Expression and lipoylation in *Escherichia coli* of the inner lipoyl domain of the E2 component of the human pyruvate dehydrogenase complex

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The dihydrolipoamide acetyltransferase subunit (E2p) of mammalian pyruvate dehydrogenase complex has two highly conserved lipoyl domains each modified with a lipoyl cofactor bound in amide linkage to a specific lysine residue. A sub-gene encoding the inner lipoyl domain of human E2p has been over-expressed in *Escherichia coli*. Two forms of the domain have

been purified, corresponding to lipoylated and non-lipoylated species. The apo-domain can be lipoylated *in vitro* with partially purified *E. coli* lipoate protein ligase, and the lipoylated domain can be reductively acetylated by human E1p (pyruvate dehydrogenase). Availability of the two forms will now allow detailed biochemical and structural studies of the human lipoyl domains.

INTRODUCTION

The pyruvate dehydrogenase complex (PDC) catalyses the oxidative decarboxylation of pyruvate to acetyl-CoA and CO₂ and is one of the most extensively studied multi-enzyme systems (for recent reviews, see [1–3]). It consists of multiple copies of three component enzymes, namely pyruvate dehydrogenase (E1p), dihydrolipoamide acetyltransferase (E2p) and lipoamide dehydrogenase (E3), which catalyse consecutive steps in the overall reaction.

Research on PDC has concentrated mainly on the E2p component, which has a central role in both the catalytic activity and the structure and assembly of the complex. The mammalian complex comprises a pentagonal dodecahedron core of 60 E2p subunits around which the E1p and E3 components are assembled: approx. 30 E1p tetramers ($\alpha_2\beta_2$) bind at the 30 edges, and at least six E3 dimers on the 12 faces of the core [4]. The E2p component also has multiple roles in catalysis. It contains two covalently bound lipoyl cofactors that are reductively acetylated by E1p, the acetyl group then being transferred to CoA at the E2p active site. After acetyl transfer, the reduced lipoamide is reoxidized by E3 to complete the catalytic cycle. Thus the E2p carries determinants for the assembly of the E2p core, the specific binding of E1p and E3, the covalent attachment of lipoic acid and the acetyltransferase active site. Analysis of the cDNA sequence of various mammalian E2p subunits [5–8] has confirmed earlier studies on the *Escherichia coli* enzyme [9,10] that the E2p is highly segmented (see Figure 1 below). Two highly conserved lipoyl domains are located in tandem repeat at the N-terminus of the protein, followed by a distinct region of polypeptide considered to be involved in binding of the E3 component, although the mammalian complexes contain an additional component, protein X, which appears to play a major role in binding of E3 [11,12]. The C-terminal catalytic domain of E2p, in addition to containing the active site, binds to other E2p polypeptides to

maintain the central core, and is thought to be responsible for binding of the E1 component of the complex. These independent domains are interconnected by flexible linker regions, characteristically rich in alanine and proline residues.

The lipoyl domains of E2p are of considerable interest. It is the lipoate-lysine residue of these domains that is central to the activity of the complex, providing a swinging arm that has sufficient rotational mobility to allow movement between the active sites of the three component enzymes [13]. However, surprisingly little is known concerning the enzymes responsible for the attachment and removal of this essential lipoic acid cofactor. The sequence surrounding the lipoate-attachment site on several bacterial and mammalian PDCs has been determined [14–16] and is highly conserved. Individual lipoyl domains of *E. coli* and *Bacillus stearothermophilus* E2p have previously been over-expressed in *E. coli*, and both lipoylated and unlipoylated gene products were recovered [17–19]. Additionally octanoylation of the *B. stearothermophilus* and *E. coli* domains was observed, the latter when a lipoic acid-deficient mutant of *E. coli* was used as the host strain [20]. A recent study has provided evidence for two protein ligase activities in *E. coli* [21]. However, the only information on the mammalian lipoylation system, to date, is its intra-mitochondrial localization [22].

Recently, considerable interest has been generated by the identification of E2p as the major mitochondrial autoantigen in the human autoimmune liver disease primary biliary cirrhosis (PBC) [23,24]. Furthermore, the major epitope of E2p has been localized to the lipoyl domains in two separate studies [25,26], and work with the E2p from *E. coli* has indicated that the lipoic acid cofactor plays a key role in the immune response [27]. Consequently these domains are of both biochemical and medical interest.

The cDNA encoding the entire human E2p has previously been cloned and sequenced [7]. To facilitate further studies on the human lipoyl domains, this cDNA was used in the present study

Abbreviations used: PDC, pyruvate dehydrogenase complex; PBC, primary biliary cirrhosis; E2p, dihydrolipoamide acetyltransferase (EC 2.3.1.12); IPTG, isopropyl β -thiogalactopyranoside; E1p, pyruvate dehydrogenase; E3, lipoamide dehydrogenase; GST, glutathione S-transferase; PMSF, phenylmethanesulphonyl fluoride.

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Figure 1 Strategy adopted for the construction and expression of a sub-gene encoding the inner lipoyl domain

(a) The cDNA encoding the entire multidomain human E2p is represented as a bold line. PCR primers P1 and P2 were designed to anneal to regions of the cDNA flanking the coding sequence of the inner lipoyl domain, the limits of which were determined by sequence alignment. The nucleotide sequence of the primers illustrates the engineered restriction sites, which allows subcloning of the PCR product into the prokaryotic expression vector pGEX-2T. As indicated, an additional tetrapeptide was incorporated at the N-terminus of the domain as a result of the cloning strategy employed. A stop codon was incorporated into P2 to direct transcription termination at the end of the lipoyl domain coding sequence. The nucleotide and derived amino acid sequences are numbered in accordance with Coppel et al. [7]. (b) The relevant features of pGEX-2T are indicated. This plasmid directs the synthesis of GST fusion proteins under the control of the IPTG-inducible *lac* promoter (Ptac). Expression of recombinant GST-fusions allows purification from crude lysates by affinity chromatography on immobilized GSH. Illustrated is the engineered nucleotide sequence at the C-terminus of GST, which contains multiple cloning sites, and an additional sequence encoding a thrombin cleavage site, which allows subsequent cleavage of GST from its fusion partner. The *EcoRI*–*Bam*HI restricted PCR product (hatched box) was cloned into the multicloning site of pGEX-2T to produce plasmid pGLIP-2T. Abbreviations: Amp^r, ampicillin-resistance gene; ori, origin; lac I^q, *lac* repressor gene.

in the construction and high-level expression in *E. coli* of a sub-gene encoding the human inner lipoyl domain. A procedure has been devised for purification of the recombinant protein from crude bacterial extracts, and it has been shown that over-expression of the sub-gene generates lipoylated and unlipoylated forms of the intact domain. We also illustrate that the unlipoylated human apo-domain can be lipoylated *in vitro* upon incubation with partially purified *E. coli* lipoate protein ligase.

EXPERIMENTAL

Materials

The cDNA encoding the entire human E2p was kindly donated by Professor M. E. Gershwin, University of California at Davis, CA, U.S.A. The expression vector pGEX-2T was obtained from

Pharmacia–LKB, and the XL1-Blue *E. coli* strain from Stratagene Ltd. Restriction endonucleases and T₄ DNA ligase were purchased from Northumbria Biologicals. Oligonucleotide PCR primers were synthesized on an Applied Biosystems 381A DNA synthesizer. Bovine thrombin was purchased from Calbiochem Corp., lipoic acid from Sigma Chemical Co., and Fluorotrans membrane from Pall Corp.

Construction of expression vector

The strategy adopted for the construction of the vector expressing human E2p lipoyl domain is summarized in Figure 1. By using the cDNA encoding the entire human E2p as a template, the region encoding the inner lipoyl domain was selectively amplified using PCR. The 330 bp PCR product generated was digested with restriction enzymes *EcoRI*/*Bam*HI, and the resulting 321 bp

fragment directionally ligated into the prokaryotic expression vector pGEX-2T [28]. The recombinant plasmid, designated pGLIP-2T, is 5259 bp in size and encodes a recombinant glutathione S-transferase (GST)-lipoyl domain fusion protein. *E. coli* strain XL1-Blue [29] was used as host for the recombinant plasmid. Preparation of the plasmid, PCR techniques, restriction-endonuclease digestion, isolation of DNA fragments, ligation and transformation were carried out by standard methods [30].

Expression of recombinant polypeptides

An overnight culture of *E. coli* XL1-Blue (100 ml) containing either pGEX-2T or pGLIP-2T was inoculated into 1 litre of Luria broth/ampicillin (50 µg/ml) medium. The culture was incubated on a rotary shaker (250 rev./min, 37 °C) for 1 h, then isopropyl β-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM, and the culture was incubated for an additional 4 h. Cells were harvested by centrifugation (10000 g, 10 min at 25 °C) washed with 50 mM Tris (pH 8.0)/25% (w/v) sucrose/10 mM EDTA/0.1 mM phenylmethanesulphonyl fluoride (PMSF) (buffer A) and resuspended in 40 ml of buffer A. Lysozyme was added (80 mg) and cells incubated on ice for 1 h. The bacteria were collected by centrifugation (10000 g, 10 min at 4 °C) and resuspended in 20 ml of 14 mM Na₂HPO₄/8 mM KH₂PO₄, pH 7.0 (buffer B). Bacterial lysis was completed by rapid freezing in liquid nitrogen and thawing twice. The bacterial debris/insoluble fraction was removed by centrifugation (12000 g, 10 min at 4 °C) and the supernatant (lysate) retained.

Purification and cleavage of recombinant fusion proteins

The bacterial lysate was mixed with 5 ml (50%, v/v) of GSH-agarose beads (prepared essentially as in [31]) on a rotating platform, for 1–2 h at 4 °C, to allow coupling of the recombinant fusion protein. After adsorption, the beads were spun down (1000 g, 1 min at 4 °C), washed five times with buffer B and once with thrombin-cleavage buffer [50 mM Tris (pH 8.0)/150 mM NaCl/2.5 mM CaCl₂/0.1% β-mercaptoethanol]. Approx. 6 mg of fusion protein, immobilized on the GSH-agarose, was resuspended in 2 ml of thrombin-cleavage buffer and incubated with 25 units of thrombin (2331 units/mg) at room temperature for 30 min. The supernatant, which contained purified human lipoyl domain, was recovered and stored at –20 °C. Protein was determined by the method of Bradford [32].

PAGE

PAGE was performed in the presence or absence of SDS with a resolving gel containing 15% acrylamide and stacking gel of 5% acrylamide [33]. The gels were stained for protein with Coomassie Brilliant Blue R, after which some were subjected to quantitative densitometric analysis.

Anion-exchange chromatography

Purified lipoyl domain (2 mg), previously dialysed against 10 mM ammonium acetate, pH 5.0, was applied at a flow rate of 1 ml/min to a 1 ml Mono Q column (Pharmacia-LKB), previously equilibrated with 10 mM ammonium acetate, pH 5.0. The column was washed with 20 column vol. of equilibration buffer, followed by 20 column vol. of a linear 10–300 mM ammonium acetate, pH 5.0, gradient. This was followed by 10 column vol. of a further ammonium acetate gradient, 300–

600 mM (based on [34]). Elution was monitored by absorbance at 280 nm, and protein-containing fractions were analysed by non-denaturing PAGE.

M.s.

Samples of lipoyl domain were prepared for analysis by electrospray m.s. by freeze-drying from NH₄HCO₃ and water twice. Samples were redissolved in methanol/water/acetic acid (50:49:1, by vol.) (final protein concentration 10–20 pmol/ml). Mass analysis was performed on a VG BioQ quadrupole mass spectrometer using myoglobin as the calibration standard.

Synthesis of DL-[2-³H]lipoic acid

DL-[2-³H]Lipoic acid (10 mCi/mmol) was prepared by a catalysed-exchange procedure involving metalation of lipoic acid with lithium at C-2, followed by treatment with tritiated water (63 mCi/mmol) ([35,36] and Lester J. Reed, personal communication).

Lipoylation *in vitro* of human apo-domain

Human lipoyl apo-domain (0.25 µg) was incubated with ATP (80 µM), DL-lipoic acid or DL-[2-³H]lipoic acid (60 µM), MgCl₂ (3.2 mM), sodium phosphate buffer, (25 mM, pH 7.0) and partially purified *E. coli* lipoate protein ligase B (specific activity 0.12 nmol of *E. coli* domain modified/min per mg of protein), in a final volume of 30 µl for 6 h at 30 °C [21]. Modified domains were detected by migration on non-denaturing PAGE or by incorporation of DL-[2-³H]lipoic acid, in which case electrophoresed proteins were blotted on to Fluorotrans membrane and exposed to Amersham Hyperfilm ³H.

RESULTS

Lysates from ampicillin-resistant XL1-Blue transformants, carrying plasmids pGEX-2T and pGLIP-2T, were subjected to SDS/PAGE after a standard 4 h induction. Strains harbouring plasmids pGEX-2T and pGLIP-2T over-express polypeptides of *M_r* approx. 27 500 and 39 000 respectively (Figure 2). The *M_r* values of these plasmid-encoded polypeptides are consistent with those of known or calculated values: *M_r* of pGEX-2T-encoded GST is 27 500 [22] and the calculated *M_r* (predicted from the cDNA sequence) of the lipoyl domain plus the additional N-terminal tetrapeptide is 11 840, giving a predicted *M_r* of approx. 39 300 for the GST-lipoyl domain fusion protein. Production of approx. 6 mg of fusion protein per litre of induced culture was consistently obtained.

The procedure devised for purifying the lipoyl domains from crude bacterial extracts of induced cells is outlined in the Experimental section. Yields of 2 mg of pure domain/litre of induced culture were routinely obtained. After thrombin cleavage, the lipoyl domain was subjected to SDS/PAGE and found to be essentially free of contaminating proteins (Figure 2, lane 3) and to have an *M_r* of approx. 11 800. The identity of the domain was verified by immunoblotting against sera from patients with PBC, which are known to contain high-titre autoantibodies against the lipoyl domains of E2p [24,25]. PBC antibodies recognized isolated domains (and GST-lipoyl-domain fusion protein), but not native GST, indicating the antigenicity to be confined to the expressed lipoyl domains (results not shown). Furthermore, automated protein sequence analysis of the domain on a Beckman Porton instrument confirmed that the first 28 residues were identical with those predicted from the cDNA (not shown). However,

Figure 2 Recombinant protein expression and purification

Expression of GST (lane 1) and GST–lipoyl-domain fusion protein (lane 2) from plasmids pGEX-2T and pGLIP-2T respectively. After a 4 h induction, cell extracts were analysed by SDS/PAGE on 15% gels, followed by protein staining with Coomassie Brilliant Blue R. Lane 3 shows purified human lipoyl domain (3 µg). The positions and sizes of M_r markers (lane M) are indicated.

upon Coomassie Blue staining the polypeptide band was somewhat diffuse in nature, indicating possible microheterogeneity.

When samples of the purified preparation were subjected to non-denaturing PAGE, two components were identified (Figure 3, inset), indicating that two forms of domain are generated on over-expression of the sub-gene [17]. These two forms were designated Ulip and Llip to correspond to their upper and lower positions (slower and faster migration) on non-denaturing PAGE. The relative amounts of the domains Ulip and Llip were approx. 3:2 with pGLIP-2T cultures after a standard 4 h induction, as determined by quantitative densitometric analysis.

In order to resolve the two forms of the domain, the mixture was fractionated by chromatography on Mono Q, the elution profile being shown in Figure 3. Two protein peaks were detected. Non-denaturing PAGE of the peak fractions indicated that the domain mixture had been resolved into single forms of the domain (Figure 3, inset). The upper band (Ulip) was consistently eluted ahead of the lower domain (Llip), suggesting that Llip carries an overall slightly higher negative charge under the conditions employed. Electrospray m.s. of the Ulip domain preparation gave a single component with an M_r of 11650 ± 2.0 (S.D.), which corresponds to the mass of the non-lipoylated domain form (calculated M_r 11651.8). Llip consistently gave a noisy spectrum, but a mass of 11851.1 ± 10.7 (S.D.) was obtained, indicating the presence of a single lipoyl residue (M_r 189.3) (results not shown). Further m.s. analysis of a domain preparation from cultures supplemented with lipoic acid gave a major component with an M_r of 11842.5 ± 1.7 (S.D.), as expected of a lipoylated domain form (calculated M_r 11841.1). Two minor components were also detected with masses of 11778.7 ± 1.7 (S.D.), corresponding to an octanoylated domain (calculated mass 11779.0), and 11995.2 ± 2.3 (S.D.). This latter component may correspond to a lipoylated domain form with an additional arginine residue at the N-terminus (calculated M_r 11997.3), indicating incorrect thrombin cleavage of the domain from the GST fusion (see Figure 1). When the Ulip/Llip domain mixture was incubated with [2- 14 C]pyruvate, thiamin pyrophosphate and Elp from human heart, radioactive acetyl groups were incorporated into Llip but not Ulip, as determined by autoradiography (results not shown), showing that Llip (at least in part), but not Ulip, contains a lipoate moiety.

Figure 3 Elution profile of purified domain preparation subjected to f.p.l.c. on a Mono Q anion-exchange column

The domain preparation was fractionated on a Mono Q column, equilibrated with 10 mM ammonium acetate (see the Experimental section). An ammonium acetate gradient (0–0.3 M) eluted two major peaks which were analysed on 15% non-denaturing polyacrylamide gels (inset). The inset shows non-denaturing PAGE of the domain preparation before chromatography (m), protein peak a and protein peak b.

Figure 4 Lipoylation *in vitro* of human apo-domain

(a) Purified apo-domain (lane 2) was incubated with lipoate ligase in the presence of both ATP and [3 H]lipoic acid (lane 3), in the presence of ATP (lane 4) and in the presence of lipoic acid (lane 5). The positions of unlipoylated (U) and lipoylated (L) domains are indicated (lane 1). (b) Corresponding autoradiograph of lane 3.

Further evidence that Ulip and Llip correspond to the non-lipoylated and lipoylated forms of the domain was obtained by incubating Ulip with partially purified *E. coli* lipoate protein ligase. After a 6 h incubation with the lipoate ligase, the majority of Ulip was converted into Llip, as detected by its increased mobility on non-denaturing PAGE. This conversion was dependent on the presence of both ATP and lipoic acid (required for ligase activity), further indicating that it resulted from lipoylation of Ulip (Figure 4a). When lipoylation of Ulip was carried out using [2- 3 H]lipoic acid, radioactivity was detected only in the Llip form (Figure 4b).

DISCUSSION

Milligram amounts of the inner lipoyl domain of the human E2p polypeptide can now be isolated from *E. coli* cells transformed with pGLIP-2T. It is of interest that the *E. coli* cells can correctly lipoylate the human recombinant domain. Indeed, it is the fusion protein that is post-translationally modified. The presence of a non-lipoylated domain is apparently a direct consequence of the amplified rate of domain synthesis exceeding the host cells'

capacity for lipoylation. Cultures supplemented with lipoic acid generate a much higher proportion of the lipoylated form, suggesting that synthesis of lipoic acid is the limiting factor in lipoylation (results not shown).

Similar studies with lipoyl-domain sub-genes of *E. coli* have shown that over-expression leads to the formation of lipoylated and unlipoylated products, except when the domain is rendered unlipoylatable by a lipoyl-lysine → glutamine substitution [17,18]. Lipoylated and unlipoylated forms of the *B. stearothermophilus* lipoyl domain are likewise produced during expression in *E. coli* [19]. In this respect it is of interest that, when the E2 component of the ox liver branched-chain-2-oxo-acid dehydrogenase complex was expressed in *E. coli*, it was apparently unlipoylated [22]. However, incubation of the recombinant protein with ox liver mitochondrial extracts resulted in lipoylation of the E2. The lipoylation of the recombinant human domain is therefore the first report of correct post-translational modification *in vivo* of a eukaryotic substrate by the *E. coli* lipoylating system. Furthermore, the availability of large amounts of human unlipoylated domain, produced in the present study, will permit investigation of the mammalian lipoylation system and of the possible presence of a mammalian lipoamidase.

The ability to over-express and isolate different forms of the human lipoyl domain also offers opportunities for structure determination by two-dimensional ^1H n.m.r. Previous structural studies on the PDC have been hindered by the large size of the complex and its components, resulting in little tertiary-structure data becoming available. A method of circumventing this problem, particularly with E2p, is by structural determination of the individual domains. The availability of homogeneous lipoyl domain from overexpression of the *B. stearothermophilus* subgene has made it possible to tackle the structure by n.m.r. spectroscopy. The 400 MHz ^1H n.m.r. spectra of lipoylated and unlipoylated forms of the domain are essentially identical [17]. Furthermore the secondary structure of the *B. stearothermophilus* lipoyl domain has been determined [37]. Over-expression of the human domain will now permit similar studies on this protein.

The inner lipoyl domains of human E2p has been identified as the major auto-epitope in the autoimmune disease PBC (see above), although at present there is conflicting evidence as to whether lipoic acid is required for antigenicity. Investigations using the recombinant human E2p indicated that site-directed mutagenesis at the lipoate-lysine residue resulted in only minor differences in antibody recognition of the resulting proteins [38]. However, it was acknowledged in that study that the lipoylation state of the 'wild-type' recombinant protein was unknown. In contradiction to this, immunoblotting and e.l.i.s.a. studies on genetically engineered *E. coli* domains indicated that the presence of a lipoyl or octanoyl residue within the domain was crucial for antibody recognition [27]. The availability of both lipoylated and unlipoylated human antigen will help resolve this controversy and, more importantly, permit further studies into the role of the antigen in disease pathogenesis.

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