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Component X of mammalian pyruvate dehydrogenase complex: structural and functional relationship to the lipoate acetyltransferase (E2) component

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The lipoate acetyltransferase (E2, M_r 70 000) and protein X (M_r 51 000) subunits of the bovine pyruvate dehydrogenase multienzyme complex (PDC) core assembly are antigenically distinct polypeptides. However comparison of the N-terminal amino acid sequence of the E2 and X polypeptides reveals significant homology between the two components. Selective tryptic release of the ^{14}C-labelled acetylated lipoyl domains of E2 and protein X from native PDC generates stable, radiolabelled 34 and 15 kDa fragments, respectively. Thus, in contrast to E2 which contains two tandemly-arranged lipoyl domains, protein X appears to contain only a single lipoyl domain located at its N-terminus.

Pyruvate dehydrogenase complex; Component X; Lipoyl domain; Homology; Component E2; (Bovine heart)

1. INTRODUCTION

Mammalian pyruvate dehydrogenase complex (PDC), which catalyses the oxidative decarboxylation of pyruvate, with the formation of acetyl CoA and NADH, is a high M_r assembly of 3 separate enzymes acting in concert. In the native complex, 20–30 $\alpha_2\beta_2$ tetramers of pyruvate dehydrogenase, E1 (EC 1.2.4.1) and 6 homodimers of dihydrolipoamide dehydrogenase, E3 (EC 1.8.1.4) are attached non-covalently to a multimeric core assembly of 60 lipoic acid-containing acetyltransferase (E2) subunits (EC 2.3.1.12), organised in the form of a pentagonal dodecahedron [1,2]. Attention has also focused on a minor core species, protein or component X [3,4], a distinct lipoyl-bearing polypeptide [5] which is tightly-bound to E2 and also participates in the acetylation reactions of the complex [6].

Recent cloning and sequence analysis of the human lipoate acetyltransferase (E2) gene [7,8] in conjunction with earlier protein-chemical studies [9] have demonstrated the presence of two tandemly-repeated lipoyl domains of approximately 125 amino acids in length, located at the N-terminus of the polypeptide. A short, highly conserved E3 binding sequence is found distal to the second lipoyl domain followed by the C-terminal intersubunit binding domain which also houses the acetyltransferase activity. This general structure for E2 was first elucidated for PDC from *E. coli* [10,11] which, however, contains 3 homologous lipoyl domains while the equivalent *S. cerevisiae* enzyme has only a single lipoyl domain [12]. This paper presents evidence that protein X is also a functional variant or isoenzyme of E2 containing a single lipoyl domain.

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2. MATERIALS AND METHODS

PDC was purified according to the method of Stanley and Perham [13] with minor modifications [14]. Buffalo rat liver (BRL), bovine kidney (NBL-1) and porcine kidney (PK-15) cells were purchased from Flow Laboratories, Irvine, Scotland.

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Subunit-specific antisera to gel-purified E2 and X subunits were prepared as described previously [3]. [3-^{14}C]Pyruvate (17.4 mCi/mmol) was obtained from New England Nuclear and [^{125}I]NaI (essentially carrier free) from Amersham. Protein A and trypsin (*N*-tosyl-L-phenylalanine chloromethyl ketone-treated) were from Sigma. Nitrocellulose (0.45 μm pore size) was the product of Schleicher and Schull, Dassel, FRG.

Protocols for routine maintenance of mammalian cell cultures, preparation of cellular and mitochondrial extracts, standard SDS-polyacrylamide gel electrophoresis, immune replica analysis and detection of immune complexes with ^{125}I-labelled protein A have been described [3,14,15]. ^{14}C-labelled peptides were detected by fluorography following the method of Chamberlain [16].

For N-terminal sequencing of E2 and X subunits, purified bovine heart PDC was resolved by SDS-polyacrylamide gel electrophoresis on a 'Mighty Small II' gel apparatus (Hoefer, San Francisco) according to the conditions described in Applied Biosystems User Bulletin no.25. The gel was then blotted onto Immobilon membrane (Millipore, Harrow, Middlesex) as described by Matsudaira [17]. Protein bands were carefully excised with a scalpel and placed in the cartridge block of an Applied Biosystems model 470A protein sequencer equipped with a 120A on-line PTH analyser, using the standard O3R PTH programme.

3. RESULTS

The monospecificity and high-titre of antiserum raised to the gel-purified lipoate acetyltransferase (E2) subunit of bovine heart PDC is demonstrated in fig.1 where immune replica analysis (panel A) is conducted against purified PDC and several cellular and mitochondrial extracts. Variations in the M_r values of the E2 subunit are evident in different cell lines. In particular, the E2 polypeptide in BRL cells (lanes 2–4) has a lower apparent M_r value of 68000 compared to 70000–72000 in bovine and porcine tissues. This finding is in agreement with the subunit molecular masses reported for lipoate acetyltransferase in purified rat liver [18] and bovine heart PDC [3]. No cross-reaction of this anti-serum with the protein X polypeptide was observed in any cell line.

In fig.1 (panel B) the reactivity of anti-X serum is illustrated against bovine heart PDC (lane 1) and whole cell (lane 2), mitochondrial (lane 5), post-nuclear supernatant (lanes 3 and 4) and nuclear fractions (lane 6) derived from BRL cells. It is evident that the antibody to component X shows a strong positive cross-reaction with an unidentified 48 kDa species which is not present in either nuclear or mitochondrial fractions. Its exact subcellular location has not yet been determined. In mitochondrial extracts, however, this antibody reacts exclusively with a 51 kDa component corresponding to protein X. Once again, anti-X IgG failed to recognise the E2 component in these cell cultures.

In fig.2 (panel A) pure bovine heart PDC, preincubated with [3-^{14}C]pyruvate in the absence of CoA to promote [^{14}C]acetylation of lipoyl groups on the proteolytically-sensitive E2 and X subunits, was then subjected to tryptic digestion for the indicated times to promote selective release of the ^{14}C-labelled lipoyl domains. Specific proteolysis of the [^{14}C]acetylated domains is observed with protein X degrading rapidly to produce a unique, stable, radiolabelled 15 kDa peptide (panel A), as confirmed by immune replica analysis (panel C). In contrast, E2 is cleaved more slowly via a transient ^{14}C-labelled 45 kDa species, finally generating a stable 34 kDa lipoyl peptide (panel A). Verification that these polypeptides were tryptic fragments of E2 and X, respectively, was again achieved by immunoblotting with subunit-specific antisera (panel B). A similar pattern of E2 and X-derived cleavage products could be obtained with several specific (e.g. V-8 protease) or non-specific (e.g. papain, elastase) proteases (not shown).

Table 1 compares the N-terminal amino acid sequences of the bovine heart E2 and protein X polypeptides with the equivalent primary structures of the human [7,8], rat [19] and *S. cerevisiae* [12] lipoate acetyltransferases derived from determination of the nucleotide sequences of the cloned genes. Protein X exhibits significant homology with all eukaryotic lipoate acetyltransferases examined to date, particularly evidenced in a highly conserved PS/ALSPTM sequence commencing at residues 9–12 from the N-terminus or at equivalent positions in the internally repeated lipoyl domains of the human and rat enzymes. There are two other positions in which complete identity is retained in all cases involving an invariant proline near the N-terminus and a glycine located in the C-terminal region of this peptide sequence. Interestingly, a conserved histidine at position 3 or 4, present in all these lipoate acetyltransferases including the yeast enzyme, is absent in protein X, as is a conserved N-terminal serine (residue 1 or 2).

The corresponding stretches of amino acid sequence from the N-terminal and internally repeated lipoyl domains of human E2 reveal sequence identity in 15 out of 24 residues while pro-

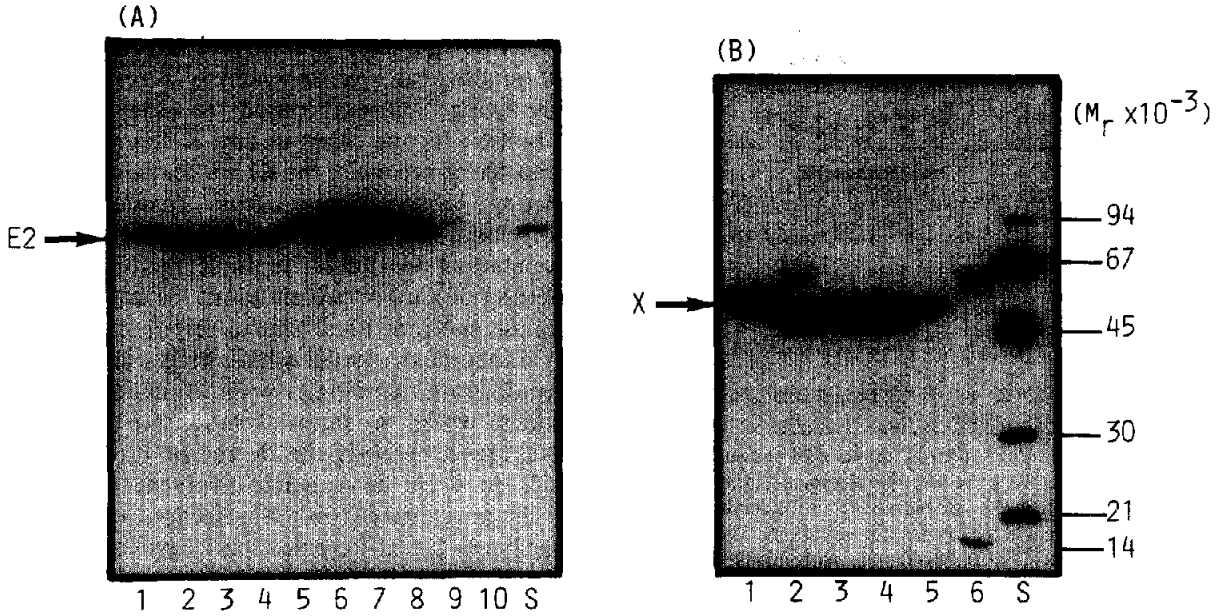

Fig.1. Immunoblotting analysis of lipoate acetyltransferase (E2) and protein X in various cellular extracts. Purified PDC and various cellular extracts were resolved by electrophoresis on 10% (w/v) SDS-polyacrylamide gels. Gels were used for immunoblotting analysis (see section 2 for details) with antibody raised against the E2 subunit (panel A) or protein X subunit (panel B). (A) Lanes: 1 and 5, 0.1 μg PDC; 2, SDS extract of BRL cells, 80 μg; 3, BRL mitochondria, 40 μg; 4, rat liver mitochondria, 40 μg; 6, ox heart mitochondria, 15 μg; 7, SDS extract of NBL-1 cells, 80 μg; 8, SDS extract of PK-15 cells, 80 μg; 9, PK-15 mitochondria, 20 μg; 10, PK-15 post-mitochondrial supernate, 60 μg; S, ^{125}I-labelled molecular mass marker, bovine serum albumin. (B) Lanes: 1, 0.5 μg purified PDC; 2, SDS extract of BRL cells; 3 and 4, post-nuclear supernate, 50 and 60 μg; 5, BRL mitochondria, 40 μg; 6, nuclear fraction, 40 μg; S, ^{125}I-labelled molecular mass markers.

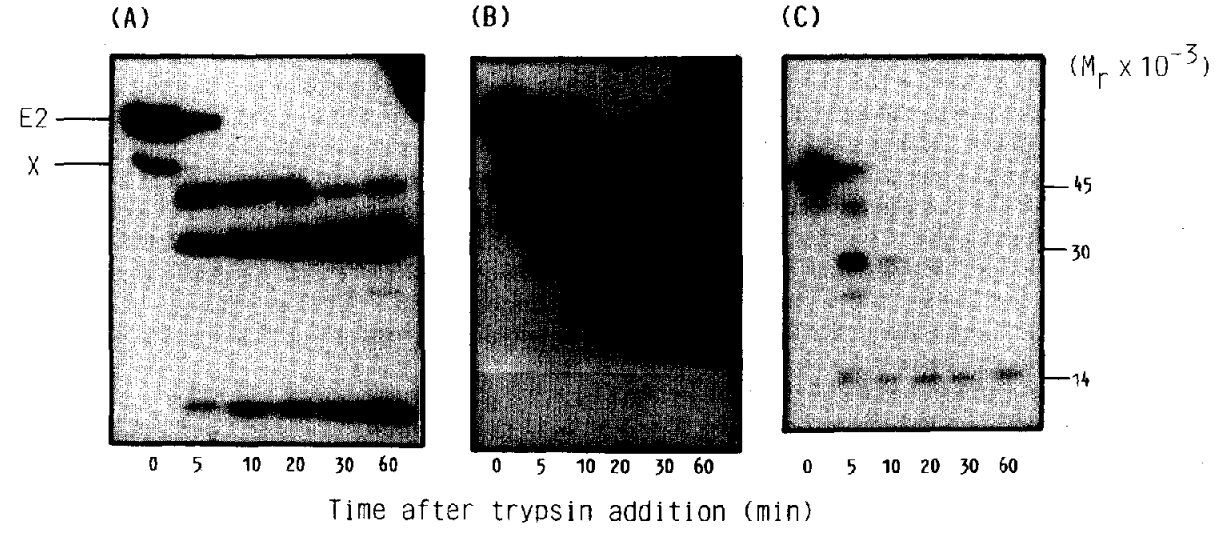

Fig.2. Selective tryptic release of [^{14}C]acetylated lipoyl domains from E2 and X polypeptides of native bovine heart PDC. Purified bovine heart PDC (500 μg) was preincubated for 15 min with non-radioactive (panels B and C) or [3-^{14}C]pyruvate (panel A) in the presence of 0.5 mM *N*-ethylmaleimide. Excess *N*-ethylmaleimide was reacted with 45 mM 2-mercaptoethanol before treatment of radiolabelled enzyme with 1% (w/w) trypsin at 25°C for the indicated times. Samples (30 μg) were removed, immediately treated with Laemmli sample buffer (2 ×) and subjected to fluorography following resolution on 12.5% (w/v) SDS-polyacrylamide gels (panel A) [^{14}C]acetylated peptides; panels (B&C) immunoblots of parallel samples with anti-E2 and anti-X sera, respectively.

Table 1

Comparison of N-terminal amino acid sequences of protein X and various lipoate acetyltransferases (E2) from eukaryotic sources

Source of Enzyme	Amino Acid Sequence
Bovine heart E2	S L P P H Q K V P L P S L S P T M Q A G T I A -
Bovine heart protein X	A D P I - - K I L M P S L S P G T M E E G N I (V) (K)
Human liver E2 (a) (7,8)	S L P P H Q K V P L P S L S P T M Q A G T I A R
E2 (b)	S Y P P H M Q V L L P A L S P T M T M G T V Q R
Rat liver (19) E2 (b)	S Y P V H M Q I V L P A L S P T M T M G T V Q R
S.cerevisiae (12) E2	A S Y P E H T I I G M P A L S P T M T Q G M L A A

Bovine heart E2 and protein X amino acid sequences were determined directly while the N-terminal human liver, E2(a), rat liver and yeast sequences were derived from the corresponding nucleotide sequences in the indicated publications. The primary structure of protein X is continuous with blank positions present only to optimise sequence alignment. For human and rat liver enzymes the corresponding internal repeat sequences, E2(b), at the start of the second lipoyl domain are included for comparison. Regions of complete or near complete sequence identity are boxed as shown. Amino acids given in parentheses have only been identified tentatively

tein X exhibits lower homology (10 out of 22 residues) with the equivalent N-terminal region from bovine heart E2. The N-terminal primary structures of human and bovine heart E2 are identical over the first 22 amino acids. Heterogeneity was also observed at residue 13 in the protein X sequence where both proline and glycine were found in comparable yields. During preparation of this manuscript, a similar, but not identical sequence, was published for the N-terminal region of protein X from bovine kidney [20]. Considerable sequence variability was noted amongst this group of enzymes outwith the highly conserved regions as evidenced by alterations in sequence length and the presence of many conservative and several non-conservative substitutions, notably in protein X which has several unique features e.g. two consecutive glutamic acid residues at positions 16 and 17.

4. DISCUSSION

4.1. *Immunology of protein X and lipoate acetyltransferases (E2)*

In view of the striking homology (45% identity) in the N-terminal sequences of the bovine heart E2 and X polypeptides, it may seem surprising that no immunological cross-reactivity is apparent employing antisera raised to the individual subunits. In one case, an anti-X serum has been reported to recognise the E2 enzyme also [4]. Conflicting observations occur frequently with polyclonal sera where the characteristics of the IgG population depend on multiple factors, e.g. the method of antigen presentation, the immunisation regime and individual animal responses.

Immunological studies have been widely used to demonstrate homologies within families of isoenzymes where a linear relationship has been established between the degree of cross-reaction and the extent of sequence divergence. However, systematic analysis of a selection of purified lysozymes [21,22] revealed that enzymes exhibiting less than 60% homology did not usually show an immunological cross-reaction in precipitation assays or by microcomplement fixation.

Antibodies to native proteins are primarily against non-contiguous epitopes (three-dimensional determinants) on the surface of the polypeptide while, for denatured antigen, linear elements of sequence (5–8 amino acids) are responsible for eliciting the major antibody response. If the common sequences are highly conserved e.g. the PS/ALSPTM sequence in E2 and X they may be very weak immunogens (see [14]) accounting for the lack of cross-reaction between these two homologous proteins. The absence of cross-reaction may suggest that the E2 and X polypeptides are encoded by separate genes rather than products arising from alternate exon splicing of a single genomic copy. In the latter case, the presence of long stretches of identical sequences in common exon segments should lead to immunological cross-recognition by anti-E2 or anti-X sera.

4.2. *Organisation, origins and function of protein X*

Limited amino acid sequence data on bovine heart protein X indicate that this protein contains a lipoyl domain located at its N-terminus and belongs to a family of homologous enzymes, all the other members of which possess lipoate acetyltransferase activity. The heterogeneity at position 13 in the protein X sequence where both

proline and glycine were present, probably represents separate allelic forms of the enzyme as the PDC was purified from a single beef heart.

There are several reasons for suggesting that, in contrast to bovine E2, protein X contains only a single lipoyl domain: (i) selective release of lipoyl domains from E2 and X by limited proteolysis with a variety of specific and non-specific proteases producing stable peptides, containing all the [^{14}C]acetylation sites, in the 32–42 kDa range for E2 and 15–20 kDa range for protein X; (ii) the subunit molecular mass of protein X, 51 kDa as opposed to 70 kDa for E2 is best accounted for by the absence of an approx. 125 amino acid lipoyl domain from protein X. These domains are known to migrate anomalously on SDS-polyacrylamide gels owing to the presence of Ala-Pro-rich linker regions [11]; and (iii) specific cross-linking studies on E2 and X with phenylene-*o*-bismaleimide via substrate-generated thiols on lipoic acid groups has provided evidence for the presence of two lipoyl groups per E2 chain and only a single lipoate on protein X [9].

Both E2 and protein X participate in the acetylation reactions of the complex [6] and apparently act as independent substrates for reductive acetylation by pyruvate dehydrogenase (E1) [23]. The inability to dissociate protein X from the E2 core assembly except under denaturing conditions also highlights the close physical and functional integration of these two components [3]. Indeed, protein X contains a C-terminal domain, equivalent to the intersubunit binding domain of E2, and may be an effective replacement for E2 within the core structure. A recent report [20] also claims that protein X is involved in binding dihydrolipoyl dehydrogenase (E3) and thus may also possess an E3 binding region which is highly conserved in both prokaryotic and eukaryotic PDCs [8]. An intriguing question, therefore, currently under investigation, relates to the precise role of component X: (i) is it an isoenzyme of the major lipoate acetyltransferase arising by gene duplication and independent evolution but with no distinctive biochemical function, or (ii) is it a variant of E2 performing an essential structural or mechanistic role within the multimeric core assembly? Cloning and sequence analysis of the protein X gene will be an important first step in endeavouring to answer these questions.

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