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Evidence for two protein-lipoylation activities in *Escherichia coli*

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The lipoate acyltransferase subunits of the 2-oxo acid dehydrogenase complexes are post-translationally modified with one or more covalently-bound lipoyl cofactors. Two distinct lipoate-protein ligase activities, LPL-A and LPL-B, have been detected in *E. coli* by their ability to modify purified lipoyl apo-domains of the bacterial pyruvate dehydrogenase complex. Both enzymes require ATP and Mg^{2+}, use L-lipoate, 8-methyllipoate, lipoyl adenylate and octanoyl adenylate as substrates, and both activate lipoyl-deficient pyruvate dehydrogenase complexes. In contrast, only LPL-B uses D-lipoate and octanoate and there are differences in the metal-ion and phosphate requirements. It is suggested that LPL-B may be responsible for the octanoylation of lipoyl domains observed previously under lipoate-deficient conditions.

Lipoate-protein ligase; Protein acylation; Lipoyl domain; Pyruvate dehydrogenase complex; Post-translational modification; *Escherichia coli*

1. INTRODUCTION

The lipoate acyltransferase subunits (E2o and E2p) of the 2-oxoglutarate dehydrogenase (ODH) and pyruvate dehydrogenase (PDH) complexes of *Escherichia coli* contain one or three lipoyl domains which are post-translationally modified by N^6-lipoylation of specific lysine residues [1–4]. Early work on the lipoate activation–ligation systems of *E. coli* and *Streptococcus faecalis* by Reed and coworkers showed that ATP, Mg^{2+} and phosphate are required for PDH apo-complex activation, and that lipoate and ATP can be replaced by lipoyl adenylate, which may serve as an enzyme-bound intermediate [5,6]. It was also shown that excess octanoyl adenylate inhibits the activation of PDH apo-complex [5] and that octanoate is used as a substrate by the mammalian lipoate-activating enzyme [7], but the formation of octanoylated protein was not explored.

More recently, the over-expression of a lipoyl-domain subgene of *E. coli* has been shown to generate lipoylated and unlipoylated domains, indicating that the amplification of domain synthesis can exceed the cell's capacity for lipoylation [8–10]. It was further observed that the lipoylated domain is replaced by an octanoylated product during over-expression in a lipoate-deficient host [10]. This novel modification is not normally observed under lipoate-sufficient conditions, although approximately half of the modified domains were octanoylated when a glycerol-containing production medium was used [9,10]. Lipoylated and unlipoylated forms of *Bacillus subtilis* lipoyl domain are likewise produced during amplification in *E. coli* and a minor fraction appears to be octanoylated [11].

Octanoylation represents a novel protein modification raising questions regarding the route of lipoate biosynthesis and the mechanism of protein lipoylation. Here, evidence is presented for the existence of two independent lipoate-protein ligase activities in *E. coli*, LPL- A and LPL-B. Both are capable of lipoylating the lipoyl apo-domains and activating PDH apo-complex, but only one (LPL-B) can utilise octanoate.

Abbreviations: LPL, lipoate-protein ligase; E2p, lipoate acetyltransferase; E2o, lipoate succinyltransferase; EDTA, diaminoethane tetra-acetate; IPTG, isopropyl β-thiogalactoside; ODH, 2-oxoglutarate dehydrogenase; PAGE, polyacrylamide gel electrophoresis; PDH, pyruvate dehydrogenase; PMSF, phenylmethylsulphonyl fluoride.

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2. MATERIALS AND METHODS

2.1. *Lipoyl apo-domain and lipoate-protein ligase (LPL)*

Lipoyl apo-domain was purified from *E. coli* JM101(pGS331) and assayed densitometrically after PAGE [9]. Lipoate-protein ligase was purified from aerobically-grown cultures of *E. coli* CAG627, harvested 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 PMSF). Clarified French press extracts were applied to heparin agarose (100 mm × 27 mm) in LPL buffer and eluted with a linear gradient of 0–300 mM $(NH_4)_2SO_4$ in 400 ml of LPL buffer. Further purification of the unbound fraction (LPL-A) was achieved by gel filtration on Sephacryl HR200 (900 mm × 30 mm) eluted with LPL buffer.

LPL was assayed by incubating lipoyl apo-domain (0.6 μg) with ATP (80 μM), DL-lipoate (60 μM), $MgCl_2$ (3.2 mM), sodium phosphate buffer (25 mM, pH 7.0) and extract, in a final volume of 30 μl, for 2–4 h at 30°C. Reactions were terminated by heating (70°C, 1 min) and analysed for modified domain by non-denaturing PAGE: lipoylated and octanoylated domains have a higher mobility than apo-domain [9,10]. Modified domain was quantified by densitometry of gels stained with Coomassie brilliant blue. Protein was estimated with the Biorad protein reagent. One unit of LPL activity was defined as the

amount of LPL needed to modify 1 nmol of lipoyl apo-domain in 1 min at 30°C.

2.2. *PDH complex assay and trypsin digestion*

PDH complexes were isolated at greater than 50% purity from *E. coli* JRG2433, an *aceEF-lpd*-deletion strain in which complexes containing one lipoyl domain per E2p chain are expressed from pGS367 by IPTG-induction. Cultures, grown in L-broth supplemented with glucose (0.2%) and ampicillin (50 μg/ml), were induced with IPTG (60 μM) at A_{650} = 0.25-0.50 and incubated for a further 6 h (aerobic) or 16 h (anaerobic). French press extracts in 20 mM potassium phosphate buffer (pH 7.8, containing 2 mM EDTA, 1 mM benzamidine and 1 mM PMSF) were clarified and the PDH complex sedimented by centrifugation for 4 h at 100 000 × *g* and 4°C. One unit of PDH complex activity [12] corresponds to 1 μmol NADH formed per min per mg protein. Samples of PDH complex (38 μg complex in 70 μg protein; 115 μl, final volume) were treated with trypsin (0.3 μg) for 1 h at 30°C in potassium phosphate buffer (20 mM, pH 7.0; containing 2.7 mM EDTA). Reactions were stopped by heating (70°C, 10 min) and the lipoyl domains detected in clarified supernatants by non-denaturing PAGE.

2.3. *Materials*

Lipoic acid (DL-6,8-thioctic acid) was purchased from Sigma and octanoic acid from BDH. D- and L-lipoic acid were kindly provided by Asta Pharma AG and 8-methylliopoic acid from American Cyanamid. Lipoyl adenylate and octanoyl adenylate were prepared as previously described [5].

3. RESULTS

3.1. *Partial purification and characterization of two LPL activities*

Two LPL activities were resolved when *E. coli* extracts were fractionated by heparin-agarose chromatography (Fig. 1). One designated LPL-A was not bound, whereas the other, designated LPL-B, was bound and eluted by 60 mM $(NH_4)_2SO_4$. Gel filtration chromatography indicated that both enzymes have the same molecular weight (47 kDa approx.) but tests with preparations of comparable specific activity showed that they had distinct substrate specificities. In both cases modification of the apo-domain required lipoate, ATP and Mg^{2+}, but LPL-B differed in using octanoate as an alternative substrate (Fig. 1).

In further studies it was found that LPL-A and LPL-B could use L-lipoate and 8-methyllipoate as substrates, but D-lipoate and octanoate were only used by LPL-B, and neither enzyme was active with hexanoic, decanoic, dodecanoic or hexadecanoic acids (Table I). Both enzymes required Mg^{2+}, which could be replaced by Mn^{2+}, Co^{2+}, Zn^{2+}, or Ni^{2+}. In addition, LPL-A used Cu^{2+} and LPL-B used Ca^{2+}, Fe^{2+} and Fe^{3+}, but neither enzyme could use K^+, Rb^+, or Mo^{6+} in place of Mg^{2+}. The enzyme required ATP, but in both cases ATP and lipoate could be replaced by lipoyl adenylate and octanoyl adenylate. It would appear that the primary difference between LPL-A and LPL-B is the failure of LPL-A to convert octanoate to octanoyl adenylate, since the preformed intermediate can be used to modify the apo-domain. At a later stage of purification, LPL-B became phosphate-dependent. The significance of this is not clear because the phosphate requirement observed previously was thought to be associated with the generation of ATP and not with the lipoylation reaction [5].

3.2. *Activation of PDH apo-complex*

The specific activity of the PDH complex is not linearly related to the degree of lipoylation of the E2p subunits [13,14], this was deduced from the relative amounts of lipoylated and unlipoylated domain released from partially-purified PDH complexes by tryptic proteolysis [2,15]. Studies on the effects of inducing PDH-complex synthesis under different conditions

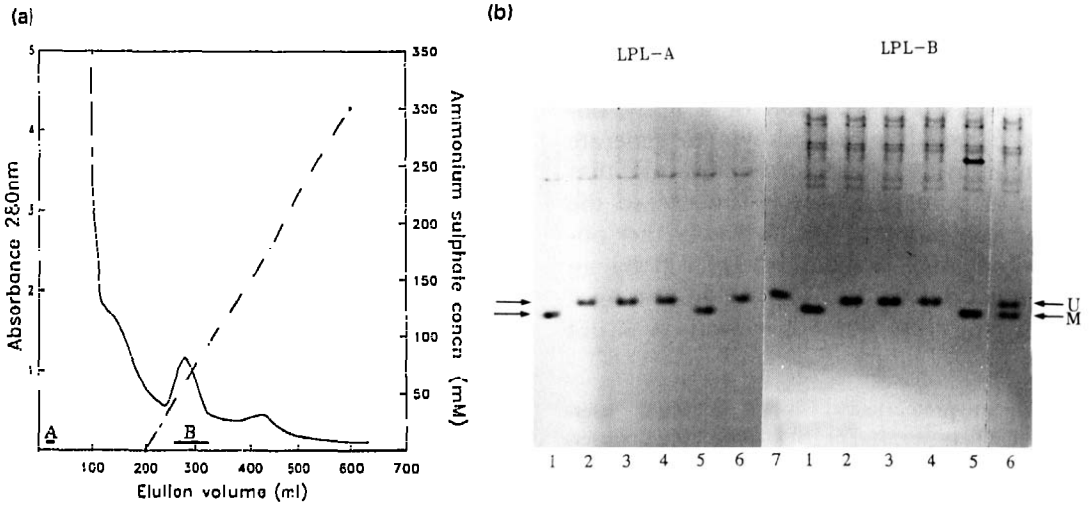

Fig. 1. Fractionation of lipoate-protein ligase on heparin agarose and substrate specificities. (a) Elution profile with bars to denote fractions containing LPL activity: A_{280} (—); ammonium sulphate gradient (- -). (b) Native polyacrylamide gels illustrating cofactor requirements and substrate specificities for LPL-A and LPL-B. The LPL-A was purified further by gel-filtration (see Table I for specific activities). The lanes represent: 1, complete system with lipoate; 2, no lipoate; 3, no ATP; 4, no Mg^{2+}; 5, no phosphate; 6, octanoate instead of lipoate; 7, lipoyl apo-domain alone. The modified and unmodified domains are denoted M and U, respectively.

Table I

Substrate specificities for LPL-A and LPL-B

Substrate	Omission	Relative activity (%)	
		LPL-A	LPL-B
DL-lipoate	none	100	100
DL-lipoate	phosphate	90	100*
DL-lipoate	Mg^{2+}	ND	ND
DL-lipoate	ATP	ND	ND
Lipoyl adenylate	ATP	35	68
Octanoyl adenylate	ATP	76	77
L-lipoate	none	115	77
D-lipoate	none	ND	25
8-Methyllipoate	none	25	54
Octanoate**	none	ND**	10**

The standard LPL assay was used: 100% corresponds to a specific activity of 0.05 U/mg for LPL-A and 0.27 U/mg for LPL-B; relative activities were reproducible within ±5% of quoted values; ND denotes that no activity (<1%) could be detected; *indicates that a phosphate requirement became apparent on further purification of LPL-B; **indicates that hexanoate, decanoate, dodecanoate and hexadecanoate were not active as substrates with either enzyme.

showed that very little lipoylation occurs during anaerobic growth, this provided a good source of PDH apo-complex for testing activation by LPL-A and LPL-B. Both enzymes activated the apo-complex and this was accompanied by increased amounts of the modified domain in the corresponding tryptic digests (Fig. 2).

4. DISCUSSION

The presence of two apparently independent lipoylating activities in *E. coli* raises questions concerning their physiological roles. Their properties show that LPL-B has a broader specificity than LPL-A since it exhibits no stereospecificity for its substrate and can use octanoate. The two enzymes can modify lipoyl domains from the PDH complex in both the free and complexed state. However, their specificities for lipoyl domains of different origin have not been investigated, it is possible that each enzyme may have a preference for lipoylation sites in the PDH or ODH complexes. In this context it is relevant that the E2 subunit of the *B. subtilis* PDH complex is lipoylated in *E. coli* [11], but that of the bovine branched chain 2-oxo acid dehydrogenase complex is not [16].

It would appear that LPL-B is responsible for the octanoylation of amplified lipoyl domains, observed under lipoate-deficient conditions [10]. During normal growth little octanoylation is detected, and this is consistent with octanoate being a poorer substrate than lipoate for LPL-B. It seems doubtful whether octanoylation plays a significant role in regulating the activities of the 2-oxo acid dehydrogenase complexes. The existence of two distinct lipoate-protein ligases needs to be confirmed at the genetic level, but the absence of lipoylation-deficient mutants amongst those

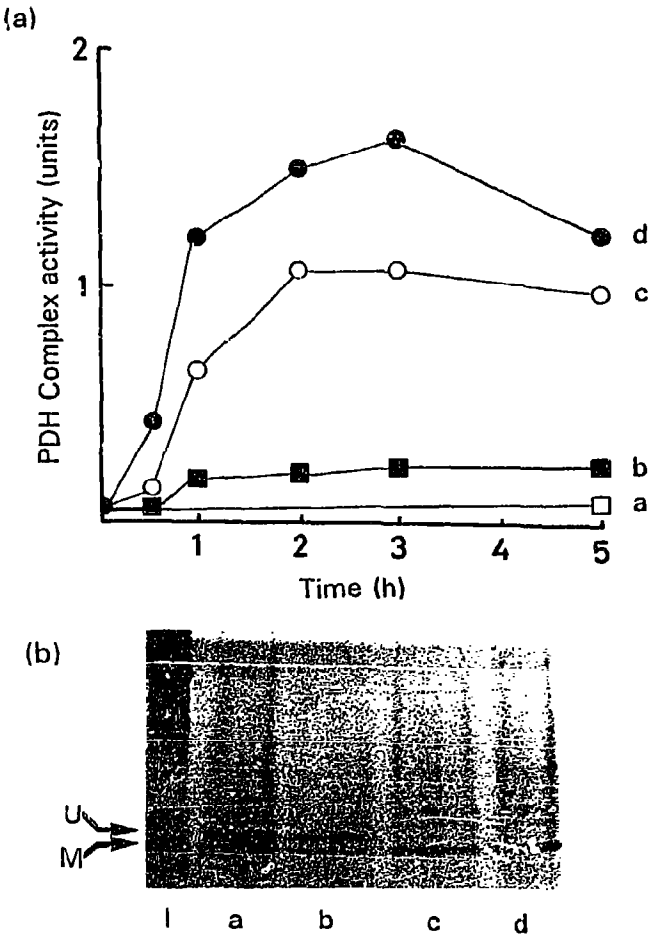

Fig. 2. Activation of PDH apo-complex by lipoate-protein ligases A and B. (a) Partially-purified PDH apo-complex was incubated with: ○, LPL-A plus cofactors; ●, LPL-B plus cofactors; ■, cofactors alone; □, no additions. Reactions contained 120 μg of protein (66 μg PDH complex) in 200 μl of LPL reaction mixture with 6 units of LPL-A or 13 units of LPL-B (when added), and samples (12 μl) were assayed for PDH complex activity [12] at different times. (b) Non-denaturing PAGE analysis of 5 h samples from (a) after trypsin treatment (see section 2): a, no additions; b, cofactors alone; c, LPL-A plus cofactors; d, LPL-B plus cofactors; l, sample derived from fully-lipoylated PDH complex. The positions of unmodified domain (U) and modified domain (M) are indicated.

with dual lesions in PDH and ODH complex activities (e.g. lipoamide dehydrogenase mutants) is consistent with the existence of 2 mutually- complementing genes.

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