The EMBO Journal vol.3 no.6 pp.1315–1319, 1984

Repeating functional domains in the pyruvate dehydrogenase multienzyme complex of *Escherichia coli*

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Communicated by R.N.Perham

Each polypeptide chain in the lipoate acetyltransferase (E2) core of the pyruvate dehydrogenase complex from *Escherichia coli* contains three repeating sequences in the N-terminal half of the molecule. The repeats are highly homologous in primary structure and each includes a lysine residue that is a potential site for lipoylation. We have shown that all three sites are lipoylated, at least in part, and that the three lipoylated segments of the E2 chain can be isolated as distinct functional domains after limited proteolysis. Each domain becomes partly acetylated in the intact complex in the presence of substrate. In the primary structure, the domains are separated by regions of polypeptide chain oddly rich in alanine and proline residues. These regions are probably the conformationally mobile segments observed in the ^{1}H-n.m.r. spectrum of the complex and which are removed by tryptic cleavage at Lys-316. The C-terminal half of the molecule contains the acetyltransferase active site and the binding sites for E1, E3 and other E2 subunits. The pyruvate dehydrogenase complex of *E. coli*, which has a heterogeneous quaternary structure, is thus far unique among the 2-oxo acid dehydrogenase complexes in possessing more than one lipoyl domain per E2 chain, but this may be a general feature of the enzyme from Gram-negative organisms.

Key words: domains/lipoyl/pyruvate dehydrogenase/multienzyme complex

Introduction

The structural core of the pyruvate dehydrogenase (PDH) complex of *Escherichia coli* (mol. wt. 4.5–6 x 10^6) is made up of 24 lipoate acetyltransferase (E2) polypeptide chains to which the pyruvate decarboxylase (E1) and dihydrolipoamide dehydrogenase (E3) subunits are bound tightly but non-covalently (Reed, 1974; Danson *et al.*, 1979). The lipoyl-lysine residues in the E2 chains, which act as 'swinging arms' in the mechanism of the complex (Reed, 1974; Bates *et al.*, 1977; Collins and Reed, 1977; Danson *et al.*, 1978; Packman *et al.*, 1983) reside in portions of the E2 subunits that protrude from an inner part of the E2 core (Bleile *et al.*, 1979; Hale and Perham, 1979a) and which contain substantial regions of conformationally mobile polypeptide chain (Perham *et al.*, 1981; Roberts *et al.*, 1983). Labelling with substrates indicates the presence of two lipoic acid residues per E2 chain (Danson and Perham, 1976; Bates *et al.*, 1977; Collins and Reed, 1977) and direct measurement by means of mass spectrometry appears to confirm this value (White *et al.*, 1980). However, PDH complex from *E. coli* grown on [^{35}S]sulphate has been found to bear three [^{35}S]lipoyl groups per E2 chain (Hale and Perham, 1979b). Since only one lipoyl-lysine-containing sequence could be identified, the presence of repeating sequences in the primary structure of E2 was suggested (Hale and Perham, 1980). The amino acid sequence of the E2 polypeptide chain inferred from the sequence of its structural gene, *aceF* (Stephens *et al.*, 1983a), reveals that three highly homologous regions of sequence, comprising some 100 residues each, are repeated in the N-terminal half of the protein. A potential lipoylation site is present in each repeat. We show here that all three such sites in the E2 chain are at least partly lipoylated, that each lipoyl group can be reductively acetylated by pyruvate and that the three lipoyl segments exist as distinct domains which can be obtained as separate functional units after limited proteolysis of the PDH complex.

Results and Discussion

Sites of lipoylation in the E2 core

Lipoic acid-containing peptides were isolated from [^{35}S]lipoic acid-labelled complex after digestion with trypsin and *Staphylococcus aureus* V8 proteinase and resolved into three pure components (Table I). The unresolved mixture of peptides gave a single N-terminal sequence of Gly-Asp-Lys(N^6-lipoyl)-Ala-Ser-Met-Glu-Val-Pro- (Hale and Perham, 1980) but the amino acid compositions of the individual components were significantly different. The presence of Ile in TA2a, of Thr and Phe in TA2b and of Phe in TA2c enabled them to be identified as deriving from positions 38–55, 141–158 and 242–259, respectively, in the amino acid sequence of E2 (Figure 1). We conclude that lipoic acid is attached to each of the lysine residues at positions 40, 143 and 244. In addition, each site is probably fully lipoylated since

Table I. Amino acid compositions of three lipoylated peptides from *E. coli* PDH complex

Amino acid	Peptide TA2a	Residues 38–55	Peptide TA2b	Residues 141–158	Peptide TA2c	Residues 242–259
Asx[a]	2.4	1	2.5	1	2.4	1
$MeSO_2$[a]		1		1		1
Thr	–	–	0.6	1	–	–
Ser	2.0	2	1.4	1	1.1	1
Glx	3.0	3	2.5	2	2.1	2
Pro	1.9	2	1.9	2	2.0	2
Gly	1.9	2	1.9	2	1.8	2
Ala	1.9	2	2.5	3	2.7	3
Val[b]	2.1	2	2.5	2	2.6	3
Ile[b]	0.8	1	–	–	–	–
Phe	–	–	0.7	1	0.9	1
Lys	2.0	2	1.7	2	1.9	2
N-terminal residue	Gly	Gly	Gly	Gly	Gly	Gly
Specific radio-activity (d.p.m./nmol)	1400		1200		1300	

[a]Asp and $MeSO_2$ were not resolved on the analyser
[b]Values given for 72 h hydrolysis

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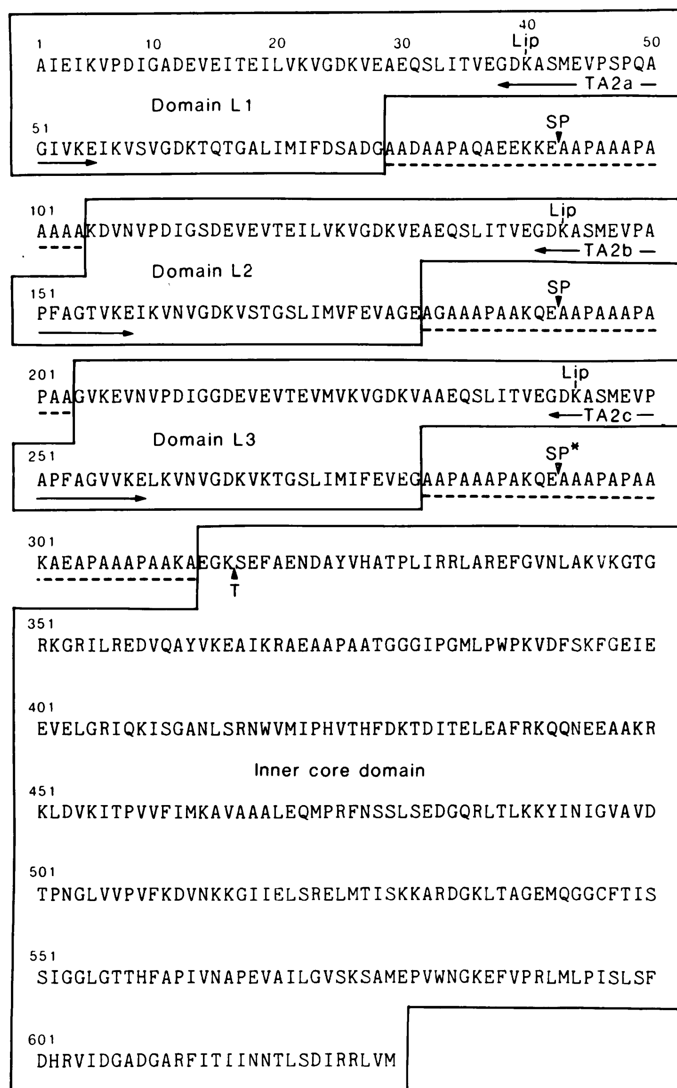

Fig. 1. The primary structure of the E2 chain of the PDH complex (Stephens *et al.*, 1983a). The locations of peptides TA2a, TA2b and TA2c are shown: Lip, lipoyl group; SP, cleavage site with *S. aureus* V8 proteinase; SP*, probable cleavage site with same proteinase; T, cleavage site with trypsin; ----, regions rich in Ala and Pro, assigned high conformational mobility. The demarcation of folded lipoyl domains (L1, L2 and L3) is still approximate, particularly at their C-terminal ends.

the specific radioactivity of each peptide was the same and a total of three lipoyl groups per E2 chain was estimated for the intact complex (Hale and Perham, 1979b).

The lipoyl domains of the E2 core

To investigate which of the lipoyl groups could be reductively acetylated, PDH complex was incubated with pyruvate and N-ethyl[2,3-^{14}C]maleimide (Danson and Perham, 1976) and subjected to limited proteolysis with *S. aureus* V8 proteinase. The radiolabelled (and hence lipoic acid-containing) fragments were isolated as a single fraction (apparent mol. wt. ~27 000) by gel filtration and this contained three major peptides (S1, S2 and S3) resolvable by polyacrylamide gel electrophoresis in the absence and presence of SDS (Figure 2). As judged by the latter technique, fragments S1, S2 and S3 had apparent mol. wts. of 11 000, 9000 and 8000, respectively, whereas gel filtration in the presence of 6 M guanidine.HCl (Packman *et al.*, 1984) indicated a single apparent mol. wt. of ~12 500. These fragments were resolved by ion-exchange chromatography (Figure 2) and subjected to amino acid and N-terminal sequence analysis to locate them in the primary structure of E2.

Fragment S1 gave the N-terminal sequence AAPAAAPAPA, recognisable as residues 193–202 of E2, which implies that fragment S1 corresponds to lipoyl segment L3 (Figure 1). Fragment S2 gave the N-terminal sequence A$^{I}_{L}$E$^{I}_{L}$KVPD$^{I}_{L}$G [I and L are not distinguished (Chang, 1983)], recognisable as the N-terminal sequence of E2, which implies that fragment S2 corresponds to lipoyl segment L1. Fragment S3 gave the N-terminal sequence AAPAAAPAAAAAKDV, recognisable as residues 93–107 of E2, which implies that fragment S3 corresponds to lipoyl segment L2. The amino acid compositions did not enable us to identify unambiguously the C-terminal ends of the fragments but it is obvious that *S. aureus* V8 proteinase has cut the N-terminal half of the E2 chain into three separate lipoyl segments, 90–100 residues in length, each of which was resistant to further proteolysis under these conditions and can be envisaged as a separately folded domain. All three lipoyl domains were radiolabelled, implying that the lipoyl-lysine residues at positions 40, 143 and 244 were reductively acetylated by the substrate, pyruvate. However, it is unlikely that this can have occurred fully at each site since a total of only 1.7–2 lipoyl groups per E2 chain can be reductively acetylated in the complex (Danson and Perham, 1976; Bates *et al.*, 1977; Collins and Reed, 1977).

Similar results were obtained when the lipoyl-lysine residues were radiolabelled by treating the complex with 2.5 mM phenylmaleimide in the presence of [2-^{14}C]pyruvate. Reaction of the [^{14}C]acetyl dihydrolipoic acid residues with phenylmaleimide was effectively complete within 90 s, as evidenced by the 90% loss of enzymic activity. The incorporation of [^{14}C]acetyl groups was only ~1.7 mol/mol E2 chain, as before (see refs. in Table II). After digestion of the radiolabelled complex with *S. aureus* V8 proteinase, the lipoyl domains were separated by polyacrylamide gel electrophoresis in a phosphate-buffered system (Packman *et al.*, 1984), pH 6.7, lacking SDS, and each domain was found to be radiolabelled. Thus, all three lipoyl domains must have reacted, at least in part, with the substrate. There was no indication of any substantial difference between the extents of the reaction of the individual domains, as judged by this technique, but we cannot rule out a preference for the order in which the lipoyl domains became reductively acetylated in the intact complex.

In a further experiment, native PDH complex was digested with *S. aureus* V8 proteinase and the mixture of lipoyl domains was isolated by gel filtration. The domains were incubated with [2-^{14}C]pyruvate and either intact PDH complex or resolved E1 (Packman *et al.*, 1984; Coggins *et al.*, 1976) and then separated by polyacrylamide gel electrophoresis under non-denaturing conditions. All three domains were found to be reductively acetylated. Thus, unlike free lipoamide, individual lipoyl domains retain their ability to function as substrates for E1 after excision from the enzyme complex.

Tryptic cleavage of the E2 core

When the PDH complex is subjected to limited proteolysis with trypsin, the complete lipoic acid-containing portion of the E2 polypeptide chain (probable mol. wt. 32 000) is removed, leaving a residual complex assembled round an octahedral inner E2 core composed of truncated E2 subunits

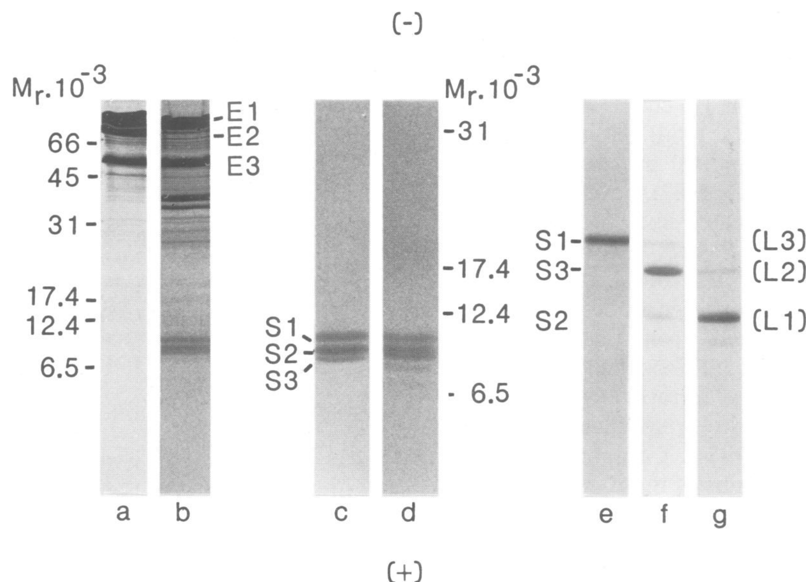

Fig. 2. Preparation of free lipoyl domains by limited proteolysis of the PDH complex. The lipoyl groups on the PDH complex were radiolabelled with N-ethyl[2,3-^{14}C]maleimide in the presence of pyruvate. The complex was subjected to limited proteolysis and the radiolabelled fragements were isolated by gel filtration and ion-exchange chromatography. **Lanes a–c**, resolving gel 27% T, 0.3% C (Packman *et al.*, 1984), stacking gel 5% T, 2.5% C, each containing 0.1% SDS. Staining was with Coomassie Brilliant Blue R. **Lane a**, intact complex. **Lane b**, complex after digestion for 2 h with *S. aureus* V8 proteinase. **Lane c**, radioactive (lipoyl domain) fraction after gel filtration of the digest on Sephadex G-75. **Lane d**, autoradiograph of **lane c**. **Lanes e–g**, gel composition as in **lanes a–c** but lacking SDS. **Lane e**, purified fragment S1. **Lane f**, purified fragment S3. **Lane g**, purified fragment S2.

with apparent mol. wt. of 36 000 by SDS-polyacrylamide gel electrophoresis (Bleile *et al.*, 1979; Hale and Perham, 1979a) and 30 000 by sedimentation-equilibrium centrifugation (Bleile *et al.*, 1979). To locate the site of tryptic cleavage, the E2 core was isolated from the PDH complex and subjected to limited proteolysis with trypsin. A precipitate of the inner core formed which was composed of a major fragment (IC1) and a minor fragment (IC2); these were separated by ion-exchange chromatography in 8 M urea (Figure 3). N-terminal sequence analysis of fragment IC1 gave SEFAXNBAY (where B = D or N; X = undetermined); this sequence corresponds to residues 317–325 (SEFAENDAY) in E2 (Figure 1). The N-terminal sequence of fragment IC2 could not be determined, but we suspect that it represents a minor tryptic cleavage inside fragment IC1 induced by the relatively harsh conditions of limited proteolysis we used to ensure a maximal yield of homogeneous fragment IC1.

A principal tryptic cleavage of E2 must occur at Lys-316 to generate fragment IC1. In the absence of C-terminal cleavages, the true mol. wt. of fragment IC1 will be 34 375. To the N-terminal side of Lys-316 are the three lipoyl domains and, as revealed by proton n.m.r. spectroscopy (Perham *et al.*, 1981), most of the mobile region(s) of the polypeptide chain. Since the n.m.r. spectra indicate a high proportion of alanine residues in the mobile polypeptide, it is likely that the regions of E2 oddly rich in alanine, proline and charged amino acids (Figure 1) are those concerned. These regions cannot as yet be defined in length exactly but, as outlined in Figure 1, the total number of residues involved (~90) agrees well with the best estimate yet made from n.m.r. spectroscopy (Perham *et al.*, 1981; Roberts *et al.*, 1983). It is interesting that similar regions of polypeptide chain have been assigned high conformational mobility in other proteins such as muscle light chains and lens crystallins (Berbers *et al.*, 1983, and refs. therein). This may turn out to be a general feature of proteins in which mobility is required.

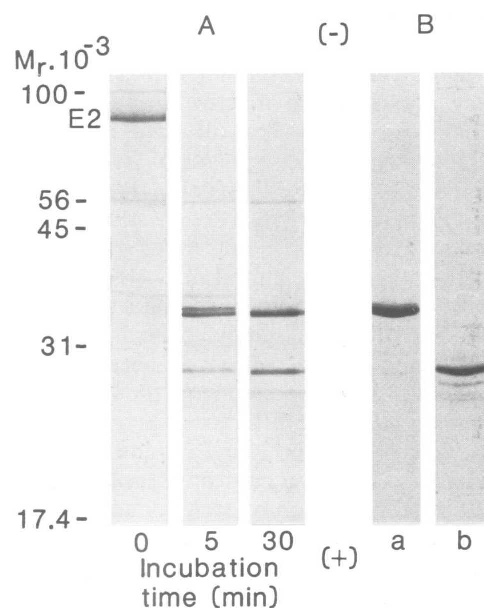

Fig. 3. Limited proteolysis of isolated E2 core with trypsin. Cleavage of the E2 chains was monitored by SDS-polyacrylamide gel electrophoresis (20% T, 0.3% C) and the major cleavage products were S-carboxymethylated and purified by ion-exchange chromatography. **Panel A**: time-course of tryptic digestion. **Panel B**: SDS-polyacrylamide gel electrophoresis of purified proteins, **Lane a**, IC1. **Lane b**, IC2.

A model for the structure of the E2 core

Our experiments demonstrate that the E2 chain of the *E. coli* PDH complex consists of three lipoyl domains, highly homologous in amino acid sequence and presumably three-dimensional structure, connected by lengthy intervening regions of mobile polypeptide chain and similarly linked to an inner core domain which contains the acetyltransferase active

site and binding sites for the E1 and E3 subunits (Bleile *et al.*, 1979; Hale and Perham, 1979a). 2-Oxo acid dehydrogenase complexes from mitochondria and Gram-positive bacteria are likely to be similar except that their E2 chains contain only one lipoyl domain (Packman *et al.*, 1984). However, PDH complexes from other Gram-negative bacteria, such as *Azotobacter vinelandii* (Bosma *et al.*, 1982), have an E2 chain large enough to accommodate repeated lipoyl domains such as we have demonstrated here for *E. coli*.

The lipoyl domain from the PDH complex of *Bacillus stearothermophilus* shows curious differences between its apparent mol. wt. values estimated from sedimentation-equilibrium centrifugation (6500), SDS-polyacrylamide gel electrophoresis (~7800) and from gel filtration (~10 000 and 20 400 in the presence and absence, respectively, of 6 M guanidine.HCl) (Packman *et al.*, 1984). Comparable results were obtained with the lipoyl domains of the *E. coli* E2 chain (see above). Similar properties have previously been reported for the large lipoic acid-containing fragments released from the E2 chains of *E. coli* (Bleile *et al.*, 1979) and ox heart (Bleile *et al.*, 1981) complexes by limited tryptic digestion, and attributed to their acidic nature and a swollen or elongated overall shape. Information about the folded structure of lipoyl domains should emerge from detailed n.m.r. studies (Packman *et al.*, 1984).

Why the E2 chain of the *E. coli* PDH complex carries a three-fold repeat of the lipoyl domain is obscure. Lipoyl-lysine residues can be modified or hydrolysed and lipoyl domains can be excised without loss of catalytic activity in the complex (Berman *et al.*, 1981; Stepp *et al.*, 1981). This can be explained by an exotic form of active site coupling, in which a lipoyl domain can visit several different E1 active sites by virtue of the highly flexible segments of polypeptide chain in E2 (Berman *et al.*, 1981; Perham *et al.*, 1981; Roberts *et al.*, 1983; Stepp *et al.*, 1981; Hackert *et al.*, 1983). Any function of an excised lipoyl domain is thus taken over by one that remains. On the other hand, a total of ~1.7 acetyl groups can be incorporated per E2 chain in the intact complex, yet we have now shown that all lipoyl domains are lipoylated, perhaps fully, and can be reductively acetylated, at least in part, both in and when separated from the PDH complex. No simple explanation for the difference, such as failure specifically to lipoylate one of the lysine residues (Stephens *et al.*, 1983a), is tenable. However, we cannot rule out the possibility of incomplete lipoylation of each domain occurring under different growth conditions of the organism.

Heterogeneity of the quaternary structure of the PDH complex

The most powerful method for determining polypeptide chain ratios in protein complexes, radioamidination and SDS-polyacrylamide gel electrophoresis (Bates *et al.*, 1975; Hale *et al.*, 1979), depends on accurate knowledge of the amino acid compositions of the proteins concerned. These are now available from the DNA sequence of the structural genes for E2, E1 and E3 in *E. coli* K12 (Stephens *et al.*, 1983a, 1983b, 1983c). As predicted (Bleile *et al.*, 1979), the apparent mol. wt. of the E2 chain inferred from SDS-polyacrylamide gel electrophoresis (~80 000) is significantly higher than the true value (66 524). We have therefore recalculated chain ratios and lipoic acid contents from previously published data (Table II).

It is clear from these that E1:E2 is always >1.0, generally in the range 1.2–1.5 (cf. Vogel *et al.*, 1972), whereas E3:E2 is <1.0, generally 0.6–0.8. Similar ratios are obtained from PDH complex isolated from *E. coli* grown on [^{35}S]sulphate (Table II). These ratios are not integral, though the method has given correct integral ratios in other protein complexes (Gray *et al.*, 1980; Bates and Thomas, 1981). Thus, the PDH complex might not necessarily be an exact, homogeneous structure in terms of its E1 and E3 subunits although the structure of the E2 core is governed by its octahedral or icosahedral symmetry (Bates *et al.*, 1975; Henderson and Perham, 1980). Evidence of heterogeneity from hydrodynamic studies of the PDH complex (Schmitt and Cohen, 1980; Gilbert and Gilbert, 1980) and electron microscopy of the 2-oxoglutarate dehydrogenase complex (Wagenknecht *et al.*, 1983) has been reported. Other workers (Oliver and Reed, 1982) have proposed a model of the PDH complex from *E. coli* Crookes strain based on chain ratios (E1:E2:E3) of 1.0:1.0:0.5, but with overlap of binding sites for E1 and E3 subunits on the E2 core. Competition for binding sites during assembly could be the basis for the type of heterogeneity we envisage, especially if there are differences in the rates of biosynthesis of different subunits under different growth conditions. Equally, there may be subtle differences in structure and lipoic acid content between PDH complexes isolated from *E. coli* K12 and Crookes strain (Bates *et al.*, 1975; Stephens *et al.*, 1983a). More work will be needed to resolve this point. At this stage it is abundantly clear that 2-oxo acid dehydrogenase complexes do not conform entirely to the design principles of simpler oligomeric systems (Perham, 1975).

Table II. Polypeptide chain ratios in PDH complex from *E. coli* K12

Chain ratios (E1:E2:E3)[a]	Lipoyl groups per E2 chain	Revised chain ratios (E1:E2:E3)	Revised estimate of lipoyl groups per E2 chain	References
1.56:1:0.78		1.37:1:0.73		Danson *et al.* (1979)
1.80:1:0.85[b]	2.0[d]	1.58:1:0.79	1.7	Bates *et al.* (1977)
1.24:1:0.95	2.1[d]	1.09:1:0.89	1.8	Danson *et al.* (1978)
1.45:1:0.77	2.0[d]	1.27:1:0.73	1.7	Packman *et al.* (1983)
1.69:1:1.03	2.0[e]	1.48:1:0.96	1.7	Danson and Perham (1976)
1.62:1:1.06[c]		1.24:1:0.76		Hale and Perham (1979b)
1.53:1:0.90[c]	3.4[f]	1.18:1:0.64	2.7	Hale and Perham (1979b)
1.67:1:0.87		1.52:1:0.80		Bates *et al.* (1975)
1.81:1:0.88		1.64:1:0.82		Bates *et al.* (1975)
1.38:1:0.85		1.25:1:0.79		Bates *et al.* (1975)

[a]Estimated by radioamidination method (Bates *et al.*, 1975; Hale *et al.*, 1979).
[b]Complex obtained by self-assembly *in vitro*.
[c]Estimated from incorporation of [^{35}S]sulphate into lipoic acid, cysteine and methionine.
[d]Estimated from incorporation of [2-^{14}C]pyruvate.
[e]Estimated from incorporation of N-ethyl[2,3-^{14}C]maleimide in the presence of pyruvate.
[f]Estimated from incorporation of [^{35}S]sulphate into lipoic acid.
Data obtained with the radioamidination method (Bates *et al.*, 1975) have been recalculated using the amino acid compositions of E2, E1 and E3 chains of *E. coli* K12 PDH complex (Stephens *et al.*, 1983a, 1983b, 1983c): E1 (mol. wt. 99 474; 49 amino groups); E2 (mol. wt. 66 524; 51 amino groups, assuming three lipoyl-lysine residues per E2 chain); E3 (mol. wt. 50 554; 40 amino groups). Chain ratios for ^{35}S-labelled complex (Hale and Perham, 1979b) have been recalculated similarly: E1 (21 Met, 6 Cys); E2 (16 Met, 1 Cys, 3 lipoic acids); E3 (9 Met, 5 Cys).

Materials and methods

Materials

PDH complex was purified from a constitutive mutant of *E. coli* K12 (Danson *et al.*, 1979). *S. aureus* V8 proteinase was from Miles Laboratories, tosylphenylchloromethylketone-treated trypsin was from Worthington, 4-N,N-dimethylaminoazobenzene 4'-isothiocyanate was from BDH. Sequencing reagents (Sequanal grade) were from Pierce and Warriner.

Protein chemical methods

Amino acid analysis, S-carboxymethylation and high-voltage paper electrophoresis were carried out as described by Perham (1978). N-terminal sequence analysis (Chang, 1983) was performed as modified by Runswick and Walker (1983).

Isolation of three lipoylated peptides from PDH complex

PDH complex was mixed with a small amount of complex containing [^{35}S]-lipoic acid and [^{35}S]cysteine which was isolated from bacteria grown in medium containing [^{35}S]sulphate and unlabelled methionine (Berman *et al.*, 1981). The lipoic acid-containing fragments were isolated by limited proteolysis with trypsin and gel filtration on Sepharose CL-6B (Hale and Perham, 1979a). The fragments were further digested with *S. aureus* V8 proteinase (2%, w/w) at 37°C for 45 min in 0.5% NH_4HCO_3 and then subjected to diagonal paper electrophoresis at pH 6.5. Performic acid oxidation was carried out between the electrophoretic steps (Hale and Perham, 1979a). A single off-diagonal radioactive spot (peptide TA2) was detected. Paper chromatography in butan-1-ol/acetic acid/water/pyridine (15/3/12/10, by vol.) resolved TA2 into three components: TA2a, TA2b and TA2c. The peptides were hydrolysed in 6 M HCl, 0.05% 2-mercaptoethanol for 24 and 72 h. The values for Thr and Ser were extrapolated back to zero time.

Isolation of lipoyl domains

PDH complex (10 mg/ml), was treated with N-ethyl[2,3-^{14}C]maleimide in the presence of pyruvate (Danson and Perham, 1976) to radiolabel the lipoyl groups, and then incubated at 0°C with 0.2% (w/w) *S. aureus* V8 proteinase in 20 mM sodium phosphate buffer, pH 7, containing 2.7 mM EDTA and 0.02% NaN_3. At timed intervals, samples were removed for analysis by SDS-polyacrylamide gel electrophoresis (Packman *et al.*, 1984) with the pH of the resolving gel adjusted to 8.3. After 2 h, the residual digest was fractionated at 0°C on a Sephadex G-75 column (132 cm x 0.7 cm) in the 20 mM sodium phosphate buffer (flow 1.7 ml/h). The bulk of the protein emerged at the void volume of the column whereas the radioactive peptides (lipoyl domains) emerged as a single peak (k_d = 0.29 ± 0.04, apparent mol. wt. 27 000) having appreciable A_{230} but no detectable A_{280}. The lipoyl domains were separated by ion-exchange chromatography on Whatman DE-52 (6 cm x 1.2 cm) at 0°C using a linear gradient (80 ml, 14 ml/h) of 10–600 mM ammonium acetate, pH 5. This yielded pure fragments S1 and S2 but fragment S3 was contaminated with the other two fragments. Further chromatography (FPLC) was performed on Pharmacia Mono Q resin using a linear gradient (20 ml) of 0.25–0.35 M ammonium acetate, pH 5, to yield pure fragment S3. The elution order on both resins was S1, S3, S2.

Limited proteolysis of the E2 core

E2 core was isolated from PDH complex (Coggins *et al.*, 1976; Reed and Willms, 1966). The protein (4 mg/ml) was digested for 30 min at 30°C with 1% (w/w) trypsin in 20 mM sodium phosphate buffer, pH 7, containing 2.7 mM EDTA and 0.02% NaN_3. Samples were removed at timed intervals for SDS-polyacrylamide gel electrophoresis (20% T, 0.3% C). E2 (apparent mol. wt. 80 000) was quickly degraded to two proteins of similar size (apparent mol. wt. 36 000). After 30 min, the upper band on the gel disappeared, leaving a mixture of two insoluble proteins (IC1 and IC2) with apparent mol. wt. values of 36 000 and 28 000. The washed precipitate was S-carboxymethylated in 5 M guanidine.HCl and, after dialysis, dissolved in 10 mM ammonium acetate, pH 5, containing 8 M urea. IC1 and IC2 were separated by ion-exchange chromatography on Whatman CM-32 (2.4 cm x 1.2 cm) developed with a linear gradient (30 ml, 10–200 mM, 5 ml/h) of the same buffer. The proteins were dialysed exhaustively against 0.5% NH_4HCO_3 and freeze-dried for sequence analysis.

Acknowledgements

We are grateful to Miss M.B.Thomas and Miss M.Mather for technical assistance and to the Science Research Council for financial support.

References

Bates,D.L. and Thomas,J.O. (1981) *Nucleic Acids Res.*, **9**, 5883-5894.

Bates,D.L., Harrison,R.A. and Perham,R.N. (1975) *FEBS Lett.*, **60**, 427-430.

Bates,D.L., Danson,M.J., Hale,G., Hooper,E.A. and Perham,R.N. (1977) *Nature*, **268**, 313-316.

Berbers,G.A.M., Hoekman,W.A., Bloemendal,H., de Jong,W.W., Kleinschmidt,T. and Braunitzer,G. (1983) *FEBS Lett.*, **161**, 225-229.

Berman,J.N., Chen,G.-X., Hale,G. and Perham,R.N. (1981) *Biochem. J.*, **199**, 513-520.

Bleile,D.M., Munk,P., Oliver,R.M. and Reed,L.J. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 4385-4389.

Bleile,D.M., Hackert,M.L., Pettit,F.H. and Reed,L.J. (1981) *J. Biol. Chem.*, **256**, 514-519.

Bosma,H.J., de Graaf-Hess,A.C., de Kok,A., Veeger,C., Visser,A.J.W.G. and Voordouw,G. (1982) *Ann. N.Y. Acad. Sci.*, **378**, 265-285.

Chang,J.-Y. (1983) *Methods Enzymol.*, **91**, 455-466.

Coggins,J.R., Hooper,E.A. and Perham,R.N. (1976) *Biochemistry (Wash.)*, **15**, 2527-2533.

Collins,J.H. and Reed,L.J. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 4223-4227.

Danson,M.J. and Perham,R.N. (1976) *Biochem. J.*, **159**, 677-682.

Danson,M.J., Fersht,A.R. and Perham,R.N. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 5386-5390.

Danson,M.J., Hale,G., Johnson,P., Perham,R.N., Smith,J. and Spragg,P. (1979) *J. Mol. Biol.*, **129**, 603-617.

Gilbert,G.A. and Gilbert,L.M. (1980) *J. Mol. Biol.*, **144**, 405-408.

Gray,J.C., Hooper,E.A. and Perham,R.N. (1980) *FEBS Lett.*, **114**, 237-239.

Hackert,M.L., Oliver,R.M. and Reed,L.J. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 2907-2911.

Hale,G. and Perham,R.N. (1979a) *FEBS Lett.*, **105**, 263-266.

Hale,G. and Perham,R.N. (1979b) *Biochem. J.*, **177**, 129-137.

Hale,G. and Perham,R.N. (1980) *Biochem. J.*, **187**, 905-908.

Hale,G., Hooper,E.A. and Perham,R.N. (1979) *Biochem. J.*, **177**, 136-137.

Henderson,C.E. and Perham,R.N. (1980) *Biochem. J.*, **189**, 161-172.

Oliver,R.M. and Reed,L.J. (1982) in Harris,R. (ed.), *Electron Microscopy of Proteins*, vol. **2**, Academic Press, London, pp. 1-48.

Packman,L.C., Stanley,C.J. and Perham,R.N. (1983) *Biochem. J.*, **213**, 331-338.

Packman,L.C., Perham,R.N. and Roberts,G.C.K. (1984) *Biochem. J.*, **217**, 219-227.

Perham,R.N. (1975) *Phil. Trans. R. Soc. Lond. Ser. B*, **272**, 123-136.

Perham,R.N. (1978) in Kornberg,H.L., Metcalfe,J.C., Northcote,D.H., Pogson,C.I. and Tipton,K.F. (eds.), *Techniques in Protein and Enzyme Biochemistry*, vol. **B110**, Elsevier/North-Holland, Amsterdam, pp. 1-39.

Perham,R.N., Duckworth,H.W. and Roberts,G.C.K. (1981) *Nature*, **292**, 474-477.

Reed,L.J. (1974) *Acc. Chem. Res.*, **7**, 40-46.

Reed,L.J. and Willms,C.R. (1966) *Methods Enzymol.*, **9**, 247-265.

Roberts,G.C.K., Duckworth,H.W., Packman,L.C. and Perham,R.N. (1983) *Ciba Found. Symp.*, **93**, 47-62.

Runswick,M.J. and Walker,J.E. (1983) *J. Biol. Chem.*, **258**, 3081-3089.

Schmitt,B. and Cohen,R. (1980) *Biochem. Biophys. Res. Commun.*, **93**, 709-712.

Stephens,P.E., Darlison,M.G., Lewis,H.M. and Guest,J.R. (1983a) *Eur. J. Biochem.*, **133**, 481-489.

Stephens,P.E., Darlison,M.G., Lewis,H.M. and Guest,J.R. (1983b) *Eur. J. Biochem.*, **133**, 155-162.

Stephens,P.E., Lewis,H.M., Darlison,M.G. and Guest,J.R. (1983c) *Eur. J. Biochem.*, **133**, 519-527.

Stepp,L.R., Bleile,D.M., McRorie,D.K., Pettit,F.H. and Reed,L.J. (1981) *Biochemistry (Wash.)*, **20**, 4555-4560.

Vogel,O., Hoehn,B. and Henning,U. (1972) *Proc. Natl. Acad. Sci. USA*, **69**, 1615-1619.

Wagenknecht,T., Francis,N. and DeRosier,D.J. (1983) *J. Mol. Biol.*, **165**, 523-529.

White,R.H., Bleile,D.M. and Reed,L.J. (1980) *Biochem. Biophys. Res. Commun.*, **94**, 78-84.

Received on 2 March 1984