                                         |
| C12        | 0.2                              | 6.2                                         |
| C14        | 0.0                              | 4.1                                         |
| C16        | 0.0                              | 0.0                                         |
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unstable; all of the lipoyl-ACP underwent spontaneous hydrolysis within 2 h. Instability of lipoyl-ACP has been reported previously (8) and to our knowledge, lipoyl-ACP has not been detected in mammalian mitochondria. Clearly lipoic acid synthase is a mitochondrial enzyme in mammals (40). What appears uncertain is whether the substrate is octanoyl-ACP or the N-octanoylated acceptor proteins. In E. coli, the preferred substrate for lipoic acid synthase (LipA), the enzyme that introduces the sulfur atoms into the octanoyl moiety is the N-octanoylated protein, not octanoyl-ACP (7). Our results indicate that in animal mitochondria too, octanoyl moieties synthesized de novo can be transferred directly to N-linkage on the acceptor protein where they presumably are the substrates for lipoic acid synthase. This pathway avoids altogether the formation of a potentially unstable lipoyl-ACP intermediate.

In microorganisms two distinct pathways for protein lipoylation are operative, one involving the diversion of octanoyl moieties from the ACP-linked pathway of de novo fatty acid synthesis, via an octanoyl-ACP:protein N-octanoyltransferase transferase (LipB) and subsequent introduction of the two sulfur atoms by lipoic acid synthase (LipA), the other utilizing exogenous free lipoic acid, which is recruited by a lipoic ligase (LplA) forming an AMP-linked lipoyl intermediate that is then transferred to the lipoylation site on the acceptor protein. Fujiiwara and colleagues (9, 19) have established that mammalian mitochondria possess the enzymes required for utilizing the exogenous lipoic acid pathway for protein lipoylation so it appears that mammalian mitochondria also have alternative routes for protein lipoylation. Previous studies have established that the de novo pathway for synthesis of lipoyl moieties is operative in the mitochondria of fungi (41, 42) and plants (10, 11, 13, 14, 43) so that this pathway appears to be ubiquitous in all mitochondria, consistent with the hypothesis that mitochondria originated from free-living bacteria. Disruption of fungal nuclear-encoded genes for mitochondrial fatty acid synthase proteins results in a respiratory-deficient phenotype (41, 42). In yeast this defect cannot be corrected by supply of exogenous lipoic acid indicating that the de novo pathway for the production of lipoyl moieties is critical for mitochondrial function (42). Recent studies in which the mouse gene for lipoic acid synthase was disrupted have revealed that endogenous lipoyl acid synthase is essential for survival of developing embryos and perinatal death cannot be prevented by supplementation of the maternal diet with lipoate (44). This finding implies that supply of the precursor for lipoic acid synthase likely is also critical for normal fetal development. Demonstration in our study that the octanoyl precursor for synthesis of lipoyl moieties can be provided by the mitochondrial pathway for de novo fatty acid biosynthesis suggests that this pathway too, likely plays an important role in embryological development. As this article was being prepared for submission, a report was published indicating that knockdown of the mitochondrial fatty acid biosynthetic system in Trypanosoma brucei drastically reduced protein lipoylation (45). Thus, it seems likely that in all animal life forms, octanoyl moieties formed in the mitochondria are the major precursors for the production of lipoyl moieties.

Experiments with the purified recombinant β-ketoacyl synthase and the reconstituted mitochondrial lipogenic pathway indicate that this system is also able to produce longer fatty acyl chains containing up to 14 carbon atoms. It is unclear at present whether this capability is important to mitochondrial function. However, the ACP associated with respiratory complex 1 in Neurospora crassa (46) and bovine mitochondria (47) reportedly carries a long chain fatty acyl moiety esterified to the phosphopantetheine thiol and in N. crassa mutants lacking the mitochondrial ACP, complex 1 does not assemble correctly (41). These observations suggest that perhaps a second function of the mitochondrial lipogenic system might be to supply specific longer chain acyl moieties that play a role in the assembly of complex 1.

Although most human mitochondrial proteins are encoded in the nucleus, only a small number have been identified and their roles defined. Mutations in more than 30 of these genes have been shown to give rise to devastating "mitochondrial diseases" including Friedreich ataxia, hereditary spastic paraplegia, and Parkinson disease and the exact causes of many diseases suspected as resulting from abnormal mitochondrial function have yet to be deciphered. Clearly, further studies are required to determine how important the linked lipogenic and lipoylation pathways are to mitochondrial function and human health.

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