Post-translational modification in the archaea: structural characterization of multi-enzyme complex lipoylation

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INTRODUCTION

Aerobic metabolism of 2-oxoacids and C1 metabolism are dependent on LA (lipoic acid) in a highly conserved manner [1]. LA is an essential co-factor of the OADHCs (2-oxoacid dehydrogenase complexes), which include the PDHC (pyruvate dehydrogenase complex), OGDHC (2-oxoglutarate dehydrogenase complex) and BCOADHC (branched-chain 2-oxoacid dehydrogenase complex), and of the GCS (glycine cleavage system). OADHCs comprise multiple copies of three proteins: 2-oxoacid decarboxylase (E1), dihydrolipoyl acetyltransferase (E2) and dihydrolipoamide dehydrogenase (E3). E2 comprises E2lipD [E2 lipoyl domain(s)], a PSBD (peripheral subunit-binding domain) and a catalytic domain. E2lipD is the post-translational modification target: LA is covalently attached to E2lipD via an amide linkage to the ε-amino group of a specific lysine located at the tip of a β-turn. Once attached, the lipoyl moiety acts as a swinging arm that shuttles substrates/intermediates between the active sites of E1, E2 and E3. In the GCS, LA is attached to a lysine of the H protein that is structurally homologous with E2lipD.

Lipoylation, the covalent attachment of lipoic acid to 2-oxoacid dehydrogenase multi-enzyme complexes, is essential for metabolism in aerobic bacteria and eukarya. In Escherichia coli, lipoylation is catalysed by LplA (lipoate protein ligase) or by LipA (lipoic acid synthetase) and LipB [lipoyl(octanoyl)transferase] combined. Whereas bacterial and eukaryotic LplAs comprise a single two-domain protein, archaeal LplA function typically involves two proteins, LplA-N and LplA-C. In the thermophilic archaeon Thermoplasma acidophilum, LplA-N and LplA-C are encoded by overlapping genes in inverted orientation (ipla-c is upstream of ipla-n). The T. acidophilum LplA-N structure is known, but the LplA-C structure is unknown and LplA-C’s role in lipoylation is unclear. In the present study, we have determined the structures of the substrate-free LplA-N–LplA-C complex and E2lipD (dihydrolipoyl acyltransferase lipoyl domain) that is lipoylated by LplA-N–LplA-C, and carried out biochemical analyses of this archaeal lipoylation system. Our data reveal the following: (i) LplA-C is disordered but folds upon association with LplA-N; (ii) LplA-C induces a conformational change in LplA-N involving substantial shortening of a loop that could repress catalytic activity of isolated LplA-N; (iii) the adenylate-binding region of LplA-N–LplA-C includes two helices rather than the purely loop structure of varying order observed in other LplA structures; (iv) LplA-N–LplA-C and E2lipD do not interact in the absence of substrate; (v) LplA-N–LplA-C undergoes a conformational change (the details of which are currently undetermined) during lipoylation; and (vi) LplA-N–LplA-C can utilize octanoic acid as well as lipoic acid as substrate. The elucidated functional inter-dependence of LplA-N and LplA-C is consistent with their evolutionary co-retention in archaeal genomes.

Key words: binding-induced folding, lipoate protein ligase, lipoyl domain, NMR spectroscopy, protein–protein interaction, X-ray crystallography.

Abbreviations used: E2lipD, E2 lipoyl domain; IPTG, isopropyl β-D-thiogalactopyranoside; GCS, glycine cleavage system; HSQC, heteronuclear single-quantum coherence; LA, lipoic acid; LipA, lipoic acid synthetase; LipB, lipoyl(octanoyl) transferase; LplA, lipoate protein ligase; LPT, lipoyltransferase; MPD, 2-methyl-2,4-pentanediol; OA, octanoic acid; OADHC, 2-oxoacid dehydrogenase complex; OGDHC, 2-oxoglutarate dehydrogenase complex; PDHC, pyruvate dehydrogenase complex; RMSD, root mean square deviation.

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Co-ordinates and the structure factor file for the T. acidophilum LplA-N–LplA-C structure and co-ordinates for the E2lipD structures are in the PDB under accession codes 3R07 and 2L5T.

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Available structures include \textit{E. coli} LpaL [13] (PDB entries 1X2G, 1X2H, 3A7A and 3A7R), streptococcal LplAs (PDB entries 2POL and 1VQZ) and a mammalian LPT [8] (PDB entries 2E5A and 3A7U). The LpaL N-terminal domain belongs to the \( a/\beta \) class of proteins [3,5] and is structurally homologous with and evolutionarily related to the central catalytic domain of biotin protein ligase and class II aminocacyl-tRNA synthetase [14,15]. LpaL C-terminal domain comprises three \( \alpha \)-helices and two \( \beta \)-helices packed against a three-stranded \(-\)sheet [5]. Bovine LPT resembles LpaL in that it comprises a larger N-terminal domain and a smaller C-terminal domain, both with similar folds to their respective \textit{E. coli} counterparts. The overall conformation of lipoyl-AMP-bound LPT is, however, stretched relative to unliganded \textit{E. coli} LpaL due to rotation of the C-terminal domain by approximately 180° with respect to the N-terminal domain [8]. A similar rotation of the C-terminal domain relative to its apo orientation was observed in the crystal structure of lipoyl-AMP-bound \textit{E. coli} LpaL [13]. In the same structure, it was noted that two important loops also undergo conformational change upon lipoate adenylation: the adenylate-binding loop (residues 165–64) is too great for initiation of lipoyl transfer. In \textit{E. coli} LpaL, the distance between octanoyl-AMP and the acceptor lysine residue (LysApoH) is too great for initiation of lipoyl transfer. In the same study, Fujiwara et al. [13] also observed that, unlike \textit{E. coli} LpaL, bovine apo-LPT adopts the same relative N- and C-terminal domain orientations as lipoyl-AMP-bound LPT [13].

Archaeal LpaL studies have been conducted largely in \textit{Thermoplasma acidophilum}, a species that possesses genes encoding individual proteins that resemble the N- and C-terminal domains of non-archaeal LpaL. We term these gene products LpaL-N and LpaL-C. Structures of \textit{T. acidophilum} LpaL-N in unliganded (PDB entries 2ARS and 2CT7), lipoyl-AMP-bound (PDB entry 2ART), LA-bound (PDB entry 2CSM) and ATP-bound (PDB entry 2ARU) forms exhibit the same overall fold as the non-archaeal LpaL N-terminal domain [3,16]. It was shown in crystal soaking experiments (1 day soaks) that LpaL-N can catalyse lipoate adenylation to form lipoyl-AMP [16], but LpaL-N is unable to catalyse lipoate transfer in vivo and an accessory protein was suggested [3]. We subsequently showed that LpaL-N requires LpaL-C to carry out lipoylation (corroborated using complementation assays in \textit{E. coli} [17]) and that lipoylation occurs in vivo [18].

Comparative genomic analyses across 115 archaeal genomes (M.G. Posner, A. Upadhyay, M.J. Danson, S. Dorsu and S. Bagby, unpublished work) show that archaeal species capable of lipoylation retain either the LplA or LipA–LipB system with 81 % (61 out of 75 species) retaining LplA. Despite the evolutionary predominance of LplA in the archaea, and the fact that LplA-C is essential for lipoylation, no mechanistic information exists concerning co-ordination of LplA-N and LplA-C function. In the present study we have used structural and biochemical methods to investigate the role of LplA-C. We present structures of the \textit{T. acidophilum} LplA-N–LplA-C complex and of E2lipD, show that LplA-C folding is driven by association with LplA-N, and that LplA-C induces localized conformational change in LplA-N, and we use NMR to monitor LplA-N–LplA-C interactions with LA/ATP and E2lipD, and to monitor E2lipD lipoylation.

**EXPERIMENTAL**

**Expression and purification of \textit{T. acidophilum} LpaL-N–LpaL-C and \textit{T. acidophilum} E2lipD**

pET19b-L-\textit{p}la and pET24a-c-t\textit{d} were co-transformed into \textit{E. coli} BL21(DE3) cells and expression was induced with 0.25 mM IPTG (isopropyl \( \beta \)-D-thiogalactopyranoside) at 16°C overnight. Harvested cells were sonicated, the lysate was centrifuged at 21000 \( g \) for 40 min and LpaL-N–LpaL-C complex was purified using His MultiTrap \( ^{TM} \) FF and His MultiTrap \( ^{TM} \) HP columns (GE Healthcare). The final LpaL-N–LpaL-C complex purity was >95 % as judged by SDS/PAGE. \textit{T. acidophilum} E2lipD was expressed and purified as described previously [18].

**Expression and purification of \textit{E. coli} LpaL and E2lipD**

\textit{E. coli} LpaL and E2lipD were expressed using TM202 and pET11c plasmids. Expression in BL21(DE3) cells was induced with 0.5 mM IPTG for 3 h at 37°C. Cells were sonicated and proteins were purified using HiTrap QFF with a 0–0.5 M NaCl gradient in 20 mM Tris/HCl (pH 7.5).

**Crystallization of \textit{T. acidophilum} LpaL-N–LpaL-C complex, data collection and structural analysis**

\textit{T. acidophilum} LpaL-N–LpaL-C was exchanged into 10 mM Tris/HCl (pH 7.5), concentrated to 20 mg/ml and centrifuged at 13000 \( g \) for 20 min at 4°C. Sitting-drop vapour-diffusion crystallization screens were set up at 18°C using Molecular Dimensions screens with a Phoenix robot (Art Robbins Instruments). Crystals in 40 % (w/w) MPD (2-methyl-2,4-pentanediol), 0.1 M sodium acetate (pH 4.6) and 0.02 M CaCl\(_2\) were suitable for X-ray diffraction without further cryoprotectant. Diffraction data were collected at Diamond Light Source (Harwell, UK) on an ADSC Q315 CCD (charge-coupled device) detector on station IO2 (\( \lambda = 0.9795 \) Å, 1 Å = 0.1 mm). In total, 360 images were collected at an oscillation angle of 1°. Raw data images were processed using HKL2000 [19].

**Model building**

Molecular replacement using BALBES [20] was followed by model building with Coot [21] and rounds of refinement using Refmac5, part of CCP4 [22]. Other software included Molprobity [23] and Procheck [24].

**Structural analysis**

Hydrogen bonds and ionic interactions were evaluated with Contact CCP4 [22], ProtorP [25] and PISA [26]. Molecular graphics Figures were prepared in PyMOL (http://www.pymol.org).

**NMR spectroscopy**

\(^{15}\)N-labelled LpaL-C and \(^{15}\)N- and \(^{15}\)N\(^{13}\)C-labelled E2lipD were produced by expression in M9 minimal medium supplemented with 1 g/l \(^{15}\)NH\(_4\)Cl as the sole nitrogen source or 1 g/l \(^{15}\)NH\(_4\)Cl and 2 g/l \(^{13}\)C-glucose respectively. His-tagged proteins were purified as described previously [18]. Most E2lipD and all LpaL-N–LpaL-C NMR data were acquired at 37 or 50°C on a 600 MHz Varian Unity Inova spectrometer with an ambient temperature probe, processed using NMRPipe/NMRDraw [27] and analysed using CCPN Analysis [28]. \(^{15}\)N-edited NOESY and \(^{13}\)C-edited NOESY spectra of E2lipD were acquired on an 800 MHz Varian Inova spectrometer at the MRC Biomedical NMR Centre (Mill Hill,
Chemical-shift perturbations were calculated as a weighted average of 
1H, 15N, and 13C chemical shifts referenced to DSS [29]. Structures were calculated as described previously [30]. (1H-15N)-HSQC (heteronuclear single-quantum coherence) spectra of uniformly 15N-labelled LplA-C, both with and without unlabelled LplA-N, were recorded in 20 mM Tris/HCl (pH 7.5) and 150 mM NaCl. E2lipD spectra were recorded in 50 mM Hepes (pH 7.5) and 50 mM NaCl. All 1H, 15N HSQC spectra in the present study were recorded with 128 increments in the nitrogen dimension, unless otherwise stated.

**NMR titration of LplA-N–LplA-C with LA, ATP and Mg²⁺, then with E2lipD**

LA (racemic mixture unless otherwise stated), ATP and Mg²⁺ were titrated in combination against an NMR sample containing LplA-N–LplA-C (unlabelled LplA-N and uniformly 15N-labelled LplA-C) in 20 mM Tris/HCl (pH 7.5) and 150 mM NaCl. The molar ratio of LplA-N–LplA-C to LA at each titration point was 1:0, 1:0.25, 1:0.50 and 1:1; a (1H-15N)-HSQC spectrum (32 scans, 128 min recording time) was recorded at each titration point. Unlabelled E2lipD was then added to the same NMR sample with ratios of LplA-N–LplA-C to E2lipD of 1:0.25, 1:0.50 and 1:1.25; a (1H-15N)-HSQC spectrum (32 scans, 128 min recording time) was recorded after each E2lipD addition.

**NMR titration of E2lipD with LplA-N–LplA-C, LA and ATP**

Catalytic quantities of LplA-N–LplA-C were added to 15N-labelled 1.1 mM E2lipD in four steps (molar ratio of E2lipD to LplA-N–LplA-C of 1:0.0025, 1:0.005, 1:0.0075 and 1:0.01), followed by two additions of LA to a final concentration of 2.25 mM, and then by two additions of ATP to a final concentration of 2.25 mM (1.5 mM Mg²⁺) in the initial NMR sample. A (1H-15N)-HSQC spectrum was recorded at each titration point (eight scans, 34 min recording time). Chemical-shift perturbations were calculated as a weighted average of 1H and 15N chemical shift changes, Δδᵦ \( \Delta \delta _{ \alpha } (p.p.m.) = [ ( \Delta \delta _{ \alpha } ^{ NN } + \Delta \delta _{ \alpha } ^{ NN } )/2 ]^{ 1/2 } \) [31].

**Lipoylation/octanoylation activity assay**

The electrophoretic mobility of E2lipD before and after lipoylation/octanoylation was analysed by non-denaturing PAGE as described previously [18]. In the lipoylation/octanoylation assays and T. acidophilum E. coli enzyme cross-reactivity assays, the ratio of lipoylated to non-lipoylated E2lipD was quantified by MS.

**Synthesis of octanoyl-AMP**

Synthesis of octanoyl-AMP was carried out as described previously [32,33].

**Model of LplA-N–LplA-C–E2lipD complex**

In order to model a possible end-point of conformational change in LplA-N–LplA-C that permits lipoylation of E2lipD, the relative orientation of LplA-N and LplA-C was first changed to that observed between the N- and C-terminal domains of E. coli LplA in its complex with apo H protein and octanoyl-AMP (PDB entry 3A7A). E2lipD was then docked with the reoriented LplA-N–LplA-C complex using ClusPro 2.0 [34,35]; in the resulting models, E2lipD orientation and acceptor lysine (LysE₂lipD[42]) position were compared with those in PDB entry 3A7A of apo H protein and Lysγ₁[37] respectively. Models comparable with PDB entry 3A7A (i.e. with LysE₂lipD[42] in proximity to and oriented towards the LplA-N active site) were selected and their quality assessed using QMean [36]. The model from this subset with the highest QMean score was selected as a representative structure.

**RESULTS**

**Structure of T. acidophilum LplA-C**

Despite the fact that LplA-C is essential for lipoylation by archaeal LplA [17,18], the functional and structural relationship between LplA-N and LplA-C is poorly understood. We examined whether LplA-N and LplA-C exist independently or form a stable complex by first studying LplA-C structure without LplA-N. In crystallization screens, LplA-C showed a high propensity to precipitate. Poor chemical-shift dispersion, variable peak intensity and low peak count (approximately 50 peaks observed compared with 84 expected on the basis of the LplA-C amino acid sequence) in (1H-15N)-HSQC NMR spectra showed that LplA-C is disordered and heterogeneous over a range of pH values (pH 6–8) and NaCl concentrations (50–150 mM NaCl) (Figure 1A). Upon stepwise addition of unlabelled LplA-N to 15N-labelled LplA-C (final LplA-N/LplA-C molar ratio of 1:1), the observed dramatic increase in dispersion, homogeneity and number of LplA-C (1H-15N)-HSQC peaks indicated that LplA-C undergoes LplA-N binding-induced folding (Figure 1B). In total, 76 distinct backbone amide NH peaks were observed in the LplA-N-bound LplA-C (1H-15N)-HSQC spectrum; this close correspondence with the expected total of 84 peaks indicated that LplA-N-bound LplA-C adopts a single dominant conformation on average, and indirectly supports the presence of a single predominant LplA-N–LplA-C complex conformation in solution. The LplA-N-induced LplA-C fold, and by inference the LplA-N–LplA-C complex, is stable to at least 50°C (Figure 1B).

**T. acidophilum LplA-N–LplA-C X-ray crystal structure: comparison with other LplAs**

Subsequent to the LplA-C NMR studies described above, crystallization screens of co-expressed LplA-N and LplA-C produced LplA-N–LplA-C complex crystals in 40% (v/v) MPD, 0.1 M sodium acetate (pH 4.6) and 0.02 M CaCl₂. The LplA-N–LplA-C structure (Figure 2A) was determined to 2.7 Å resolution by molecular replacement with LplA-N (PDB entry 2ARS) (Table 1). The overall fold of LplA-N [3,16] is maintained in the presence of LplA-C, as confirmed using Dali [37] (Table 2). The β-strands in LplA-N are β₁ (residues 1–7), β₂ (residues 35–39), β₃ (residues 44–47), β₄ (residues 68–71), β₅ (residues 64–66) respectively. Models comparable with LplA-N–LplA-C were selected and their quality assessed using QMean [36]. The model from this subset with the highest QMean score was selected as a representative structure.

**Structure of an archaeal lipoylation system**

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LplA-N–LplA-C is structurally similar to single polypeptide apo-LplAs from Streptococcus pneumoniae and E. coli, including similar domain orientations (Figure 2C and Table 2). Bovine (Bos taurus) LPT, however, has a different arrangement of domains in both apo and lipoyl-AMP-bound forms, as does lipoyl-AMP-bound E. coli LplA; in these cases, the C-terminal domain has undergone a 180° rotation (Figure 2C) [13]. The structures of E. coli LplA C-terminal domain and T. acidophilum LplA-C agree well, with both forming a canopy above the tunnel-like entry to the active site. With respect to the inverted gene orientation in T. acidophilum (lpla-c is upstream of lpla-n with a TATA box upstream of lpla-c but no cis-regulatory sequence in the proximity of lpla-n) [18], it is important to note that the LplA-C C-terminus and LplA-N N-terminus are located at opposite ends of the LplA-N–LplA-C complex, approximately 56 Å apart (Figure 2A), confirming that LplA-N and LplA-C are made as separate polypeptides.

The LplA-N–LplA-C interface has a buried surface area of 805 Å² compared with 993 Å² between the N- and C-terminal domains of the closest single protein homologue, S. pneumoniae LplA [3,18]. The LplA-N–LplA-C interface involves 50 residues and includes 12 hydrogen bonds plus five salt bridges involving three pairs of residues [26]. These include a five-residue network that forms salt bridges (GluLplA-N 56–ArgLplA-C 17, GluLplA-N 55–HisLplA-C 31) and three hydrogen bonds (Glu LplA-N 55–HisLplA-C 31, GluLplA-N 55–SerLplA-C 23, GluLplA-N 55–ArgLplA-C 23) (Figure 2B). In the corresponding location, S. pneumoniae LplA has an interdomain three-residue (Arg45–Asp284–His46) network with two interdomain ionic interactions involving Asp 284, and E. coli LplA has no obvious ionic interaction. In addition, the LplA-N–LplA-C interface has a substantial hydrophobic component with approximately 25 hydrophobic residues contributing to the interface.
LplA-C-induced conformational change of LplA-N: ‘capping’ loop and adenylate-binding loop

LplA-N undergoes a substantial local structural rearrangement upon binding LplA-C. In isolated LplA-N (i.e. without LplA-C), β8 consists of residues 138–141 and is connected to β9 (residues 144–154) by a short β-turn, whereas strands β7 and β8 are connected by a long loop consisting of residues 124–137 (orange and labelled as the ‘capping loop’ in Figure 3). Interestingly, this loop makes several contacts with lipoyl-AMP in PDB entry 2ART, and may play a role in ensuring that isolated LplA-N is catalytically inert. This region is reorganized in the LplA-N–LplA-C structure such that residues 128–131 form β8, and a short turn comprising residues 125–127 connects β7 to β8, whereas β8 is connected to β9 by a disordered loop comprising residues 132–142, for most of which electron density is not observed (Figure 3).

This structural shift seems to be facilitated by the similarity of the two motifs that alternate as β8: residues 128–131 are Asp-Val-Ile, whereas residues 138–141 are Asp-Ile-Met-Ala. It is of note that in E. coli LplA, β8 is a fixed motif, connected to β7 and β9 by short loops on either side.

The lipoylated binding loop adopts the same conformation with and without LplA-C (Figure 3), whereas in LplA-N–LplA-C a substantial portion of the region corresponding to the adenylate-binding loop strikingly forms contiguous α-helices (α4 and α5; residues 181–183 and 184–194). In other LplA structures, this region is either a loop or is largely disordered such that electron density is absent. In several structures of isolated LplA-N, for example, much of the adenylate-binding loop region is disordered (e.g. PDB entries 2ARS and 2C7I, both unliganded; PDB entry 2C8M, LA-bound; and PDB entry 2ART, lipoyl-AMP bound), although it is of note that following an electron density gap in the structures 2ARS, 2ART and 2C8M, there is a nascent α-helix that overlaps with part of LplA-N–LplA-C α5 (e.g. the region shown in purple in Figure 3). In the structure of unliganded E. coli LplA (PDB entry 1X2G), the adenylate-binding loop occupies a similar position to LplA-N–LplA-C helices α4 and α5, whereas there is again missing electron density in unliganded bovine LPT. The adenylate-binding loops of lipoyl-AMP-bound E. coli LplA and bovine LPT overlap closely with each other and are shifted towards the active site relative to the adenylate-binding loops of the respective unliganded enzymes.

LplA-N–LplA-C interaction with lipoyl-AMP and E2lipD

We used NMR titrations to investigate LplA-N–LplA-C interactions, monitoring the ([H-15N]-HSQC spectrum of 15N-labelled LplA-C in complex with unlabelled LplA-N. In one titration (Titration 1), LA, ATP and Mg2+ were added together to LplA-N–15N-LplA-C, then E2lipD was added (Figures 4A and 4B). Upon addition of LA, ATP and Mg2+ (and presumably therefore upon formation of the lipoyl-AMP-bound form of LplA-N–LplA-C), 15 out of the 76 distinct backbone amide NH peaks in the ([H-15N]-HSQC spectrum of LplA-N–15N-LplA-C were significantly broadened (intermediate timescale exchange), five exhibited slow exchange (two peaks observed per backbone amide NH), four underwent a chemical-shift change, four exhibited both...

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Table 1 Data collection and structural refinement statistics for the crystal structure of the LplA-N–LplA-C complex (PDB entry 3R07)

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Table 2 Summary of Dali similarity searches

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<td>26.3 (2.1)</td>
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<tr>
<td>B. taurus</td>
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<td>2E5A</td>
<td>27.2 (1.2)</td>
<td>7.7 (2.5)</td>
</tr>
</tbody>
</table>

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However, when LA, ATP and Mg²⁺ residues identified by structure-based alignment to form the adenylate binding region are some had not (Figure 4D).

In order to facilitate modelling studies of the complete T. acidophilum lipoylation system (see below), the T. acidophilum E2lipD structure was determined by NMR (Table 3). T. acidophilum E2lipD is similar overall to other lipoyl domains [Dalilite Z-score of 6.8 and RMSD (root mean square deviation) over all Ca atoms of 2.7 Å compared with E. coli E2lipD (PDB entry 1QJO; 27% sequence identity)]. In an NMR titration to monitor E2lipD lipoylation, T. acidophilum E2lipD (H⁻¹-N⁻¹) HSQC did not change upon step-wise addition of catalytic quantities of LplA-N–LplA-C (final molar ratio of 100:1, E2lipD/LplA-N–LplA-C), or upon addition of LA (final molar ratio approximately 1:2, E2lipD/LA) (Figure 5). Upon subsequent addition of ATP (final molar ratio approximately 1:2, E2lipD/ATP), however, several E2lipD backbone amide peaks underwent chemical-shift perturbation (Figures 5A and 5B); the largest chemical-shift perturbations were observed for E2lipD residues 42–44 ([Ỹ₈₆₂₅ₙ₀] is the lipoylation target residue), followed by residues 9–10; the Thr₆₇₄₄ peak was broadened. When mapped onto the E2lipD structure, the pattern of largest chemical-shift perturbations (plus broadening for residue 40) indicates that lipoylation induces a localized conformational change in E2lipD (Figure 5C).

**Figure 3** LplA-C-induced conformational change of LplA-N

The structure of the LplA-N–LplA-C complex (PDB entry 3R07) is shown with LplA-N predominantly in grey and LplA-C in blue. The structure of isolated (i.e. without LplA-C) LplA-N (PDB entry 2ART) is superimposed on the LplA-N–LplA-C complex and is also shown predominantly in grey. Structural features of structure 3R07 are highlighted in green and structural features of structure 2ART are highlighted in yellow, orange and purple. Lipoate-AMP from structure 2ART is shown in red. Secondary structure elements of structure 3R07 are labelled. The lipoate-binding loop conformations are almost identical in structures 3R07 (green) and 2ART (yellow). In the absence of LplA-C, residues 124–137 form a long loop (orange, labelled as ‘capping loop’); the corresponding loop (green) is much shorter in the LplA-N–LplA-C complex. Instead, the subsequent loop is much longer in the LplA-N–LplA-C complex than in isolated LplA-N (electron density is lacking between Val₁₃₃ and Gly₁₄₂ in the LplA-N–LplA-C complex). Residues identified by structure-based alignment to form the adenylate binding region are shown in green (PDB entry 3R07) and purple (PDB entry 2ART), although in 2ART much of the adenylate-binding region lacks defined electron density.

In a separate titration (Titration 2), unlabelled E2lipD was first titrated into a LplA-N–¹⁵N-LplA-C NMR sample, most of the peaks that had been perturbed (Figure 4A) reverted to a state the same as or close to that observed before the addition of LA, ATP and Mg²⁺ (Figure 4B), consistent with lipoyl transfer to E2lipD and hence consumption of substrate.

In the absence of LplA-C, residues 124–137 form a long loop (orange, labelled as ‘capping loop’); the corresponding loop (green) is much shorter in the LplA-N–LplA-C complex. Instead, the subsequent loop is much longer in the LplA-N–LplA-C complex than in isolated LplA-N (electron density is lacking between Val₁₃₃ and Gly₁₄₂ in the LplA-N–LplA-C complex). Residues identified by structure-based alignment to form the adenylate binding region are shown in green (PDB entry 3R07) and purple (PDB entry 2ART), although in 2ART much of the adenylate-binding region lacks defined electron density.

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**Substrate promiscuity and recognition of E2 lipoyl domains by LplA-N–LplA-C**

As its bipartite nature may affect substrate recognition and specificity, we tested T. acidophilum LplA-N–LplA-C activity with different acceptor domains and substrates, including LA, OA and octanoyl-AMP, with E. coli LplA serving as a positive control. In gel-shift lipoylation assays [18] with LA, co-expressed LplA-N–LplA-C and a 1:1 mixture of individually expressed and purified LplA-N and LplA-C showed equal activity. LplA-N–LplA-C showed activity with both OA and octanoyl-AMP, in agreement with previous findings that LplAs can catalyse the formation and transfer of octanoyl-AMP [38]. As judged by MS, E2lipD modification efficiency by LplA-N–LplA-C with OA as the substrate was 20–30% of that with LA as the substrate.

E2lipD cross-reactivity was analysed next. E2lipD residues both N-terminal (−) and C-terminal (+) of the target lysine residue are important for efficient lipoylation [39]. Previous large-scale sequence alignments identified a highly conserved aspartate residue at −1, hydrophobic residues at +1, +5 and −4, glutamate/aspartate enrichment at −3 and +4, and serine/alanine at +7 [40]. A glutamate residue at −3 and glycine residue at −16 are involved in LplA recognition. Sequence alignment with E. coli E2lipDs and E. coli H protein showed that a glycine residue at −16 is conserved in T. acidophilum E2lipD. A glutamate residue at −3 is conserved in E. coli E2lipDs and H protein, whereas T. acidophilum E2lipD has a methionine residue at −3; residues other than glutamate at −3 reduce lipoylation efficiency in E. coli [41]. Correspondingly, E. coli LplA lipoylated T. acidophilum E2lipD with approximately 50% efficiency relative to E. coli E2lipD, and T. acidophilum LplA-N–LplA-C lipoylated E. coli E2lipD with approximately 15–20% efficiency relative to T. acidophilum E2lipD.

**NMR analysis of T. acidophilum E2lipD structure and lipoylation**

In order to facilitate modelling studies of the complete T. acidophilum lipoylation system (see below), the T. acidophilum E2lipD structure was determined by NMR (Table 3). T. acidophilum E2lipD is similar overall to other lipoyl domains [Dalilite Z-score of 6.8 and RMSD (root mean square deviation) over all Ca atoms of 2.7 Å compared with E. coli E2lipD (PDB entry 1QJO; 27% sequence identity)]. In an NMR titration to monitor E2lipD lipoylation, T. acidophilum E2lipD (H⁻¹-N⁻¹) HSQC did not change upon step-wise addition of catalytic quantities of LplA-N–LplA-C (final molar ratio of 100:1, E2lipD/LplA-N–LplA-C), or upon addition of LA (final molar ratio approximately 1:2, E2lipD/LA) (Figure 5). Upon subsequent addition of ATP (final molar ratio approximately 1:2, E2lipD/ATP), however, several E2lipD backbone amide peaks underwent chemical-shift perturbation (Figures 5A and 5B); the largest chemical-shift perturbations were observed for E2lipD residues 42–44 ([Ỹ₈₆₂₅ₙ₀] is the lipoylation target residue), followed by residues 9–10; the Thr₆₇₄₄ peak was broadened. When mapped onto the E2lipD structure, the pattern of largest chemical-shift perturbations (plus broadening for residue 40) indicates that lipoylation induces a localized conformational change in E2lipD (Figure 5C).

**Model of the T. acidophilum LplA-N–LplA-C–E2lipD complex**

In E. coli LplA, lipoate adenylation causes conformational changes, including an 180° rotation of the C-terminal domain (Figures 2C and 6A), that prime the system for lipoyl transfer [13]. In T. acidophilum apo-LplA-N–LplA-C, LplA-C forms a canopy over the active site in a similar manner to the C-terminal domain of other LplAs, obstructing access to lipoate of LysE₂lipD [42], the lipoate acceptor residue (Figure 6A). Hence, a substantial change to the apo-LplA-N–LplA-C structure is required for lipoylation to occur. Using the E. coli LplA–octanoyl-AMP–ApoH protein complex crystal structure as a template [13] (Figure 6A), we have modelled a possible end-point of such a change with E2lipD.
The results of two titrations are shown. In (A) and (B), LA, ATP and Mg\(^{2+}\) were added before E2lipD. In (C) and (D), E2lipD was added before LA, ATP and Mg\(^{2+}\). (A) LA, ATP and Mg\(^{2+}\) were added together in a step-wise fashion to an NMR sample containing LplA-N–LplA-C (unlabelled LplA-N and uniformly \(^{15}\)N-labelled LplA-C) in 20 mM Tris/HCl (pH 7.5) and 150 mM NaCl. A \(^{(1H-15N)}\)-HSQC spectrum was recorded at each titration point. The molar ratio of LplA-N–LplA-C complex to LA at each titration point was 1:0, 1:0.25, 1:0.50 and 1:1.25. The initial spectrum is added together in a step-wise fashion to an NMR sample containing LplA-N–LplA-C (unlabelled LplA-N and uniformly \(^{15}\)N-labelled LplA-C) in 20 mM Tris/HCl (pH 7.5) and 150 mM NaCl. A \(^{(1H-15N)}\)-HSQC spectrum of the same sample as in (A) was recorded after leaving the sample overnight at 4°C. The peak at around 6.4 p.p.m., 113 p.p.m. labelled with an asterisk moved to 6.1 p.p.m., 113 p.p.m. Peaks subject to smaller chemical-shift changes have not been highlighted. Perturbed LplA-C peaks are highlighted using the same scheme described for (A). In total, 35 LplA-N residues, eight LplA-C residues and 34 E2lipD residues are involved in the interface [24].

### DISCUSSION

Biochemical data from our laboratory and elsewhere indicate that LplA-C is essential for lipoylation of E2 in \(T.\) acidophilum [17,18]. Our genomic profiling, moreover, indicates that bipartite LplA-N–LplA-C is the evolutionarily predominant lipoylation system in the archaea (M.G. Posner, A. Upadhyay, M.J. Danson, S. Dorus and S. Bagby, unpublished work). There is, however, conflicting evidence as to the exact role of LplA-C: although LplA-N by itself was reported to catalyse lipoylation de novo to form lipoyl-AMP [16], it was more recently reported that LplA-C is required for lipoylation de novo [17]. Further structural and mechanistic analysis of LplA-C function is therefore warranted. The structures of \(T.\) acidophilum LplA-N–LplA-C and E2lipD described in the present study represent the first structural analysis of a complete archaean lipoylation system and the first of a bipartite lipoyl protein ligase.

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The current evidence indicates that the observed interface between LplA-N and LplA-C is a biological rather than crystal packing interface, and that LplA-N and LplA-C exist permanently as a complex. This evidence includes the observations that isolated LplA-C is disordered, the LplA-N–LplA-C complex is stable to at least 50°C (Figure 1), LplA-N and LplA-C associate strongly only during lipoylation. We now know that LplA-C is probably not functional by itself as it is disordered and undergoes LplA-N-induced folding. The C-terminal domain of \(E.\) coli LplA, on the other hand, was found by limited proteolysis to be structurally stable [3]. We do not know, however, whether LplA-C retains its fold once it is released from LplA-N as we have been unable to establish a non-denaturing procedure to dissociate the LplA-N–LplA-C complex.

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thus mechanistic differences from the LipA–LipB system in which LipA and LipB operate sequentially, although we note that E. coli LipA and LipB have been found to form a tight non-covalent association with the E2 components of PDHC and OGDHC [4], presumably with resulting potential for greater processivity and for interaction between LipA and LipB themselves. The inherent robustness of T. acidophilum LplA, and presumably LplA from other thermophiles, makes these ligases attractive starting points for biotechnological and chemical biology applications, such as have been demonstrated for E. coli LplA [42,43].

The T. acidophilum LplA-N–LplA-C complex adopts the same fold and the same spatial arrangement of domains as the structurally characterised bacterial apo-LplAs (PDB entries 1X2G, 2POL and 1VQZ; Figure 2). LplA-N–LplA-C does, however, possess at least two distinctive local conformational features that could be functionally important. First, in E. coli LplA and bovine LPT, the adenylate-binding region is a loop, often at least partially disordered, whereas in LplA-N–LplA-C the equivalent region includes two contiguous \( \alpha \)-helices (\( \alpha4 \) and \( \alpha5 \); Figure 3). We cannot say whether these helices persist at the optimum temperature (55°C) for T. acidophilum, but their presence reduces the probability that the LplA-N adenylate-binding region undergoes the same transition as the E. coli adenylate-binding region upon lipoate adenylation, which includes formation of a new \( \beta \)-strand anti-parallel to \( \beta13 \) of the C-terminal domain [13]. Secondly, LplA-C-induced conformational changes in LplA-N around strands \( \beta7 \) and \( \beta8 \) result in substantial
Figure 6 Lipoyl protein ligase complexes with E2lipD or ApoH protein

(A) Superimposition of E. coli octanoyl-5′-AMP-bound LpLA (yellow) in complex with E. coli ApoH (grey) (PDB entry 3A7A) and T. acidophilum LpLA-N (green)–LpLA-C (blue) (PDB entry 3R07).

(B) Comparison of the acceptor lysine residue positions in the E. coli LpLA–ApoH complex and in the T. acidophilum LpLA-N–LpLA-C–E2lipD complex in which LpLA-C has undergone a change in position and orientation relative to LpLA-N–LpLA-C. E. coli ApoH is positioned as in the LpLA–ApoH complex, but E. coli LpLA has been omitted for clarity. The lipoyl acceptor residue of E. coli ApoH, LpLA-Cys42, is shown in black. T. acidophilum E2lipD is in magenta with its lipoyl acceptor residue, LysE2lipD42, in cyan. Octanoyl-5′-AMP (red) is positioned as in the E. coli LpLA–ApoH complex.

Table 3 Structural statistics for the ensemble of NMR-derived structures of E2lipD

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shortening of a loop (that we label as the capping loop in Figure 3) that in isolated LpLA-N partially occupies the space that the adenylate-binding loop occupies in E. coli LpLA. It is likely that the capping loop functions at least to repress catalytic activity of LpLA-N in the absence of LpLA-C. It would be interesting in future to establish whether replacement of the capping loop with the equivalent short loop from E. coli LpLA shows gain-of-function effects in isolated LpLA-N.

E. coli LpLA undergoes significant structural changes upon lipoate adenylation, including reorientation of the C-terminal domain to produce a more stretched overall conformation (PDB entry 3A7R). Bovine LPT adopts this stretched capping domain in both apo- and lipoyl-AMP-bound forms (PDB entries 2E5A and 3A7U). We have been unable to produce crystals of LpLA-N–LpLA-C complexes with lipoyl-AMP and with E2lipD that diffract to sufficient resolution to investigate structural changes in LpLA-N–LpLA-C. We have, however, studied LpLA-N–LpLA-C interactions with LA/ATP/Mg2+ and E2lipD using (1H-15N)-HSQC NMR spectra, which are highly sensitive to conformational changes and interactions. We performed two titerations in which at each step we recorded (1H-15N)-HSQC spectra of 15N-labelled LpLA-C in complex with unlabelled LpLA-N. In Titration 1, LA/ATP/Mg2+ were added before E2lipD such that lipoate adenylation and lipoate transfer are monitored separately. The observed NMR spectral changes (Figure 4A) are not immediately suggestive of a substantial LpLA-C conformational change upon formation of the lipoyl-AMP intermediate, but a LpLA-C positional change, like the 180° rotation of the C-terminal domain observed in E. coli LpLA [13], cannot be ruled out as the observation of exchange in approximately 25 peaks indicates that nearly a third of LpLA-C residues sample more than one chemical environment.

In Titration 2, E2lipD was added before LA/ATP/Mg2+. The lack of change upon E2lipD addition indicates that in apo-LpLA-N–LpLA-C, LpLA-C does not interact with E2lipD and, further, if there is any E2lipD interaction with LpLA-N in apo-LpLA-N–LpLA-C, it does not occur in the vicinity of LpLA-C. Chemical-shift perturbations were observed in more than one-third of LpLA-C (1H-15N)-HSQC peaks when LA/ATP/Mg2+ were then added to the mixture of apo-LpLA-N–LpLA-C and E2lipD (Figure 4C). Thus a more substantial change in LpLA-C occurs when E2lipD is already present at the time of adding lipoate adenylation ingredients. A reliable explanation of this observation would require extensive further investigation, but for now we note that lipoate adenylation and lipoate transfer are monitored simultaneously in Titration 2, rather than separately as in Titration 1. We note also that at least 75% of peaks perturbed in Titration 1 were also perturbed in Titration 2, indicating the involvement of substantially overlapping regions of LpLA-C in any conformational/positional changes occurring during the two titerations.

Our NMR data, particularly from Titration 2, indicate that LpLA-C does undergo significant conformational change at one or more stages of lipoylation. This is consistent with our structure-based hypothesis that rearrangement of the LpLA-N–LpLA-C complex, akin to that seen in E. coli LpLA [13], is required to allow E2lipD access to the LpLA-N active site and hence to allow lipoate transfer. In our model of a possible end-point of such a rearrangement, LpLA-C has rotated through approximately 120°, compared with the approximately 180° rotation observed for E. coli LpLA C-terminal domain upon lipoate adenylation [13]. One potential flaw in our model, in common with the E. coli LpLA–octanoyl-AMP–ApoH protein complex crystal structure, is that...
the distance between the adenylated intermediate and the acceptor lysine (LysE2lipD) is too great for initiation of lipoyl transfer. It remains to be seen whether, as Fujiwara et al. [13] suggest, this is rectified if a version of a LplA–E2lipD/H protein complex with ‘true substrates’ can be crystallized.

Assuming that LplA-C becomes disordered if it is released from LplA-N, there is no evidence from our NMR data that LplA-C dissociates from LplA-N during lipote adenylation or lipote transfer; we detect only folded LplA-C species during catalysis of both steps, suggesting that LplA-C is not competed off LplA-N by incoming substrate and that LplA-C remains bound to LplA-N during any rearrangement of the complex. There remains the possibility, however, of minor populations of disordered LplA-C species at any one time that are not detected by the techniques used in the present study. On the other hand, if LplA-C remains structured upon dissociation from LplA-N, then rearrangement of the LplA-N–LplA-C complex could clearly involve a simple release and rebind mechanism.

We also used NMR to monitor the effect of lipoylation on E2lipD. We believe that the observed chemical-shift perturbations upon addition of the lipoylation ingredients (Figure 5) are more likely to result from lipoylation-induced localized conformational change in E2lipD than from non-covalent E2lipD interaction with LplA-N–LplA-C (present only in catalytic quantities) or substrate. Previous NMR analysis did not indicate any conformational change in E2lipD from Bacillus stearothermophilus PDHC upon lipoylation [44]. The reason for the difference is not obvious, but it is clear that the results of this titration represent further evidence that the T. acidophilum LplA-N–LplA-C complex is functional.

We have previously described features of the genes encoding LplA-C and LplA-N, including the facts that their genes overlap by a single base pair, and a TATA box is readily identifiable upstream of lpla-c; however, no cis-regulatory sequence is observed in the proximity of lpla-n, suggesting that the genes are transcriptionally coupled. Given that the gene order is lpla-c then lpla-n [18], our structure-based observation that the LplA-C C-terminus and LplA-N N-terminus are located at opposite ends of the LplA-N–LplA-C complex confirms that LplA-N and LplA-C are made as separate polypeptides.

We have shown that, like other LpLAs, T. acidophilum LplA-N–LplA-C can use OA and octanoyl-AMP as substrates, albeit less efficiently than LA. At first glance this may be unsurprising, but fatty acid synthesis, the source of the OA precursor, is thought to be absent in the archaea [45]. It has long been hypothesized that ancient enzyme promiscuity gave rise to the specialized lipoylation pathways that are absent in the archaea [45]. It has long been hypothesized that ancient enzyme promiscuity gave rise to the specialized lipoylation pathways that are absent in the archaea [45]. The reason for the difference is not obvious, but it is clear that the results of this titration represent further evidence that the T. acidophilum LplA-N–LplA-C complex is functional.

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REFERENCES


AUTHOR CONTRIBUTION

Mareike Posner and Abhishek Upadhayay did all of the protein expression, purification, crystallization and NMR sample preparation. Mareike Posner, Abhishek Upadhayay and Susan Crennell acquired, processed and analysed X-ray diffraction data. Mareike Posner and Stefan Bagby did all of the protein expression, purification, crystallization and NMR sample preparation. Mareike Posner and Stefan Bagby synthesized octanoyl-AMP. Steve Dorus and Stefan Bagby did the competitive genomic analysis. Mareike Posner, Abhishek Upadhayay, Michael Danson, Steve Dorus and Stefan Bagby designed experiments and interpreted the results. All authors contributed to writing of the paper, with Mareike Posner and Stefan Bagby making the major contributions.