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Unraveling the Pathway of Lipoic Acid Biosynthesis

Lipoic acid is almost universally required for aerobic metabolism. However, the mechanism for its synthesis and incorporation into proteins has remained elusive. A groundbreaking study published in the December issue of *Chemistry & Biology* [20] uncovers critical features of the lipoic acid biosynthetic pathway.

Learning about the pyruvate dehydrogenase complex (PDC) has evolved into a rite of passage for every beginning biochemist. Few have been spared the analogy of the lipoyl group acting as a crane in a construction yard, methodically removing intermediates from one subunit of the complex and successively delivering them to the active sites of others. Pyruvate and coenzyme A (CoA) enter the complex, while CO_2 and acetyl-CoA exit. In the process, NAD^+ is reduced to NADH. Other very worthy cofactors, such as thiamin diphosphate (TDP) and flavin adenine dinucleotide (FAD), participate in the reaction; however, the lipoyl group and its associated subunit (E2) serve as the core of this gigantic factory. The lipoyl group makes repeat performances in several other multienzyme complexes that are involved in primary and secondary metabolism. These include the α-ketoglutarate dehydrogenase complex (KGDC) of the citric acid cycle, the branched-chain 2-oxo acid dehydrogenase complex (BCDC), which is important in the metabolism of several of the branched-chain amino acids, and the glycine cleavage system (GCS), which degrades glycine to CO_2 and ammonia, while using the α carbon of the amino acid to generate N^5,N^{10}-methylene-tetrahydrofolate. Again, all of this takes place with concomitant reduction of NAD^+ to NADH [1].

The Biosynthesis of Lipoic Acid

Given the importance of the lipoyl group in central metabolism, it should not be surprising that many organisms elaborate more than one pathway to incorporate it into those complexes that require it. In the cell, very little lipoate exists as the free acid; almost all is tethered to the ϵ-amino group of a conserved lysine residue on lipoyl-accepting domains of target complexes. Pioneering studies from John Cronan's laboratory indicate that *E. coli* maintain at least two pathways for attaching the lipoyl group to these target lysine residues (Figure 1) [2]. Lipoic acid that the organism obtains from the medium is first activated by ATP, and then transferred and appended with concomitant release of AMP. In *E. coli*, both steps are catalyzed by a lipoate-protein ligase, which is designated LplA [3]. LplA also will use octanoic acid as a substrate, albeit with reduced efficiency [4]. Alternatively, the lipoyl group can be synthesized endogenously as an offshoot of fatty acid biosynthesis. The exact details of this pathway have not been completely illuminated; however, the major players have been identified and are currently being characterized. LipB is a lipoyl (octanoyl)-transferase; it can transfer either a lipoyl or octanoyl group from a bacterial type II acyl carrier protein (ACP) to lipoyl-accepting domains [4, 5]. LipA catalyzes sulfur insertion into the octanoyl group, forming the lipoyl appendage [6]. Evidence for similar endogenous pathways in several eukaryotes has also surfaced, providing rationale for the previously unexplained

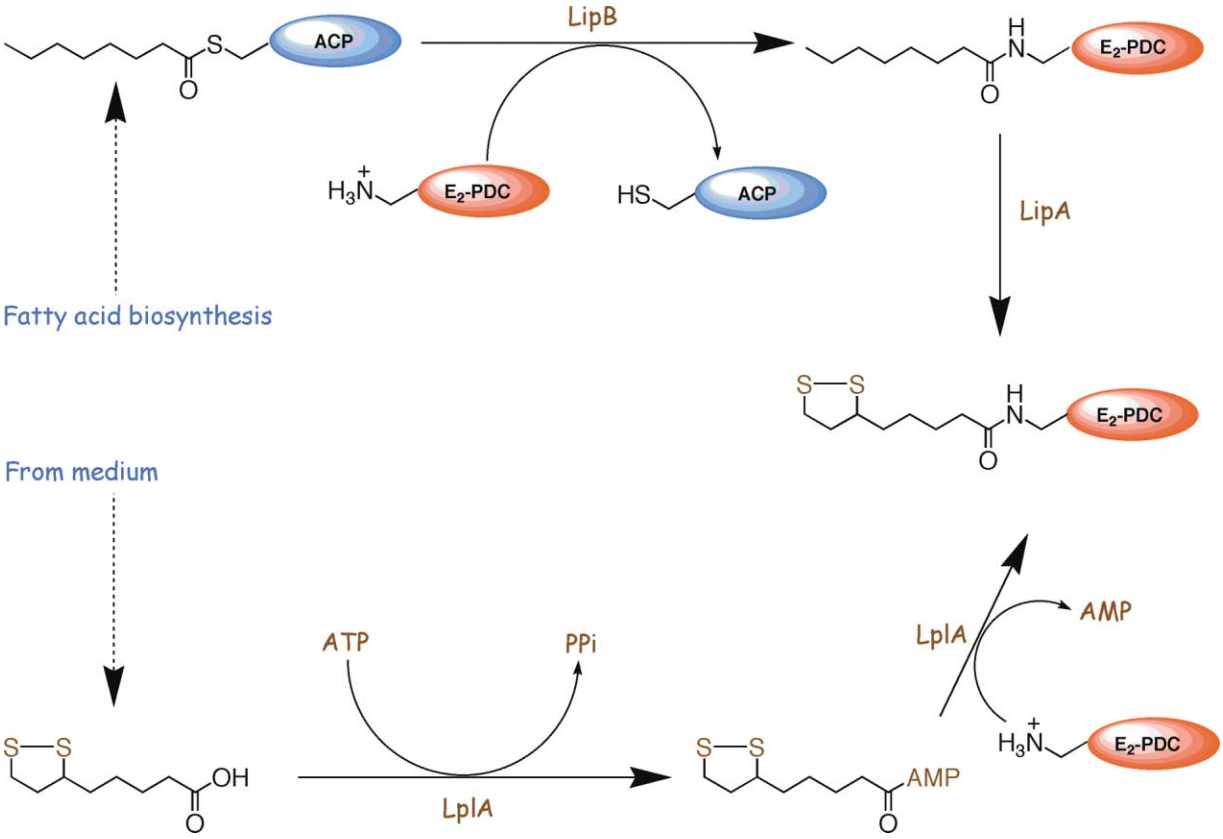

Figure 1. Pathways for Lipoyl Incorporation in *Escherichia coli*

presence of bacterial type-II ACPs in mitochondria of various eukaryotes, since primary fatty acid biosynthesis takes place in the cytoplasm via a type I fatty acid synthase [5, 7–12].

LipA and the Radical SAM Superfamily

Genomic and biochemical studies indicate that LipA is a member of a recently recognized class of metalloenzymes called the Radical SAM Superfamily [6, 13–15]. These proteins use S-adenosyl-L-methionine (SAM) as the source of a 5′-deoxyadenosyl 5′-radical (5′-dA•), which is a requisite intermediate in each enzyme's mechanism of catalysis. The 5′-dA• is generated via a reductive cleavage of SAM, yielding methionine as the remaining product. The reaction requires the input of one electron, which is supplied by a 4Fe-4S cluster that is bound to the protein via cysteine ligands that reside in a CXXXCXXC motif common to all Radical SAM enzymes. In vivo, the iron-sulfur cluster obtains its reducing equivalents from flavodoxin, a flavin-containing redox-active protein. In vitro, artificial reductants such as sodium dithionite or 5-deazaflavin plus light can satisfy the requirement for a reducing agent. In all cases, the role of the 5′-dA• is simply to remove a key hydrogen atom (H•) from the substrate, which initiates the catalytic cascade [16]. In lipoyl biosynthesis, it is speculated that the 5′-dA• abstracts one hydrogen atom from both C-8 and C-6 of a protein-derived octanoyl group, allowing insertion of sulfur atoms at each of these positions [6, 17]. It is believed that the source of the sulfur atom also is an iron-sulfur cluster that is bound to the protein [6, 18]. These hypotheses are working models and have yet to be substantiated experimentally.

Will the Real Substrate Please Stand Up?

Recently, the laboratories of John Cronan, Michael Marletta, and their collaborators were the first to establish in vitro turnover with purified LipA [6]. They devised a clever and sensitive method to assay the protein, linking the synthesis of lipoic acid to the formation of a functional E2 subunit on PDC. The activity of PDC could then be determined spectrophotometrically by observing the time-dependent reduction of an analog of NAD^+ in the presence of pyruvate, coenzyme A, thiamin diphosphate, and cysteine. The extent of lipoyl-PDC formation catalyzed by LipA was then quantified by comparing the activity of PDC to corresponding activities in a standard curve containing known and graded concentrations of lipoyl-PDC.

Using this assay, the authors were able to determine the minimum requirements for LipA-dependent lipoyl biosynthesis. As expected, LipA and AdoMet were essential, as was a source of reducing equivalents to generate the active form of the iron-sulfur cluster. The nature of the assay required the presence of LipB, since ACP was the octanoyl source and PDC was the complex that was assayed. The synthesis and attachment of the lipoyl group to apo-PDC was also verified by mass spectroscopy. Although earlier in vivo studies had hinted that octanoyl-ACP was the substrate into which LipA in-

serted sulfur atoms [19], these in vitro studies were not wholly consistent with this premise. The authors were unable to show formation of a lipoyl-ACP intermediate by conducting the reaction in the absence of LipB and/or apo-PDC.

The article by Zhao et al. in last month's issue of *Chemistry & Biology* firmly establishes that the preferred substrate for LipA is not octanoyl-ACP but octanoyl-E2 [20]. By extension of this finding, it can be assumed that LipA has multiple substrates, which are the lipoyl-accepting domains of PDC, KGDC, BCDC, and the H protein of GCS. The strength of their work lies in the inclusion of both in vivo and in vitro experiments in establishing this finding. The authors use strains of *E. coli* with null mutations in *lipA*, *lipB*, and *fadE* to incorporate exogenous deuterated octanoic acid into the lipoyl-accepting domains of a plasmid-encoded PDC. The authors then induce lipoyl synthase activity via transduction of the culture with phage λ particles containing a *lipA* cosmid. Analysis of the isolated E2 domains by mass spectroscopy revealed that the deuterium-containing octanoyl groups had become lipoylated. No labeled lipoyl domains were observed in cultures that were not transduced with the *lipA* cosmid.

The authors arrive at the same conclusion in in vitro studies using purified LipA. They monitored cleavage of SAM into methionine and 5′-deoxyadenosine as well as lipoyl-E2 formation. Significant production of 5′-deoxyadenosine was observed when octanoyl-E2 was the starting substrate, while only trace amounts were observed when octanoyl-E2 was replaced with octanoyl-ACP. Moreover, lipoyl-E2 production was observed only in the presence of octanoyl-E2.

The enzymology of sulfur insertion into unactivated C-H bonds is unchartered territory and portends new and exciting chemistry to be unraveled. The seminal experiments of Miller et al. suggest that the immediate sulfur donor is already associated with the protein, since activity was observed in the absence of exogenous sulfur sources [6]. Lipoyl synthase has two sets of conserved cysteine residues. One set contains the motif that is common to all Radical SAM enzymes, while the second set resides in a CXXXXCXXXXXC motif, which is common only to lipoyl synthases. In analogy to biotin synthase, this motif could house a second iron-sulfur cluster that acts as the sulfur donor in the reaction [18]. The intermediates and sequence of events associated with sulfur insertion are presently only speculative in both of these enzyme systems. In addition, the exact mechanism by which SAM is cleaved to generate a 5′-dA• is currently unknown in all Radical SAM enzymes [16]. The conclusions reached by Zhao et al. now enable these questions to be addressed because they resolve the major issue and limitation associated with lipoyl synthase: the nature of the true substrate.

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Profound Insights into Squalene Cyclization

In this issue of *Chemistry & Biology*, our understanding of the formation of pentacyclic hopene from the linear squalene is enhanced by an X-ray structure of a complex between squalene-hopene cyclase and the substrate analog 2-azasqualene [13].

A classic example of the interface between chemistry and biology is the carbocationic transformation of squalene and (*3S*)-2,3-oxidosqualene to polycyclic triterpenes. In 1955, seminal papers were published that described the chemical mechanism of the cyclization reaction [1, 2]. These studies highlighted important reac-