Crystal Structure of Lipoate-Protein Ligase A Bound with the Activated Intermediate

**INSIGHTS INTO INTERACTION WITH LIPOYL DOMAINS**

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Do Jin Kim, Kyoung Hoon Kim, Hyung Ho Lee, Sang Jae Lee, Jun Yong Ha, Hye Jin Yoon, and Se Won Suh

From the Department of Chemistry, College of Natural Sciences, Seoul National University, Seoul 151-742, Korea

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Lipoic acid is the covalently attached cofactor of several multi-component enzyme complexes that catalyze key metabolic reactions. Attachment of lipoic acid to the lipoyl-dependent enzymes is catalyzed by lipoate-protein ligases (LPLs). In *Escherichia coli*, two distinct enzymes lipoate-protein ligase A (LplA) and *lipB*-encoded lipoamidoreductase (LipB) catalyze independent pathways for lipoylation of the target proteins. The reaction catalyzed by LplA occurs in two steps. First, LplA activates exogenously supplied lipoic acid at the expense of ATP to lipoyl-AMP. Next, it transfers the enzyme-bound lipoyl-AMP to the e-amino group of a specific lysine residue of the lipoyl domain to give an amide linkage. To gain insight into the mechanism of action by LplA, we have determined the crystal structure of *Thermoplasma acidophilum* LplA in three forms: (i) the apo form; (ii) the ATP complex; and (iii) the lipoyl-AMP complex. The overall fold of LplA bears some resemblance to that of the biotinyl ligase module of the *E. coli* biotin holoenzyme synthetase/bio repressor (BirA). Lipoamidyl-AMP is bound deeply in the bifurcated pocket of LplA and adopts a U-shaped conformation. Only the phosphate group and part of the ribose sugar of lipoyl-AMP are accessible from the bulk solvent through a tunnel-like passage, whereas the rest of the activated intermediate is completely buried inside the active site pocket. This first view of the activated intermediate bound to LplA allowed us to propose a model of the complexes between Ta LplA and lipoyl domains, thus shedding light on the target protein/lysine residue specificity of LplA.

Lipoic acid (6,8-thioxic acid or 1,2-dithiolane-3-pentanonic acid), a covalently bound cofactor, is essential for function of several key enzymes involved in oxidative metabolism in most prokaryotic and eukaryotic organisms (1). The lipoatedyl proteins include pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase, branched-chain 2-oxoacid dehydrogenase, and the glycine cleavage system (2). In the reaction catalyzed by the lipoamidylating enzymes, i.e., lipoate-protein ligases (LPLs),3 the free carboxyl group of lipoic acid is attached via an amide linkage to the e-amino group of a specific lysine residue of the lipoate-accepting protein domains (termed the lipoyl domains) of these multi-enzyme complexes (3). The lipoidamyl arm protruding from a tight β-turn of the structure of the lipoyl domains shuttles reaction intermediates among different active sites of the multi-enzyme complexes (2).

Although the general role of lipoic acid as the covalently attached coenzyme has been known for decades, the mechanisms by which lipoic acid is synthesized and becomes linked to its cognate proteins continue to be elucidated. In *Escherichia coli* two independent LPL enzymes modify lipoyl domains (4). The best characterized lipoylating enzyme is *E. coli* lipoate-protein ligase A (LplA). LplA utilizes exogenously supplied free lipoic acid to modify the specific lysine of the lipoyl domain. In the first step, LplA catalyzes synthesis of the activated intermediate lipoyl-5'-adenylate (lipoyl-AMP) from lipoic acid and ATP. In the next step, it catalyzes the attack of enzyme-bound lipoyl-AMP by the e-amino group of the lysine of the lipoyl domain to give an amide linkage. The lipoylation reaction catalyzed by LplA is analogous to that of the biotinylation reaction mediated by biotinyl protein ligase (BPL) (2, 3). The second lipoylation pathway is dependent on the lipB and the lipA gene products (5). LplA, octanoyl-[acyl carrier protein]-protein N-octanoyltransferase, uses the octanoyl thioester of the acyl carrier protein of fatty acid synthesis as the acyl donor *in vivo*. After LplB transfers the octanoyl group to its target protein, the sulfur insertion into the octanoyl moiety is carried out by LplA, the sulfur insertion protein. It was shown that the transfer of lipoic acid and octanoic acid from their acyl carrier protein thioesters to lipoyl domains can also be catalyzed, albeit very poorly, by LplA (6). Despite poor sequence conservation, an evolutionary relationship among LplA, LplB, and BPL has been detected (2).

LplA and LplB counterparts of the *E. coli* enzymes are present in many organisms, but little is known about their three-dimensional structures. Lipoyltransferases showing a 31–35% sequence identity with *E. coli* LplA have been purified from human and bovine mitochondria (7, 8). In this study, we have determined the crystal structure of *Thermoplasma acidophilum* (Ta) LplA, a 262-residue protein, by the multi-wavelength anomalous diffraction (MAD) method of x-ray crystallography to provide the missing structural information. The amino acid sequence of Ta LplA shares 28%, 27%, and 25% identity with those of *E. coli* LplA, and the human and bovine mitochondrial lipoyltransferases, respectively. We report here the structure of Ta LplA in three different forms: (i) the apo form; (ii) the ATP complex; and (iii) the lipoyl-AMP complex. This study provides essential structural data on the ligand binding site of LplA and useful insights into binding mode of the activated intermediate. It also allowed us to propose a model of the complexes between LplA and lipoyl domains.

**EXPERIMENTAL PROCEDURES**

Protein Expression and Purification—The *lplA* gene from *T. acidophilum* (TA0514) encoding LplA was cloned into the expression vector pET-28b(+) (Novagen). The recombinant protein fused with a
hexastidine-containing tag at its amino terminus was overexpressed in *E. coli* Rosetta(DE3) cells using Terrific broth culture medium. Protein expression was induced by 0.5 mM isopropyl 1-thio-β-D-galactopyranoside, and the cells were incubated for an additional 6 h at 37 °C following growth to mid-log phase at 37 °C. The cells were lysed by sonication in lysis buffer (50 mM Tris-HCl at pH 7.9, 500 mM NaCl, and 10% (v/v) glycerol) containing 50 mM imidazole. The crude lysate was centrifuged at ~36,000 × g for 60 min. The supernatant was applied to an affinity chromatography column of nickel-nitrilotriacetic acid-agarose (Qiagen). The protein was eluted with lysis buffer containing 500 mM imidazole, and the eluted sample was diluted 5-fold with buffer A (50 mM Tris-HCl at pH 7.2, 5% (v/v) glycerol, and 10 mM β-mercaptoethanol). The diluted sample was applied to a Source 15Q ion-exchange column (Amersham Biosciences), which was previously equilibrated with buffer A. The protein was eluted with a linear gradient of 0–1.0 M NaCl in buffer A. The next step was gel filtration on a HiLoad 16/60 Superdex-200 prep-grade column (Amersham Biosciences), employing an elution buffer of 20 mM Tris-HCl at pH 7.2 and 100 mM NaCl. For overexpressing the selenomethionine (SeMet)-substituted protein in *E. coli* Rosetta(DE3) cells, we used the M9 cell culture medium that contained extra amino acids, including SeMet. When the cell culture reached an *A*<sub>600</sub> of 0.6, SeMet was added at 50 mg/liter, and at the same time the synthesis of Met was repressed by the addition of Phe, Thr, and Lys at 100 mg/liter, and Leu, Ile, Val, and Pro at 50 mg/liter (9). After 15 min, expression of the SeMet-substituted protein was induced with 0.4 mM isopropyl 1-thio-β-D-galactopyranoside, and the culture was grown for an additional 20 h at 20 °C. The procedure for purifying the SeMet-substituted protein was the same except for the presence of 10 mM dithiothreitol in all buffers used during purification steps besides buffer A.

**Crystallization**—Crystals were grown by the hanging-drop vapor diffusion method at 24 °C by mixing equal volumes (2 μl each) of the protein solution (at 18 mg ml<sup>−1</sup> concentration in 20 mM Tris-HCl at pH 7.2 and 100 mM NaCl) and the reservoir solution. To grow crystal of the native protein in the apo form, we used a reservoir solution consisting of 100 mM sodium citrate at pH 5.5, 20% (v/v) iso-propanol, 20% (w/v) polyethylene glycol 3350, and 100 mM NaCl. Rod-shaped crystals grew to approximate dimensions of 0.1 × 0.1 × 0.3 mm within a few days. When we added ATP, lipoic acid, or octanoic acid to the protein solution, the enzyme aggregated rapidly and co-crystallization was not possible. Therefore, we used the soaking method to obtain crystals of the native protein bound with the ligands. To obtain crystals of the ATP complex, we soaked the apo crystals of the native protein in the reservoir solution containing ATP in 50-fold molar excess for 1 day. To obtain crystals of the lipoyl-AMP complex, we transferred the ATP-soaked crystals into the reservoir solution containing lipoic acid in 50-fold molar excess and soaked them for 1 day. Apparently the reaction took place in the crystal, as evidenced by the clear electron density for the activated intermediate, lipoyl-AMP (Fig. 1A, left). When we transferred the ATP-soaked crystals into the biotin-containing reservoir solution, the reaction did not occur in the crystal, as evidenced by the lack of electron density for biotinyl-AMP. The difference Fourier map showed only the electron density of ATP. When we soaked the native crystals in the reservoir solution containing lipoic acid or octanoic acid alone in 50-fold molar excess for 1 day, the crystal quality deteriorated and we could not collect x-ray diffraction data to sufficiently high resolution. The SeMet-substituted protein in the apo form was crystallized under conditions identical to those for the apo crystals of the native protein except for the presence of 10 mM dithiothreitol in the protein solution.
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The electron density of the bound ATP was a little weaker than that of lipoyl-AMP (Fig. 1A, right), and we arbitrarily fixed the occupancy of ATP at 0.5. We suspect that the binding affinity of Ta LplA for ATP is weaker than that for lipoyl-AMP, and incomplete occupation of the ATP binding site resulted from a partial loss of the bound ATP during quick soaking of the crystals in the cryoprotectant solution.

Ta LplA exists as a monomer in solution according to our dynamic light scattering analysis. The LplA monomer is oblate-shaped, with approximate dimensions of 40 Å × 40 Å × 32 Å (Fig. 1B). The central core of Ta LplA comprises two β-sheets: a larger eight-stranded β-sheet and a smaller three-stranded β-sheet. The larger sheet consists of the strands β1 (residues 2–6), β2 (35–39), β6 (85–94), β7 (122–123), β8 (139–141), β9 (144–154), β10 (156–165), and β11 (225–229) with topology ↑ β11 ↑ β1 ↑ β2 ↑ β6 ↑ β10 ↓ β9 ↓ β8 ↓ β7. The smaller sheet consists of the strands β3 (residues 44–47), β4 (68–71), and β5 (79–81) with topology ↓ β4 ↓ β3 ↓ β5 (Fig. 1C). The two sheets are nearly orthogonal, diverging from each other at the lipoyl-AMP binding site (Fig. 1B). Eight α-helices (α1 (residues 14–27), α2 (53–56), α3 (59–64), α4 (99–116), α5 (170–176), α6 (207–222), α7 (233–245), and α8 (250–254)) surround the central β-sheets. The residues 192–195 form a 3_{10} helix in the apo and the ATP complex structures, but not in the lipoyl-AMP complex structure.

### Table One

| Data collection, phasing, and refinement statistics | Data collection and phasing |
|---------------------------------------------------|-----------------------------|
| Unit cell parameters: a = 109.35 Å, b = 62.95 Å, c = 46.86 Å, α = γ = 90°, β = 111.57° (Space group: C2) | **X-ray source:** PF BL-6A* |
| **Data set** | **SeMet A1 (peak)** | **SeMet A2 (edge)** | **SeMet A3 (remote)** |
| **Resolution range (Å)** | 50–2.04 | 50–2.04 | 50–1.98 |
| **Completeness (%)** | 99.8 (97.8)* | 100 (99.8)* | 100 (100)* |
| **R_{sym} (%)** | 7.5 (29.4)* | 6.0 (30.2)* | 6.7 (40.4)* |
| **R_{int} (%)** | 6.8 (11.5)* | 8.0 (16.4)* |
| **F^2 / F^2 (e)** | -7.19/5.77 | -9.46/3.08 | -3.03/4.03 |
| **Figure of merit** for MAD phasing (30–2.04 Å): 0.50/0.72 (before/after density modification) | |

*PF, Photon Factory; PLS, Pohang Light Source; BL-6A, beamline 6A.

Numbers in parentheses are for the last shells (2.11–2.04 Å or 2.05–1.98 Å).

د (1–180 and 191–257). The electron density of the bound ATP was a little weaker than that of lipoyl-AMP (Fig. 1A, right), and we arbitrarily fixed the occupancy of ATP at 0.5. We suspect that the binding affinity of Ta LplA for ATP is weaker than that for lipoyl-AMP, and incomplete occupation of the ATP binding site resulted from a partial loss of the bound ATP during quick soaking of the crystals in the cryoprotectant solution.

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### Structural Comparison—DALI structural similarity searches (17)

With the apo structure of Ta LplA revealed two close relatives. The highest Z-score is obtained with a putative LplA from Streptococcus pneumoniae, a 329-residue protein (unpublished deposition; PDB code 1VQZ). A root mean square (r.m.s.) deviation of 2.5 Å for 218 equivalent Cα positions, a Z-score of 25.6, and a sequence identity of 29%. This structure was solved without any bound ligand in the active site. S. pneumoniae LplA is longer than Ta LplA in its C terminus by 67 residues. A search of the protein sequence database indicates that LplAs fall into two size categories; the shorter ones have ~260–270 residues and the longer ones ~330–340 residues. Ta LplA in the crystal catalyzed the
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A. Electron density of the bound ligands and overall fold of Ta LplA. A. 2Fobs - Fcal electron density maps of the bound ligands. Atoms of the ligands are also labeled. B. ribbon diagram of Ta LplA. Secondary structure elements were assigned by PROMOTIF (26). α-Helices, β-strands, and loops are colored in red, blue, and yellow, respectively. Lipoyl-AMP bound near the center of LplA is shown in sticks. All the figures except Fig. 3 are drawn with PyMOL (DeLano, 2002, The PyMOL Molecular Graphics System, www.pymol.org). C. topology diagram of Ta LplA. β-Strands are shown as triangles and α-helices as circles. D. stereo Ca trace of Ta LplA. Every tenth residue is marked by a black dot, and every twentieth residue is labeled. Three signature sequence motifs are highlighted in colored lines: motif II (RRXGGG/V/F/YHD) at positions 71–82 in red, motif III (KHXLG/AA at positions 145–150) in green, and motif IV (HXX/L/M) at positions 161–177) in blue, respectively.

formation of the activated intermediate (lipoyl-AMP) from ATP and lipoic acid. This suggests that the extra C-terminal region of the longer LplAs may not be required for catalyzing the activation step.

The next highest Z-score is obtained with the E. coli biotin holoenzyme synthetase/bio repressor (BirA) (PDB code 1BIA; an r.m.s. deviation of 2.7 Å for 134 equivalent Cα positions, a Z-score of 9.7, and a sequence identity of 13%) (18). The structure of BirA consists of three domains. The central domain II represents the BPL module, and houses both the biotin and the ATP binding sites. In E. coli BirA, the binding site of biotin was located using biotinyl-lysine, but there was no direct crystallographic data for the ATP binding site. A region of sequence homology to the BPL module of E. coli BirA can be identified in all biontinylating enzymes, whereas other domains are retained only in some bacterial counterparts of the E. coli enzyme (3). The equivalent of the smaller β-sheet of Ta LplA is missing from the domain II of E. coli BirA. Thus the β-sheet of the domain II of E. coli BirA is largely solvent-exposed on one side, where biotin was observed to bind (18). In comparison, in Ta LplA, the additional smaller β-sheet covers the lipoate portion of the bound lipoyl-AMP (Fig. 1B). A careful sequence analysis showed that LplA enzymes as well as LipB enzymes bear very low but detectable homology to the BPL module of biontinylating enzymes (3), suggesting an evolutionary relationship among them.

Binding of Lipoyl-AMP and ATP at the Active Site—After submission of this report, the crystal structure of LplA from E. coli was reported (19) (PDB codes 1X2G for the apo enzyme and 1X2H for the lipoic acid-complexed enzyme). This structure as well as those of E. coli BirA (18) and S. pneumoniae LplA does not provide detailed information about the binding modes of either the activated intermediate or ATP within the active site. To gain further insight into the ligand binding by LplA, we have determined the structures of Ta LplA in complex with either ATP or lipoyl-AMP, in addition to the apo structure. The apo, the ATP-bound, and the lipoyl-AMP-bound structures of Ta LplA are highly similar to each other. The r.m.s. deviation between the apo and the ATP-bound structures is 0.23 Å for 245 Ca atoms (residues 1–178 and 191–257), whereas that between the apo and the lipoyl-AMP-bound structures is 0.39 Å for 245 Ca atoms (residues 1–178 and 191–257). Between the ATP-bound and the lipoyl-AMP-bound structures, the r.m.s. deviation is 0.30 Å for 247 Ca atoms (residues 1–180 and 191–257).

The lipoyl-AMP binding pocket is bifurcated, and the conformation of the bound lipoyl-AMP is U-shaped (Fig. 2A). The phosphate group and part of the ribose sugar are accessible from the bulk solvent; they are accessible through an ~10 Å-deep, tunnel-like entrance that accommodates the lysine side chain protruding from the lipoyl domain (Fig. 2A). The rest of the activated intermediate is buried completely inside the bifurcated pocket, with the dithiolane ring of lipoyl-AMP being located roughly in the center of Ta LplA (Fig. 1B). The dithiolane ring and the aliphatic chain of lipoyl-AMP are surrounded by aliphatic side chains of four hydrophobic residues (Leu18, Ile46, Val79, and Ala163), the side chains of two histidine residues (His135 and His145), and the aliphatic part of the side chain of an arginine residue (Arg72). Four conserved glycine residues (Gly48, Gly75, Gly79, and Gly83) also surround them (supplemental Fig. S1). The imidazole ring of His135 rotates about 90° and moves ~1 Å away from the dithiolane ring of lipoyl-AMP in the lipoyl-AMP complex structure, relative to the apo structure. When we tried to incorporate biotin into the ATP-soaked crystals, the enzymes in the crystal did not catalyze the reaction and biotinyl-AMP was not formed. The present structure shows that the terminal double rings of biotin are too big to fit into the lipoate binding pocket of LplA. They are also more polar than the dithiolane ring of lipoic acid and are probably not readily accommodated in the lipoic acid binding pocket whose surface is lined largely with hydrophobic residues.

The phosphate moiety of lipoyl-AMP interacts with both Lys135 and Lys145 (Fig. 2, B and C). The distance from the O2A oxygen atom of lipoyl-AMP to the Nζ of Lys135 is 2.60 Å. Lys145 corresponds to the strictly conserved lysine residue among the members of LplA, LipB, and BPL families (3). The distance from the Nζ of Lys135 to the carboxyl oxygen atom of the lipoyl part is 3.29 Å, whereas those to the O4* and O5* atoms of the ribose, and the OAF atom of the phosphate are 3.02 Å, 3.31 Å, and 3.10 Å, respectively (Fig. 2, B and C). The side chain of Lys135 also makes a hydrogen bond with the side chain of Asp138 (2.77 Å between Nζ of Lys135 and Oδ1 of Asp138) (Fig. 2, B and C). Asp138 of Ta LplA is part of a somewhat variable region (Fig. 3). However, its role could possibly be played by an equivalent negatively charged residue, because there is at least one Asp or Glu near this region of other LplAs. Similar interactions were observed in E. coli BirA (18), where the conserved Lys183 makes hydrogen bonds with the carboxyl group of biotin as well as with the strictly conserved Asp176. In Ta LplA, the side chain O61 atom of Asp138 interacts with the Nζ atoms of both Lys135 (2.92 Å) and Lys145 (3.03 Å). A water molecule (Wat78) is hydrogen-bonded to the phosphate O1A oxygen atom (2.55 Å).
The ribose sugar of lipoyl-AMP is hydrogen-bonded to two water molecules (Wat34 and Wat76 in Fig. 2B). Wat34 is hydrogen-bonded to the ribose O3* atom (2.65 Å). Wat76 is hydrogen-bonded to the O2* and O3* oxygen atoms of the ribose ring (2.68 and 3.05 Å); it also interacts with the side chain O\textsubscript{\alpha}/H\textsubscript{\gamma} atom of Thr192 (3.02 Å). The side chain of the strictly conserved Val196 contacts the nonpolar portion of the ribose (supplemental Fig. S1).

The adenine ring of lipoyl-AMP makes three hydrogen bonds with Tyr80 and Asp85 (Fig. 2C). The adenine N6 atom makes two hydrogen bonds with the main chain carbonyl oxygen atom of Tyr80 (3.13 Å) and the side chain O\textsubscript{\beta}1 atom of Asp85 (3.03 Å), whereas the N7 atom forms a hydrogen bond with the amide nitrogen atom of Tyr80 (3.13 Å). Two hydrogen bonds involving the N6 atom will be lost if the adenine ring is substituted with the guanine ring. The adenine ring is also surrounded by hydrophobic residues (Val79, Ala150, Ala163, Leu165, Leu173, and Leu177). All these hydrophobic residues, except Ala163, as well as the above-mentioned histidine and glycine residues (His81, His161, Gly48, Gly75, Gly76, and Gly77) are strictly conserved among bacterial LplA proteins whose sequences are aligned in Fig. 3.

ATP binds to the same site as the AMP portion of lipoyl-AMP. Its β- and γ-phosphate groups point inward, and the β-phosphate occupies roughly the same position as the carboxyl portion of lipoyl-AMP. The bent conformation of the triphosphate group is maintained through a strong interaction with the side chain of Arg72 inside the lipoic acid binding pocket. When lipoic acid enters into its binding pocket of the ATP-bound Ta LplA, we expect that the β- and γ-phosphate groups of the bound ATP would be released from the lipoic acid binding pocket, because the environment surrounding the β- and γ-phosphate groups of the bound ATP is largely hydrophobic, and thus it would preferentially bind lipoic acid. If lipoic acid were bound prior to ATP binding, Arg72 would neutralize the negative charge of the carboxylate of lipoic acid.

**Conserved Sequence Motifs and Roles of Conserved Residues**—The sequence alignment of LplAs, including the human mitochondrial lipoate-transferase, shows that there exist at least three conserved sequence motifs (Fig. 3). The sequence motif I (RRXXGGGXXV[F/Y]HD), encompassing Arg72–Asp85, is most highly conserved (boxed in red in Fig. 3; highlighted in red lines in Fig. 1D). Motif II (KhXGX)
covers Lys<sup>145</sup>–Ala<sup>150</sup> and contains the strictly conserved Lys<sup>145</sup> (boxed in green in Fig. 3; highlighted in green lines in Fig. 1), whereas motif III (H<sub>V</sub>XX(L/M)XXX(L/D)XXhL) covers His<sup>161</sup>–Leu<sup>177</sup> (boxed in blue in Fig. 3; highlighted in blue lines in Fig. 1D). X stands for any amino acid, h is a hydrophobic residue, and the strictly conserved residues are in boldface. The three motifs provide key residues that are involved in...
Highly conserved residues that line the surface of the lipoyl-AMP bind Ta LplA, Lys145 makes key interactions with the phosphate moiety of Gly148 and lipoyl-AMP are drawn as sticks. Electrostatic potential representation of the surface of Ta LplA and the modeled complex with the lipoyl domain of the pyruvate dehydrogenase complex from A. vinelandii (drawn as an orange tube). Blue and red colors correspond to positive and negative potentials, respectively. The residues that belong to the lipoyl domain are labeled in white.

FIGURE 4. Modeling of the complexes between Ta LplA and lipoyl domains. A, modeled complexes between Ta LplA and each of the two lipoylated proteins, the lipoyl domain of the pyruvate dehydrogenase complex from A. vinelandii and the glycine cleavage system H protein from T. thermophilus. The target lysine residue and the bound lipoyl-AMP are drawn as sticks. B, electrostatic potential representation of the surface of Ta LplA and the modeled complex with the lipoyl domain of the pyruvate dehydrogenase complex from A. vinelandii (drawn as an orange tube). Blue and red colors correspond to positive and negative potentials, respectively. The residues that belong to the lipoyl domain are labeled in white.

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The formation of the lipoyl-AMP binding pocket (Figs. 1D and 2) as well as possibly a key residue (Arg71) for interaction with the lipoyl domain. Highly conserved residues that line the surface of the lipoyl-AMP binding pocket are Leu18, Ile64, Gly48, [Arg72, Gly75, Gly76, Gly77, Val79, Tyr80, His81], Asp82, [Lys145, Ala156, His161, Leu165, Leu166, Leu173, Leu177], and Val186 (marked with blue rectangles in Fig. 3). The strictly conserved residues are in boldface, and the residues belonging to the conserved sequence motifs are in square brackets. Arg74 of motif I appears to play a key role in recognizing the lipoyl domain, because our modeling of the complex between Ta LplA and the lipoyl domain of the pyruvate dehydrogenase complex from Azotobacter vinelandii (20) (PDB code 1IYU) indicates that it could interact with the strictly conserved Glu17 and Glu36 of the lipoyl domain (discussed below). Other highly or strictly conserved residues of Ta LplA such as Ala19, Leu36, Leu60, Ala70, and Gly148 seem to play structural roles. Asp92, Thr168, and Asn198 are involved in a hydrogen-bond network, whose significance is not clear.

Glu56 and Asp169 are located on the molecular surface.

Despite low levels of overall sequence similarity among the LplA, LipB, and BPL proteins, a lysine residue is strictly conserved in all members of these protein families (3). It corresponds to Lys145 of Ta LplA. In Ta LplA, Lys145 makes key interactions with the phosphate moiety of lipoyl-AMP, as discussed above. Besides this role in recognizing the activated intermediate, it may also play a critical role in transferring the lipoyl group from the activated intermediate to the specific lysine residue of the lipoyl domains in the second step of the LplA-catalyzed reaction. The equivalent residues of the BPL (for example, Lys165 of E. coli BirA) and LipB enzymes are expected to play similar roles. In the case of the reaction catalyzed by LipB, lipoyl-acyl carrier protein corresponds to the activated intermediate.

Lipoic transferases present in human and bovine mitochondria show 31–35% sequence identity with E. coli LplA, but they cannot catalyze the initial activation of lipoic acid to form lipoyl-AMP (7, 8). Instead, the medium-chain fatty acid CoA ligase-III in bovine liver mitochondria was found to activate lipoic acid utilizing GTP (21). Further studies are needed to clarify why the human and bovine lipoic transferases are not capable of activating lipoic acid.

Modeling of the Complexes Between Ta LplA and Lipoyl Domains—The biotinyl domains and lipoyl domains are closely related in their threedimensional structures, and there must be one or more key differences between them to ensure their correct selection. To understand how discrimination between lipoylation and biotinylation is achieved, we made an attempt to model the complexes between Ta LplA and two lipoylated proteins, the lipoyl domain of the pyruvate dehydrogenase complex from A. vinelandii (20) (PDB code 1IYU) and the apo form of the glycine cleavage system H protein from Thermus thermophilus (22) (PDB code IONL). The glycine cleavage system is a multienzyme complex consisting of four different components (the P-, H-, T-, and L-proteins). The present structure of the lipoyl-AMP complex allowed us to derive plausible models of the complexes between Ta LplA and the lipoylated proteins (Fig. 4). In our modeling attempts, we placed the protruding lysine of the lipoyl domains (Lys79 or Lys83 in each of the above-mentioned lipoylated proteins, respectively) in the tunnel-like entrance to the lipoyl-AMP pocket of Ta LplA (Fig. 2A), and optimized the orientation and position of the lipoyl domain in such a way that the two proteins make reasonable contacts with each other. The interface solvent-accessible surface area for each of the resulting models of the complexes is ~1,000 Å², comparable to the values for typical heterodimers (Protein-Protein Interaction Server at www.biochem.ucl.ac.uk/bsm/pp/server). The interface between the two proteins has also excellent charge and shape complementarities, even though the interacting partners are from different sources. Interestingly, we note that the large central β-sheet of Ta LplA is extended by one of the β-sheets of the lipoyl domain in the modeled complexes (Fig. 4A).

Furthermore, our modeling predicts interactions between highly conserved residues of LplA and its target proteins (Fig. 5). In our modeled complex between Ta LplA and the lipoyl domain of the pyruvate dehydrogenase complex from A. vinelandii, Arg71 in the conserved sequence motif I of Ta LplA lies within a hydrogen bonding distance from the side chains of Glu17 and Glu36 of the lipoyl domain. The latter two residues are strictly conserved among lipoyl domains of pyruvate dehydrogenases from different sources. Similarly, the modeled complex between Ta LplA and the glycine cleavage system H protein from T. thermophilus places Arg73 of Ta LplA within a hydrogen bonding distance from the side chains of Glu42 and Glu60 of the H protein. Glu42 is highly conserved as either Asp or Glu, whereas Glu60 is strictly conserved among the H proteins. This indicates that Glu42 and Glu60 of the T. thermophilus H protein are structurally equivalent to Glu17 and Glu36 of the A. vinelandii lipoyl domain, respectively, even though the two protein sequences are too dissimilar to be aligned. In our proposed...
models of the complexes, Lys$^{155}$ of Ta LplA, which is not strictly conserved but is either Lys or Arg in many other LplAs, also interacts with another negatively charged, conserved residue, i.e. Glu$^{63}$ of the A. vinelandii lipoyl domain and Asp$^{67}$ of the T. thermophilus H protein. A structural comparison of the T. thermophilus and pea H proteins revealed two negative surface regions, designated I and II, that are highly conserved between the two species (22). The negative charges of the surface region I of the T. thermophilus H-protein are attributed to Glu$^{42}$, Glu$^{60}$, and Asp$^{67}$. This region was previously proposed to constitute an interaction surface with T-protein (22). Our modeling of the complex between LplA and the H-protein from T. thermophilus (drawn as a cyan tube). The residues that belong to the lipoyl domain are labeled in magenta. B, stereo C$_{a}$ trace of the modeled complex between Ta LplA and H-protein from T. thermophilus (drawn as a cyan tube). The residues that belong to the lipoyl domain are labeled in cyan.

Mutational studies of the E. coli biotinyl domain indicated that one of the key structural determinants of protein specificity for biotinylation by BPL in E. coli is a thumb-like protrusion comprising Thr$^{94}$–Lys$^{100}$ between strands $\beta$2 and $\beta$3 of the E. coli biotinyl domain (23), which is an insertion relative to the lipoyl domains (2). When the residues Thr$^{94}$–Lys$^{100}$ were deleted from the E. coli biotinyl domain, the mutant protein was efficiently lipoylated (23). It was concluded that the protruding thumb between strands $\beta$2 and $\beta$3 is not critical for the interaction with
BPL, but its presence is sufficient to prevent the *E. coli* biotinyl domain from becoming lipoylated (23). This effect may be limited to *E. coli*, because amino acids that constitute the thumb are not present in most other biotinyl domains (23, 24). When we superimpose the biotinyl domain of acetyl-coenzyme A carboxylase from *E. coli* (25) (PDB code 1BDO) with the lipoyl domain of the modeled complex, the protruding domain of acetyl-coenzyme A carboxylase from *E. coli* biotinyl domain clashes severely with Ta LplA (mostly the carboxyl-terminal side of α7) and, thus, binding of the biotinyl domain to LplA would be prevented. Deletion of the residues Thr94–Lys100 from the *E. coli* biotinyl domain would remove the severe overlap between Ta LplA and the biotinyl domain from the modeled complex. When we overlay the structure of the *E. coli* BirA onto that of Ta LplA, the thumb of the biotinyl domain does not block binding of the biotinyl domain to BirA. Therefore, our modeling is consistent with the previously proposed role of the thumb-like protrusion in preventing the *E. coli* biotinyl domain from being lipoylated (23).

Of the seven lysine residues of the *A. vinelandii* lipoyl domain (or six of the *T. thermophilus* H protein), only Lys39 (or Lys63) is lipoylated. Discrimination of the target lysine from other lysine residues may be understood from our modeling, which indicates that Lys39 (or Lys63) is most protruding and can fit into the entrance to the lipoyl-AMP binding pocket of Ta LplA without causing severe clashes between the two proteins, whereas all other lysine residues cannot.

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