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Lipoylation of the E2 components of the 2-oxo acid dehydrogenase multienzyme complexes of *Escherichia coli*

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The number of functional lipoyl groups in the dihydrolipoyl acetyltransferase (E2) chain of the pyruvate dehydrogenase multienzyme complex from *Escherichia coli* has been re-assessed by means of a combination of protein-chemical and mass-spectrometric techniques. (1) After the complex had been treated with *N*-ethyl[2,3-^{14}C]maleimide in the presence of pyruvate, the lipoyl domains were excised from the complex, treated with $NaBH_4$ and re-exposed to *N*-ethyl[2,3-^{14}C]maleimide. All the chemically reactive lipoyl groups in the native complex were found to be catalytically active. (2) Proteolytic digests of the separated lipoyl domains were examined for the presence of the lipoylation-site peptide, GDKASME, with and without the lipoyl group in N^6-linkage to the lysine residue. Only the lipoylated form of the peptide was detected, suggesting that all three lipoyl domains are fully substituted at this site. (3) The behaviour of each lipoyl domain was examined on ion-exchange chromatography in response to alkylation with 4-vinylpyridine after either chemical reduction of the lipoyl group with dithiothreitol or reductive acetylation by the pyruvate dehydrogenase complex in the presence of pyruvate. All three domains exhibited a quantitative shift in retention time, confirming that each domain was fully substituted by an enzymically reactive lipoyl group. (4) When subjected to electrospray mass spectrometry, each domain gave a mass consistent with a fully lipoylated domain, and no aberrant substitution of the target lysine residue was detected. The same result was obtained for the lipoyl domain from the *E. coli* 2-oxoglutarate dehydrogenase complex. (5) Previous widespread attempts to assess the number of functional lipoyl groups in the pyruvate dehydrogenase multienzyme complex, which have led to the view that a maximum of two lipoyl groups per E2 chain may be involved in the catalytic mechanism, are in error.

INTRODUCTION

The 2-oxo acid dehydrogenase complexes are among the most extensively studied multienzyme systems, in terms of both structure and mechanism (for reviews, see Perham *et al.*, 1987; Perham & Packman, 1989; Guest *et al.*, 1989; Patel & Roche, 1990). The dihydrolipoyl acyltransferase (E2) component is particularly important because it is the second of three enzymes (E1, E2, E3) that must act sequentially in the overall reaction and it also serves as the structural core of the complexes (Reed, 1974). The domain structure of the E2 component in several organisms has been elucidated by means of the combined techniques of limited proteolysis, electron microscopy, sequence analysis (DNA and protein) and n.m.r. spectroscopy (reviews cited above; Reed & Hackert, 1990). It consists of one to three *N*-terminal lipoyl domains, each of which bears a lipoyl prosthetic group bound in amide linkage to the N^6-amino group of a lysine residue; the lipoyl domains are linked to each other and thence to an E3-binding domain by segments of flexible polypeptide chain; the E3-binding domain is in turn linked to an inner core domain, which assembles to form the structural core (octahedral or icosahedral) of the complex and which, in some instances, binds E1. The lipoyl-lysine side chain is responsible for shuttling acyl groups and reducing equivalents between the active sites of E1 (2-oxo acid dehydrogenase), E2 and E3 (dihydrolipoamide dehydrogenase), with the acyltransferase active site being located in the inner-core domain of the E2 component.

The stoicheiometry of the lipoylation of the three lipoyl domains of the E2p component (EC 2.3.1.12) of the PDH complex of *Escherichia coli* has been measured in many ways. Danson & Perham (1976) first demonstrated the existence of more than one acetylatable lipoyl group per E2p chain and, over the years, numerous studies in this and other laboratories came to suggest values of 1.7–2.0 [reviewed by Packman *et al.* (1984*a*)]. Similar values were derived from alkylation of reductively acetylated E2p with *N*-ethyl[2,3-^{14}C]maleimide, as well as from alternative approaches based on isotope-dilution methods (White *et al.*, 1980). However, Hale & Perham (1979) isolated the complex from bacteria grown on ^{35}S-containing medium, and, from the incorporation of radiolabel into the complex, estimated a stoicheiometry of three lipoyl groups per E2p chain. Finally, the amino acid sequence of the E2p chain, derived from the sequence of the encoding *aceF* gene, demonstrated the presence of three potential lipoylation sites (Stephens *et al.*, 1983) and each potential lipoyl-lysine residue was shown to inhabit a functional lipoyl domain (Packman *et al.*, 1984*a*; Allen *et al.*, 1989). The extent of substitution of the E2o chain (EC 2.3.1.61) of the *E. coli* 2-oxoglutarate dehydrogenase complex has been less controversial, estimates being close to 1 mol of lipoyl group per chain (Collins & Reed, 1977; White *et al.*, 1980; Perham & Roberts, 1981).

We have now carried out a series of further experiments designed to resolve the puzzle of the number of lipoyl groups present in the *E. coli* E2p chain. These are based in part on our recent study of the lipoylation of a lipoyl domain of the E2p chain of the *Bacillus stearothermophilus* PDH complex expressed in *E. coli* (Dardel *et al.*, 1990). Some of these methods have also been applied to the E2o chain of the *E. coli* 2-oxoglutarate dehydrogenase complex. The results indicate that the three lipoyl domains of the *E. coli* E2p chain are fully lipoylated and that all

Abbreviations used: DTT, dithiothreitol, PDH, pyruvate dehydrogenase; Nbs_2, 5,5′-dithiobis-(2-nitrobenzoic acid).

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these groups are functional in the enzymic reaction. The single lipoyl domain of the *E. coli* E2o chain also appears to be fully lipoylated.

MATERIALS AND METHODS

Materials

PDH and 2-oxoglutarate dehydrogenase complexes were purified from *E. coli* as described previously (Danson *et al.*, 1979). *N*-Ethyl[2,3-^{14}C]maleimide and [2-^{14}C]pyruvate were from Amersham International, Amersham, Bucks., U.K., and methyl [^{3}H]acetimidate was synthesized as described by Armstrong *et al.* (1980). The sources of proteinases and sequencing reagents are given in Packman *et al.* (1984*a*). Reagents used in all procedures were analytical grade or better.

Borohydride reduction and alkylation of lipoyl domains

PDH complex was labelled with *N*-ethyl[2,3-^{14}C]maleimide in the presence of pyruvate (Danson & Perham, 1976). The radiolabelled lipoyl domains were excised from the complex by limited proteolysis with *Staphylococcus aureus* V8 proteinase and purified as a mixture by gel filtration (Packman & Perham, 1987). The domains (58 μg in 200 μl of 50 mM-sodium phosphate buffer, pH 7.0) were dried and resuspended in 50 μl of 8 M-guanidine hydrochloride. The thiol group of the acetylated lipoyl group of each domain was regenerated by adding 15 μl of 3 M-$NaBH_4$ in water. After 15 min at room temperature, the mixture was acidified with 20 μl of 20% (v/v) acetic acid, which lowered the pH from 9.5 to 5.5. After 15 min, the solution was buffered with 50 μl of 1.5 M-Tris/HCl, pH 8.4, containing 25 μM-DTT; the resulting pH was 7.5. (In some experiments, the original lipoyl domains were in 50 μl of 50 mM-sodium phosphate buffer, pH 7.0; these incubations required 80 μl of Tris/DTT.) A control, lacking protein, was tested with Nbs_2 (see below) and excess borohydride was shown to have been destroyed. Alkylation with *N*-ethyl[2,3-^{14}C]maleimide [4 μl of 42 mM reagent in acetonitrile (final concn. 1 mM)] was allowed to proceed at room temperature under N_2 protection for 60 min, followed by a second addition of fresh reagent and incubation for a further 60 min. The reaction was quenched with 2 μl of 2-mercaptoethanol (final concn. 20 mM). The incorporation of radiolabel was assessed by collecting trichloroacetic acid-precipitated protein on glass-fibre filters, followed by scintillation counting (Graham *et al.*, 1989), or by exhaustive dialysis against 0.25% (w/w) NH_4HCO_3 at 4 °C followed by scintillation counting, either directly or after acid precipitation. As controls, samples of domain were reduced and alkylated with non-radioactive *N*-ethylmaleimide or treated with *N*-ethyl[2,3-^{14}C]maleimide without prior reduction. These procedures confirmed that the incorporated radiolabel was stable to borohydride treatment and that further incorporation of *N*-ethyl[2,3-^{14}C]maleimide was dependent on reduction. Further controls of known amounts of free lipoamide (95 nmol) were included to check the efficiency of the reduction procedure; after the neutralization step with Tris buffer, the thiol content of the mixture was determined by making the volume up to 1 ml with 1 mM-Nbs_2 in 20 mM-Tris/HCl, pH 8.4, and reading the absorbance at 412 nm (ϵ_{1cm} 13600).

Protein-chemical techniques

Protein concentrations were determined by amino acid analysis (Packman *et al.*, 1988). Lipoyl domains LpV1, LpV2 and LpV3 were purified individually from *E. coli* PDH complex as described previously (Packman & Perham, 1987). Amidination of lipoyl domains with methyl [^{3}H]acetimidate was carried out under denaturing conditions at pH 10 designed to modify the lysine side chains specifically (Hale *et al.*, 1979). The proteins were dialysed against several changes of 10 mM-NH_4HCO_3 in Spectrapor 3 dialysis tubing until no ^{3}H was detectable in the diffusate.

Samples of amidinated lipoyl domain were digested with proteinase, and the peptides were fractionated as described in the text. Peptides were analysed by amino acid and manual sequence analysis (Packman & Perham, 1987). Peptide GDKASME was prepared from a digest of the parent synthetic peptide TVEGDKASME (Graham *et al.*, 1989) and purified by reverse-phase h.p.l.c. under conditions described above. The peptide TVEGD([^{3}H]amidino)KASME was prepared by amidination of the parent peptide with methyl[^{3}H]acetimidate, as above, and purified by h.p.l.c. It was then cleaved with *S. aureus* V8 proteinase to generate the peptide GD([^{3}H]amidino)KASME. Similarly, the N^{6}-lipoylated peptide GD(lipoyl)KASME was prepared from TVEGD(lipoyl)KASME (Graham *et al.*, 1989) and purified by h.p.l.c.

Analytical ion-exchange chromatography of purified lipoyl domains was performed on a Polypore DEAE column (2.1 mm × 30 mm; Brownlee Labs) equilibrated with 10 mM-NH_4HCO_3 at 0.2 ml/min; elution of protein was by a gradient of 0–0.2 M-NH_4HCO_3 (5 min), then with 0.2–0.4 M-NH_4HCO_3 (15 min). Samples (1 nmol) of domain were reduced (40 mM-DTT, 2 h) and alkylated (105 mM-4-vinylpyridine, 3 h) in 10–15 μl of 40 mM-Tris/HCl, pH 8.3, under Ar, at room temperature, and applied directly to the column. The high concentration of DTT used in this experiment was necessary to reduce the lipoyl group fully in a reasonable time. Reductive acetylation of lipoyl domains was achieved by incubating 1 nmol of domain at 37 °C for 20 min with 4 μg of PDH complex in 25 μl of 10 mM-sodium phosphate buffer, pH 7.0, containing 0.3 mM-thiamin diphosphate, 1.3 mM-$MgCl_2$ and 4 mM-sodium pyruvate. Subsequent alkylation with 20 mM-4-vinylpyridine was carried out for 30 min at 37 °C under Ar protection; the mixture was then applied directly to the ion-exchange column.

Mass spectrometry

Samples of lipoyl domain were prepared for analysis by electrospray mass spectrometry by freeze-drying from 50 mM-NH_4HCO_3 and freeze-drying again twice from water. The samples were redissolved in a solution of 2% acetic acid and diluted with an equal volume of methanol to give a final concentration of approx. 25–50 pmol/μl. Mass analysis was performed on a prototype BioQ quadrupole mass spectrometer, with myoglobin and bovine ubiquitin as the calibration standards.

RESULTS AND DISCUSSION

The first aim of this work was to determine whether all the lipoyl groups in *E. coli* E2p that were susceptible to chemical reduction and alkylation were also susceptible to enzymic reduction and alkylation. PDH complex was isolated from *E. coli* grown under the same conditions as used by Hale & Perham (1979), but omitting ^{35}S from the medium, to ensure that the enzyme was comparable with the ^{35}S-labelled complex in which were detected three lipoyl groups per E2p chain. Assay of the lipoyl content by reductive acetylation with [2-^{14}C]pyruvate (Bates *et al.*, 1977) gave a value of 1.6 mol/mol of E2p, closely similar to the value of 1.7 mol/mol of E2p seen with complex isolated from cells grown in rich medium (Packman *et al.*, 1984*a*, and references therein). A sample of PDH complex that was reductively acetylated with unlabelled pyruvate and then exposed to *N*-ethyl[2,3-^{14}C]maleimide (Danson & Perham, 1976) was found to incorporate radioactivity to the same level. The three

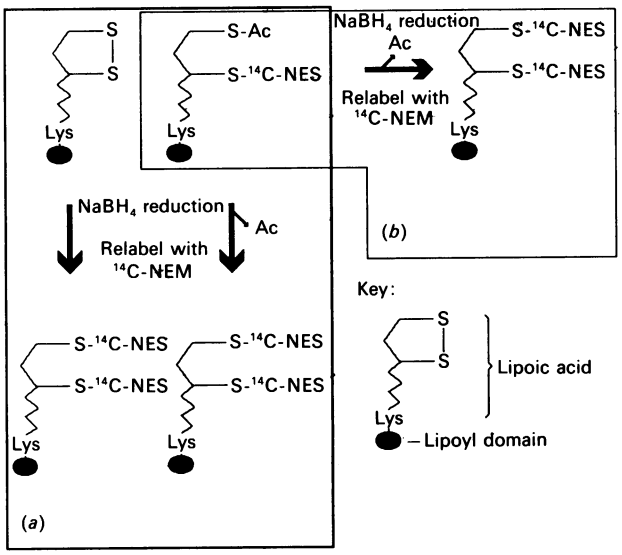
Table 1. Incorporation of *N*-ethyl[2,3-^{14}C]maleimide (NEM) into reductively acetylated lipoyl domains

Values for ^{14}C incorporation from duplicate samples of the mixture of LpV1–3 are given as mol/mol of single domain and therefore represent an average value. Protein was estimated by amino acid analysis. ^{12}C-NEM denotes the use of non-radioactive *N*-ethylmaleimide in a control experiment.

Treatment	Method of counting...	^{14}C incorporated mol/mol of domain		Ratio of ^{14}C incorporation*	
		Solution†	Precipitation	Solution	Precipitation
None		1.07	0.43±0.01	1.0	1.0
BH_4^-, ^{12}C-NEM		1.20	0.41±0.03	1.12	0.95
BH_4^-, ^{14}C-NEM		2.20	0.84±0.01	2.06	1.95
^{14}C-NEM		1.27	0.48±0.04	1.19	1.12

* Relative to reductively acetylated domain.

† The values obtained from solution counting were variable between different experiments, giving, for example, a range of 0.9–1.3 mol/mol for untreated domain, but the ratios of incorporation were more consistent; the data shown for solution counting are from a single representative set of experiments.



Scheme 1. Models for the reaction of *N*-ethyl[2,3-^{14}C]maleimide with reductively acetylated and *N*-ethyl[2,3-^{14}C]maleimide-labelled lipoyl domains after reduction with $NaBH_4$

(*a*) If, say, half the population of lipoyl domains has been reductively acetylated and alkylated and half remains catalytically unreactive, $NaBH_4$ reduction and further alkylation lead to a 4-fold increase in the incorporation of radiolabel. (*b*) If the whole population of lipoyl domains has been reductively acetylated and alkylated, $NaBH_4$ reduction and further alkylation lead to a 2-fold increase in the incorporation of radiolabel. Abbreviations: NEM, *N*-ethylmaleimide; NES, *N*-ethylsuccinimidyl.

lipoyl domains were excised from the *N*-ethyl[2,3-^{14}C]maleimide-labelled complex by treatment with *S. aureus* V8 proteinase and purified as a mixture of LpV1, LpV2 and LpV3. By liquid-scintillation counting of the radiolabelled domains, ^{14}C incorporation was estimated to be 0.9–1.3 mol of lipoyl group/mol of domain; by scintillation counting of the radioactivity of the lipoyl domains after acid precipitation, the estimate of ^{14}C incorporation reproducibly decreased to 40–50% of this value, more in line with the values estimated from acid precipitation and scintillation counting of the intact PDH complex (Packman *et al.*, 1984*a*). We have observed a similar decrease (up to 30%) in ^{14}C estimation between counting *N*-ethyl[2,3-^{14}C]maleimide-labelled PDH complex in solution (after dialysis to remove excess ^{14}C reagent) and after acid precipitation (results not shown).

The mixture of ^{14}C-labelled lipoyl domains were treated with $NaBH_4$ (*a*) to reduce any lipoyl groups refractory to enzymic

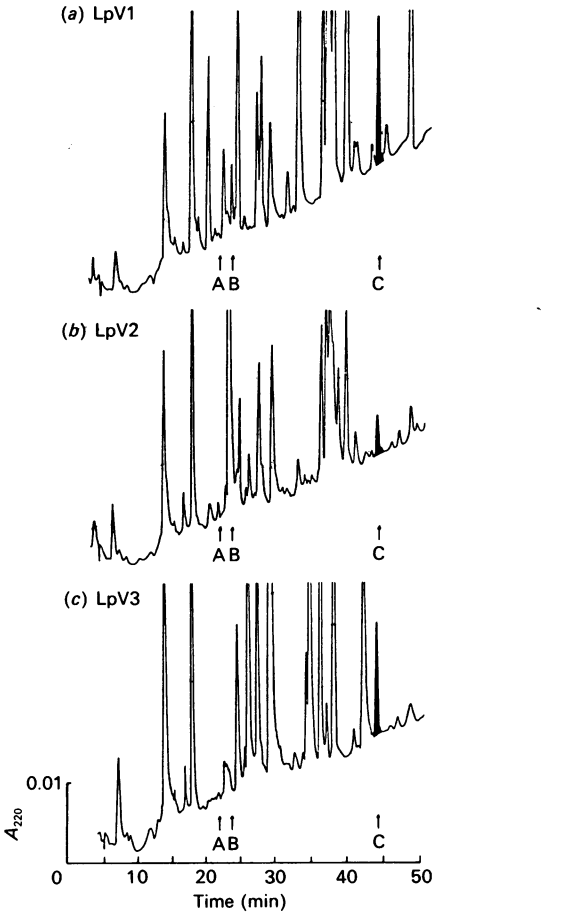


Fig. 1. Reverse-phase h.p.l.c. separation of the peptides from *S. aureus* V8 proteinase digests of [^{3}H]amidinated lipoyl domains LpV1–3 (*a–c*)

Samples (4–8 nmol) of amidinated lipoyl domain were digested at 37 °C for 18 h with 2% (w/w) *S. aureus* V8 proteinase in 0.5% (w/w) NH_4HCO_3. The resulting peptides were fractionated on a Chromspher C_{18} reverse-phase column (3 mm × 20 cm, Chrompack) in 0.1% trifluoroacetic acid with acetonitrile as organic modifier (0–35% in 45 min) at a flow rate of 0.5 ml/min. The elution positions of the model peptides are shown: (A) GDKASME; (B) GD([^{3}H]amidino)KASME; (C) GD(lipoyl)KASME. The peak in each digest identified as GD(lipoyl)KASME by sequence analysis is highlighted. The yield of all the peptides in the digest of LpV2 is lower than in the digests of LpV1 and LpV3; LpV2 appears to be more resistant to degradation by the proteinase. All peptides close to the elution positions (A) and (B) were shown by further h.p.l.c. in 10 mM-ammonium acetate, pH 5.8, with acetonitrile as organic modifier, to be different from the model peptides (see the text).

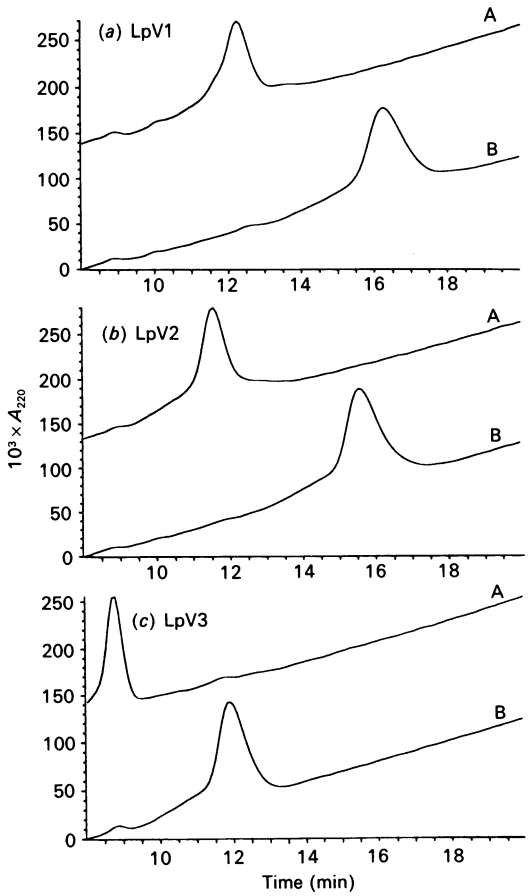


Fig. 2. Reaction of lipoyl domains LpV1–3 (*a–c*) with DTT and 4-vinylpyridine

Samples (1 nmol) of lipoyl domain were reduced with 40 mM-DTT for 2 h and then alkylated with 105 mM-4-vinylpyridine as described in the text. The samples were applied to a Polypore DEAE column and eluted with a gradient of NH_4HCO_3. (A) Lipoyl domain treated with DTT only; (B) lipoyl domain treated with DTT and then alkylated with 4-vinylpyridine.

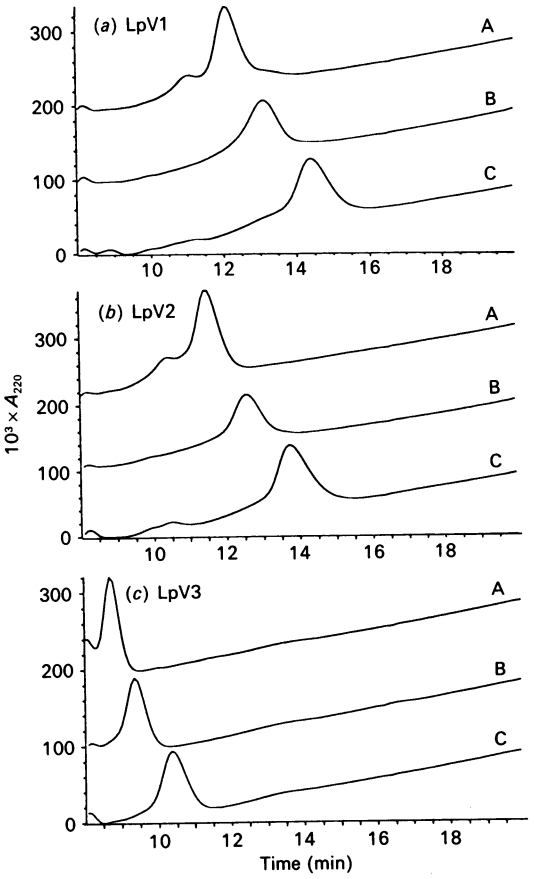


Fig. 3. Reductive acetylation of lipoyl domains LpV1–3 (*a–c*) and subsequent alkylation with 4-vinylpyridine

Samples (1 nmol) of lipoyl domain were reductively acetylated by treatment with pyruvate in the presence of catalytic amounts of PDH complex and then alkylated by treatment with 25 mM-4-vinylpyridine, as described in the text. The samples were applied to a Polypore DEAE column and eluted with a gradient of NH_4HCO_3. The minor peak preceding the untreated lipoyl domain responds to either reductive acetylation (this Figure) or chemical reduction (Fig. 2) to become part of the main peak. Its origin is unknown. Lipoyl domain: (A) untreated; (B) after reductive acetylation; (C) after reductive acetylation and alkylation with 4-vinylpyridine.

reduction and alkylation and (*b*) to remove the acetyl groups from the S^6-acetyl,S^8-(*N*-ethyl-[2,3-^{14}C]succinimidyl)lipoyl groups; the same conditions were shown to reduce over 93% of a sample of free lipoic acid. After acidification to destroy excess $NaBH_4$, and then neutralization, the domains were treated again with *N*-ethyl[2,3-^{14}C]maleimide. The reduction treatment led to a doubling of the amount of *N*-ethyl[2,3-^{14}C]maleimide incorporated into the domains (Table 1). In the absence of reduction, no significant further incorporation of radiolabel occurred. This experiment showed that all lipoyl groups in the enzyme complex must have been reductively acetylated by E1 in the presence of pyruvate: if any of the lipoyl groups had been left in an unacetylated (oxidized) form, their presence would have been detected by an increase in radiolabel incorporated after borohydride reduction, in excess of 2-fold. For example, the incorporation of ^{14}C would have risen 4-fold if only half the lipoyl groups had been available for enzymic reductive acylation (Scheme 1).

All the above experiments were carried out on a mixture of the three lipoyl domains. If some of the lipoylation sites were only partly substituted with lipoic acid, a proportion of the lipoylation sites should remain as free lysine side chains, or perhaps carry a different substituent inert to reductive acetylation. To investigate this possibility, the lipoyl domains LpV1, LpV2 and LpV3 were isolated from native PDH complex prepared from *E. coli* cells grown on rich medium, and purified separately to homogeneity. Each domain was treated with methyl [^{3}H]acetimidate to modify any free lysine residues and then digested with *S. aureus* V8 proteinase for 4 h and 18 h; on the basis of the known amino acid sequence of the lipoyl domains, this procedure should release from each domain the peptide GDKASME, the lysine residue of which is the potential site of attachment of lipoic acid. The peptides were separated by reverse-phase h.p.l.c. (Fig. 1) and the 18 h digest was seen to give the higher yield of products. Reference peptides GDKASME, GD([^{3}H]amidino)KASME and GD(lipoyl)KASME were generated from a synthetic peptide, TVEGDKASME (Graham *et al.*, 1989), and subjected to h.p.l.c. under the same conditions. The peptide GD(lipoyl)KASME was readily located in the digests of all three domains and its identity confirmed by a second dimension of h.p.l.c. and by sequence analysis. For each domain there were significant peaks corresponding closely in retention time to those of the peptides GD([^{3}H]amidino)KASME and GDKASME. However, each of these peaks was shown by a second dimension of h.p.l.c. to differ markedly from the reference peptides, and amino acid and sequence analysis of all peaks obtained in sufficient yield

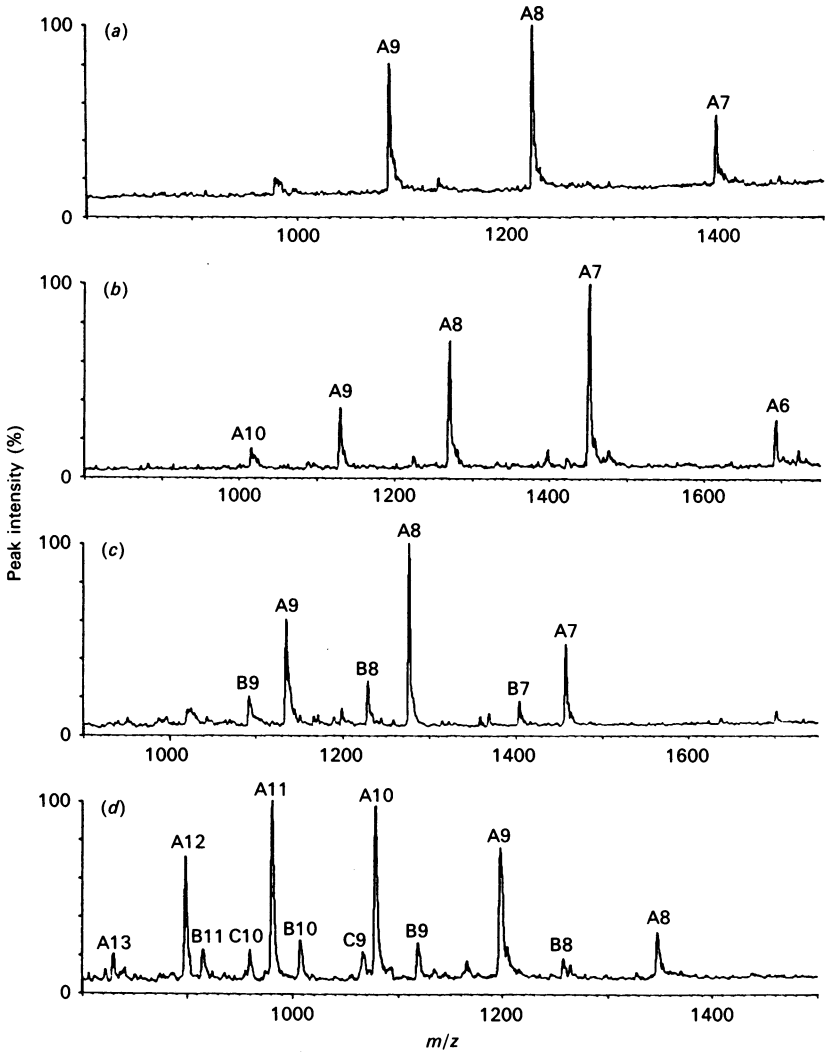


Fig. 4. Electrospray mass spectra of lipoyl domains

LpV1–3 are from the PDH complex and LoT1 is from the 2-oxoglutarate dehydrogenase complex. The numbers in the lettered series denote the charge state (n) of the $(M+nH)^{n+}$ ions. The mass scales are not the same for each spectrum. (*a*) LpV1, A series: 9764.4 Da, S.D. 0.92; (*b*) LpV2, A series: 10149.7 Da, S.D. 1.2; (*c*) LpV3, A series: 10210.2 Da, S.D. 0.2; B series: 9829.8 Da, S.D. 0.48; (*d*) LoT1, A series: 10755.6 Da, S.D. 1.97; B series: 10044.3 Da, S.D. 2.84; C series: 9572.4 Da, S.D. 1.2 (S.D. = standard deviation for the family of peaks in a series).

confirmed the absence of any unlipoylated GDKASME sequences. These experiments suggested that all potential lipoylation sites were fully lipoylated.

A further test came from characterizing the elution positions of the reduced lipoyl domains on an ion-exchange resin, Polypore DEAE, before and after alkylation. Each lipoyl domain was reduced with DTT and then alkylated with 4-vinylpyridine. Each bis(pyridylethyl)lipoyl domain was eluted at a much higher salt concentration than the unalkylated domain, presumably because of increased hydrophobic interactions with the support. Most importantly, the alkylation-dependent shift in elution position appeared to be quantitative, strongly suggesting that the domains were capable of being fully reduced and bis-pyridylethylated, and were thus fully lipoylated (Fig. 2).

To confirm that all the chemically reactive lipoyl groups were capable of acting as substrates in the reaction of the whole enzyme complex, the individual domains were reductively acetylated by treatment with pyruvate and a catalytic amount of the PDH complex, and then allowed to react with 4-vinylpyridine. The reductive acetylation alone caused a significant shift in the elution position of the domain on Polypore DEAE (Fig. 3), unlike the reduction with DTT (Fig. 2). The change in elution time was dependent on the presence of both pyruvate and enzyme in the prior treatment; clearly the interaction with the Polypore resin is sensitive to small changes in the hydrophobicity of the domain induced by acetylation. Subsequent alkylation with 4-vinylpyridine again quantitatively increased the elution time of each domain, although less markedly than with the DTT-reduced domain, since only a single pyridylethyl group had been incorporated (Fig. 3). These results all strongly suggest that the three lipoyl domains are fully lipoylated and catalytically functional.

In a final experiment, each lipoyl domain was examined by electrospray mass spectrometry. The observed masses (LpV1, 9764.4 Da; LpV2, 10149.7 Da; LpV3, 10210.2 Da) were consistent with the values expected for fully lipolated domains (LpV1, 9765.8 Da; LpV2, 10147.3 Da; LpV3, 10210.4 Da) (Fig. 4). No preparation of domain was found to contain detectable amounts of unlipoylated protein, which would be 188.3 Da smaller, or aberrantly substituted protein; the preparation of LpV3 did contain a small amount of an unidentified component of mass 9829.8 Da, but this was expected, as examination of the

preparation by non-denaturing gel electrophoresis (Packman *et al.*, 1984*b*) showed it to be slightly impure (results not shown). This evidence for full lipoylation of the lipoyl domains of the *E. coli* PDH complex is significant in the light of two recent reports describing octanoylation of lipoyl domains as an alternative to lipoylation (Dardel *et al.*, 1990; Ali *et al.*, 1990). As both these reports derive from protein that has been purified from *E. coli* cells over-expressing a cloned subgene encoding the lipoyl domain of the *B. stearothermophilus* (Dardel *et al.*, 1990) and *E. coli* (Ali *et al.*, 1990) PDH complexes, it seems likely that this aberrant substitution arises principally when the lipoylation system of the organism is unable to cope with the excessive amounts of protein generated by the expression vectors used.

The single lipoyl domain of the E2o chain of the 2-oxoglutarate dehydrogenase complex was also examined by mass spectrometry. The fragment LoT1 (residues 1–100) was generated by limited proteolysis of the complex with trypsin (Packman & Perham, 1987). Its observed mass (10755.6 Da) closely matched the value expected for fully lipoylated domain (10754.8 Da). No peak corresponding to an unlipoylated or aberrantly modified domain was present (Fig. 4). Minor peaks in the spectrum were detected which corresponded to the lipoylated forms of the shorter tryptic fragments, LoT2 (residues 1–93, calculated mass 10043.1 Da, observed 10044.3 Da) and LoT3 (residues 1–89, calculated mass 9569.6 Da, observed 9572.4 Da) (Packman & Perham, 1987), and their presence in the preparation was confirmed by non-denaturing gel electrophoresis (results not shown).

Conclusions

We conclude that previous attempts to determine the lipoylation stoicheiometry of the E2p chains of the PDH complex of *E. coli* by measuring the incorporation of ^{14}C from [2-^{14}C]-pyruvate or *N*-ethyl[2,3-^{14}C]maleimide have been misleading. Perhaps this has arisen from the difficulty in achieving quantitative precipitation of the radiolabelled enzyme complex for scintillation counting, or an underestimation of the level of radiation quenching when counting on filters. As shown by our present experiments, the E2p chain does indeed contain three enzymically reactive lipoyl groups (one per lipoyl domain), in accord with the estimate of the lipoyl content based on ^{35}S-labelling (Hale & Perham, 1979). The E2o chain contains one lipoyl group. In neither chain was there any evidence of aberrant substitution, e.g. octanoylation, of the lipoylated lysine residue(s).

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