Protein moonlighting elucidates the essential human pathway catalyzing lipoic acid assembly on its cognate enzymes

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The lack of attachment of lipoic acid to its cognate enzyme proteins results in devastating human metabolic disorders. These mitochondrial disorders are evident soon after birth and generally result in early death. The mutations causing specific defects in lipoic assembly map in three genes, LIAS, LIPT1, and LIPT2. Although physiological roles have been proposed for the encoded proteins, only the LIPT1 protein had been studied at the enzyme level. LIPT1 was reported to catalyze only the second partial reaction of the classical lipoate ligase mechanism. We report that the physiologically relevant LIPT1 enzyme activity is transfer of lipoic moieties from the H protein of the glycine cleavage system to the E2 subunits of the 2-oxoacid dehydrogenases required for respiration (e.g., pyruvate dehydrogenase) and amino acid degradation. We also report that LIPT2 encodes an octanoyl transferase that initiates lipoic group assembly. The human pathway is now biochemically defined.

lipoic acid | mitochondrial disorder | inborn errors | glycine cleavage system | 2-oxoacid dehydrogenases

Although lipoic acid was discovered over 60 y ago as a covalently bound enzyme cofactor required for aerobic metabolism (1–3), it is only in recent years that the mechanisms of its biosynthesis have become understood (4–6). The importance of protein lipoylation is illustrated by disorders of this mitochondrial pathway, which result in grave metabolic defects and early death.

Lipoic acid biosynthesis is best described as an assembly process because lipoic moieties are constructed on the enzyme subunits of the cognate enzymes via a markedly atypical pathway (7) (Fig. 1). Lipoic acid is an eight-carbon fatty acid in which sulfur atoms replace the hydrogen atoms of carbons 6 and 8 of the acyl chain (oxidation of the resulting disulfide gives lipoic acid). Genetic and biochemical studies in Saccharomyces cerevisiae showed that an octanoate moiety diverted from fatty acid synthesis by the LipB octanoyl transferase becomes attached to the ε-amino group of a specific lysine residue of the cognate enzyme proteins (4). The octanoylated proteins then become substrates for sulfur insertion by the S-adenosyl-l-methionine radical enzyme, LipA (Fig. 1). The lipoil-modified proteins are the GCVH protein of glycine cleavage (8) and the small universally conserved protein domains located at the amino termini of the E2 subunits of the 2-oxoacid dehydrogenases required for aerobic metabolism and other reactions (4). The LipB-LipA pathway (Fig. L4) is the simplest, but not the only lipoil assembly pathway (4). Another bacterium, Bacillus subtilis, requires four proteins for lipoyl assembly rather than the two that accomplish the task in E. coli (4, 9, 10) (Fig. 1B). In contrast to E. coli where the lipoil assembly pathway directly modifies each of the cognate proteins, B. subtilis assembles lipoil moieties only on the H protein of the glycine cleavage system (4, 9, 10). The other lipoate-dependent enzymes obtain lipoil moieties only upon transfer from the H protein. Essentially, the same pathway has recently been documented in Staphylococcus aureus (11). Thus, the small H protein (127 residues) has two functions in central metabolism: the glycine cleavage pathway of single carbon metabolism and lipoylation of the 2-oxoacid dehydrogenases required for aerobic metabolism and branched chain fatty acid synthesis (12). Indeed, B. subtilis strains that lack the H protein are unable to grow without lipoate (or supplements that bypass function of the key 2-oxoacid dehydrogenases) and cannot cleave glycine to serve as the sole nitrogen source (9, 12). The yeast, Saccharomyces cerevisiae, is thought to have a similar pathway (13), although little in vitro enzymology has been done due to the intractable nature of the yeast proteins.

In mammals, all proteins involved in lipoyl assembly are located in the mitochondria. In humans, the first indicator of defective lipoal assembly is generally the presence of abnormally elevated levels of lactate (derived by reduction of pyruvate accumulated due to pyruvate dehydrogenase deficiency) in urine and plasma. Subsequent measurements of glycine levels in body fluids allow these individuals to be divided into two groups (14, 15). Normal glycine levels indicate that the glycine cleavage system is functional, and thus the glycine cleavage H protein (GCSH) is lipoylated, whereas abnormally high glycine levels indicate a lack of GCSH lipoylation. Elevated brain glycine levels result in a host of neurological disorders, including neurodegeneration, encephalopathy, and neonatal-onset epilepsy (14, 15), whereas the lack of 2-oxoacid dehydrogenase lipoylation short-circuits function of the tricarboxylic acid cycle, resulting in defects of energy production, accumulation of toxic levels of certain amino acids, and early death. The different pathways for lipoic acid synthesis put forth have not been validated by direct analysis of the postulated enzyme reactions, excepting a protein called LIPT1. Unfortunately, the enzyme activity reported for LIPT1 is misleading and seems to be an evolutionary remnant. We report that LIPT1 has a second “moonlighting” enzyme activity that fully explains the physiology of individuals lacking LIPT1 activity. We also document the postulated activity of LIPT2, another essential enzyme of the pathway.

**Significance**

Lipoic acid is an enzyme cofactor found throughout the biological world that is required for key steps in central metabolism. In humans, defective lipoic acid synthesis results in defective energy production, accumulation of toxic levels of certain amino acids, and early death. The different pathways for lipoic acid synthesis put forth have not been validated by direct analysis of the postulated enzyme reactions, excepting a protein called LIPT1. Unfortunately, the enzyme activity reported for LIPT1 is misleading and seems to be an evolutionary remnant. We report that LIPT1 has a second “moonlighting” enzyme activity that fully explains the physiology of individuals lacking LIPT1 activity. We also document the postulated activity of LIPT2, another essential enzyme of the pathway.

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severe respiratory deficiency and extreme muscle weakness (14, 15).

Human individuals having severely decreased levels of all lipoylated proteins have mutations in either LIAS, which encodes a lipoyl synthase known to functionally replace E. coli LipA (16), or LIPT2, which is proposed to encode an octanoyl transferase (14, 15). The patients who selectively retain GCSH lipoylation have mutations in a third gene, LIPT1 (14, 15). For decades, the reported LIPT1 enzymatic function has muddled interpretation of the human disorders. The reported activity for LIPT1 protein is transfer of a lipoyl moiety from lipoyl-adenylate to both GCSH and to the 2-oxoacid dehydrogenase E2 subunits (17, 18) (Fig. 1D). Modification of both acceptor proteins directly conflicts with the LIPT1 biochemical phenotype because individuals lacking LIPT1 activity should lack GCSH lipoylation whereas GCSH lipoylation (hence glycine cleavage) is normal in LIPT1 patients. A second argument against the physiological relevance of the reported LIPT1 “half-ligase” activity is that there seems to be no valid source of the lipoyl-adenylate required for the reaction. ACSM1, an extraordinarily promiscuous acyl-CoA synthetase (19), was reported to synthesize lipoyl-adenylate (20). However, LIPT1 utilizes both isomers of lipoate, whereas lipoylated proteins contain only the R isomer (20), which strongly argues against a role for ACSM1 in lipoyl attachment. Despite these shortcomings, LIPT1 and ACSM1 have been ascribed roles in disorders of human lipoyl metabolism, generally in uptake and attachment of dietary lipoic acid (21, 22). However, dietary lipoic acid supplementation has no effect on survival of mammals or tissue culture cells defective in lipoyl assembly (see below). The close analogy of the human lipoyl metabolism defects to those of B. subtilis mutant strains defective in lipoylation led to the hypothesis that the relevant LIPT1 activity is transfer of lipoyl moieties from lipoyl-GCSH to the 2-oxoacid dehydrogenase subunits (4).

In our first experiments to test LIPT1 amidotransferase activity (Fig. 2), we constructed three compatible plasmids, each of which expresses one of the relevant human proteins: GCSH, LIPT1, or a hexahistidine-tagged lipoyl domain (LD) derived from the E2 subunit of pyruvate dehydrogenase. Expression of each codon-optimized protein was placed under a tightly controlled promoter. The plasmids were transformed into an E. coli ΔlipB strain to attempt construction of the human lipoyl transfer pathway in this bacterium (Fig. 2). We first induced GCSH expression from an IPTG-inducible promoter led LipM. Expression of LipT1 and the PDH inner E2 domain were suspended in fresh medium lacking lipoyl and IPTG (supplementation with acetate and succinate allowed growth to proceed). Expression of LipT1 and the PDH inner E2 domain were then induced with arabinose to provide the enzyme and its putative substrate for lipoyl transfer from GCSH to the E2 domain.
Fig. 2. (A) Flow chart of the reconstitution of LIPT1-lipoic transfer from lipoic-GCSH to a human LD acceptor in the *E. coli* ΔlipB strain, XC.127. The cultures were grown in glycerol minimal medium with differing supplements as shown in the figure. Supplementation with succinate and acetate bypasses the need for 2-oxoacid dehydrogenase lipoylation (4). Strain XC.127 transformed with the plasmid encoding the His<sub>6</sub>-LD acceptor protein was additionally transformed with the GCSH plasmid plus the LIPT1 plasmid, the GCSH plasmid alone, or the LIPT1 plasmid alone. The resulting cultures were induced with IPTG in the presence of lipoate to allow the host LplA ligase to synthesize lipoic-GCSH (if present). The cells of each culture were then collected by centrifugation and washed to remove lipoate and IPTG. After resuspension in glycerol minimal medium containing acetate and succinate, arabinose was added to the three cultures to induce expression of LIPT1 and His<sub>6</sub>-LD. The cultures were incubated to allow further growth and accumulation of the His<sub>6</sub>-LD. The cells were then collected and lysed, and the His<sub>6</sub>-LD of each culture was purified by Ni<sup>2+</sup> chelate chromatography. The purified samples were then submitted for mass-spectrometric analysis and the proteins expressed in each sample are summarized in B, where + or − denotes expression. The electrospray mass-spectrometric scans for each culture are given in C–E. (C) Mass-spectrometric analysis of the His<sub>6</sub>-LD acceptor accumulated in the absence of the LIPT1 plasmid. The LD remained in the apo form (m/z 13,796.2 Da). Note that the apo LD mass was 18 Da less than that calculated (13,814.3 Da), consistent with either dehydration (−18 Da) or deamidation (−17 Da) during mass spectrometry (Protein Prospector, prospector.ucsf.edu). Dehydration seems more probable since almost a third of the protein is composed of residues (S, T, E, and D) known to undergo water loss. (D) Mass-spectrometric analysis of the His<sub>6</sub>-LD acceptor accumulated in the absence of the GCSH plasmid. The LD remained in the apo form (m/z 13,797.2 Da). (E) Mass-spectrometric scan of the His<sub>6</sub>-LD accumulated when both LIPT1 and GCSH were expressed. The mass of the lipoylated LD form (13,983.1 Da) agrees well with the calculated value (14,001.5 Da). The change in mass upon modification (calculated for lipoic modification, 188 Da; observed, 187 Da) is within the accuracy of the instrument utilized. a.u., arbitrary units; Intens., intensity.
The E2 domain was then purified by Ni-chelate chromatography and analyzed by mass spectrometry. The apo-E2 domain (m/z of 13,814.3) was converted to lipoyl-E2 domain (m/z of 14,001.5) (Fig. 2E). The delta mass between the two forms was 187, whereas a lipoyl moiety is 188. The 14,001.5 species was not present when either the LIPT1 or GCSH-encoding plasmid was omitted (Fig. 2 C and D), indicating that the washing steps effectively removed lipoylate and thereby rendered the host LplA lipoylate ligase inactive.

**LipT1 Has Lipoyl Amidotransferase Activity but Lacks Octanoyl Amidotransferase Activity.** Given these encouraging results, we expressed each of the genes in *E. coli* and purified the proteins (Fig. 3A). The substrates needed to assay lipoyl amidotransferase activity were also prepared in pure form. These consisted of an acceptor domain consisting of the inner LD of the human pyruvate dehydrogenase E2 subunit and a lipoyl (or octanoyl) donor protein consisting of the modified human GCSH protein. Lipoyl-GCSH was synthesized from the apo protein using the *B. subtilis* LpIJ lipoylate ligase (Fig. 1D), ATP, and lipic acid, whereas octanoyl-GCSH labeled with 14C in the octanoyl moiety was prepared with 14C-octanoic acid using the same enzymatic reaction.

Lipoyl-GCSH purified free of ATP and LpIJ was incubated with LIPT1 plus the LD acceptor protein. Three different assays were used (Fig. 3). Fig. 3C shows a mobility shift assay using gel electrophoresis in the presence of urea. In this assay, modification of the LD results in an increased rate of migration of the protein due to loss of the positive charge of the modified lysine (both LDs and glycine cleavage H subunits are unusually acidic proteins). In reactions that contained all of the reaction components, the LD was largely converted to a faster-migrating species, whereas no such shift was seen when a reaction component was omitted. A second assay evaluated transfer of lipoyl moieties from purified lipoyl-GCSH to the human LD. These reaction products were separated by SDS/PAGE followed by Western blotting with an anti-lipoate antibody (Fig. 3B). A species having the same mobility as the lipoyl-LD standard was formed in the complete reaction mixture but not when a component was omitted. Finally, a portion of the complete reaction mixture was analyzed by electrospray mass spectrometry. The two peaks observed were the remaining unmodified apo-LD substrate and lipoyl-LD. The difference in mass values of the two peaks was 188.5, whereas a lipoyl moiety has a mass of 188. Note that traces of LD modification were seen in the absence of lipoyl-GCSH (Fig. 3B and C). Since prior workers demonstrated that LipT1 purified from *E. coli* contained bound lipoyl-adenylate (28) and can transfer the lipoyl-adenylate lipoyl moiety to LD domains (17), this low level of LD modification is attributed to bound lipoyl-adenylate that accompanied LIPT1 through purification of the protein.

We also tested the ability of LIPT1 to transfer 14C-octanoyl moieties from octanoyl-GCSH to the human LD (Fig. 4) and found no detectable transfer. Note that the LD preparation was the same as that used in the lipoyl transfer experiments and was active with LpIJ.

**The Putative LIPT2 Octanoyl Transferase Catalyzes Transfer from Octanoyl-ACP to GCSH.** Human genetics investigators have postulated that LIPT2 is an octanoyl transferase (5, 15, 27) based on sequence alignments with the octanoyl transferases of *E. coli* (29, 30) and *Mycobacterium tuberculosis* (31) (Fig. 5A). However, sequence alignments within Pfam PF03099 protein family are not a trustworthy predictor of function and must be viewed with considerable caution (Fig. 5B). For example, the *B. subtilis* genome has been annotated as encoding three lipoate ligases (www.microbesonline.org). However, only one protein had ligease activity; the other two proteins catalyzed octanoyl transfer and lipoyl amidotransfer (9, 10). These considerations indicated that validation by direct assay of the postulated LIPT2 activity was required. The lack of such evidence became a more serious shortcoming when the first LIPT2 mutations were detected in human patients (27).

**Fig. 3.** LIPT1 catalyzes transfer of lipoyl moieties from GCSH to an LD derived from human pyruvate dehydrogenase. (A) Purification of the lipoate assembly proteins. The proteins were purified as described in Experimental Procedures and analyzed by SDS/PAGE on 4–20% polyacrylamide gels. The molecular weights of the Bio-Rad broad-range protein standards are indicated. (B) Western blot analysis of SDS/PAGE assay of LIPT1 lipoyl amidotransferase activity using anti-lipoate antibody and *B. subtilis* LpIJ-generated lipoyl-GCSH as the substrate. Lipoyl-GCSH was synthesized with LpIJ plus ATP and lipoyc acid and then purified using anion exchange chromatography to remove LpIJ and residual ATP. Lanes: 1, standard lipoyl-LD prepared by LplJ modification of human LD (Hs apo-LD); 2, lipoyl-GCSH; 3, lipoyl-GCSH plus the human LD; 4 and 5, lipoyl-GCSH incubated with LIPT1 and LD. (C) LIPT1 lipoyl amidotransferase activity analyzed by urea gel electrophoresis. Loss of the positive charge of the modified lysine ε-amino group of the LD results in faster migration of the modified form on these gels. The gel was stained with Coomassie Blue. (D) Electrospray mass-spectrometric analysis of lipoylated human LD and the remaining apo LD from the reaction of gel (B, lane 4). The calculated difference in mass (delta mass) between the apo and lipoylated forms was 188, whereas the observed delta mass was 188.5. Note that, in A and B, trace levels of LD lipoylation were seen in the absence of GCSH, which is attributed to LIPT1-bound lipoyl-AMP that survives purification and crystallization (28). The traces of lipoylation that appears without LIPT1 in B seems likely to be due the lipoate assembly pathway of the wild-type *E. coli* strain used for protein production.
We began by asking whether LIPT2 could functionally replace the *E. coli* LipB octanoyl transferase, an enzyme essential for lipoyl assembly (29, 32). A synthetic LIPT2 gene with codons optimized for *E. coli* expression was inserted into the medium copy vector pBAD322A to be transcribed from the arabinosinducible araBAD promoter and translated using the vector ribosome binding site. The mouse LIPT2 sequence was used because an unambiguous human sequence was not available when this work was initiated. The gene encoding the primary translation product was used because the *E. coli* and *Arabidopsis* LipBs are inactivated by small N-terminal truncations (29, 33). The LipT2 plasmid was transformed into an *E. coli* strain carrying deletions of the genes encoding both LipB and the LplA lipoate ligase. The latter mutation was included to avoid bypass of LipB by LplA mutations (34). The Δ*lplA* Δ*lphiA* strain was grown on glycerol minimal medium containing acetate and succinate (which bypass the lack of lipoic proteins) and then streaked on glycerol minimal medium plates containing various supplements. Growth proceeded only when the medium was supplemented with arabinose, the inducer of the *araBAD* promoter (Fig. 6). Slow growth was observed, which we attribute to the requirement that LIPT2 function with three bacterial protein substrates: ACP and the two 2-oxoacid dehydrogenase E2 subunits. These complementation results strongly suggest that LIPT2 was indeed an octanoyl transferase and that *E. coli* octanoyl-ACP and *E. coli* E2 LDs should function in vitro as the octanoyl donor and acceptor, respectively.

We then expressed a hexahistidine-tagged version of LIPT2 in *E. coli* and purified the enzyme to homogeneity (Fig. 3). LIPT2 readily transferred the octanoyl moiety from *E. coli* [1-C]octanoyl-ACP to human GCSe but was unable to transfer an octanoyl moiety to the human LD (Fig. 7A). Note that the human LD domain was an excellent substrate for the *B. subtilis* LplJ ligase and as an acceptor in lipoic amidotransfer assays, indicating that it had native structure. As expected from the observed functional replacement of LIPT2 with LipB, LIPT2 modified an *E. coli* LD and also the GcvH proteins of *E. coli* and two other bacteria (Fig. 7B). In the LipB reaction, an octanoyl moiety is transferred from ACP to the LipB active-site cysteine thiol (30). This acyl-enzyme intermediate is then attacked by the ε-amino group of the target lysine residue to give the octanoylated acceptor protein (30, 35, 36). Based on the alignments of Fig. 5, Cys185 of LIPT2 was expected to be the site of acyl enzyme formation. To test this hypothesis, Cys185 was replaced with either alanine or serine. As expected from prior results with LipB (30), both mutant proteins lacked octanoyl transferase activity (Fig. 7C). Finally, the results obtained using the radioactive assay were validated by mass-spectroscopic analysis of

**Fig. 4.** Ability of LIPT1 to transfer octanoyl moieties from GCSe to an LD derived from human pyruvate dehydrogenase. (A) Autoradiograms of urea-PAGE gels of assays testing LipT1-catalyzed octanoyl amidotransfer from purified [1-14C]octanoyl-GCSe to the lipoyl domain (LD). Lanes: 1, [1-14C]octanoyl-GCSe synthesized using *B. subtilis* LplJ plus ATP and [1-14C]octanoic acid; 2, [1-14C]octanoyl-LD standard synthesized with LD as an acceptor. The mouse LIPT1-labeled LD band indicates that residual ATP remaining from the in vitro reaction of lanes 1 and 2 of the gel in A, respectively. (B) Schematic representation of the LIPT1-catalyzed octanoyl amidotransfer reaction. The octanoyl-labeled LD band indicates that residual ATP remaining from the [1-14C]octanoyl-GCSe synthetic reaction was used by LplJ to modify the LD. (6) Octanoyl amidotransferase urea-PAGE gel assays performed in the presence of an ATP trap (hexokinase plus α-glucose) to prevent LplJ modification of the LD. Lanes: 1 and 2 are a repetition of the experiment of lanes 1 and 2 of the gel in A, respectively; 3, LD added to the [1-14C]octanoyl-GCSe synthetic reaction. The [1-14C]octanoyl-labeled LD band indicates that residual ATP remaining from the [1-14C]octanoyl-GCSe synthetic reaction was used by LplJ to modify the LD. (8) Octanoyl amidotransferase urea-PAGE gel assays performed in the presence of an ATP trap (hexokinase plus α-glucose) to prevent LplJ modification of the LD. Lanes: 1 and 2 are a repetition of the experiment of lanes 1 and 2 of the gel in A, respectively; 3, LD added to the [1-14C]octanoyl-GCSe synthetic reaction. The [1-14C]octanoyl-labeled LD band indicates that residual ATP remaining from the [1-14C]octanoyl-GCSe synthetic reaction was used by LplJ to modify the LD.
LIPT2-catalyzed modification of human GCSH with a non-radioactive octanoyl moiety (Fig. 7D).

As first seen with the mouse LIPT2 (Fig. 6), a synthetic gene expressing the full-length human LIPT2 (84% identical to the mouse LIPT2) restored growth of the E. coli ΔlipB ΔlipA strain in the presence of arabinose (Fig. 8). However, the putative protease-processed mature form of human LIPT2 recently reported (26) (a deletion of residues 2–31) failed to complement (Fig. 8). Hence, although the cell death observed (26) was attributed to a lack of mitochondrial targeting, the LIPT2 construct also lacked activity. Indeed, LIPT2 is not processed. Several mass-spectral analyses of the human proteome report detection of LIPT2 peptides corresponding to the N-terminal seven residues plus residues 14–24 and 29–40 of the primary translation product (37–39) (the peptide data are collected at www.proteomicsdb.org), which would be lacking in the putative mature form (26). Note that, consistent with human LIPT2, loss of the 22 N-terminal E. coli LipB residues inactivated the protein (29). This might be explained by the similarities (underlined) seen in the N-terminal sequences of LIPT2 (residues 5–19, AVRLVRGLGRVPYAEEL, and LipB residues 5–14, LVRLQGL—LYEFP).

Discussion

Our evidence that LIPT1 has lipoyl amidotransferase activity renders inoperative the models of the human disorders that include the partial ligase activity. This, plus the demonstration that LIPT2 is an octanoyl transferase, defines a straightforward pathway that fully explains each of the phenotypes of the human disorders. Individuals having mutations that result in loss of function of LIAS or LIPT2 are unable to assemble lipoyl moieties, and hence all cognate proteins remain in their unmodified and inactive apo forms. These individuals suffer high levels of body fluid glucose, lysis, and branched chain amino acids plus defective energy metabolism (14, 15). In contrast, although patients carrying LIPT1 mutations have normal lipoyl-GCSH and glycine cleavage levels, they suffer from defective energy metabolism plus high levels of lysine and branched chain amino acids (14, 15). Note that decreased 2-oxoacid dehydrogenase activities would additionally result in severely decreased levels of acetyl-CoA and succinyl-CoA, and thus modification of histones and other proteins could be compromised (40).

Our data plus prior work on mitochondrial fatty acid synthesis put the pathway (Fig. 1C) on a solid basis. Following demonstration that mammalian mitochondria contain soluble ACP in addition to the ACP molecules that become subunits of respiratory complex I (41), a complete type II fatty acid synthesis pathway in mammalian mitochondria was demonstrated by Smith and coworkers (42–44), who went on to show that this pathway provides the octanoyl-ACP required for lipoyl moiety synthesis (42–44). Moreover, these workers showed that blocking mitochondrial fatty acid synthesis in transgenic mice blocked protein lipoylation and resulted in a variety of serious physiological abnormalities (including early death) due to disruption of energy metabolism (45).

A similar but less severe decrease in lipoyl protein assembly due to deficient mitochondrial fatty acid synthesis was recently reported in human patients (46). GCSH is the only gene of the pathway in which no human disorder maps, although mutations in the other genes specific to glycine cleavage (AMT and GLDC) are fairly abundant (47). This disparity seems due to the small size of the GCSH coding sequence (173 codons), a comparatively small mutational target relative to the coding sequences of AMT (403 codons) and GLDC (1020 codons). Mutations inactivating GCSH should have the same phenotype as mutations in LIAS or LIPT2 and thus would have more profound effects that those individuals having AMT or GLDC mutations, which retain normal energy metabolism and protein modification ability (47).

Our finding that LIPT1 has two mechanistically discrete enzyme activities fits the concept of enzyme evolution called “moonlighting” that has received strong support in recent years (23–25, 48–52), including early death) due to disruption of energy metabolism (45). A similar but less severe decrease in lipoyl protein assembly due to deficient mitochondrial fatty acid synthesis was recently reported in human patients (46). GCSH is the only gene of the pathway in which no human disorder maps, although mutations in the other genes specific to glycine cleavage (AMT and GLDC) are fairly abundant (47). This disparity seems due to the small size of the GCSH coding sequence (173 codons), a comparatively small mutational target relative to the coding sequences of AMT (403 codons) and GLDC (1020 codons). Mutations inactivating GCSH should have the same phenotype as mutations in LIAS or LIPT2 and thus would have more profound effects that those individuals having AMT or GLDC mutations, which retain normal energy metabolism and protein modification ability (47).

Our finding that LIPT1 has two mechanistically discrete enzyme activities fits the concept of enzyme evolution called “moonlighting” that has received strong support in recent years (23–25, 48–52), including its importance in diagnosis of inborn errors of metabolism (53, 54). It has been shown that a protein can acquire a second (moonlighting) function without concomitantly losing all or part of its original function. That is, mutations can enhance the moonlighting function without necessarily eliminating the ancestral function (23–25). The original ancestor of LIPT1 seems likely to have encoded a fully functional lipoyl ligase analogous to E. coli LpIA and B. subtilis LplI (Fig. 1) rather than the present defective (half) ligase. In this scenario, evolution of the LIPT1 gene has resulted in a protein that has lost the ability to activate lipote while acquiring amido-transferase activity and (temporarily?) retaining the lipoyl transfer activity of the defective ligase. Indeed, LIPT1 is a member of Pfam PF03099, a group of enzymes that are constructed on the same structural scaffold (4), albeit from diverse sequences and extra domains in the ligases. Surprisingly, despite...
their conserved structural scaffold, these enzymes perform chemically distinct reactions: They can be lipoate (or biotin) ligases, octanoyl transferases, or lipoamide amidotransferases (4). Even PF03099 enzymes that catalyze the same reaction via the same chemical mechanism can be divergent. The LipB and LipM octanoyl transferases share almost no sequence conservation, and their active-site cysteine residues are found on different loops of the common scaffold (35) (Fig. 5B). It would be interesting to produce a mutant LIPT1 that lacks the partial ligase activity while retaining its amidotransferase activity (assuming that the same lipoate binding site is used in both reactions). A straightforward approach would be to mutate the LIPT1 residues that are hydrogen bonded to the lipoate adenylate adenosine moiety. However, this is problematical because those bonds are primarily formed with backbone atoms (28). It should be noted that, although both LIPT1 reactions involve transfer of a lipoate moiety, the energetics of transfer are strikingly different. Lipoamide adenylate contains a “high-energy” mixed anhydride linkage, and thus lipoal transfer is extremely facile. Indeed, adenylates are known to readily modify protein amino groups without enzymatic assistance (55, 56). In contrast, the amide linking the lipoate moiety to GCSH is among the lowest of “low-energy” linkages found in biology, and thus lipoal transfer from this linkage is kinetically and chemically challenging.

Although modification of lipoal proteins by incorporation of exogenously supplied lipoal acid has been invoked in models of human lipoal disorders (21, 22), there is a large body of evidence indicating that mammals are unable to use exogenous lipoal acid to bypass loss of the lipoal assembly pathway. Dietary lipoal readily enters the bloodstream and tissues. Radioactive lipoal acid has been administered to mammals and its fate followed (57, 58). The labeled cofactor was quickly reduced and degraded by the $\beta$-oxidation pathway and no evidence for attachment of exogenously fed lipoal to proteins was reported. Moreover, studies of homozygous lipoal synthase (LIAS) knockout mice (59), of LIAS, LIPT1, and LIPT2 patients (plus fibroblasts derived from patients) (14–16, 27, 60), and mammalian tissue culture cells blocked in synthesis of the lipoal backbone (42) invariably report that lipoal acid supplementation is without benefit. Moreover, lipoalic acid supplementation did not significantly increase the levels of lipoal-modified 2-oxaloacid dehydrogenases or GCSH (14–16, 60).

Our finding that LIPT2 is unable to catalyze octanoyl transfer from octanoyl-GCSH to the human pyruvate dehydrogenase LD (Fig. 7A) is expected from the phenotype of the LIPT1 disorder. If octanoylation of the 2-oxaloacid dehydrogenase E2 subunits did occur, this could provide a substrate for LIAS-catalyzed sulfur insertion as suggested (26). However, if these were the case, loss of
LIPT1 activity would be bypassed and no LIPT1 metabolic disorder would exist.

**Experimental Procedures**

**Chemicals and Growth Media.** The antibiotics and most chemicals used in this study were purchased from Millipore, Sigma, and Fisher, unless noted otherwise. American Radiolabeled Chemicals provided [1-14C]octanoic acid. DNA manipulation enzymes were from New England Biolabs. DNA sequencing was performed by AGCT. Invitrogen provided the Ni²⁺-agarose column. Growth media were as in prior publications.

**Plasmids and Bacterial Strains (Table 1).** Human genes synthesized with optimized *E. coli* codons encoding GCSH, the inner LD of the E2 component of the human pyruvate dehydrogenase complex (E2p), LIPT1, and human or mouse LIPT2 were from Epoch Life Science or Integrated DNA Technologies. All constructs were verified by sequencing. The human LD and LIPT1 genes were inserted into vector pET28b (Table 1) to generate plasmids pXC.065 and pXC.067 using restriction sites NdeI plus BamHI and NdeI plus HindIII, respectively. Mouse LIPT2 was amplified with primers oXC288/oXc289 and inserted into the NdeI plus SalI restriction sites of vector pQE-2 to give pXC.066. The human GCSH gene was amplified with primers oXC159/oXC160 (Table 2), which added BspHI and HindIII sites to allow ligation into NcoI plus HindIII-cut vector pEH1 downstream of an IPTG-inducible promoter to give pXC.067. Plasmid pXC.068 was generated by excising LIPT1 directly from pXC.065 with restriction enzymes XbaI plus HindIII and ligation into pBAD33 using the same restriction sites. Plasmid pXC.068 carries the pET28b ribosome binding site.

**Table 1. Bacterial strains and plasmids**

| Strains/plasmids | Relevant genotype or description | Reference or derivation |
|------------------|----------------------------------|-------------------------|
| E. coli strains  |                                  |                         |
| BL21(DE3)        | *E. coli* b pmpT hsdS8 gal dcm (DE3) | Lab stock               |
| DH5α             | Δ(argF− lacZU169 φ80 Δlac2)M15 recA1 endA1 | Lab stock               |
| MG1655           | Wild-type *E. coli* K-12          | Lab stock               |
| BL21(Tuner)      | pmpT hsdS8 lacY1 gal dcm (DE3)    | Lab stock               |
| QC145            | lipB::cml ΔλplA-kan of MG1655     | Ref. 68                 |
| QC146            | ΔλplB ΔλplA of MG1655             | Ref. 68                 |
| XC.080           | BL21 (DE3)/pXC.065               | This study              |
| XC.083           | BL21 (Tuner)/pXC.044             | This study              |
| XC.127           | ΔλplB of MG1655                  | This study              |
| XC.131           | XC.127/pXC.067, pXC.068, pXC.069 | This study              |
| XC.139           | XC.127/pXC.067 and pXC.069       | This study              |
| XC.184           | QC146/pXC.064 and pTARA          | This study              |
| XC.213           | DH5α/pXC.066                    | This study              |
| Plasmids         |                                  |                         |
| pET28b           | T7 promoter expression vector, KanR | Novagen                 |
| pQE-2            | T5 promoter expression vector, AmpR | Qiagen                 |
| pTARA            | T7 RNA polymerase expression     | Ref. 76                 |
| pE1             | lacUV5 promoter expression vector, KanR | Ref. 77                 |
| pBAD33           | araBAD promoter expression, p15 Ori CmlR | Ref. 78              |
| pBAD1031G        | araBAD promoter expression vector, p1031 Ori GmR | Ref. 79             |
| pkD46            | Recombineering phage λred genes  | Ref. 62                 |
| pkP20            | Yeast Flp recombinase gene       | Ref. 62                 |
| pXC.004          | pET28b encoding native *S. coelicolor* GcvH | This study |
| pXC.044          | pET28b encoding N-terminal His₆-human GCSH | This study |
| pXC.065          | pET28b encoding N-terminal His₆-human LIPT1 | This study |
| pXC.066          | pQE-2 encoding N-terminal His₆-mouse LIPT2 | This study |
| pXC.067          | pE1 encoding native human GCSH   | This study              |
| pXC.068          | pBAD33 encoding human LIPT1      | This study              |
| pXC.069          | pBAD1031G encoding N-terminal His₆-human LD of pyruvate dehydrogenase E2 | This study |
| pCY754           | pBAD322A encoding full length mouse LIPT2 | This study |
| pCY1108          | pBAD322A encoding human LIPT2 lacking the 31 N-terminal residues. | This study |
| pCY1110          | pBAD322A encoding full-length human LIPT2 | This study |

**Table 2. Oligonucleotides**

| Oligonucleotide | Sequence, 5′→3′ |
|-----------------|-----------------|
| Mouse *lipT2*, forward (Ndel) | ATTCACATATGAGCCTGCCGGTGGTGG7G |
| Mouse *lipT2*, reverse (SalI) | TATAGTCGACTTAGCTCGGGCTATCTTCGCTAATCAG |
| Human gcsH, forward (BspHI) | ATAATTCATGAGCGTGCGCAAATTCA |
| Human gcsH, reverse (HindIII) | TAGATAAGCTTTCACTCCTCAATGG |
| P1 priming site for *E. coli* lipB | AAGTGGTACAGCGCCATGCGC |
| P2 priming site for *E. coli* lipB | TATATGAGGCTGCATCTCAGTACATAGCAAT |
| lipB 250 bp upstream, forward | TTTCCCCCACTTTTATCTCATGTCCTCCACCGAGAATGCGCGTGGTTTT |
| lipB 250 bp downstream, reverse | GTACGGAATAAAATTGTTAGTGAGGAGCGCTCCTCATGTCCTCCACCGAGAATGCGCGTGGTTTT |
| lipB 250 bp upstream, forward | AGATATTATAGGATAGACCAGTAAATGTGAGGAGCGCTCCTCATGTCCTCCACCGAGAATGCGCGTGGTTTT |
| lipB 250 bp downstream, reverse | TTTCCCCCACTTTTATCTCATGTCCTCCACCGAGAATGCGCGTGGTTTT |

LIPT1 activity would be bypassed and no LIPT1 metabolic disorder would exist.

**Experimental Procedures**

**Chemicals and Growth Media.** The antibiotics and most chemicals used in this study were purchased from Millipore, Sigma, and Fisher, unless noted otherwise. American Radiolabeled Chemicals provided [1-14C]octanoic acid. DNA manipulation enzymes were from New England Biolabs. DNA sequencing was performed by AGCT. Invitrogen provided the Ni²⁺-agarose column. Growth media were as in prior publications.

**Plasmids and Bacterial Strains (Table 1).** Human genes synthesized with optimized *E. coli* codons encoding GCSH, the inner LD of the E2 component of the human pyruvate dehydrogenase complex (E2p), LIPT1, and human or mouse LIPT2 were from Epoch Life Science or Integrated DNA Technologies. All constructs were verified by sequencing. The human LD and LIPT1 genes were inserted into vector pET28b (Table 1) to generate plasmids pXC.064 and pXC.065 using restriction sites NdeI and SalI and inserted into the Ndel plus Sall restriction sites of vector pQE-2 to give pXC.066. The human GCSH gene was amplified with primers oXC159/oXC160 (Table 2), which added BspHI and HindIII sites to allow ligation into NcoI plus HindIII-cut vector pEH1 downstream of an IPTG-inducible promoter to give pXC.067. Plasmid pXC.068 was generated by excising LIPT1 directly from pXC.065 with restriction enzymes XbaI and HindIII and ligation into pBAD33 using the same restriction sites. Plasmid pXC.068 carries the pET28b ribosome binding site.
Plasmid pXC669 was generated by excision of the human Hls3-LD gene from pXC664 via the Ncol and HindIII sites followed by ligation into the same sites of pBAD1030G (Table 1). Construction of the lipoyl auxotroph strain XC127 (MG1655ΔlipB) was performed as previously described using pKD3 (Table 1) as the template and P1–P2 as the primers (Table 2) (61, 62), and the chloramphenicol marker was excised by pCP20-encoded Flp recombinase encoded by pCP20 (Table 1) to yield XC127. The ΔlipB construct was verified by sequencing a PCR product obtained using primers oXC134 and oXC135 (Table 2).

Protein Expression and Purification. Hexahistidine-tagged versions of Homo sapiens GCSH and LIPT1 were expressed in E. coli BL21, whereas Mus musculus LIPT2 was expressed in DH5α. These strains were grown in 1 L of LB medium containing the antibiotics required for plasmid maintenance. Expression was induced by the addition of 25 μM IPTG at the start of the culture. Cells were harvested by centrifugation after incubation at 30 °C for 22 h. The proteins were purified by nickel affinity and anion exchange chromatographic steps as previously described (63). Protein concentrations were determined both by the Bradford assay (64) and at 280 nm using extinction coefficients calculated from the ProtParam program of the ExPASy tool website. Protein purity was monitored by SDS-PAGE.

To purify the hexahistidine-tagged human LD (E2p) in the purely apo form, plasmids pXC664 and pTARA were co-transformed into the lipoyc acid auxotrophic strain QC146 to yield strain QC184. The strain was grown at 30 °C in M9 minimum medium with 0.8% glycerol, 5 mM acetate, and 5 mM succinate (pH 7.0), and 0.2% arabinose was added at culture initiation. IPTG was added to 100 μM when the culture reached an absorbance of 0.6 at 600 nm. The culture was incubated for another 6 h before the cells were frozen at −80 °C. The protein was purified by Ni2⁺ affinity chromatography followed by anion exchange chromatography as previously described (65). Bacillus subtilis LipM and GcvH, E. coli holo-acyl carrier protein (ACP) and LD, the Vibrio harveyi AasS, and the Streptomyces coelicolor GcvH and LD proteins were purified as described previously (12, 35, 65–67). Electrospray mass spectrometry was carried out as described previously (65).

Preparation of [1-14C]Octanoyl-GCSH and Assay of LIPT1-Catalyzed Octanoyl Transfer. [1-14C]Octanoyl-labeled GCSH was prepared using B. subtilis lipoyc acid protein ligase (LpII) and purified by nickel affinity plus ion exchange chromatography as previously described (10). The 100–μL reaction mixture contained 50 mM sodium phosphate (pH 7.8), 2 mM MgCl2, 10 mM ATP, 0.5 mM sodium [1-14C]octanoate, 0.5 mM human GCSH, and 5 mM LpII. The reaction was allowed to proceed for 4 h at 37 °C.

To test whether LIPT1 transfer from octanoyl-GCSH to apo LD, the substrate was [1-14C]octanoyl-GCSH (see above). Each reaction (30 μL) contained 20–μL [1-14C]octanoyl-GCSH mixtures (containing LpII and residual ATP), 50 mM sodium chloride, 2 μM purified LipT1, 20 μM apo human LD, 2 units of hexokinase, and 10 mM d-glucose. Hexokinase plus d-glucose served as an ATP trap to remove any ATP remaining from the LpII-catalyzed reaction.

After incubation at 37 °C for 1 h, each reaction was loaded on a 15% native polyacrylamide gel containing 2 M urea, and separated by electrophoresis. The gel was stained with Coomassie R-250, soaked in Amplify (GE Healthcare), dried on filter paper, and exposed to preflashed Biomax XAR film (Kodak) at −80 °C for 24 h.

Preparation of Lipoyl-GCSH and Assay of LIPT1-Catalyzed Lipoyl Transfer. To directly measure lipoyl amido-transfer by LIPT1, lipoyl-GCSH was synthesized as a substrate using LpII. The reaction contained 50 mM sodium phosphate (pH 7.8), 10 mM ATP, 2 mM MgCl2, 1 mM sodium lipidate, and 10 μM LpII, and was incubated at 37 °C for 4 h. The reaction was diluted 20-fold in 50 mM sodium phosphate buffer (pH 8.0) and purified by anion exchange chromatography using an AKTA Purifier10 (GE Healthcare) with a 5-mL POROS QE anion exchange column with a flow rate of 2.5 mL per min. Proteins were eluted with a 0–2 M NaCl gradient. Lipoyl-GcvH eluted at about 400 mM NaCl. Lipoyl amidotransfer to apo human LD protein in reactions (20 μL) containing 50 mM sodium phosphate (pH 7.8), 50 mM sodium chloride, 20 μM lipoyl-GCSH, 20 μM apo human LD, and 2 μM LIPT1. The reactions (20 μL) were incubated at 37 °C for 1 h, loaded on a 2 M urea–PAGE gel (15% polyacrylamide) for gel shift analysis, or loaded onto 15% SDS-PAGE gels for Western blot analysis.

Western Blot Analysis of LIPT1 Amidotransferase Activity. Anti-lipoyl protein primary antibody was utilized to probe protein lipoylation, as described previously (65). Briefly, LIPT1-catalyzed amidotransfer reactions (20 μL) were loaded onto SDS-PAGE gel and transferred by electrophoresis to immobilon-P membranes (Millipore) for 30 min at 60 V. The membranes were pre-blocked with TBS buffer (100 mM Tris base and 0.9% NaCl, pH 7.5) containing 0.1% Tween 20 and 5% nonfat milk powder. The membranes were probed for 1 h with an anti-lipoyl protein primary antibody (Calbiochem) diluted 1:10,000 in the above buffer. Following incubation with anti-rabbit secondary antibody (diluted 1:5,000; GE Healthcare Life Sciences), the labeled prods (Human LD) were detected using Quantity One software.

Coupled Assay/LIPT2 Octanoyl Transfer Reaction. The coupled reaction mixture (25 μL) contained 100 mM sodium phosphate (pH 7.2), 50 mM NaCl, 5 mM disodium ATP, 0.25 mM [1-14C]octanoate, 50 μM holo-ACP, 2.5 μM of the V. harveyi AasS acyl-ACP synthetase, 2 μM LIPT2, and −20 μM human GCSH or another acceptor protein. The reaction was performed at 37 °C for 2 h. The products were electrophoresed on 2 M urea–PAGE (15% acrylamide), and then dried under vacuum at 65 °C for 2 h and exposed to preflashed Biomax XAR film (Kodak) at −80 °C for 24 h.

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