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Biosynthesis and processing of the large and small subunits of succinate dehydrogenase in cultured mammalian cells

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Monospecific polyclonal antisera have been raised to purified bovine heart succinate dehydrogenase and to the individual large and small subunits of this enzyme. These antisera exhibit cross-reactivity with the corresponding polypeptides in rat liver (BRL), pig kidney (PK-15) and bovine kidney (NBL-1) cell lines, and were employed to investigate some of the events involved in the biogenesis of succinate dehydrogenase in the PK-15 cell line. Newly-synthesized forms of the large and small subunits of succinate dehydrogenase were detected in cultured PK-15 and BRL cells labelled with [^{35}S]methionine in the presence of uncouplers of oxidative phosphorylation. In PK-15 cells, the precursor forms of the large and small subunits exhibit M_r values approx. 1000–2000 and 4000–5000 greater than those of the corresponding mature forms. When the uncoupler is removed in pulse–chase experiments, complete conversion of the precursors to the mature forms occurs within 45 min. Studies on the kinetics of processing and stability of the large subunit precursor revealed that reversal of precursor accumulation is rapid, with processing occurring with a half-time of 5–7.5 min, and that the accumulated precursor exhibits long-term stability when PK-15 cells are maintained in the presence of 2,4-dinitrophenol.

INTRODUCTION

Succinate dehydrogenase (SDH) occupies a unique position in energy-yielding metabolism, being a component of both the tricarboxylic acid cycle and the electron transport chain. Although the purified enzyme is capable of reducing artificial electron acceptors such as ferricyanide, it requires to be associated with one or two additional polypeptide components of complex II to interact with its physiological acceptor ubiquinone (Capaldi *et al.*, 1977; Ackrell *et al.*, 1980; King, 1982).

The enzyme from bovine heart mitochondria has been isolated in water-soluble form and consists of two subunits with M_r values of 70000 and 27000 (Davis & Hatefi, 1971). The M_r 70000 polypeptide contains one molecule of covalently-attached FAD (Walker & Singer, 1970). Biophysical methods have revealed the existence of Fe–S clusters of the [2Fe–2S], [3Fe–*x*S] and [4Fe–4S] types, although definitive evidence concerning their subunit location has yet to emerge [for a review, see Singer & Johnson (1985)]. The enzyme is tightly associated with the mitochondrial inner membrane and exhibits an asymmetrical distribution within the lipid bilayer; ferricyanide-binding, immunological and chemical-labelling studies have indicated that SDH is located both physically and functionally on the matrix side of the inner membrane (Klingenberg & Buchholz, 1970; Merli *et al.*, 1979; Girdlestone *et al.*, 1981).

The process whereby nuclear-coded mitochondrial polypeptides are imported into mitochondria from their cytoplasmic site of synthesis has been widely studied in recent years. In the majority of cases, these polypeptides are synthesized as higher-M_r precursor forms which possess a cleavable *N*-terminal signal sequence (Reid, 1985). Uptake of precursors which are destined for insertion into or translocation across the mitochondrial inner membrane requires an electrochemical potential gradient across the inner membrane (Schleyer *et al.*, 1982; Gasser *et al.*, 1982) and is accompanied by processing of the precursor to the mature polypeptide. The final step in the biogenesis of an imported mitochondrial polypeptide is the acquisition of a functional conformation in the correct submitochondrial location.

The biosynthesis of SDH is of special interest since generation of the holoenzyme involves covalent attachment of FAD to the large subunit and insertion of the three Fe–S clusters. However, to date only one study on the biosynthesis of mammalian SDH, concerning the M_r 70000 subunit, has been reported (Ono & Tuboi, 1986). This paper describes the production and characterization of antisera to the bovine heart holoenzyme and to the individual large and small subunits. These antisera have been employed to monitor the biosynthesis and processing of SDH *in vivo* in cultured mammalian cells.

MATERIALS AND METHODS

Materials

Pig kidney (PK-15), bovine kidney (NBL-1) and Buffalo rat liver (BRL) cell lines were obtained from Flow Laboratories. Cell culture media were purchased from Gibco, and Pansorbin, a 10% (w/v) suspension of formalinized *Staphylococcus aureus* cells, Cowan I strain, was the product of Calbiochem–Behring.

L-[^{35}S]methionine (> 1100 Ci/mmol) and Na^{125}I (carrier-free) were obtained from Amersham International, and X-ray film (X-Omat S or XAR-5) was obtained from Kodak.

Abbreviations used: DNP, 2,4-dinitrophenol; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; NGM, normal growth medium; SDH, succinate dehydrogenase.

Low-M_r marker proteins were obtained from Pharmacia. Nitrocellulose paper (0.45 μm pore size) was from Schleicher und Schüll, Dassel, West Germany. Phenylmethanesulphonyl fluoride, *p*-aminobenzamidine HCl and DNP were obtained from Sigma. FCCP came from Aldrich. Iodogen was purchased from Pierce and Warriner (U.K.), Chester, U.K. All other reagents were of the highest grades available commercially.

SDH was prepared according to the method previously reported (King, 1967) with the following modifications. Succinate cytochrome *c* reductase was used instead of the heart muscle preparation. The succinate-preincubated reductase was cleaved by anaerobic incubation at pH 10.4 (0° C). The supernatant solution separated by ultracentrifugation was adjusted to pH 9.0, adsorbed on calcium phosphate and the procedure was continued as described (King, 1967). SDH thus prepared was > 99% reconstitutively active and contained about 9.5 nmol of FAD/mg of protein.

SDS/polyacrylamide-gel electrophoresis

SDS/polyacrylamide slab gel electrophoresis was performed by using the discontinuous buffer system of Laemmli (1970) on 10% (w/v) resolving gels (19.0 cm × 9.5 cm × 0.15 cm) or, when greater resolution was required, on 6% (w/v) resolving gels (16.0 cm × 14.0 cm × 0.15 cm). For preparative purposes, 10% (w/v) gels were cast with a thickness of 0.3 cm and the stacking gel was cast with a single large well (16.2 cm × 1.2 cm × 0.3 cm). Further details of sample preparation, staining and M_r estimation have been described previously (De Marcucci *et al.*, 1985).

Preparation of antisera

Antiserum to the SDH holoenzyme was raised in a New Zealand White rabbit by following a previously described regime (De Marcucci *et al.*, 1985). After resolution of the subunits of SDH by preparative SDS/polyacrylamide-gel electrophoresis, subunit-specific antisera to the individual large and small subunits (anti-L and anti-S sera, respectively) were produced by adopting the protocol described by Hunter & Lindsay (1986).

Cell culture

PK-15 cells were routinely maintained in Glasgow-modified Eagle's medium supplemented with non-essential amino acids and 5% (v/v) newborn calf serum (NGM). BRL and NBL-1 cells were maintained in the same medium supplemented with 10% (v/v) newborn calf serum.

Metabolic labelling of cultured cells

PK-15 cells were grown in 10 cm diameter plastic petri dishes in NGM (10 ml) at an initial concentration of 3×10^5 cells/ml. When semi-confluent, the cell monolayers were transferred to low-methionine medium for 90 min (NGM diluted 1:20 with 'minus-methionine medium' consisting of Glasgow-modified Eagle's medium without L-methionine and L-glutamine, supplemented with 2 mM-L-glutamine and newborn calf serum at the appropriate concentration). After addition of L-[^{35}S]methionine (200 μCi/dish), the cells were incubated for a further 3–4 h, then radiolabelled cell lysates were prepared as described by Hunter & Lindsay (1986).

To accumulate precursors, DNP and FCCP was added to the medium 10 min before the addition of isotope. For pulse–chase experiments, monolayers were labelled in the presence of uncouplers as above, then the cells were transferred to warm (37 °C) NGM with or without uncoupler as required. After the appropriate chase period, cell lysates were prepared as above.

Immunological methods

Immune blotting was performed essentially as described by Towbin *et al.* (1979). Briefly, protein samples were resolved by SDS/polyacrylamide-gel electrophoresis and then transferred electrophoretically onto nitrocellulose paper. The transfer buffer was supplemented with 0.02% (w/v) SDS. After blocking remaining binding sites by the modification described by Batteiger *et al.* (1982), the nitrocellulose was incubated with either anti-SDH, anti-L or anti-S sera at dilutions of 1:100, 1:100 and 1:50 respectively. Immune complexes were detected by incubation with ^{125}I-labelled protein A followed by autoradiography at −80 °C in the presence of a tungstate intensifying screen. Immunoprecipitation of precursor and mature forms of the subunits of SDH from [^{35}S]methionine-labelled cell lysates was performed as described in a previous paper from our group (Hunter & Lindsay, 1986).

Other methods

Fluorography was performed by the method of Chamberlain (1979). Protein was determined by a modification of the Lowry procedure using bovine serum albumin as a standard (Markwell *et al.*, 1978). ^{125}I-labelled protein A and low-M_r markers were prepared by the method of Salacinski *et al.* (1981), as described by De Marcucci *et al.* (1985).

RESULTS

Fig. 1(*a*) shows the Coomassie Blue profile of different amounts of the purified SDH preparation after electrophoresis on a SDS/10% (w/v) polyacrylamide slab gel. In addition to the major M_r 70000 and M_r 27000 bands (corresponding to the large and small subunits of SDH, respectively), the preparation contains several minor contaminants with intermediate M_r values. By the criterion of densitometric scanning, the preparation was judged to be 85–90% pure.

Figs. 1(*b*)–1(*d*) show the results of immune blot analysis of the purified SDH with anti-SDH serum (*b*) or subunit-specific anti-L (*c*) and anti-S (*d*) sera, after detection of immune complexes with ^{125}I-labelled protein A. In addition to reaction with their parent antigens, anti-SDH and anti-L sera show cross-reaction with all the minor bands of intermediate M_r values in the preparation. Fig. 1(*d*) shows that, at higher loadings of SDH, anti-S serum also exhibits weak cross-reactivity with the M_r 70000 subunit and with an M_r 50000 large subunit fragment.

Immunoblotting analysis of mitochondrial fractions (results not shown) from rat liver, bovine kidney and pig kidney indicated that anti-SDH, anti-L and anti-S sera all exhibited strong and specific cross-reaction with their parent antigens in each case. Moreover, no striking differences in the M_r values of either subunit of SDH were observed when compared with the bovine heart enzyme.

For detection of higher-M_r precursor forms of the large and small subunits of SDH (Fig. 2), PK-15 cells

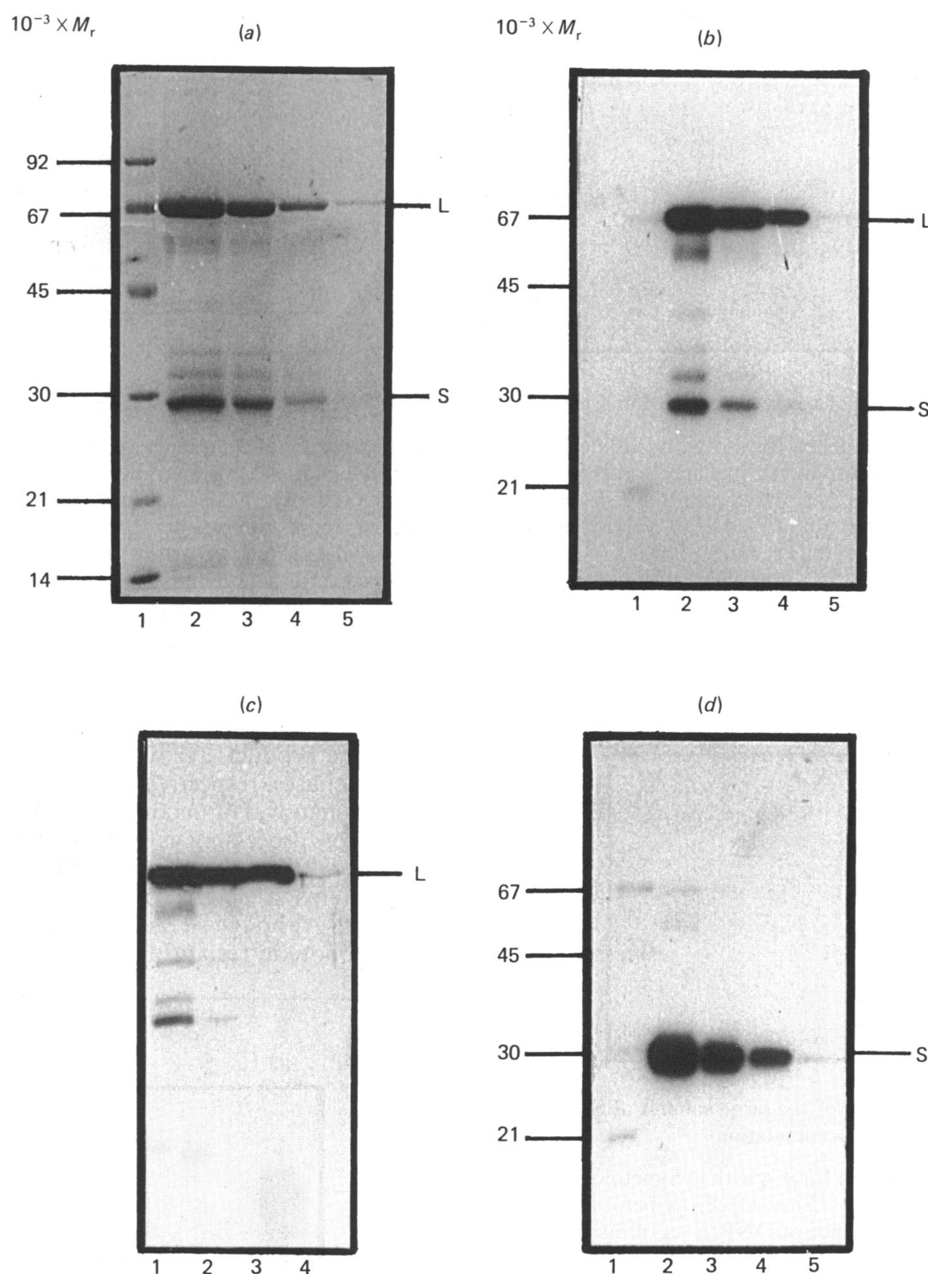

Fig. 1. Immune blot analysis of purified SDH with anti-SDH serum and subunit-specific anti-L and anti-S sera

Various amounts of purified succinate dehydrogenase were electrophoresed on a 10% (w/v) SDS/polyacrylamide slab gel. One portion of the gel (*a*) was stained with Coomassie Blue. Polypeptides on replicate portions were transferred electrophoretically onto nitrocellulose paper for incubation with anti-SDH (*b*), anti-L (*c*) or anti-S (*d*) sera. Immune complexes were visualized by autoradiography after incubation with ^{125}I-labelled protein A (see the Materials and methods section). Lanes 2–5 in (*a*), 25, 10, 5 or 1 μg of enzyme, respectively. Lanes 2–5 in (*b*) and (*d*) and lanes 1–4 in (*c*), 5, 2, 1 or 0.2 μg of enzyme, respectively. Lane 1, low-M_r marker proteins (*a*) or ^{125}I-labelled low-M_r marker proteins (*b*) and (*d*).

were pulse-labelled for 4 h in the presence or absence of the uncouplers DNP or FCCP. Detergent extracts of the cells were subjected to immunoprecipitation with anti-L (Fig. 2*a*) or anti-S (Fig. 2*b*) sera and the recovered [^{35}S]methionine-labelled polypeptides were analysed by SDS/polyacrylamide-gel electrophoresis and fluorography.

It is apparent that, on incubation with either DNP (panel *a*, lane 4; panel *b*, lane 3) or FCCP (panel *a*, lane 6; panel *b*, lane 5) newly-synthesized (precursor) forms of the large and small subunits accumulate with M_r values approx. 1000–2000 and 4000–5000 greater than those of the corresponding mature forms. After removal of uncouplers (panel *a*, lanes 5 and 7; panel *b*, lanes 4 and 6), complete conversion of subunit precursors to the mature products can be achieved within a 45 min chase period. Precursor states of similar, if not identical, M_r values were also observed in BRL (rat liver) cells (results not shown) although, in this case, inhibition of processing is complete in the presence of 10 μM-FCCP

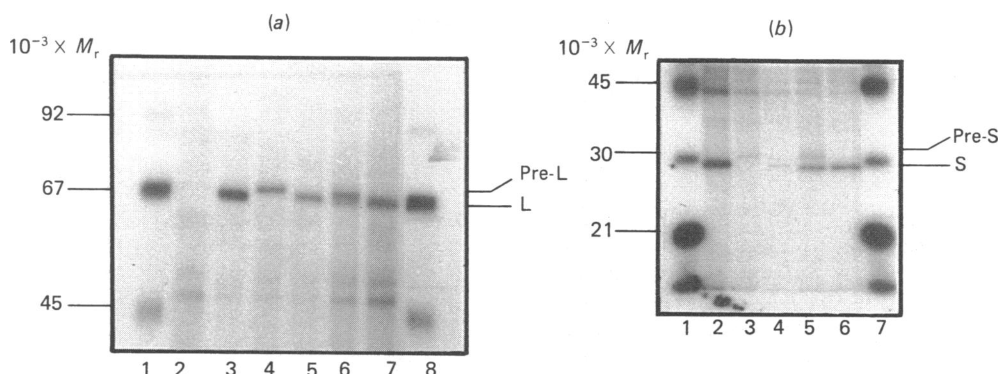

Fig. 2. Immunoprecipitation of newly-synthesized and processed subunits of SDH in pulse-labelled and pulse–chased PK-15 cells

PK-15 cells were pulse-labelled for 4 h with [^{35}S]methionine both in the absence and presence of uncoupler, or pulse-labelled in the presence of uncoupler and then chased for 45 min after uncoupler removal. After preparation of radiolabelled cell lysates, immunoprecipitation was performed using anti-L serum (*a*) and anti-S serum (*b*). The resulting immunoprecipitates were analysed on 6% (w/v) or 10% (w/v) SDS/polyacrylamide gels (panels *a* and *b*, respectively) and visualized by fluorography (see the Materials and methods section). Panel (*a*): lanes 1 and 8, ^{125}I-labelled low-M_r markers; lane 2, pulse (no uncoupler), non-immune serum; lanes 3–7, immunoprecipitates with anti-L serum; lane 3, pulse, no uncoupler; lane 4, pulse + 2 mM-DNP; lane 5, as 4, but with 45 min chase; lane 6, pulse + 10 μM-FCCP; lane 7, as 6 but with 45 min chase. Panel (*b*): lanes 1 and 7, ^{125}I-labelled low-M_r markers; lanes 2–6, same as lanes 3–7 in (*a*) except with anti-S serum.

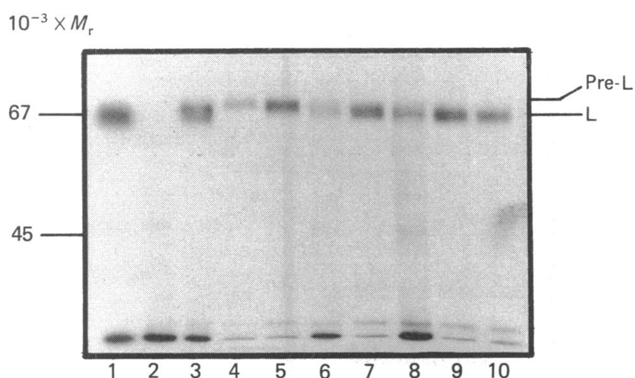

Fig. 3. Kinetics of processing of the large subunit of SDH after reversal of precursor accumulation

PK-15 cells were incubated for 4 h with [^{35}S]methionine in the absence or presence of 2 mM-DNP, as before. After pulse-labelling in the presence of DNP, a set of dishes was utilized to perform chases of increasing duration in the absence of uncoupler. After preparation of cell lysates at the appropriate time points, immunoprecipitation was performed with anti-L serum. Analysis of the resulting immunoprecipitates was as described in Fig. 2. Lane 1, ^{125}I-labelled low-M_r markers; lane 2, pulse (no uncoupler), non-immune serum; lane 3, pulse (no uncoupler), anti-L serum; lane 4, pulse + 2 mM-DNP, anti-L serum; lanes 5–10; as 4, but with 2 min, 5 min, 7.5 min, 10 min, 15 min and 30 min chase respectively.

while only partial accumulation of precursors occurs in the PK-15 cell line (panel *a*, lane 6; panel *b*, lane 5) at this concentration.

The pig kidney cell line was utilized further to investigate the stability and kinetics of processing of the SDH large subunit precursor. Fig. 3 shows the results of an experiment in which large subunit precursor accumulation in the presence of 2 mM-DNP was followed by chases of increasing duration in the absence of uncoupler. Lane 4 shows the precursor accumulated at the start of the chase after labelling in the presence of DNP; lanes 5–10 show the products recovered after 2, 5, 7.5, 10, 15 and 30 min chases, respectively, in the absence of uncoupler.

After removal of uncoupler from the cells, the onset of precursor processing is rapid. The immunoprecipitate obtained after a 2 min chase in the absence of DNP (lane 5) contains a small amount of M_r 70000 (mature) subunit. Although it is difficult to achieve complete electrophoretic resolution of the precursor and mature

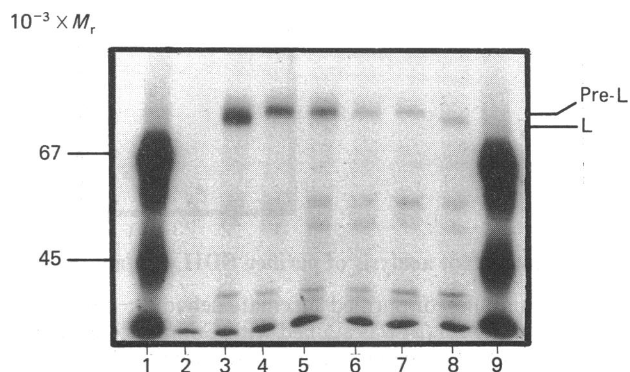

Fig. 4. Stability of the accumulated precursor to the large subunit of SDH

PK-15 cells were pulsed for 4 h with [^{35}S]methionine in the absence or presence of 2 mM-DNP and then chased in the presence of DNP for the times indicated. In addition, cells which had been subjected to a 5 h chase in the presence of DNP were incubated for a further 45 min in medium minus uncoupler. Immunoprecipitates were obtained from radiolabelled cell lysates with anti-L serum and analysed by SDS/polyacrylamide gel electrophoresis and fluorography (see Fig. 2). Lanes 1 and 9, ^{125}I-labelled low-M_r markers; lane 3, pulse (no uncoupler), non-immune serum; lane 3, pulse (no uncoupler), anti-L serum; lane 4, pulse + 2 mM-DNP; lanes 5–7, same as 4, but with 1 h, 3 h and 5 h chases, respectively; lane 8, same as 7, but with additional 45 min chase minus uncoupler.

forms even on the 6% (w/v) gel used in this experiment, we estimate that the half-life of the large subunit precursor is between 5 and 7.5 min. Longer chase period (lanes 9 and 10) revealed that processing was complete after 15 min.

To monitor stability of the large subunit precursor accumulated in the presence of [^{35}S]methionine, a chase in non-radioactive medium was performed for different time periods in the presence of uncoupler (Fig. 4). Lane 4 shows the precursor recovered after a 4 h pulse with 2 mM-DNP, while lanes 5–7 show the precursor recovered after a 4 h pulse with uncoupler followed by a 1, 3 and 5 h chase, respectively, also in the presence of uncoupler. Although the intensity of the precursor band decreases over this time period, a substantial proportion remains detectable even after an additional 5 h chase. When the 5 h chase in DNP-containing medium was followed by a 45 min chase in the absence of uncoupler, complete conversion of precursor to mature large subunit was again observed (lane 8).

DISCUSSION

The data presented in Fig. 1 establish that the minor components present in the purified SDH preparation cross-react specifically with antiserum raised against the large subunit excised from an SDS/polyacrylamide gel. These species are most probably proteolytic fragments of the M_r 70000 polypeptide generated during the isolation procedure. Similar immunologically-reactive fragments of the large subunit can be detected after limited proteolysis of submitochondrial particles in experiments designed to probe the organization of SDH within the lipid bilayer (G. H. D. Clarkson & J. G. Lindsay, unpublished work).

Antiserum was produced also to the small subunit of SDH isolated in a similar manner. The weak cross-reactivity of anti-(small subunit) serum with the large subunit may reflect genuine immunological similarity between the M_r 70000 and M_r 27000 subunits. This situation is not entirely unlikely, since both polypeptides contain sequences which participate in the formation of Fe–S clusters in the holoenzyme. However, the possibility that the material used for production of anti-(small subunit) serum contained a M_r 27000 large subunit fragment cannot be excluded.

Several points have emerged from our experiences using immune blot analysis. Firstly, this technique can be used to monitor potential loss of a polypeptide by proteolysis during its purification. Secondly, the ability to detect immunoreactive proteolytic fragments in pure preparations offers an alternative criterion by which to assess the homogeneity of a purified protein. Clearly, the purity of the preparation used for immunization in this case was greater than the original estimate. Finally, the cross-reactivity exhibited by our various antisera to SDH with the corresponding parent antigens from other tissues and species illustrates a potential immunologically-based method of identifying equivalent proteins in closely or even distantly related sources.

The energy-dependent nature of the import of cytoplasmically-synthesized mitochondrial polypeptides was exploited to accumulate newly-synthesized forms of the subunits of SDH in cultured mammalian cells. Fig. 2 shows that when PK-15 cells are incubated with [^{35}S]methionine and uncouplers of oxidative phosphorylation, larger precursor forms of both subunits can be immunoprecipitated. The precursors to the large and small subunits exhibit M_r values which are approx. 1000–2000 and 4000–5000 higher than those of the corresponding mature forms. The data for the precursor to the large subunit is in agreement with the estimate obtained by Ono & Tuboi (1986) either *in vitro* by using rat liver mRNA to direct a cell-free translation system, or *in vivo* in ascites hepatoma cells. Our data concerning the biosynthesis of the small subunit provide the first demonstration that the M_r 27000 polypeptide is also synthesized as a higher-M_r precursor. Weak immunoprecipitation of the small subunit (Fig. 2) may be the result of several factors: (*a*) this subunit is less immunogenic than the large polypeptide (Fig. 1), (*b*) it has a much lower methionine content, and (*c*) accumulation of precursor depends on the long-term stability of this species in the cytoplasmic compartment (see later in the Discussion). In addition, the M_r 45000 band observed with anti-S serum represents actin, which has been reported previously to be commonly associated with immunoprecipitates (Barber & Delovitch, 1978). Radiolabelled actin can be competed out by addition of small amounts (5–20 μg) of purified, non-radioactive, actin to the incubation mixture.

It is of interest to consider the existence of higher-M_r precursor forms of the subunits of SDH in relation to the precursor forms of other mitochondrial inner membrane components. A range of inner membrane polypeptides which exhibit 'sidedness' with respect to the lipid bilayer have been studied. The majority of these are synthesized as higher-M_r precursors. In contrast, fewer inner membrane components which exhibit transmembrane activity have been studied. Three of these, the adenine nucleotide translocase (O'Malley *et al.*, 1982; Hatalová & Kolarov, 1983), thermogenin (Freeman *et al.*, 1983) and the phosphate–hydroxyl antiporter (G. M. Gibb & J. G. Lindsay, unpublished observations) are synthesized without a cleavable signal sequence. Whether the lack of such a signal sequence in these proteins is related to their topographical arrangement remains to be determined.

The reversible nature of uncoupler-induced precursor accumulation was demonstrated in experiments where complete conversion of precursor to mature form was observed after a 45 min chase in the absence of uncoupler. Chases of shorter duration revealed that onset of precursor processing after uncoupler removal is rapid, and occurs with an estimated half-life of 5–7.5 min. Similar half-lives have been reported for other mammalian mitochondrial polypeptide precursors (Fenton *et al.*, 1984; Mori *et al.*, 1981; Raymond & Shore, 1981).

A noteable feature of the pig kidney large subunit precursor is its stability when import into the mitochondrion is inhibited. An appreciable amount of this polypeptide persists for at least 5 h after synthesis. Furthermore, the accumulated precursor remains in an import-competent form, since processing is still observed when PK-15 cells are reintroduced to uncoupler-free medium after a 5 h chase plus uncoupler. Similar long-term stability has been observed in the case of methylmalonyl-CoA mutase (Fenton *et al.*, 1984).

What is the basis of this long-term stability? Although the possibility exists that DNP acts on the cells to inhibit general proteolysis, we do not favour this explanation for two reasons. Firstly, pre-(aspartate aminotransferase) exhibits marked instability, being rapidly destroyed with

a half-life of approx. 5 min when the accumulated precursor is chased in the presence of uncoupler (Jaussi *et al.*, 1982). Secondly, in an experiment analogous to that shown in Fig. 4, the precursors to the three component enzymes of the mitochondrial 2-oxoglutarate dehydrogenase complex were found to exhibit differing stabilities (Hunter, 1985). Thus, we propose that observed long-term stability is an intrinsic property of the individual precursor, rather than a reflection of the experimental conditions employed.

In this report an initial description of the biogenesis of mammalian SDH is presented. We are now in a position to investigate additional aspects of the process, e.g. the involvement of FAD attachment or Fe–S cluster insertion in the generation of functional enzyme.

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