Biosynthesis, import and processing of precursor polypeptides of mammalian mitochondrial pyruvate dehydrogenase complex

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An immunological analysis has been conducted of early events in the biosynthesis, import and assembly of the mammalian pyruvate dehydrogenase complex (PDC). For this purpose, monospecific polyclonal antisera were produced against the intact assembly from ox heart, M_r 8.5×10^6 , and each of its component polypeptides, E1 α , E1 β , E2, E3 and protein X. Optimal detergent-based incubation mixtures were developed for obtaining clean immunoprecipitation of PDC polypeptides and their precursors from [35 S]methionine-labelled extracts of PK-15 (pig kidney), NBL-1 (bovine kidney) and BRL (Buffalo Rat liver) cells. In PK-15 cells, independent higher M_r species, corresponding to precursors of the E2, E1 α and E1 β subunits of PDC, could be detected by immune precipitation and fluorography after incubation of intact cells for 4 h with [35 S]methionine and 1–2 mM 2,4-dinitrophenol or 10–15 μ M carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. Similar precursor states could be observed in uncoupler-treated BRL or NBL-1 cells. Pre-E1 α , pre-E1 β and also pre-E3, have signal sequences in the M_r range 1500–3000 while pre-E2 contains a long additional segment of M_r 7000–9000. All of these forms exhibit similar kinetics of processing to the mature subunits with a transit time of 10–12 min. In NBL-1 cells, E3 is present in the immune complexes formed with anti-PDC serum whereas this is not the case in PK-15 cells. Thus, there are significant variations in the affinity of lipoamide dehydrogenase (E3) for the E2 core structure in different species. Pre-E1 α accumulates only poorly in PK-15 cells and is aberrantly processed on removal of uncoupler. This precursor is markedly more stable in NBL-1 and BRL cells. The lack of detection of a precursor form of component X is also discussed.

INTRODUCTION

The initial reaction of the tricarboxylic acid cycle requires the conversion of pyruvate, on its entry into mitochondria, to acetyl-CoA for subsequent condensation with oxaloacetate, the end product of the cycle. This important step is regulated by the pyruvate dehydrogenase multi-enzyme complex (PDC), a high M_r assembly [(7–8.5) $\times 10^6$; approximately the size of a mammalian ribosome], which is situated in the matrix inner-membrane compartment of the organelle (Reed & Pettit, 1981; Yeaman *et al.*, 1986).

Despite its high overall M_r value, PDC has a relatively simple polypeptide composition, comprising only three separate enzymes which are present in multiple copies in the intact complex. An additional polypeptide, component X (M_r 50000), has been described recently and may play an active role in the regulation of the complex (De Marcucci & Lindsay, 1985; De Marcucci *et al.*, 1986a; Rahmatullah *et al.*, 1986). In ox heart, there are apparently 30 $\alpha_2\beta_2$ tetramers of pyruvate dehydrogenase (E1) (EC 1.2.4.1) and six homodimers of lipoamide dehydrogenase (E3) (EC 1.8.1.4) associated with the central core of the complex, a multimeric aggregate of 60 lipoate acetyltransferase (E2) (2.3.1.12) molecules, organized in the form of a pentagonal dodecahedron. After the initial decarboxylation of substrate, E1 also promotes transfer of two carbon units via hydroxyethylthiamine diphosphate to covalently bound

lipoic acid groups on the E2 core, producing an *S*-acetyldihydrolipoamide intermediate before final transfer to CoASH. Reducing equivalents from dihydrolipoamide are transferred to NAD $^+$ by the action of E3, an FAD-linked enzyme. Thus a total of five co-factors and prosthetic groups are involved in catalysing the complete reaction.

The small size of the mitochondrial genome in higher animals, sufficient to code for only a dozen polypeptides, means that the vast majority of mitochondrial proteins, including the constituent polypeptides of PDC, must be nuclear-coded with a site of synthesis on cytoplasmic ribosomes (Hay *et al.*, 1984; Reid, 1985). Rapid advances in our understanding of the import mechanisms employed by cytoplasmically synthesized polypeptides have established many of the general features of the process: (a) most proteins destined for this organelle appear transiently as higher M_r cytosolic precursors with *N*-terminal extensions of variable length; (b) this transit peptide is required to direct the precursor to the mitochondrion where it is cleaved by a specific matrix protease during or shortly after entry; (c) normal uptake is dependent on the electrochemical gradient ($\Delta\mu_{H^+}$) across the mitochondrial inner membrane which can be abolished by uncouplers of oxidative phosphorylation. The essential features of signal sequences which are required for correct targeting to the organelle and its sub-compartments form an area of active investigation at present (Hurt *et al.*, 1985).

Abbreviations used: 2,4-DNP, 2,4-dinitrophenol; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; PDC, pyruvate dehydrogenase complex; OGDH, 2-oxoglutarate dehydrogenase complex.

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In this communication, we report: (a) optimal conditions for accumulation and immunoprecipitation of precursor forms of PDC subunits; (b) an analysis of their kinetics of processing to the mature states; (c) comparative aspects of the characteristics of PDC precursors from different cell lines and with their counterparts from the analogous 2-oxoglutarate dehydrogenase complex (OGDC).

MATERIALS AND METHODS

Bovine kidney (NBL-1) cells, pig kidney (PK-15) cells and Buffalo Rat liver (BRL) cells were all purchased from Flow Laboratories. Cells were passaged routinely in Glasgow-modified Eagle's medium supplemented with 10% (v/v) newborn-calf serum (for BRL cells) and 5% or 10% (v/v) fetal-calf serum (for PK-15 and NBL-1 cells, respectively). Methionine-free medium for isotopic incorporation studies was obtained from Gibco-BRL (Paisley, U.K.). Pansorbin, a 10% (w/v) suspension of formalinized *Staphylococcus aureus* cells, Cowan 1 strain, standardized for IgG binding capacity, was the product of Calbiochem-Behring.

Protein A was obtained from Sigma and low- M_r marker proteins from Bio-Rad Laboratories. Lipoamide dehydrogenase, E3, from pig heart was commercially available from Calbiochem-Behring.

L-[35 S]Methionine (1330 Ci/mmol) and Na 125 I (carrier-free) were purchased from Amersham International. *N*-[2- 3 H]Ethylmaleimide was the product of New England Nuclear. X-ray film, either X-Omat S or X-Omat XAR 5, was available from Kodak (U.K.). Lightning Plus intensifying screens were the product of MAS (Stirling, U.K.) and film cassettes were obtained from Sky Plastics (Sutton-in-Ashfield, Notts., U.K.).

Cycloheximide, phenylmethanesulphonyl fluoride, *p*-aminobenzamidine, 1,10-phenanthroline, leupeptin and 2,4-dinitrophenol (2,4-DNP) were from Sigma. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) came from Aldrich. IodogenTM (1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycouril) was bought from Pierce and Warriner (Chester, U.K.).

Preparation of PDC and component polypeptides

PDC from bovine heart was purified to near homogeneity ($\geq 95\%$) essentially as described by Stanley & Perham (1980) with some minor modifications (De Marcucci *et al.*, 1985). Enzymic activity was assayed by following NADH formation at 340 nm (Brown & Perham, 1976). Protein was measured by a modified Lowry procedure with bovine serum albumin as standard.

For preparation of the E2-X subcomplex, and the combined E1 plus E3 components, PDC (50 mg) was dissociated in 1 M-NaCl/1 mM-MgCl₂/2 mM-dithiothreitol/0.1 mM-EDTA/0.1 M-glycine buffer, pH 9.0, before gel-filtration chromatography on a Sepharose CL-6B column (80 cm $\times$ 1.5 cm), basically as described by Linn *et al.* (1972). Peak 2 from this step was fractionated further by (NH₄)₂SO₄ precipitation to yield pyruvate dehydrogenase, E1, collected at 30% (w/v) saturation and a supernatant fraction containing most of the lipoamide dehydrogenase. E1 (5 mg) was separated into its α and β subunits by resolution on phosphocellulose in 8 M-urea/0.05 M-2-mercaptoethanol/0.02 M-imidazole/HCl buffer, pH 6.0, according to Barrera *et al.* (1972). Component X was purified by elution of this

polypeptide from SDS/polyacrylamide gels (De Marcucci *et al.*, 1986a). In all cases, isolated polypeptides were purified to near homogeneity ($\geq 98\%$) as estimated by densitometric scanning of Coomassie Blue-stained 10% (w/v) SDS gels.

SDS/polyacrylamide-gel analyses, fluorographic and immunological procedures

SDS/polyacrylamide-gel electrophoresis and fluorographic analyses were performed on 10% (w/v) slab gels basically employing the Laemmli system. Fluorographic detection of [35 S]methionine-labelled polypeptides was achieved by the method of Chamberlain (1979).

Full details of the methodology for production of antisera to intact PDC, OGDC and the individual denatured subunits have been reported previously as has the protocol for immunoprecipitation of radiolabelled antigens (De Marcucci & Lindsay, 1985; Hunter & Lindsay, 1986). Optimal conditions for immunoprecipitation of PDC or individual subunits were determined in pilot studies employing (a) 0.5 μ g of purified *N*-[2- 3 H]-ethylmaleimide-modified PDC or (b) [35 S]methionine-labelled cellular extracts in 1% (v/v) Triton X-100/1% (w/v) sodium deoxycholate/0.5% (w/v) SDS/0.1 M-KCl/5 mM-MgCl₂/0.1 M-Tris/HCl buffer, pH 8.2, which contained 10⁷ d.p.m. per incubation. In both cases quantitative (88–95%) immunoprecipitation of PDC subunits occurred as judged by the complete disappearance of the appropriate polypeptide(s) from the supernatant fraction (a) or lack of detection of PDC components in a second precipitation of the original samples (b). The preparation of *N*-[2- 3 H]ethylmaleimide-labelled PDC (E1 α labels prominently) for M_r estimations has also been published elsewhere (De Marcucci & Lindsay, 1985).

For pulse-chase experiments, 2,4-DNP or FCCP was present in the cultures during the 4 h initial incubation with [35 S]methionine. At this stage, medium was removed and replaced with warm, standard, growth medium for varying times. At the indicated times, cell monolayers were washed rapidly in ice-cold phosphate-buffered saline (0.15 M-NaCl/20 mM-sodium phosphate buffer, pH 7.4) to inhibit processing before harvesting immediately in 1% (v/v) Triton X-100/5 mM-MgCl₂/0.1 M-KCl/Tris/HCl buffer, pH 8.2, containing 4 mM-1,10-phenanthroline/0.4 mM-phenylmethanesulphonyl fluoride/0.3 μ M-leupeptin. These proteinase inhibitors were present in all subsequent steps during the immunoprecipitation procedure.

Measurement of [35 S]methionine incorporation into total cellular protein

Duplicate petri dishes (3 cm) containing PK-15 cells (2×10^5 cells/ml) were incubated overnight at 37 °C in normal growth medium before addition of 2,4-DNP or FCCP at the indicated concentrations plus 5 μ Ci of [35 S]methionine for a 4 h period. After this time, the radioactive medium was rapidly removed, cell monolayers rinsed three times in 0.15 M-NaCl/20 mM-sodium phosphate buffer, pH 7.4, and then similarly with 10% (w/v) trichloroacetic acid to release acid-soluble pools. Cellular protein was dissolved overnight at 4 °C in 0.1 M-NaOH. After neutralization, 0.5 ml portions were mixed with 5 ml of scintillation fluid consisting of 35% (v/v) Triton X-100 and 5 g of 2,5-diphenyloxazole/l in toluene before assay on a Beckman IS 6800 scintillation counter.

Fig. 1. Effects of FCCP and 2,4-DNP on [35 S]methionine incorporation into total cellular protein or mitochondrially coded proteins in cultured mammalian cells

PK-15 cells at an initial inoculum of 2×10^5 cells/ml were then pre-treated for 5 min with varying concentrations of FCCP (●) or 2,4-DNP (○) as indicated before adding 5 μ Ci of [35 S]methionine to each dish and continuing cell growth for a further 4 h at 37 °C. Incorporation of [35 S]methionine into cell protein was measured as described in the Materials and methods section. Samples were analysed in triplicate with each dish varying by less than $\pm 10\%$. For analysis of mitochondrially coded proteins in BRL cells (3×10^5 /ml, inset) normal medium was replaced by warm, low-methionine medium (see Materials and methods section) 2 h before supplementing the medium with inhibitors of uptake/processing of nuclear-coded mitochondrial polypeptides and 250 μ Ci of [35 S]methionine. After a 4 h incubation at 37 °C samples were analysed on 10% (w/v) SDS/polyacrylamide gels and mitochondrially synthesized proteins detected by fluorography as described in the Materials and methods section. Lane 1, cycloheximide (1 mg/ml) + 1 μ M-FCCP; lane 2, cycloheximide (1 mg/ml) + 10 μ M-FCCP; lane 3, cycloheximide (1 mg/ml) + 0.3 mM-2,4-DNP; lane 4, cycloheximide (1 mg/ml) + 2 mM-2,4-DNP; lane 5, cycloheximide (1 mg/ml); lane 6, cycloheximide (1 mg/ml) + chloramphenicol (300 μ g/ml).

RESULTS

Initial efforts were directed towards the establishment of optimal conditions for precursor detection in cultured PK-15 or NBL-1 cells (see also Hunter & Lindsay, 1986). As shown in Fig. 1, the effects of varying concentrations of the uncouplers, 2,4-DNP or FCCP, on the rate of [35 S]methionine incorporation into PK-15 cells is measured to determine their general effects on protein synthesis. Pronounced inhibition of [35 S]methionine incorporation into total cellular protein occurs at concentrations above 20 μ M-FCCP or 2 mM-2,4-DNP. An SDS/polyacrylamide-gel analysis is shown in Fig. 1 (inset) of the biosynthesis of the major translation products of the mitochondrial genome which are charac-

Fig. 2. Identification of mature subunits of PDC in NBL-1 cells by immunocompetition

Semi-confluent monolayers of NBL-1 cells were labelled for 18 h in 8.5 cm petri dishes with 100 μ Ci of [35 S]methionine per dish. Cells were disrupted and portions of the extracts (10^7 d.p.m.) were subjected to immunoprecipitation with 10 μ l of anti-PDC serum as described in the Materials and methods section. Before addition of the antiserum, various components of PDC were added at the levels indicated below. Radiolabelled PDC components present in the immune complexes were adsorbed onto *S. aureus* cells and analysed by fluorography after resolution on 10% (w/v) SDS/polyacrylamide gels. Lane S, 3 H-labelled PDC (E2, E3, E1 α and E1 β subunits with respective M_r values of 70 000, 55 000, 42 000 and 36 000) as markers; lane 1, anti-PDC immunoprecipitate with no additions; lane 2, plus 5 μ g of PDC; lane 3, plus 5 μ g of component X; lanes 4 and 5, plus 2.5 μ g and 5 μ g of E1 respectively; lanes 6 and 7, plus 5 μ g and 10 μ g of pig heart E3, respectively; lane 8, plus 5 μ g of E1 β subunit; lanes 9 and 10, plus E1 component, 6 μ g and 12 μ g, respectively; lane 11, plus 10 μ g of E2-X subcomplex.

terized by their resistance to cycloheximide, a potent inhibitor of protein synthesis on cytoplasmic ribosomes (Mason & Schatz, 1974). Synthesis of this class of mitochondrial protein is indirectly dependent on the electrochemical-potential gradient ($\Delta\psi$) across the inner mitochondrial membrane which is required for intramitochondrial ATP production. The capacity of 2,4-DNP and FCCP to abolish $\Delta\psi$ is demonstrated by their specific inhibitory effects on mitochondrial protein synthesis. These levels were chosen for subsequent studies on precursor accumulation since they arrest mitochondrial protein synthesis without inducing a dramatic decline (20–40%) in the rate of [35 S]methionine uptake into total cellular protein.

Fig. 2 illustrates the identification of the mature [35 S]methionine-labelled polypeptides following immunoprecipitation of PDC in detergent extracts of NBL-1 cells. Maximal immunoprecipitation with low levels of background contamination was observed in a complex detergent mixture containing 5 mM-MgCl₂/0.1 M-KCl/0.1 M-Tris/HCl (pH 8.2)/1% (v/v) Triton X-100/1% (w/v) deoxycholate/0.5% (w/v) SDS. This incubation mixture has two further advantages, stemming from the observation in trial immunoprecipitations of pure *N*-[3 H]ethylmaleimide-modified PDC, that this detergent system promotes only partial dissociation of the complex. Such dissociation was

Fig. 3. Immunological detection of higher M_r precursor states of individual PDC subunits in NBL-1 and PK-15 cell lines

Semi-confluent monolayers of NBL-1 cells (a) or PK-15 cells (b) were labelled, normally for 4 h in low methionine medium plus 200 μ Ci of [35 S]methionine in the presence or absence of FCCP (a) or 2,4-DNP (b), as indicated. Immune precipitation with anti-PDC serum, electrophoretic analysis of dissociated immune complexes and fluorography were performed as described earlier. (a), (b) Lane S, 3 H-labelled PDC (E1 α subunit, M_r 42000 only); lane C, immunoprecipitate with control serum. (a) Lane 1, anti-PDC immunoprecipitates from cells labelled for 4 h without uncouplers; lanes 2 and 3, immunoprecipitates from NBL-1 labelled for 4 h in the presence of 5 μ M- or 10 μ M-FCCP respectively; lane 4, as lane 3 after 40 min chase minus FCCP. (b) Lanes 1 and 2, anti-PDC immunoprecipitate from PK-15 cells labelled for 4 h or 18 h with [35 S]methionine without uncouplers; lanes 3 and 4, immunoprecipitates after 4 h incubation plus 1 mM- or 2 mM-2,4-DNP respectively; lanes 5 and 6, as lanes 3 and 4 plus 40 min chase in normal non-radioactive medium in the absence of 2,4-DNP.

necessary for detection of individual polypeptides with subunit-specific antisera (see Fig. 4) which were incapable of inducing precipitation of intact, native PDC; however, the presence of fully or partially assembled PDC also permits the analysis of polypeptides which are immunoprecipitated by virtue of their association *in vivo* with the complex.

Characterization of the four polypeptides present in the final immune complex as the E2, E3, E1 α and E1 β subunits of PDC was achieved by immunocompetition studies in which radiolabelled antigens were competed out with excess highly purified ox heart complex, E2-X

subcomplex, native E1 or E3, E1 β subunit or component X. Lane 2 of Fig. 2 demonstrates initially that the four major immunoprecipitated polypeptides of PDC could be competed out with excess (5 μ g) purified complex. Immunocompetition analysis with specific polypeptide antigens of PDC [native E1 (2.5, 5.0, 6.0 and 12.0 μ g, lanes 4, 5, 9 and 10, respectively); E1 β (5.0 μ g, lane 8); E3 (5 and 10 μ g, lanes 6 and 7, respectively) and E2-X subcomplex (10 μ g, lane 11)] confirms the identity of the individual subunits of the enzyme. The E2 component (lane 11), was found to be competed out only poorly unless larger amounts of competing antigen (25–50 μ g) were employed (not shown).

Two important differences were observed in comparing these immunoprecipitation profiles with earlier immunoblotting analysis of NBL-1 cell extracts using the same anti-PDC serum (De Marcucci & Lindsay, 1985). Firstly, no 50000- M_r species corresponding to protein X could be detected in immunoprecipitates although immune replicas indicate the presence of a high titre of antibody against this component. The absence of protein X was confirmed by the inability of excess antigen to affect the immunoprecipitation of any of the four major subunits (Fig. 2, lane 3). Secondly, the presence of the E3 subunit, as confirmed by immunocompetition analysis (Fig. 2, lanes 6 and 7), was unexpected since it has been reported previously (De Marcucci & Lindsay, 1985) that an extremely low titre of antibody is elicited against lipoamide dehydrogenase relative to the other constituent polypeptides of PDC.

Fluorographic detection of higher M_r precursor states of individual PDC subunits in NBL-1 and PK-15 cell lines is illustrated in Figs. 3(a) and 3(b), respectively. Inhibition of processing to the mature forms was attempted by a 4 h incubation with 5–10 μ M-FCCP in NBL-1 cells (Fig. 3a) or 1–2 mM-2,4-DNP in PK-15 cultures (Fig. 3b), both in the presence of [35 S]-methionine. There are several noteworthy points in these results: (a) Standard immune precipitation of the mature PDC polypeptides from PK-15 cells yields, in contrast to NBL-1 cells, only three major components corresponding to the E2, E1 α and E1 β subunits. (b) Incubation of cultures for 4 h plus FCCP or 2,4-DNP leads to the accumulation of stable, higher M_r forms as evidenced by their slower migration on SDS/polyacrylamide gels (Fig. 3a, lane 3; Fig. 3b, lanes 3 and 4). (c) The level of uncouplers can markedly influence the accumulation of precursors over a narrow range of concentrations. In Fig. 3(a), FCCP (5 μ M, lane 2) causes no accumulation of higher M_r species while complete inhibition of processing is achieved with 10 μ M-FCCP (Fig. 3a, lane 3). Similarly, 2,4-DNP (1 mM) induces partial inhibition in PK-15 cells (Fig. 3b, lane 3) leading to the simultaneous appearance of both precursor and mature states while incubation with 2,4-DNP at 2 mM is sufficient to achieve complete inhibition (Fig. 3b, lane 4). (d) The reversible mode of action of both uncouplers is confirmed by placing inhibitor-treated cultures in fresh non-radioactive medium for a further 40 min before immune precipitation (Fig. 3a, lane 4; Fig. 3b, lanes 5 and 6). Complete conversion to mature-sized subunits occurs in this time, confirming the precursor-product relationship between the two separate states of each polypeptide. (e) While mature E3 is readily detectable in immune complexes from NBL-1 cells (Fig. 3a, lanes 1 and 4), no significant immunoprecipitation of

Fig. 4. Immunoprecipitation of precursor and mature forms of the E1 α , E1 β and E2 polypeptides with subunit-specific antisera

Semi-confluent monolayers of BRL cells (a) or NBL-1 cells (b and c) were labelled with [35 S]methionine in the presence or absence of 2,4-DNP or FCCP as indicated and cell extracts were processed for fluorography (see Fig. 2 for details) after immunoprecipitation with anti-E1 α (a), anti-E1 β (b) or anti-E2 specific (c) sera. Lane S (a and b), 3 H-labelled ox heart PDC as marker showing E2, E3, E1 α and E1 β subunits with M_r values of 70000, 55000, 42000 and 36000 respectively; lane C (b and c) immunoprecipitates with control serum. (a) Lanes 1 and 4, anti-E1 α immunoprecipitate from cells incubated with [35 S]methionine for 4 h or 18 h, respectively; lanes 2 and 3, as lane 1 except that cells were incubated in the presence of 2 mM- or 1 mM-2,4-DNP, respectively. (b) Lane 1, anti-PDC immunoprecipitate after 4 h incubation of NBL-1 cells with [35 S]methionine; lane 2, as lane 1 except that 5 μ g of purified E1 β was added before antiserum; lane 3, anti-E1 β immunoprecipitate after 4 h labelling of cells treated with 2 mM-2,4-DNP; lane 4, as lane 3 after a 30 min chase in non-radioactive medium minus uncoupler. (c) Lane 1; anti-PDC immunoprecipitate from NBL-1 cells labelled for 4 h minus uncoupler; lane 2, as lane 1 using anti-E2 serum; lane 3, as lane 2 except for the presence of 2 mM-2,4-DNP during the incubation; lane 4, as lane 2 after 40 min chase period minus uncoupler.

a band corresponding to pre-E3, M_r 56 500, (De Marcucci *et al.*, 1986b; J. G. Lindsay, unpublished observations) is seen in uncoupler-treated cultures (Fig. 3a, lane 3).

Unambiguous assignment of the higher M_r species observed in Fig. 3 as precursors to individual subunits of PDC was attempted by a similar immunoprecipitation study with subunit-specific antisera raised against gel-purified E1 α , E1 β and E2 polypeptides (Fig. 4). In the case of pre-E1 α in BRL cells (Fig. 4a), a single polypeptide of M_r 44 500 is immunoprecipitated from uncoupler-inhibited cell extracts (Fig. 4a, lanes 2 and 3) compared

Table 1. Comparison of M_r values of precursors to PDC and OGDC polypeptides as estimated by SDS/polyacrylamide-gel electrophoresis

All M_r values are the averages of three independent determinations which differed by no more than ± 1000 . Mature and precursor sizes for the distinct enzymes of the 2-oxoglutarate complex and the common E3 enzyme were taken from Hunter & Lindsay (1986) and De Marucci *et al.* (1986b) respectively.

Polypeptide	Apparent M_r	
	Mature form	Precursor form
Pyruvate dehydrogenase		
E1 α subunit	42 000	44 500
E1 β subunit	36 000	39 000
2-Oxoglutarate dehydrogenase, E1	96 000	98 000
Dihydrolipoyl acetyltransferase, E2	70 000*	78 000*
Dihydrolipoyl succinyltransferase, E2	48 000	56 000
Component X	51 000	N.D.
Lipoamide dehydrogenase, E3	55 000	56 500

* In rat liver cells, these values correspond to 68 000 and 76 000 ± 1000 for the mature and precursor forms, respectively.

with 42 000 for the native subunit. For E1 β (Fig. 4b, lanes 3 and 4) and its precursor, the corresponding M_r values are 39 000 and 36 000 respectively. In immune complexes of the putative pre-E1 β , precursor (Fig. 4b, lane 3), an additional polypeptide is found with an M_r of similar mobility to pre-E1 α . The specificity of the E1 β antiserum for its parent antigen only, is confirmed in Fig. 4(b), lane 4, in immunoprecipitates of the mature E1 β subunit.

Lipoyl acetyltransferase (E2), M_r 70 000, is synthesized initially as a large precursor (M_r 78 000 $\pm$ 1000) in NBL-1 cells (Fig. 4c, lane 3). Anti-E2 serum, however, also precipitates an unidentified polypeptide of M_r 45 000 $\pm$ 1000 from extracts of untreated NBL-1 cells in addition to mature E2 (Fig. 4c, lanes 2 and 4). We have eliminated the possibility that this species is component X which may be present by virtue of its association with E2 from immunocompetition studies with purified non-radioactive component X and from comparative M_r analyses. It is possible that this prominent 45 000 M_r species is the stable lipoyl peptide which can be easily generated from the proteolytically sensitive, mature E2 subunit during incubation with the anti-E2 serum. The contractile protein actin, M_r 44 000, has also been reported to be a major interfering component, often associated with immune complexes (Barber & Delovitch, 1978). In a recent analysis (not shown), the weak doublet seen in uncoupler-treated NBL-1 cells (Fig. 4c, lane 3 and control lane) can be eliminated by the inclusion of excess non-radioactive actin during immunoprecipitation. In the case of both anti-E2 and anti-E1 β sera, their absolute monospecificity for the parent antigens were also checked by immune replica analysis against native PDC or SDS extracts of the individual cell lines (not shown).

A complete review of the M_r values of the major enzymes of the PDC and OGDC (Hunter & Lindsay, 1986) together with those of their respective precursor states is presented in Table 1. The M_r value for pre-E3

Fig. 5. Kinetics of processing of precursor forms to mature PDC subunits in pulse-chase analysis

PK-15 cells were labelled for 4 h with 200 μ Ci of [35 S]-methionine in the presence of 2 mM-2,4-DNP. At this stage, the medium was replaced with normal growth medium containing no uncouplers. Cell extracts were prepared at various times thereafter and subjected to immunoprecipitation with anti-PDC serum followed by SDS/polyacrylamide-gel electrophoresis and fluorography. Lane S, 3 H-labelled ox heart PDC (see Fig. 4 for subunit M_r values); lane 1, anti-PDC immunoprecipitate after 4 h labelling without uncoupler; lanes 2–6, anti-PDC immunoprecipitates from uncoupler-treated cultures chased for 0, 5, 10, 15 and 25 min respectively after release of inhibition.

was obtained in a separate study utilizing specific antibody raised against purified lipoamide dehydrogenase from pig heart (De Marucci *et al.*, 1986b). Each individual polypeptide in these complexes is formed initially as a separate higher M_r form which is processed rapidly to the mature species. Considerable variability is seen in the apparent size of the presequences which are in the M_r range 1500–3000 for the vast majority of polypeptides. In the case of the E2 components, however, these transit regions are exceptionally large with apparent M_r values of 7000–9000. It is not surprising that only a single precursor state of E3 is detected since it is believed that the same gene product is responsible for this activity in these two multi-enzyme complexes and in the related branched-chain 2-oxo acid dehydrogenase complex.

In Fig. 5 the kinetics of disappearance of precursor polypeptides of PDC were determined after incubating PK-15 cells with [35 S]-methionine in the presence of 2 mM-2,4-DNP, followed by different times of chase on release of inhibition. Accumulated precursors at the start of the chase are illustrated in lane 2 of Fig. 5 whereas lanes 2–5 depict the products which were recovered at various times thereafter. On removal of uncoupler, newly processed forms of E2 and E1 β , in particular, appeared within 10 min with a concomitant decrease in the amount of precursor. No intermediate states were detected during the conversion of precursor to mature forms of these two subunits, which was complete within 25 min with a half-time of 10–12 min. In the case of the E1 α subunit, inefficient or incomplete processing is evident in this cell line with the generation of an additional minor species of intermediate M_r value. However, this effect was confined to the pig kidney cell line since normal processing of pre-E1 α to E1 α occurs in BRL and NBL-1 cells.

DISCUSSION

In this communication, we have defined some of the early events in the biosynthesis, transport and maturation of mammalian mitochondrial PDC. Stable, higher M_r states of two of the major enzymes have been detected including separate precursor forms of the E1 α and E1 β subunits of pyruvate dehydrogenase. Their kinetics of processing to the mature-size subunits have also been monitored. Some interesting variations in the size of the individual presequences and in the affinity of E3 for the intact complex have been observed in differing cell types.

Characteristics of antisera in relation to the detection of mature and precursor states of PDC polypeptides

A specific property of our antibody preparation to intact PDC is that it has a negligible titre to the E3 component although it does elicit a strong cross reaction with the other four major polypeptides including component X on immune replica analysis. A similar response was observed on preparing antibody to native OGDC (De Marcucci *et al.*, 1985). This is not unexpected as lipoamide dehydrogenase, E3, catalyses the identical reaction in both complexes and is believed to be the same gene product. The highly conserved nature of E3 polypeptide is apparent in comparing the amino acid sequence of the *Escherichia coli* enzyme with tryptic peptides of E3 from pig heart (Stephens *et al.*, 1983) which may account for its poor immunogenicity.

In view of the relative titres of the anti-PDC serum to individual subunits of PDC, it was surprising to find in NBL-1 cells that component X was not detectable and that E3 represented a major product in the final immunoprecipitates. However, in PK-15 cultures, only three major bands corresponding to E2, E1 α and E1 β polypeptides were apparent. Our interpretation is that E3, by virtue of its association with the E2 core structure, at least in NBL-1 cells, is co-precipitating with the other components of the complex. The absence of E3 from immunoprecipitates in PK-15 cells also suggests that there is variable affinity of this component for the E2 core assembly between these cell types. An interesting corollary to this result is seen in Fig. 3(a) (compare lanes 1 and 3), which shows that pre-E2, pre-E1 α and pre-E1 β are readily visualized in uncoupler-inhibited NBL-1 cells after immune precipitation with anti-PDC serum whereas there is almost no detectable band corresponding to pre-E3, M_r 56 500, under these conditions. This suggests that there is no association of pre-E3 with precursor forms of E1 or E2 polypeptides in the cytoplasmic compartment before entry into the mitochondrion. In contrast, the presence of an additional polypeptide corresponding in M_r value to pre-E1 α , as well as the presumed pre-E1 β precursor in immune complexes formed with subunit-specific sera to E1 β (Fig. 4b), may indicate that these precursors are capable of interacting in the cytosol, especially as anti-E1 β serum detects only its parent antigen in extracts from uninhibited cultures. Further experiments are desirable to confirm the possible association of pre-E1 α and pre-E1 β under these conditions.

General properties of PDC precursors

Mitochondrial N-terminal extensions vary considerably in length and no homologies are apparent between those which have been sequenced at present

(Reid, 1985). In the case of pre-E1 α , pre-E1 β and pre-E3, the transit peptides are in the expected M_r range of 1500–3500. However, a large extension sequence exists on the lipoyl acetyltransferase pre-E2 component, similar to the situation previously reported for lipoyl succinyltransferase (EC 2.3.2.61), the analogous E2 core species of OGDC (Hunter & Lindsay, 1986). In addition to targeting polypeptides to the surface of the organelle, 'signal' peptides may be involved in (a) regulating uptake into specific mitochondrial compartments, e.g. outer membrane (Hase *et al.*, 1984); (b) maintaining hydrophobic integral membrane proteins in a soluble state (Viebrock *et al.*, 1982) and (c) perhaps interacting with soluble cytosolic recognition factors (Ohta & Schatz, 1984). Immunological data have revealed that pre-E2 for OGDC is conformationally dissimilar to mature, multimeric enzyme, leading to the possibility that a secondary function of the long signal peptide may be to prevent premature aggregation of this subunit before entry into the organelle (Hunter & Lindsay, 1986).

The inability to detect either component X or its precursor with anti-PDC sera which has a high titre of antibody against this component may be influenced by several factors including the rate of turnover of this species, its abundance in comparison with the other PDC polypeptides and its content of methionine. It appears that there are between 6 and 12 mol of component X per mol of complex (De Marcucci & Lindsay, 1985; De Marcucci *et al.*, 1986a), i.e. only 10–20% of the number of copies of E1 α , E1 β and E2 molecules. Although E3 is present at only six homodimers/complex in PDC, it is also a common subunit of OGDC and the branched-chain 2-oxoacid dehydrogenase complex. In addition, preliminary amino acid analysis (J. A. Hodgson & J. G. Lindsay, unpublished observations) reveals that protein X contains only 1–2 methionine residues/50 000- M_r polypeptide subunit.

Characteristics and kinetics of precursor processing

As shown in Fig. 5, precursors of E1 α , E1 β , E2 (and E3, not shown) are all transported into the organelle and proteolytically cleaved to their mature counterparts with similar lag times. Although this observation may suggest that uptake of the constituent enzymes of PDC is a co-operative process, all available evidence is to the contrary. Individual components of complex multi-subunit enzymes, e.g. α - and γ -subunits of F₁-ATPase, are readily transported when synthesized in a *Saccharomyces cerevisiae* mutant which lacks the ability to produce the β -subunit (Burns & Lewin, 1986). Moreover, the complete absence of the E2 component of branched-chain 2-oxoacid dehydrogenase complex has been observed in a patient with Maple Syrup Urine disease (Daume *et al.*, 1985). Despite this defect, normal processing of the E1 α and E1 β polypeptides of branched-chain 2-oxoacid dehydrogenase appears to occur in this patient.

The aberrant proteolytic cleavage of the pre-E1 α subunit in PK-15 cells after reversal of uncoupler inhibition almost certainly reflects the relative instability of the subunit in this cell line, since normal processing and stronger immunoprecipitation of the precursor form can be achieved using either NBL-1 or BRL cultures. We have also established the low intrinsic stability of pre-E1 α

in pig kidney cells by monitoring its rapid non-specific degradation in the cytosolic compartment in FCCP-inhibited cultures maintained in non-radioactive medium after an initial stage of accumulation of the [³⁵S] methionine-labelled precursor.

Current research is concerned with further analysis of the states of aggregation of cytosolic precursors, their possible interactions, and the role of co-factor insertion in the maturation and assembly of PDC polypeptides.

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