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Protein Expression and Purification 28 (2003) 140–150

Protein Expression & Purification

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Expression and purification of the dihydrolipoamide acetyltransferase and dihydrolipoamide dehydrogenase subunits of the *Escherichia coli* pyruvate dehydrogenase multienzyme complex: a mass spectrometric assay for reductive acetylation of dihydrolipoamide acetyltransferase

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Received 27 August 2002, and in revised form 1 November 2002

Abstract

Plasmids were constructed for overexpression of the *Escherichia coli* dihydrolipoamide acetyltransferase (1-lip E2, with a single hybrid lipoyl domain per subunit) and dihydrolipoamide dehydrogenase (E3). A purification protocol is presented that yields homogeneous recombinant 1-lip E2 and E3 proteins. The hybrid lipoyl domain was also expressed independently. Masses of $45{,}953 \pm 73$ Da (1-lip E2), $50{,}528 \pm 5.5$ Da (apo-E3), $51{,}266 \pm 48$ Da (E3 including FAD), and 8982 ± 4.0 (lipoyl domain) were determined by MALDI–TOF mass spectrometry. The purified 1-lip E2 and E3 proteins were functionally active according to the overall PDHc activity measurement. The lipoyl domain was fully acetylated after just 30 s of incubation with E1 and pyruvate. The mass of the acetylated lipoyl domain is 9019 ± 2 Da according to MALDI–TOF mass spectrometry. Treatment of the 1-lip E2 subunit with trypsin resulted in the appearance of the lipoyl domain with a mass of $10{,}112 \pm 3$ Da. When preincubated with E1 and pyruvate, this tryptic fragment was acetylated according to the mass increase. MALDI–TOF mass spectrometry was thus demonstrated to be a fast and precise method for studying the reductive acetylation of the recombinant 1-lip E2 subunit by E1 and pyruvate.

The *Escherichia coli* pyruvate dehydrogenase multienzyme complex (PDHc)[3] is a member of the family of 2-oxo acid dehydrogenase complexes and catalyzes the decarboxylation of pyruvate with the formation of acetyl-CoA and NADH [1,2]. The *E. coli* PDHc consists of multiple copies of three enzymes: pyruvate dehydrogenase (E1, EC 1.2.4.1), dihydrolipoamide acetyltransferase (E2, EC 2.3.1.12), and dihydrolipoamide dehydro- genase (E3, EC 1.8.1.4). The E1 component is a homodimeric enzyme with a subunit molecular mass of 99,474 Da [3]. The crystal structure of E1 has been reported to a resolution of 1.85 Å by our laboratory and our collaborators [4]. The E2 subunit has a multidomain structure. Starting from the N-terminus, it consists of three lipoyl domains

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[1] Supported at Rutgers by NIH-GM 62330, the NSF Training Grant-BIR 94/13198 in Cellular and Molecular Biodynamics (F.J., PI).
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[3] *Abbrevations used*: PDHc, pyruvate dehydrogenase multienzyme complex; 1-lip PDH complex, pyruvate dehydrogenase complex with a single lipoyl domain per E2 subunit; E1, pyruvate dehydrogenase; E2, dihydrolipoamide acetyltransferase; 1-lip E2, dihydrolipoamide acetyltransferase with a single lipoyl domain per subunit; 3-lip E2, dihydrolipoamide acetyltransferase with three lipoyl domains per subunit; E3, dihydrolipoamide dehydrogenase; ThDP, thiamin diphosphate; MALDI–TOF, mass spectrometry, matrix-assisted laser desorption/ionization with time-of-flight mass spectrometry; IPTG, isopropyl-β-D-thiogalactopyranoside; AEBSF, 4-(2-aminoethyl)-benzensulfonyl fluoride; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol.

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doi:10.1016/S1046-5928(02)00674-5

(of approximately 80 amino acids each), a peripheral subunit-binding domain (4 kDa), and a large core-forming acetyltransferase domain (28 kDa), all linked together by flexible (25–30 residues) segments rich in alanine, proline, and charged amino acids [5,6]. The subgene encoding a single lipoyl domain, representing a hybrid between the first and third lipoyl domains of the *E. coli* E2 subunit, was cloned and expressed in *E. coli* [7]. Its structure was determined by NMR spectroscopy [8]. The structure of the innermost lipoyl domain from *E. coli* was also determined by NMR spectroscopy [9] and was found to be very similar to that of the hybrid lipoyl domain; however, no complete structure of the entire E2 subunit from any sources has yet been determined. Nor is the crystal structure of the E3 component from *E. coli* yet available. The nucleotide sequence and the primary protein sequence of the *E. coli* E3 subunit were reported in the literature [10]. In the present study, plasmids were constructed for expression of the 1-lip E2 and E3 subunits. The purification of homogeneous 1-lip E2 and E3 components is presented and their masses were determined by MALDI–TOF mass spectrometry, along with that of the hybrid lipoyl domain. The recombinant 1-lip E2 and E3 subunits could be successfully reconstituted with independently expressed recombinant E1 subunits, according to an assay measuring the overall PDHc activity. We have used MALDI–TOF mass spectrometry to detect reductive acetylation of either lipoyl domain or of 1-lip E2 subunit by pyruvate in the presence of E1 independently expressed and with active center variants of E1. This method is fully capable of providing information about this protein–protein communication event, and the time-course of the reductive acetylation. The method not only obviates the need for radioactive pyruvate used earlier for the same purpose, but also offers a rapid and precise method to identify substitutions at either E1 or E2 that impair the reductive acetylation.

Materials and methods

Bacterial strains, plasmids, and growth conditions

The *E. coli* strain JRG1342 carrying the plasmid pGS501 that expresses the 1-lip PDH complex, the *E. coli* strain JM 101 carrying the plasmid pGS331 encoding the hybrid lipoyl domain, and strain JRG3456 carrying the plasmid pGS878 encoding the E1 subunit were all provided by Professor John Guest of the University of Sheffield.

Construction of pET-22b(+)-1-lip-E2 and pET-22b(+)-E3 plasmids

The plasmid pGS501 was purified according to the protocol described for the Wizard 373 DNA purification system (Promega, Madison, WI). The *Nde*I restriction site was created at the start codon of the 1-lip E2 or E3 genes on the pGS501 plasmid, using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) (Fig. 1). The following synthetic oligonucleotides (and their complements) were used as mutagenic primers (mismatched bases are underlined, while the *Nde*I sites created are shown in boldface type):

5′-GCGTCTGGCGTAAGAGGTAAAAGA**CATA**
TGGCTATCG-3′ (for 1-lip E2) and
5′-TATAGAGGT**CATATG**AGTACTGAAATCAA
AACTCAGGTCG-3′ (for E3)

The unique *Xho*I restriction site on the pGS501plasmid located 295 bp downstream of the 1-lip E2 gene stop codon and the unique *Sal*I restriction site located 85 bp downstream of the E3 gene stop codon were selected as second restriction sites (Fig. 1). The 1.58 kb *Nde*I–*Xho*I fragment encoding 1-lip E2 or 1.5 kb *Nde*I–*Sal*I fragment encoding E3 was subcloned into the pET-22b(+) vector (Novagene, Madison, WI) that had been pretreated with the corresponding restriction enzymes. The entire 1-lip E2 and E3 genes were sequenced using the ABI Prism Ready-Reaction dye terminator cycle sequencing kit (Applied Biosystems, Forster City, CA). The following specific primers were created for the 1-lip E2 gene sequence:

T7P 5′-CCGCGAAATTAATACGACTCACTATA-3′

T7T 5′-GTTATGCTAGTTATTGCTCAGCGGT-3′

1. 5′-GAAGGCGCAGCGCCTGC-3′
2. 5′-GGTGAAATCGAAGAAGTGTAACTG-3′
3. 5′-CAACAAGAAAGGCATCATCGAGC-3′

The following primers were created for the E3 gene sequence (T7P and T7T were the same as for E2 gene):
1. 5′-GCTGAACACGGTATCGTCTTCG-3′
2. 5′-CGGTATCATCGGTCTGGAAATG-3′
3. 5′-GTGTGCCGAACGGTAAAAACC-3′
4. 5′-CTGAGAAAGAAGCGAAAGAGAAA-3′

Expression and purification of 1-lip E2

E. coli BL21(DE3) cells (Novagene, Madison, WI) transformed with the pET-22b(+)-1-lip-E2 plasmid were grown for 16 h at 37 °C on an LB plate containing 50 μg/mL ampicillin. A single colony was used to inoculate 1000 mL LB medium containing 50 μg/mL ampicillin and 0.1 mM DL-α-lipoic acid. Cells were grown to $A_{650} = 0.4$–0.6 and then induced with IPTG (final concentration = 1 mM), harvested after 3–4 h, washed with 20 mM KH_2PO_4 (pH 7.0) containing 1 mM EDTA, and stored at −20 °C. All purification steps were carried out at 4 °C, unless otherwise indicated. For purification of 1-lip E2, 1.75 g cells from 1000 mL culture were resus-

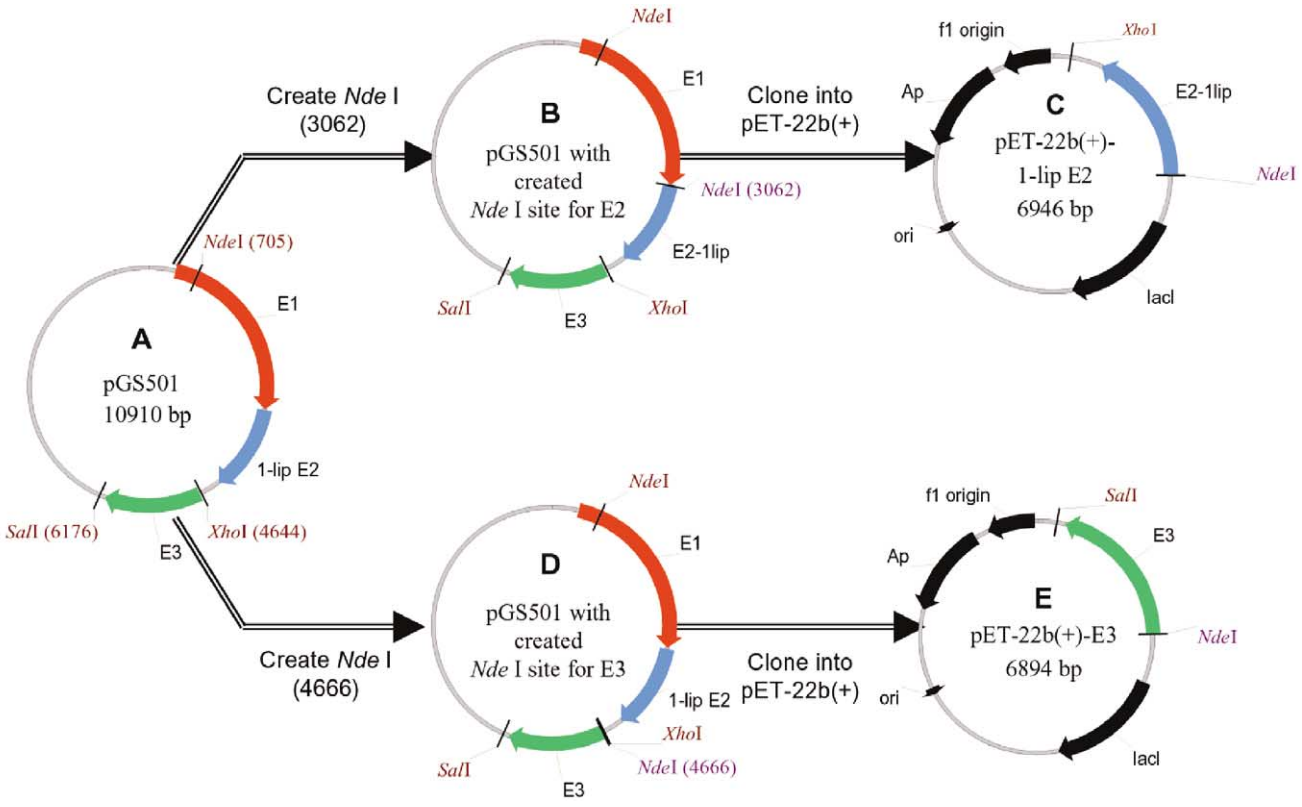

Fig. 1. Scheme for the construction of the expression vectors pET-22b(+)-1-lip E2 (C) and pET-22b(+)-E3 (E). The orientation of genes and promoters is indicated with arrows. (A) pGS501 plasmid encoding *E. coli* PDHc with a single lipoyl domain per E2 subunit. (B) pGS501 plasmid with *Nde*I site introduced at the start codon of the 1-lip E2 gene. (D) pGS501 plasmid with *Nde*I site introduced at the start codon of the E3 gene. The construction of the expression vectors is described under Materials and methods.

pended in 17.5 mL of 20 mM Tris–HCl buffer (pH 8.5) containing 1 mM EDTA, 0.5 mM AEBSF, and 20 μM leupeptin (to inhibit adventitious serine and cysteine proteases). The cells were destroyed by French press treatment at 24,000 psi and the extract was clarified by 20 min of centrifugation at 30,000*g*. Ammonium sulfate was added to 0–25% and 25–55% saturation. The pellet collected at 25–55% saturation was dissolved and dialyzed against 20 mM Tris–HCl buffer (pH 8.5), containing 5 μM AEBSF and 0.2 μM leupeptin. The 1-lip E2 was applied at room temperature to a HiLoad Q-Sepharose High Performance column (Amersham Pharmacia Biotech, Piscataway, NJ), pre-equilibrated with 20 mM Tris–HCl buffer (pH 8.5). The protein was eluted with a linear gradient (0–1.2 M) of NaCl in 20 mM Tris–HCl buffer (pH 8.5) and placed on ice immediately after collection. Fractions containing 1-lip E2 were combined and concentrated at 0–55% saturation of ammonium sulfate. The pellet was dissolved in 20 mM Tris–HCl buffer (pH 8.5) containing 5 μM AEBSF, 0.2 μM leupeptin, and 25% ammonium sulfate and dialyzed against the same buffer. The dialyzed protein was applied to a phenyl-Sepharose High Performance hydrophobic column (Amersham Pharmacia Biotech, Piscataway, NJ) at room temperature and the protein was eluted with a decreasing gradient of ammonium sulfate. The 1-lip E2 was eluted at 5% saturation of ammonium sulfate. Fractions containing 1-lip E2 were combined and dialyzed against 20 mM Tris–HCl buffer (pH 8.5) containing 0.3 M NaCl, 1 mM EDTA, 50 μM AEBSF, and 0.2 μM leupeptin. For long-term storage of 1-lip E2, the concentrations of AEBSF and leupeptin were adjusted to 0.5 mM and 20 μM, respectively.

Expression and purification of E3

The cell growth and expression of E3 were the same as described above for 1-lip E2, except the DL-α-lipoic acid was not included in the LB medium. Cells (1.97 g) from a 500 mL culture were resuspended in 50 mM KH_2PO_4 buffer (pH 8.0) also containing 1 mM EDTA and 1 mM PMSF. The cells were disrupted by French press treatment at 24,000 psi. The supernatant was clarified by centrifugation at 30,000*g* and ammonium sulfate was added to 40% of saturation. The clarified supernatant was applied to a phenyl-Sepharose High Performance hydrophobic column (Amersham Pharmacia Biotech, Piscataway, NJ) at room temperature, pre-equilibrated with 50 mM KH_2PO_4 (pH 8.0) containing 1 mM EDTA and 40% ammonium sulfate. A

decreasing gradient of ammonium sulfate was applied and E3 was eluted at 15% saturation of ammonium sulfate. Fractions containing the E3 were collected and analyzed using SDS–PAGE. Finally, the E3 was dialyzed against 50 mM KH_2PO_4 (pH 8.0) containing 1 mM EDTA and 50% glycerol was added to the buffer for storage at −20 °C.

Expression and purification of lipoyl domain

E. coli cells (JM101) transformed with pGS331 plasmid were used for expression of hybrid lipoyl domain. Cells were grown at 37 °C in 4000 mL LB medium supplemented with 0.1% glucose and 100 μg/mL ampicillin until an A_{650} of 0.5–0.7 was achieved. The IPTG (0.042 mM) and DL-α-lipoic acid (0.5 mM) were added to induce the expression and to generate the lipoylated form of lipoyl domain and cells were grown at 37 °C for 16 h. The harvested bacteria were washed with 20 mM KH_2PO_4 (pH 7.0) containing EDTA (2 mM), NaN_3 (0.02%), PMSF (1 mM), and benzamidine hydrochloride (1 mM) and stored at −20 °C. The lipoyl domain was purified according to Ali and Guest [7]. Cells were resuspended in 20 mM KH_2PO_4 (pH 7.0) containing EDTA (2 mM), NaN_3 (0.02%), PMSF (1 mM), and benzamidine hydrochloride (1 mM) and disrupted by sonication. The pH was lowered to 4.0 and insoluble material was removed by centrifugation (30,000*g* for 20 min). The supernatant was concentrated by ultrafiltration on Centriprep YM-3 (Millipore, Bedford, MA) and applied to a 1 mL Mono-Q column (Amersham Pharmacia Biotech, Piscataway, NJ). The lipoyl domain was eluted with 10–600 mM ammonium acetate buffer (pH 5.0) gradient over 20 column volumes. The fractions containing the lipoyl domain were concentrated, dialyzed against 20 mM KH_2PO_4 (pH 7.0), and stored at −20 °C.

Activity measurements

1-lip E2 activity was measured in the overall PDHc reaction after reconstitution with E1 and E3. E1 (2 μg) and E3 (1 μg) were preincubated with 1-lip E2 (in the range of 0.5–8.0 μg) in 100 μL of 20 mM KH_2PO_4 (pH 7.0) at 25 °C. After 30–40 min of incubation, 880 μL reaction medium containing 0.1 M Tris–HCl (pH 8.0), 2 mM pyruvate, 0.2 mM ThDP, 1 mM $MgCl_2$, 2.6 mM DTT, and 2.5 mM NAD^+ was added and the reaction was initiated by addition of CoA (0.2 mM final concentration). The formation of NADH product was monitored at 340 nm at 30 °C. A similar value of PDHc activity was measured when the reaction was started by addition of a mixture of E1, 1-lip E2, E3, and CoA. Steady-state velocities were taken from the linear portion of the progress curves. One unit of activity is defined as the amount of 1-lip E2 required to produce 1.0 μmol/min NADH (μmol/min/mg of protein). One unit of PDHc activity corresponds to 1 μmol NADH formed per min per mg total protein.

E3 activity was measured in both the forward and reverse reactions according to the literature [11]. In the forward reaction, E3 catalyzes the oxidation of dihydrolipoamide to lipoamide and of NAD^+ to $NADH + H^+$. The reaction medium contained in a 1 mL test volume 0.1 M KH_2PO_4 (pH 8.0), 3 mM NAD^+, 3 mM DL-dihydrolipoamide, and 1.5 mM EDTA at 25 °C. The reaction was initiated by the addition of E3. One unit of activity is defined as the amount of NADH produced (μmol/min/mg protein). In the reverse reaction, E3 catalyzes the oxidation of NADH to NAD^+ in the presence of lipoamide. The reaction medium contained in a 1 mL test volume 0.1 M KH_2PO_4 (pH 8.0), 0.1 mM NAD^+, 0.1 mM NADH, 3.0 mM DL-lipoamide, and 1.5 mM EDTA. The reaction was initiated by adding the E3. One unit of activity is defined as the amount of E3 required to oxidize NADH (μmol/min/mg protein). DL-Dihydrolipoamide was synthesized by reducing DL-lipoamide with $NaBH_4$ [11,12].

MALDI–TOF mass spectrometry of lipoyl domain, 1-lip E2, and E3

Samples of lipoyl domain were desalted with $ZipTip_{C18}$ (Millipore, Bedford, MA), while samples of 1-lip E2 and E3 were desalted with $ZipTip_{C4}$ (Millipore, Bedford, MA) according to manufacturer's instructions, or were diluted 10–30 times from the stock. One μL of sample was mixed with 1 μL matrix solution (sinapinic acid) directly on the target plate. Mass spectra were acquired on a Voyager—DE PRO MALDI–TOF (PerSeptive Biosystems, Framingham, MA) mass spectrometer equipped with a nitrogen laser to desorb and ionize samples. The accelerating voltage used was 25 kV. The spectrometer was calibrated using bovine serum albumin (molecular mass = 66,579 Da) for mass determination of the E3 and 1-lip E2 subunits, or ubiquitin (molecular mass = 8566 Da) for mass determination of lipoyl domain.

Reductive acetylation of the lipoyl domain and of 1-lip E2 monitored by MALDI–TOF mass spectrometry

For the reductive acetylation of lipoyl domain, E1 (0.1 μM) was incubated in 20 mM KH_2PO_4 (pH 7.0) with 2 mM $MgCl_2$, 0.2 mM ThDP, 2.0 mM pyruvate, and 0.6 mM of lipoyl domain in a total volume of 0.10 mL at 25 °C. After 30 min, the sample was desalted using a $ZipTip_{C18}$ and 1 μL sample was mixed with 1 μL sinapinic acid on the target plate. For the reductive acetylation of 1-lip E2, E1 (0.1 μM) was incubated in 50 mM Tris–HCl (pH 8.2), containing 0.3 M NaCl, 2 mM $MgCl_2$, 0.2 mM ThDP, 2.0 mM pyruvate, and 1-lip E2 (2 mg/mL) in a total volume of 0.1 mL at 25 °C.

After 30 min, trypsin was added to digest 1-lip E2 at 25 °C (wt/wt ratio of 1-lip E2: trypsin = 200:1). After 2 h, benzamidine hydrochloride (0.15 mg/mL) was added to terminate the reaction. Samples were desalted with $ZipTip_{C18}$ and 1 μL was mixed with 1 μL of sinapinic acid on the target plate.

The synthesis of pyruvate-d_3[(C_3–$^2H_3$)-pyruvate] was according to the literature [13].

Results

Expression of the 1-lip E2 and E3 proteins

In this paper, procedures are presented for construction of pET-22b(+)-1-lip E2 and pET-22b(+)-E3 vectors (Fig. 1—see Materials and methods for details). The pGS 501 plasmid encoding the PDHc with a single lipoyl domain per E2 subunit was used for construction of the 1-lip E2 and E3 vectors [14]. The *E. coli* BL21(DE3) competent cells were used for expression of 1-lip E2 and E3 proteins. Significant amounts of 1-lip E2 and E3 proteins were already expressed after 2 h of IPTG induction according to the SDS–PAGE (data not shown). Lipoic acid (0.1 mM) was added to the medium for cell growth to ensure full lipoylation of the 1-lip E2 subunit. The primary sequence of the 1-lip E2 subunit is presented in Fig. 2. The initiating methionine residue is removed post-translationally, as was confirmed by N-terminal peptide sequencing of the 1-lip E2 subunit in our laboratory: Ala–Ile–Glu–Ile–Lys–Val–Pro–Asp–Ile. The lipoyl domain in the E2 subunit is a hybrid lipoyl domain. The 85 amino acid residues starting from the N-terminus of the 1-lip E2 subunit comprise residues 1–33 of the first lipoyl domain and residues 238–289 of the third lipoyl domain of the E2 subunit (see Fig. 2). The hybrid lipoyl domain was also expressed independently from the pGS331 plasmid following literature procedures [7]. The primary sequence of the hybrid lipoyl domain is available [7] and is similar to that of the hybrid lipoyl domain in the 1-lip E2 subunit (Fig. 2).

```
     1                  10                  20
     A I E I K V P D I G A D E V E I T E I L V K V G D K V

   30    33 238        *
E A E Q S L I T V E G D K A S M E V P A P F A G V V K E L K

                                                    289
V N V G D K V K T G S L I M I F E V E G A A P A A A P A K Q

E A A A P A P A A K A E A P A A A P A A K A E G K S E F A E

N D A Y V H A T P L I R R L A R E F G V N L A K V K G T G R

K G R I L R E D V Q A Y V K E A I K R A E A A P A A T G G G

I P G M L P W P K V D F S K F G E I E E V E L G R I Q K I S

G A N L S R N W V M I P H V T H F D K T D I T E L E A F R K

Q Q N E E A A K R K L D V K I T P V V F I M K A V A A A L E

Q M P R F N S S L S E D G Q R L T L K K Y I N I G V A V D T

P N G L V V P V F K D V N K K G I I E L S R E L M T I S K K

A R D G K L T A G E M Q G G C F T I S S I G G L G T T H F A

P I V N A P E V A I L G V S K S A M E P V W N G K E F V P R

L M L P I S L S F D H R V I D G A D G A R F I T I I N N T L

S D I R R L V M
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Fig. 2. Primary sequence of the 1-lip E2 subunit. The lipoyl domain (marked with the solid line) comprises residues 1–33 from the first and residues 238-289 from the third lipoyl domain of the E2 subunit (reprinted from [7]). The Lys-244 is the site of lipoylation (*). The additional amino acid residues (marked with the broken line) contribute to the mass of the lipoyl domain obtained by tryptic digestion of the 1-lip E2 subunit.

Purification of 1-lip E2 and E3 proteins

The 1-lip E2 was purified, as described under Materials and methods and as presented in Table 1. 1-lip E2 (17.9 mg) was obtained from 1000 mL culture, representing 6.3% of total protein in the clarified extract. Homogeneous 1-lip E2 was obtained by using a HiLoad Q-Sepharose HP anion exchange column, followed by phenyl-Sepharose HP hydrophobic chromatography. The resulting 1-lip E2 was pure, as judged by SDS–PAGE (Fig. 3A). The purified 1-lip E2 exhibited a tendency to aggregate, as was reported for 3-lip E2 separated form *E. coli* PDHc [15]. It was reported that freezing and thawing, a pH < 6.6, a high protein concentration, and room temperature all promote aggregation of 3-lip E2 [15]. However, use of room temperature for the FPLC steps was unavoidable, and in fact, for the hydrophobic column it is a must. In this study, the 1-lip E2 at a concentration of 6.9–9.0 mg/mL dissolved in 20 mM Tris–HCl (pH 8.5) containing 0.3 M NaCl, 1 mM EDTA, 0.5 mM AEBSF, and 20 μM leupeptin was stored at −20 °C. The molecular mass of 1-lip E2 subunit determined by MALDI–TOF mass spectrometry was 45,953 ± 73 Da, as compared with the theoretical molecular mass of 45,802 Da determined from the nucleotide sequence of 1-lip E2 subunit and its amino acid composition (425 amino acid residues with a lipoylated lysine residue) (Fig. 4A).

The activity of 1-lip E2 measured in the overall PDHc reaction after reconstitution with E1 and E3 was in the range of 9–13.6 units/mg total protein (23–34 units/mg 1-lip E2). The maximal PDHc activity was observed at a mass ratio of E1:E2:E3 = 1:1:0.5 (2 μg:2 μg:1 μg). These data are in good agreement with the activity of 6.86 and 8.43 units/mg total protein reported earlier from our laboratory for 1-lip PDHc and 3-lip PDHc [16] and an activity of 14 units/mg total protein reported for 1-lip PDHc from the Guest laboratory [14]. Preincubation of all three recombinant subunits, E1, 1-lip E2, and E3 at 25 °C for 30–40 min, was required to attain maximal PDHc activity. According to the overall PDHc assay, the presence of 0.1 mM lipoic acid in the culture for cell growth increased the activity by sixfold.

Table 1
Purification of 1-lip E2

Fractions	Total protein (mg)	Specific activity (μ/mg of E2)[a]	Total activity (units)[a]	Yield (%)
Clarified cell lysate	287.0	—	—	100
25–55% $(NH_4)_2SO_4$	221.4	12.2	2701	77
HiLoad Q-Sepharose HP column	48.4	39.5	1912	16.8
Phenyl-Sepharose HP column	17.9	34.1	611	6.3

[a] The specific and total activities of the clarified cell lysate were very low. 1-lip E2 activity was measured in the overall PDHc reaction after reconstitution with E1 and E3. The mass ratio of E1:1-lip E2:E3 was 1:1:0.5 (2 μg:2 μg:1 μg).

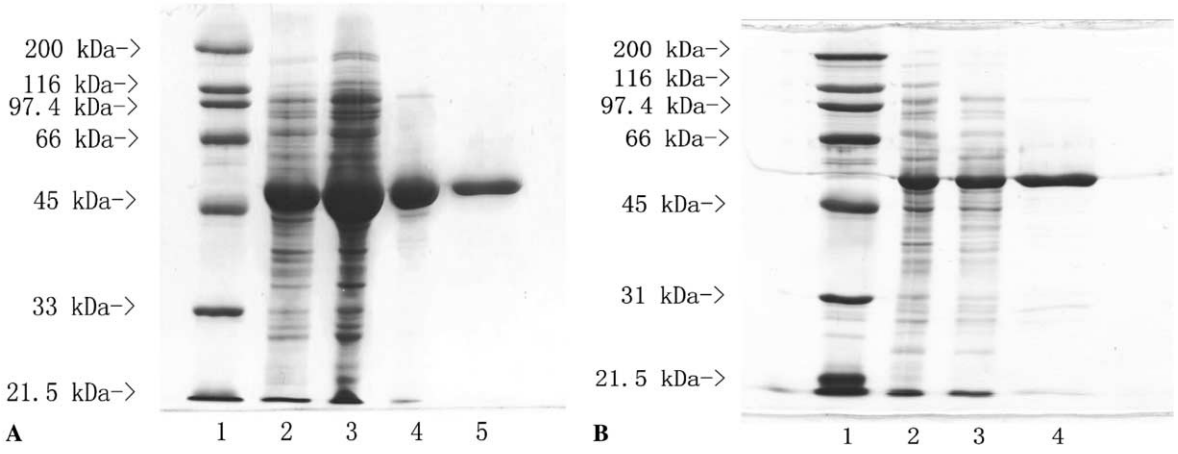

Fig. 3. SDS–PAGE (10%) showing the purification of 1-lip E2 and E3 proteins. (A) Lane 1, Bio-Rad standard protein markers; lane 2, cell lysate; lane 3, pellet at 25–55% $(NH_4)_2SO_4$ saturation; lane 4, 1-lip E2 from HiLoad Q-Sepharose HP anion exchange column; and lane 5, 1-lip E2 from phenyl-Sepharose HP hydrophobic column. (B) Lane 1, Bio-Rad standard protein markers; lane 2, cell lysate; lane 3, supernatant at 40% $(NH_4)_2SO_4$ saturation; and lane 4, E3 from phenyl-Sepharose HP hydrophobic column.

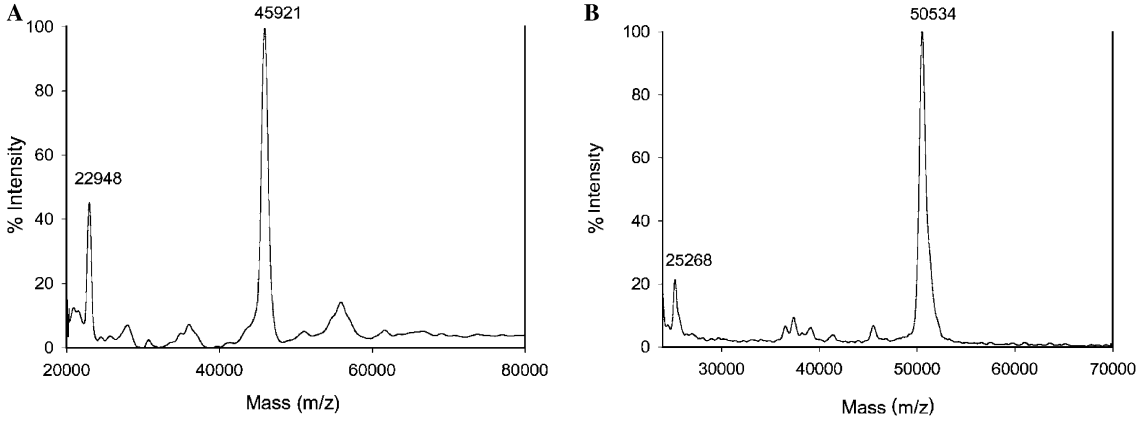

Fig. 4. (A) MALDI–TOF mass spectrum of the 1-lip E2 subunit. The spectrum shows singly 45,921 Da $(M + H^+)$ and doubly 22,948 Da $(M + 2H)^{2+}$ charged molecular ions for 1-lip E2 subunit. Sample preparation is described under Materials and Methods. (B) MALDI–TOF mass spectrum of the E3 subunit. The protein was diluted 20-fold from the stock (2 mg/mL). One μL sample was mixed with 1 μL sinapinic acid directly on the target plate. The spectrum shows singly 50,534 Da $(M + H)^+$ and doubly 25,268 Da $(M + 2H)^{2+}$ charged molecular ions for the E3 subunit without FAD (apo E3).

The E3 protein was purified, as described under Materials and methods. The protein yield and activity are presented in Table 2. E3 (10.9 mg) was obtained from 500 mL culture (4.2% total protein) with a specific activity of 301.6 μ/mg of E3 measured in the forward reaction and 29.6 μ/mg E3 measured in the reverse reaction. Homogeneous E3 resulted from phenyl-Sepharose High Performance hydrophobic column chromatography, as judged by SDS–PAGE (Fig. 3B). Apparent molecular masses, of $50{,}528 \pm 5.5$ Da and $51{,}266 \pm 48$ Da were determined for purified E3 by MALDI–TOF mass spectrometry, in good agreement with the mass of 50,554 Da (apo-E3) and a mass of 51,274 (including the noncovalently bound FAD)

Table 2
Purification of E3

Fractions	Total protein (mg)	Specific activity (μ/mg of E3)[a]	Specific activity (μ/mg of E3)[b]	Total activity (units)[a]	Total activity (units)[b]	Yield (%)
Clarified cell lysate	258.4	52.8	6.5	13644	1680	100
40%$(NH_4)_2SO_4$	199.8	55.5	6.2	11089	1239	77
Phenyl-Sepharose HP column	10.9	301.6	29.6	3287	323	4.2

[a] Forward reaction: Dihydrolipoamide + $NAD^+ \rightarrow$ Lipoamide + NADH + H^+.
[b] Reverse reaction: Lipoamide + NADH + $H^+ \rightarrow$ Dihydrolipoamide + NAD^+.

determined from the nucleotide sequence and amino acid composition of the E3 subunit (473 amino acid residues and N-terminal methionine residue removed post-translationally) (Fig. 4B) [10]. Peaks corresponding to both apo-E3 and E3 containing FAD were in evidence in the MALDI–TOF mass spectrum for protein twice diluted from the stock (2 mg/mL), but only one peak with a molecular mass corresponding to apo-E3 was detected for 20-fold diluted protein (Fig. 4B).

Reductive acetylation of the lipoyl domain

The hybrid lipoyl domain expressed from the pGS331 plasmid was purified as described under Materials and methods following the protocol described in the literature [7]. The purified lipoyl domain was in the lipoylated form, as indicated by MALDI–TOF mass spectrometry (Fig. 5A) and by nondenaturing PAGE (not shown). The molecular mass of lipoyl domain determined by MALDI–TOF mass spectrometry was 8982 ± 4 Da (the lipoic acid is covalently attached), in good agreement with the theoretical molecular mass of 8975 Da [7]. According to reports in the literature, the E2p lipoyl domain is a good substrate for its cognate E1 [17]. To confirm the fact that the purified lipoyl domain is functionally active, its reductive acetylation was studied, as described under Materials and methods. The final product, acetylated lipoyl domain with a molecular mass of 9019 ± 2, was detected by MALDI–TOF mass spec-

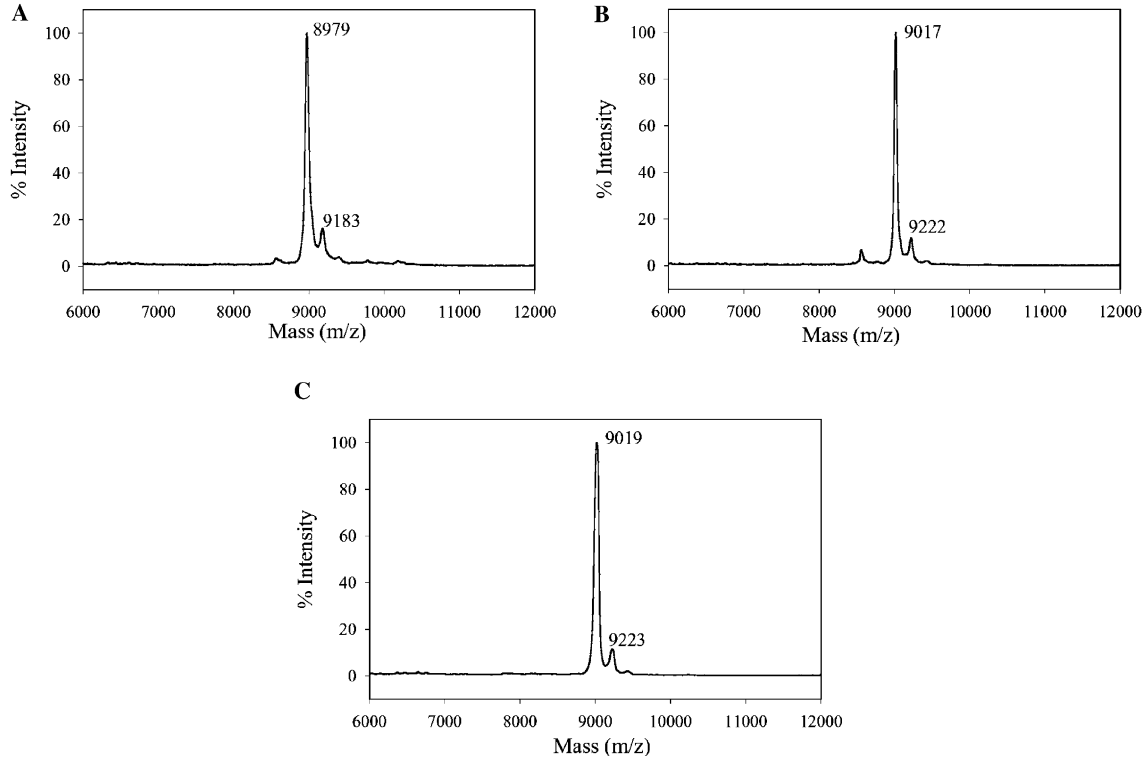

Fig. 5. (A) MALDI–TOF mass spectrum of the lipoyl domain. The spectrum shows the molecular ions for singly (mass = 8979 Da) and doubly (mass = 9183 Da) lipoylated forms of the domain. (B) MALDI–TOF mass spectrum of acetylated lipoyl domains. The spectrum shows the acetylation of singly (mass = 9017 Da) and doubly (mass = 9222 Da) lipoylated forms of the lipoyl domain at 30 s of incubation with E1 and pyruvate. The conditions of the experiment are described under Materials and methods. (C) MALDI–TOF mass spectrum of the acetylated lipoyl domains at 30 min incubation with E1 and pyruvate.

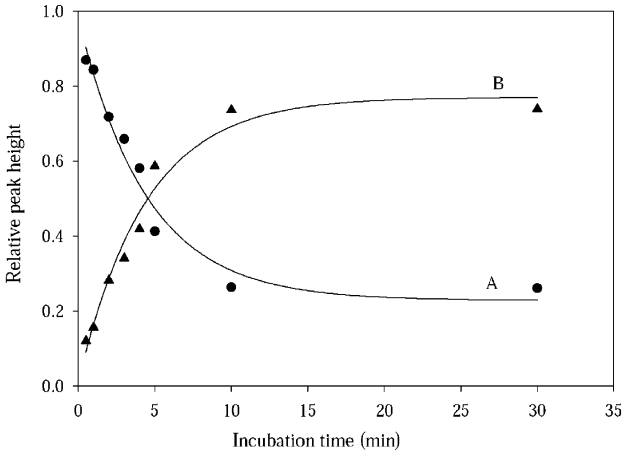

Fig. 6. Curves of the time-dependent reductive acetylation of the lipoyl domain by pyruvate and the H407A E1 variant based on the relative peak height determined for each form. Curve (A) exponential depletion of the unacetylated lipoyl domain; curve (B) formation of the reductively acetylated lipoyl domain.

trometry (the molecular mass of lipoyl domain is 8975 Da and the mass increased by 44 Da). The time dependence of reductive acetylation established the fact that within 30 s of incubation with E1, the lipoyl domain was in the fully acetylated form. No peaks corresponding to unacetylated lipoyl domain could be detected with pyruvate as a substrate (Figs. 5B and C present data for 30 s and 30 min of incubation). However, as illustrated in Fig. 6, for the lipoyl domain incubated with the His407Ala active-center variant of E1, the time dependence of acetylation could be monitored by MALDI–TOF mass spectrometry. With this E1 variant, the unacetylated lipoyl domain (mass = 8982 Da) coexisted with the reductively acetylated form (mass = 9019 Da; theoretical mass increase resulting from reductive acetylation is 44 Da), clearly showing the gradual loss of the unacetylated protein and the appearance of the acetylated one during the extended incubation time. To our knowledge, this is the first demonstration of the ability of MALDI–TOF mass spectrometry to monitor the time-course of reductive acetylation in a 2-oxo acid dehydrogenase multienzyme complex. In the widely used earlier protocol to monitor this reaction, the incorporation of ^{14}C from [2-^{14}C]-pyruvate into the lipoyl domain is measured in the absence of CoA. Protein is precipitated on a filter paper disk and the amount of radioactivity bound is counted [18]. Electrospray mass spectrometry has already been used for molecular mass determination of *E. coli* lipoyl domain and of the acetylated form of the lipoyl domain [9].

Reductive acetylation of lipoyl domain in 1-lip E2

As mentioned above, the lipoyl domain used in our study is a hybrid lipoyl domain, constructed in vitro. To study the specificity of interaction between the E1 and 1-lip E2, it would be important to establish the fact that the 1-lip E2 can indeed undergo the reductive acetylation. However, the error in determination of the mass of 1-lip E2 subunit using MALDI–TOF was 73 Da (see above), even larger than the mass increase expected on reductive acetylation (44 Da). It had been reported that the lipoyl domain of the *E. coli* E2 subunit is stable to limited proteolysis by trypsin [5]. Therefore, we subjected the 1-lip E2 subunit to tryptic digestion. Preincubation of 1-lip E2 subunit with trypsin (see conditions under legend to Fig. 7) for 5, 10, 30, 60, 90, and 120 min resulted in the appearance of two major fragments with M_r's of 35,000 and 10,000 (according to SDS–PAGE) (Fig. 7). These two fragments were stable and not cleaved by trypsin even after 120 min of incubation. The fragment with M_r of 10,000 was assigned to the lipoyl domain and its mass is $10{,}112 \pm 3$ Da according to MALDI–TOF mass spectrometry (Fig. 8A), higher than the expected theoretical mass 8975 Da, indicating that trypsin cleaved the 1-lip E2 subunit at a lysine residue, 12 amino acid residues upstream from the C-terminus of the lipoyl domain (see Fig. 2 for the lipoyl domain primary sequence). Treatment of the E1 subunit with trypsin under the same conditions led to the appearance of numerous small fragments with masses smaller than 10,000 after 120 min of incubation (SDS–PAGE and MALDI–TOF mass spectrometry, data not shown). Preincubation of 1-lip E2 with E1, ThDP, Mg^{2+}, and pyruvate for 30 min at 25 °C led to the appearance of a fragment with a mass $10{,}159 \pm 3$ Da, which we assign to reductively acetylated lipoyl domain (Fig. 8B). To confirm the fact that the increased mass resulted from reductive acetylation, the acetyl group being derived from pyruvate, we used a 1:1 (wt/wt) mixture of pyruvate and pyruvate-d_3. Two masses at 10,161 and 10,163 Da were

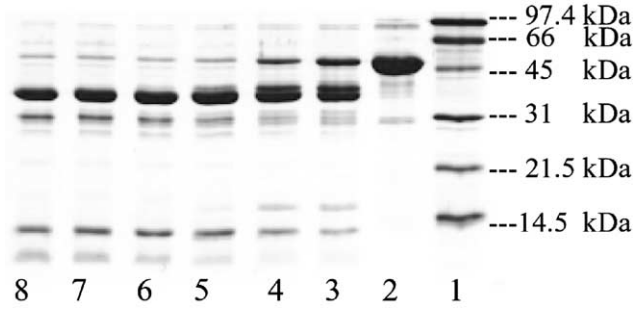

Fig. 7. Time-course of limited proteolysis of 1-lip E2, as monitored by SDS–PAGE. 1-lip E2 (1 mg/mL) was subjected to tryptic digestion for 5–120 min. Digestion was carried out in 50 mM Tris–HCl (pH 8.2) containing 0.3 M NaCl in a total volume of 0.15 mL at 25 °C and a trypsin/substrate ratio of 1:200 (w/w). At the indicated times, aliquots (20 μL) were withdrawn and subjected to SDS–PAGE (5 μg protein per lane). Lane 1, Bio-Rad standard protein markers; lane 2, 1-lip E2 before the trypsin digestion; and lanes 3–8, time-course of tryptic digestion of 1-lip E2 for 5, 10, 30, 60, 90, and 120 min, respectively.

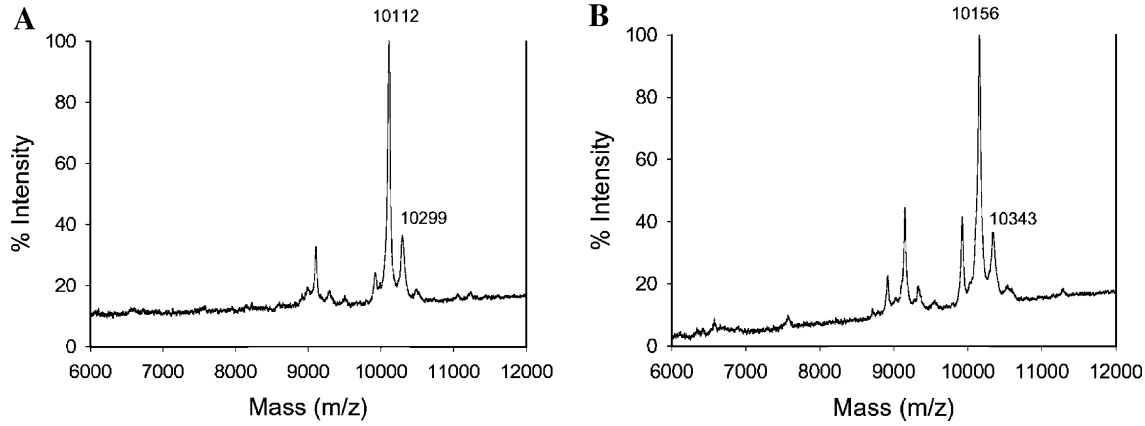

Fig. 8. (A) MALDI–TOF mass spectrum of the tryptic digest of 1-lip E2. The 1-lip E2 (1 mg/ mL) was subjected to tryptic digestion for 120 min, as described in the legend to Fig. 7. Next, benzamidine hydrochloride (0.15 mg/mL) was added to terminate the reaction. Samples were desalted with $ZipTip_{C18}$ and 1 μL tryptic digest was mixed with 1 μL sinapinic acid on the target plate. The fragment with a mass of 10,112 Da was assigned to the lipoyl domain cleaved from the 1-lip E2 subunit. (B) MALDI–TOF mass spectrum of the tryptic digest of acetylated 1-lip E2. The reductive acetylation of 1-lip E2 with E1 and pyruvate was carried out as described under Materials and methods. The fragment with a mass of 10,156 Da was assigned to the acetylated lipoyl domain.

detected (data not shown), affirming that the increased mass corresponding to addition of a CH_3CO-group originated from pyruvate and that acetylation of lipoyl domain does indeed take place under the experimental conditions.

Discussion

In this paper, we describe for the first time an expression system for the *E. coli* E2 subunit of PDHc with a single lipoyl domain. Plasmids encoding the PDHc with no lipoyl domain and up to nine lipoyl domains per E2 subunit were created in the Guest laboratory [19]. It was shown that PDHc activity of purified complexes decreased once the number of lipoyl domains exceeded four. The stoichiometry of E1:E2:E3 subunits in purified complexes was within (0.7–1.2):1.0:(0.8–1.3) [19]. In previous studies, we used the pGS501 plasmid encoding 1-lip PDHc to study the role of substitutions in the ThDP-fold on PDHc activity [16]. It was shown that the kinetic behavior of 1-lip PDHc was very similar to that of 3-lip PDHc. Since the 1-lip E2 construct here reported is independently expressed, it was important to show that in all respects it functions as it does when expressed in the complex. The following evidence has been obtained, indicating that the independently expressed 1-lip E2 subunit is correctly folded: (1) The purified 1-lip E2 is cleaved by trypsin into two major fragments with M_r's of 35,000 and 10,000 (the latter has a mass of 10,112 Da and it encompasses the lipoyl domain), similar to that presented in the literature for *E. coli* PDHc and its 3-lip E2 subunit [5]; (2) The 1-lip E2 subunit is reconstituted with E1 and E3 subunits with an overall PDHc activity of 9–13 units/mg total protein, which is not very different from the activities of 1-lip PDHc and 3-lip PDHc published from our laboratory [16] and the Guest laboratory [14]; (3) The 1-lip E2 subunit is acetylated by pyruvate in the presence of E1, according to MALDI–TOF mass determination for acetylated lipoyl domain. The combined evidence indicates that functionally active recombinant 1-lip E2 was purified. In addition, using E2 subunit with a single lipoyl domain (instead of three as found in the wild-type PDHc) significantly simplifies the interpretation of kinetic studies of the E1 variants with substitutions in the active site.

The lipoyl domain expressed independently and excised from the 1-lip E2 subunit was a hybrid lipoyl domain. A similar lipoyl domain was used for three-dimensional structure determination by NMR spectroscopy (an additional modification was that the lipoic acid-bearing lysine residue in the hybrid lipoyl domain was substituted by glutamine) [8]. As shown by NMR, the three-dimensional structure of the hybrid lipoyl domain was similar to that of the wild-type lipoyl domain from *Bacillus stearothermophilus* PDHc [8] and the innermost lipoyl domain from *E. coli* [9]. With this in mind, the hybrid lipoyl domain was purified and characterized in this paper using MALDI–TOF mass spectrometry and E1 expressed independently. According to MALDI–TOF mass spectrometry and FT-ICR mass spectrometry (data in progress), the purified lipoyl domain was in a fully lipoylated form. No unlipoylated form of the domain could be detected with 0.5 mM lipoic acid added to the medium for the cell growth, similar to that published for human lipoyl domain [20].

There was an additional minor component with a mass of 9190 ± 4.79 Da detected in the mass spectra of the lipoyl domain and its mass also increased by 44 Da

under the conditions of reductive acetylation (see Figs. 5A–C). We believe that the species represented by this larger mass may be assigned to the doubly lipoylated domain (additional increase in mass of lipoylated domain by 188 Da). The recognition sequence for lipoylation is DKA (AspLysAla). As seen in Fig. 2, lipoylation is at K244 (shown by Guest and collaborators). Additionally, there are two sites with partial recognition sequences D25K26 and D266K267; one of these two lysine residues is a possible minor site for lipoylation. According to the literature, the minor component detected in the spectrum of lipoyl domain may be assigned to the octanoylated form of domain [20]. That this is not the case in our experiments is attested to by the fact that for the octanoylated form, an increase in mass of the lipoyl domain by 127.2 Da would be expected. The observations are different from that published for the lipoyl domain from *B. stearothermophilus* [21]. The additional lysine residues introduced in *B. stearothermophilus* lipoyl domain by replacement of Asp or Ala residues in the conserved Asp–Lys–Ala fold were not lipoylated according to electrospray mass spectrometry [21]. Shifting the Lys residue at the β-turn of the lipoyl domain towards the N-terminus hindered lipoylation, indicating that the exact positioning of the lipoyl-bearing lysine is essential for correct post-translational modification of *B. stearothermophilus* lipoyl domain [21].

The important feature of the *E. coli* lipoyl domain expressed independently and excised from the 1-lip E2 subunit is its reductive acetylation by pyruvate and E1, as monitored by MALDI–TOF mass spectrometry. The time dependence of reductive acetylation of lipoyl domain by pyruvate and E1, as monitored by MALDI–TOF mass spectrometry (Fig. 6), is to our knowledge the first instance in the literature and provides a useful tool for all 2-oxo acid dehydrogenase multienzyme complexes.

In this paper, we also report the expression and purification of the *E. coli* E3. An expression system for the wild-type *E. coli* E3 and its variants had been presented in the literature; however, the protein was not purified and activity was measured in the cell-free extracts [22]. As was shown with our data, the *E. coli* E3 is a highly hydrophobic protein and is tightly bound to the hydrophobic column, similar to E3 from *Azotobacter vinelandii* [23]. The purified E3 is a dimer according to preliminary data obtained using light scattering measurements (experiments in progress), as found for E3 proteins with known three-dimensional structures [2]. The activity of the *E. coli* E3 in the forward reaction was 302 units/min/mg protein, compared with 540 units/mg protein for human E3 [24], and 117 units/mg protein for *B. stearothermophilus* E3 [25]. In the reverse reaction, the activity of *E. coli* E3 was about 30 units/mg protein and was lower than the 117 units/mg protein reported for E3 from *A. vinelandii* [23] and 127.1 units/mg protein for E3 from *Pseudomonas fluorescens* [26]. The *E. coli* E3 has a high degree of identity with E3 from PDHc and oxoglutarate dehydrogenase (OGDH) complexes of *Haemophilus influenzae*, PDHc of *Alcaligenes eutrophus*, PDHc and OGDH complexes of *Neisseria meningitis* (the structure has been determined), PDH and OGDH complexes of *Bacillus subtilis*, and PDHc of *Staphylococcus aureus* according to a BLAST search of sequences.

Acknowledgments

The authors gratefully acknowledge the receipt of plasmids from Professor John Guest of Sheffield University and Mr. Farzad Sheibani for developing the protocol for purification of the lipoyl domain.

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