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The pyruvate dehydrogenase multi-enzyme complex of *Escherichia coli*: genetic reconstruction and functional analysis of the lipoyl domains

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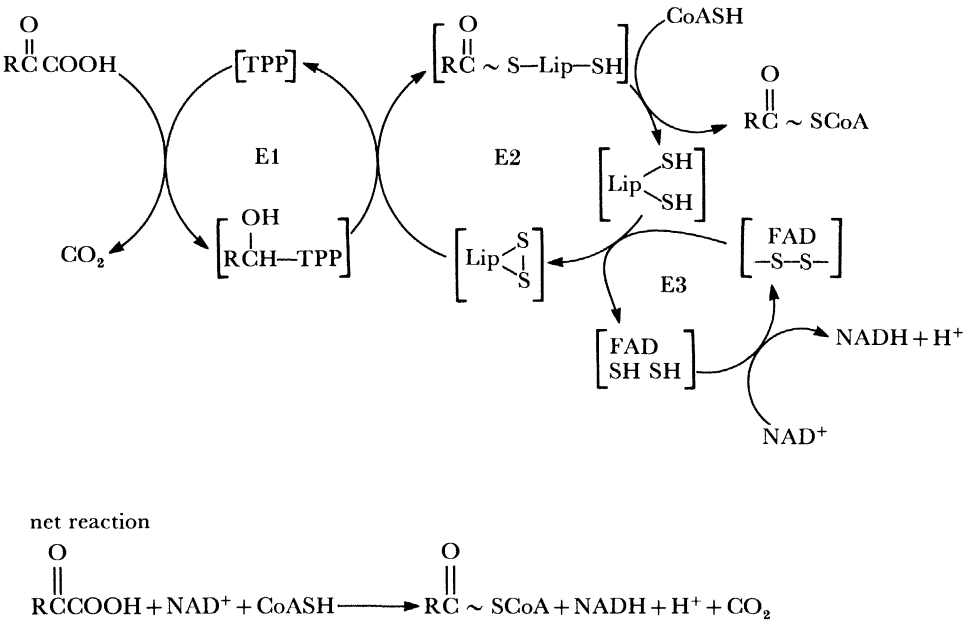
The dihydrolipoamide acetyltransferase (E2p) component of the pyruvate dehydrogenase complex of *Escherichia coli* contains three highly homologous lipoyl domains (*ca.* 100 residues) that are tandemly repeated to form the N-terminal half of the polypeptide chain. These lipoyl domains are linked to a much larger (*ca.* 300 residues) subunit-binding domain that aggregates to form the octahedral inner core of the complex and also contains the acetyltransferase active site. Selective *in vitro* deletions in the E2p gene (*aceF*) have allowed the creation of truncated E2p chains in which one or more of the lipoyl domains has been excised. Site-directed mutagenesis has been used to change individual residues. The effects of these deletions and mutations on the assembly, catalytic activity and active-site coupling in the complex are assessed.

1. Introduction

The 2-oxo acid dehydrogenase complexes consist of multiple copies of three enzymes which function successively to catalyse the reaction shown schematically in figure 1. For the pyruvate dehydrogenase (PDH) complex, the enzymes are pyruvate dehydrogenase (lipoamide) (E1p, EC 1.2.4.1), dihydrolipoamide acetyltransferase (E2p, EC 2.3.1.12) and dihydrolipoamide dehydrogenase (E3, EC 1.6.4.3). Corresponding enzymes comprise the 2-oxoglutarate dehydrogenase and branched-chain 2-oxo acid dehydrogenase complexes.

The PDH complex of *Escherichia coli* ($M_r \approx 5 \times 10^6$) has a structural core made up of 24 E2p polypeptide chains arranged with octahedral symmetry, which binds the E1p and E3 subunits tightly but non-covalently (Reed 1974; Danson *et al.* 1979). The substrate is transferred between the physically separate active sites by lipoyl-lysine 'swinging-arms' on which it is retained in thioester linkage (Reed 1974; Ambrose & Perham 1976; Grande *et al.* 1976). The lipoyl-lysine residues are located in the E2p subunits, in segments of the E2p chains that protrude between the E1p and E3 subunits from an inner part of the E2p core (Bleile *et al.* 1979; Hale & Perham 1979). There exists an extensive network of coupling reactions which permits the intramolecular transfer of acetyl groups between lipoic acid residues of different E2p subunits in the same enzyme core (Bates *et al.* 1977; Collins & Reed 1977; Packman *et al.* 1983) and, moreover, a lipoyl group can visit the active site of several E1p subunits in the same complex (Stepp *et al.* 1981; Berman *et al.* 1981; Hackert *et al.* 1983). These remarkable forms of active-site coupling are thought to be facilitated by particular regions of the E2p polypeptide chains which, to judge from ^{1}H n.m.r. spectroscopy, enjoy substantial conformational mobility (Perham *et al.* 1981; Roberts *et al.* 1983).



net reaction

$$RCCOOH + NAD^+ + CoASH \longrightarrow RC \sim SCoA + NADH + H^+ + CO_2$$

FIGURE 1. The reaction mechanism of the 2-oxo acid dehydrogenase multi-enzyme complexes. R = CH_3 for pyruvate dehydrogenase. TPP, thiamin pyrophosphate; Lip, lipoic acid.

The PDH complex of *E. coli* is encoded by three genes, *aceE* (E1p), *aceF* (E2p) and *lpd* (E3), which comprise the *aceEF-lpd* operon (Spencer & Guest 1985) and whose complete nucleotide sequences have been determined (Stephens *et al.* 1983*a*, *b*, *c*). The primary structure of the E2p chain inferred from the DNA sequence of the *aceF* gene (Stephens *et al.* 1983*b*) is notable for three highly homologous sequences of *ca.* 100 amino-acid residues (designated lip1–3), that are tandemly repeated to form the N-terminal half of the protein (figure 2). Each repeat contains a lysine residue which is a potential site for lipoylation (Hale & Perham 1980) and all three sites are lipoylated, at least in part, in the native enzyme (Packman *et al.* 1984*a*).

The lipoylated region of each E2p chain is released from the PDH complex by limited tryptic cleavage of E2p at Lys-316 and can in fact be isolated as three distinct functional entities (domains) after limited proteolysis of the complex with *Staphylococcus aureus* V8 proteinase (see figure 2). The lipoyl group of each domain becomes reductively acetylated in the intact complex in the presence of substrate (Packman *et al.* 1984*a*). The lip segments also contain lengthy C-terminal regions of polypeptide chain (20–30 amino-acid residues) that are unusually rich in residues of alanine, proline and charged amino acids (Stephens *et al.* 1983*b*). It is likely that these regions are the sources of some major sharp resonances in the n.m.r. spectra of the complex and they may be associated with the conformational mobility expected of the lipoyl segments (Packman *et al.* 1984*a*; Spencer *et al.* 1984). The large domain of the E2p chain that remains aggregated to form the inner core of the complex and which retains both the acetyltransferase active site and the binding sites for E1p and E3 subunits (Bleile *et al.* 1979; Hale & Perham 1979) is provided by the C-terminal half of the sequence (Stephens *et al.* 1983*b*; Spencer *et al.* 1984). This section of the E2p chain (and the *aceF* gene) is designated cat (*cat*). Thus, in addition to its visits to the active sites of E1 and E3 subunits, an E2 lip domain must be capable of visiting an E2 cat domain of the complex inner core.

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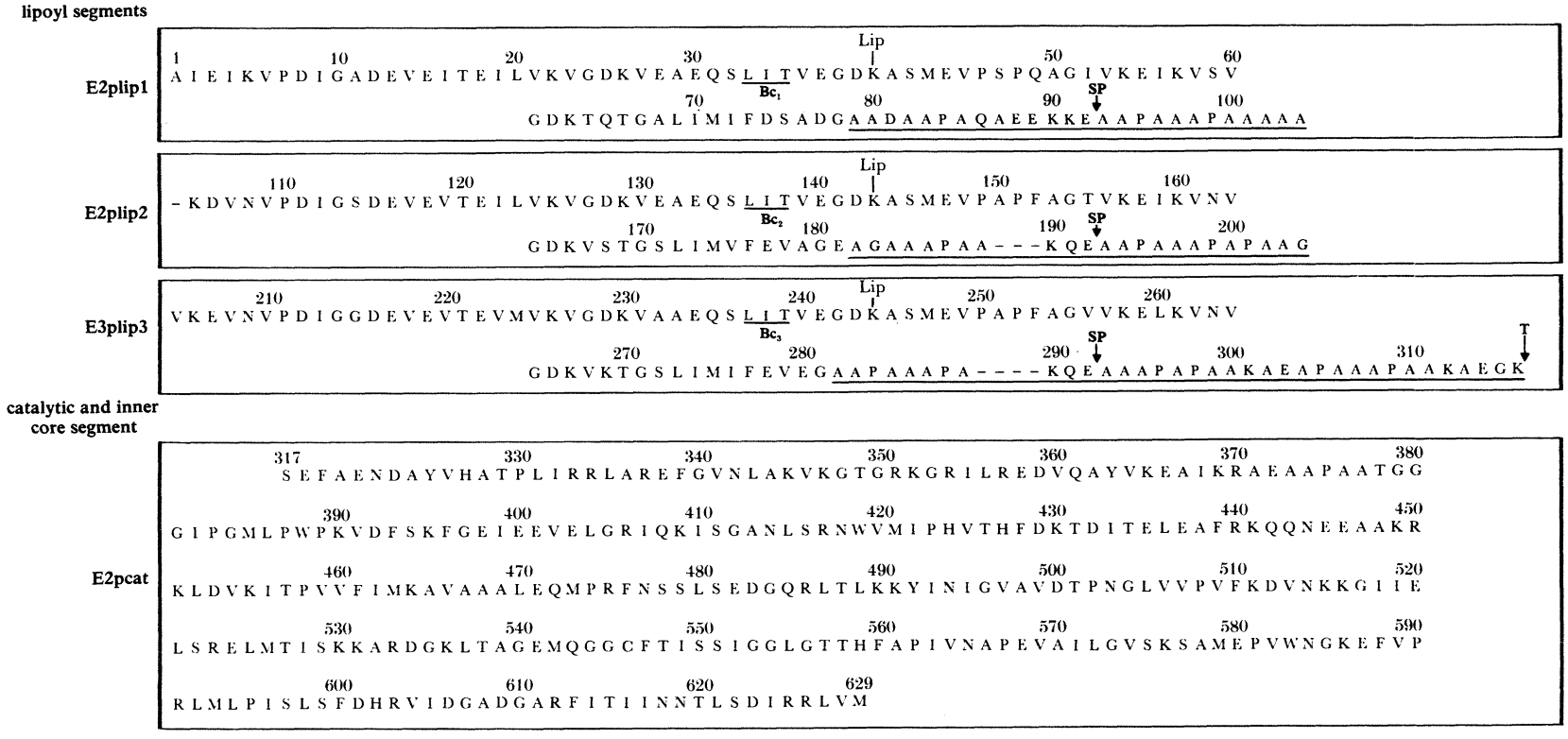

FIGURE 2. Primary structure of the E2p chain of the PDH complex of *E. coli* showing the repeating lipoyl segments and the catalytic and inner core segment. The sites of covalent attachment of the lipoyl groups (Lip), the (alanine+proline)-rich sequences (underlined), and the highly sensitive bonds cleaved by trypsin (**T**) and *S. aureus* V8 proteinase (**SP**) are marked. The positions of the *Bcl*I restriction sites (**Bc_1**, **Bc_2**, **Bc_3**) in the corresponding *aceF* gene are also indicated.

The structure and mechanism of other 2-oxo acid dehydrogenase complexes are thought to be essentially the same but there are some significant differences. For example, the E2 cores of the PDH complexes of mammalian mitochondria (Reed 1974) and *Bacillus* species (Henderson *et al.* 1979) comprise 60 E2p chains arranged with icosahedral symmetry and each E2p chain appears to contain only one lipoyl group (White *et al.* 1980; Bleile *et al.* 1981; Stanley *et al.* 1981), housed in a single lipoyl domain (Packman *et al.* 1984*b*). On the other hand, although the E2 core of the 2-oxoglutarate dehydrogenase complex of *E. coli* comprises 24 E2o chains arranged with octahedral symmetry (Reed 1974), thus resembling the *E. coli* PDH complex, it too has only one lipoyl group per E2o chain (White *et al.* 1980). Yet the corresponding lipoylated region (Perham & Roberts 1981) has an amino-acid sequence that is homologous to the three lip segments of the *E. coli* E2p chain (Guest *et al.* 1984; Spencer *et al.* 1984).

In this paper we describe the use of precise reconstructions of the *E. coli* E2p gene (*aceF*) to tackle three important enzymological problems:

(i) the need for repeating lipoyl domains in the E2p polypeptide chain;

(ii) the role of the alanine- and proline-rich regions at the C-terminal ends of the three lip segments;

(iii) the role of the lipoyl-lysine residue in the lipoyl domain.

2. Restructuring and deletion of the lipoyl domains

An obvious question to ask of the PDH complex of *E. coli* is whether all three lip segments per E2p chain are essential for catalytic activity. There is a high degree of amino-acid sequence homology in them (figure 2) which derives from corresponding nucleotide sequence homology in the *lip* segments of the *aceF* gene (Stephens *et al.* 1983*b*). Consequently, restriction targets, such as those for *Bcl*I (figure 2), occur at the same relative positions in adjacent *lip* segments. This offers a strategy for selective *in vitro* deletion in the *aceF* gene, leading to deletions in the E2p polypeptide chain equivalent to one or two lip segments and the formation of a hybrid lipoyl domain closely resembling the original ones (figures 2, 3).

This was achieved with the use of the two plasmids shown in figure 3: pGS87, which expresses all three components of the PDH complex from a 10.2 kbp *aceEF–lpd* fragment inserted between the *Hind*III and *Sal*I sites of the parent vector, pBR322; and pGS101, which contains a unique 2.28 kbp *Kpn*I–*Sph*I fragment encoding the three tandem lip segments of the E2p chain (Guest *et al.* 1985). Because it has no *Bcl*I sites other than those in the *lip* regions of the *aceF* gene, pGS101 could be manipulated by partial *Bcl*I digestion and religation of pGS101, and new *aceEF–lpd* operons with modified *aceF* genes could be created by recloning the shortened *Kpn*I–*Sph*I fragments in pGS87 (figure 3). Three different deletion derivatives were isolated, pGS102–104, and used to construct the corresponding *aceEF–lpd* plasmids, pGS108–110 (figure 3). Digestion at the three *Bcl*I sites (3884, 4193, 4496) produces precise 'in-phase' deletions equivalent to one or two lipoyl domains and generates hybrid domains from the flanking segments as illustrated in figure 4. The amino-acid sequences of these hybrid domains can be deduced from the primary structure shown in figure 2. Thus the E2p components encoded by specific plasmids should lack the following residues: pGS108, Δ33–135; pGS109, Δ136–236; and pGS110, Δ33–236.

The structures of the plasmids pGS108–110 were confirmed by restriction analysis and their abilities to direct the synthesis of truncated E2p polypeptides, as well as the normal E1p and

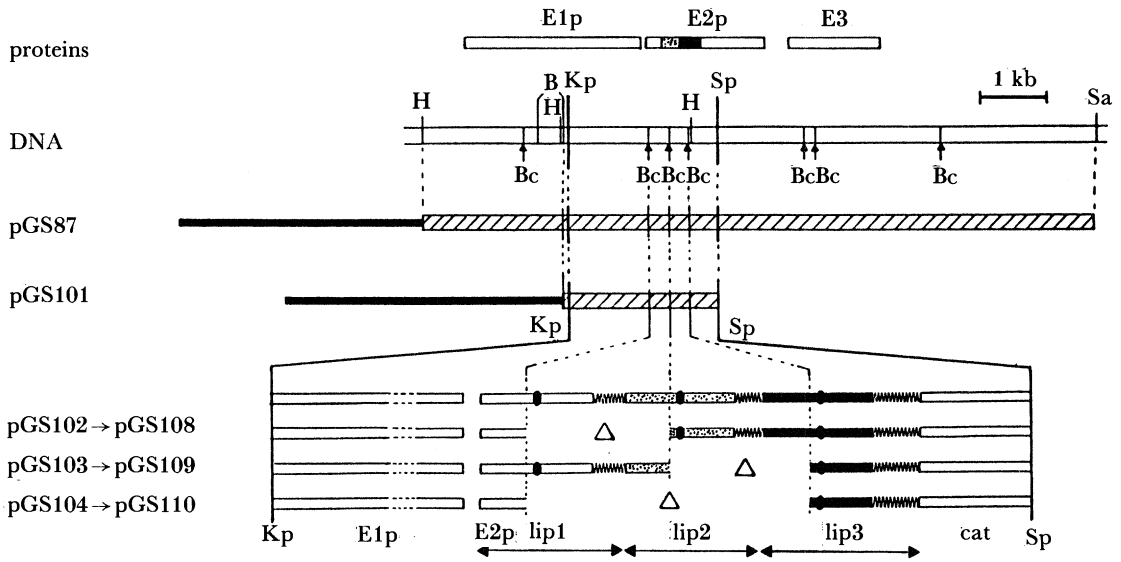

FIGURE 3. Strategy for selective deletion of lipoyl segments from the E2p chain of the PDH complex. The fragments of bacterial DNA (hatched) cloned in pBR322 (solid bars) in the primary plasmid (pGS87), which expresses all three components (E1p, E2p, and E3) of the PDH complex, and in the intermediate plasmid (pGS101) which encodes the three lipoyl segments (lip1–3), are shown below the physical map of the *aceEF–lpd* region. The details of the *Bcl*I-induced deletions (Δ) affecting the lipoyl segments are illustrated in the expanded section. The positions corresponding to the lipoylation sites (filled ovals) and (alanine + proline)-rich regions (zigzag lines) are indicated for each derivative of the intermediate and primary plasmids. Abbreviations: Bc, *Bcl*I; Bg, *Bgl*II; H, *Hin*dIII; Kp, *Kpn*I; Sa, *Sal*I; Sp, *Sph*I.

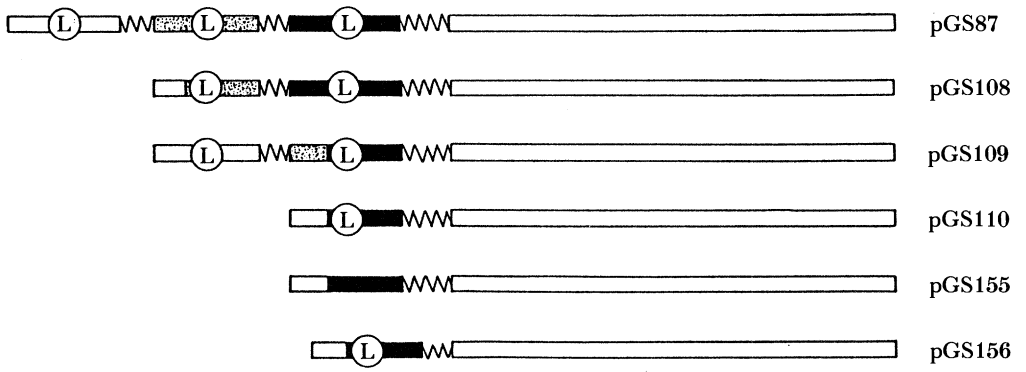

FIGURE 4. Schematic structures of the E2p chains expressed by the modified *aceEF–lpd* operons of different plasmids. The lipoyl segments are differentially shaded, and the lipoylation sites (encircled L), (alanine + proline)-rich regions (zigzag lines), and catalytic or inner core segments (open bars) are indicated.

E3 polypeptides, were demonstrated by the maxicell transcription–translation procedure of Sancar *et al.* (1979). Functional tests were performed with Amp^R transformants of a strain of *E. coli* that is deleted for the *aceEF–lpd* operon (JRG1342; Δ(*aroP–lpd*) *recA*). All of the *Bcl*I-generated deletion derivatives restored the wild-type (Ace^+Lpd^+) nutritional phenotype and the enzymic activities in cell-free extracts were not significantly different from those generated by the primary plasmid, pGS87 (table 1).

Detailed studies were performed with the PDH complexes purified from reconstructed strains

TABLE 1. *IN VITRO* MUTATIONS OF THE LIPOYL DOMAINS OF E2p

plasmid	mutation	lipoyl domains no.	lipoyl domains type	M_r of E2p DNA	M_r of E2p SDS–PAGE[a]	effects on Ace⁻Lpd⁻ host (JRG1342) Ace phenotype	specific activity of enzyme[b] PDHC	E1p	E2p	E3
pGS87&107	—	3	1, 2, 3	65959	83000	+	2.5	1.0	5.1	2.7
pGS108	lip deletion	2	1.2, 3	55592	69000	+	3.4	0.6	5.9	2.4
pGS109	lip deletion	2	1, 2.3	55978	—	+	2.6	0.8	3.4	2.2
pGS110	lip deletion	1	1.3	45611	52000	+	2.5	0.9	5.5	2.6
pGS156	lip deletion & Ala+Pro deletion	1	1.3	44533	47000	+	1.5	0.7	6.0	1.3
pGS155	lip deletion & Lys-244→Gln	1	1.3	45610	53000	–	<0.1	<0.02[c]	8.2	1.5
pBR322	—	—	—	—	—	–	<0.02	<0.02	0.3	0.05

[a] Purified component.
[b] Enzyme assays in cell-free extracts: specific activities in micromoles per milligram protein per hour. PDHC, pyruvate-dependent reduction of 3-acetylNAD; E1p, pyruvate-dependent reduction of ferricyanide; E2p, formation of S-acetyldihydrolipoamide from dihydrolipoamide and acetyl-CoA, and E3, dihydrolipoamide-dependent reduction of 3-acetylNAD.
[c] Active in purified enzyme.

of *E. coli* containing plasmid pGS108 (two lipoyl domains per E2p chain) and pGS110 (one lipoyl domain per E2p chain). Their specific activities were measured in the NAD^+-reduction assay and found to have values (27–30 μmol min^{-1} mg^{-1}), which are not very different from those of a wild-type complex (30–35 μmol min^{-1} mg^{-1}) isolated from a PDH-constitutive mutant of *E. coli* (Danson *et al.* 1979). Their behaviour as large macromolecular assemblies during ultracentrifugation was also little changed: for example, wild-type complex and the pGS110-complex gave values for $s_{20,w}$ of 55.3S and 52.9S, respectively, at identical protein concentrations of 2.0 mg ml^{-1}.

When subjected to SDS–polyacrylamide gel electrophoresis, the wild-type and reconstructed complexes differed only in the Coomassie Blue staining and apparent M_r values of their E2p polypeptide chains (figure 5). The Coomassie staining of the E2p chain increased and the band sharpened as the number of lip segments decreased; the differential staining effect did not persist when the silver stain (Morrissey 1981) was applied. The estimated M_r values were as follows: for the wild-type E2p chain, $M_r \approx 83000$; for the two lipoyl-domain E2p chain (pGS108), $M_r \approx 69000$; and for the one lipoyl-domain E2p chain (pGS110), $M_r \approx 52000$. These are in good agreement with the values estimated for the 'maxicell' translation products but differ substantially from the true values (66000, 56000 and 46000, respectively) calculated from the DNA sequence (table 1). Each lip segment appears to contribute some 6000 to the discrepancy. Anomalous electrophoretic mobility and poor Coomassie Blue staining of lipoylated regions of the E2p chain have previously been reported, and attributed to their acidic nature and a swollen or elongated shape (Bleile *et al.* 1979). A further explanation is suggested by the lengthy (alanine+proline)-rich sequence in each lip segment because somewhat similar sequences present at the N-termini of oxy β-crystallin B_1-chains confer on them anomalously low electrophoretic mobilities during SDS–polyacrylamide gel electrophoresis (Berbers *et al.* 1983).

Samples of wild-type PDH complex and of the reconstructed complexes were subjected to limited proteolysis with *S. aureus* V8 proteinase and the products were separated by means of polyacrylamide gel electrophoresis (figure 6). The three distinct lipoyl domains of wild-type

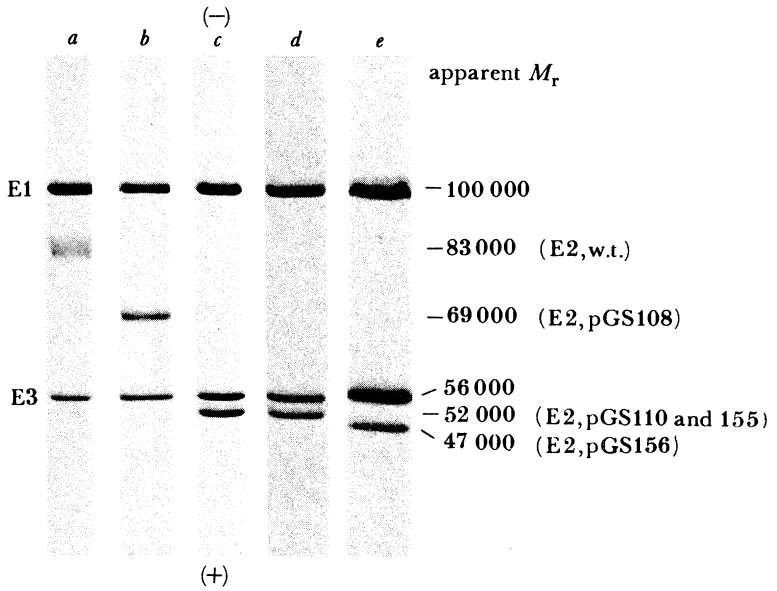

FIGURE 5. Separation of polypeptide chains of wild-type and restructured PDH complexes by means of SDS–polyacrylamide gel electrophoresis. Wild-type complex purified from a constitutive strain of *E. coli* K12, (track *a*): restructured complexes encoded by plasmids pGS108 (track *b*), pGS110 (track *c*), pGS155 (track *d*) and pGS156 (track *e*). Staining was with Coomassie Brilliant Blue.

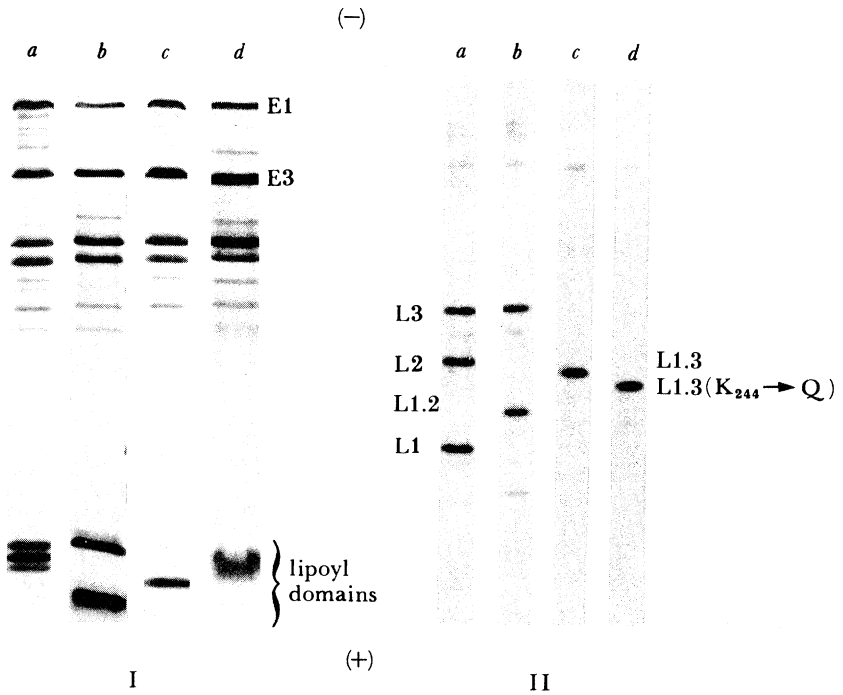

FIGURE 6. Limited proteolysis, with *S. aureus* V8 proteinase, of wild-type (*E. coli* K12) and restructured (plasmids pGS108, 110 and 155) PDH complexes. Panel I shows how the products of proteolysis were separated by SDS–polyacrylamide gel electrophoresis and visualized by staining with Coomassie Blue followed by silver. Wild-type *E. coli* K12 complex (track *a*); restructured complexes encoded by plasmids pGS108 (track *b*), pGS110 (track *c*) and pGS155 (track *d*). Panel II: the products of proteolysis were separated by means of polyacrylamide gel electrophoresis in the absence of SDS and visualized by silver staining. In this system, the resolution of lipoyl domains is enhanced and material of higher M_r or lower negative charge remains near the top of the gel (not shown). Tracks *a*–*d* as in Panel I.

complex were replaced by two lipoyl domains and one lipoyl domain in the digests of the pGS108 and pGS110 complexes, respectively, and their electrophoretic mobilities were consistent with the hybrid lipoyl domains formulated in figure 4. Prior treatment with radio-labelled substrate revealed that the normal and reconstructed lipoyl domains were reductively acetylated in their respective PDH complexes.

Despite the existence of three lipoyl domains in the E2p chain of the normal PDH complex, a total of only 1.7–2.0 lipoyl groups per E2p chain can be reductively acetylated in the presence of pyruvate (Danson & Perham 1976; Bates *et al.* 1977; Collins & Reed 1977; Speckhard *et al.* 1977). This anomaly remains in the restructured pGS108 and pGS110 complexes: the extents of reductive acetylation with [2-^{14}C]pyruvate (Packman *et al.* 1983) were found to be *ca.* 1.0–1.1 and 0.5–0.6 acetyl groups per E2p chain, respectively. These values are roughly two-thirds and one-third of the value observed for the wild-type complex.

The PDH complex from *E. coli* K12 (Danson *et al.* 1979) and the complexes encoded by plasmids pGS108 and pGS110 were also compared for their ability to undergo reductive acetylation in the presence of the E1 transition state analogue, thiaminthiothiazolone pyrophosphate (Gutowski & Lienhard 1976), as described by Packman *et al.* (1983). The acetylation curves (figure 7) for all three complexes were found to be indistinguishable. Because the departure of the curve from linearity is a measure of the extent of active-site coupling within a complex (Bates *et al.* 1977; Stanley *et al.* 1981), it is clear that genetic removal of one or two lip segments per E2p chain has not significantly impaired the ability of probably all twenty-four E2p chains in the E2 core to participate.

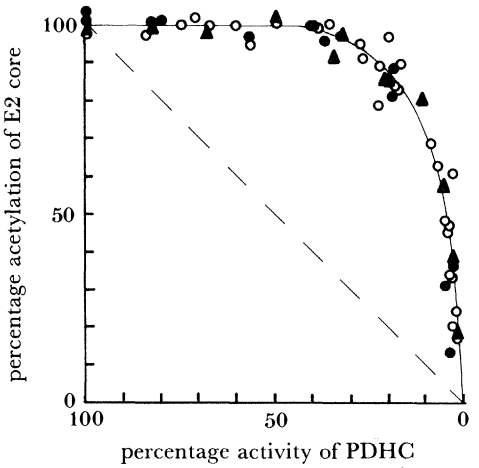

FIGURE 7. Acetylation curves to demonstrate active-site coupling in wild-type and restructured PDH complexes. The PDH complex activity was progressively inhibited by treating samples of complex with increasing amounts of thiaminthiothiazolone pyrophosphate, which selectively inhibits the E1p component. In each sample, the extent of reductive acetylation of the E2p component induced by incubating the complex with [2-^{14}C]pyruvate at 37 °C was measured: ○, wild-type *E. coli* K12 complex; ●, restructured complex encoded by pGS108; and ▲, restructured complex encoded by pGS110.

3. A DELETION IN THE (ALANINE + PROLINE)-RICH SEQUENCE

The 'one-lipoyl domain' PDH complex encoded by pGS110 is, as we have just seen, fully active by all the criteria thus far applied. Its simpler *aceF* gene made it the obvious choice for further studies by using the *in vitro* mutagenic approach. In this section we describe the deletion

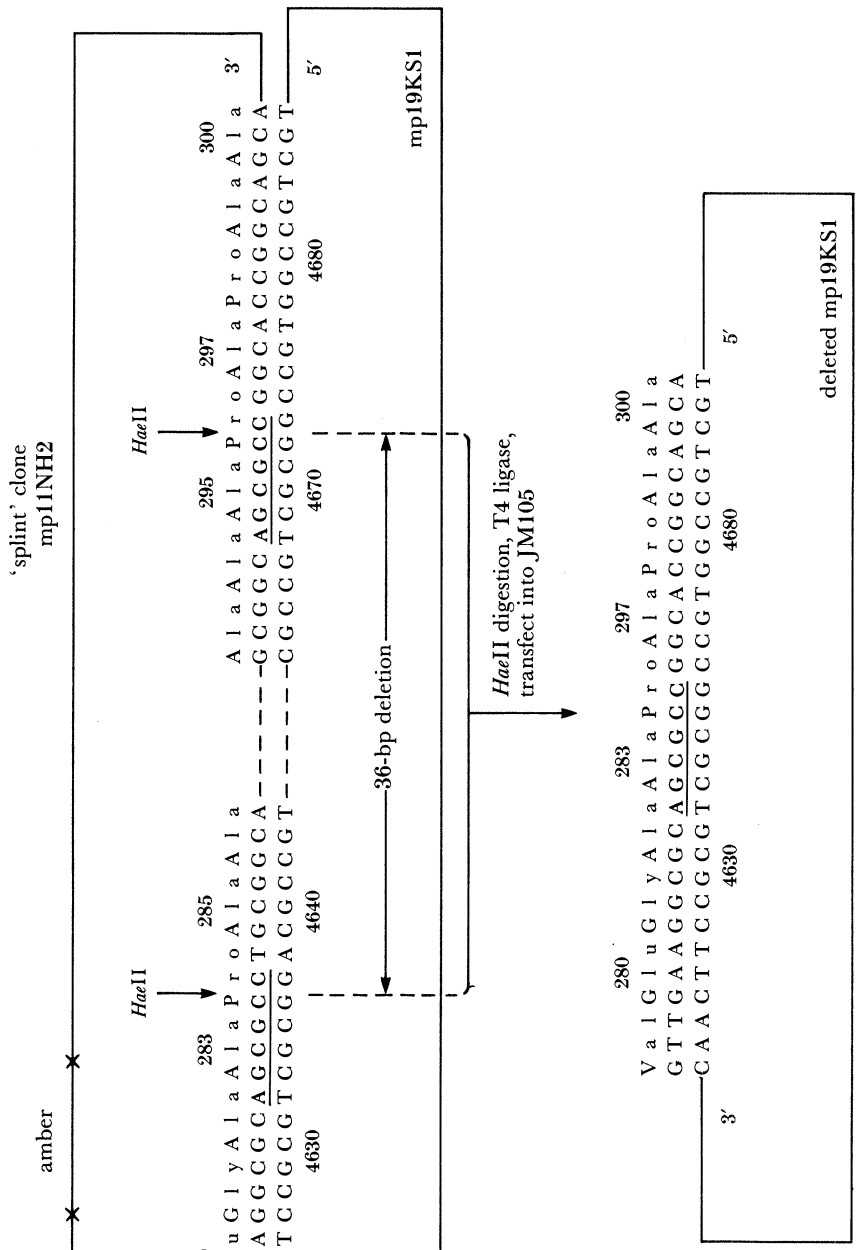

FIGURE 8. Deletion of part of the (alanine + proline)-rich region with the use of the 'splint-clone' procedure. Cleavage at the *Hae*II sites (underlined), removal of the intervening DNA fragment and subsequent religation generates a precise in-phase deletion of 36 base pairs of coding region.

of a further specific section of the *aceF* gene, encoding approximately one-third (residues 284–295 inclusive; see figure 2) of the single (alanine + proline)-rich region (residues 282–313) of this simplified E2p chain.

The 'splint-cloning' technique (Waye *et al.* 1983) was used, as shown in figure 8, with the 1.68 kb *Kpn*I–*Sph*I fragment (2653–4937) of pGS110 subcloned into bacteriophage M13mp19 (Norrander *et al.* 1983) to give mp19KS1, and a 0.3-kb *Nar*I–*Hae*III fragment (4536–4834) subcloned into the 'amber' M13mp11 (Messing 1983) as the 'splint' (mp11NH2). Two out of fourteen M13 clones recovered in a suppressor-free host following *Hae*II digestion of the partial duplex, lacked the 36-base segment (4637–4672) that encodes residues 284–295 of the (alanine + proline)-rich region.

The double-stranded *Kpn*I–*Sph*I fragment containing the deletion mutation was recovered from the recombinant M13 after primer extension and recloned into the 11.5 kb *Kpn*I–*Sph*I receptor fragment of pGS110 to generate plasmid pGS156. This plasmid contains an *aceEF–lpd* operon that encodes an E2p chain lacking 12 of the 32 residues of the (alanine + proline)-rich region of its one and only lip segment (figure 4).

Plasmid pGS156 restored the Ace$^+$ phenotype to *E. coli* JRG 1342 (Δ(*aroP–lpd*) *recA*) to an extent comparable with pGS110. The enzymic activities of cell-free extracts of the two plasmid-containing strains are rather similar (table 1), suggesting that plasmid pGS156 encodes a functional PDH complex. This complex was purified and its specific activity in the NAD^+-reduction assay was found to be 32 μmol min^{-1} $(mg)^{-1}$, similar to the values found for the wild-type enzyme from *E. coli* K12 and for pGS110 complex (§2). When subjected to SDS–polyacrylamide gel electrophoresis (figure 5), the E2p chain migrated a little faster than that of pGS110 complex. Its estimated M_r was 47000, compared with the value of 44500 calculated from the DNA sequence (table 1). The discrepancy between real and apparent M_r values (i.e. 2500) is smaller than that (6000) found for the E2p chain of the pGS110 complex (§2), which also suggests that the (alanine + proline)-rich sequence is a major contributor to the anomalously low electrophoretic mobility of E2p chains that contain a lip segment.

The E2p chain of the pGS156 complex was resistant to limited proteolysis with *S. aureus* V8 proteinase, which accords with the deletion of residues 284–295 inclusive (figure 8) because these include the site (Glu-292; figure 2) at which the proteinase normally severs the lip segment from the cat segment (Packman *et al.* 1984*a*, unpublished work).

Preliminary experiments to determine the acetylation curve of the pGS156 complex (see figure 7) indicate that the deletion engineered in the (alanine + proline)-rich region of its E2p chain has, by this criterion, left the intramolecular coupling of active sites unimpaired.

4. Substitution of the lipoyl-lysine residue

Unlike free lipoic acid or lipoyl-lysine, the lipoyl-lysine residue in each of the three lipoyl domains of the E2p chain released by cleavage with *S. aureus* V8 proteinase remains a good substrate for reductive acetylation by free E1 (Packman *et al.* 1984*a*). Presentation of the lipoamide to the E1 active site by the lipoyl domain is evidently an important part of the catalysis. To investigate the part played by the protein, we have performed a site-directed mutagenesis of the pGS110 complex to replace the lysine residue that carries the lipoyl group. Again, the pGS110 complex was used for these experiments because of its simpler E2p chain.

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The M13 clone containing the 1.68 kb *Kpn*I–*Sph*I fragment from pGS110 (mp19KS1) was used as the ssDNA template for oligonucleotide-directed mutagenesis. An oligonucleotide (16-mer), hybridizing to positions 4509–4524 and containing a single C–T mismatch (position 4517, figure 9), was used to direct the Lys244→Gln mutation by the 'all-the-way-round'

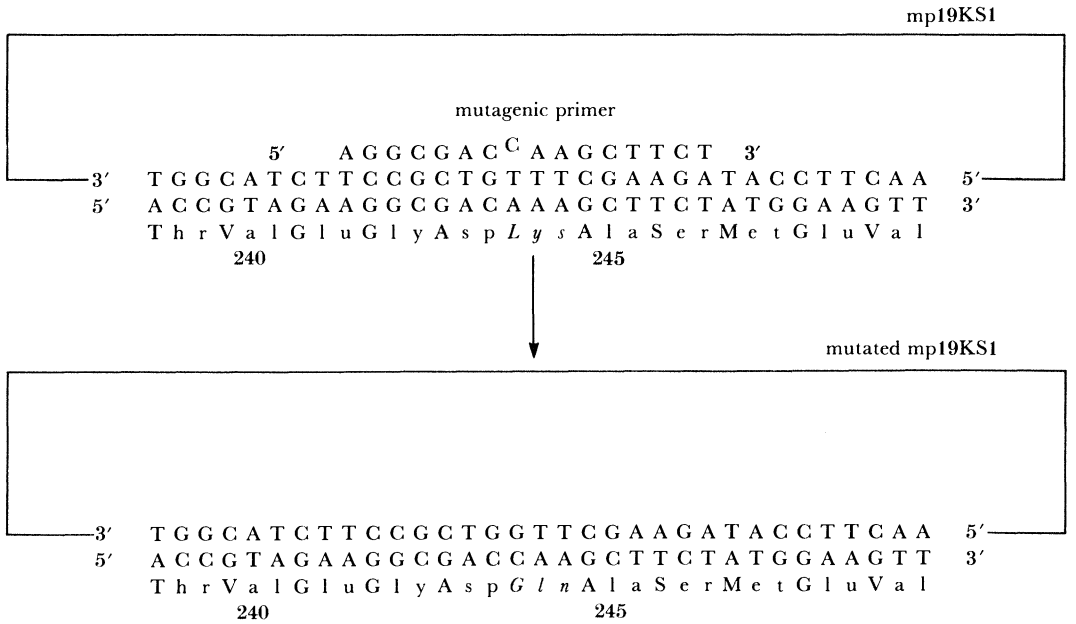

FIGURE 9. Strategy for inducing a Lys→Gln mutation at the lipoylation site (Lys 244) of the 'one-lipoyl segment' complex expressed by pGS110 with the use of a 16-base mutagenic primer.

primer extension plus ligation method (Zoller & Smith 1983). Of the eighty plaques screened by a phage dot-hybridization procedure, three gave positive signals at their estimated T_m. DNA sequencing confirmed that all three clones contained the expected A→C mutation at position 4517. The point mutation was transferred into the 11.5 kb receptor fragment of pGS110 (§3 above) to generate plasmid pGS155. This plasmid contains an *ace–lpd* operon encoding the E2p chain in which a glutamine residue replaces the Lys-244 residue at the lipoyl binding site of its single lipoyl segment (figure 4).

The Ace$^+$ phenotype was not restored in *E. coli* JRG1342 by transformation with pGS155. Enzymic assays of cell-free extracts of the plasmid-containing strain (table 1) showed that the E2p and E3 activities were similar to those encoded by pGS110, but no E1p activity or overall PDH complex activity could be detected. A structurally intact PDH complex was purified from the transformed strain by assaying for the activity of the E3 component, which was unimpaired. The complex was inactive in the overall PDH complex assay and its E1 component exhibited a normal activity when assayed by the DCPIP-reduction (Lowe *et al.* 1983) and ferricyanide methods.

When subjected to SDS–polyacrylamide gel electrophoresis (figure 5), the E2p chain of the pGS155 complex had a mobility similar to that of the E2p chain of the pGS110 complex. Treatment of the pGS155 complex with *S. aureus* V8 proteinase released a single lipoyl domain from its E2p chain. This fragment migrated more slowly during SDS–polyacrylamide gel

electrophoresis than the lipoyl domain of the pGS110 complex (figure 6), which was unexpected because there is a slight fall in M_r and no change in charge when a lipoyl-lysine residue is replaced by glutamine. Perhaps the lipoyl-lysine residue has some effect on the binding of SDS, a point that remains to be investigated further. The small but detectable increase in mobility of the pGS155–lipoyl domain compared with its pGS110 counterpart during polyacrylamide gel electrophoresis in the absence of SDS (figure 6), is consistent with the slight fall in true M_r.

Thus replacement of the lipoyl-lysine residue by a glutamine residue in the lipoyl domain of an E2p chain inhibits the overall PDH complex activity without apparent effect on the E1 or E3 components. Experiments to investigate the interaction of the modified lipoyl domain with the other active sites of the PDH complex are now being pursued.

5. Discussion

The genetic reconstructions of the *E. coli* PDH complex we have described allow unambiguous answers to at least some of the questions posed at the beginning. For example, we have shown that lowering the number of lipoyl segments from three to two or to one per E2p chain still permits the assembly of functional PDH complexes. This is consistent with the view that the three lipoyl domains which can be isolated as separate functional entities after limited proteolysis of the wild-type complex (Packman *et al.* 1984*a*) are folded as independent units. It is also apparent that complexes having such truncated E2p chains are, by the tests applied so far, unimpaired in catalytic activity and in the intramolecular coupling of active sites. Thus, in the complex encoded by plasmid pGS110, we have created an active *E. coli* PDH complex (octahedral symmetry) with one lipoyl segment per E2p chain that resembles the PDH complexes (icosahedral symmetry) of *B. stearothermophilus* (Packman *et al.* 1984*b*), *B. subtilis* (Hodgson *et al.* 1983) and ox-heart mitochondria (Reed 1974; Bleile *et al.* 1981; Stanley *et al.* 1981).

In one sense, therefore, the two extra lipoyl domains per E2p chain in the wild-type *E. coli* PDH complex can be regarded as redundant, with no obvious role attributable to them. Perhaps a more stringent kinetic analysis of the restructured complexes will reveal what that role might be. It is unlikely that the extra lipoyl domains would be retained in *E. coli* unless they conferred some selective advantage, because the highly homologous and tandemly repeated *lip* segments of the *aceF* gene would otherwise seem to be good candidates for recombinational instability and deletion.

The (alanine + proline)-rich regions of the E2p chains have been tentatively identified as the source of major sharp resonances in the n.m.r. spectrum of the *E. coli* PDH complex (Packman *et al.* 1984*a*; Spencer *et al.* 1984). The high-resolution n.m.r. spectrum of the pGS110 complex shows that the sharp signals are indeed depleted commensurate with the loss of two lip segments in the E2p chain (unpublished work in collaboration with Dr G. C. K. Roberts). It is likely that the (alanine + proline)-rich regions contribute to the conformational flexibility required of the E2p chain. However, we have shown that deletion of a large section of the (alanine + proline)-rich region of the single lip segment of the E2p chain of the pGS110 complex does not abolish catalytic activity or active-site coupling. Further experiments on complexes containing reconstructed E2p chains should throw more light on the function of these unusual sequences.

The need for the lipoic acid residue to be incorporated into a lipoyl domain if it is to become

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a good substrate for reductive acetylation by E1 is another enzymic curiosity of the 2-oxo acid dehydrogenase complexes. The creation of an *E. coli* E2p chain in which the lipoyl domain has lost its lipoylatable lysine residue opens the door to an investigation of the role of the protein in this part of the enzymic reaction. Similarly, we are now in a position to create an E2p chain in which one lipoyl domain is capable of being lipoylated and another in the same chain is not. An investigation of the structural and catalytic properties of a PDH complex assembled round a core of such E2p chains will enable us to pose more sophisticated tests of the mechanism, such as whether there is a preferred or even mandatory order of reductive acetylation of lipoyl domains in a given E2p chain. It is evident that protein engineering can teach us much more yet about the assembly and mechanism of these complex structures.

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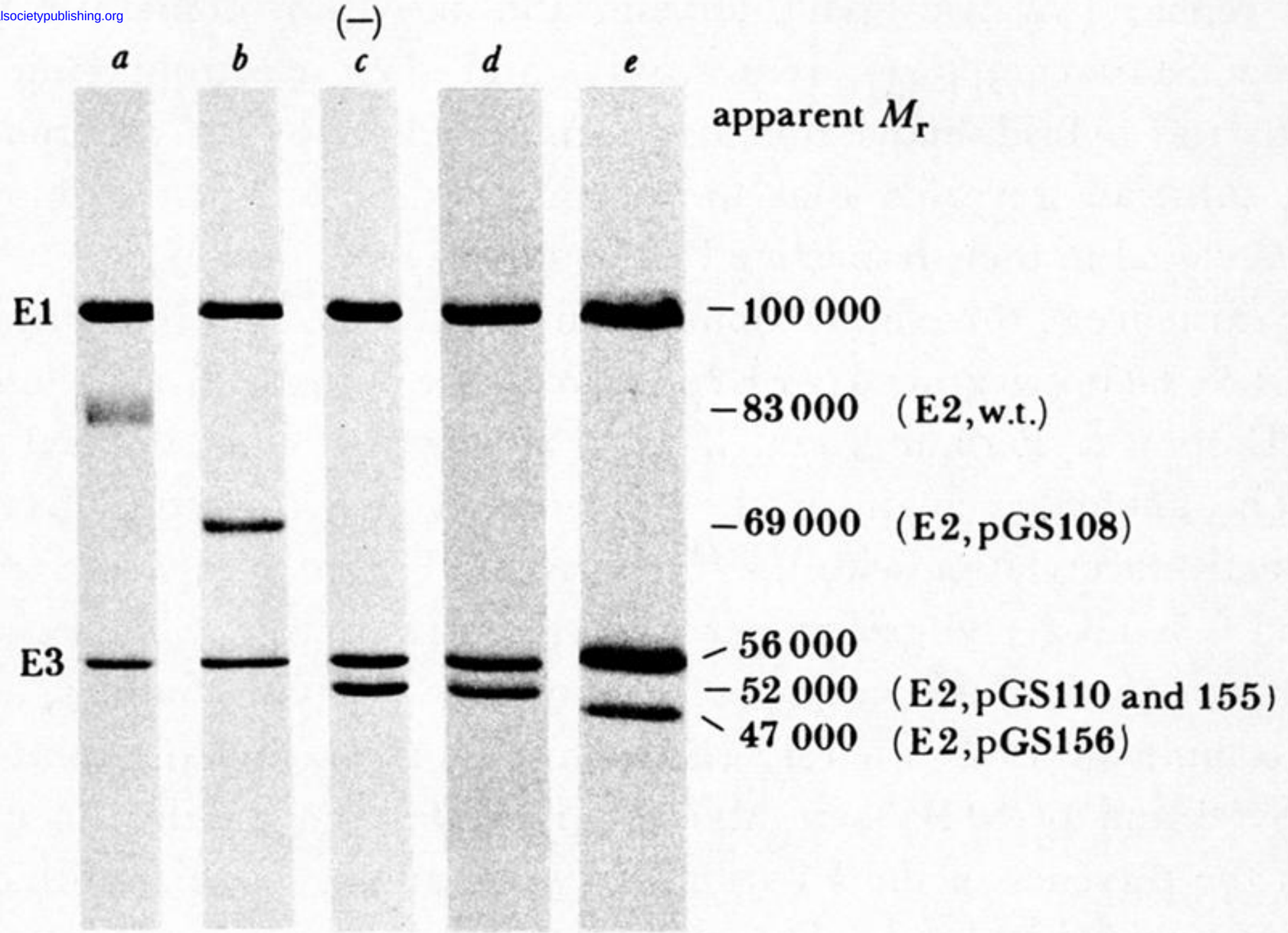

FIGURE 5. Separation of polypeptide chains of wild-type and restructured PDH complexes by means of SDS–polyacrylamide gel electrophoresis. Wild-type complex purified from a constitutive strain of *E. coli* K12, (track *a*): restructured complexes encoded by plasmids pGS108 (track *b*), pGS110 (track *c*), pGS155 (track *d*) and pGS156 (track *e*). Staining was with Coomassie Brilliant Blue.

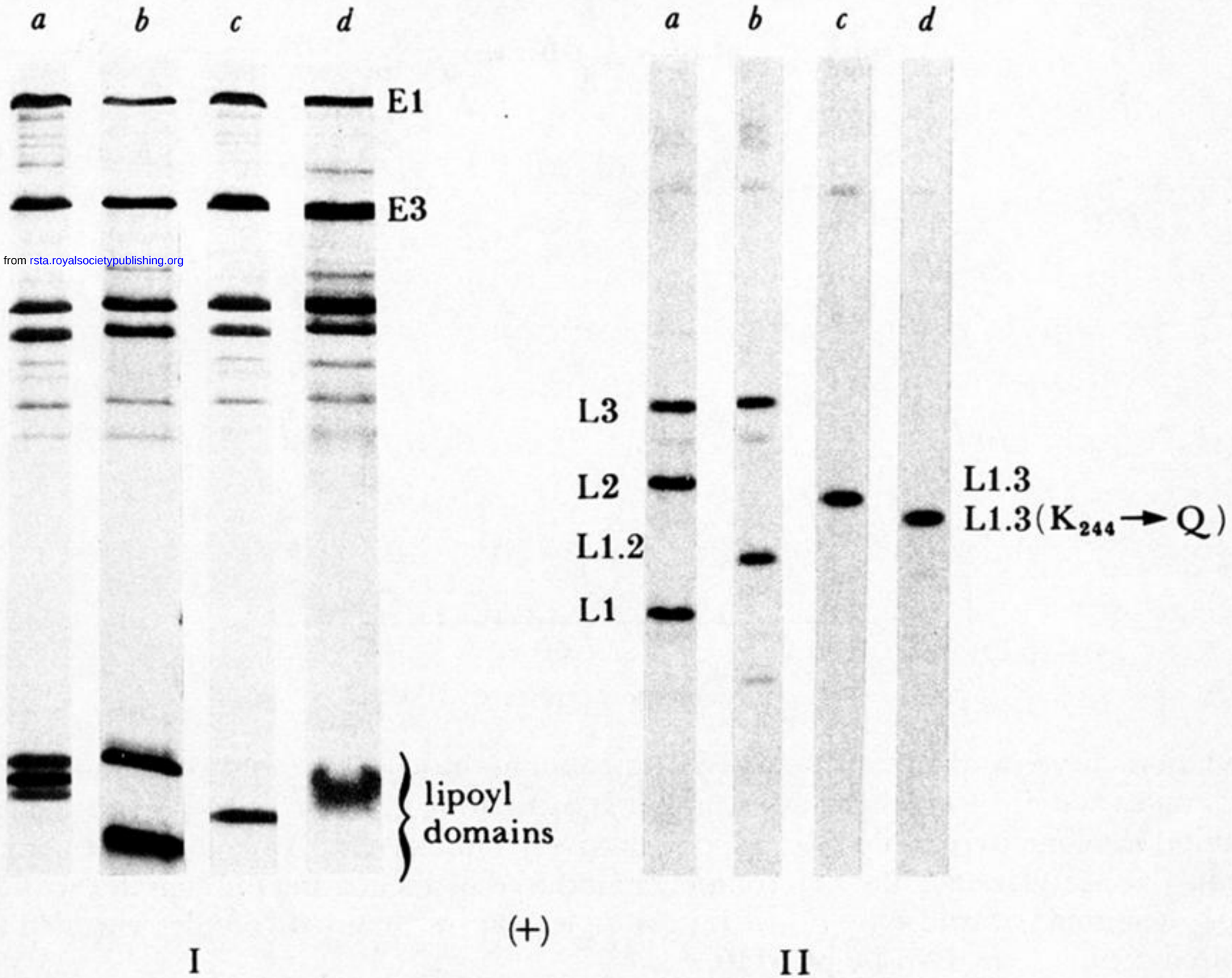

FIGURE 6. Limited proteolysis, with *S. aureus* V8 proteinase, of wild-type (*E. coli* K12) and restructured (plasmids pGS108, 110 and 155) PDH complexes. Panel I shows how the products of proteolysis were separated by SDS–polyacrylamide gel electrophoresis and visualized by staining with Coomassie Blue followed by silver. Wild-type *E. coli* K12 complex (track *a*); restructured complexes encoded by plasmids pGS108 (track *b*), pGS110 (track *c*) and pGS155 (track *d*). Panel II: the products of proteolysis were separated by means of polyacrylamide gel electrophoresis in the absence of SDS and visualized by silver staining. In this system, the resolution of lipoyl domains is enhanced and material of higher M_r or lower negative charge remains near the top of the gel (not shown). Tracks *a*–*d* as in Panel I.