Import of rat liver mitochondrial transhydrogenase

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The biosynthesis of pyridine dinucleotide transhydrogenase has been studied in isolated rat hepatocytes and in a rabbit reticulocyte-lysate translation system supplemented with either intact isolated rat liver mitochondria or the soluble matrix fraction from isolated mitochondria. In intact hepatocytes, the transhydrogenase precursor was short-lived in the cytosol and was efficiently imported into the membranous fraction. When the cell-free translation mixture was incubated with intact mitochondria, the transhydrogenase precursor was processed to the mature form, to an extent that depended on the amount of added mitochondria. Incubation of the translation mixture with the soluble mitochondria matrix fraction converted the precursor to a mature-sized protein with 75% efficiency, this being blocked by various proteinase inhibitors such as EDTA, 1,10-phenanthroline and leupeptin.

INTRODUCTION

Most mitochondrial proteins are encoded by nuclear DNA, synthesized in the cytosol and imported into mitochondria (Hay *et al.*, 1984). Those proteins destined for import into the inner membrane and matrix are generally higher-molecular-mass precursors that are cleaved to their mature forms post-translationally by a matrix-processing proteinase (Conboy *et al.*, 1982). About 20 nuclear genes encoding mitochondrial proteins have been cloned, and thus the primary amino acid sequences of the mature proteins and their cleavable pre-sequences have been revealed (Douglas & Takeda, 1985). Although their pre-sequences do not show extensive primary sequence homology, a few common features have been noted. Pre-sequences are (a) rich in the positively charged amino acids arginine and lysine, (b) rich in the hydroxylated amino acids serine and threonine and (c) devoid of acidic residues (Hurt & van Loon, 1986).

Pyridine dinucleotide transhydrogenase is a mitochondrial-inner-membrane redox-linked proton pump. The enzyme couples the reversible transfer of a hydride ion between matrix NADPH and NAD⁺ to vectorial proton translocation from the matrix to the cytosol (Rydstrom, 1981; Fisher & Earle, 1982). Bovine heart transhydrogenase possesses a simple subunit structure consisting of a dimer of apparently identical 110 kDa subunits (Wu & Fisher, 1983). A previous study on the biosynthesis of transhydrogenase by isolated rat hepatocytes and by rabbit reticulocyte lysates programmed with partially purified rat liver mRNA has shown that transhydrogenase is synthesized as a precursor having an approx. 2 kDa extension (Wu *et al.*, 1985). Here we report further data on kinetics of the processing of transhydrogenase precursor.

EXPERIMENTAL

Materials

Rat liver mitochondria and the matrix fraction were prepared as described by Schnaitman & Greenawalt

(1968). Polyclonal antibodies were raised against homogeneous purified bovine heart transhydrogenase as described by Anderson *et al.* (1981). Total rat liver RNA was isolated by the guanidinium thiocyanate extraction method (Chirgwin *et al.*, 1979). Poly(A)⁺ RNA was fractionated by passage through oligo(dT)-cellulose columns (Aviv & Leder, 1972). Further sucrose-gradient fractionation of poly(A)⁺ RNA was performed, and RNA was recovered after repeated ethanol precipitation (Lizardi, 1983; Wu *et al.*, 1985). Translations *in vitro* were performed at 30 °C for 90 min, and the incorporation of [³⁵S]methionine radioactivity was determined (Wu *et al.*, 1985). Rat hepatocytes were prepared (Seglen, 1976) and diluted to a concentration of 4 × 10⁷ cells/ml with suspension buffer containing 68 mM-NaCl, 1.2 mM-CaCl₂, 30 mM-Hepes, 5.4 mM-KCl, 1 mM-MgCl₂, 1.1 mM-KH₂PO₄, 30 mM-Tes, 36 mM-Tricine and 10 mM-dextrose, pH 7.6. Other materials were obtained from the cited sources: [³⁵S]methionine (1000 Ci/mmol), du Pont; glutaraldehyde-fixed *Staphylococcus aureus* cells, Miles; digitonin, Triton X-100, Lubrol WX, pepstatin, leupeptin, PMSF, 1,10-phenanthroline, rhodamine 123, rhodamine 6G, zincon and cycloheximide, Sigma; rabbit reticulocyte lysate, BRL.

Cellular processing of transhydrogenase precursor

Hepatocytes in 0.5 ml of suspension buffer were supplemented with 5% (v/v) of dialysed fetal-calf serum and 0.05 ml of a solution containing 1 mM each of 19 amino acids (but lacking methionine). The suspension was preincubated at 37 °C for 10 min with shaking in a clinical rotator at 250 rev./min. Radiolabelling of the cells was initiated by addition of [³⁵S]methionine to 600 µCi/ml, and the incubation was continued for 10 min before adding 5 µl of 10 mM unlabelled methionine and 5 µl of 70 mM-cycloheximide. At various times during the chase, the labelled cell suspension (100 µl) was diluted to 1 ml with a solution containing 150 mM-NaCl, 4 mM-EDTA, 2 mM-1,10-phenanthroline, antipain (20 µg/ml), leupeptin (20 µg/ml), 1 mM-PMSF, 30 µM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazide, 40 mM-Tris/HCl, pH 7.5, and digitonin (1 mg/ml). The

Abbreviations used: PMSF, phenylmethanesulphonyl fluoride; poly(A)⁺, polyadenylated; zincon, 2-carboxy-2'-hydroxy-5'-sulphoformazylbenzene.

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digitonin mixture was incubated for 10 min at 4 °C with occasional shaking. The sample was then diluted to 3 ml by addition of the same buffer lacking digitonin, centrifuged at 3000 *g* for 5 min in a Beckman JA-20 rotor and the supernatant was separated from the pellet. To the supernatant was added 30 μ l of 20% (w/v) SDS and 15 μ l of 20% (v/v) Triton X-100. The pellet was solubilized in 0.4 ml of a buffer composed of 150 mM-NaCl, 5 mM-EDTA, 2 mM, 1,10-phenanthroline, 1 mM-PMSF, leupeptin (25 μ g/ml), antipain (25 μ g/ml), 2% (w/v) SDS, 1% (v/v) Triton X-100, 50 mM-Tris/HCl, pH 7.5, and diluted to 3 ml with the same buffer lacking detergent. Both the particulate and soluble extracts were centrifuged at 105000 *g* in a Beckman Ti-50 rotor for 30 min and immunoprecipitated as described by Wu *et al.* (1985).

Processing by mitochondria *in vitro*

Freshly isolated rat liver mitochondria (60 μ l in 440 mM-D-mannitol/140 mM-sucrose/4 mM-Hepes, pH 7.6) were added to the translation mixture and incubated for 1 h at 30 °C. Mitochondria were separated from the processing mixture by centrifugation in an Eppendorf centrifuge (model 5414) for 4 min, and both the mitochondrial pellet and the supernatant fraction were immunoprecipitated.

Processing by the matrix fraction *in vitro*

The translation mixture (30 μ l) was incubated with 65 μ l of the matrix fraction (300 μ g of protein in 220 mM-D-mannitol/70 mM-sucrose/2 mM-Hepes, pH 7.6) in the presence of 200 μ M-ZnCl₂ at 30 °C for 1 h or as specified. To prepare the sample for immunoprecipitation, 15 μ l of 10% SDS (w/v), 5 μ l of leupeptin (5 mg/ml) and 5 μ l of pepstatin (2.4 mg/ml) were added to the processing mixture. The samples were placed in a boiling-water bath for 3 min, diluted to 1 ml and then immunoprecipitated.

SDS/polyacrylamide-gel electrophoresis

Immunoprecipitates were analysed as described by Laemmli (1970) on 5–15%-(w/v)-polyacrylamide gradient gels, but electrophoresed for twice the time required for the tracking dye to run off the gel. The gels were analysed by fluorography.

RESULTS

Import of transhydrogenase precursor by intact hepatocytes and by isolated mitochondria

To demonstrate the relationship between the native transhydrogenase precursor and its mature form, a pulse-chase experiment was performed in which labelled hepatocytes were fractionated into soluble (cytosolic) and particulate (membrane) components before immunoprecipitation. The enzyme was immunoprecipitated from detergent extracts of labelled cells by addition of heart anti-transhydrogenase, followed by adsorption to Protein A-bearing *Staphylococcus aureus* cells. The precursor would be expected to predominate in the cytosol, whereas the mature enzyme should be found associated with the membrane fraction of the cell. This is illustrated in Fig. 1(a), where the precursor was found to disappear from the soluble fraction (odd-numbered lanes) with concurrent appearance of the mature enzyme in the particulate fraction (even-numbered lanes). The apparent half-life of the precursor was about 2 min. The addition of purified heart transhydrogenase to the labelled cell extract totally prevented the immunoprecipitation of both forms of the enzyme. A minor contaminating band of fastest mobility present in lanes 1, 3, 5 and 7 might arise from the non-specific binding occurred during immunoprecipitation. Note that the contaminant band is only present in the cytosol fraction and cannot be imported into the mitochondrial membrane fraction.

Import of transhydrogenase was also investigated in a cell-free system, where translation products were subsequently exposed to differing amounts of intact rat liver mitochondria. After incubation, the mitochondria were separated from the suspending medium by centrifugation, and transhydrogenase was immunoprecipitated from both fractions. Processing of transhydrogenase was dependent on the concentration of added mitochondria, as Fig. 1(b) shows. The amount of *mature* transhydrogenase present in the mitochondrial pellet (lanes 1 and 3) increased, whereas the amount of transhydrogenase *precursor* present in the supernatant fraction (lanes 2 and 4) decreased.

Fig. 1. Processing of transhydrogenase precursor *in vivo* and *in vitro*

(a) Hepatocytes were pulsed for 10 min and then 'chased' in the presence of 1 mM-unlabelled methionine and 1 mM-cycloheximide. At various times during the chase, aliquots were taken and fractionated into particulate and soluble components. Samples were then detergent-extracted, immunoprecipitated and analysed by SDS/polyacrylamide-gel electrophoresis and fluorography. Lanes 1 and 2 contain the immunoprecipitate from the soluble and particulate fractions respectively after the pulse. Similarly, lanes 3+4, 5+6 and 7+8 contain immunoprecipitates obtained after 2, 4 and 6 min of chase respectively. (b) Translation mixture (60 μ l) was incubated with rat liver mitochondria (60 μ l) for 1 h at 30 °C containing 0.36 mg (lanes 1 and 2)- and 1.08 mg (lanes 3 and 4) of protein. The mixture was centrifuged to separate the mitochondrial pellet (lanes 1 and 3) from the supernatant fraction (lanes 2 and 4), and both portions were immunoprecipitated. All samples were analysed by gel electrophoresis and fluorography.

Processing of transhydrogenase precursor by the matrix fraction

A mitochondrial matrix processing proteinase that cleaves pre-(ornithine carbamyltransferase) to an intermediate form (M_r 37000) has been partially purified from rat liver and has been shown to be sensitive to EDTA, 1,10-phenanthroline, zincon and leupeptin (Miura *et al.*, 1982). However, Conboy *et al.* (1982) discovered that the processing proteinase responsible for converting pre-(ornithine transcarbamylase) to the mature size (M_r 36000) required 0.1 mM- Zn^{2+} for activity and was inhibited by 1,10-phenanthroline. Whether these two proteinases are different is not known at present. Sites of cleavage differ significantly among many precursors. For example, in rat liver ornithine transcarbamylase the cleavage site (denoted by an arrow) is Gln¹-Ser-Gln (Takiguchi *et al.*, 1984), in cytochrome *c* oxidase subunit IV it is Arg¹-Ile-Ser (Lomax *et al.*, 1984), and in bovine adrenal cytochrome *P*-450 it is Gly¹-Ile-Ser (Mosohashi *et al.*, 1984). This suggests that different processing proteinases are operative.

Here a comparable study was performed on the specificity of processing of the transhydrogenase precursor by a soluble matrix fraction in the presence or absence of inhibitors. Mitoplasts were isolated from rat liver mitochondria after solubilization with digitonin (Schnaitman & Greenawalt, 1968). These mitoplasts were then extracted with Lubrol WX, and the supernatant fraction obtained after centrifugation at 135000 *g* for 1 h was used as the matrix fraction. The translation mixture was incubated with the matrix fraction at times specified in Fig. 2. A time-course study showed that about 75 % of the precursor was converted into the mature size in 1 h. When the incubation was performed in the presence of

Fig. 2. Processing of transhydrogenase precursor by the mitochondrial matrix fraction

Translation mixture (30 μ l) was incubated at 30 °C with the matrix fraction (65 μ l) containing 300 μ g of protein in the presence of 200 μ M- $ZnCl_2$ for various times as specified below. Lane 1, translation mixtures, no matrix protein added; lane 2, matrix protein added, 10 min incubation; lane 3, same as lane 2, 20 min; lane 4, 40 min; lane 5, 60 min; lane 6, 60 min with 2 mM-EDTA added; lane 7, 60 min with only matrix isolation buffer (220 mM-D-mannitol/70 mM-sucrose/2 mM-Hepes, pH 7.6) added. All samples were subjected to immunoprecipitation, gel electrophoresis and fluorography. Abbreviations: pTH, transhydrogenase precursor; TH, transhydrogenase.

Fig. 3. Effects of inhibitors on processing of transhydrogenase precursor by mitochondrial matrix fraction

Processing was performed for 1 h at 30 °C as described in Fig. 2 in the presence of the following compounds: lane 1, 1,10-phenanthroline (2 mM); lane 2, rhodamine 123 (10 μ g/ml); lane 3, rhodamine 6 G (10 μ g/ml); lane 4, zincon (1 mM); lane 5, PMSF (0.5 mM); lane 6, pepstatin (12.5 μ g/ml); lane 7, leupeptin (25 μ g/ml); lane 8, no matrix protein, no addition. All samples were subjected to immunoprecipitation, gel electrophoresis and fluorography. pTH and TH are defined in the legend to Fig. 2.

2 mM-EDTA for 1 h at 30 °C, the precursor remained the predominant band and processing was largely prevented (cf. lanes 5 and 6). That the precursor was stable during the incubation is evidenced by the retained intensity of the precursor band (lane 7) if the translation mixture was incubated for 1 h with the matrix isolation medium in the absence of the matrix fraction. Occasionally, some higher- or lower- M_r bands were present after immunoprecipitation. However, only the pre-transhydrogenase and native band could be blocked from precipitation by addition of pure transhydrogenase. Thus these extraneous bands appear to arise from non-specific binding of other proteins to the protein A membrane during immunoprecipitation.

Inhibitors (PMSF, pepstatin and leupeptin), metal chelators (EDTA, 1,10-phenanthroline, zincon) and mitochondrial disruptors (rhodamine 123, rhodamine 6G) was tested for their effect on the processing activity of the matrix fraction under the same conditions as those described in Fig. 2 (Fig. 3). In the presence of 2 mM-1,10-phenanthroline the matrix processing activity was completely inhibited (lane 1). Leupeptin was also capable of suppressing this conversion (lane 7). Other compounds exerted very little influence; the precursor and mature transhydrogenase were distributed equally.

DISCUSSION

The radiolabelled transhydrogenase precursor directly synthesized in isolated hepatocytes was rapidly converted into the mature form with a half-life of only 2 min. When the 'in vitro'-translated transhydrogenase precursor was incubated with isolated rat liver mitochondria, the extent of import and processing to mature transhydrogenase varied from preparation to preparation. On average, processing of the precursor by mitochondria required about 60 min, and the efficiency of the process was 20–40 %. Generally, a substantial amount of the precursor was not imported into mitochondria and remained undegraded in the translation medium. In some instances,

the precursor bound to mitochondria, but was not converted into mature transhydrogenase.

There may be several reasons for this inefficient import and processing *in vitro*. First, the intactness of rat liver mitochondria may not be adequate for uptake. While a normal respiratory control ratio of 5 was determined for each mitochondrial preparation, this characteristic alone may not be sufficient for judging the competency of mitochondria required for import. Secondly, the '*in vitro*'-translated precursor may not have the proper conformation for rapid import, perhaps lacking secondary structure required for recognition by mitochondria. The presence of 'amphiphilic helices' in mitochondrial pre-sequences has been suggested to play a crucial role in transporting proteins into mitochondria (Von Heijne, 1986; Ohta & Schatz, 1984; Roise *et al.*, 1986).

Thirdly, translocation factors may be involved in import. The observation that adequate binding occurs between the transhydrogenase precursor and mitochondria *in vitro*, in contrast with the fact that little processing was accomplished by mitochondria, suggests that the blockage occurs at the import step. It is important to realize that the pre-sequence of the transhydrogenase precursor must thread through both the mitochondrial outer and mitochondrial inner membranes during import, and the final processing to the mature enzyme is accomplished by a matrix-located proteinase. In our import experiments it is possible that the transhydrogenase pre-sequence penetrated partly into the intermembrane space (or the outer-membrane/inner-membrane junction), but was unable to cross through the mitochondrial inner membrane. In such a situation, a major portion of the precursor may remain located at the outer face of the mitochondrial outer membrane, and subsequent processing by the matrix proteinase would be prevented. If this type of blockage of import does occur, pre-sequences may recognize and bind to mitochondria, but there may be a lack of an additional translocation factor needed for targeting precursors to the mitochondrial inner membrane. Some components mediating the transport of proteins into mitochondria have been identified. For example, recent studies indicated that cytosolic factors (Ohta & Schatz, 1984) and an RNA species (Firgaira *et al.*, 1984) might be involved in import of precursors into mitochondria.

The '*in vitro*'-translated precursor was exposed to an isolated mitochondrial matrix fraction in the presence or absence of inhibitors in order to investigate the specificity of the matrix-located processing proteinase. When the transhydrogenase precursor was incubated with the soluble mitochondrial matrix fraction, conversion to the mature-sized form was improved by the presence of 200 μ M-ZnCl₂. Subsequently all matrix-processing experiments were investigated in the presence of ZnCl₂. EDTA and 1,10-phenanthroline were effective in inhibiting the activity of the soluble matrix fraction. Leupeptin, a trypsin inhibitor, was equally capable of significantly diminishing the conversion of the precursor into the mature-sized protein. These observations suggest that the matrix processing proteinase may be dependent

on Zn²⁺ for its activity and may have an arginine-binding site.

The efficient import ($t_1 \approx 2$ min) and processing of the transhydrogenase precursor by the '*in vivo*' system (in contrast with processing into mitochondria *in vitro*, which required at least 1 h) demonstrates that the '*in vivo*' precursor must be present in a conformation ideal for mitochondrial recognition and possibly that the import machinery is better preserved in the '*in vivo*' system.

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