Primary structure around the lipoate-attachment site on the E2 component of bovine heart pyruvate dehydrogenase complex

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Bovine heart pyruvate dehydrogenase complex was acetylated by using [3-¹⁴C]pyruvate in the presence of *N*-ethylmaleimide, with approx. 1 mol of acetyl groups being incorporated per mol of E2 polypeptide. After peptic digestion, lipoate-containing peptides were purified by high-voltage electrophoresis and ion-exchange and reverse-phase h.p.l.c. The amino acid sequence around the lipoic acid-attachment site of E2 was determined by automated Edman degradation. Acetylation of a lipoate cofactor bound to a lysine residue was verified by fast-atom-bombardment m.s.

INTRODUCTION

Pyruvate dehydrogenase complex (PDC) is one of three related multienzyme complexes present in mammalian mitochondria that are responsible for the oxidative decarboxylation of 2-oxo acids [1,2]. Each complex consists of three component enzymes, termed E1, E2 and E3. E1 is a thiamin pyrophosphate-dependent 2-oxo acid dehydrogenase, and E3 is an FAD-dependent lipoamide dehydrogenase. E2, which forms the central core of the complexes, is an acyltransferase that utilizes lipoic acid as an essential cofactor. The lipoic acid cofactor is covalently attached to a flexible domain of the E2 polypeptide [3,4], the mobility of the region allowing the functional end of the lipoic acid to interact with three different active sites on each complex.

Current evidence indicates that each E2 of mammalian PDC contains one lipoic acid residue [5], although a second group capable of undergoing slow acetylation is also present on the E2 polypeptide [6]. In contrast, nucleotide sequence work indicates that PDC from *Escherichia coli* contains three lipoic acid residues per E2 polypeptide, present in three repeating highly conserved domains [7]. This is consistent with work on PDC isolated from *E. coli* grown on [³⁵S]sulphate [8], but enzymological studies have shown that only approx. 2 mol of acetyl groups can be incorporated from pyruvate per mol of E2 polypeptide [9,10]. The primary structure around the lipoate residues of the *E. coli* enzyme is known [7,11], but there is no sequence information available concerning the mammalian enzyme.

Recently an additional polypeptide component of mammalian PDC has been identified, termed protein X [12,13]. Immunological data and peptide-mapping studies indicate that it is distinct from E2, although it has been demonstrated that protein X also contains a lipoic acid residue capable of undergoing

acetylation [14]. However, protein X is present in only approx. 10% of the amount of E2 and its function is not yet clear.

MATERIALS AND METHODS

PDC was purified from bovine heart by a minor modification of the method in ref. [15]. [3-¹⁴C]Pyruvate was from New England Nuclear. Pepsin was from Boehringer, and *N*-ethylmaleimide from Sigma Chemical Co. H.p.l.c. was carried out on a Beckman 420 system, with TSK DEAE 3SW ion-exchange (LKB) and Vydac C₁₈ reverse-phase columns. Chromatography paper (3MM) for high-voltage electrophoresis was from Whatman, and Liquiscint scintillation cocktail from National Diagnostics.

For ion-exchange h.p.l.c. the column was equilibrated in 30 mM-imidazole/HCl buffer, pH 6.0, and eluted with a linear gradient of NaCl (0–400 mM) in the same buffer. Absorbance was monitored at 280 nm, the flow rate was 1 ml/min and 1 ml fractions were collected. Radioactivity was determined by counting samples of fractions in scintillant. Fractions from ion-exchange chromatography were applied to the C₁₈ column equilibrated in 0.1% (v/v) trifluoroacetic acid and eluted with a linear gradient of acetonitrile (0–30%, v/v) in 0.1% trifluoroacetic acid. Peptides were detected by absorbance at 206 nm. [¹⁴C]Lipoyl-peptides were pooled, freeze-dried, resuspended in 10 mM-ammonium acetate and re-freeze-dried to ensure complete removal of trifluoroacetic acid. Finally peptides were re-run on the Vydac column equilibrated in 10 mM-ammonium acetate and eluted with an acetonitrile gradient (0–40%, v/v).

Sequence analysis was carried out in an Applied Biosystems 477 pulsed liquid sequencer and amino acid analysis on an Applied Biosystems 420A derivative-former with on-line 130A analyser. Fast-atom-bombardment mass spectra were recorded on a VG ZAB-SE

Abbreviations used: PDC, pyruvate dehydrogenase complex; OGDC, 2-oxoglutarate dehydrogenase complex.

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Fig. 1. Acetylation of PDC with [3-¹⁴C]pyruvate in the presence of *N*-ethylmaleimide

PDC (2 mg/ml) was incubated at 4 °C in 50 mM-potassium phosphate buffer, pH 7.4, with 0.4 mM-thiamin pyrophosphate, 2 mM-MgCl₂, 0.5 mM-*N*-ethylmaleimide and 0.2 mM-[3-¹⁴C]pyruvate (30000 c.p.m./nmol) in a final volume of 0.5 ml. At the indicated time points, samples were removed for determination of enzyme activity (●) and protein-bound radioactivity (■) [16]. No significant loss in PDC activity was observed in incubations lacking either pyruvate or *N*-ethylmaleimide (results not shown).

high-field mass spectrometer at an operating voltage of 8 kV. A standard Iontech fast-atom-bombardment source was used to generate an 8 kV xenon beam.

RESULTS

When purified PDC from bovine heart was incubated in the presence of [3-¹⁴C]pyruvate and *N*-ethylmaleimide, the complex became rapidly acetylated, with incorporation reaching a maximum value of approx. 7 nmol/mg of protein (Fig. 1). This corresponds to approx. 1 mol of acetyl group/mol of E2 polypeptide, allowing for incorporation also into polypeptide X. Acetylation was accompanied by a slower loss of activity due to modification by *N*-ethylmaleimide with the free thiol group generated by the reductive acetylation of the lipoic acid.

Lipoic acid-containing peptides were purified as follows. PDC (50 mg) was acetylated as described in Fig. 1 and the reaction terminated by addition of trichloroacetic acid to a final concentration of 10% (w/v). After standing on ice for 5 min, the resulting precipitate was collected by centrifugation at 10000 *g* for 2 min. The pellet was washed once with acetone, twice with diethyl ether and resuspended in 7% (w/v) formic acid. Subsequent digestion with pepsin (1:50, w/w) for 1 h at 20 °C solubilized more than 90% of the radioactivity. Any remaining insoluble material was removed by centrifugation, and the supernatant was subjected to high-voltage electrophoresis at pH 1.9 for 1 h at 2.5 kV. Analysis of the electrophoretogram by autoradiography indicated the presence of two major acetylated peptides,

Table 1. Amino acid sequences of lipoate-containing peptides from bovine heart PDC

Peptides	Sequence
A1 _a , A1 _b	Val-Glu-Thr-Asp-Lys [*] -Ala-Thr-Val-Gly-Phe
B1 _a , B1 _a '	Lys [*] -Ala-Thr-Ile-Gly-Phe
B1 _b , B1 _b '	Lys [*] -Ala-Thr-Val-Gly-Phe

* Denotes lipoyl-lysine residue.

Fig. 2. Sequence analysis of peptide A1_b

The peptide (900 pmol, 37000 c.p.m.) was applied to a Polybrene-treated glass-fibre disc for sequencing. One-third of the amino acid phenylthiohydantoin derivative obtained at each cycle of Edman degradation was directly analysed, and the rest of the sample was recovered in the fraction collector of the sequencer, transferred to plastic vials containing scintillant and ¹⁴C radioactivity was determined. Radioactivity (c.p.m.) and amount (pmol) were corrected to 100% sample.

termed A and B (with mobilities of 0.30 and 0.37 relative to serine). These were further purified by ion-exchange and reverse-phase h.p.l.c.

Ion-exchange h.p.l.c. of peptides A and B yielded in each case a major and a minor radioactive peak, designated A1, A2, B1 and B2 respectively. (Peptides A2 and B2 were not analysed further.) Reverse-phase h.p.l.c. of peak A1 yielded a single major radioactive peak, which was resolved into two closely spaced absorbance peaks (A1_a and A1_b) on the second reverse-phase chromatography. Sequence analysis of the material in

Fig. 3. Fast-atom-bombardment m.s. of peptide A1_a.

The peptide (approx. 500 pmol) was resuspended in 10 μ l of h.p.l.c.-grade methanol and transferred to the probe tip. After partial evaporation, 1 μ l of acetic acid was added (to induce protonation), followed by 2 μ l of a 1:1 α -thioglycerol/glycerol matrix. This method gave excellent $[M + H]^+$ ions.

peaks A1_a and A1_b showed them to be identical ten-residue peptides (Table 1).

On reverse-phase h.p.l.c. of peak B1, two radioactive peaks (B1_a and B1_b) were detected, each of which was subsequently resolved into two absorbance peaks by the second reverse-phase chromatography. These pairs of six-residue peptides (B1_a and B1_a', and B1_b and B1_b') were shown to differ in valine/isoleucine at position 4 but to be otherwise identical (Table 1).

The sequence of the isolated peptides was determined and is shown in Table 1. Each peptide gave a single unambiguous sequence. For peptides A1_a and A1_b, no residue was detected at position 5; however, a peak of radioactivity was released at this position (Fig. 2), consistent with the presence of an acetylated residue. Amino acid analysis (not shown) of each peptide revealed the presence of a lysine residue, which was not detected during sequence analysis. Similarly, no residue was detected at position 1 in peptides B1_a, B1_a', B1_b and B1_b', but again a peak of radioactivity was released at this position.

Fast-atom-bombardment m.s. verified the presence of an acetylated lipoyl-lysine (modified with *N*-ethylmaleimide) at the expected positions on the different peptides (Fig. 3). Pseudomolecular ions in the positive ion mode $[M + H]^+$ were seen as follows: 1425 for peptides A1_a and A1_b; 994 for peptides B1_a and B1_a'; 908 for B1_b and B1_b'. These correspond to the molecular

mass of the protonated form of the acetylated *N*-ethylmaleimide-modified lipoyl-peptides. No evidence for differences in structure of the pairs of peptides was obtained that could explain their separation on reverse-phase h.p.l.c., their molecular masses being identical. One possibility is *cis* and *trans* addition of the *N*-ethyl group of *N*-ethylmaleimide, with respect to the CH₃CO-S-lipoyl bond; alternatively, the peaks may represent the 6- and 8-S-acetyl derivatives of the lipoyl-lysine residue [17,18].

DISCUSSION

We report here the amino acid sequence surrounding the lipoic acid cofactor on E2 of mammalian PDC. The sequences of the different lipoyl-containing peptides are internally consistent, with the exception of the finding of an isoleucine residue instead of valine at position 4 in peptides B1_a and B1_a'. No decapeptide containing an isoleucine residue was recovered, and it is possible that other differences may exist at the *N*-terminal side of the lipoyl-lysine. There are several possible explanations for the presence of the two different sequences. E2 may contain two or more lipoate residues, but isotope-dilution studies [5] and the observed stoichiometry of acetylation (Fig. 1) argue against this possibility. It is important to re-iterate, however, that, although three lipoate residues are thought to be present on each E2 polypeptide of *E.*

Table 2. Amino acid sequences flanking lipoyl-lysine residues of lipoate-containing proteins

Source	Enzyme	Sequence
Bovine heart (present work)	PDC E2	Val-Glu-Thr-Asp-Lys [*] -Ala-Thr-Val-Gly-Phe and Lys [*] -Ala-Thr-Ile-Gly-Phe
<i>E. coli</i> [11]	PDC E2	Val-Glu-Gly-Asp-Lys [*] -Ala-Ser-Met-Glu-Val
<i>E. coli</i> [19]	OGDC E2	Ile-Glu-Thr-Asp-Lys [*] -Val-Val-Leu-Glu-Val
Chicken liver [20]	Glycine-cleavage H protein	Leu-Glu-Ser-Val-Lys [*] -Ala-Ala-Ser-Glu-Leu

* Indicates lipoyl-lysine residues.

coli PDC, a maximum of two acetyl groups can be incorporated [9,10]. Furthermore in E2 from *E. coli* PDC the sequence around the three lipoate residues is identical in each domain [7]. Although a second group on bovine kidney E2 can undergo slow acetylation [6], this does not occur when the reaction is carried out in the presence of *N*-ethylmaleimide (Fig. 1). Alternatively, peptides B1_a and B1_a' may be derived from component X and not from E2. However, this seems unlikely, as protein X is thought to be present in only approx. 10% of the amount of E2 whereas isoleucine-containing peptides B1_b and B1_b' constituted approx. 30% of the recovered acetylated peptides. A further possibility is that there is microheterogeneity in the gene for the E2 polypeptide present in the bovine population.

The sequences reported here (Table 2) demonstrate significant homology with the corresponding region of E2 of PDC and 2-oxoglutarate dehydrogenase complex (OGDC) from *E. coli* and with the lipoate-containing region of the H protein of the chicken liver glycine-cleavage system. Furthermore as far as we are aware it is the first sequence information available for E2 of any the mammalian 2-oxo acid dehydrogenase complexes.

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