PRIMARY STRUCTURE OF THE SWINGING ARMS OF THE PYRUVATE DEHYDROGENASE COMPLEX OF *ESCHERICHIA COLI*

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1. Introduction

Lipoate acetyltransferase (E2) forms the structural core of the pyruvate dehydrogenase multienzyme complex of *Escherichia coli,* to which are bound multiple copies of the two other components, pyruvate decarboxylase (E1) and lipoamide dehydrogenase (E3) (reviewed [1,2]). Lipoate acetyltransferase comprises 24 polypeptide chains [1,3], each of which has mol. wt ~80 000 [4–7] and carries 2 covalently-bound lipoic acid residues which become reductively acetylated during the course of the normal enzymic reaction [8–11]. These lipoic acid residues, in amide linkage with the N^6-amino groups of lysine residues [12], act as 'swinging arms' rotating among the catalytic sites of the 3 enzymes [13–15].

The native complex is readily cleaved by endogenous proteinases [4,5,16] and by trypsin [17], the E2 component being particularly sensitive. A detailed study of limited proteolysis with trypsin showed that the major product is an enzyme complex of high molecular weight in which the E2 chains have been degraded to fragments with mol. wt ~36 000. These fragments form stable folding domains to which E1 and E3 subunits bind and they may be represented twice in each E2 chain [17]. Independent evidence from peptide mapping experiments for the existence of two large homologous domains within the E2 chain has been presented [16], indicative of a gene duplication and fusion [4,16,17].

The peptide bonds in lipoate acetyltransferase most susceptible to trypsin involve lysine residues, probably on exposed loops of polypeptide chain [17]. We show here that both of the lipoic acid residues reductively acetylated by substrate can be excised from the complex by limited proteolysis with trypsin and describe the single amino acid sequence that we have obtained from the resulting lipoyl peptides.

Abbreviations: E1, pyruvate decarboxylase (EC 1.2.4.1); E2, lipoate acetyltransferase (EC 2.3.1.12); E3, lipoamide dehydrogenase (EC 1.6.4.3); SDS, sodium dodecylsulphate; Mes, methionine sulphone

2. Materials and methods

Pyruvate dehydrogenase complex was isolated from a mutant of *E. coli* K12 constitutive for production of the complex [3]. The lipoic acid residues were labelled by incubating the complex with 0.3 mM *N*-ethyl[2,3-^{14}C]maleimide in the presence of pyruvate [8]. The ^{14}C-labelled enzyme (5 mg) was mixed with unlabelled complex (380 mg) in 27 ml 20 mM sodium phosphate buffer (pH 7.0) containing 2 mM EDTA. Limited digestion with trypsin [17] was carried out by adding trypsin (4 mg) and incubating for 60 min at 0°C. The overall enzyme activity, assayed in the direction of NAD^+ reduction [18], fell to <10% of the original value and soybean trypsin inhibitor (10 mg) was then added. The mixture was gel filtered on a column of Sepharose 6B (980 × 30 mm) in 0.5% (w/v) ammonium bicarbonate. Fractions were monitored for protein content by their A_{280}, for E3 enzyme

activity [18], and for radioactivity by scintillation counting [8].

SDS–polyacrylamide gel electrophoresis was as in [4]. Peptides were digested with 2% (w/w) *Staphylococcus aureus* proteinase at 37°C in 0.5% (w/v) NH_4HCO_3 for 4 h [19]. Paper electrophoresis, amino acid analysis, dansyl-Edman degradation of peptides and diagonal paper electrophoresis were carried out as in [19].

3. Results

3.1. *Excision of lipoyl peptides by trypsin*

Native pyruvate dehydrogenase complex mixed with a small amount of complex in which the lipoic acid residues were labelled with *N*-ethyl[2,3-^{14}C]-maleimide (to act as marker) was subjected to limited proteolysis with trypsin. The product was gel-filtered on Sepharose 6B (fig.1). Most of the protein (A_{280}) and 80% of the E3 enzyme activity was eluted at the same position as native, unproteolysed complex would have been [17], but <10% of the radioactivity was associated with this peak. Instead, ~80% of the total radioactivity was eluted as a peak just after the small peak of free E3 activity. These fractions were presumed to include the lipoyl-lysine-containing peptides, a small proportion of which were labelled with *N*-ethyl[2,3-^{14}C]maleimide.

3.2. *Sequence analysis of lipoyl-lysine peptides*

SDS–polyacrylamide gel electrophoresis showed that the radioactive fractions contained a variety of polypeptide chains with mol. wt 5000–20 000. Autoradiography of the gels revealed that several of these were radiolabelled. No attempts were made to purify these peptides. The unlabelled lipoyl-lysine-containing peptides, which were present in much greater quantities, were more readily purified by diagonal paper electrophoresis as follows.

The radioactive fractions obtained by gel filtration of the trypsin treated complex (fig.1) were pooled and freeze-dried. A sample (10 mg) was digested with *S. aureus* proteinase and the digest was submitted to paper electrophoresis at pH 6.5. After exposure to performic acid vapour, the paper was

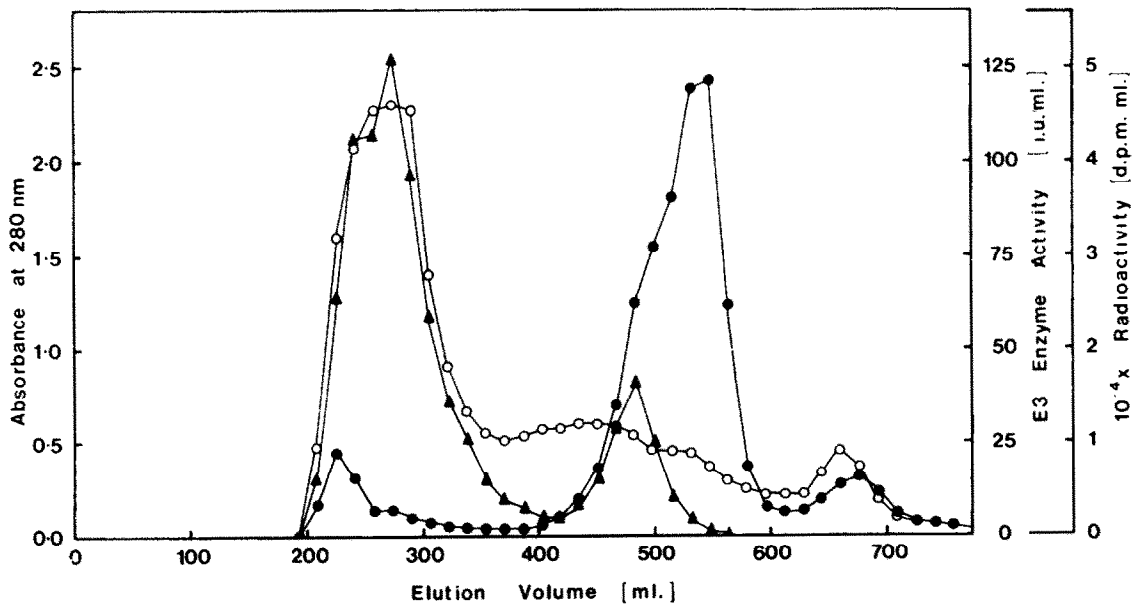

Fig.1. Gel filtration of trypsin-treated pyruvate dehydrogenase complex. A mixture of native pyruvate dehydrogenase complex and complex labelled with *N*-ethyl[2,3-^{14}C]maleimide was incubated with trypsin for 1 h at 0°C as described in the text. The mixture was applied to a column of Sepharose 6B (980 × 30 mm) and eluted with 0.5% (w/v) NH_4HCO_3. (○) A_{280}; (▲) lipoamide dehydrogenase (E3) activity; (●) radioactivity.

Table 1
Properties of peptide TA1

Amino acid	Mol/mol peptide	From sequence
Aspartic acid	2.0[a]	1
Methionine sulphone		1
Serine	0.9	1
Glutamic acid	1.1	1
Glycine	0.8	1
Alanine	1.1	1
Lysine	0.7	1
Electrophoretic mobility at pH 6.5 [19]	Before oxidation −0.57 After oxidation −0.99	
Sequence established by dansyl-Edman degradation	Gly–Asp–Lys(N^6-Lip)–Ala– Ser–Mes–Glu	

[a] Aspartic acid and methionine sulphone were not resolved on the Rank Chromaspek amino acid analyser

submitted to electrophoresis at right-angles to the first dimension [19]. Under these conditions the lipoyl groups should be oxidized to 6,8-disulphooctanoyl groups [12,20], causing a net charge change of −2 at pH 6.5. When the paper was stained with fluorescamine [19], a major off-diagonal peptide (TA1) was observed.

The properties of peptide TA1 are summarized in table 1. The N-terminal residue, established by dansylation, was glycine, which may account for the slightly low yield of this amino acid since the peptide was eluted from paper after being stained with fluorescamine [19]. No N^6-Dns-lysine was detected, consistent with the N^6-amino group being acylated. The sequence, determined by dansyl-Edman degradation (table 1), was Gly–Asp–Lys(N^6-lipoyl)–Ala–Ser–Mes–Glu. The electrophoretic mobility of the peptide at pH 6.5 before and after performic acid oxidation indicated the presence of two unamidated acidic residues and one lipoyl-lysine residue [19].

On some occasions a further off-diagonal peptide (TA2) was also observed, with an electrophoretic mobility of –0.35 before oxidation and –0.63 after oxidation. The N-terminal sequence of this peptide was identical with that of peptide TA1 and further digestion of it with *S. aureus* proteinase yielded a peptide that co-migrated with peptide TA1 on electrophoresis. Peptide TA2 appears to be an extension of peptide TA1 on the C-terminal side.

4. Discussion

Several lines of evidence suggest that, in the presence of pyruvate, *N*-ethylmaleimide reacts with two lipoic acid residues on each E2 polypeptide chain of the pyruvate dehydrogenase complex of *E. coli* [8–11]. We showed earlier that limited proteolysis of the complex with trypsin causes a loss of overall enzymic activity without any substantial change in the quaternary structure [17]. Our present results demonstrate that this treatment with trypsin excises the two acetylatable lipoic acid residues, which may account for the accompanying loss of catalytic activity. The sites of attachment of the lipoyl groups are therefore not included in the folding domains of approximate M_r 36 000 and are likely to be found on exposed, flexible loops of the polypeptide chain [17]. If these loops were sufficiently flexible, the effective radius of a swinging arm might be greatly increased beyond its normally assumed value of ~1.4 nm. This in turn might account, at least in part, for the large distances separating the active sites of the enzymes in the complex calculated from fluorescence energy-transfer measurements [21,22].

An interesting feature of our experiments is that only one lipoyl-lysine-containing amino acid sequence was found. This sequence agrees with and extends by one residue (the C-terminal glutamic acid) that obtained by Reed's group from studies of complex labelled with [^{35}S]lipoic acid [23] or [^{14}C]pyruvate [24]. Since two lipoic acid residues per E2 chain become reductively acetylated by pyruvate [8–11], it is conceivable that all sequence analysis thus far has failed to reveal the expected second sequence for undetermined technical reasons. An attractive alternative, especially in view of the possibility of gene duplication events in the evolution of the E2 polypeptide chain [16,17], is that the sequence we have determined appears twice in each chain. Much longer amino acid sequences will be needed to resolve this question.

Other experiments involving ^{35}S-labelled *E. coli* complex have suggested that there may even be a

third lipoic acid residue attached to the E2 chain [20]. Since this residue appears not to become reductively acetylated by substrate, the experiments we describe above shed no further light on its existence or biological role.

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