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Isolation and characterization of lipoylated and unlipoylated domains of the E2p subunit of the pyruvate dehydrogenase complex of *Escherichia coli*

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The dihydrolipoamide acetyltransferase subunit (E2p) of the pyruvate dehydrogenase complex of *Escherichia coli* has three highly conserved and tandemly repeated lipoyl domains, each containing approx. 80 amino acid residues. These domains are covalently modified with lipoyl groups bound in amide linkage to the N^6-amino groups of specific lysine residues, and the cofactors perform essential roles in the formation and transfer of acetyl groups by the dehydrogenase (E1p) and acetyltransferase (E2p) subunits. A subgene encoding a hybrid lipoyl domain was previously shown to generate two products when overexpressed, whereas a mutant subgene, in which the lipoyl-lysine codon is replaced by a glutamine codon, expresses only one product. A method has been devised for purifying the three types of independently folded domain from crude extracts of *E. coli*, based on their pH- (and heat-)stabilities. The domains were characterized by: amino acid and *N*-terminal sequence analysis, lipoic acid content, acetylation by E1p, tryptic peptide analysis and immunochemical activity. This has shown that the two forms of domain expressed from the parental subgene are lipoylated (L203) and unlipoylated (U203) derivatives of the hybrid lipoyl domain, whereas the mutant subgene produces a single unlipoylatable domain (204) containing the Lys-244→Gln substitution.

INTRODUCTION

The pyruvate dehydrogenase (PDH) multienzyme complex of *Escherichia coli* catalyses the oxidative decarboxylation of pyruvate to acetyl-CoA and CO_2 [reviewed by Perham *et al.* (1987), Yeaman (1989) and Guest *et al.* (1989)]. The complex contains multiple copies of three enzymic subunits. The dihydrolipoamide acetyltransferase subunits (E2p) form the structural cores (24-mers), which bind the pyruvate dehydrogenase (E1p) and dihydrolipoamide dehydrogenase (E3) subunits to produce complexes of somewhat variable stoichiometry (E1p/E2p/E3 proportions generally 1.4:1.0:0.8; Packman *et al.*, 1984).

The E1p, E2p and E3 subunits are encoded by the *aceE–aceF–lpd* operon, which is located at 2.8 min (130 kb) in the *E. coli* linkage map (Kohara *et al.*, 1987). The operon has been cloned and sequenced (Stephens *et al.*, 1983*a,b,c*), and site-directed mutagenesis has been used to analyse the structure–function relationships of the E2p (*aceF*) and E3 (*lpd*) components (Allison *et al.*, 1988; Guest *et al.*, 1989; Russell *et al.*, 1989*a*).

The E2p subunit has a highly segmented structure comprising five independently folded domains connected by interdomain linker sequences of different lengths and characteristically rich in alanine and proline residues (Stephens *et al.*, 1983*b*; Perham *et al.*, 1987). The *N*-terminal half of this subunit contains three highly conserved tandemly repeated lipoyl domains each containing a lysine residue that is specifically modified by amide linkage to lipoic acid. This lipoyl-lysine residue is central to the overall catalytic activity of the complex, providing a swinging arm that has sufficient rotational mobility to allow movement between the active sites of the three enzymic components (Ambrose & Perham, 1976). The lipoyl domains protrude from the core, and it would appear that the inherent conformational mobility of the alanine plus proline-rich linkers, as judged by ^{1}H-n.m.r. spectroscopy (Perham *et al.*, 1987; Texter *et al.*, 1988), is also required for full catalytic activity and active-site coupling in the complex (Miles *et al.*, 1988). The number of lipoyl domains per E2p chain can be reduced from three to one without impairing catalytic activity, but activity is lost when all three domains are removed or deleted, and when the lipoyl-lysine residue of a 'one-lip' E2p subunit is replaced by glutamine (Guest *et al.*, 1985; Graham *et al.*, 1986; Angier *et al.*, 1987). Studies with E2p subunits containing different arrangements of lipoylatable and unlipoylatable (Lys→Gln) domains have shown that each lipoylated domain can function independently (Allen *et al.*, 1989). It has also been shown that a 'no-lip' PDH complex can be re-activated *in vivo* by independently expressed lipoyl domains (Russell *et al.*, 1989*b*).

The proteolytic stability of the lipoyl domains has been used to isolate individual domains from purified PDH and ODH complexes for studies on their acylation kinetics and specificities (Bleile *et al.*, 1981; Packman *et al.*, 1984; Graham *et al.*, 1989). These have shown that the intact lipoyl domain is essential for the El-catalysed reductive acylation of the lipoyl cofactor, because the proteolytically excised lipoyl domains are acylated whereas lipoate, lipoamide and a lipoylated decapeptide are not. Furthermore, the lipoyl domain considerably influences the specificity of the lipoyl cofactor for reductive acylation. Thus the lipoyl domain from the ODH complex is virtually inactive as a substrate for reductive acetylation, and the lipoyl domains from

Abbreviations used: PDH complex, pyruvate dehydrogenase multienzyme complex; E1p, pyruvate dehydrogenase (EC 1.2.4.1); E2p, dihydrolipoamide acetyltransferase (EC 2.3.1.12); E3, dihydrolipoamide dehydrogenase (EC 1.8.1.4); ODH complex, 2-oxoglutarate dehydrogenase multienzyme complex; E1o, 2-oxoglutarate dehydrogenase (EC 1.2.4.2); f.a.b.-m.s., fast-atom-bombardment mass spectrometry; g.c.–m.s., gas chromatography–mass spectrometry; T, % (w/v) total acrylamide, C, % (w/v) bisacrylamide/acrylamide.

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the PDH complex are only poor substrates for reductive succinylation by E1o. This situation contrasts with the E2p-catalysed and the E3-catalysed reactions, where free lipoic acid and lipoamide function as model substrates (Reed *et al.*, 1958).

In order to obtain amplified and homogeneous sources of unique lipoyl domains for structural analysis and functional studies, *aceF* subgenes encoding single hybrid lipoyl domains were cloned and overexpressed from thermo-regulated λ promoters (Miles & Guest, 1987). The amino acid sequence of the hybrid domain is shown in Fig. 1. It has already been established that this domain is functional in a 'one-lip' PDH complex and that it is capable of folding into a proteolytically stable form (Guest *et al.*, 1985). Expression of the subgene generated two products whereas an analogous subgene encoding a mutant domain containing the Lys-244→Gln substitution generated only one (Miles & Guest, 1987). It was suggested that the two wild-type domains could represent lipoylated and unlipoylated species, but other explanations, such as differential acetylation, oxidation and mixed-dithiol formation (which are also consistent with the absence of heterogeneity in the unlipoylatable mutant form) or proteolytic processing of the wild-type domain, were not ruled out. In the present paper the amplification of lipoyl domains has been assessed by using different vectors and a procedure has been devised for purifying the wild-type and mutant forms from crude cell-free extracts of *E. coli*. It has also been shown that the wild-type *aceF* subgene expresses lipoylated and unlipoylated forms of the intact domain.

METHODS AND MATERIALS

E. coli strains and plasmids

Two strains of *E. coli* K12 were used as plasmid hosts: CAG629 (lac_{am} trp_{am} pho_{am} $htpR_{am}$ *mal rpsL lon* $supC_{ts}$, kindly provided by Dr. C. A. Gross) and JM101 (*ΔproAB–lac thi supE* /F′*traD36 proA*$^+$*B*$^+$ *lacI*^{q}*Z*). Strain JRG26 (*lip2 supE42*; A.T.C.C. 25645, formerly W1485*lip2*; Herbert & Guest, 1968) was used in the turbidimetric assay for lipoic acid. The two plasmids, pGS203 and pGS204, which express functional and mutant (Lys-244→Gln) hybrid lipoyl domains (respectively) from *aceF* subgenes coupled to thermo-inducible λ promoters, have been described previously (Miles & Guest, 1987). An analogous pair of plasmids (pGS331 and pGS332) was constructed by transferring the *aceF* subgenes from pGS203 and pGS204 (respectively) to the ptac85 expression vector described by Marsh (1986). The *aceF*-containing 0.6 kb *Nco*I–*Sal*I fragments of pGS203 and pGS204 were gel-purified and ligated with the corresponding receptor fragment of ptac85. Ampicillin-resistant transformants of strain JM101 were selected and screened for the desired products. Another plasmid, pGS253, a pACYC184 derivative that expresses the *aceF* subgene from the *tet* promoter (Russell *et al.*, 1989*b*), was also used.

Expression of lipoyl domains

All strains were grown in L broth (Lennox, 1955), with ampicillin (100 μg/ml) or chloramphenicol (20 μg/ml) as required, for plasmid maintenance. Cultures for the isolation of lipoyl domains were grown in 16-litre batches in an LH fermenter supplied with air at a rate of 10 litres/min and inoculated to a starting A_{650} value of 0.1. For transformants containing pGS203 or pGS204 the medium was supplemented with 0.1% glucose and growth was at 30 °C until an A_{650} value of 0.5 was reached, whereupon the temperature was increased to 42 °C and maintained until early stationary phase (A_{650} = 2.2). Transformants of strain JM101 containing pGS331 and pGS332 were grown at 37 °C for 16 h in L broth containing isopropyl β-D-thiogalactoside (10 μg/ml).

Isolation of lipoyl domains

The harvested bacteria (50 g) were resuspended (0.6 g/ml) in 20 mM-sodium phosphate buffer, pH 7.0, containing EDTA (2 mM), NaN_3 (0.02%), phenylmethanesulphonyl fluoride (1 mM) and benzamidine hydrochloride (1 mM). Cells were disrupted by ultrasonic treatment (ten 2 min bursts) with cooling to 4 °C between bursts. The ultrasonic extract was cleared by centrifuging (38000 *g* for 40 min) and lipoyl domain was isolated from the cell-free supernatant. The pH was lowered to 4.0 with 1 M-HCl, and insoluble material removed by centrifuging (38000 *g* for 20 min) before returning the pH to 7.0 with 1 M-NaOH. The resultant extract (90 ml) was concentrated approx. 8-fold by ultrafiltration on a Filtron Omegacell 3 K membrane. If required, the extract was heated at 65 °C for 10 min and the insoluble precipitate removed by centrifugation. The supernatant was subjected to gel filtration in 20 mM-sodium phosphate buffer, pH 7.0, on a Sephadex G-75 column (90 cm × 44 mm) (Amicon Corp.) and the appropriate fractions (180 ml) were pooled and concentrated 36-fold by ultrafiltration. This sample (5 ml) was subjected to f.p.l.c. anion-exchange chromatography on a 1 ml Mono-Q column (Pharmacia–LKB) with a 10–600 mM-ammonium acetate buffer, pH 5.0, gradient, based on the procedures of Packman *et al.* (1984). The gradient was generated over 20 column volumes and homogeneous lipoyl domain was recovered.

Assay of the lipoyl domain and lipoic acid

The lipoyl domain was assayed by non-denaturing PAGE, [stacking gel (pH 6.8) 5% T, 2.5% C; resolving gel (pH 8.3) 20% T, 0.6% C] followed by quantitative densitometry of Coomassie Brilliant Blue-stained gels. Reference samples of purified domain, quantified by total amino acid analysis, were included. Protein determination was by the Lowry method, with BSA as standard. Lipoic acid was assayed by the turbidimetric method of Herbert & Guest (1975) except that BSA was added during the extraction procedure, as recommended by Bothe & Nolteernsting (1975). Purified domain, 10 μg in 0.3 ml of 3 M-H_2SO_4 containing BSA (2%, w/v), was hydrolysed at 121 °C for 2 h to release protein-bound lipoic acid. The resultant hydrolysates were neutralized to pH 7.0 with 4 M-NaOH and adjusted to a final volume of 1 ml before being assayed. Duplicate samples of several independent hydrolysates were assayed over a range of concentrations, and the average values (with standard deviations) are quoted after correction for a consistent loss of 70% observed with standard lipoic acid under these hydrolytic conditions.

Reductive acylation of the lipoyl domains

The presence of active domain was tested qualitatively by reductive acetylation with sodium [2-^{14}C]pyruvate and with *N*-ethyl[2,3-^{14}C]maleimide (0.1 or 0.3 μCi per reaction) by the procedures of Bleile *et al.* (1981) and Packman *et al.* (1984), respectively. Purified domain (0.5 nmol) was incubated at 18 °C for 10 min in a reaction mixture (100 μl) containing 0.5 mM-thiamin pyrophosphate, 5 mM-$MgCl_2$, 20 mM-sodium phosphate buffer, pH 7.0, plus 0.5 mM-*N*-ethylmaleimide and 0.5 mM-sodium pyruvate (only one being labelled), and 'one-lip' PDH complex (0.01 nmol) as a source of E1p. Reductive succinylation (3-carboxypropionylation) was carried out with sodium 2-oxo-[5-^{14}C]glutarate (1.25 μCi per reaction) and ODH complex (0.1 nmol) as a source of E1o (Graham *et al.*, 1989). Reaction mixtures were analysed directly by non-denaturing PAGE and autoradiography.

Isoelectric focusing and SDS/PAGE

Samples of purified domain were run on precast Servalyt

Precotes (pH 3–6) isoelectric-focusing gels according to the manufacturer's instructions. Denaturing SDS/PAGE was as for non-denaturing PAGE (above) except that all solutions contained 0.1% SDS and samples were heated (5 min at 100 °C) in dissociation buffer (Laemmli, 1970).

Amino acid and *N*-terminal sequence analysis

Domains and peptides were hydrolysed with 5.7 M-HCl containing 15 μM-phenol (110 °C, for 16 h *in vacuo*) and compositions were determined with an LKB4400 amino acid analyser or a Waters Picotag system. The *N*-terminal sequences were determined by using an Applied Biosystems 470A gas-phase sequencer.

Peptide cleavage and separation

Lipoyl domain (2 mg) was denatured by performic acid oxidation by the procedure of Hirs (1956). Samples were freeze-dried, resuspended at 2 mg/ml in 0.5% NH_4HCO_3, pH 8.0, and digested with trypsin (1%, w/w) at 37 °C for 17 h. Peptides were separated as described previously (Ali *et al.*, 1990).

G.c.–m.s. and f.a.b.-m.s.

Purified domains (2 mg of protein in 0.1 ml of 3 M-H_2SO_4) were hydrolysed for 2 h at 121 °C, extracted with dichloromethane (five 0.1 ml portions) and analysed by g.c.–m.s. as described previously (Ali *et al.*, 1990), except that a directly coupled 4 m BP10 column was used with a flow rate of 1.5 ml of He/min and a temperature gradient of 100 °C to 240 °C at 20 °C/min. F.a.b.-m.s. of purified peptides was by previously reported methods (Ali *et al.*, 1990).

Immunology and enzyme assays

Purified domains U203 and L203 were each mixed with Freund's complete or incomplete adjuvant for injection into rabbits to produce anti-(lipoyl domain) serum. Antiserum was titred for by its ability to inhibit PDH-complex activity. Crude extracts, having complex activities of 3.2 μmol/h, were incubated at room temperature for 20 min with 0–50 μl of antiserum. Samples were centrifuged at 10000 g (4 °C) for 20 min, where appropriate, and supernatants tested for residual enzyme activity (Langley & Guest, 1974). Enzyme activities for the PDH and ODH complexes, E1p, E2p and E3 were measured as described previously (Jeyaseelan & Guest, 1980).

Materials

BSA (fraction V) and trypsin were from Sigma Chemical Co. Radiochemicals were purchased as follows: *N*-ethyl[2,3-^{14}C]maleimide (331 MBq/mmol) and sodium 2-oxo[5-^{14}C]-glutarate (895 MBq/mmol) from Amersham International, and sodium [2-^{14}C]pyruvate (592 MBq/mmol) from New England Nuclear. Lipoic acid was from Koch–Light Laboratories. Restriction enzymes were purchased from Pharmacia–LKB and T_4 DNA ligase was from Bethesda Research Laboratories. Servalyt Precotes 3–6 precast gels were purchased from Serva Feinbiochemica. Partially purified 'one-lip' PDH complex was generously provided by G. C. Russell and purified ODH complex was kindly supplied by S. J. Angier.

RESULTS AND DISCUSSION

Overexpression of lipoyl domains

The 85-residue hybrid lipoyl domain (Fig. 1) is encoded by an *aceF* subgene constructed *in vitro* in which the codons for residues 1–33 and 238–289 of the first and third lipoyl domains of the E2p subunit were fused between an *Nco*I-containing translational initiator and tandem stop codons (Miles & Guest, 1987). The lipoyl domain and a mutant derivative containing a Lys-244→Gln substitution were originally expressed from thermo-inducible λ promoters by using two plasmids, pGS203 and pGS204 respectively. With large-scale cultures the domains were amplified to 0.6–1.0% of the soluble protein. Enrichments approaching 8% were obtained when the *aceF* subgenes were expressed from the *tac* promoter in plasmids pGS331 and pGS332 (see the Methods and materials section). With another plasmid, pGS253, in which the *aceF* subgene is expressed from the *tet* promoter (see the Methods and materials section), the lipoyl domain amounted to only 0.1–0.2% of the soluble protein. This

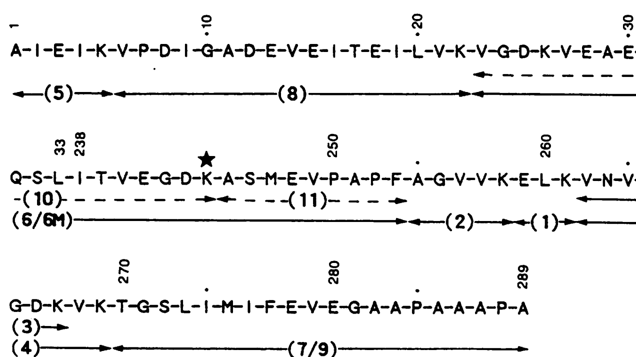

Fig. 1. Primary structure of the hybrid lipoyl domain

The lipoyl domain encoded by the *aceF* subgene of pGS203 comprises residues 1–33 and 238–289 from the first and third lipoyl domains of the E2p component. Peptides isolated after tryptic digestion of domain L203 are numbered (1 to 9) to denote their order of elution. The site of lipoylation (domain L203) or substitution (domain 204) at Lys-244 is marked (★). Two peptides (10 and 11) generated by tryptic cleavage at Lys-244 in domain U203 are indicated by broken lines.

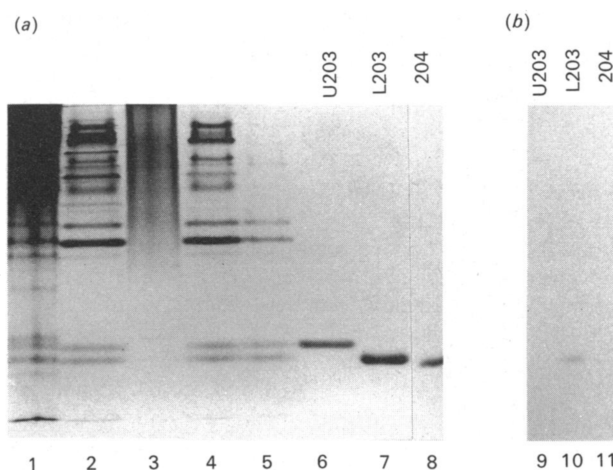

Fig. 2. Purification and reductive acetylation of lipoyl domains

(*a*) Coomassie Brilliant Blue-stained non-denaturing 20%-PAGE gel showing samples from sequential steps in the purification of the lipoyl domains expressed from pGS203: crude extract (lane 1); pH 4.0 treatment (lane 2, supernatant; lane 3, precipitate); heat treatment (lane 4, supernatant); gel filtration (lane 5); ion-exchange chromatography (lane 6, domain U203; lane 7, domain L203). Lane 8 contains a sample of purified mutant domain, 204. (*b*) Autoradiograph of three forms of the lipoyl domain (approx. 2–3 μg) after reductive acetylation with [2-^{14}C]pyruvate (lane 9, domain U203; lane 10, domain L203; lane 11, domain 204). No incorporation was obtained if pyruvate or PDH complex (the source of E1p) was omitted.

Table 1. Purification of the lipoyl domains

The effectiveness of each step in a typical purification of the pGS203-encoded domains is shown. The total protein at each step is indicated as is the lipoyl domain (%, estimated by densitometry of Coomassie Brilliant Blue-stained gels). The overall purification in this experiment is 117-fold.

Purification step	Total protein (mg)	Lipoyl domain (%)	Purification factor (fold)	Total domain (mg)	Yield (%)
Crude extract	4100	0.85	–	35.0	100
pH 4.0 supernatant	400	8.6	10.1	34.4	98
Heat treatment	327	10.3	1.2	33.7	96
Gel filtration	68	45.0	4.4	30.8	88
Ion-exchange					
U203	15	100.0	2.2	15.0	60
L203	6	100.0	2.2	6.0	

level corresponds to the total amount of lipoyl domain normally synthesized with the PDH complex. Plasmids pGS203 and pGS331 produced two forms of domain, designated U203 and L203, to denote their upper and lower positions (i.e. slower and faster migrations) in non-denaturing PAGE (Fig. 2). The relative amounts of domains U203 and L203 were normally 2:1 with pGS203 cultures and 3:1 with pGS331 cultures. This contrasted with pGS253, which produced only the L203 form of domain, and pGS204 and pGS332, which produced a single but unique form that migrated faster than domains U203 and L203 and was designated 204 (Fig. 2).

Purification of lipoyl domains

The procedure devised for purifying the lipoyl domains in relatively high yield from crude cell-free extracts of induced cultures is outlined in the Methods and materials section. It exploits the high stability of the domains at low pH (and high temperature), in which most *E. coli* proteins are insoluble or denatured. The two forms of lipoyl domain, U203 and L203, co-purified without any change in relative abundance until the final step, and combined recoveries of approx. 60% were routinely obtained. A summary of a typical purification of the pGS203-encoded lipoyl domains is shown in Table 1, and the corresponding PAGE profiles of the products at each stage are shown in Fig. 2.

Effects of $(NH_4)_2SO_4$, pH and heat on domain solubilities. $(NH_4)_2SO_4$ precipitation was not satisfactory as a first step because the domains were precipitated across a wide range above 30% saturation and there was considerable variation between experiments. The domains remained soluble over a wide pH range (3.0–11.3), whereas most of the contaminating proteins were insoluble at pH 4.0 (Fig. 2, lanes 2 and 3). Treating the crude amplified extracts for up to 1 h at 55, 60, 65 and 70 °C indicated that the lipoyl domains remained soluble even after 1 h at 70 °C, much of the contaminating protein being precipitated within 10 min. A pH treatment giving a 10–12-fold enrichment was chosen as the first purification step (Table 1). This was followed by a heat step (65 °C for 10 min), which was often omitted because it contributed little to the overall purification (Table 1 and Fig. 2, lane 4).

Gel filtration and ion-exchange chromatography. Previous studies have shown that Sephadex G-75 and ion-exchange chromatography can be used for the isolation of lipoyl domains released by proteolysis from purified PDH complex (Packman *et al.*, 1984). Gel filtration of the acid-treated and concentrated material gave a 4–5-fold enrichment of the domains (Table 1 and Fig. 2, lane 5). The pooled and concentrated fractions were further fractionated by ion-exchange chromatography, and an analysis of the eluate fractions by denaturing and non-denaturing PAGE showed that the lipoyl domain could be separated from all contaminants (Fig. 2, lanes 6, 7 and 8). The two forms of the lipoyl domain expressed from pGS203 were also resolved, indicating that they differ in overall charge. The upper band (U203) was always eluted ahead of the lower domain (L203), and mutant domain (204) emerged at an intermediate position.

Characterization of the lipoyl domains

The domains each have a very weak absorption spectrum owing to the absence of strongly absorbing aromatic amino acids: the average molar absorption coefficient at the absorption maximum (260 nm) was 2020 $M^{-1} \cdot cm^{-1}$.

M_r and isoelectric point. The lipoyl domains migrate as unique but rather diffuse bands in SDS/PAGE, domain L203 having a slightly higher mobility than domains U203 and 204. Their mobilities correspond to apparent M_r values of 9200–9400, in reasonable agreement with the predicted values: 8787 for domains U203 and 204 and 8975 for domain L203. Isoelectric-focusing studies with the three domains confirmed that they are very acidic (pI < 3.5), domain U203 being slightly less acidic than domains L203 and 204.

Table 2. Amino acid compositions of purified domains

The compositions of the three types of domain are compared with the composition deduced from the nucleotide sequence. The differences predicted for the pGS204-encoded domain, due to the Lys→Gln substitution, are shown in parentheses.

Amino acid	Composition (mol/mol of domain)			
	Predicted	U203	L203	204
Asx	6	6.6	6.6	6.0
Thr	3	3.3	3.1	2.6
Ser	3	3.4	3.1	2.8
Glu	12 (13)	12.2	12.5	13.3
Pro	5	4.9	4.6	5.5
Gly	7	7.2	7.6	7.6
Ala	12	11.7	12.1	12.0
Val	13	12.4	12.4	12.5
Met	2	2.1	2.1	1.8
Ile	8	7.6	7.5	8.5
Leu	4	4.1	4.0	4.5
Phe	2	2.0	1.9	2.2
Lys	8 (7)	7.9	7.7	6.5
Total	85			

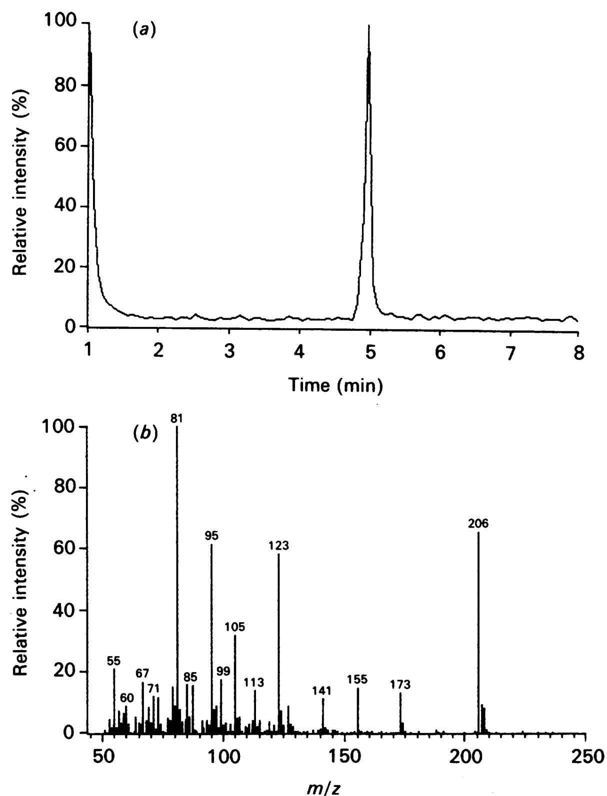

Fig. 3. G.c. and m.s. of lipoic acid

(a) G.c. elution profile of the dichloromethane extract of hydrolysed domain L203 showing a major component with the same retention time as lipoic acid. (b) Mass spectrum of material eluted at 5 min in (a), which is identical with lipoic acid.

Amino acid composition and N-terminal analysis. The compositions of the three types of purified lipoyl domain agreed with those deduced from the nucleotide sequences (Table 2). The N-terminal sequences for the first eight residues of each type of domain were the same (Fig. 1). These results indicate that domains U203 and L203 have the same primary structures and are not differentially proteolysed forms.

Radiolabelling studies. The functionality of the three types of domain was investigated by testing their ability to serve as substrates for reductive acetylation (see the Methods and materials section). Only domain L203 was reductively acetylated (Fig. 2). Similar results were obtained with either [2-^{14}C]pyruvate or N-ethyl[2,3-^{14}C]maleimide as the radioactive substrate. The latter reacts with the thiol groups of reduced and acetylated lipoyl cofactors, and the failure to react in the absence of E1p or pyruvate further indicates that the cofactor is in the oxidized (disulphide) state in domain L203. The results show that domain L203 is functional but the other domains are not, presumably because they are unlipoylated (U203) or unlipoylatable (204). None of the domains was reductively succinylated in comparable tests with ODH complex as the source of E1o and 2-oxo[5-^{14}C]glutarate. This contrasts with the results reported by Graham et al. (1989), who observed reductive succinylation at 9–12% of acetylation with lipoyl domains from purified PDH complex. It is not known whether this discrepancy is due to the insensitivity of the present tests or to a unique specificity of the hybrid lipoyl domain relative to the natural domains. It seems unlikely that contamination of the purified PDH complex with ODH complex or E1o could be responsible for the cross-reactivity of the complex-derived domains.

Lipoic acid bioassay and g.c.–m.s. The three types of domain were assayed for lipoic acid after hydrolysis with or without BSA. Controls containing BSA with a domain-equivalent of DL-lipoic acid were also hydrolysed. Lipoic acid was only detected in samples containing domain L203 or added cofactor. The recovery of lipoic acid was reproducibly 30% for the standard samples plus BSA, and after correction the observed values corresponded to 1.03 ± 0.03 mol of lipoic acid/mol of domain L203 and less than 0.005 ± 0.001 mol/mol of domains U203 and 204. In the absence of BSA only 6% of the lipoic acid was recovered with domain L203. These findings indicate that domain L203 is fully lipoylated and that domains U203 and 204 are unlipoylated.

The presence of lipoic acid in domain L203 but not domains U203 or 204 was confirmed by g.c.–m.s. Dichloromethane extracts of samples of domain L203, hydrolysed at a concentration of 20 mg/ml (without BSA), contained a major component having an elution time (5.0 ± 0.1 min) and mass spectrum identical with those of authentic lipoic acid (Fig. 3). The mass spectrum for lipoic acid is quite similar to that of the methylated derivative of the cofactor released from the ODH complex (Pratt et al., 1989). In quantitative g.c. analysis the recovery of standard lipoic acid from the column increased from

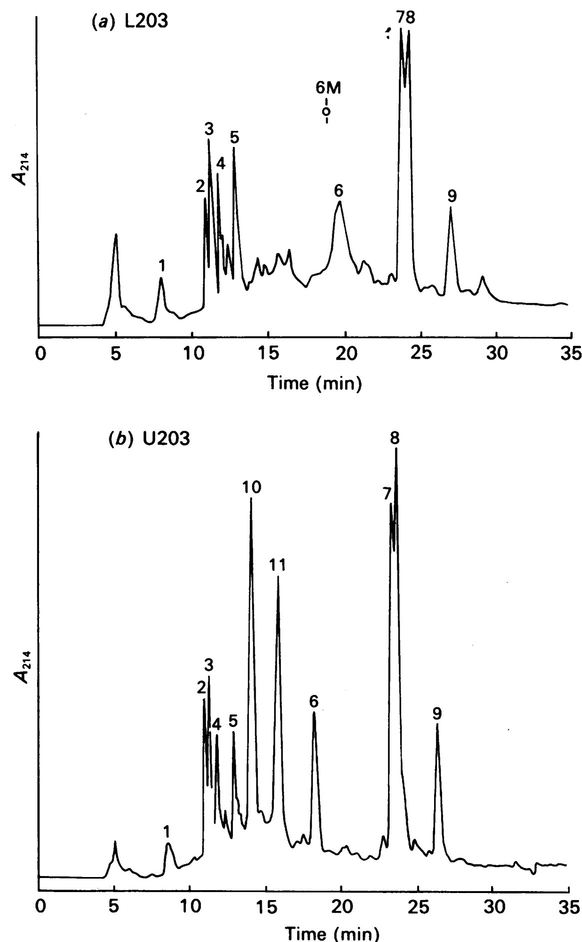

Fig. 4. Elution profiles of tryptic digests of lipoyl domains

(a) Profile for tryptic digest of performic acid-oxidized domain L203. The same major products (1 to 9) were also obtained with domains U203 and 204. The position of the mutant variant (6M) of peptide 6 from domain 204 is indicated (○). (b) Profile for domain U203 showing two additional peptides (10 and 11) arising from cleavage at the unlipoylated lysine residue (Lys-244). The compositions of individual peak fractions are listed in Table 3 and the corresponding peptides are identified in Fig. 1.

Table 3. Amino acid compositions of tryptic peptides

The compositions of the major tryptic peptides separated by h.p.l.c. from domain L203 are listed in order of elution (see Fig. 4) except for the extra products (peptides 10 and 11), derived from domain U203, and a unique peptide (6M) from the mutant domain containing the Lys244→Gln substitution. Methionine sulphone, generated during performic acid oxidation, was not resolved.

		Amino acid composition (mol/mol of peptide)											
Amino acid	Peptide ...	1	2	3	4	5	6	6M	7	8	9	10	11
Asx				1.91	1.80		1.83	1.94		1.88		1.74	
Thr							1.06	0.90	1.04	0.97	0.97	0.90	
Ser							1.77	1.74	1.01		0.94	0.94	0.99
Glx		0.94				0.90	5.04	6.23	2.27	2.84	2.18	4.15	1.23
Pro							2.11	1.96	1.96	1.21	1.93		1.81
Gly			1.13	1.02	1.12		2.23	1.98	2.06	1.25	2.08	1.86	
Ala			1.08			1.03	3.65	3.22	6.10	2.15	6.23	1.47	2.12
Val			1.64	2.04	3.08		3.54	3.57	1.11	2.42	1.09	2.38	1.09
Ile		1.06				2.08	1.22	0.98	1.80	2.58	1.88	1.63	
Leu							1.04	1.03	1.03	0.96	1.01	0.90	
Phe							0.78	0.94	0.89		0.90	0.99	
Lys		1.00	1.15	1.04	2.02	0.98	1.85	0.91		0.76		1.99	

10% to 50% over the range 0.2–20 μg of lipoic acid. Within this range estimates for the L203 extract corresponded to 0.75 ± 0.10 mol of lipoic acid/mol of domain.

Proteolytic digestion and peptide analysis. To further confirm that domain L203 is lipoylated and that it is modified at Lys-244, tryptic digests of samples of the three types of domain were subjected to reverse-phase h.p.l.c. and the amino acid compositions of the major components were determined. The native domains were resistant to proteolytic degradation, but good digestion was obtained with performic acid-treated samples. A typical elution profile for domain L203 is shown in Fig. 4(*a*), and analyses for nine major products are listed, in the order of their elution, in Table 3. The compositions correspond to the peptides identified in Fig. 1, and they account for all of the residues in the domain. A similar profile was obtained with domain 204 except for one peptide, which was eluted at 19.0 min rather than 19.8 min (L203) and contained the Lys→Gln substitution (Fig. 4*a* and Table 3). The fact that both digests contain single peptides corresponding to residues 23–253 indicates that tryptic cleavage is blocked at Lys-244, presumably because it is modified (lipoylated, L203) or replaced (204). It is apparent that peptides 6 and 6M have arisen by cleavage at Phe-253, possibly due to the autocatalytic loss of tryptic specificity (Keil-Dlouha *et al.*, 1971). The lipoylated peptide was eluted as a broad peak whereas the peaks of the mutant and overlapping unlipoylated peptides were sharp (Fig. 4): this could be due to incomplete performic acid oxidation of the lipoyl cofactor.

An elution profile for a comparable digest of domain U203 is shown in Fig. 4(*b*). It contains two additional components, 10 and 11, corresponding to residues 23–244 and 245–253 (Table 3 and Fig. 1). These peptides clearly arise by cleavage at Lys-244, and confirm that Lys-244 is unmodified in domain U203. The compositions of all of the other components, including peptide 6, were identical with their L203 counterparts. In different digests of domain U203 the amounts of peptide 6 varied inversely with those of peptide 10 and 11, and this is consistent with their common origin. The extent of peptide 6 cleavage was greater with higher concentrations of trypsin (up to 5%), further indicating that Lys-244 is not modified in domain U203. It is interesting that domain U203 contains three potential trypsin-sensitive sites that are only partially cleaved, Lys-26, Lys-244 and Lys-267 (Fig. 1). In each case the lysine residue is immediately preceded by aspartic acid; there are precedents for such refractility (Allen, 1989). Peptides 7 and 9 have identical compositions, corresponding to the alanine-rich *C*-terminal peptide (Table 3 and Fig. 1). Elution at two different positions may reflect some special feature of the *C*-terminal peptide, e.g. a strong interaction with peptide 8 from the *N*-terminal region (Fig. 1).

F.a.b.-m.s. Further attempts to characterize the substituent on domain L203 were made by f.a.b.-m.s. of a peptide (residues 242–253) released from the performic acid-treated domain with chymotrypsin and staphylococcal V8 proteinase. Unfortunately, no major mass ion could be detected, even though the analogous peptides from domains U203 and 204 produced good signals at the predicted mass, 1281. Failure to detect the modified peptide could in part be due to its distribution between four oxidation states corresponding to those observed for performic acid-treated lipoic acid.

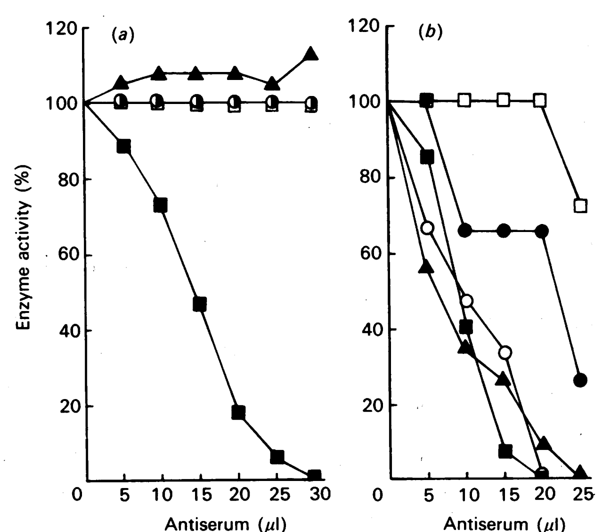

Fig. 5. Effects of anti-(lipoyl domain) serum on the activities of the PDH and ODH complexes

The effects of increasing amounts of anti-(domain U203) serum on the enzyme activities in cell-free extracts were determined (*a*) before and (*b*) after centrifuging: ■, PDH complex; □, ODH complex; ○, E1p; ▲, E2p; ●, E3. Similar results were obtained with the anti-(domain L203) serum.

Immunological studies with anti-(lipoyl domain) sera. In unsedimented samples the antisera raised against lipoylated (U203) and unlipoylated (L203) domains inactivated the PDH complex without affecting the component activities or the ODH complex (Fig. 5*a*). This shows that the antisera are specifically interfering with the acetyl-carrying function of the corresponding lipoyl domain. Studies with sera raised against an interdomain linker have given similar results (Radford *et al.*, 1989). In the sedimented samples it was apparent that the antisera removed the PDH complex, E1p and E2p activities, and part of the E3 activity, before affecting the ODH complex and its associated E3 activity (Fig. 5*b*). Moreover, higher concentrations of antisera were needed to inactivate the PDH complex than to produce a sedimentable antibody–antigen complex. This would be expected, since it is known that a substantial fraction of the lipoyl domains or cofactors can be removed enzymically or genetically before the catalytic activity is seriously affected (Berman *et al.*, 1981; Stepp *et al.*, 1981; Guest *et al.*, 1985; Allen *et al.*, 1989). The anti-(domain L203) serum was also more effective than anti-(domain U203) serum in its ability to precipitate the ODH complex (results not shown). This is presumably because the lipoate cofactor provides a common antigenic site in both complexes, but there are other epitopes in both of the isolated domains that are specific for the PDH complex.

Conclusion

The properties of the isolated domains are entirely consistent with their predicted primary structures. They appear to retain an active folded state because of their trypsin-resistance and because domain L203 is acetylated but not succinylated. The observed differences in behaviour would appear to stem from the presence of lysine, lipoyl-lysine or glutamine at position 244. The unlipoylated domain was always 2–3-fold more abundant with strong promoters ($\lambda P_R P_L$ or *tac*), but full lipoylation occurred when synthesis failed to exceed normal levels, indicating that overexpression outstrips the cell's capacity for lipoylation. The observed heterogeneity is reminiscent of the multiple E2p bands observed in post-infection labelling studies with λ*aceEFlpd* derivatives (Guest *et al.*, 1981). This could not be ascribed to differences in the degree of lipoylation resulting from short-term high expression from a λ promoter. Recently it has been found that overexpression in a lipoate-deficient strain produces octanoylated and unlipoylated domains (Ali *et al.*, 1990). No octanoylated domain was detected in Lip$^+$ hosts, except with a glycerol-containing production medium, which gave equivalent amounts of octanoylated and lipoylated forms. Thus growth conditions may affect both the degree and nature of domain modification.

The ability to overexpress and isolate different forms of lipoyl domain in quantity offers opportunities for structure determination by two-dimensional ^{1}H n.m.r., for identifying the E1p active site and defining their interactions with the three types of active site in the PDH complex and for studying the process of lipoylation and investigating the final steps in lipoate biosynthesis. The isolated domains have been used recently to show that the lipoyl group is an essential component of the main immunogenic region recognized in the autoimmune disease primary biliary cirrhosis (Fussey *et al.*, 1990).

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