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HHS Public Access
Author manuscript
EcoSal Plus. Author manuscript; available in PMC 2015 January 06.

Published in final edited form as:
EcoSal Plus. 2014 May ; 6(1): . doi:10.1128/ecosalplus.ESP-0001-2012.

Biotin and Lipoic Acid: Synthesis, Attachment and Regulation

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Summary

Two vitamins, biotin and lipoic acid, are essential in all three domains of life. Both coenzymes function only when covalently attached to key metabolic enzymes. There they act as "swinging arms" that shuttle intermediates between two active sites (= covalent substrate channeling) of key metabolic enzymes. Although biotin was discovered over 100 years ago and lipoic acid 60 years ago, it was not known how either coenzyme is made until recently. In *Escherichia coli* the synthetic pathways for both coenzymes have now been worked out for the first time.

The late steps of biotin synthesis, those involved in assembling the fused rings, were well-described biochemically years ago, although recent progress has been made on the BioB reaction, the last step of the pathway in which the biotin sulfur moiety is inserted. In contrast, the early steps of biotin synthesis, assembly of the fatty acid-like "arm" of biotin were unknown. It has now been demonstrated that the arm is made by using disguised substrates to gain entry into the fatty acid synthesis pathway followed by removal of the disguise when the proper chain length is attained. The BioC methyltransferase is responsible for introducing the disguise and the BioH esterase for its removal.

In contrast to biotin, which is attached to its cognate proteins as a finished molecule, lipoic acid is assembled on its cognate proteins. An octanoyl moiety is transferred from the octanoyl-ACP of fatty acid synthesis to a specific lysine residue of a cognate protein by the LipB octanoyl transferase followed by sulfur insertion at carbons C6 and C8 by the LipA lipoyl synthetase. Assembly on the cognate proteins regulates the amount of lipoic acid synthesized and thus there is no transcriptional control of the synthetic genes. In contrast transcriptional control of the biotin synthetic genes is wielded by a remarkably sophisticated, yet simple, system, exerted through BirA a dual function protein that both represses biotin operon transcription and ligates biotin to its cognate protein.

1. INTRODUCTION

Biotin (vitamin H, viamin B_7 or 5-[(3a*S*,4*S*,6a*R*)-2-oxo-1,3,3a,4,6,6a-hexahydrothieno[3,4-d]imidazol-4-yl]pentanoic acid) and lipoic acid (5-[(3*R*)-dithiolan-3-yl]pentanoic acid, also called 6,8-thioctic acid) share many similarities (Fig. 1). Both vitamins are essential for aerobic growth of *E. coli* and *S. enterica* whereas biotin is also required for growth of these

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bacteria under anaerobic conditions. Both biotin and lipoic acid must be covalently attached to their cognate proteins to perform their roles in cellular enzymology; the free vitamins are not physiologically useful (although free biotin plays an indirect regulatory role). The protein domains to which biotin and lipoic acid are attached have very similar 3-dimensional structures and the enzymes that perform the attachment of the two cofactors are members of the same protein family based on their structures. Thus, the speculation made many years ago (1) that biotin and lipoic arose together "late" in evolution is germane. Moreover, although the two molecules look to have little similarity when drawn as in Fig. 1, both are chiral. Biotin has a chair shape due to the C-N bonds whereas the ring of lipoic acid is skewed by the C-S bonds. Proteins recognize these structures in somewhat similar manners since the biotin binding protein, avidin, also shows significant (albeit much weaker) binding of lipoic acid and antibodies raised against one of the molecules as a hapten often bind to proteins modified with the other cofactor (2).

Biotin and lipoic acid also share the property that they are attached to very few protein species. *E. coli* has only a single biotinylated protein, the AccB subunit of the essential enzyme, acetyl-CoA carboxylase whereas *S. enterica* has a second inducible biotinylated protein, the α subunit of oxalacetate carboxylase (3–5). *E. coli* has three lipoylated proteins, these are subunits of pyruvate dehydrogenase (PDH) and 2-oxoglutarate dehydrogenase (2-OGDH), enzymes essential for aerobic growth, plus a third lipoylated protein induced by the presence of glycine that is a subunit of the glycine cleavage system of single carbon metabolism (6–8). In each of these proteins the cofactor is attached to a lysine residue ε-amino group of a domain of highly conserved structure. This domain is the N-terminal part of a lipoylated protein and the C-terminal part of a biotinylated protein and is connected to the remainder of the protein by a long proline plus alanine-rich linker region (9). The modified subunits form noncovalent interactions with other members of a protein complex of the three or four protein species that constitute the active enzyme. The cofactor-modified domains then shuttle intermediates between the multiple active sites of the enzyme complex (9). The mobility of the domains is due to the proline-alanine linkers and the domains constitute the distal ends (the "hands") of the swinging arms long ago postulated for these enzyme complexes. These arrangements can be considered as providing substrate channeling via covalent attachment (9). Finally both biotin and lipoic acid are needed in only trace quantities. In *E. coli* only a few hundred molecules of biotin per cell are sufficient for growth (10) and the requirement for lipoic acid is similar. Therefore, the enzymes of these pathways are expressed at very low levels (< 350 molecules/cell [11]) and the enzymes have generally low turnover numbers.

Synthesis of Biotin

The early steps of biotin biosynthesis are not well understood in any organism, but clearly differ between *E. coli* and *Bacillus subtilis*. In both cases a seven carbon dicarboxylic acid, pimelic acid is assembled with one of its carboxyl groups in thioester linkage. Pimeloyl-CoA has long been thought to be the thioester-activated form of pimelic acid, but recent evidence indicates a role for the acyl carrier protein (ACP) of fatty acid synthesis as the thiol moiety (12, 13). In contrast the steps that follow formation of the pimeloyl-thioester are well conserved throughout biology even in organisms (e.g., *Saccharomyces cerevisiae*) that lack

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the ability to perform any early biosynthetic steps. In *E. coli* the atoms of biotin are derived from rather disparate sources, acetate, alanine, CO_2, S-adenosylmethionine (SAM) and sulfide. Two groups have traced the origins of the biotin and dethiobiotin carbon atoms by ^{13}C labeling followed by analysis by ^{13}C NMR (14, 15). Using the numbering system of Figure 1, the C-3, C-5, and C-7 carbon atoms of biotin are derived from C-1 of acetate whereas the C-2 of acetate contributes the biotin C-2, C4, and C-6 carbon atoms. Acetate labeled in both carbon atoms is incorporated intact as shown by ^{13}C coupling. Biotin carbon atoms C-9 and C10 are contributed by L-alanine. The C-1 and ureido (C-2′) carbon atoms are derived from CO_2 (14). The nitrogen atom adjacent to C7 is from SAM whereas the other nitrogen atom is from alanine. The labeling pattern is consistent with formation of a pimelic acid moiety by head to tail incorporation of three intact acetate units as is the case in fatty acid (or polyketide) synthesis (14, 15) and the labeling pattern eliminates other plausible pathways from tryptophan, lysine, diaminopimelic acid or elongation of 2-oxoglutarate (15). Moreover, the ^{13}C labeling results eliminate free pimelic acid as an intermediate in biotin biosynthesis. Pimelic acid is a symmetrical dicarboxylic acid whose carboxyl groups cannot be stereochemically distinguished and if free pimelic acid is an intermediate, then biotin carbon atoms C-1 and C-7 would have the same labeling pattern. This is not the case (14, 15) and thus, the pimelate moiety must be assembled with one of the carboxyl groups covalently linked to another moiety. A thioester seems the most likely linkage (14, 15). It should be noted that biotin is required for synthesis of malonyl-CoA, the postulated source of all of the carbon atoms of the pimelate moiety. Hence, we are presented with an evolutionary conundrum, biotin is required for biotin synthesis.

The Genes of Biotin Synthesis

Biotin requiring mutants of *E. coli* were first isolated many years ago. All of the mutants isolated as biotin auxotrophs were clustered at min 17 of the genetic map and defined five genes, called *bioABCDF,* based on mapping, cross-feeding and complementation studies (16–18). However, during deletion analysis of the maltose utilization genes, a strain that required biotin was isolated and called *bioH* (19). More recently, strains having a nonfunctional *pfs* (now called *mtn*), the gene encoding 5′-methylthioadenosine/S-adenosylhomocysteine nucleosidase were shown to require biotin due to accumulation of an inhibitory metabolite (see below) (20, 21). The fact that biotin auxotrophs due to mutations in the *bioH* or *mtn* genes were not isolated by classical means is probably due to the fact that *E. coli* biotin auxotrophs require only miniscule amounts of this cofactor (supplementation with biotin at concentrations of a few nanomolar) and that conventional undefined growth media are often very rich in biotin. Therefore, often several platings on biotin-free media are required to detect the biotin requirement of auxotrophs.

The Pathway and Proteins of Biotin Synthesis

The late steps of biotin biosynthesis (Fig. 2) were worked out many years ago whereas the early steps have only recently been elucidated. The proteins (BioA, BioB, BioD and BioF) of the late steps are well-studied enzymes of known crystal structures whereas the proteins of the early steps, BioC and BioH, were much more poorly understood. The sequence of steps in the late pathway was readily deduced since *E. coli* takes up each of the late

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intermediates. Growth of mutants with lesions in *bioC* and *bioH* proceeds when the medium is supplemented with 7-keto-8-amino pelargonic acid (KAPA) or any of the later intermediates in the pathway. No cross-feeding is observed between *bioC* and *bioH* mutant strains suggesting that the early intermediates may not pass through cell membranes, perhaps because they are protein-bound. In recent years the functions of BioC and BioH has become clear more then 50 years after the genes were discovered. The question was how to assemble a seven-carbon dicarboxylic acid in *E. coli.* BioC was annotated as an *S*-adenosyl-L-methionine (SAM)-dependent methyltransferase whereas BioH had been shown to have esterase activity on a series of short and medium chain acyl *p*-nitrophenyl esters(13, 22, 23) and on the methyl ester of dimethylbutyryl-*S*-methyl mercaptopropionate (24). The BioC annotation was especially puzzling because biotin contains no methyl groups and (as discussed above) all of the pimeloyl moiety carbon atoms are derived from acetate, alanine and CO_2. Thus, it seemed that assembly of the pimeloyl moiety must require enzymes of another biosynthetic pathway that are somehow assisted in this task by BioC and BioH. Many years ago Lynen and coworkers (25) suggested that pimeloyl-CoA could be formed by the enzymes of fatty acid synthesis. They proposed that three molecules of malonyl-CoA would be condensed with the primer malonyl moiety retaining the carboxyl group introduced by acetyl-CoA carboxylase fixation of CO_2. The other two malonyl-CoA molecules would lose their free carboxyl groups in the course of the two decarboxylative Claisen reactions required to give the C7 dicarboxylate. This scheme is consistent with the ^{13}C labeling studies and is chemically reasonable because type III polyketide synthases are known that use such a malonyl-primed mechanism to make dicarboxylic acids of odd carbon lengths in which one the two carboxyl groups is in thioester linkage (26, 27). However, in fatty acid synthesis the growing chains are attached to ACP rather than CoA and unlike polyketides, where the keto groups are retained or consumed in subsequent rearrangements of the carbon chain (e.g., cyclization), pimelate synthesis requires that the keto groups be converted to methylenes. Although the condensation, reduction and dehydration enzymes of fatty acid synthesis could perform the net reduction of the keto groups to methylenes required for assembly of the pimeloyl moiety, it seemed most unlikely that the fatty acid synthetic enzymes would be active on substrates having a carboxyl group in place of the usual terminal methyl group because the fatty acid synthetic enzymes sequester the growing fatty acyl chains in tunnels or clefts that are strongly hydrophobic (28). Recently it has been shown that this conundrum is avoided by “disguising” the terminal carboxyl group such that it can be recognized by the fatty acid synthesis enzymes (Fig. 2). Introduction of the disguise is the role of BioC which converts the free carboxyl group to its methyl ester by transfer of a methyl group from SAM. Methylation cancels the carboxyl group charge and provides a methyl carbon that mimics the methyl of the normal acyl chains. This methylated species has properties (chain length, hydrophobicity) approximating those of the substrates normally accepted by the enzymes of fatty acid synthesis. Following completion of the pimelic acid moiety the methyl ester would then be cleaved by BioH to give pimeloyl-ACP. This in turn would react with L-alanine in the BioF reaction to give 7-keto-8-aminopelargonic acid (KAPA), the first intermediate in assembly of the biotin ring structures (Fig. 2). BioH thus acts to free the carboxyl group that will eventually be used to attach biotin to the metabolic enzymes where it performs its key metabolic roles (29).

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BioC

Prior to the recent work nothing was known of the function of BioC, a protein of 28.3 kDa. It is highly conserved among the proteobacteria and is often annotated as a SAM-dependent methyl transferase. It had been proposed that BioC acts as a carrier protein that carries an intermediate transferred by BioH (30), but recent work disproves this notion. The BioC protein had not been studied biochemically probably because it invariably forms inclusion bodies upon overexpression (31). This recalcitrant property of BioC has precluded its direct analysis, although some activity was obtained upon denaturing and refolding the protein (13). The BioCs of close relatives of *E. coli* were as intractable as *E. coli* BioC and thus the BioCs of more diverse bacteria were tested. Expression of the BioC of *Bacillus cereus* in *E. coli* restored biotin synthesis to an *E. coli* *bioC* strain and this monomeric protein could be expressed in soluble form in *E. coli* and purified to homogeneity (32). In disagreement with prior scenarios that favored malonyl-CoA as the methyl acceptor, malonyl-ACP was a far better acceptor of methyl groups from S-adenosyl-L-methionine than was malonyl-CoA. BioC was specific for the malonyl moiety and was inhibited by S-adenosyl-L-homocysteine and sinefungin. Indeed, although the BioC k_{cat} values of ~200 s^{-1} are modest, they not nearly as low as those of the enzymes late in the pathway. For example BioD and BioB, the last two enzymes of the pathway, are notably poor catalysts having reported k_{cat} values of 0.06 and 0.002 s^{-1}, respectively. Hence, when compared to BioD and BioB, BioC is an effective catalyst. A rationale for the disparity between the first and concluding enzymes of the biotin synthetic pathway is that BioC must have reasonable activity in order to effectively compete with the 3-ketoacyl-ACP synthases for malonyl-ACP. However, if BioC is overly active, it would convert too much malonyl-ACP to the methylated species and thereby block fatty acid synthesis. Indeed, BioC overproduction provides a very effective means to block *E. coli* fatty acid synthesis (32).

BioH

In contrast to *E. coli* BioC, *E. coli* BioH is a well-behaved monomeric 28.5-kDa protein which allowed determination of its crystal structure at 1.7 Å (23). BioH is a monomeric two-domain protein (23, 31). A putative catalytic triad (Ser-82, His-235, and Asp-207) similar to that of the catalytic triad of hydrolases was identified. Moreover, in the BioH crystals the serine residue was found to have been modified by a protease inhibitor. Consistent with these indications of hydrolase activity, BioH had weak esterase activity on several model substrates (23), although this activity was not shown to depend on the Ser, His, Asp triad. Others had noted two Gly-Xaa-Ser-Xaa-Gly motifs in BioH that are characteristic of acyltransferase and thioesterase proteins (30). However, the crystal structure gave no clues as to the identities of the substrates of BioH. BioH has been reported to bind CoA in vitro (31), but the significance of this finding remains unclear. BioH has recently been shown to act prior to BioF in an *in vitro* system and thus acts as the gatekeeper that prevents methyl pimeloyl-ACP from being elongated to azelayl-ACP methyl ester, a physiologically useless product (33). This was buttressed by 2.05 Å resolution co-crystal structure of a complex of a catalytically inactive BioH with Me-pimeloyl-ACP. The BioH-ACP interface contacts identified in the structure (four salt bridges between BioH arginine sidechains and ACP acidic residues) were demonstrated to be required for binding of its substrate by BioH (33).

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The BioH proteins that lacked these contacts were inactive *in vitro* and *in vivo* indicating that Me-pimeloyl-ACP is the physiological substrate of BioH, and that BioH is the gatekeeper (33). As will be further discussed below, it should be noted that in the *E. coli* genome the *bioH* gene is well removed from the other genes of the pathway and is not regulated by the BirA repressor (see below) whereas in other proteobacteria (e.g., the pseudomonads) *bioH* is found in a apparent biotin synthetic gene operon.

BioF

BioF is 7-keto-8-amino pelargonic acid (KAPA) synthase, a pyridoxal phosphate-dependent homodimer of 41.6 kDa of known crystal structure (34, 35). The enzyme condenses alanine with pimeloyl-CoA to give 7-keto-8-amino pelargonic acid (formal name, 8-amino-7-oxononanoic acid) plus CoA and CO_2 (resulting from decarboxylation of alanine). BioF is a two-domain protein with the pyridoxal phosphate bound in a crevice between the two domains formed by residues of both domains. The mechanism of the enzyme has been studied in some detail (36). Historically the enzyme has been assayed using pimeloyl-CoA although pimeloyl-ACP could be the physiological substrate in *E. coli* (ACP-requiring enzymes will often use the analogous CoA compound as a model substrate). Consistent with this notion the *E. coli* 7-keto-8-amino pelargonic acid synthase has a much higher Michaelis constant for pimeloyl-CoA than the analogous enzyme from *Bacillus sphaericus* (37), an organism in which pimeloyl-CoA is thought to be the physiological substrate due to the presence of pimeloyl-CoA synthetase.

BioA

BioA is 7,8-diaminopelargonic acid (DAPA) aminotransferase (the formal name of DAPA is 7,8-diaminononanoate) that has many similarities to BioF, the preceding enzyme in the pathway. Although the BioA subunit (47.3 kDa) is slightly larger than that of BioF, it is also a homodimeric pyridoxal phosphate-dependent enzyme. Indeed, the overall structure of BioA is very similar to that of BioF (38) and this is reflected in a weak sequence homology. BioA is a transaminase that converts KAPA to DAPA and as such is not a particularly interesting enzyme (39, 40). However, the amino donor is not a standard amino acid, but rather the highly activated amino acid SAM (39, 41) which requires three ATP equivalents for its synthesis. The deaminated product derived from SAM, S-adenosyl-2-oxo-4-thiomethylbutryate, spontaneously degrades *in vitro* (39), and thus it seems likely that three ATP equivalents are consumed in what is an otherwise simple transamination reaction. The expense of this perplexing choice of amino donor may provide a rationale for the known tight regulation of biotin synthesis. However, it could be argued that use of a more pedestrian amino donor (*B. subtilis* uses lysine (42)) could alleviate the need for tight regulation.

BioD

In contrast to the preceding enzymes BioD (dethiobiotin synthase or DTBS) catalyzes an unusually interesting step, the formation of the ureido moiety of biotin (43, 44). The BioD reaction is the ATP-dependent formation of dethiobiotin from DAPA and CO_2. The enzyme is a homodimeric protein (subunit of 24.1 kDa) that is structured into a single well folded

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domain (45–48). X-ray crystallographic studies have shown that the reaction proceeds by carbamoylation of N-7 of DAPA (45, 46) (Fig. 3). Independent NMR evidence for carbamate formation has also been obtained (49). The second partial reaction is also unusual. In this reaction the carbamate is activated by transfer of the γ-phosphoryl moiety of ATP to a carbamate oxygen to form a mixed anhydride (Fig. 3). This mixed anhydride species has been demonstrated by time-resolved crystallography (50). The final step of the dethiobiotin synthase reaction is a nucleophilic attack by the N-8 nitrogen of DAPA on a carbamoyl oxygen of the mixed anhydride (Fig. 3). This results in release of the phosphate group and formation of the ureido ring of dethiobiotin.

BioB

The bête noire of biotin synthesis has long been the last step, insertion of the sulfur atom into DTB to form the thiophane ring of biotin. For many years this activity was ascribed to BioB by genetic means (biotin auxotrophs unable to grow on DTB) and could be assayed only by the ability of intact cells to convert DTB to biotin. Extensive attempts to obtain sulfur insertion *in vitro* all failed until Ifuku and coworkers (51) succeeded in showing biotin synthesis from DTB in a cell extract. The reaction required DTB, SAM, NADPH, BioB, and an unknown protein or proteins later shown to be flavodoxin (FldA) and flavodoxin reductase (Fpr) (51–53). This breakthrough was soon followed by demonstration of activity in a defined system containing NADPH, flavodoxin and flavodoxin reductase as the electron transfer system plus DTB, SAM, and a BioB preparation plus a reducing environment (54). BioB (a homodimer of a 38.6 kDa subunit) was found to be a very labile protein that is best purified and assayed under anaerobic conditions. The discovery that SAM was absolutely required for biotin synthesis and was not the sulfur donor (55, 56) strongly suggested that BioB was a member of the (then) small family of “radical SAM” enzymes. It has recently become apparent that this is a large family of proteins that catalyze a range of reactions that invariably involve difficult reactions often accessible only by radical chemistry. The radical is generated by reductive cleavage of SAM to give a deoxyadenosyl radical (DOA·) plus methionine. The DOA radical then cleaves a C—H bond to generate a carbon radical that allows the chemistry to proceed. The electron donor in the single electron reduction of SAM is a [4Fe-4S] cluster liganded to the cysteine residues of a perfectly conserved CXXXCXXC motif. Consistent with this picture, the BioB reaction is chemically difficult since it requires cleavage and sulfur insertion into two unreactive C—H bonds. The mechanism currently accepted by most workers in the field (57–59) is given in Fig. 4.

The BioB species involved in the mechanism of Fig. 4 contains two [Fe-S] clusters. The number and composition of these clusters has been the subject of much disagreement in the literature. However, a variety of spectroscopic techniques plus a recent BioB crystal structure give a consistent picture. BioB contains two different clusters, the [4Fe-4S] cluster characteristic of radical SAM enzymes and a [2Fe-2S] cluster located at a different site (the [2Fe-2S] cluster was often thought to be a degradation product of a [4Fe-4S] cluster until mutagenesis experiments suggested otherwise). The crystal structure shows that BioB to be a α/β_8 (TIM) barrel protein with the two [Fe-S] clusters located at either end of the barrel (58). The [4Fe-4S] cluster is located at the open end of the barrel whereas the [2Fe-2S] cluster (which utilizes an unusual arginine ligand) is at the closed end of the barrel. The

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crystal structures contain both SAM and DTB. The SAM is positioned such that reductive cleavage by the [4Fe-4S] cluster could readily occur while the DTB is positioned such that the C-9 carbon can accept a sulfur atom from the [2Fe-2S] cluster (58). Indeed, 9-mercaptodethiobiotin is a catalytic intermediate (60–62) This latter finding fits with the belief of most workers in the field that the [2Fe-2S] cluster is the immediate source of the biotin sulfur atom. This belief is supported by experiments in which each of the sulfur-containing small molecules of the defined *in vitro* reaction mixture was labeled with ^{35}S and incorporation of the isotope into biotin was measured (see ref. (63) and references therein). No radioactive biotin was obtained. Isotopically labeled biotin was obtained only when BioB was labeled with ^{35}S *in vivo* (63) or with ^{34}S by reconstitution of the [Fe-S] clusters *in vitro* (64). More recent reports have shown that BioB reconstituted with Se in place of S gave selenobiotin derived from the (2Fe-2Se) cluster (65). Spectroscopic studies indicate that the [2Fe-2S] cluster disappears concomitantly with sulfur insertion (66, 67) and more recently evidence for that reduction of the [2Fe-2S] cluster accompanies formation of 9-mercaptodethiobiotin (62) consistent with a mechanism in which the [2Fe-2S] cluster simultaneously provides and oxidizes sulfide during carbon-sulfur bond formation.

For many years one of the few points of agreement in the literature was the finding that BioB itself is the sulfur source impinges, that the BioB reaction is not catalytic *in vitro* (57, 59, 68). Numerous and diverse justifications were put forth for the observed lack of catalysis (69–71), but no general agreement emerged. The favored and most provocative explanation for the lack of catalysis was that given above, the [2Fe-2S] cluster of the protein donates the biotin sulfur atom and this donation inactivates BioB. In this view BioB would be a reactant or substrate rather than an enzyme and, in the absence of repair of the [2Fe-2S] center, the protein would be sacrificed. The scenario of protein sacrifice was not completely unreasonable because there is no need for *E. coli* biotin synthase to be an efficient catalyst because *E. coli* (like most other organisms) requires only minuscule quantities of biotin for growth. *E. coli* can grow with only 100–200 molecules of biotin per cell (10, 72) and thus sacrifice of a few hundred molecules of a medium sized protein would not be a major drain on cellular resources. However, it was shown that Choi-Rhee and Cronan (73) demonstrated that *E. coli* BioB is catalytic *in vivo*. Such *in vivo* measurements are difficult since the endogenous expression level of biotin synthase is very low and because biotin may be split between pools of free and protein bound cofactor. The first issue was overcome by overexpressing hexahistidine-tagged biotin synthase under control of an arabinose-inducible promoter. The second issue was overcome by massively overexpressing, under control of an IPTG-inducible T7 promoter, biotin ligase (BirA) and a truncated, hexahistidine-tagged form of the acetyl CoA carboxylase biotinyl domain that can accept biotin but does not form an active enzyme complex. These investigators then used a combination of anti-pentahistidine antibodies, [^{35}S]methionine labeling, and streptavidin to quantify the levels of each protein and of total biotinylated protein separated by denaturing and nondenaturing gel electrophoresis. The use of the two gel systems allowed the turnover number of BioB is be calculated in an unusually straightforward manner. The ratio of biotinylated domain to BioB monomer gives 20–60 equivalents of biotin produced per initial biotin synthase monomer (73). Very recently Jarrett and coworkers reported that in their *in vitro* assay system they observed that BioB is catalytic, 11 μM BS dimer produced 35 μM biotin over 4 h indicating

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at least three consecutive turnovers (74). Biotin production showed burst kinetics, a burst phase of $k = 0.12\ min^{-1}$, followed by a steady-state phase with a turnover number of $k = 0.0089\ min^{-1}$. The rate of the burst phase observed *in vitro* is similar to that observed *in vivo* suggesting that *in vivo* activity is not limited by FeS cluster reassembly but rather by the chemistry of biotin formation. The key to obtaining catalysis in vitro was preparation of SAM free of the contaminents present in commercial preparations and addition of Mtn to cleave the inhibitory 5′-deoxyadenosine produced in the reaction.

It should be noted that the *in vivo* measurement of BioB catalysis was complicated by the unexpected finding that enzyme turnover renders the enzyme susceptible to proteolytic degradation (73). A 50%–90% depletion of the level of His6-BioB was observed after incubation. This depletion was not observed in the absence of DTB or in the presence of biotin (73). The observed degradation of BioB was proposed to result from collapse of the enzyme [2Fe-2S] center due to donation of a sulfur atom to DTB. The [2Fe-2S] centre of BioB is located deep within the barrel of this α/β_8 (TIM) protein (58) and thus it seems probable that a substantial unfolding of the protein would be required to allow rebuilding of the [2Fe-2S] cluster. Such unfolding would allow restoration of the [2Fe-2S] center, but at the cost of exposure of the protein to proteolytic attack while unfolded. Therefore, in this scenario catalysis by a molecule of BioB would require the protein to run a gauntlet of proteolysis until restoration of normal folding (with concomitant resistance to proteolysis) by rebuilding of the [2Fe-2S] center expended in biotin synthesis (73). The turnover numbers observed may thus be viewed as the products of a stochastic process. If the [2Fe-2S] cluster of a BioB molecule is rebuilt before proteolysis occurs, that protein will perform another turnover. If not, the protein molecule perishes and must be re-synthesized *de novo*. More recent work done *in vitro* has shown that loss of iron-sulfur clusters from BioB as a result of catalysis promotes unfolding and degradation (75). Hence, some BioB molecules may catalyze only one or a few turnovers in their lifetimes whereas others may complete >100 turnovers. The steady state level following the burst phase in the optimized *in vitro* system (74) may reflect the loss of active BioB molecules.

The biotin requirement of *mtn* (*pfs*) mutant strains (20) is due to inhibition of BioB by the byproduct of sulfur insertion, 5′-deoxyadenosine (21). The *mtn* gene encodes the 5′-methylthioadenosine/S-adenosylhomocysteine nucleosidase which was shown to also cleave 5′-deoxyadenosine to adenine plus 5′-deoxyribose (21, 74, 76). Mutants lacking Mtn activity precisely mimic BioB mutants in that they grow on biotin, but not on DTB or DAPA, and excrete DTB (21).

Remaining problems in biotin synthesis

The *E. coli bioH* gene differs from the other genes in the pathway in that it is neither located within the *bio* operon nor regulated by the BirA repressor/biotin protein ligase (77, 78). This is in contrast to many other bacteria where *bioH* resides within the biotin operon and is generally located immediately upstream of *bioC* (79). Moreover, BioH is a rather promiscuous hydrolase that also cleaves the ethyl, propyl and butyl esters of pimeloyl-ACP plus adipoyl-ACP methyl ester, although it is unable to cleave the thioester bond of these substrates. Others have reported that BioH cleaves the methyl ester of dimethylbutyryl-*S*-

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methyl mercaptopropionate (24) and a series of short and medium chain acyl *p*-nitrophenyl esters (22, 23, 80). Thus, the *E. coli bioH* gene may encode a protein that is less specific than those encoded by the more "domesticated" *bioH* genes. Note that the BioH function seems something of a "wild card" among biotin synthetic enzymes since in some bacteria the gene has been displaced from its site upstream of *bioC* by other genes (79) that have been shown functionally replace BioH (81).

The BioB reaction also requires additional study. This very intricate enzyme cannot yet be considered understood. There remain several loose ends. For example, the sequence of events that follow destruction of the BioB [2Fe-2S] cluster thought to donate the biotin sulfur atom remains unclear (67). Marquet and coworkers (67) reported that cluster destruction is accompanied by biotin formation whereas others (66) report that biotin formation is 10- to 1,000-fold slower than cluster destruction and is biphasic. It therefore seems possible that there may be several steps in the formation of biotin by BioB and different steps may be rate limiting in different enzyme preparations. A more extreme case is the claim that BioB has an intrinsic pyridoxal phosphate-dependent cysteine desulfurase activity responsible for generating the sulfur atom of biotin which would enter DTB via a persulfide (71, 82). This claim is countered by the finding that no pyridoxal phosphate is visible in the BioB crystal structure (58), other laboratories have been unable to demonstrate pyridoxal phosphate binding or cysteine desulfurase activity (27, 83) and the finding that biotin synthesis from DTB proceeds normally in cultures of *E. coli* starved for pyridoxal (84). It should be noted that although the BioB crystal structure is a major step forward, crystallization necessarily selects for a single protein species. Thus, the crystallized form of BioB may not fully represent all of the active enzyme species. Moreover, the present structure is of only moderate resolution (3.4 Å). An unsolved difficulty with the stoichiometry given by the BioB structure is that it contains only a single SAM molecule and there is no room for a second molecule (58). Therefore, the enzyme seems equipped to form only a single C-S bond. Using deuterium labeled DTB species it was shown that both C6 and C9 of biotin become labeled and thus it seems clear that 2 mol of AdoMet are necessary to break the positions 6 and 9 C-H bonds (85). Thus, the most likely scenario is that following synthesis of the first C-S bond, the methionine and 5′-deoxyadenosine products are released in order that a second molecule of SAM can bind. Another complication is that it is not completely clear how 9-mercaptodethiobiotin is bound in the active site. In the BioB structure DTB is located in a position where 9-mercaptodethiobiotin seems an unlikely intermediate in biotin formation. In seems probable that in order to complete the reaction BioB must attain a structure that is differs markedly from that of the extant crystal structure.

The recent finding that BioB undergoes burst kinetics during catalysis also deserves attention. Are the slow turnovers follwing the burst due to extraction of a sufur atom from the [2Fe-2S] cluster? How is the BioB [2Fe-2S] cluster rebuilt *in vivo* and would addition of the cellular rebuilding factors prevent decay of the enzyme to the less active state? Although BioB has recently been reported to accept a [4Fe-4S] center from two *E. coli* Fe-S center scaffold proteins, SufA and IscA, no [2Fe-2S] center was formed (86). It should be noted that the BioB [2Fe-2S] has a novel ligand, an arginine residue rather the Cys or His residues

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commonly used as ligands (58). This unusual ligand implied specificity for the guanidium ligand, but recent results indicated that substitution of Cys, Ala, His or Met for the arginine residue failed to inactivate BioB (87). Moreover, prior mutagenesis experiments indicated that two of the three conserved [2Fe-2S] cluster cysteine residues must be removed before BioB activity is lost (86, 88). The plasticity of this cluster suggests that the usual sulfur insertion pathways (the Isc and Suf systems) may not apply and, thus far, this seems to be the case. Inclusion of IscS does not allow BioB to become catalytic *in vitro* (69). The [2Fe-2S] cluster cannot be assembled by the Suf system *in vitro* (86) and *E. coli* strains with null mutations of either the *suf* or *isc* operons are not biotin auxotrophs (J. Imlay, personal communication). Unfortunately, *suf isc* double mutants are inviable so the possibility that biotin is synthesized due to redundant functions of the two systems cannot be tested.

Regulation of Biotin Synthesis

Expression of the *Escherichia coli* biotin synthetic (*bio*) operon is controlled by a simple, yet remarkably sophisticated, regulatory system in which the rate of transcription of the operon responds not only to the supply of biotin, but also to the supply of proteins (called biotin acceptor proteins) that become modified by covalent attachment of biotin (Fig. 5) (29, 89–94). This regulatory system is understood in considerable detail thanks to a combination of genetic, physiological, biochemical and biophysical investigations. The biotin operon of *E. coli* and other enteric bacteria is a striking example of regulation in which the transcriptional regulatory protein (BirA) is also an enzyme, in this case the biotin-protein ligase, that catalyzes the covalent attachment of the biotin to certain proteins involved in key metabolic carboxylation and decarboxylation reactions. Moreover, regulation of the *E. coli* biotin operon is probably the best understood example of transcriptional regulation by an enzyme unrelated to nucleic acid metabolism. Superficially, the system resembles the classical TrpR regulation of the *E. coli* tryptophan operon where the Trp repressor protein binds to the *trpEDCBA* operator only when complexed with the co-repressor, tryptophan. However in *bio* operon regulation, the repressor is also the biotin-protein ligase and the co-repressor is not biotin, but biotinoyl-5′-AMP (bio-AMP), the product of the first half-reaction of the ligase reaction. It is these novel features that give this regulatory system its unusually subtle properties. The *bio* operon is actually two transcriptional units (*bio*A and *bio*BFCD) controlled by a common operator.

The Model

Maximal rates of *bio* operon transcription (derepression) occur when the biotin supply is severely limited (e.g., biotin starvation of a *bio* auxotroph) (Fig. 5A) or when high levels of a biotin acceptor protein are present (Fig. 5B). Under these conditions any bio-AMP synthesized is rapidly consumed in biotinylation of the acceptor protein (apo AccB) and hence no significant levels of the BirA-bio-AMP complex accumulate. Hence, BirA remains largely monomeric so the *bio* operator is seldom occupied and transcription is maximal. Repression of *bio* operon transcription occurs when the supply of biotin is in excess of that needed to biotinylate apoAccB. Under these conditions apo-BCCP is fully biotinylated, the BirA:bio-AMP complex accumulates, followed by dimerization of the protein to form the repressor species. The dimers then bind to the *bio* operator and represses transcription from

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both promoters. The two derepression conditions act by a common mechanism in that both decrease the levels of the BirA:bio-AMP complex available to bind the *bio* operator (Fig. 5C). Hence, the degree of repression of *bio* operon transcription can be most simply viewed as an antagonism between retention of bio-AMP in the BirA active site versus consumption of the bio-AMP bound to BirA by transfer of the biotinyl moiety to unmodified acceptor proteins (93). The model of Beckett and coworkers (91) in which the unmodified acceptor protein binds monomeric BirA and thereby inhibits formation of BirA dimers, the species required for effective repression, provides a structural context for this antagonism. Because the rate of *bio* operon transcription is sensitive not only to the intracellular concentration of biotin, but also to the supply of the protein to which the biotin must be attached, the net result of accumulation of the unmodified protein is an increase in the rate of synthesis of the small molecule needed for the post-translational modification. The evidence for this model is strong and is discussed below.

BirA protein

The evidence that the ligase and repressor are the same protein was very firmly established by data from several laboratories. The key genetic observation was that of Campbell and co-workers who showed that *E. coli* mutants defective in intracellular retention of biotin (called *birA*) were allelic to mutants defective in repression of the *bio* operon (called *bioR*; the *birA* designation has been retained). Since biotin is retained in *E. coli* only as the protein-bound species, it followed that the *birA* gene encoded biotin-protein ligase activity and this was demonstrated (89, 90). Furthermore, these workers also showed that a partially purified BirA protein preparation protected a specific segment of *bio* operon DNA (Fig. 6B) from nuclease digestion. This DNA segment contained a region of degenerate dyad symmetry previously defined as the operator of the *bio* operon (see below) by transcriptional (95) and mutational studies (96). As expected (see below), protection by BirA required the presence of bio-AMP. At about the same time, Eisenberg and co-workers showed that the purified repressor protein bound to *bio* operon DNA and catalyzed the biotin-protein ligase reaction (97). These workers also found that binding of the repressor protein to *bio* operon DNA *in vitro* required either biotin or bio-AMP but that bio-AMP was 1,000-fold more effective than biotin and biotin was active only at non-physiological concentrations (98). Bio-AMP was also shown to be 1,000-fold more efficient than biotin in repression of *bio* operon transcription in a coupled transcription-translation system (99). Since these pioneering studies, it has become possible to obtain large amounts of BirA (normally a very non-abundant protein) (100, 101) that has led to biophysical studies as well as crystal structures of the unliganded (apo) protein (102) and of complexes of BirA with biotinoyl-lysine (102), biotin (103), or biotinoyl-AMP, a non-hydrolyzable analogue of bio-AMP (104). Although we lack the structure of the tertiary complex of BirA, the *bio* operator and bio-AMP (or an analogue), these studies show that BirA is a winged helix-turn-helix protein (102, 105) of 35.2 kDa (Fig. 6). The winged helix-turn-helix is located at the extreme N-terminus of the protein and is one of the three BirA domains, the others being a large central domain where is active site is found and a small C-terminal domain. The latter two domains show high levels of structural similarity with biotin-protein ligases from throughout biology (106). More recent work has shown that BirA requires bio-AMP to dimerize at physiological

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concentrations (107) and only the BirA dimer can efficiently bind the operator (108–111). Bio-AMP binding activates the assembly of the BirA-operator complex by increasing the extent of dimerization by three orders of magnitude (112, 113).

The biotin attachment activity of BirA (Fig. 7) proceeds through the bio-AMP intermediate formed from biotin and ATP (106). Enzyme bound bio-AMP is then attacked by the ε-amino group of a specific lysine of the acceptor protein to give the biotinylated acceptor protein (106) (Fig. 7). In the absence of an appropriate acceptor protein the bio-AMP intermediate remains bound within the BirA active site where it is protected from solvent and is quite stable (100). BirA shows very high specificity for biotin. The discrimination in favor of biotin versus DTB is *ca.* 50,000-fold (73, 114) although BirA-catalyzed attachment of DTB can be demonstrated (114). Both DTB and the oxidized form of biotin, biotin sulfoxide, show very weak abilities to derepress transcription of the biotin operon (115). A large number of *birA* mutants have been isolated based on their transcriptional phenotypes (using *bio-lacZYA* fusions) (77) and the mutational alterations of a considerable number of these have been determined by DNA sequencing (116). These fall into three main classes, mutants defective in regulation (the classical *bioR* phenotype), mutants defective in binding biotin and/or bio-AMP (the classical *birA* phenotype, (117)), and those having temperature-sensitive growth (77). However, there is considerable overlap among these phenotypes and some mutant proteins show all three phenotypes (77). All BirA crystal structures including that with a bio-AMP analogue show the N-terminal DNA binding domain markedly protruding from the body of the protein (Fig. 6A) and thus it is surprising that deletion of this domain has a profound effect on the ligase activity of the truncated protein due to poor binding of biotin and/or bio-AMP (118). It should be noted that BirA is an essential gene (77, 119, 120) since it is required for fatty acid synthesis and hence, membrane lipid synthesis (121).

The biotin acceptor protein

AccB protein, the sole biotin acceptor protein of *E. coli*, is an unusual protein, the N-terminal half appears largely unstructured (although the extreme N-terminus is known to interact with the AccC subunit (122) whereas the C-terminal half of the protein is folded into a compact and stable structure called the biotin domain (Fig. 7). This domain has a structure very similar to that of lipoyl domains (see below). The AccB biotinoyl domain is as efficient a biotin acceptor as the full-length protein (123) and is often used for *in vitro* work to avoid the problems with aggregation of the full-length protein (122, 123). The structure of biotinoyl domains is strongly conserved throughout biology and expression of foreign biotinoyl domains in *E. coli* can derepress *bio* operon transcription (124). Mutants of the AccB biotinoyl domain have been isolated that are defective in interaction with BirA (125) and mutations have been introduced that allow the protein to accept lipoic acid in place of biotin (126). The work on biotin and functions is intimately involved with (and is historically derived from) that on lipoyl domain structure and will be discussed in that context below.

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The biotin operator

The enzymes of *E. coli* biotin synthesis are encoded (with the exception of *bioH*) by a cluster of genes located adjacent to the attachment site of phage λ called the biotin (bio) biosynthetic operon (Fig. 5 and 6). Transcription of these *bio* genes is from two partially overlapping face-to-face promoters controlled by a common operator site of 40 bp that binds a dimer of the BirA protein (91, 95, 96, 127) (Fig. 6B). The leftward promoter transcribes *bioA* whereas the rightward promoter transcribes *bioBFCD*. The 5'ends of the transcripts have been mapped and mutations within the operator that ameliorate repression of either rightward or leftward transcription (or both) are known (95, 96, 128, 129). The operon and operator sequence are conserved in *S. enterica*, and *Citrobacter freundii* (129). A long-standing puzzle is that *bioH* is not under BirA regulation (77) especially since in other proteobacteria (e.g., pseudomonads) *bioH* seems part of a biotin biosynthetic operon. Also, unlike many repressors, BirA does not appear to be autoregulated because it is cotranscribed with an essential gene (*murB*) of peptidoglycan biosynthesis.

Physiological aspects of *bio* operon regulation

E. coli contains only a single species of biotin acceptor protein, the AccB subunit of acetyl-CoA carboxylase (ACC), which is the first enzyme of fatty acid biosynthesis (10, 94, 130, 131) and is therefore essential for growth. The response of the *E. coli* biotin regulatory system to the supply of biotin acceptor proteins is readily rationalized by the fact that biotin attains biological function only when the vitamin is covalently attached to AccB; the free vitamin cannot support ACC activity (121). AccB, which is also called biotin carboxyl carrier protein (BCCP), forms an unstable complex with AccC, the subunit that catalyses the biotin carboxylase partial reaction of acetyl-CoA carboxylase. The chromosomal locations of the genes (*accA* and *accD*) that encode the other two ACC subunits are well removed from the *accBC* operon and each other(10, 94, 130, 131). The AccB-AccC complex was recently shown to consist of an AccC dimer plus four copies of AccB (122). This complex is thought to bind an $\alpha_2\beta_2$ heterotetramer of the AccA and AccD subunits to give active ACC, the enzyme required for production of malonyl-CoA, the key precursor of fatty acid synthesis (121). The rates of transcription of all four genes are controlled by cellular growth rate (132) which is physiologically reasonable since lipids (hence fatty acids) constitute a constant fraction of the cell mass. The fact that *bio* operon transcription is derepressed by increased synthesis of AccB nicely ties biotin synthesis to growth rate. This is because increased growth rates require increased flux through the fatty acid synthetic pathway in which ACC catalyzes a rate-limiting step (133). Indeed, biotin consumed by increased protein biotinylation has been shown to be restored by increased biotin synthesis (92).

The fact that the only *acc* genes that are cotranscribed are *accB* and *accC* and that this gene arrangement is very widely conserved in bacteria raised the question of its relevance to the regulation of biotin synthesis (134). It seems possible that the defined stoichiometry given by cotranscription of *accB* and *accC* might function to aid efficient biotinylation of AccB. It seemed possible that an excess of AccC might tie up apo-AccB in a complex that would be a poor substrate for BirA and thereby disrupt the regulatory system (Fig. 5D). This has been shown to be the case (134). Overproduction of AccC gave almost maximal repression at

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biotin concentrations that normally give only slight repression and inhibited biotinylation of AccB. As expected overproduction of both AccB and AccC to restore the normal ratio of the two proteins relieved the down-regulation given by overproduction of AccC alone and this relief required that the overproduced AccB species be competent to interact with AccC (134).

What is the regulatory switch in BirA regulation?

The present model of *bio* operon regulation has a very solid experimental basis obtained by both *in vivo* and *in vitro* approaches. However, there are two contrasting views of the mechanism whereby accumulation of the unmodified biotin domain derepresses transcription of the operon. In one view this is simply a competition for bio-AMP between its consumption by protein biotinylation versus its presence in the BirA active site where it triggers dimerization and subsequent operator binding (93). In the second view the biotin domain forms a heterodimeric complex with a monomer of BirA. The BirA surface used to form the heterodimer is proposed to be the same surface as that used in forming the BirA homodimer. Hence, in this view competing protein-protein interactions are responsible for derepression upon accumulation of unmodified biotin domain (91). However, a major caveat to the model is that no direct detection of the postulated AccB plus BirA:bio-AMP heterodimer has been reported and only indirect evidence for its existence is available (135) (6) (135). The two models have a conceptual distinction, the lifetime of the BirA-biotin domain interaction. In the bio-AMP competition model the interaction is ephemeral, the two proteins associate, biotin is transferred and the complex rapidly dissociates as in most enzyme reaction whereas in the competing protein-protein interaction model the BirA-biotin domain interaction is long lived.

An approach that distinguishes these models utilized the small peptides that are substrates for biotinylation by BirA (136). These peptides, which were isolated by screening large peptide libraries, are quite diverse in sequence and have as few as two residues (one being the reactive lysine residue) that are found in naturally biotinylated proteins (136). Due to their small sizes (14 residues is sufficient, (136, 137)) and diverse sequences, it seems unlikely that stable peptide-BirA complexes are made. If these sequences (attached to a partner protein) are expressed in *E. coli* they should derepress *bio* operon expression, the bio-AMP competition model is supported. If they fail to derepress, but are efficiently biotinylated, then the competing protein-protein interaction model would be supported. Although the most studied of these peptides (Pep-85) is reported to be as good a biotin acceptor as the AccB biotin domain, this peptide remains enigmatic because it seems to lack structure in solution (137) and can only be biotinylated by BirA (124). Biotin ligases from six other organisms fail to use this peptide as a biotin acceptor, although these ligases readily utilize the AccB domain as a substrate (124, 138). Thus, if the most studied peptide sequence somehow mimicked structural attributes of AccB, the sequence should be biotinylated by BPLs other than BirA. However, BirA is the only ligase known to biotinylate the Pep-85 sequence.

Two fusion proteins containing synthetic biotin accepting peptide sequences (one being Pep-85) were as efficient in derepression of *bio* operon transcription as the when the natural

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acceptor, AccB-87, was the fusion partner (138). These results argue strongly against the competing protein-protein interaction model. As noted above the strict specificity of Pep-85 for BirA argues against the peptides being structural mimics of the natural acceptor domain. Moreover, even if this were somehow the case the the peptides would have to interacted with BirA:bio-AMP with the same binding strength and kinetics as that of the natural acceptor protein despite their small size and markedly diverged sequences. Indeed, the peptides lack several residues postulated to play important roles in forming the putative heterodimer and have other residues, some of which cannot participate in hydrogen bonding) in place of resides thought to play roles in heterodimer formation. It it follows that the rules governing biotinylation are markedly different for AccB and the peptide sequences (138). The possibility that the competing protein-protein interactions model is the regulatory switch seems extremely remote. The classical work on BirA mutants did not include BirA super-repressing mutants. These would be mutants that would repress transcription under all conditions including biotin limitation and apo-domain overexpression. Some of the possible classes of mutants are: (i) BirA proteins unable to bind the biotin acceptor protein, (ii) BirA proteins that bind the acceptor protein but are unable to biotinylate it, (iii) BirA proteins that form very tight homodimers (perhaps even in the absence of bio-AMP) and (iv) BirA proteins that cannot dissociate from the operator DNA. Some of the mutants might be genetically dominant. Most of these mutants could be nonviable because fatty acid synthesis would be blocked due to lack of biotinylation of AccB thereby account for the fact that such mutants were not reported in the early investigations. Hence, the isolation of super-repressor mutants was done in a strain where expression of a heterologous biotin protein ligase active on AccB allowed fatty acid synthesis to proceed (139). This allowed mutant strains having the super-repressor phenotype by a combined selection-screening approach and resolved multiple mutations to give several *birA* super-repressor alleles each having a single mutation all of which showed repression dominant over the wild type allele. All of these mutant strains repressed *bio* operon transcription *in vivo* at biotin concentrations that gave derepression of the wild type strain and retained sufficient ligation activity for growth when overexpressed (139). All mutant strains except G154D BirA showed derepression of *bio* operon transcription upon overproduction of a biotin accepting protein. The G154D BirA was a lethal mutation in single copy and the purified protein was unable to transfer biotin from enzyme bound biotinoyl-adenylate either to the natural acceptor protein or to a biotin accepting peptide sequence. Consistent with the transcriptional repression data, each of the purified mutant proteins showed increased affinity for the biotin operator DNA in electromobility shift assays. Surprisingly although most of the mutations were located in the catalytic domain all those tested excepting G154D BirA had normal ligase activity. Most of the mutations that gave super-repressor phenotypes altered residues located close to the dimerization interface of BirA. However, two mutations were located at sites well removed from the interface. The properties of the super-repressor mutants strengthen and extend other data indicating that BirA function entails extensive interactions among the three domains of the protein and shows that normal ligase activity does not ensure normal DNA binding (139).

Finally, the crystal structure of BirA complexed with the *bio* operator and bio-AMP (or an analogue) seems likely to very informative. This may give information on the

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conformational changes in BirA that accompany bio-AMP binding (140). Co-crystals of the BirA-biotinoyl domain complex would also be of great interest. The super-repressor mutant proteins may stabilze the BirA-operator contacts and thereby facilitate crystallization of the complex.

Lipoic acid synthesis

Lipoic acid (Fig. 1) is a sulfur-containing cofactor found in most prokaryotic and eukaryotic organisms. In *Escherichia coli* and other organisms lipoic acid is essential for function of several key enzymes involved in oxidative and single carbon metabolism including pyruvate dehydrogenase (PDH), 2-oxoglutarate dehydrogenase (2-OGDH), branched-chain 2-oxoacid dehydrogenase, acetoin dehydrogenase and the glycine cleavage system (141). In each enzyme, a specific subunit is modified by attachment of lipoic acid to specific lysine residues within conserved domains of these subunits. In each of these domains an amide linkage is formed between the carboxyl group of lipoic acid and the ε–amino group of the specific lysine residue (142). During catalysis, the protein-bound lipoamide moieties serve as carriers of reaction intermediates among the multiple active sites of these multienzyme complexes (141).

Our knowledge of the pathways of lipoic acid synthesis, attachment and function has progressed rapidly in the last 10 years largely due to complementary genetic and biochemical analyses in *E. coli.* I shall first discuss the enzymes that carry and require the cofactor because they are derived from diverse areas of metabolism. Next, the mechanisms of attachment of lipoic acid and its precursor, octanoic acid, to these proteins will be reviewed. Finally, the synthesis of the cofactor itself will be discussed. This organization was chosen because the unusual biosynthetic pathway of lipoic acid is mechanistically intertwined with attachment of the cofactor.

Lipoic acid-dependent enzymes

Pyruvate dehydrogenase (PDH)

The PDH reaction mechanism is probably the most thoroughly characterized lipoic acid-dependent enzyme. PDH catalyzes the oxidative decarboxylation of pyruvate to the key metabolic intermediate, acetyl-CoA. This very large enzyme complex consists of multiple copies of each of three subunits encoded by the *aceE aceF lpd* operon. The first subunit (AceE) is a thiamine diphosphate-dependent decarboxylase (E1p) that catalyzes both the decarboxylation of pyruvate and the reductive acetylation of the lipoyl group that is covalently attached to the second subunit, E2p (AceF). The E2p subunit is a dihydrolipoyl acetyltransferase responsible for the transfer of the acyl group from lipoyl moiety to CoA to form acetyl-CoA. The third subunit, E3 (Lpd), is a dihydrolipoyl dehydrogenase that serves to regenerate the disulfide bond of the lipoyl moiety of E2p (143) and thereby prepares the enzyme for another cycle of catalysis. The E2p subunit to which E1 and E3 are bound strongly (but noncovalently) forms the structural core of the multienzyme complex. The oxidative decarboxylation of pyruvate to form acetyl-CoA is the link between glycolysis and the citric acid cycle and therefore PDH activity is essential to cells that rely upon respiration to provide metabolic energy. In most aerobically respiring organisms the PDH complex also

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supplies the acetyl-CoA necessary to sustain essential biosynthetic pathways, especially those of fatty acid and amino acid synthesis (144). Synthesis of the PDH complex varies over a 7- to 10-fold range depending on the growth conditions (145–147). It is induced by exogenous pyruvate or when pyruvate is generated endogenously e.g. by thiamine starvation, and it is partially repressed by excess glucose and during growth on acetate or on citric acid cycle intermediates. Regulation by pyruvate or a derivative of pyruvate proceeds through the PdhR repressor (148, 149). PDH synthesis is repressed during anaerobic fermentative growth where the catalytic activity is also inhibited. Under these conditions the conversion of pyruvate to acetyl-CoA is mediated by the derepression and activation of pyruvate formate lyase (146, 150).

2-Oxoglutarate dehydrogenase (2-OGDH)

The mechanism of 2-OGDH is essentially the same as that of PDH as is the structure of the complex. Indeed, the 2-OGDH complex has been reported to contain low levels of PDH subunits (151). The 2-OGDH complex contains three subunits, a 2-oxoglutarate decarboxylase component (E1o), a trans-succinylase component (E2o) and a dihydrolipoyl dehydrogenase (E3). The E1o and E2o subunits are different proteins from the corresponding subunits of the PDH complex and are encoded by the *sucA* and *sucB* genes, respectively. However, the E3 subunit is the same protein, Lpd, found in the PDH complex. In aerobically grown *E. coli*, this complex catalyzes a key step in the citric acid cycle and also supplies succinyl-CoA for biosynthesis of two amino acids, methionine and lysine (152). Under the appropriate conditions, *E. coli* strains lacking functional 2-OGDH can be supplemented with succinate or methionine plus lysine to provide metabolic bypasses of loss of this enzyme complex (152). Expression of the 2-OGDH is highly induced during aerobic growth on acetate and citric acid cycle intermediates and is severely repressed during fermentative growth where succinyl-CoA is generated by succinyl-CoA synthetase (144) although 2-OGDH is synthesized by cells gown in anaerobic media containing an electron acceptor such as nitrate or fumarate (153).

Glycine cleavage system

The third lipoylated protein of *E. coli* is the H protein of the glycine cleavage system, an enzyme widely distributed in bacteria and in the mitochondria of plants (where it is called glycine decarboxylase), fungi and mammals (154–156). The glycine cleavage system catalyzes the reversible cleavage of glycine, yielding carbon dioxide, ammonia, 5,10-methylenetetrahydrofolate plus a reduced pyridine nucleotide. It consists of four component proteins termed the T, H, P and L proteins. The first three proteins are encoded by the *gcvT gcvH gcvP* operon while L protein is the same as Lpd, the E3 protein of the 2-oxo acid dehydrogenases as discussed above (157). P protein catalyzes the pyridoxal phosphate-dependent decarboxylation of glycine and transfers the remaining methylamine moiety to one of the sulfhydryl groups of the lipoyl prosthetic group of H protein. T protein catalyzes the release of ammoniate and transfer of the one-carbon unit to tetrahydrofolate from the lipoyl residue. L protein is a lipoamide dehydrogenase that catalyzes the reoxidation of the dihydrolipoyl residue of H protein and reduction of NAD^+. Thus, the lipoic acid moiety of H protein interacts with the active sites of three different enzymes in a manner analogous to that found for 2-oxoacid dehydrogenase complexes.

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Structures of lipoylated and biotinylated proteins

In all 2-oxoacid dehydrogenase complexes, the core of the structure is provided by the E2 subunit to which the E1 and E3 components are bound tightly but noncovalently. In the PDH and 2-OGDH complexes of *Escherichia coli* and other gram-negative bacteria (158, 159) plus the 2-OGDH and branched-chain 2-oxoacid dehydrogenase complexes of mammals (160, 161), the core consists of 24 copies of the E2 chain arranged with octahedral symmetry, whereas in the PDH complexes of mammals and Gram-positive bacteria (162–165), the core comprises 60 E2 chains arranged with icosahedral symmetry. In all 2-oxoacid dehydrogenase complexes, the E2 component has a multi-domain structure comprising (from the N terminus): lipoyl domain (or domains of ca. 9 kDa), a small peripheral subunit-binding domain (ca. 4 kDa) and a much larger catalytic domain (ca 28 kDa) that houses the acyltransferase activity and aggregates to form the inner core of the complexes. These domains are separated by long (25–30 residue) segments of polypeptide chain, characteristically rich in alanine, proline and charged amino acids that form flexible but extended linkers (143).

The numbers of PDH lipoyl domains per E2 subunit varies from one to three. In the PDH complexes of Gram-negative bacteria, the number is usually three (e.g., *E. coli* and *Azotobacter vinelandii*) or two (e.g. *Haemophilus influenzae, Neisseria meningitidis, Alcaligenes eutrophus*, and *Thiobacillus ferrooxidans*) (9). All of the 2-OGDH E2o subunits described to date contain a single lipoyl domain, as is also the case for the E2b chains of all BCDH complexes (9, 141, 143, 166). A generally applicable explanation for the variation in the number of lipoyl domains has not yet been worked out. Protein engineering experiments have eliminated the straightforward explanations. In *E. coli* PDH, selective deletion of one or two lipoyl domains has no detectable effect on the overall catalytic activity, the system of active site coupling or the ability to complement pyruvate dehydrogenase complex mutants (167). As expected the catalytic activity is abolished when all three lipoyl domains are deleted or when the lipoyl domains are rendered unlipoylatable by conversion of the lipoylated lysine residue to glutamine (167, 168). There is no mandatory order of reductive acetylation of the repeated lipoyl domains within E2p polypeptide chains because complexes containing mixtures of wild-type and mutant lipoyl domains (+/−; −/+; +/+/−) are fully active, although the complex containing the −/−/+ version of the E2p polypeptide chain showed a 50% reduction in specific activity (168). Activity is also impaired (but not abolished) by increasing the lipoyl domain content to four to nine per E2p chain, possibly due to under-lipoylation of the domains participating in catalysis and interference from unlipoylated domains (169). High-field NMR studies were carried out with variants containing zero to nine lipoyl domains per E2p subunit. These studies suggest an explanation for the presence of three lipoyl domains per E2p subunit in the wild-type PDH complex that is based on the greater inherent mobility and thus potentially more efficient active-site coupling of this arrangement (170). The superiority of the three lipoyl domain-PDH complex has since been confirmed by physiological studies from which it was concluded that decreased lipoyl domain contents adversely affect growth rate and growth yield (171). The physiological consequences of increasing the number of lipoyl domains from three to nine per E2p chain, and the effects of inserting up to seven unlipoylated

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(mutant) domains between a wild-type N-terminal lipoyl domain and the E3-binding domain were also investigated and indicate that three lipoyl domains per E2p chain are optimal and that only the outermost lipoyl domain needs to be lipoylated to obtain full catalytic activity (172). It was concluded that the reason for retention of three lipoyl domains is to extend the reach of the outermost lipoyl cofactor rather than to provide extra cofactors for catalysis (172). However, given this advantage why then do many lipoylated proteins contain only a single lipoyl domain?

The conserved structure of lipoyl domains (Fig. 8A) is directly related to catalytic functions of the domain in substrate channeling and active-site coupling. First of all, although free lipoic acid is a substrate for E2p and E3 *in vitro*, lipoylated domain is a much better substrate (Graham et al., 1989). Attachment of the lipoyl group to the conserved lysine at the tip of the protruding β–turn gives a dramatic reach to the "business end". Moreover, the flexible and extended linker regions that connect the lipoyl domain(s) with the catalytic domain contribute increased mobility to the swinging arm since deletion of the linker region in a modified "single lipoyl domain" E2p chain caused an almost total loss of overall activity without substantially affecting the individual enzymatic activities (173). Second E1p and E1o of *E. coli* (85, 114) and *A. vinelandii* (174) can only transfer acyl groups to their cognate E2 protein thereby providing an accurate substrate channeling mechanism such that the reductive acylation only occurs on the lipoyl group covalently attached to the appropriate E2 subunit. Third, although the attached lipoate was once thought to be freely rotating (175, 176), recent structural data showed that the lipoyl-lysine β–turn of the domain became less flexible after lipoylation of the lysine residue (177). The restricted motion of the lipoyl group would facilitate the effective E1 and E2 interaction by presenting the lipoyl group in the preferred orientation to the active sites of E1 and thereby enhance catalysis. This is in agreement with the recent crystal structure of the E1 component of the BCDH complex from *P. putida* (178). According to this structure, the active site where thiamine diphosphate binds is at the bottom of a long funnel-shaped tunnel, which suggests that the lipoyl group attached to the lipoyl domain must be fully extended and accurately positioned in order to reach the thiamine diphosphate cofactor. Amino acid side chains responsible for this specific positioning have been mapped to two residues that flank the lipoyl-lysine (179). Finally, the prominent surface loop connecting β–strands 1 and 2 (which lie close in space to the lipoyl-lysine) is another major determinant of the interactions of the lipoyl domain with E1 (180). Deletion of this loop results in a partially folded domain and almost completely abolishes lipoylation and reductive acylation indicating that the loop is involved in maintaining the structural integrity of the domain, post-translational modification and catalytic function (177). It is believed that the loop structure is important for stabilizing the thioester bond of the acyl-lipoyl intermediate (177, 181).

Subgenes that encode the lipoyl domains from a wide range of bacteria, including *E. coli* E2p (182) and E2o (183), *Bacillus stearothermophilus* E2p (184), human E2p (185), *Azotobacter vinelandii* E2p (166) and E2o (186), and *Neisseria meningitidis* E2p (187) have been overexpressed in *E. coli* and sufficient recombinant protein has been obtained for the domain structures to be determined by multidimensional nuclear magnetic resonance (NMR) spectroscopy. The archetypical structure, that of the single apo lipoyl domain of the E2p

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chain of *B. stearothermophilus* (188), is composed largely of two four-stranded β–sheets, with the N- and C-terminal residues of the domain close together in space in one sheet and the lysine residue earmarked for lipoylation in an exposed position in a tight type I β–turn generated by β–strand 4 and 5 in the other sheet. There is a well-defined hydrophobic core, the least well-defined regions being the exposed β–turn where the lipoyl-lysine resides and, most notably, the nearby large surface loop that connects β–strands 1 and 2 (Fig. 8A). Consistent with the high level of sequence similarity between lipoyl domains of 2-oxoacid dehydrogenase multienzyme complexes, all other lipoyl domains conform to the same structural pattern. Given the small differences in the NMR spectra of the lipoylated and unlipoylated forms of the *B. stearothermophilus* (175) and *A. vinelandii* (189) E2p domains, the structures of holo- and apo-domains have been inferred to be substantially the same.

The determination of lipoyl domain structures has allowed prediction of the structure of another lipoylated protein: the H protein of the glycine cleavage system. H proteins are about 130 resides in length (190). Although the overall sequence identity was low (<20%) (191), the conservation of key residues indicated that there was likely to be considerable structural similarity between the H protein of glycine cleavage system and the lipoyl domains of 2-oxo acid dehydrogenase complexes (192). Indeed, the X-ray crystal structure of the lipoylated pea leaf H protein agreed well with the theoretical predictions. The biotinyl domains of biotin-dependent enzymes have structures strikingly similar to those of lipoyl domains (Fig. 8B) as originally predicted by Brocklehurst and Perham (192). This is particularity true of biotin domains from enzymes other than bacterial and plant plastid acetyl-CoA carboxylases. The biotinylated subunits of the bacterial and plastid acetyl-CoA carboxylase contain a characteristic thumb structure not found in other biotinoyl domains or in lipoyl domains (10). The structure of the biotin domain of *E. coli* AccB has been established by X-ray crystallography (193) and NMR spectroscopy (Fig. 8B) (194–196). The structure closely resembles those of the lipoyl domain in the E2 component of 2-oxoacid dehydrogenase complexes and of the H protein in the glycine cleavage system. Like these lipoylated proteins the AccB domain is a flattened β-barrel, comprising two 4-stranded anti-parallel β–sheets, with the biotinyl-lysine residue located in the exposed β–turn between β–strands 4 and 5 (Fig. 8B). The high-resolution NMR structure of another biotinoyl domain, that of *Propionibacterium shermanii* transcarboxylase, has also been determined (197). This structure more closely resembles the lipoyl domain structures since it lacks the protruding thumb of the *E. coli* biotin domain (to which it is otherwise quite similar). Depending on the pair of domains chosen for comparision the root mean square deviation of biotinoyl and lipoyl domain backbone atoms can be as low as 1 Å and hence these proteins define a protein family (PF00364). Other work has shown that one of the proline/alanine-rich linker regions that lie between the domains of *E. coli* PDH can functionally replace the proline/alanine-rich linker region that lies upstream of the biotin domain of *E. coli* BCCP (130) underlining the interrelatedness of the biotin and lipoic acid acceptor proteins.

Protein lipoylation pathways

Post-translational modification of apoproteins with lipoic acid occurs by several mechanisms. In *E. coli*, two complementary systems for protein lipoylation have been characterized, by genetic and subsequent biochemical analyses. Exogenous lipoate or

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octanoate is transferred to unlipoylated apoproteins in an ATP-dependent process by lipoate-protein ligase (LplA) (198, 199). The second *E. coli* pathway requires the *lipB* gene product (octanoyl-ACP:protein-N-octanoyltransferase) to transfer endogenously synthesized octanoate to apoproteins which is then becomes the substrate for sulfur insertion (Figure 6) (199–203).

Lipoate-protein ligase (LplA)

Lipoate-protein ligase activity was first described by Reed and coworkers (204) in *Enterococcus faecalis* as well as in *E. coli* and these workers postulated that lipoate was attached to protein by a two-step ATP-dependent reaction with lipoyl-AMP as an activated intermediate. The reaction is exactly the same as that of BirA (Fig. 7) with the substitution of lipoic acid for biotin (hence this is not shown). Although the lipoate-protein ligases were key reagents in demonstration of the role of lipoic acid in the 2-oxoacid dehydrogenase reactions (142, 205), neither protein had been purified to homogeneity and thus the proposed mechanism could not be proved. The *E. coli lplA* gene was the first lipoate-protein ligase gene to be isolated and LplA was the first such ligase purified to homogeneity (199, 206). Mutants in *lplA* were isolated by two different approaches. In the first approach a *lipA* strain was mutagenized by transposon insertion and the mutagenized cells were supplemented with a mixture of succinate and acetate to bypass the lipoate requirement. The supplement was then switched to lipoate and an ampicillin enrichment was performed followed by plating onto the succinate-acetate supplemented medium. The resulting colonies were screened for strains able to grow on succinate-acetate supplemented medium, but not on lipoate supplemented medium. Three classes of such mutant strains could have resulted from this scheme, strains lacking the ligase (*lplA*), strains defective in lipoate transport and *lpd* mutants that lack the E3 subunit common to all of the lipoate-dependent enzymes of *E. coli*. Indeed, the selection was an unwitting repeat of the selection used for *lpd* mutants (207). Surprisingly, all of the mutants isolated were *lplA* mutants. It is unclear why no *lpd* mutants were isolated in the *lplA* selection and *vice versa*. The lack of lipoate transport mutants suggests that there may be no lipoate transporter in *E. coli* (as is believed to be the case for short chain fatty acids). Given the small size, hydrophobicity and the miniscule amount of the cofactor needed for growth no transporter may be needed. Indeed it has been reported that both enantiomers of ^{35}S-lipoate were taken up by *E. coli*, although only R-lipoic acid became attached to the 2-oxoacid dehydrogenases (208). Since a protein transporter would be expected to discriminate between enantiomers, this finding argues strongly against the existence of a lipoate transporter. Mutants mapping in *lplA* were also isolated by a direct selection, resistance to selenolipoic acid. Selenolipoic acid is a growth-inhibitory lipoate analogue in which the sulfur atoms are replaced with selenium (209). These mutants proved to encode a ligase of somewhat compromised activity that was able to discriminate against the selenium analogue (198).

The purified LplA enzyme is a 38 kDa monomeric protein (206). Assays with a fully purified apoprotein acceptor have demonstrated that purified LplA plus lipoate and Mg-ATP are sufficient to reconstitute lipoylation *in vitro* (126, 199, 206, 210). Thus, it is clear that LplA catalyses both the ATP-dependent activation of lipoate to lipoyl-AMP as well as the transfer of this activated lipoyl species to apoprotein with concomitant release of AMP. This

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conclusion is consistent with the early findings of Reed and coworkers (204) that the *E. coli* enzyme could not be fractionated into separable lipoate activation and lipoyl transferase components. LplA has been shown to be capable of utilizing lipoate and several lipoate analogs as donors for the post-translational modification of E2 apoproteins *in vivo* (199). This rather-broad substrate specificity *in vivo* matches the similarly broad substrate specificity observed (211).

Very recently several crystal structures of LplA and of LplA homologues have been reported including structures of *E. coli* LplA (212) plus an LplA-lipoic acid complex (212). The reported structures agree well and show *E. coli* LplA to by two-domain protein consisting of a large N-terminal domain and a small C-terminal domain. The *E. coli* LplA-lipoic acid complex was difficult to interpret because lipoic acid was bound to different LplA molecules within the crystals with different modes and with poor resolution. For example in one case the lipoic acid carboxyl was hydrogen bonded to Ser-72 whereas in the other case Arg-140 was the hydrogen bond donor (212). Since enzymes rarely show such plasticity and lipoic acid is a hydrophobic molecule, it seemed possible that the observed association with a hydrophobic LplA surface in the interdomain cavity was artifactual. Moreover, in prior work Reed and coworkers had isolated LplA mutants resistant to selenolipoic acid (209). Since this is a very discrete modification of the LplA substrate, the mutant protein would be expected to have an alteration close to the pocket that binds the lipoic acid thiolane ring. However, the site of this mutation (Gly-76 to serine, (198)) was distal from the lipoate-binding site reported. This dilemma appeared resolved first by two lipoic acid-containing structures of an LplA homologue from the Archaeon, *Thermoplasma acidophilum* (213, 214) that can be readily superimposed on the *E. coli* LplA structure except that the *T. acidophilum* protein lacks the LplA C-terminal domain. In both *T. acidophilum* structures the lipoate thiolane ring was close to the glycine residue that corresponds to *E. coli* Gly-76, the residue giving resistance to the selenium analogue and a plausible reorganization of the molecule to prevent binding of the larger selenolipoic acid was proposed (214). Moreover, addition of lipoic acid to a complex of the *T. acidophilum* with ATP gave lipoyl-AMP thereby showing that the lipoic acid was bound in a physiologically meaningful manner (213). Lipoyl-AMP was bound in a U-shaped pocket and was well shielded from solvent. The *T. acidophilum* LplA was reported to be inactive in catalyzing the overall LplA reaction (214), although lipoyl-AMP synthesis was demonstrated (213). Since *T. acidophilum* LplA lacks the C-terminal domain of *E. coli* LplA (213, 214) this suggested that the missing domain plays a key role in transfer of the lipoyl moiety from lipoyl-AMP to the acceptor domain Indeed, a second protein has been proposed to interact with *T. acidophilum* LplA and allow the complete reaction (214) and this proved to be the case (215, 216). The fact that the lipoate of one of the *T. acidophilum* LplA structures was converted to lipoyl-AMP and that the locations of lipoate moieties of the two *T. acidophilum* LplA structures agreed well argues that these represent the catalytically competent lipoate binding site. It therefore follows that in the first *E. coli* LplA structure the lipoate molecule was bound in a catalytically inappropriate manner. Indeed, in a later report from the same group cocrystals of LplA with lipoyl-AMP and LplA with octyl-5′-AMP and the apo form of GcvH. These structures define the LplA tructural mechanism. Three large scale conformational changes occur upon completion of the lipoate adenylation reaction i) the adenylate-binding, ii) the

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lipoate-binding loops move to maintain the lipoyl-5′-AMP reaction intermediate and iii) the C-terminal domain rotates by about 180 degrees. These changes are prerequisites for LplA to accommodate the apoprotein for the lipoate transfer reaction. The invariant Lys133 residue plays essential roles in both lthe ipoate adenylation and transfer steps.

3.2 Octanoyl-ACP:protein N-octanoyltransferase (LipB)

During the characterization of *E. coli lplA* null mutant strains compelling evidence was found for a second protein lipoylation pathway that did not require the *lplA* gene product. When independently derived *lplA* null alleles were transduced into wild-type strains, the resulting mutant strains showed no growth defects on minimal glucose medium indicating that these strains possessed functional (therefore lipoylated) 2-oxoacid dehydrogenases. This was directly confirmed by bioassays that showed *lplA* null mutants to contain normal levels of lipoylated proteins (198). Thus, it was clear that *E. coli* has an *lplA*-independent lipoylation pathway. This was first attributed to a second ligase that had somehow been missed in the biochemical analyses perhaps due to the *in vitro* conditions chosen. However, no such second ligase could be found (206) and thus alternative pathways were considered. The most straightforward alternative pathway was that the fatty acid synthesis intermediate, octanoyl-ACP would be converted either directly or indirectly to lipoylated proteins. That is, lipoate synthesis would occur without a free carboxyl group. The carboxyl group would be bound in the thioester bond that links fatty acids to ACP and this bond would then be attacked by the ε-amino group of the lipoyl domain lysine residue to give the amide linkage. Several lines of evidence demonstrated that this alternative protein lipoylation pathway was dependent on the *lipB* gene product. The *lipB* gene was originally isolated as a class of lipoic acid auxotrophs (6). These mutants showed residual (leaky) growth in the absence of lipoic acid despite having putative null mutations due to transposon insertions into *lipB* (6, 217). This leakiness was reflected in their 2-oxo acid dehydrogenase activities and lipoylated protein contents. These strains retain about 20% of the enzyme activities and about 10% of lipoyl protein content of wild type strains (217). The leaky growth of *lipB* strains in the absence of lipoate was eliminated by introduction of an *lplA* mutation suggesting that *lipB* was involved in lipoyl domain modification as well as lipoate biosynthesis (198). Indeed, bioassays demonstrated that the low lipoyl protein content of the *lipB* null mutants was further depressed to undetectable levels in the *lipB lplA* double mutants (198). This finding suggested that the attenuated, but still detectable, accumulation of protein-bound lipoate by *lipB* null mutants was entirely due to the action of the *lplA* gene product. Moreover, overexpression of LplA allowed normal growth of *lipB* null mutant strains in the absence of lipoate thus clearly demonstrating the redundant roles of these two genes (198). Genetic and biochemical evidence demonstrated that *lipB* encoded the octanoyl-ACP:protein N-octanoyltransferase (which is also an lipoyl-ACP:protein N-lipoyltransferase) (Fig. 9). An enzyme activity was detected in *E. coli* cell extracts that catalyzed the transfer of octanoic acid (lipoic acid) from octanoyl-ACP (lipoyl-ACP) to E2 apo-domains (Fig. 9). This activity was also found in extracts of *E. coli lplA* null mutants and, unlike LplA, this activity was not dependent on ATP. However, transferase activity was absent in *E. coli lipB* mutants (218). A temperature-sensitive *lipB* strain was obtained and found to encode a transferase of decreased thermal stability (200) indicating that *lipB* encoded the transferase rather than playing a regulatory role. Finally, His-tagged LipB was purified and the purified protein had

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high levels of octanoyltransferase and lipoyltransferase activities (200). The untagged protein has recently been purified by conventional means (219).

Based on these observations, a two pathway *E. coli* lipoylation system was proposed (198, 201, 218) (Fig. 10). When presented with free lipoic acid in the medium *E. coli* incorporates extracellular lipoate (217, 220) via the LplA-dependent scavenging pathway which utilizes ATP to activate lipoic acid in the form of lipoyl-AMP. When lipoate is absent from the medium lipoyl groups must be made by *de novo* synthesis. An octanoyl group is first transferred from octanoyl-ACP to the apo-proteins by LipB. LipA then acts on the protein-bound octanoyl groups to catalyze the sulfur insertion step (Fig. 10). This model explains why *lplA* null mutants showed no growth defects unless the strain also carried a *lipA* or *lipB* mutation as well as the unidirectional redundancy between LipB and LplA functions. LplA was reported to utilize octanoyl-ACP as substrate with low but detectable efficiency (200) which was puzzling given the very different chemistries of the two reactions. However, more recent work showed that this was due to traces of ATP and octanoate present in the octanoyl-ACP preparations (F. Hermes and J. E. Cronan, unpublished data). Recently it was shown that the ability of LplA overexpression to complement *lipB* strains is due to increased scavanging of cytosolic octanoic acid. In the absence of LplA overexpression suppression by chromosomal mutations was observed. These suppressor mutations map in LplA and result in proteins having reduced Km values for free octanoic acid which allows efficient scavanging of cytosolic octanoic acid (221).

It should be noted that strains having null mutations in both *lplA* and *lipB* contain no detectable lipoylated proteins indicating that LplA and LipB are the only *E. coli* enzymes capable of modifying lipoyl domains (198).

Three assays have been used to detect attachment of lipoic acid to apo forms of PDH and 2-OGDH *in vitro*. The first assay measured the conversion of radioactive lipoate (or octanoate) to a protein-bound form defined as being insoluble in organic solvents (199). This is a sensitive and quantitative assay, but applicable only to LplA since both the LipB substrate and product are protein bound. The second assay relies on the use of inactive unmodified apo-PDH or 2-OGDH complex purified from a *lipB lplA* strain completely deficient in modification of the E2 proteins. Lipoylation of the purified apo-PDH or 2-OGDH complex was detected by assay of the products of ligation reactions for either PDH or 2-OGDH activity (222). The third assay is a gel mobility shift assay (218). It follows the acylation-dependent shift in the electrophoretic mobility of a purified 87 residue apo-lipoyl domain from the *E. coli* PDH complex (223). This assay is much less sensitive than the other two 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. With this assay it was found that purified LipB could convert the apo form of lipoyl domain to the holo domain with either octanoyl-ACP or lipoyl-ACP as the substrate. When LipB was tested for the ability to use ATP plus lipoic acid or octanoic acid, no modification was detected.

Another *lipB* phenotype is that multiple copies of the gene confer resistance to selenolipoic acid. An analogue-resistant mutant that did not map at the *lplA* locus (209) was shown to be a chromosomal amplification of the *lipA lipB* region of the chromosome (224). The

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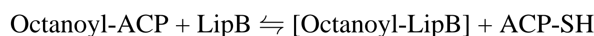
increased *lipB* dosage resulted in greater LipB activity that resulted in increased levels of lipoylation by endogenously synthesized lipoic acid that competed with the utilization of exogenous selenolipoic acid via the LplA-dependent pathway. A very modest (two- to threefold) increase in *lipB* dosage was sufficient to give resistance which was explained by the known highly nonlinear relationship between the degree of protein lipoylation and the activity of the 2-oxoacid dehydrogenase complexes plus the fact that *E. coli* does not require full activity of the 2-oxoacid dehydrogenases for growth on minimal medium containing glucose (224). Thus, synthesis of sufficient lipoic acid to modify a few percent of the 2-oxo acid dehydrogenase complexes allowed growth in presence of selenolipoic acid.

As mentioned above the LipB reaction was recently shown to proceed via an acyl enzyme intermediate (225). The octanoyl group is transferred from the ACP thiol to the thiol of Cys-169. This protein-bound thioester is then attacked by the ε-amino group of the lipoyl domain lysine to give the modified domain.


Octanoyl-ACP + LipB ⇋ [Octanoyl-LipB] + ACP-SH

[Octanoyl-LipB] + Apo Lipoyl Domain * Octanoyl-E2 Domain

The fact that LipB could transfer either octanoate or lipoate from ACP to a lipoyl domain raised the question of the true intracellular substrate of the enzyme. This has been answered by overexpression of LipB and isolating the enzyme under conditions that retained any acyl-enzyme interemediate present. Mass spectrometry showed that only octanoate was attached to the enzyme thereby demonstrating the specificity of the enzyme (226).

The reactivity of the cysteine residue seems responsible for the both LipB crystal structure scurrently available, those of the *Mycobacterium tuberculosis* and *T. acidophilum* enzymes (227, 228). The *M. tuberculosis* LipB, expression which complements growth of *E. coli lipB* mutant strains, was crystallized in a covalent complex with decanoic acid. Surprisingly, although the acyl chain was bound to the sulfur atom of a cysteine residue corresponding to Cys-169 of *E. coli* LipB, the bond was a thioether linkage to C3 of decanoate rather than a thioester link to the carboxyl group (227). This unexpected finding seems likely to be the result of a Michael addition of the cysteine thiol to the unsaturated bond of *trans*-2 decenoyl-ACP or *cis*-3-decenoyl-ACP, a key intermediate in *E. coli* unsaturated fatty acid biosynthesis. Consistent with this interpretation no such adduct was seen upon expression of the protein in *Mycobacterium smegmatis*, which forms unsaturated fatty acids by a pathway that does not involve decenoyl intermediates (227). However, the protein lacking the adduct failed to crystallize and thus adduct formation trapped LipB into a form amenable to crystallization. *T. acidophilum* LipB structure also required covalent trapping I to form crystals, an intermolecular disulfide (228). Based on the *M. tuberculosis* LipB crystal structure and mutagenesis studies LipB is thought to function as a novel cysteine/lysine dyad acyltranferase, in which the dyad residues function as acid/base catalysts (227).

Biosynthesis of lipoic acid

Although the functions of lipoic acid in the multienzyme complexes have been well studied over the past forty years, an understanding of lipoic acid biosynthesis pathway has only

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recently been achieved. Such studies have focused on *E. coli*. Early studies had established that octanoic (properly n-octanoic) acid (Fig. 1) is the precursor of the lipoic acid carbon chain (229). Analysis of the conversion of specifically labeled forms of octanoic acid to lipoic acid by *E. coli* cultures showed that sulfur atoms are introduced with loss of only two hydrogen atoms from the chain, one from C-6 and the second from C-8 (230, 231). Additional metabolic feeding studies demonstrated that *E. coli* lipoic acid biosynthesis does not involve either desaturation or hydroxylation of octanoic acid, but does result in inversion of stereochemistry at C-6 (231, 232). Sulfur is introduced at C-8 with racemization in agreement with the formation of an intermediate carbon radical at C-8 (230, 232–234). 8-Thiooctanoic acid and 6-thiooctanoic acid were readily converted to lipoic acid, although 6-thiooctanoic acid was converted only 10–20% as efficiently as the other positional isomer (234). Genetic studies identified a single *E. coli* gene responsible for the sulfur-insertion steps of lipoic acid biosynthesis, first called *lip* (152) and now called *lipA* which encodes a protein called lipoic acid synthase. *E. coli* strains having null mutations in *lipA* do not synthesize lipoic acid and the phenotypes of these mutants suggested that LipA was responsible for the formation of both C-S bonds (6] which encodes a protein called lipoic acid synthase. E. coli strains having null mutations in lipA do not synthesize lipoic acid and the phenotypes of these mutants suggested that LipA was responsible for the formation of both C-S bonds {Herbert, 1968 #114, 217, 235, 236) and encodes a protein now called lipoic acid synthase. There are strong parallels between LipA and biotin synthase (BioB), the enzyme discussed above that catalyzes the final step in the biotin biosynthesis (Fig. 4). LipA like BioB makes two C-S bonds and also removes two unactivated hydrogen atoms. The similarity in chemistry between the biosynthesis of the dithiolane ring of lipoate and the thiophane ring of biotin strongly suggests functional parallels in the mechanisms of the enzymes that produce these compounds. Indeed, the amino acid sequences of the *E. coli* LipA and BioB proteins show marked similarities; 40% sequence similarity and 17% sequence identity (217).

As discussed above BioB has both a [4Fe-4S] cluster and a [2Fe-2S] cluster. The canonical iron-sulfur cluster binding motif CXXXCXXC is also found in the LipA sequence leading to early predictions that it is an iron-sulfur protein (217, 236). The LipA protein has been overexpressed in *E. coli* and purified from both soluble lysates and insoluble aggregates that were subsequently refolded and reconstituted with ferrous iron and sulfide (217, 237, 238). The purified dimeric protein (237) has a dark reddish-brown color and displays a band at 420 nm in its light absorption spectrum, characteristic of a sulfide to iron charge transfer. Resonance Raman, electronic absorbance and Mössbauer spectroscopic results were consistent with the presence of an iron-sulfur cluster in LipA. It was suggested that LipA contains [2Fe-2S] clusters that during reduction are converted into [4Fe-4S] clusters (237, 238). The Fe-S cluster of LipA was first suggested to be a [4Fe-4S] cluster bridging the two subunits (237). However, in a different report it was suggested that the limited amount of Fe and S atoms and the presence of [2Fe-2S] clusters in the previous preparation of LipA were a direct consequence of aerobic isolation. It was reported that under strictly anaerobic conditions LipA could bind one [4Fe-4S] cluster per subunit (239). Recently it was reported that LipA contains two distinct [4Fe-4S] clusters per polypeptide (203). Thus, the types of

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disagreements seen in the BioB literature are also apparent for LipA showing the obvious difficulties of working with this family of proteins

Direct involvement of LipA in the sulfur insertion reaction of lipoic acid biosynthesis was difficult to establish due to the lack of an *in vitro* assay. Much of this difficulty was due to the assumption that free octanoic acid was the sulfur acceptor. The first indication that this was not the case was the demonstration by Jordan and Cronan (218) of the LipB transferase activity. Miller and coworkers (240) were the first to report synthesis of lipoic acid *in vitro*. This was based on the discovery of LplA and LipB which led to development of a defined *in vitro* lipoic acid synthesis system and an assay that was much more sensitive and quantitative than prior assays (240). Lipoic acid synthesis was assayed indirectly using (i) the apo form of pyruvate dehydrogenase complex (apo-PDH) as a lipoyl-accepting protein, (ii) purified LipA, and either (iii) purified LplA, ATP, octanoic acid as a substrate (for lipoic acid synthesis) or iv) LipB and octanoyl-ACP as a substrate. Activation of apo-PDH upon lipoylation was monitored spectrophotometrically via reduction of an NAD^+ analogue. Within a finite range, the rate of reduced pyridine dinucleotide formation was directly dependent upon the amount of lipoylated PDH. This assay showed that LipA is responsible for both of the sulfur insertions and that octanoyl-ACP (or a derivative of octanoyl-ACP), but not octanoic acid, was a LipA substrate. Moreover, this work showed that, as suspected, the LipA reaction requires iron-sulfur clusters and SAM to perform the radical chemistry. The principal disadvantage of this assay was its indirect nature and detection of lipoylation of apo-PDH rather than of the primary lipoyl protein species *per se*. All attempts to isolate a free lipoyl-ACP product in the assay were unsuccessful. Thus, the exact identity of the immediate product of the LipA reaction could not be determined by this assay. Recent studies demonstrate that LipA acts on octanoylated derivatives of lipoyl-accepting proteins (201, 241, 242).

Lipoic acid synthesis proceeds by an unexpected and extraordinary pathway

The first evidence that octanoyl-domain rather than octanoyl-ACP was the substrate for sulfur insertion was the finding that *lipB* mutants grew well when supplemented with octanoic acid in place of lipoic acid (201). Octanoate supplementation of *lipB* strains required function of both the *lipA* and *lplA* genes; both *lipB lipA* and *lipB lplA* doubly mutant strains failed to grow on octanoate. Moreover, growth was specific to octanoate, fatty acids of 6, 7, 9 and 10 carbons were inactive (201). These observations argued for the existence of an LplA-dependent pathway that bypassed LipB function in the presence of octanoate. In the postulated bypass pathway (Fig. 10) LplA would attach octanoate derived from the growth medium to the unmodified E2 domains of the PDH and 2-OGDH E2 subunits. LipA would then insert two sulfur atoms into the covalently bound octanoyl moiety and thereby convert the octanoyl-E2 domains to lipoyl-E2 domains *in situ*. That is, lipoic acid would be assembled on its cognate proteins. The resulting active enzymes would account for the observed growth of *lipB* strains on octanoate (Fig. 10). This pathway was tested *in vivo* (201). First, an 87 residue E2 domain derived from *E. coli* PDH was expressed in a host strain that carried null mutations in *lipA* (to prevent lipoic acid synthesis), *lipB* (to block octanoate transfer from fatty acid synthesis) and *fadE* (to block β-oxidative degradation of octanoate). The use of the domain allowed detection of modification by the

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electrophoretic mobility shift assay and by mass spectroscopy. When this strain was cultured in a medium supplemented with octanoic acid about half of the domain became modified. In addition the LipB-dependent modification pathway was assayed in a *lipA lplA* null mutant strain grown in the absence of exogenous octanoate. In agreement with prior work using a *lipA* strain (223) octanoyl-E2 domain accumulation was detected. Therefore, the E2 domain could be octanoylated *in vivo* either by LplA using exogenously added octanoate or by LipB using *de novo* synthesized octanoate. In order to assay conversion of octanoyl-E2 domain to lipoyl-E2 domain the *lipA lipB fadE* strain was supplemented with deuterated octanoic acid to allow accumulation of octanoyl d_{15}-E2 domain that was readily distinguished by mass spectroscopy from domain modified with endogenously-synthesized octanoate. Following accumulation of the d_{15}-labeled octanoyl-E2 domain LipA function was restored by transduction with cells with phage λ particles containing a *lipA* cosmid that had been packaged *in vivo*. Using this approach two types of labeling experiments were done. In the first protocol E2 domains were labeled *in vivo* by growth in the presence of octanoic d_{15} acid. Following removal of the labeled octanoate the cells were then resuspended in growth medium and transduced with the packaged *lipA*-encoding cosmid. Following incubation to allow lipoate synthesis samples were taken and the E2 domain species were isolated, purified, and analyzed by electrospray mass spectroscopy (Fig. 11). In the cultures to which LipA activity was restored a readily detectable conversion of the E2 domain modified with octanoate d_{15} to a species of 60 additional mass units was seen. This was exactly the increase in mass (gain of two sulfur atoms of mass 32 and loss of two deuterium atoms of mass two) expected for conversion of the d_{15} labeled octanoyl-E2 domain to the d_{13}-labeled lipoyl-domain. In the second protocol (a variation of the first protocol) the octanoic d_{15} acid was removed by washing the cells and replaced with normal (non-deuterated) octanoate. This experiment gave essentially the same result; the d_{15} labeled octanoyl-E2 domain was converted to d_{13} labeled lipoyl-E2 domain (Fig. 11). A modification of these experiments also showed that octanoyl-PDH accumulated *in vivo* in a *lipA* strain was converted to its active form upon restoration of LipA activity (201). The conversion of octanoyl-domain to lipoyl domain was also observed *in vitro* (201), although the extant of conversion was much less than stoichiometric with LipA. These result were recently confirmed using octanoyl-H protein as the substrate with an eight-fold increase in the yield of lipoic acid formed/LipA monomer (202).

As mentioned above lipoic acid synthase is a member of the radical-SAM enzyme superfamily which utilize a reduced iron-sulfur cluster and SAM to generate 5'-deoxyadenosyl 5′-radicals (5′-dA) for further radical-based chemistry (59, 243–246). In the lipoic acid synthase reaction (Fig. 12), it is generally believed that the role of the 5′-dA is to remove one hydrogen atom from each of the C-6 and C-8 positions of octanoic acid thereby allowing for subsequent sulfur insertion (202, 240). Consistent with this prediction two molecules of SAM are required to synthesize one mole of lipoyl cofactor (202). This stoichiometry is similar to that obtained in the two studies in the BioB reaction (247, 248) and suggests that the abortive cleavage of SAM observed in these systems might result from some innate reactivity associated with this subclass of radical SAM enzymes (202).

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As in the case of biotin the source of the sulfur atoms of lipoic acid is thought to be a [Fe-S] cluster distinct from the SAM radical [4Fe-4S] cluster. In the first successful in vitro lipoic acid synthesis assays, lipoic acid was formed in the absence of exogenous sulfur-containing compounds in the *in vitro* assay (202, 240). This suggested that, like biotin synthase, the protein itself has some mobilizable sulfur atoms, either from an Fe-S cluster, a persulfide or some other species. The currently favored sulfur source is an iron-sulfur cluster (240, 249). Recent work from the Booker group reported that lipoic acid synthase contains two [4Fe-4S] clusters (203). One cluster is coordinated by the radical SAM CXXXCXXC motif that functions in 5′-dA generation. The second cluster is coordinated by the CXXXXCXXXXXC motif, which, thus far, is unique to lipoic acid synthases and suggested to be the source of the sulfur atoms. This has been addressed (as in BioB (63)) by preparation of LipA from *E. coli* cells grown on an isotopically labeled sulfur source (^{34}S) (250). As expected the lipoic acid formed using this enzyme *in vitro* was isotopically labeled with ^{34}S. Moreover, when the reactions were performed with equimolar amounts of ^{32}S-labeled LipA and ^{34}S-labeled LipA the lipoic acid molecules formed contained either two ^{32}S atoms or two ^{3}S-atoms (250). Thus both sulfur atoms emanate from the same polypeptide thereby eliminating the possibility that the monothiolated species are released and the second sulfur atom is inserted following rebinding to LipA. If release and rebinding occurred, half of the lipoic acid formed would have one atom each of ^{32}S and ^{34}S. Such mechanistic experiments are facilitated if an octanoylated peptide substrate can be substituted for the octanoylated lipoyl domain as is the case for an octanoyl-tripeptide in the *Sulfolobus solfataricus* LipA assay (242). This system has recently been used to show that *S. solfataricus* LipA catalyzed lipoate biosynthesis in a stepwise manner with sulfur first being inserted at C-6 of the octanoyl chain (251). However, this intermediate remained tightly bound to LipA. This is consistent with the finding that both sulfur atoms are derived from the same LipA polypeptide and implies that the sulfur atom of the intermediate may still exist as part of the Fe/S cluster. Incorporation of the second sulfur is much slower perhaps due to rearrangement of the Fe/S cluster. A current model of the LipA reaction is given in Fig. 12.

It should be noted that the finding that lipoic acid synthesis proceeds through an octanoyl-domain intermediate explains a previously puzzling observation first made by Ail and Guest (182, 223) and subsequently by others (252). These workers found that upon overproduction of a lipoyl domain in *E. coli* three species of domain were obtained, the expected apo and lipoylated domains plus a third species subsequently shown to be octanoylated domain. Based on conventional biochemistry in which LipA would produce free lipoic acid, the octanoylated domain was thought to be an anomalous product resulting from the lack of sufficient free lipoic acid to modify the overexpressed domain plus a lack of specificity of the attachment enzyme (182, 223). From the present pathway it now seems clear that LipA was limiting (as was proposed), but the octanoylated domain was an accumulated intermediate rather than the aberrant byproduct of overproduction.

Finally as expected from the BioB data LipA is inhibited by the 5′-deoxyadenosine producd in the reaction. The first indication was that addition of lipoic acid and biotin to a *mtm* (*pfs*) strain gave better growth than biotin alone (21). 5′-Deoxyadenosine inhibition of

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LipA and BioB that is potentiated by methionine and reversed by addition of Mtn has recently been demonstrated *in vitro* (76).

LipA and LipB form stable complexes with the 2-oxoacid dehydrogenases of *E. coli.*

The experiment discussed above in which the LipB-acyl enzyme intermediate was isolated from growing cells showed an unexpected result. The intact pyruvate and 2-oxoglutarate dehydrogenase complexes specifically copurified with affinity tagged LipB (226). Similar experiment showed that LipA, but not LplA, also interacted with the 2-oxoacid dehydrogenases. Proteomic, genetic, and dehydrogenase activity data indicate that all of the 2-oxoacid dehydrogenase components are present. The interaction is specific to the dehydrogenases in that GcvH did not copurify with either LipA or LipB. Studies of LipB interaction with engineered variants of the E2 subunit of 2-oxoglutarate dehydrogenase indicate that the binding sites for LipB reside both in the lipoyl domain and catalytic core sequences (226). These results indicate that lipoic acid is not only assembled on the dehydrogenase lipoyl domains but that the enzymes that catalyze the assembly are also present "on site." The puzzling finding that LipA and LipB do not interact with GcvH is interesting in view of bioinformatic indications that GcvH falls into a different clade than do the dehydrogenase lipoyl domains despite their similar structures (253).

Remaining questions in lipoic acid synthesis

As in the case of BioB, LipA has not been shown to be catalytic *in vitro*, the best preparations to date form only about 0.4 molecules of lipoic acid per LipA molecule and thus the sacrificial protein scenario of BioB might pertain to this protein. The question of whether or not LipA is catalytic *in vivo* remains to be tested. If results analogous to those of BioB are obtained, the question of how the novel LipA CXXXXCXXXXXC motif becomes liganded by a [4Fe-4S] cluster. In this regard coexpression of LipA with the Isc proteins results in increased LipA activity (254). However, it remains to be seen which clusters are built by this manipulation. The crystal structure of LipA with SAM and octanoylated domain would be of great benefit. This would be facilitated if an octanoylated peptide substrate can be substituted for the octanoylated lipoyl domain as is the case for *Sulfolobus solfataricus* LipA (242). It has been predicted that LipA is a $\alpha_6\beta_6$ barrel protein (a three-quarters barrel) rather than a full TIM ($\alpha_6\beta_6$) barrel like BioB (245). Moreover, LipA is reported to contain distinct two [4Fe-4S] clusters whereas BioB is believed to contain one [4Fe-4S] cluster and one [2Fe-2S] cluster. This difference in the secondary FeS clusters is presumably due to the need for two sulfur atoms to make lipoate versus one to make biotin. Therefore, although many of the questions that have bedeviled the BioB literature are germane to LipA, it seems clear that there are significant differences in how the two enzymes accomplish their reactions. A LipA crystal structure would be most useful in understanding these differences. Another major question concerns the regulation of lipoic acid synthesis. Although the *lipB* and *lipA* genes lie close to one another on the *E. coli* chromosome and are transcribed in the same direction, the genes are separated by 1.4 kbp and this spacer region contains *ybeF*, an open reading frame that encodes a possible LysR-type transcription factor. Strains carrying transposon insertions into and deletions of *ybeF* have no phenotype indicating that *lipB* and *lipA* are not in an operon (6, 120). Is expression

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of these genes regulated? Lipoic acid is clearly synthesized during aerobic growth and anaerobic function of the glycine cleavage enzyme indicates that it is also made under fermentative conditions (6). Moreover, a recent report that *E. coli* contains high levels of 2-oxoglutarate dehydrogenase when grown anaerobically with an electron acceptor such as nitrate (153) indicates that lipoic acid synthesis must also proceed under these growth conditions. The transcription of *lipA* in *S. enterica* has been assayed by transcriptional fusions to β-galactosidase and was found to be unaffected by catabolite repression conditions or by addition of lipoic acid (255). Therefore, the lipoic acid synthesis pathway may be constitutively expressed. Given the unusually sophisticated *bio* operon transcriptional regulatory system, it might seem unlikely that lipoic acid synthesis is unregulated. Indeed, biotin and lipoic acid are synthesized at similar levels in *E. coli.* However, biotin synthesis requires six enzymes and several of the reactions of the pathway require input of metabolically expensive molecules which might justify regulation of biotin synthesis enzyme production. In contrast octanoate, the precursor of the lipoic acid carbon chain, is derived from fatty acid biosynthesis in an already activated form, octanoyl-ACP, and lipoate synthesis consumes only a tiny fraction of the total cellular fatty acid synthetic capacity. Since LipB uses a preformed activated intermediate, the only further energetic input (in the form of SAM) occurs in the LipA sulfur insertion reaction. Therefore, relative to biotin synthesis, lipoic acid synthesis has low metabolic price. Another consideration is that the lipoic acid synthesis pathway is limited by the amount of apo-lipoyl domain available and thus unlike biotin synthesis lipoate synthesis is “hard wired” and cannot “run wild” to overproduce and excrete the cofactor as is the case when the *bio* operon is deregulated (117). Thus, it can be reasonably argued that regulation of the lipoic acid synthetic pathway might well be more expensive than the alternative of simply allowing constitutive expression of the genes. A perplexing observation is the report that LipB acts as a negative regulator of deoxyadenosine methyltransferase (*dam*) gene expression in *E. coli* (256). These workers speculate that LipB may inactivate a repressor protein by lipoylation. However, all of the proteins that become labeled with exogenous radioactively-labeled lipoate or octanoate *in vivo* are known subunits of the enzymes discussed above (6, 199). Hence, the putative lipoylated repressor would have to be modified by LipB, but not by LplA. Further studies of this interesting phenomenon are needed.

How do the protein biotinylation and lipoylation reactions remain discrete?

It is gratifying that the recent crystal structures have resulted in LplA, LipB and BirA being recognized as a new protein family (PFAM 03099.13) as predicted by Reche (210) although these proteins share only a single conserved residue. Indeed the Cα carbons of *E. coli* LplA and BirA (minus the DNA binding domain) structures can be aligned with a root mean square deviation of 2.8Å over much of their lengths (104) whereas *E. coli* LplA and *M. tuberculosis* LipB can be aligned over the length of LipB (the smaller protein) with a Cα value of 2.5Å (227). Moreover, the cofactor ligands in these crystal structures are in register indicating similar geometries of binding. These findings together with the even more similar structures of the domains that are the substrates of these enzymes raises the question of how the cell avoids the catastrophic effects on metabolism that would result if biotinylated proteins became lipoylated and *vice versa*. This question of accurate modification has been addressed by Reche and Perham (126, 257) who showed that the wild type biotinoyl domain

can be lipoylated by LplA *in vitro* but the reactions proceeds only with a molar excess of enzyme and very slowly. They also showed that mutations of the residues adjacent to the lysine residue of the biotinoyl domain (the protruding β-turn) to those found in the protruding β-turn of the lipoyl domain allow modification by lipoic acid attachment. Indeed, conversion of a single residue of the biotinoyl domain of *E. coli* allowed some lipoylation by LplA. Moreover, some of the hybrid domains remained substrates for biotinylation. However, these workers also found that they could leave the protruding β-turn region intact and obtain lipoylation by removal of the biotinoyl domain thumb structure which is not found in lipoyl domains (126, 257) (Fig. 8). Thus, the thumb structure seems to be the major "gate-keeper" that prevents lipoylation of AccB. This seems an appropriate choice since the thumb structure is required for function of AccB in fatty acid synthesis, but not for biotinylation of the protein (10). The determinants that prevent biotinylation of lipoyl domains have yet to be explored.

Conclusions

In the two prior editions of these volumes the biotin and lipoic acid synthetic pathways have been placed into the same chapter. It seems probable that this was because both are sulfur-containing and covalently-attached enzyme cofactors plus there was insufficient information on lipoic acid synthesis to justify a separate chapter. However, in the 16 years since the second edition of this publication the lumping of these cofactors together has come to seem prescient. We now know that there are many commonalities. Both cofactors are made as offshoots of the fatty acid synthetic pathway, sulfur insertion is done by SAM radical enzymes, the sulfur atoms inserted are derived from an enzyme iron-sulfur center, the cofactors are attached to very similar domains and the enzymes that attach these cofactors constitute a protein family despite the low sequence conservation and differing mechanisms of the enzymes. However, it should be noted that although the *E. coli* pathways seem to explain the synthesis of these two cofactors in most bacteria, there are well-documented exceptions. Indeed the biotin and lipoate pathways of *Bacillus subtilis* differ markedly from those of *E. coli* (253, 258, 259).

Acknowledgments

The preparation of the manuscript and the experimental work from our laboratory were supported by grant AI15650 from the National Institute of Allergy and Infectious Diseases.

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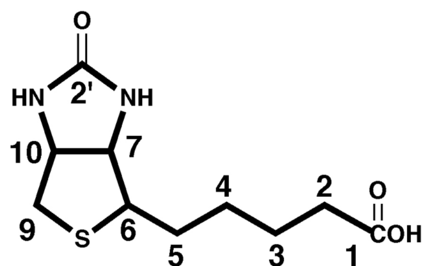
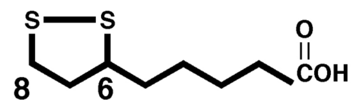
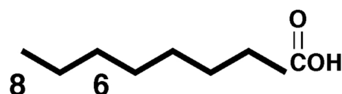
A.



Biotin



Lipoic Acid



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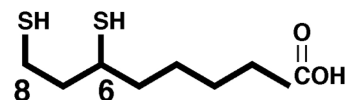
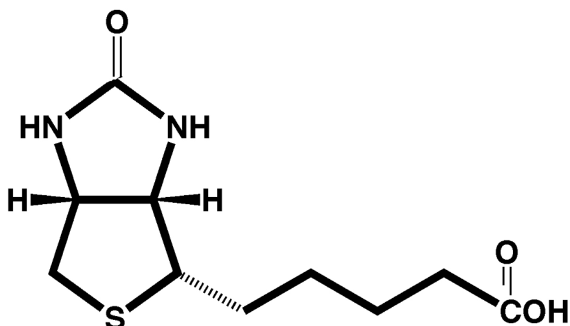


Dihydrolipoic Acid

B.



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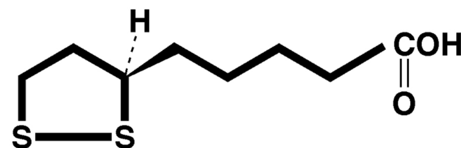

Lipoic Acid

Figure 1.
Structures of biotin, lipoic acid, n-octanoic acid, and the reduced form of lipoic acid, dihydrolipoic acid. (A) All biotin carbon atoms are numbered as are the relevant carbons of the other molecules. (B) Stereochemistry of biotin and lipoic acid showing that both molecules have non-planar structures. The lipoic acid dithiolane ring would emerge from and protrude below the plane of the page whereas biotin has a chair structure (the viewer is looking at the back of the chair). Note that lipoic acid structure is rotated relative to that in panel A to conform with the Cahn-Ingold-Prelog rules and since biotin has three chiral

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centers the hydrogen atoms attached to carbon atoms 7 and 10 can be depicted as either above or below the plane of the page depending on the chiral center chosen as primary (the ring centers were chosen in this depiction). For simplicity the stereochemistry will not be given (except as relevant) in the subsequent figures of this review.

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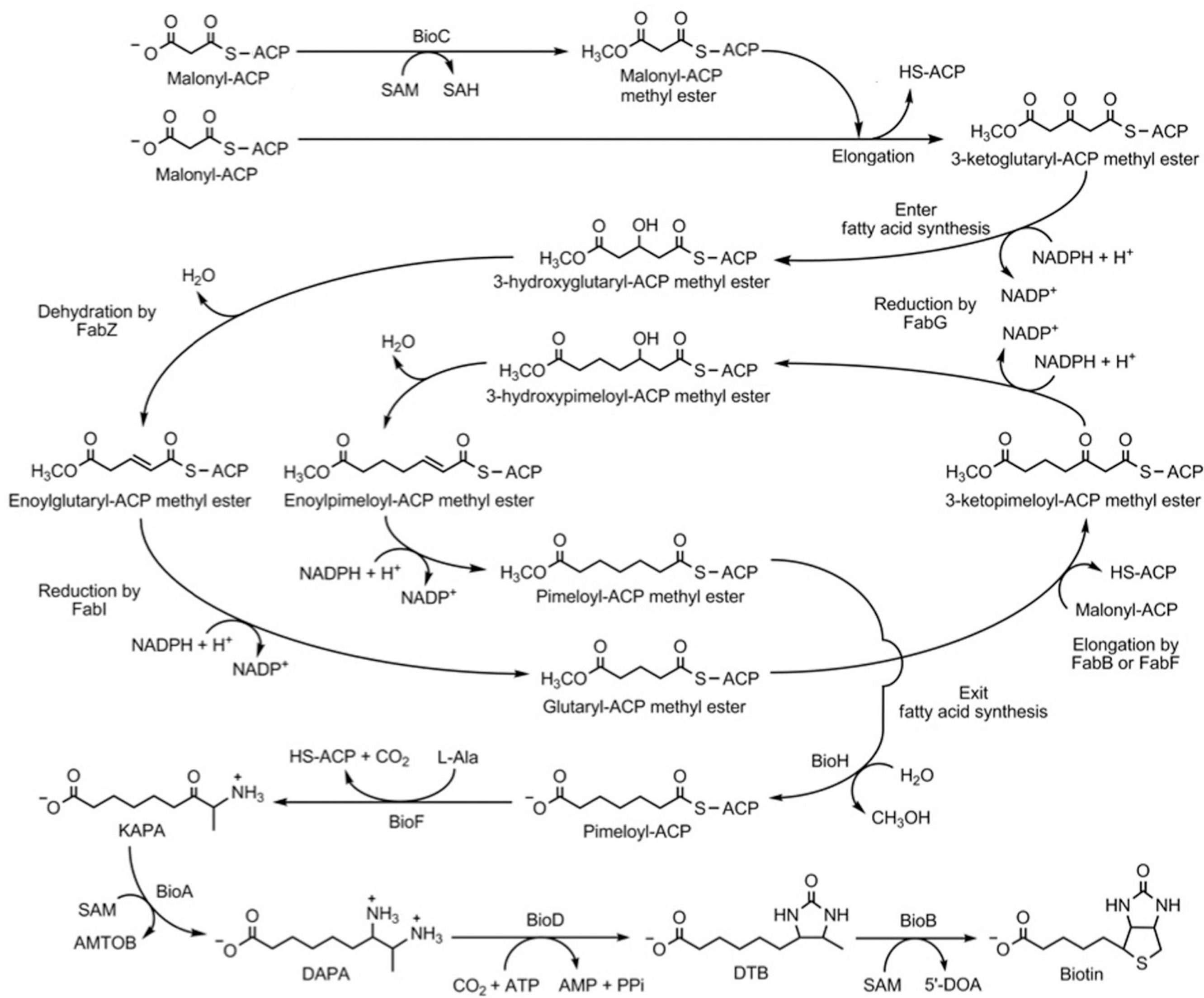

Figure 2.
The current pathway of biotin synthesis in *E. coli.*

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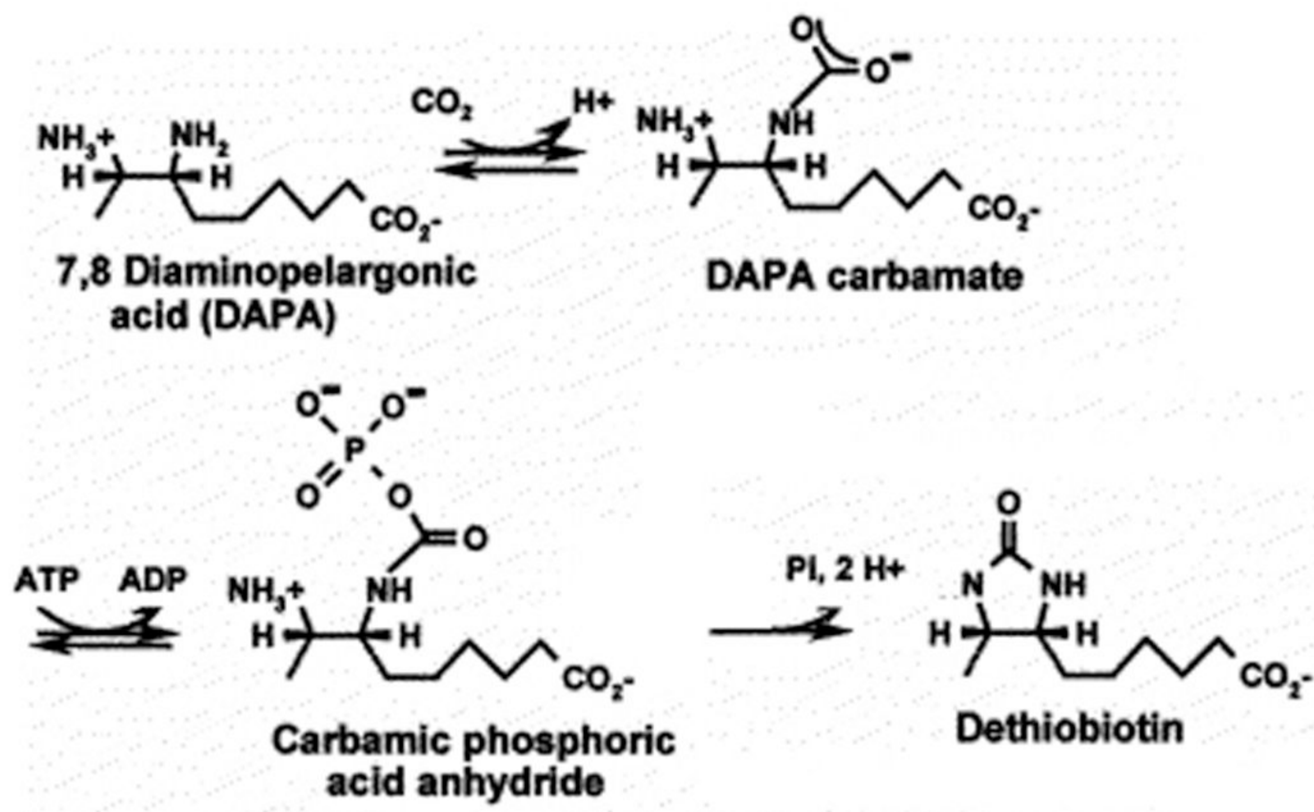

Figure 3.
The BioD reaction.

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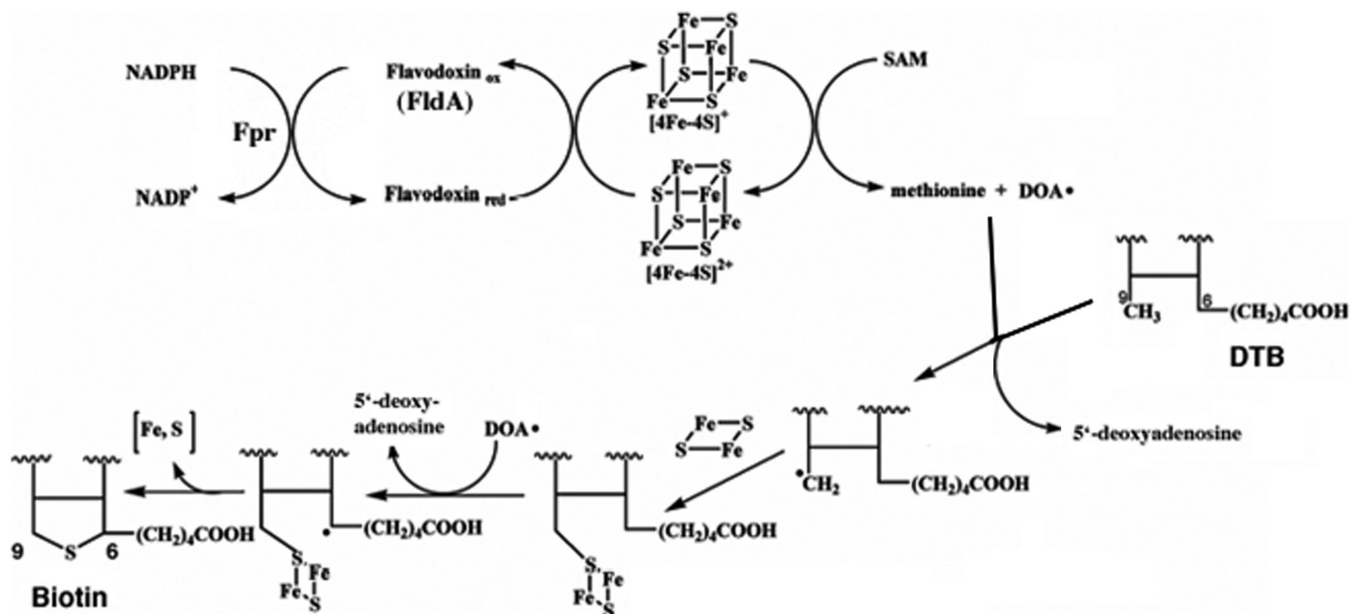

Figure 4.

The current model of the BioB reaction. For simplicity only DTB carbon atoms 6, 7, 9, and 10 (Fig. 1) are shown of which only carbons 6 and 9 are labeled. The reaction is shown as proceeding with the initial attack on C-9 because a derivative of DTB carrying a thiol group on C-9 has been shown to be converted to biotin both in vitro and in vivo (56, 260) and the crystal structure (24) shows C-9 in an appropriate position for the primary sulfur insertion.

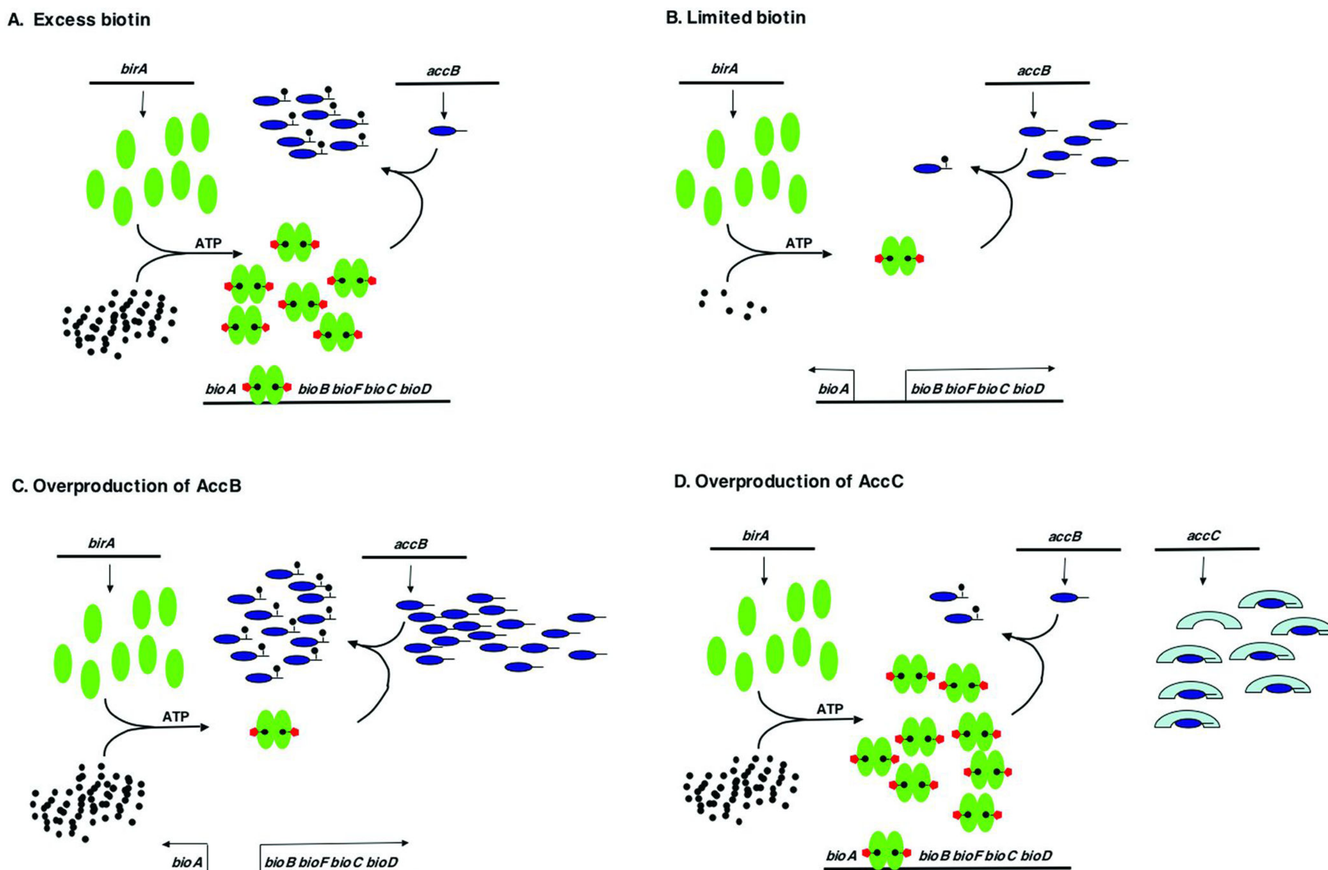

Figure 5.

The biotin regulatory system of *E. coli*. BirA is represented by green ovals, biotin by black circles, the AMP moiety by red pentagons, AccB by dark blue ovals and AccC by light blue crescents. The arrows denote transcription from the leftward and rightward *bio* promoters. (A to C) BirA switches from biotin ligation function to repressor function in response to the intracellular biotin requirement which is monitored by the level of unbiotinylated AccB. If the levels unbiotinylated AccB are high, the protein functions as a biotin ligase. Once the unbiotinylated AccB has been converted to the biotinylated form, the bio-AMP is no longer consumed and remains bound to BirA. This liganded form of BirA accumulates to levels sufficiently high to form dimers that fully occupy the *bio* operator iresulting in transcriptional repression of the biotin biosynthetic genes. (D) Overproduction of AccC ties up unbiotinylated AccB into a complex that is a poor biotinylation substrate. Therefore, high levels of the liganded form of BirA accumulate resulting in repression of *bio* operon transcription.

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Figure 6.

The structure of BirA (A) and the sequence of the *bio* operon operator/promoter region (B). Panel A. The BirA structure is that of the protein liganded with a bio-AMP analogue (which for simplicity was omitted) (235). Panel B. The boxed region is the operator to which a BirA dimer binds. P_A and P_B are the promoters of the *bioA* and *bioBFCD* transcriptional units, respectively. The –10 and –35 promotor regions are denoted by underlines.

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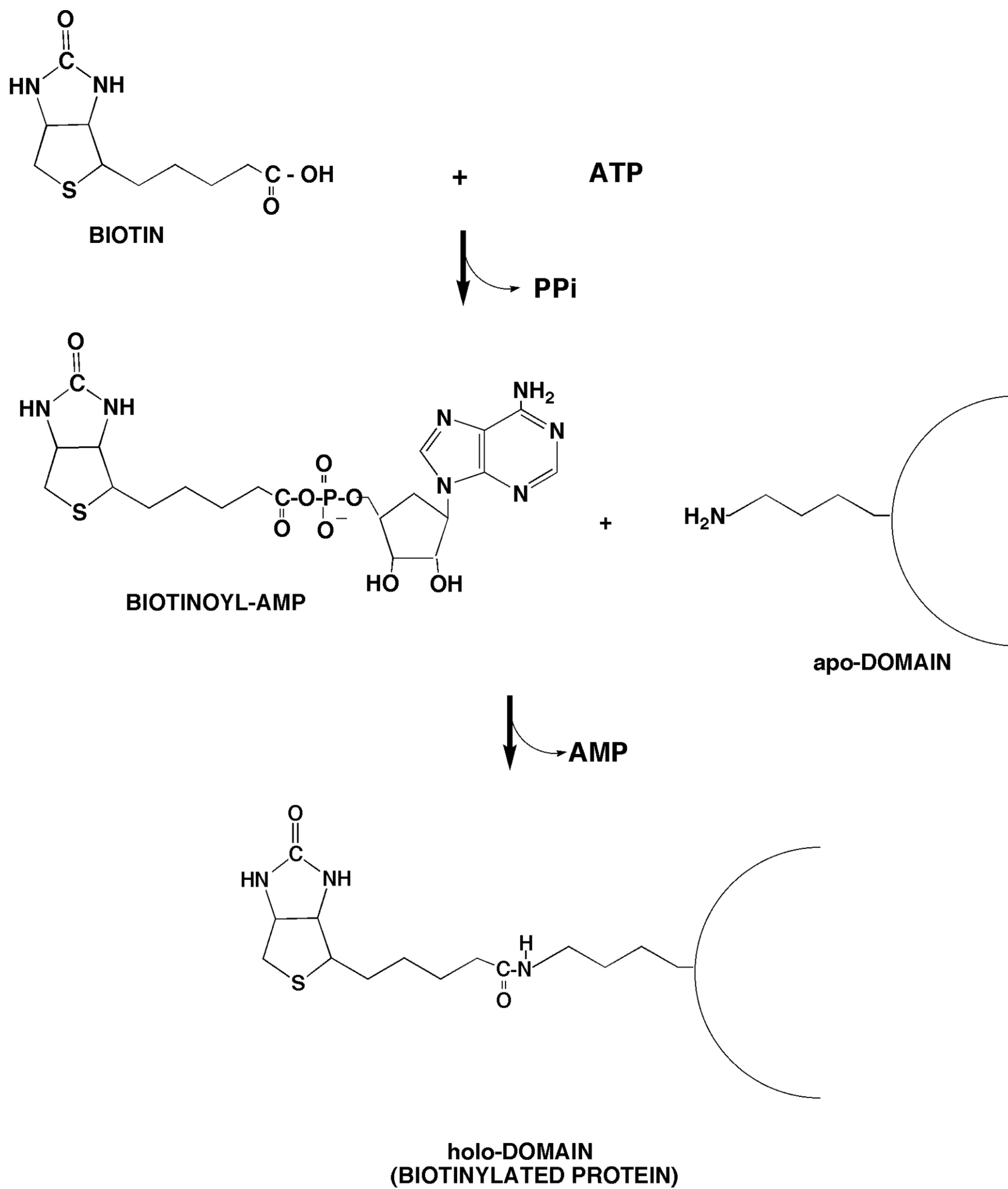

Figure 7.

The BirA reaction is shown. This is the general reaction of biotin protein ligases (38). The lipoic acid ligase LplA has the same reaction mechanism given substitution of lipoic acid for biotin.

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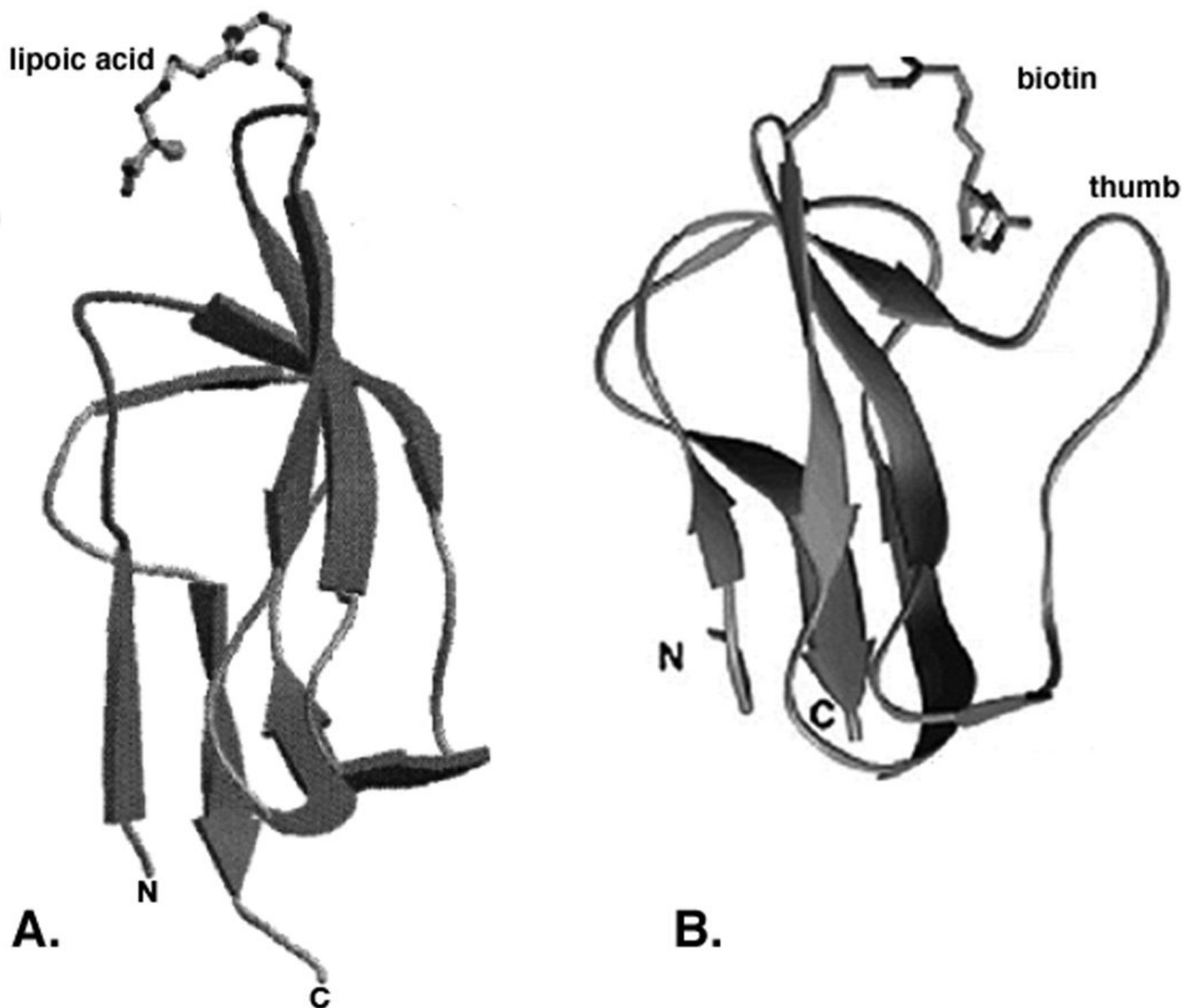

Figure 8.

Three-dimensional structures of *E. coli* lipoyl and biotinyl domains. Panel A. The innermost lipoyl domain of *E. coli* PDH. Panel B. The BCCP biotinyl domain of *E. coli* acetyl-CoA carboxylase. The images are MOLSCRIPT drawings from the NMR data of Jones and coworkers (177) and the diffraction data of Athappilly and Hendrickson (193), respectively.

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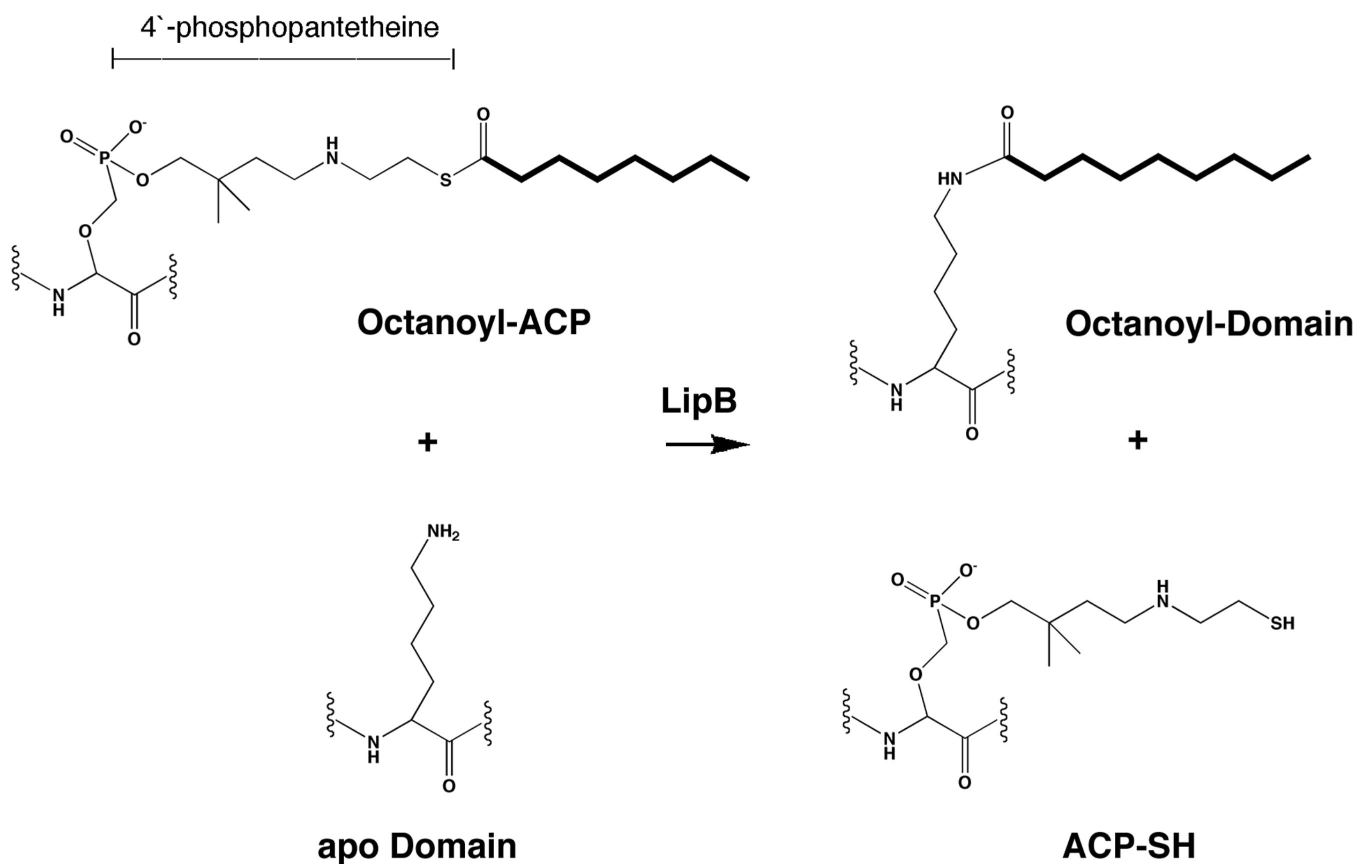

Figure 9.

Overview of the LipB reaction. The thioester linkage of octanoic acid attached to the thiol of the 4′-phosphopantetheine group of ACP (the product of fatty acid synthesis) is attacked by the ε-amino group of the target lysine of a lipoyl domain resulting in the modified protein plus the free thiol form of ACP. The enzyme also uses lipoyl-ACP although this is thought to be of no physiological importance. The reaction proceeds through an octanoyl-LipB acyl enzyme intermediate (not shown).

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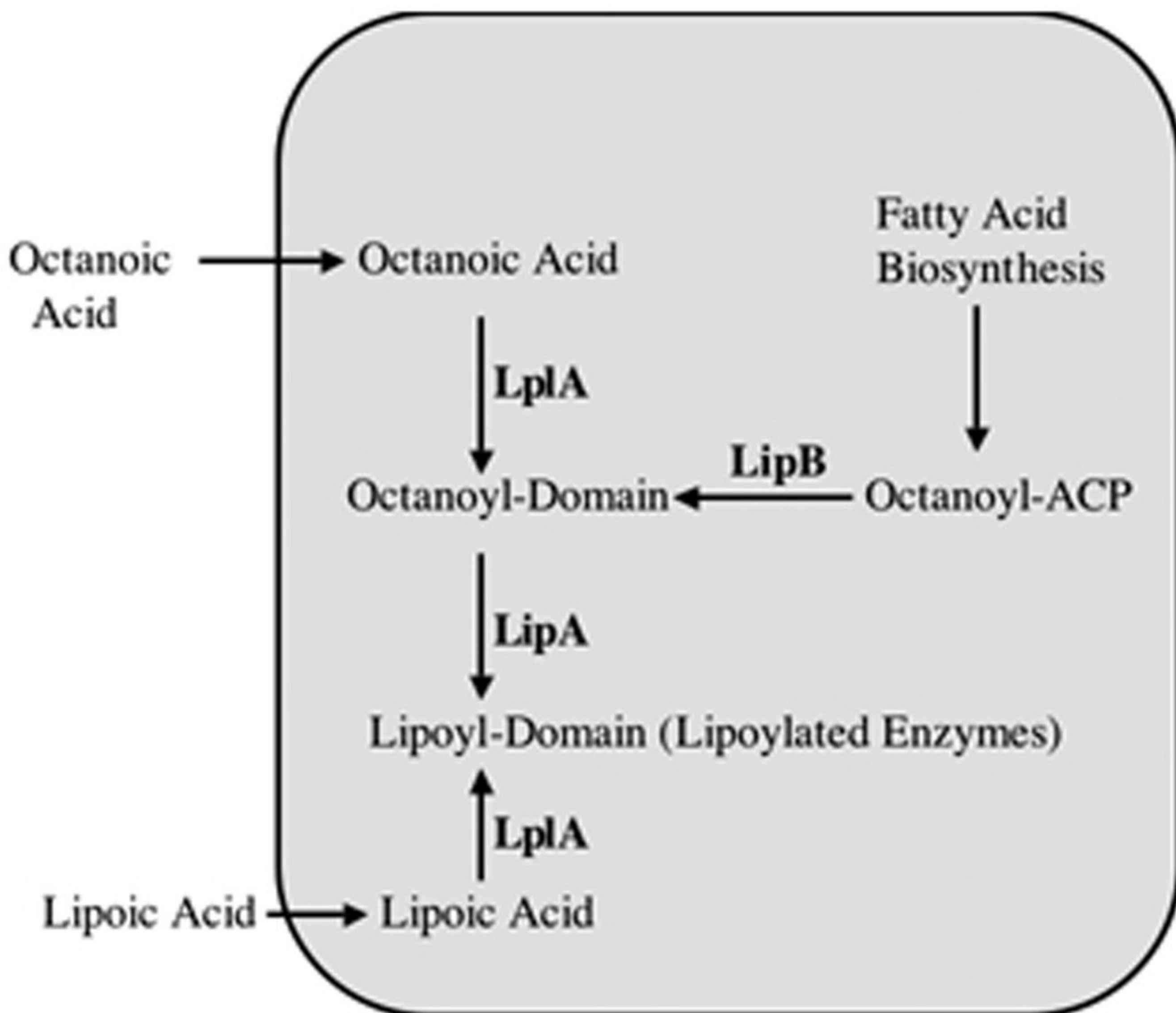

Figure 10.

Current model for lipoic acid synthesis and utilization in *E. coli*. The rounded rectangle denotes an *E. coli* cell. Exogenous lipoic acid or octanoic acid enter by diffusion and are attached to the 2-oxo acid lipoyl domains and to H protein by LplA. The domains modified with exogenously derived octanoate can be converted to lipoyl domains by LipA, although this is probably not a reaction of physiological significance because high levels of octanoic acid are required for significant modification by this route. In contrast LplA-catalyzed attachment of lipoate is very efficient and provides a salvage or scavenging pathway for utilization of exogenous lipoic acid and seems likely to be physiologically significant. The major (and probably sole) route of lipoic acid synthesis is LipB-catalyzed transfer of octanoate from octanoyl-ACP to the 2-oxo acid lipoyl domains and H protein followed by LipA-catalyzed sulfur insertion to give lipoate.

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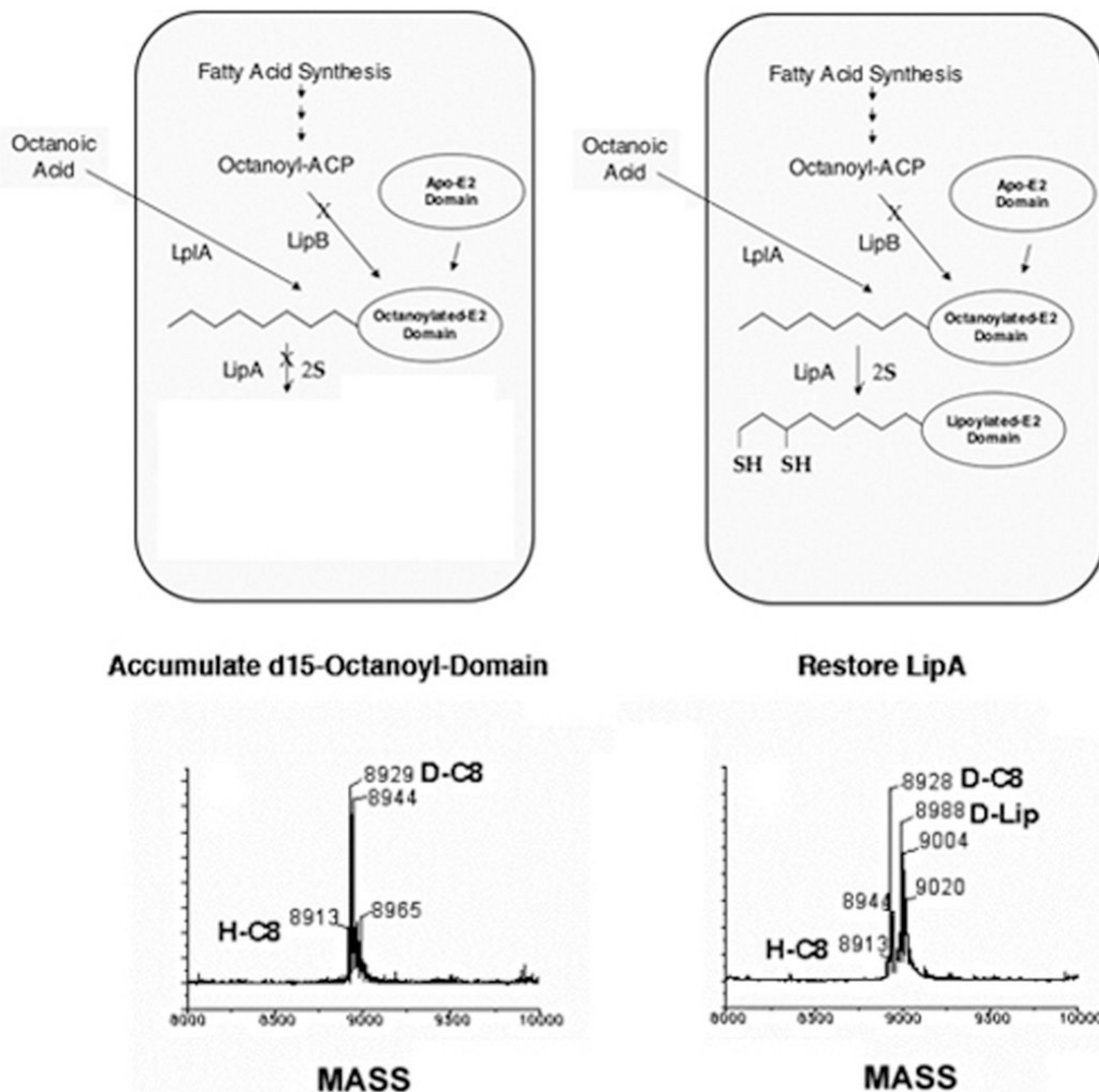

Figure 11.

Lipoate synthesis proceeds by sulfur insertion into octanoyl-domain. The bypass pathway accounting for growth of *lipB* mutants on octanoate is shown in the upper right cartoon. The experimental protocol scheme and mass spectral data for testing the pathway is also shown. In the left cartoon octanoylation of the lipoyl domain by endogenously synthesized octanoyl moieties is blocked by a *lipB* mutation and the cells use LplA and exogenously supplied *d15* octanoate to octanoylate the domain. LipA is also blocked so deuterated lipoylated domain is not made. Following accumulation of the deuterated octanoyl domain LipA function is restored (right cartoon). Following incubation to allow lipoate synthesis, the cells are

harvested and the modified domains were purified then analyzed by electrospray mass spectrometry. Note the accumulation of deuterated lipoylated domain (D-Lip) in the right hand spectrum and that the mass change between deuterated octanoylated domain (D-C8) and D-Lip is 60 mass units indicating loss of two deuterons and gain of two sulfur atoms. For details see (201).

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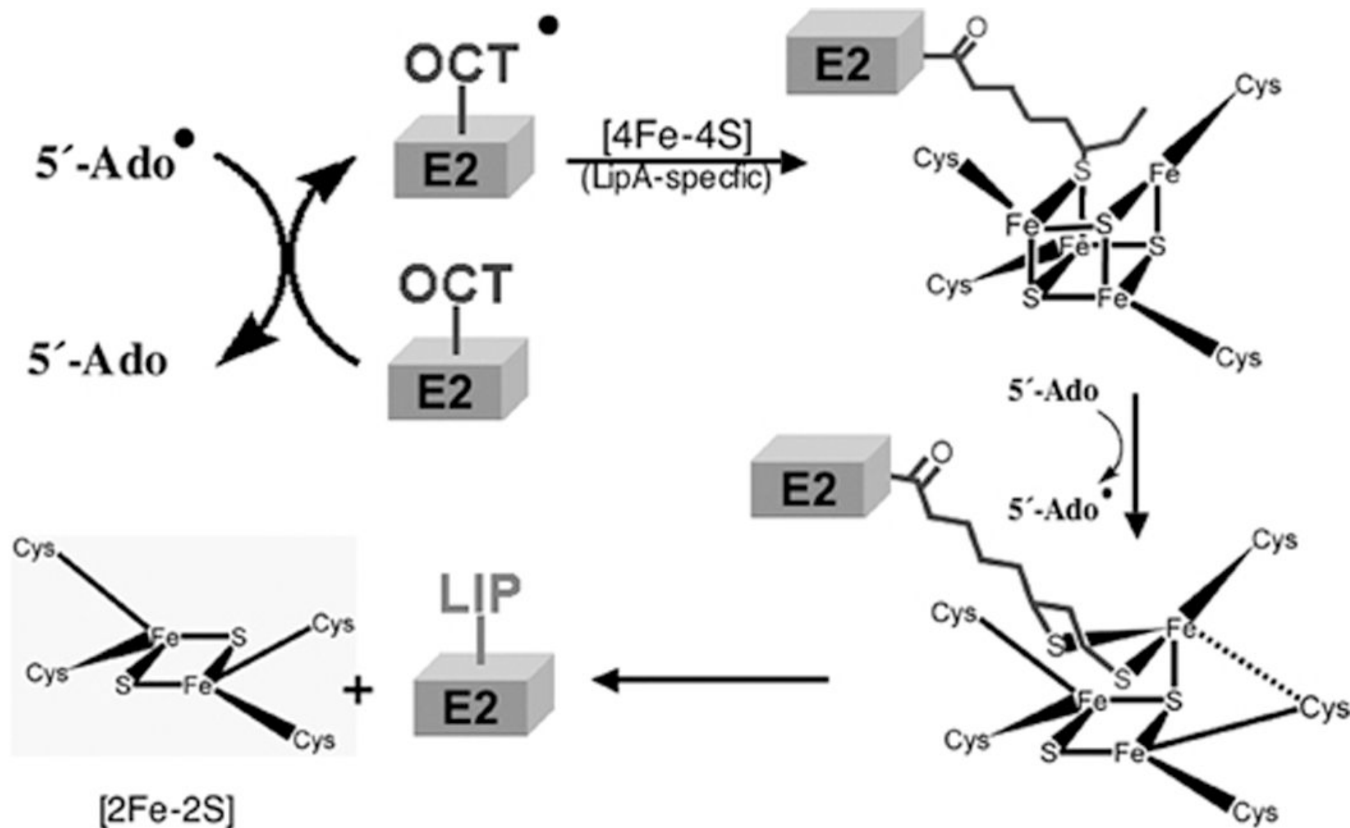

Figure 12.

A current model of the lipoate synthase (LipA) reaction (202, 203, 240). The canonical SAM radical [4Fe-4S] cluster of LipA reduces SAM to generate the deoxyadenosine radical (5′-Ado) as seen previously in the BioB reaction (Fig. 4). The radical then removes a hydrogen atom from the C6 methylene of the octanoate moiety of an octanoyl domain (Oct-E2 on the Figure) (229) to give a carbon radical that then attacks the lipoyl synthase-specific [4Fe-4S] cluster and abstracts a reduced sulfur atom. This process is repeated at the methyl carbon (C8) to give lipoyl-domain (Lip-E2, probably as the dihydrolipoyl form due to the strongly reducing conditions under which the reaction proceeds).