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Purification and properties of the lipoate protein ligase of *Escherichia coli*

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Lipoate is an essential component of the 2-oxoacid dehydrogenase complexes and the glycine-cleavage system of *Escherichia coli*. It is attached to specific lysine residues in the lipoyl domains of the E2p (lipoate acetyltransferase) subunit of the pyruvate dehydrogenase complex by a Mg^{2+}- and ATP-dependent lipoate protein ligase (LPL). LPL was purified from wild-type *E. coli*, where its abundance is extremely low (< 10 molecules per cell) and from a genetically amplified source. The purified enzyme is a monomeric protein (M_r 38000) which forms irregular clusters of needle-like crystals. It is stable at −20 °C, but slowly oxidizes to an inactive form containing at least one intramolecular disulphide bond at 4 °C. The inactive form could be re-activated by reducing agents or by an as-yet unidentified component (re-activation factor) which is resolved from LPL at the final stage of purification. The pI is 5.80, and the K_m values for ATP, Mg^{2+} and DL-lipoate were determined. Selenolipoate and 6-thio-octanoate were alternative but poorer substrates. Lipoylation was reversibly inhibited by the 6- and 8-seleno-octanoates and 8-thio-octanoate, which reacted with the six cysteine thiol groups of LPL. LPL was inactivated by Cu^{2+} ions in a process that involved the formation of inter- and intra-molecular disulphide bonds. Studies with *lplA* mutants lacking LPL activity indicated that *E. coli* possesses another distinct lipoylation system, although no such activity could be detected *in vitro*.

INTRODUCTION

The lipoate acetyltransferase subunit (E2p) of the pyruvate dehydrogenase (PDH) complex of *Escherichia coli* possesses three N-terminal tandemly repeated lipoyl domains, each of which can be modified post-translationally by the covalent addition of lipoic acid to specific lysine residues in N^6-amide linkage [1–3]. Two further lipoylated proteins have been identified in *E. coli*, the lipoate succinyltransferase subunit (E2o) of the 2-oxoglutarate dehydrogenase (ODH) complex, and the H-protein of the glycine-cleavage system [4]. In the protein bound state, lipoic acid performs an essential catalytic role in central metabolic pathways, but surprisingly little is known about the process by which lipoylation of apoproteins is achieved. Early studies on the lipoate activating systems of *E. coli* and *Streptococcus faecalis* by Reed and co-workers revealed that lipoate, ATP, Mg^{2+} and P_i are required for apo-PDH-complex activation. It was further shown that lipoate and ATP can be replaced by lipoyladenylate, a potential enzyme-bound intermediate, and that octanoyl-adenylate inhibits lipoylation [5,6]. The lipoylation reaction therefore resembles that of the well-characterized biotin protein ligase [7]. Studies by Tsunoda and Yasunobu [8] showed that the mammalian lipoylation system uses a similar reaction mechanism, and that octanoate can be used as a substrate.

Overexpression of a lipoyl-domain subgene of *E. coli* produces both lipoylated and unlipoylated forms, suggesting that amplification of domain synthesis exceeds the cell's capacity for lipoylation [9–11]. It was further shown that the domain is octanoylated during amplification in a lipoate-deficient host [11]. Octanoylation has also been observed in a wild-type host, e.g. when lipoyl domains are overproduced in a glycerol-containing production medium [10,11] and when the lipoyl domain of *Bacillus stearothermophilus* is overproduced in *E. coli* [12]. Similarly, it has been found that 80% of the H-protein of the bovine glycine-cleavage system is unlipoylated and inactive when overproduced in *E. coli*, whereas 10% is lipoylated, and the remainder contains another substituent, possibly octanoate [13]. The fact that octanoylation occurs raises questions concerning the relationship between lipoate biosynthesis and the mechanism of protein lipoylation, particularly with respect to the stage in lipoate biosynthesis at which the protein is modified [11].

Studies on the lipoylation system of *E. coli* previously indicated that the lipoate protein ligase (LPL) exists in two forms (LPL-A and LPL-B), both exhibiting distinct substrate and metal-ion specificities [14]. Interestingly, both forms used D-lipoate (the natural enantiomer), lipoyladenylate and octanoyladenylate, but only one (LPL-B) used octanoic acid and L-lipoate as substrates, the latter being used at only one-third the rate of D-lipoate. In this context it should be noted that the L and D enantiomers were erroneously interchanged in Brookfield et al. [14]. The *E. coli* enzyme (LPL-B) was also shown to lipoylate the lipoyl apo-domain of the human PDH complex [15]. It was further observed that the *E. coli* PDH complex is poorly lipoylated after amplification during anaerobic growth, but the resultant apo-PDH complex could be re-activated by LPL-mediated lipoylation of the complex [14].

More recently, mutants lacking LPL activity have been isolated by starting with a lipoate-deficient mutant (*lipA*) and screening for resistance to selenolipoate and for the inability to use exogenous lipoate, while retaining the ability to grow on glucose minimal medium with acetate and succinate as replacement supplements [16,17]. The corresponding gene (*lplA*) was subsequently mapped, cloned by virtue of its ability to restore lipoate as a permissive growth supplement to *lipA lplA* double mutants, sequenced, and overexpressed [17].

Here the *E. coli* LPL, corresponding to the LPL-B activity of Brookfield et al. [14] and product of the *lplA* gene, has been purified from both amplified and unamplified sources, and its

Abbreviations used: DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); DTT, dithiothreitol; LPL, lipoate protein ligase; ODH, 2-oxoglutarate dehydrogenase; PDH, pyruvate dehydrogenase.

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biochemical and enzymological properties have been investigated.

EXPERIMENTAL

Bacterial strains, plasmids and growth conditions

Lipoyl apo-domain was purified from *E. coli* JM101(pGS331) and assayed densitometrically after non-denaturing PAGE [10]. *E. coli* W3110 (wild-type) and JRG2854, a pGS199 (*fnr*$^+$) transformant of CAG627 (*lacZ trp pho supC*$_{ts}$ *mal rpsL lon*) [18], served as unamplified sources of LPL; TM202, a pTM70 transformant of BL21 λ DE3 [17], was the source of overproduced LPL; RB979 (*leu lacY supE44 rpsL tonA ΔdeoD-serB-trpR-arcA-thr*) [19] was the *arcA* (and *lplA* deletion strain); and JRG1728 [Δ*lac*(*IPOZYA*) X74 *galU galK rpsL* Δ(*ara-leu*) Δ(*tyrR-fnr-rac-trg*) *zdd-230*::Tn*9*] was the *fnr* deletion strain [20]. All cultures were grown at 37 °C.

Unamplified LPL was isolated from cultures of *E. coli* W3110 and related strains, grown aerobically in L broth [21], harvested in early stationary phase, and resuspended at 1 g wet wt./ml in LPL buffer [20 mM Tris/HCl, pH 7.5, containing 1 mM EDTA, 10% (v/v) glycerol and 0.1 mM phenylmethanesulphonyl fluoride]. It was more frequently isolated from highly aerated cultures of JRG2854 (normally used as a source of FNR) after growth in a 15-litre Biolafitte fermenter using a glycerol-based production medium [10]. Anaerobic cultures were grown in freshly autoclaved phosphate-buffered yeast extract/peptone medium [22] in sealed bottles. Cultures of the LPL-amplifiable source, TM202, were grown with vigorous shaking, in L broth containing 50 μg of ampicillin/ml to D_{650} = 0.6–1.0, at which point T7 RNA polymerase synthesis was induced by adding isopropyl β-D-thiogalactopyranoside (0.4 mM final concn.), followed after 45 min by rifampicin (100 μg/ml), and aerobic incubation was then continued for 2.5 h before harvesting.

Purification of LPL from *E. coli*

(a) Purification from the unamplified source

Bacteria resuspended in LPL buffer were disrupted by three passages through a French pressure cell (137 MPa) and the extract was clarified by centrifuging (25000 *g* for 2 h at 4 °C). One-fifth of the crude extract, containing about 1.2 g of protein in 40 ml, was applied to a heparin–agarose column (1.5 cm × 25 cm) pre-equilibrated with LPL buffer. The column was washed with 150 ml of LPL buffer and eluted with a 200 ml linear gradient of 0–300 mM $(NH_4)_2SO_4$ in LPL buffer. This step was repeated five times until all of the extract had been chromatographed. Fractions containing LPL activity were pooled and dialysed against LPL buffer (16 h at 4 °C) and then concentrated to 10–20 ml by ultrafiltration. This sample was applied to a Q-Sepharose column (1 cm × 30 cm) pre-equilibrated with LPL buffer, then washed with 100 ml of buffer and eluted with a 120 ml linear gradient of 0–120 mM $(NH_4)_2SO_4$ in LPL buffer. Fractions containing LPL were pooled and dialysed and then applied to a Green HE4BD–Sepharose dye affinity column (1.5 cm × 11 cm), equilibrated with LPL buffer containing 5 mM $MgCl_2$. The column was washed with 75 ml of the starting buffer, and eluted with a 125 ml linear gradient of 0–200 mM NaH_2PO_4 in the same buffer. Finally, the pooled and dialysed LPL-containing fractions were chromatographed on Mono-Q (1 ml; Pharmacia LKB) by using two gradients, 0–140 mM $(NH_4)_2SO_4$ in LPL buffer (20 ml) followed immediately by 140–400 mM $(NH_4)_2SO_4$ in LPL buffer (6 ml).

(b) Purification from the amplified source

Suspensions of LPL-amplified TM202 bacteria were disrupted, and the cell-free extract (310 mg of protein) was fractionated by heparin–agarose chromatography as described above. The LPL-containing fractions were pooled, dialysed against LPL buffer and concentrated by ultrafiltration before purification on Q-Sepharose (see above). Any minor contaminants were removed by repeating the Q-Sepharose step.

PAGE and protein estimation

Protein samples obtained during the purification of LPL were analysed by SDS/PAGE in a 15% gel (0.1% SDS in the gel and running buffer) as described by Laemmli [23]. Proteins were stained with Coomassie Brilliant Blue (0.1%) or silver-stained [24]. The purity of the samples was determined by quantitative densitometry of stained gels (BioProfil imaging system, Vilber-Lourmat). Protein was estimated by the dye-binding method of Bradford [25] with BSA as the standard.

Electrophoretic assay for LPL

LPL activity was assayed at 30 °C by incubating the lipoyl apo-domain (2.1 μM) with DL-lipoic acid (60 μM), ATP (80 μM), $MgSO_4$ (3.2 mM), sodium phosphate buffer (25 mM, pH 7.0) and extract (reaction volume 30 μl) for an appropriate period depending on the concentration and activity of LPL. The reaction was terminated by heating at 70 °C for 1 min, and the samples were analysed by non-denaturing PAGE [10,11]. The amount of modified domain was determined densitometrically, and 1 unit of LPL activity was defined as the amount of LPL required to modify 1 nmol of lipoyl apo-domain in 1 min at 30 °C.

Radioactive assay for LPL

Modification of lipoyl apo-domain was also determined by monitoring incorporation of DL-[2-^{3}H]lipoic acid (10 mCi/mmol; kindly supplied by Professor S. J. Yeaman and Dr. A. Masters) as described previously [17,26]. The reaction conditions were identical with those of the electrophoretic method except for the use of [^{3}H]lipoate. After termination, samples (30 μl) of the reaction mixtures were spotted on 3MM filter discs and air-dried before three solvent washes with chloroform/methanol/acetic acid (3:6:1, by vol.). Once dry, the discs were immersed in 5 ml of the scintillant Safefluor S and counted for radioactivity in a Beckman LS1801 scintillation counter. The amount of lipoate incorporated was determined after correcting for incorporation in the absence of LPL. The LPL specific activities were calculated and expressed in the units described above. Even in the linear range, the activities obtained by the radioactive assay were only one-tenth of those obtained by the electrophoretic assay, in experiments designed to assay the same reaction by both methods. Furthermore, the rates measured by the radioactive assay were linear for shorter periods, so the discrepancy between the two assays increased with time. The reason for the difference is not clear, but it may be due to changes in quenching such that the counting efficiency of radioactive lipoate is lower when bound to the lipoyl domain. This makes the radioactive procedure less useful than anticipated for determining absolute rates, although in other respects the results obtained with the two assays are entirely comparable. The activities reported here derive from the electrophoretic assay unless stated otherwise.

Assays for the PDH and ODH complexes

The specific activities of the PDH and ODH complexes were determined by monitoring substrate-dependent reduction of 3-acetylpyridine–adenine dinucleotide (APAD) in crude extracts at 30 °C [27].

Other methods

The native M_r of LPL was determined by gel filtration on a calibrated Sephacryl S200 column (1.6 cm × 30 cm) equilibrated with LPL buffer [28]. The pI of LPL was determined with a Phast system (Pharmacia LKB) using precast Phast gels IEF 3-9. Thiol-group titrations were carried out as described by Thelander [29]. Proteolytic cleavage was studied by using trypsin (0.1 %, w/w) and chymotrypsin (0.5 %, w/w), either alone (for 7 h) or in combination (for 17 h) at 30 °C. The resultant peptides were resolved by SDS/PAGE (22.5 % gel) and then transferred to a Fluorotrans membrane for N-terminal sequence analysis by using an Applied Biosystems 477A liquid-pulse sequencer.

LPL was crystallized from a solution of LPL (9.9 mg/ml) in 20 mM Tris, pH 7.5, set down at 20 °C against the Crystal Screen kit (Hampton Research) by the Sandwich Drop method. Crystallization was initially observed in Reagent 36 [0.1 M Tris/HCl, pH 8.5, and 8 % (w/v) poly(ethylene glycol) (PEG) 8K]. The components of this reagent were altered in further trials, resulting in 24 different conditions varying in pH (7.5–9.0) and the percentage of precipitant, PEG 8K (6.5–12.0 %), to optimize the crystallization conditions.

Materials

DL-Lipoic acid (DL-6,8-thio-octic acid) and dihydro-DL-lipoic acid were purchased from Sigma, and octanoic acid was from BDH. D-Lipoic acid [(*R*)(+)] and L-lipoic acid [(*S*)(−)] were kindly provided by Asta Pharma AG, and 8-methyl-lipoic acid was from American Cyanamid. Lipoyladenylate, octanoyl-adenylate and the lipoate analogues were synthesized as described previously [5,16]. Chromatographic media were obtained from Pharmacia LKB, the Green HE4BD dye was from ICI, and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) from Sigma.

RESULTS

Purification of LPL

(a) Choice of starting material and regulation of LPL synthesis

Because previous studies had indicated that LPL is present in extremely low abundance in *E. coli* [30], several different strains and growth conditions were tested to ensure that the most enriched starting material was used for purifying LPL. In each case the LPL activities were estimated by the electrophoretic procedure starting with extracts that had been purified by heparin–agarose chromatography in order to remove contaminating protease activity (see below). No striking induction or repression of LPL activity was observed in aerobic cultures of *E. coli* W3110 in different media, with or without added lipoate (24 μM), or at different stages in the growth cycle. The best activity (0.21 unit/mg of protein) was observed in samples taken during the exponential phase, and partially purified extracts from all of the strains tested had activities similar to that of W3110.

LPL activity was absent from extracts of anaerobically grown W3110. The anaerobic repression of LPL was further studied with strains lacking the anaerobic transcription regulators FNR (JRG1728) and ArcA (RB979), to investigate whether the absence of an anaerobic repressor might enhance aerobic expression, as is sometimes observed for tricarboxylic acid-cycle enzymes [31]. LPL activity was detected in aerobically grown JRG1728 (0.18 unit/mg of protein), but not after anaerobic growth (< 0.004 unit/mg of protein), indicating that FNR is not involved in regulating LPL synthesis. In contrast, no LPL activity could be detected in comparable extracts of either aerobically or anaerobically grown cultures of the ArcA-deficient strain, RB979. This suggested that the gene encoding LPL (*lplA*) might be located near the *arcA* gene in the segment of DNA that is deleted in RB979 (Δ*deoD-thr*, 99.4–100 min). An alternative explanation, that ArcA functions as an aerobic activator of the *lplA* gene, seemed less plausible, because ArcA usually functions as an anaerobic repressor. The first possibility was subsequently confirmed when the *lplA* gene was located at approx. 99.6 min in the *E. coli* linkage map [17]. However, despite the lack of LPL activity, extracts of aerobically grown RB979 retained normal PDH- and ODH-complex activities (results not shown). These observations strongly indicate that *E. coli* contains an alternative lipoylation system. This conclusion is substantiated by the fact that *lplA* mutants lack a nutritional phenotype [17]. However, no lipoylation could be detected in extracts of RB979 by using both assay systems and several substrates, namely DL-lipoate, octanoate, lipoyladenylate and octanoyladenylate (results not shown).

(b) Purification of LPL from unamplified *E. coli*

A typical purification of LPL from *E. coli* strain JRG2854 is summarized in Table 1(a) and an SDS/PAGE fractionation of samples taken at each step is shown in Figure 1(a). Two distinct peaks of LPL activity were resolved by chromatography on heparin–agarose, as observed previously [14]. The minor component (formerly designated LPL-A) was not bound to the resin, whereas the major component (LPL-B) was bound and subsequently eluted at an $(NH_4)_2SO_4$ concentration of approx. 120 mM. Despite exhibiting distinct substrate and metal-ion specificities [14], LPL-A was progressively converted into LPL-B by repeated fractionation on heparin–agarose, and it was concluded that LPL-B is a modified form of LPL-A (or vice versa), and that both are products of the *lplA* gene. The LPL-B component was further purified (see the Experimental section) and a good correlation was observed between the LPL activity profile and the intensity of a polypeptide of M_r 38000 during fractionation on Green HE4BD–Sepharose (Figure 1a, lane 4). Then, after chromatography on Mono-Q, densitometric analyses of silver-stained SDS/PAGE gels indicated that LPL is apparently homogeneous (Figure 1a, lane 5), although the presence of minor contaminating proteins cannot be ruled out, due to the very small amount of LPL protein available for analysis.

The yield of pure LPL protein was only 7.2 μg from 5.9 g of soluble bacterial protein (Table 1a). The overall purification factor (5100) may be significantly underestimated, due to the effects of proteolysis on quantifying LPL activities in crude extracts. Proteolysis resulted in the modified and unmodified forms of the lipoyl domain becoming less well defined or even indistinguishable, such that the degraded apo-domain co-migrates with the modified domain. This causes substantial overestimation of the LPL activity in crude extracts, which in turn may account for the high apparent loss of LPL activity (87 %) during heparin–agarose chromatography. The actual purification factor at this stage (50-fold) could therefore be underestimated by a further factor, which could be as great as 8-fold, if it is assumed that there is little or no loss of LPL activity at the heparin step. This assumption is substantiated to a large extent by observations made when purifying LPL from an

Table 1 Purification of LPL from *E. coli*

The enzyme was purified from (**a**) the unamplified source JRG2854 and (**b**) the genetically amplified source TM202. The electrophoretic assay was used throughout; 1 unit of LPL activity is defined as the amount of LPL required to modify 1 nmol of lipoyl apo-domain in 1 min at 30 °C.

Step	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Specific activity (units/mg)	Total activity (units)	Overall purification	Overall yield (%)
(**a**)							
Crude extract	192.0	31.0	5952.0	5.4×10^{-3}	32.1	1	100.0
Heparin–agarose chromatography (combined fractions)	190.0	0.08	15.2	0.27	4.1	50	12.8
Heparin–agarose chromatography (concentrated)	14.0	0.91	12.7	0.22	2.8	41	8.7
Q-Sepharose chromatography (combined fractions)	20.0	0.15	3.0	0.33	1.0	61	3.1
Green HE4BD chromatography (combined fractions)	15.0	0.0014	0.02	31.4	0.6	5800	1.9
Mono-Q chromatography (combined fractions)	4.0	0.0018	0.0072	27.6	0.2	5100	0.6
(**b**)							
Crude extract	20.0	15.50	310	36.8	11400	1.0	100.0
Heparin–agarose chromatography (combined fractions)	50.0	0.98	49	45.3	2200	1.2	19.5
Q-Sepharose chromatography (combined fractions)	30.0	0.86	26	9.4	2050	2.2	18.0

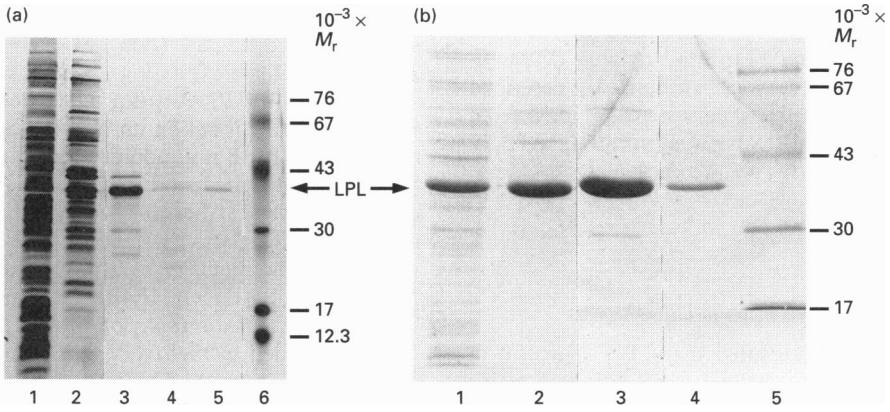

Figure 1 Purification of LPL from unamplified and amplified sources

Pooled samples from consecutive steps in the purification of LPL were analysed by SDS/PAGE (15% gel). **(a)** Purification from the unamplified source, JRG2854. Silver-stained gel with (μg of protein): lane 1, crude extract (4.5 μg); lane 2, heparin–agarose (0.7 μg); lane 3, Q-Sepharose (0.75 μg); lane 4, Green HE4BD–Sepharose (0.03 μg); lane 5, Mono-Q (0.02 μg); lane 6, molecular-mass markers. **(b)** Purification from the amplified source. Coomassie-Blue-stained gel with (μg of protein): lane 1, crude extract (10 μg); lane 2, heparin–agarose (6 μg); lane 3, Q-Sepharose (6 μg); lane 4, second Q-Sepharose (7 μg); lane 5, molecular-mass markers. Samples of LPL from both sources co-migrated with a mobility corresponding to an apparent M_r 38000.

amplified source (see below), so the overall purification factor could likewise exceed 5100 by a further factor of up to 8-fold.

Purification of LPL from a genetically amplified source, TM202

LPL was overproduced in TM202 and purified by a two-step chromatographic procedure (see the Experimental section). A typical purification is summarized in Table 1(b) and Figure 1(b). Densitometric analyses of Coomassie-Brilliant-Blue-stained SDS/PAGE gels indicated that LPL comprises approx. 16% of soluble protein in cell-free extracts of the amplified strain. Only one peak of LPL activity was recovered from heparin–agarose chromatography. It resembled LPL-B by being eluted at an $(NH_4)_2SO_4$ concentration of approx. 125 mM. The lack of LPL-A activity could be attributed to the strain or to the growth conditions employed. After further purification on Q-Sepharose,

Table 2 Kinetic parameters for LPL

Kinetic parameters were determined by the electrophoretic assay with pure overproduced LPL of specific activity 48.7 units/mg of protein (as estimated electrophoretically) and by the radioactive assay with partially purified unamplified LPL of specific activity 0.23 unit/mg of protein (as estimated electrophoretically). Each assay was performed in duplicate, and the concentration of the relevant reactant was varied. The concentration ranges of the varied substrates were: 0.25–100 μM for ATP, 2.5–3200 μM for $Mg^{2+}$and 0.3–100 μM for DL-lipoic acid. The concentrations of the fixed substrates were: 80 μM ATP, 3.2 mM $Mg^{2+}$and 60 μM DL-lipoic acid. The data were analysed by Lineweaver–Burk (LB) and Eadie–Hofstee (EH) plots with linear regression analysis (correlations within the range 0.999–0.980).

	Electrophoretic assay				Radioactive assay			
	K_m (μM)		$V_{max.}$ (units/mg)		K_m (μM)		$V_{max.}$ (units/mg)	
	LB	EH	LB	EH	LB	EH	LB	EH
ATP	1.9	2.8	40	55	3.0	3.1	0.007	0.007
DL-Lipoic acid	1.7	1.7	24	39	1.7	3.3	0.038	0.027
Mg^{2+}	152	179	28	31	450	483	0.007	0.005

the overproduced LPL was $\geqslant$ 98% pure and had a subunit M_r of 38000 (Figure 1b, lane 4). The similarity between the heparin–agarose and Q-Sepharose elution profiles obtained with the amplified and unamplified proteins, and their co-migration during SDS/PAGE, indicates that the overproduced LPL and LPL-B are the same protein. The proteolytic activity in crude extracts again made it difficult to estimate the degree of purification achieved at the heparin–agarose stage. The overall purification could therefore be as high as 11-fold rather than 2.2-fold (Table 1b), if it is assumed that there is no loss of activity at the first stage. Factors of about 6.3-fold obtained from densitometric analyses fell between the two limits, and may thus more accurately reflect the overall degree of purification.

The final specific activity of the amplified enzyme (79 units/mg of protein) was approx. 3-fold higher than that of the pure enzyme from the unamplified source. This could be because the latter suffers a higher degree of oxidative inactivation during its protracted purification, or to the presence of undetected contaminating protein in the preparations from the unamplified source. The addition of reducing agents did not significantly improve the specific activity of the unamplified enzyme, except in the restoration of activity to samples inactivated during Mono-Q chromatography (see below). Some of the experiments reported below were in consequence done with enzymes having different specific activities, but there were no significant differences in their properties.

Physico-chemical characterization of purified LPL

The subunit and native M_r values were estimated as 38000 by SDS/PAGE and 41700 by gel filtration (respectively), indicating that LPL is a monomeric protein. The pI of LPL determined by isoelectric focusing was 5.80, in good agreement with the pI value of 6.02 predicted from the amino acid composition of the protein. Crystallization trials have so far yielded small clusters (0.07 mm in diameter) of very fine needle-like crystals. They were not of sufficient quality for structural studies, and attempts to improve the crystallization conditions are continuing. The UV–visible spectrum of LPL was unremarkable: absorption maximum 280 nm ($\epsilon_{280} = 46250\ M^{-1}\cdot cm^{-1}$) and unchanged by pre-incubation with lipoate, ATP and Mg^{2+}.

Kinetic characterization and pH optimum

The basic substrate requirements for lipoic acid, ATP, Mg^{2+} and lipoyl apo-domain were confirmed for LPL purified from both amplified and unamplified sources. The apparent K_m and $V_{max.}$ values for the reaction components (ATP, DL-lipoic acid and Mg^{2+}) were determined by both the electrophoretic and radioactive assays (Table 2). The K_m values for ATP (1.9–3.1 μM) and DL-lipoic acid (1.6–5.0 μM) are very similar to those observed with the *S. faecalis* enzyme [5]. The high affinity of LPL for lipoate should ensure that most of the lipoate available to the cell is capable of being utilized. The K_m values for ATP and Mg^{2+} are comparable with those of other ATP-utilizing enzymes, e.g. kinases, synthetases and adenyltransferases.

The pH optimum was investigated by replacing the routine sodium phosphate buffer by citrate/phosphate, phosphate or Tris buffers, to cover the pH range 3–9. Two activity peaks were observed with oxidized lipoate as the substrate, one at approx. pH 4.0 and the other at pH 6.8. This was replaced by a broad peak when reduced DL-lipoate was provided (under anaerobic conditions): $\geqslant$ 70% of the maximum activity was retained from pH 5.5 to 8.0 and none was observed below pH 4.3. The significance of these observations is not known, unless it indicates that lipoylation normally involves a reduced substrate.

Substrate specificity

Lipoate protein ligase purified from unamplified sources catalysed the modification of lipoyl apo-domains with several substrates at different specific activities: DL-lipoate (30 units/mg); dihydro-DL-lipoate (24 units/mg); D-lipoate (25 units/mg); L-lipoate (11 units/mg); octanoate (3.9 units/mg) and 8-methyl-lipoate (22 units/mg); and when DL-lipoate and ATP are replaced by lipoyladenylate (17 units/mg) or octanoyladenylate (29 units/mg). This shows that, *in vitro*, the enzyme is significantly more active with the naturally occurring D-lipoate than with the L-enantiomer. This contrasts with the observation that, although both enantiomers are taken up by *E. coli* (D-lipoate being twice as effective as L-lipoate), only D-lipoate is incorporated into the PDH complex *in vivo* [32].

The specific activity observed for pure amplified enzyme with DL-lipoate and lipoyl apo-domain from the *E. coli* PDH complex (66 units/mg of protein) was 11-fold higher than that observed with apo-domain from the human mitochondrial PDH complex. Also, when the same enzyme was tested with the 6- and 8-thio-octanoates, the 6- and 8-seleno-octanoates and selenolipoate as substrates, modification of the *E. coli* apo-domain was only detected with 6-thio-octanoate (98 units/mg) and selenolipoate (3.7 units/mg). However, these substrates had lower affinities than lipoate for LPL (Table 3). So, despite the high modification

Table 3 Kinetic parameters for LPL with lipoate analogues

The electrophoretic assay for LPL activity was used with the lipoate analogue in place of DL-lipoic acid. The concentration of the analogue was varied within the range 12–500 μM for 6-thio-octanoate and 1–5 mM for selenolipoate; the other substrate concentrations and analytical methods were as described in the legend to Table 2. Each assay was performed in duplicate, and linear regression analysis gave correlations in the range 0.997–0.980.

	$V_{max.}$ (units/mg)		K_m (mM)		$V_{max.}/K_m$ (units · mg^{-1} · mM^{-1})	
	LB	EH	LB	EH	LB	EH
6-Thio-octanoate	207	150	1.6	1.1	129	136
Selenolipoate	6.2	5.1	13×10^{-3}	10×10^{-3}	476	510
DL-Lipoic acid	24	39.1	1.6×10^{-3}	1.6×10^{-3}	15000	24400

Table 4 Re-activation of lipoate-analogue-treated LPL

Pure LPL (1 unit; 0.02 mg of overproduced protein in LPL buffer) was inactivated by preincubating with 8-thio-octanoate (2.8 mM), 6-seleno-octanoate (0.7 mM) or 8-seleno-octanoate (2.8 mM), for 10 min at 20 °C in a final volume of 650 μl. Analogous control samples of LPL were treated in the same way, but without analogue. After reaction, samples were then dialysed against LPL buffer at 4 °C for 16 h to remove excess analogue and, where stated, samples were incubated with reducing agents at 0 °C, before assay of LPL activity by the standard electrophoretic method. ND indicates that no LPL activity could be detected (< 2 units/mg).

Reducing agent (mM)	Preincubation time (min)	Activity after treatment (units/mg of protein)			
		Untreated	8-Thio-octanoate	6-Seleno-octanoate	8-Seleno-octanoate
None	30	9	ND	ND	ND
None	330	9	ND	ND	ND
DTT (10)	30	17	ND	ND	14
DTT (10)	330	> 42	ND	ND	> 42
DTT (200)	30	9	ND	ND	10
β-Mercaptoethanol (200)	30	> 42	4	15	> 42

rate with 6-thio-octanoate, its very low affinity for LPL suggests that this substrate would not be used in preference to exogenous lipoate.

The lipoate analogues that failed to modify the lipoyl apodomain were found to inhibit lipoylation by DL-lipoate, the most potent inhibitor being 6-seleno-octanoate. A 10 min treatment with this analogue at 0.28 mM completely inhibited LPL activity. Similar treatments with 8-thio-octanoate or 8-seleno-octanoate caused 50% or 63% inhibition respectively. The LPL activity was not restored by dialysing analogue-treated samples, indicating that inhibition is not simply due to competition between analogue and lipoate for the active site. Indeed, analogue-treated LPL failed to enter the resolving gel (20%) in native gel electrophoresis, suggesting that the inhibitory analogues cause protein aggregation. The aggregates dispersed during SDS/PAGE, without pretreatment with β-mercaptoethanol, indicating that they are non-covalent aggregates.

Analogue-inactivated samples of LPL could be re-activated by treating with reducing agents (Table 4). Samples inactivated by 8-seleno-octanoate were most readily re-activated by DTT (10 mM for 330 min at 4 °C) or β-mercaptoethanol (200 mM for 30 min at 4 °C). It is also apparent that the thiol compounds restore activity to the untreated samples which declines from 50 to 9 units/mg of protein during the experiment (Table 4). These observations suggest that reversible inactivation involves the thiol groups associated with one or more of the six cysteine residues in the LPL subunit. Titrations with DTNB showed that there are six reactive thiol groups per native LPL subunit, but none could be detected in either native or denatured samples of LPL after inactivation with 8-thio-octanoate or 8-seleno-octanoate. Thus it appears that all six thiol groups of LPL are free to react with inhibitory analogues, and that inactivation is probably due to the formation of disulphide or seleno-sulphide bonds between the enzyme and substrate analogue at the active site. This primary modification may induce further conformational changes, which in turn promote reactions between the analogue and remaining cysteine thiol groups, leading to denaturation of the protein. Newly exposed regions of denatured LPL could then undergo hydrophobic and/or ionic interactions with the formation of inactive protein aggregates.

Metal-ion requirement of the lipoylation reaction and inhibition by Cu^{2+}

The reaction catalysed by amplified and unamplified LPL required Mg^{2+}, which could be replaced by Mn^{2+}, Co^{2+}, Fe^{2+} or Ni^{2+}, but not by Cu^{2+}, Cs^{+} or K^{+}. These specificities confirm that purified LPL corresponds to the previously identified LPL-B enzyme, rather than LPL-A, which used Cu^{2+} but not Fe^{2+} [14]. Minor discrepancies concerning the replacement of Mg^{2+} by Ca^{2+} and Fe^{3+} are probably due to the use of partially pure LPL-B at the limits of detectable activity. It was further observed that lipoylation is 50% inhibited by 130 μM Cu^{2+} and completely inhibited by 3.2 mM Cu^{2+}, even in the presence of 3.2 mM Mg^{2+}.

Transition metals are known to participate in many biological redox reactions, including the oxidation of thiol groups to disulphides [33]. The Cu^{2+}-catalysed oxidation and inactivation of LPL was therefore investigated by thiol-group titration with DTNB (Table 5). Untreated LPL had a full complement of six reactive thiol groups per molecule of protein, and hence no

Table 5 Effects of Cu^{2+} on LPL cysteine thiol-group reactivity

Thiol-group titrations with DTNB were carried out after treating purified LPL (1.33 nmol) with different concentrations of Cu^{2+}. The A_{412} was monitored until the reaction was complete; the change in A_{412} was then related to the amount of DTNB reacted.

Treatment (mol of Cu^{2+}/mol of protein)	Reactive thiol groups (mol/mol of protein)
0	6.4
0 (+8 M urea)	5.6
7.5	5.8
7.5 (+8 M urea)	3.9
1600	4.8
1600 (+8 M urea)	1.2

Table 6 Re-activation of LPL activity

Purified LPL, stored for 3 months at 4 °C to lower its activity from 55 to 9 units/mg of protein, was re-activated by treatment with reducing agents and assayed immediately for LPL activity by the electrophoretic method. Re-activation factor was added directly to the assay mixture without preincubation.

Treatment or addition to the assay	Activity (units/mg of protein)
None	9
DTT (10 mM; 30 min at 4 °C)	17
DTT (10 mM; 330 min at 4 °C)	> 41
DTT (200 mM; 30 min at 4 °C)	9
β-Mercaptoethanol (200 mM; 30 min at 4 °C)	> 41
Re-activation factor (5 μl)	> 41

disulphide bonds, but the complement was significantly decreased by treating with Cu^{2+}, especially in the presence of 8 M urea. Furthermore, SDS/PAGE analysis in the absence of β-mercaptoethanol showed that samples of native protein treated with 7.5 mol of Cu^{2+}/mol of protein had the same mobility as untreated protein, whereas treatment with 1600 mol of Cu^{2+}/mol of protein resulted in substantial polymerization, probably due to the formation of one or more intermolecular disulphide bonds (Figure 2). The latter sample also contained a species of lower apparent M_r (34000), which probably represents an oxidized form of LPL containing at least one intramolecular disulphide bond (Figure 2, lane 3). These observations suggest that Cu^{2+} inhibition involves the formation of inter- and intramolecular disulphide bonds, and hence inactive protein conformations.

Stability and re-activation

Samples of LPL purified from the amplified source were stable for several months at −20 °C. The activity declined steadily during storage at 4 °C, e.g. from 55 to 9 units/mg of protein over 3 months, but it could be substantially restored by preincubation with 200 mM DTT or β-mercaptoethanol (Table 6). Parallel SDS/PAGE analyses showed that the stored enzyme (9 units/mg of protein) contains two polypeptides of apparent M_r 38000 (40 %) and 34000 (60 %), the latter reverting entirely to M_r 38000 upon re-activation with β-mercaptoethanol (Figure 2, lane 5). Thus it seems likely that the M_r-34000 component of the aged sample contains an intramolecular disulphide bond, and that it corresponds to the analogous component in the Cu^{2+}-treated enzyme (Figure 2, lane 3). This correlation between LPL inactivation and appearance of the M_r-34000 species suggests that the inactivation of LPL during storage is due to its progressive conversion into an oxidized form having a higher electrophoretic mobility. Storage at 4 °C for 5 months led to an almost irreversible loss of activity that was accompanied by conversion into the putative oxidized form (M_r 34000) and into a degraded or proteolysed form of M_r 22000 (Figure 2, lanes 7 and 8), possibly due to the presence of contaminating proteases in the preparation.

The purification of LPL from unamplified sources was accompanied by unaccountable activity losses with increasing purity. Indeed, activity was sometimes completely lost during

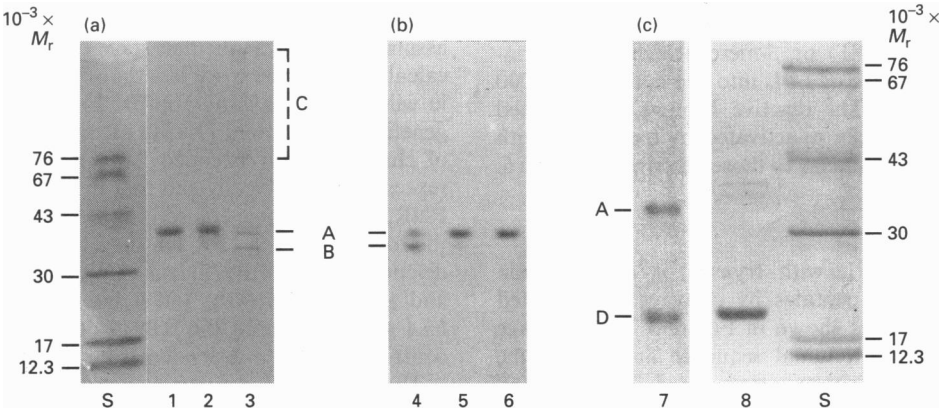

Figure 2 Effects of Cu^{2+} on stability of LPL

Coomassie-Blue-stained SDS/PAGE gels (15%) containing variously treated samples of LPL. **(a)** Pure LPL (1.33 nmol) treated with Cu^{2+} for 10 min at 30 °C before analysis in the absence of β-mercaptoethanol: lane 1, no treatment; lane 2, 7.5 mol of Cu^{2+}/mol of protein: lane 3, 1600 mol of Cu^{2+}/mol of protein. **(b)** Pure LPL (0.4 μg) stored at 4 °C for 3 months: no treatment before analysis (lane 4); treated for 30 min at 4 °C with 200 mM β-mercaptoethanol (lane 5); and heated for 3 min at 100 °C in the presence of 200 mM β-mercaptoethanol (lane 6). **(c)** Pure LPL (2 μg) stored at 4 °C for 5 months (lane 7) and 7 months (lane 8) before heating for 3 min at 100 °C in the presence of 200 mM β-mercaptoethanol. Individual components are marked: A, native LPL (M_r 38000); B, putative oxidized form of LPL containing an intramolecular disulphide bond (M_r 34000); C, polymerized forms of LPL; and D, degraded form of LPL. The molecular masses of standard proteins are indicated (lane S).

Figure 3 Proteolytic cleavage of LPL

The amino acid sequence of LPL, showing potential cleavage sites for trypsin (**bold**) and chymotrypsin (***bold italic***). The sites of actual cleavage observed by N-terminal analysis of fractionated digestion products are denoted: t (trypsin) and c (chymotrypsin). The LPL amino acid sequence was deduced from the nucleotide sequence of the *lplA* gene [17]. The amino acid sequence containing the three α-helices in the DNA-binding domain of BirA [41,45] and the ATP-binding site of methionyl-tRNA synthetase (MetRS [44]) are aligned with the LPL sequence; the helices in BirA (α), and the conserved (★) and conservatively substituted (:) residues, are indicated.

fractionation on Mono-Q. In such circumstances, the inactive LPL fractions could be re-activated by undiluted portions of a specific fraction eluted at 350 mM $(NH_4)_2SO_4$ and having no inherent LPL activity. The 'so-called' re-activation factor retained full activity after treatment with heat (100 °C for 10 min) or trypsin (0.01 mg/ml for 1 h at 37 °C) and after storage for 2.5 years at 4 °C. It had no UV–visible spectrum and was not detected on silver-stained SDS/polyacrylamide gels in the range $M_r \geqslant 10000$, but it did contain DTNB-reactive material. The re-activation factor remains uncharacterized, but it seems unlikely to be a protein. It is more probably a potent reducing agent, because it restored activity to aged (oxidized) LPL without any need for preincubation (Table 6) and because it mimics the action of dithiothreitol (DTT) or β-mercaptoethanol in converting the M_r-34000 species of LPL into the active M_r-38000 form (results not shown). The inactive Mono-Q-fractionated protein could also be partially re-activated by treatments with DTT or β-mercaptoethanol similar to those described in Table 6.

Proteolysis

Proteolytic cleavage of LPL with trypsin or chymotrypsin generated different sets of peptides by cleavage at a limited number of the potential sites shown in Figure 3. The cleavage sites were identified by N-terminal sequence analysis of the fractionated products, whose sizes (M_r 20000–22000) corresponded to protease-resistant C-terminal segments of the protein. Apart from undigested protein having an N-terminal sequence that lacks the initiating methionine residue, no other large peptides ($M_r > 10000$) were detected. This suggests that the N-terminal segment is substantially proteolysed to small peptides, leaving a C-terminal core, or, more probably, that the folded LPL protein has a particularly susceptible central region, whereat cleavage allows extensive degradation of the N-terminal region. Interestingly, the protease-resistant core has the same size (M_r 22000) as the degradation product observed after prolonged storage of LPL at 4 °C (Figure 2, lanes 7 and 8).

DISCUSSION

The covalent modification of the PDH complex and other proteins by lipoate has been neglected until recently. Purification of LPL from an unamplified source of *E. coli* proved to be extremely difficult, due to its very low abundance. However, the recent cloning and over-expression of the corresponding *lplA* gene [17] has provided a highly enriched source and facilitated the characterization of the LPL protein. Based on the specific activity of the pure enzyme (79 units/mg of protein), and assuming no loss at the first stage of purification, it can now be calculated that there are less than 10 molecules of LPL per cell in wild-type *E. coli* and approx. 2×10^5 molecules per cell after genetic amplification. During attempts to maximize expression of chromosomally encoded LPL, it was noted that activity is repressed anaerobically and that this response is independent of FNR, the anaerobic transcription regulator. Parallel studies on the role of ArcA in LPL synthesis showed that the chosen *arcA* deletion mutant (RB979) lacks LPL activity during both aerobic and anaerobic growth, and it later became apparent that the *lplA* gene lies within the RB979 deletion [17]. The mechanism controlling oxygen-dependent LPL synthesis remains unknown.

The lack of LPL activity in anaerobically grown cultures could provide a means of down-regulating PDH-complex activity so as to favour pyruvate metabolism via pyruvate formate-lyase. However, active PDH complex is synthesized during both fermentative and anaerobic (nitrate) respiratory growth [34–36]. This indicates that *E. coli* possesses a second lipoylating system which is capable of operating under these conditions, but even this activity must be limiting because the PDH complex is poorly lipoylated when amplified during anaerobic growth [14]. The existence of an alternative mechanism for lipoylation is also

evident from the lack of a nutritional phenotype in *lplA* mutants [17] and the fact that *lplA* mutants and *lplA* deletion strains produce catalytically active 2-oxoacid dehydrogenase complexes. Lipoylation of the glycine-cleavage system of *E. coli* has also been observed under conditions of complete anaerobiosis [37], i.e. conditions in which LPL activity is absent. The second lipoylating activity of *E. coli* appears to require the product of the *lipB* gene [17], although it is as yet undetectable *in vitro*. The presence of two or more enzymes catalysing the same tricarboxylic acid-cycle reaction is not unusual in *E. coli*, where multiple forms of fumarase, aconitase, citrate synthase, succinate dehydrogenase and lipoamide dehydrogenase have been identified (reviewed in [38]). In view of its intimate connection with the operation of the tricarboxylic acid cycle, and the existence of more than one lipoylatable enzyme, it is probably not too surprising that lipoylation is catalysed by at least two enzymes. It will be interesting to see whether the two processes exhibit different specificities for the target apo-enzymes.

It is becoming clear that LPL and the unidentified *lipB*-dependent enzyme use different lipoyl donors [17,39]. Current evidence clearly indicates that exogenously supplied D-lipoate is the preferred substrate for LPL, and that octanoyl – and thiooctanoyl–PDH complexes are not intermediates in the lipoylation process mediated by LPL, whereas the *lipB*-dependent activity relies on an endogenous source of lipoate supplied via the *lipA*-dependent biosynthetic route [39]. This again raises questions concerning the relationship between lipoate biosynthesis and protein lipoylation, particularly with respect to the stage at which lipoate or a precursor is bound to the lipoyl apo-domain [11]. Studies with extracts of *lplA* mutants have so far failed to detect apo-domain modification with lipoate, octanoate, octanoyl-CoA, octanoyladenylate or lipoyladenylate, so the nature of the substrate used by the *lipB*-dependent activity is unknown (results not shown). It seems likely that this activity is responsible for most of the octanoylation that occurs under lipoate-deficient conditions [39], so the precursor may be an intermediate or product of fatty acid biosynthesis. The identification and characterization of the second lipoylating activity clearly provides an interesting research priority.

The lipoate-activating system of *Streptococcus faecalis* resolves into two components [5]. One is heat-labile and is thought to catalyse the formation of lipoyladenylate, which is used by the heat-stable second component for lipoyl transfer to apo-PDH complex. The LPL activity purified from *E. coli* is a single monomeric entity. However, when purified from an unamplified source, the 'so-called' re-activation factor was often required to restore activity after Mono-Q chromatography. The effect of the re-activation factor was mimicked by reducing agents, even with Mono-Q inactivated LPL, and it is possible that the heat-stable component found in *S. faecalis* is an essential cofactor or reductant corresponding to the re-activation factor observed here. Conversion of the fraction of crude-extract LPL activity that fails to bind to heparin–agarose (LPL-A) into the binding form (LPL-B) during rechromatography might involve removal of some unidentified factor from LPL-A. It is not known whether the re-activation factor has a role in this conversion. However, it is clear that LPL-A and LPL-B are products of the same gene (*lplA*) and that they do not correspond to the two lipoylating activities discussed above.

The LPL-catalysed reaction resembles that catalysed by the aminoacyl-tRNA synthetases, some of which contain essential thiol groups [40]. The correlation between lipoate-analogue-induced inactivation of LPL and loss of thiol reactivity further strengthens the relationship between these enzymes. LPL and BirA (biotin protein ligase) likewise catalyse analogous reactions in the respective ligations of lipoate and biotin to specific apoproteins via adenylated intermediates [7]. Both proteins are monomeric, and it is possible that LPL resembles BirA in functioning additionally as a transcription regulator that responds to the adenylated intermediate. The structure of BirA has been defined [41]. It contains a small N-terminal domain with a DNA-binding helix–turn–helix motif, a central catalytic domain which binds biotin and ATP, and a small C-terminal domain. Amino acid sequence alignments for BirA and LPL show little sustained similarity. No motif resembling the ATP-binding site of BirA (GRGRRGR) or the corresponding consensus (GxGxGxER) of Class II aminoacyl-tRNA synthetases was found in LPL [41,42]. However, there is a putative DNA-binding domain displaced about 60 residues from the N-terminus (Figure 3). Here, three conserved and three conservatively substituted residues align with residues in the DNA-recognition helix (α_C) of BirA, and secondary-structure predictions further indicate that LPL contains helices aligning with α_A and α_B of BirA. Despite the apparent conservation of a DNA-binding domain, preliminary gel-retardation assays using pure LPL and labelled DNA fragments containing the promoter regions of the *lplA*, *lipA*, *lipB* genes and the *smp* gene (immediately upstream of *lplA*) failed to detect any LPL-dependent DNA-binding activity with or without lipoate plus ATP and lipoyladenylate (results not shown). However, further experiments, including footprinting studies, will be needed before a role for LPL in transcription regulation can be excluded. The lack of sustained sequence similarity between LPL and BirA does not preclude the possibility that these enzymes have the same basic structure. Indeed, the catalytic domains of BirA and serine tRNA synthetase (a Class II aminoacyl-tRNA synthetase) have recently been found to exhibit a striking structural similarity, even though there is no significant sequence similarity [43]. In this context it may be significant that the LPL sequence contains a motif resembling the ATP-binding motif of Class I aminoacyl-tRNA synthetases, e.g. methionyl-tRNA synthetase (Figure 3) [42,44]. It is therefore possible that LPL may be more related to the Class I enzymes than to BirA and the Class II enzymes. Further comparative studies with BirA and both classes of aminoacyl-tRNA synthetase could therefore shed more light on the catalytic and other potential activities of LPL.

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