A New Metabolic Link

THE ACYL CARRIER PROTEIN OF LIPID SYNTHESIS DONATES LIPIOIC ACID TO THE PYRUVATE DEHYDROGENASE COMPLEX IN ESCHERICHIA COLI AND MITOCHONDRIA

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Lipoic acid is an essential enzyme cofactor that requires covalent attachment to its cognate proteins to confer biological activity. The major lipoylated proteins are highly conserved enzymes of central metabolism, the pyruvate and α-ketoglutarate dehydrogenase complexes. The classical lipoate ligase uses ATP to activate the lipoate carboxyl group followed by attachment of the cofactor to a specific subunit of each dehydrogenase complex, and it was assumed that all lipoate attachment proceeded by this mechanism. However, our previous work indicated that *Escherichia coli* could form lipoylated proteins in the absence of detectable ATP-dependent lipoate transferase activity (6, 8). We report that *E. coli* and mitochondria contain lipoate transferases that use lipoyl-acyl carrier protein as the lipoate donor. This finding demonstrates a direct link between fatty acid synthesis and lipoate attachment and also provides the first direct demonstration of a role for the enigmatic acyl carrier proteins of mitochondria.

Lipoic acid is a cofactor required for function of the citric acid cycle (1). Two key enzyme multienzyme complexes use covalently bound lipoic acid to carry reaction intermediates between the active sites of the complexes. These enzymes are the pyruvate dehydrogenase complex (PDC) (1) that is responsible for the synthesis of the acetyl-CoA needed to prime the cycle and the α-ketoglutarate dehydrogenase complex (KGDC) that catalyzes the C5 to C4 step of the cycle (2). Covalently attached lipoic acid not only provides substrate channeling between the different active sites of these complexes but also maintains the activated acyl carrier group in thioester linkage for delivery to coenzyme A. Covalent attachment of lipoic acid to the E2 subunits of these enzyme complexes is required for activity in *vivo*. The cofactor is attached via an amide linkage formed between the lipoate carboxyl group and the ε-amino group of a specific lysine residue (2). The first enzyme shown to attach lipoic acid to the lipoyl domains of PDC catalyzes a two-step reaction in which ATP activates lipoic acid to lipoyl-AMP followed by transfer of the lipoyl moiety to the appropriate E2 lysine residue (3). Lipoylation is also required for activity of the glycine cleavage enzyme (GCV, the plant enzyme is often called glycine decarboxylase) (4, 5) and the branched chain α-keto acid dehydrogenase complex (2).

All lipoate attachment was thought to proceed by the classical ATP-dependent mechanism, and *Escherichia coli* contains such a lipoate ligase (LplA protein) that functions primarily in utilization of exogenously supplied lipoic acid (6, 7). *E. coli* strains having null mutations in the *lplA* gene lack the ability to attach *exogenously* added lipoic acid to protein and also lack detectable ATP-dependent lipoate ligase activity (6, 8). However, these strains retain the ability to attach *endogenously* synthesized lipoate to PDC and KGDC *in vivo* indicating the presence of a second enzyme (6, 7). We report that this enzyme represents a second class of lipoate attachment enzyme that utilizes the lipoate thioester of the fatty acid synthetic protein, acyl carrier protein (ACP), as the source of lipoic acid. ACPs are an extensive family of small (70–80 residues) acidic proteins modified by covalent attachment of 4'-phosphopantetheine to a centrally located serine residue. ACPs carry acyl groups via thioester linkage to the 4'-phosphopantetheine sulfhydryl group and were first demonstrated to function in the fatty acid synthetic pathways of bacteria and plants (reviewed in Refs. 9 and 10). Subsequently, the ACPs of fatty acid synthesis have been shown to act as acyl donors in the synthesis of phospholipids (9), lipid A (11), the acylated homoserine lactone signaling molecules of bacteria (12), and in the activation of bacterial toxins (13). Other ACPs function in the synthesis of polyketide (14) and polypeptide antibiotics (15), cell wall polymers (16), and compounds regulating bacteria-plant interactions (17).

The reaction we report not only provides a new role in central metabolism for ACP but is an unusual reaction in that an enzyme cofactor (lipoic acid) is first bound by thioester linkage to another cofactor, 4'-phosphopantetheine, the covalently attached prosthetic group of acyl carrier protein. This lipoyl-ACP is then used to donate lipoic acid to the enzyme subunits of PDC, KGDC, and GCV. An alternate way of viewing the reaction is that lipoic acid is transferred from one protein to another. We have also demonstrated the presence of this new lipoate transferase (the formal name is lipoyl-[acyl-carrier-protein]-protein N-lipoyltransferase) in mitochondria of both plant and fungi thus providing a physiological role for the ACPs reported in mitochondria of mammals (18), plants (19–21), and fungi (19, 22, 23).

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains**—The *E. coli* K-12 strains used were derived from *JK1* (a spontaneous *rplL* mutant of the wild type strain, W3110) by *P1* transductions with selection for internal or closely linked antibiotic resistance determinants. The mutant alleles used are all null alleles (deletions or transposon insertions) (6, 7). SWJ39 (*lplA*) is a tetracycline-sensitive (24) derivative of TM134 (*lplA::tet*) and was transduced with a *P1* stock grown on TM122 to give strain SWJ43 (*lplA ΔaroP-aceF*) *cca::Tn10*. Strain TM122 (*ΔaroP-aceE*) *cca::Tn10* and strain SWJ43 were transduced with a *P1* stock grown on

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RESULTS

Detection of a Novel Lipoate Transferase Activity—Two assays were used to detect attachment of lipoic acid (lipoylation) to the apo forms of PDC and KGDC. In the first assay lipoic acid attachment was measured by conversion of the inactive apo forms of PDC or KGDC (Fig. 1) to the active holo forms whereas the second assay followed the acylation-dependent shift in the electrophoretic mobility of a purified 80-amino acid residue apolipoyl domain from the E. coli PDC (Fig. 2A). The second assay is much less sensitive but has the advantage that it can be used with any acyl donor because the mobility shift is due to loss of the positive charge of the lysine residue. Both assays detected an enzyme activity in E. coli cell extracts that catalyzed the transfer of lipoic acid from lipoic-ACP to lipoyl domains. Moreover, this activity was present in extracts of E. coli lipA null mutants which lack detectable ATP-dependent ligase activity (7, 8) (Figs. 1 and 2B), and unlike the ATP-dependent ligase (8) the activity was not inhibited by chelating agents. As anticipated from prior in vitro studies (6) the enzyme was also active with octanoyl-ACP (Fig. 2C). [3H]Octanoyl-ACP was used to demonstrate direct transfer of the acyl chain from ACP to the lipoyl domain (Fig. 3), and the stoichiometry of the reaction was appropriate (1 mol of octanoyl-ACP was consumed per 1.4 mol of lipoyl domain modified, the discrepancy is attributed to hydrolysis of a portion of the octanoyl-ACP thioester linkages during electrophoresis). Conversion of the apo form of E. coli PDC to the enzymatically active holo form requires attachment of lipoate to a specific lysine residue of the lipoyl domains (1, 29). Therefore, activation of apoPDC (Fig. 1) demonstrated accurate modification of the lipoyl domains (activation of KGDC was also readily detected).

Specificity of the Lipoate Transferase—The gel mobility shift assay was used to determine the chain length specificity of the...
enzyme. The lipoate transferase was found to be much more active with the C6 acid than with acids of shorter or longer chain lengths (Fig. 2C). The C2 acid showed some activity, but since odd chain length acids are not present in vivo the transferase has an appropriate chain length specificity. The enzyme had no detectable activity with octanoyl-CoA (data not shown). Moreover, octanoyl-CoA failed to inhibit domain modification by octanoyl-ACP, indicating that the detergent character of the acyl-CoA could not explain the lack of activity (data not shown). The specificity of the lipoate transferase for the protein acceptor was tested by use of the apo form of the 87-residue biotin-accepting domain of E. coli acetyl-CoA carboxylase (28). Although this protein has a structure remarkably similar to the lipo domain structure (30), upon substitution of the biotin-accepting domain for the lipo domain no modification of the biotin acceptor protein was detected by a gel shift assay (28) (data not shown; the biotin domain gave the expected mobility shift upon biotinylation in the presence of E. coli biotin protein ligase, biotin, and ATP).

E. coli lipB Mutants Lack Lipoate Transferase Activity—In vivo studies from this laboratory had indicated that E. coli strains having null mutations in both lipA and a second gene (lipB) were completely defective in the modification of lipoated proteins (6). Moreover, lipB mutants were specifically defective in the attachment of endogenously synthesized octanoate (6). These data indicated that lipB mutants should be deficient in the transferase activity, and this prediction was confirmed. We were unable to detect lipoate transferase activity in extracts of lipB strains (Figs. 1 and 2B). Introduction of multicopy plasmids in which lipB was appropriately oriented downstream of a variety of powerful promoters gave only small increases in transferase activity. Moreover, in agreement with prior results from this laboratory (31) we were unable to detect high level expression of a protein having the molecular weight of LipB. Attempted optimization of translational initiation also failed to markedly increase transferase activity. Thus, we attribute our failure to detect a protein matching the LipB sequence to the very low level of lipB expression such that we failed to detect the protein band in our purified preparations (i.e. the visible bands are contaminating proteins). This very low cellular abundance is consistent with the codon bias of lipB and may be characteristic of vitamin attachment enzymes. We expect that lipB encodes an essential component of the lipoate transferase, although it remains possible that LipB is a positive regulator required for expression of the enzyme. We think this latter possibility is remote since other organisms including diverse bacteria and yeast contain DNA sequences that potentially encode proteins closely similar to LipB (data not shown). The sequences of regulatory proteins are seldom conserved among diverse organisms whereas enzymes often show strong sequence conservation. It should be noted that LipB may be only one subunit of a multisubunit transferase. If so, this would account for only modest increases in transferase activity upon introduction of multicopy lipB plasmids.

Mitochondria Contain Both Lipoate Transferase Activity and a Lipoate Donor—Several laboratories have reported that mitochondria contain ACPs (18–23), and nuclear genes encoding ACP-like proteins having mitochondrial targeting sequences have been detected in plants (20) and fungi (32). The role of these proteins has been a puzzle since the major fatty acid synthetic enzymes reside in other cellular compartments.

Since mitochondria contain lipoated proteins as well as ACP we tested lysates of plant (from pea, P. sativum) and fungal (N. crassa) mitochondria for a lipoate transferase active with lipoyl-ACP. Activity was readily detected in these preparations using either the gel shift assay (data not shown) or the E. coli apoPDC activation assay (Fig. 4). In the latter case we inactivated the endogenous mitochondrial PDCs by addition of ATP. In the presence of ATP an endogenous PDC kinase converts the plant and fungal mitochondrial PDCs to their phosphorylated and inactive forms (33, 34) (the kinase has no effect on E. coli PDC). The N. crassa transferase was also active with octanoyl-ACP. We also used apoPDC activation to test for the presence of lipoyl-ACP in the mitochondrial lysates and detected appreciable levels of endogenous lipoyl donors in both the plant and fungal preparations (Fig. 4). To demonstrate a stronger dependence of the N. crassa transferase activity on added lipoyl-ACP we serially diluted the lysate and proportionally extended the incubation time of each dilution (to compensate for dilution); the rationale being to dilute the endogenous lipoyl-ACP to concentrations at which the transferase could not efficiently bind the endogenous substrate. Under these conditions addition of E. coli lipo-ACP gave a 6.6-fold increase in apoPDC activation (data not shown). The endogenous lipoal donor present in N. crassa lysates may be a better transferase.

![Figure 3](image1.png)

**FIG. 3.** Transfer of tritium-labeled octanoate from ACP to lipo domain. The assays were done as described under "Experimental Procedures" with detection by fluorography. The complete reaction contained all required assay components (see "Experimental Procedures") whereas in the other lanes the indicated component was omitted from the reaction. ACP indicates octanoyl-ACP, and lipo domain indicates the octanoylated lipo domain.

![Figure 3](image2.png)

**FIG. 4.** Lipoyl-ACP-dependent activation of E. coli apoPDC in E. coli and mitochondrial lysates. Solid bars represent activities of an E. coli extract whereas open bars denote N. crassa mitochondrial lysates and shaded bars denote pea mitochondrial lysates. All lipoate transferase assays were performed as described under "Experimental Procedures" except that assay components were omitted or substituted as given. The octanoyl-ACP reactions contained 80 μg of octanoyl-ACP in place of lipoyl-ACP, and the low lipoyl-ACP reactions contained 5 μg (rather than 80 μg) of lipoyl-ACP. All PDC assays were performed in the presence of 2 mM ATP to inhibit the endogenous PDC activity of the mitochondrial lysates (33, 34).
substrate than E. coli lipoate-ACP, since addition of a low concentration of E. coli lipoate-ACP (22 \mu m) decreased the rate of apoPDC activation, presumably through occupation of the transferase active site by the less active E. coli lipoate-ACP substrate. Moreover, E. coli octanoyl-ACP (but not nonacylated ACP) was a potent inhibitor of the endogenous donor-dependent transferase reaction (22 \mu m gave complete inhibition in the presence of excess apoPDC) consistent with identity of the endogenous donors as lipoate thioesters of the mitochondrial ACPS.

**DISCUSSION**

Although lipoic acid attachment is required for activity of several enzymes essential to central metabolism (1, 2) and this cofactor is synthesized by most organisms (including plants and probably mammals), the mechanism of lipoic acid synthesis is not understood. Octanoic acid has been shown to be the precursor of the carbon chain (35), but neither the origin nor the mechanism whereby the sulfur atoms are inserted into the hydrocarbon chain are known. Our finding that lipoate-ACP donates lipoate to PDC suggests that lipoate acid synthesis may proceed through ACP-bound intermediates, the first of which (octanoyl-ACP) is produced by the fatty acid synthetic pathway. The amino acid sequences of PDC and KGDC and of their lipoyl domains are highly conserved throughout biology, and the conservation extends to function in that mammalian and plant mitochondria also have appropriately lipoylated upon expression in E. coli (4, 5, 36). Therefore, it seems reasonable to propose that the lipoate synthetic pathway is also conserved.

We suspect that the low rates of de novo fatty acid synthesis reported for isolated mitochondria and mitochondrial lysates (20–23) represents (at least in part) lipoic acid synthesis. Although the lipoylated enzymes, PDC, KGDC, GCV, and the branched chain \( \alpha \)-keto acid dehydrogenase complex can comprise major fractions of the mitochondrial proteins (e.g. more than one-third of the total plant mitochondrial soluble proteins; Ref. 3), these are very large protein assemblies (often larger than ribosomes) with each complex carrying only a few hundred lipoate molecules. Thus, what appears to be a low rate of fatty acid synthesis could readily suffice for synthesis of lipoic acid. Indeed, a major fatty acid chain synthesized by isolated mitochondria is octanoic acid, some of which is found in thioester linkage to the mitochondrial ACPS (21, 23). Moreover, Wada et al. (21) have recently reported that a portion of the octanoic acid synthesized by purified pea and N. crassa mitochondria is covalently attached to the H subunit of GCV, the subunit that normally carries the lipoate cofactor. Since the typical lipoylated enzymes of eucaryotes are located within the mitochondria, we propose that lipoic acid synthesis and attachment proceed within this cellular compartment and that mitochondrial ACP functions as an essential cofactor in this pathway.

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