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The synthesis of murine ferrochelatase *in vitro* and *in vivo*

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Ferrochelatase (protohaem ferro-lyase, EC 4.99.1.1), the terminal enzyme of the haem-biosynthetic pathway, is an integral membrane protein of the mitochondrial inner membrane. When murine erythroleukaemia cells are labelled *in vivo* with [^{35}S]methionine, lysed, and the extract is immunoprecipitated with rabbit anti-(mouse ferrochelatase) antibody, a protein of M_r 40000 is isolated. However, when isolated mouse RNA is translated in a cell-free reticulocyte extract, a protein of M_r 43000 is isolated. Incubation of this M_r 43000 protein with isolated mitochondria resulted in processing of the M_r 43000 precursor to the M_r 40000 mature-sized protein. Addition of carbonyl cyanide *m*-chlorophenylhydrazone and/or phenanthroline inhibits this processing. These data indicate that ferrochelatase, like most mitochondrial proteins, is synthesized in the cytoplasm as a larger precursor and is then translocated and processed to a mature-sized protein in an energy-required step.

INTRODUCTION

The terminal three steps of the haem-biosynthetic pathway, which catalyse the conversion of coproporphyrinogen III into protohaem IX, are located in the mitochondria (Jones & Jones, 1969; Poulson, 1976; Elder & Evans, 1978). Of these three enzymes, one is an extrinsic membrane protein and two are intrinsic proteins of the mitochondrial inner membrane. Ferrochelatase (protohaem ferro-lyase, EC 4.99.1.1), the terminal enzyme that catalyses the insertion of ferrous iron into protoporphyrin IX to form protohaem IX, is an intrinsic membrane protein that spans the mitochondrial inner membrane, with its active site facing the mitochondrial matrix (Harbin & Dailey, 1985). Ferrochelatase has been purified and characterized from a variety of eukaryotic sources (Taketani & Tokunaga, 1981; Dailey & Fleming, 1983; Hanson & Dailey, 1984; Dailey *et al.*, 1986), but its site of synthesis in the cell has not been examined to date.

Ferrochelatase is encoded for by nuclear genes and must be imported into the mitochondria to reach its functionally active site. A number of generalizations can be made concerning the import of nuclear-encoded proteins into mitochondria (Hay *et al.*, 1984; Douglas *et al.*, 1986; Hartl *et al.*, 1986). These proteins are usually synthesized as precursors on cytoplasmic ribosomes, and contain *N*-terminal extensions which target the proteins to the mitochondria. Insertion and translocation into the mitochondrial inner membrane requires an electrical membrane potential and, on entering the mitochondria, the proteins are processed by a proteinase which cleaves off the N-terminal extension.

To examine the synthesis and transport of ferrochelatase, we chose to compare the mouse liver system and murine erythroleukaemia (MEL) cells. MEL cells are virus-transformed murine erythroblasts that can be induced to differentiate by treatment with various chemicals such as dimethyl sulphoxide. This differentiation results in induction of the enzymes involved in haem biosynthesis (Sassa, 1976).

The data presented below indicate that ferrochelatase, like most mitochondrial-inner-membrane proteins, is synthesized as a higher-M_r precursor and is translocated into the mitochondria post-translationally in an energy-requiring step.

MATERIALS AND METHODS

Materials

All chemicals were reagent grade unless otherwise noted. Digitonin and *Staphylococcus aureus* cells (Pansorbin) were supplied by Calbiochem. Rabbit reticulocyte-lysate translation kit was obtained from Promega Biotech. HRP colour-development reagent was purchased from Bio-Rad. [^{35}S]Methionine (Amersham) had a specific radioactivity of greater than 1000 Ci/mmol.

Cell culture and radiolabelling

Friend-virus-transformed murine erythroleukaemia (MEL) DS-19 cells (supplied by D. Brenner, University of California, San Diego, CA, U.S.A.) were cultured as described previously (Sassa *et al.*, 1978) in Dulbecco's Modified Eagle Medium containing 15% (v/v) heat-inactivated fetal-bovine serum. For radiolabelling experiments, cells were seeded in fresh medium at 5×10^5 cells/ml, and 24 h later 2% (v/v) dimethyl sulphoxide (final concn.) was added to induce the cells to differentiate. Concurrent with induction, cells were radiolabelled with 5 μCi of [^{35}S]methionine/ml. At 24 h after induction, cells were harvested, washed three times in Hanks balanced salt solution, and then suspended to 10^8 cells/ml and lysed in lysis buffer [20 mM-Tris/HCl (pH 7.6), 150 mM-NaCl, 10 mM-EDTA, 2 mM-methionine, 1% Triton X-100, 0.5% cholate, 0.1% SDS, 0.4 mM-digitonin].

Immunoprecipitation and electrophoresis

Lysed radiolabelled cells (10^7 cells per sample) were immunoprecipitated by using antibodies raised in rabbits against mouse ferrochelatase. Immunoprecipitation was

Abbreviations used: MEL cells, murine erythroleukaemia cells; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

accomplished by a modification of the method of Ivarie & Jones (1979), with *S. aureus* cells (Pansorbin) as an immunoadsorbent. To decrease non-specific binding, samples were preadsorbed with 100 μl of *S. aureus* per sample. After removal of *S. aureus* by centrifugation (10 min at 10000 *g*), antibody was added and the mixture incubated overnight at 4 °C. Immune complexes were isolated by centrifugation (10 min at 10000 *g*) after addition of 50 μl of *S. aureus*. The pellet was washed four times in immunoprecipitation buffer (150 mM-NaCl, 20 mM-Tris/HCl, pH 7.6, 1% Triton X-100, 10 mM-EDTA, 0.4% SDS, 2 mM-methionine). Immune complexes were then eluted from *S. aureus* by resuspension of the pellet in SDS/polyacrylamide-gel sample buffer and boiling for 2 min. Radiolabelled proteins were analysed by SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970), followed by flourography (Matocha & Waterman, 1984).

Translation and processing

Total mouse liver RNA isolated by centrifugation through CsCl (Foster *et al.*, 1980) was translated in nuclease-treated rabbit reticulocyte lysate as per the manufacturer's instructions. After translation, radiolabelled proteins were either immunoprecipitated as described above after the addition of $\frac{1}{5}$ vol. of 5-fold-concentrated immunoprecipitation buffer or processed by isolated mouse liver mitochondria. For processing experiments, mitochondria were prepared as described by Guerra (1974) and were added directly to the translation assay after translation. To determine if dissipation of a proton gradient inhibited processing, 100 μM-carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (an uncoupler), 100 μM-phenanthroline and/or 1 mM-EDTA were added to mitochondria before incubation with translation products. Processing was carried out for 30 min in the presence and the absence of the above listed componds at 30 °C and stopped by the addition of $\frac{1}{5}$ vol. of 5-fold-concentrated immunoprecipitation buffer. Samples were then immunoprecipitated as described above.

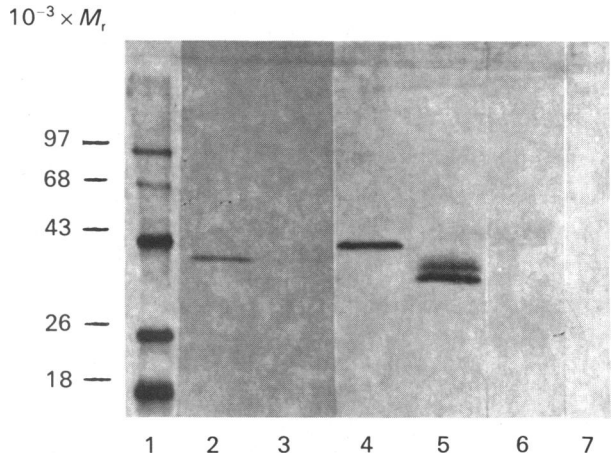

Fig. 1 Fluorogram of SDS/polyacrylamide gel of whole-cell-labelled mouse ferrochelatase

Lane 1, ^{14}C-labelled protein standards: phosphorylase *b* (M_r 97400), bovine serum albumin (68000), ovalbumin (43000), λ-chymotrypsinogen (25700), β-lactoglobulin (18400); lane 2, MEL-cell immunoprecipitate; lane 3, excess unlabelled ferrochelatase added to radiolabelled lysed MEL cells before immunoprecipitation; lane 4, cell-free translation immunoprecipitate; lane 5, limited digestion with trypsin of translation immunoprecipitate; lane 6, excess unlabelled ferrochelatase added to tranlation before immunoprecipitation; lane 7, control immunoprecipitate of radiolabelled MEL cells by using non-immune sera. Details of all procedures are in the text.

Peptide mapping

Peptide mapping of translation and cell immunoprecipitate was carried out as described by Cleveland *et al.* (1977). Before electrophoresis, 1 μg of unlabelled ferrochelatase was added to samples as a carrier.

In addition to peptide mapping, radiolabelled ferrochelatase immunoprecipitated from translation assays was subjected to limited digestion by trypsin (120 μg/ml; 30 min at 30 °C) and analysed by SDS/polyacrylamide-gel electrophoresis.

Protein blotting

Purified mouse liver ferrochelatase was electrophoresed in a Bio-Rad SDS/polyacrylamide mini-gel apparatus and transferred to nitrocellulose paper essentially as described by Towbin *et al.* (1979), except that transfer was carried out for 1 h at 30 V, 4 °C. After incubation with antibody, immune complexes were detected by using Protein A conjugated with peroxidase, and Bio-Rad HRP colour-development reagent.

RESULTS

Identification of metabolically labelled ferrochelatase

Ferrochelatase is a low-abundance protein comprising less than 0.1% of the protein of the hepatic mitochondrial membrane. This, along with the fact that it is poorly antigenic and elicits low antibody response, makes the identification of the protein by radiolabelling and immunoprecipitation difficult. Two cell types that are known to contain the highest amount of ferrochelatase are hepatocytes and differentiating erythropoietic cells. In the current study we used cultured differentiating murine erythroleukaemia (MEL) cells for a source of metabolically labelled ferrochelatase.

MEL cell cultures radiolabelled with [^{35}S]methionine and induced with dimethyl sulphoxide were harvested (approx. 2×10^6 cells/ml) and immunoprecipitated. Analysis of immunoprecipitates by SDS/polyacrylamide-gel electrophoresis revealed the presence of a single band, of M_r 40000 (Fig. 1, lane 2), which co-migrates with mature ferrochelatase purified from mouse livers. The immunological identity was further demonstrated by the fact that addition of unlabelled purified ferrochelatase decreased the amount of radiolabelled ferrochelatase that was immunoprecipitated. Addition of excess unlabelled ferrochelatase eliminated the protein band, as shown in lane 3 (Fig. 1).

Protein blotting

The specificity of antibody to ferrochelatase was also examined by protein blotting and it was found that the antibody is specific for ferrochelatase, as determined by the detection of a single band at $M_r = 40000$ (results not shown). This result is in agreement with the data obtained from cell immunoprecipitates.

Identification of ferrochelatase synthesized cell-free *in vitro*

For the translation of ferrochelatase *in vitro*, mRNA was isolated from both differentiating MEL cells and mouse liver. Although it was expected that MEL cells would contain relatively high amounts of mRNA for ferrochelatase, we found that mouse liver RNA yielded much higher amounts of translated ferrochelatase (results not shown). The reason for this is unknown at present, and requires investigation.

Isolation of RNA from a single fresh mouse liver resulted in the recovery of milligram quantities of total RNA. For translation in rabbit reticulocyte lysates, 80 μg of RNA per 100 μl of translation assay was found to be optimal. In contrast with cell immunoprecipitates, immunoprecipitation of translation assays, followed by gel eletrophoresis, revealed the presence of a sightly larger protein (M_r 43000) (Fig. 1, lane 4). Also, no protein of M_r corresponding to mature ferrochelatase could be immunoprecipitated from translation assays. As with cell immunoprecipitates, the ^{35}S-labelled protein band at M_r 43000 from translations could be decreased or eliminated by the addition of excess unlabelled ferrochelatase to the immunoprecipitation mixture (Fig. 1, lane 6).

Comparison of ferrochelatase synthesized *in vivo* and *in vitro*

In addition to antigenic identity, peptide mapping was used to demonstrate the similarity of precursor ferrochelatase to mature ferrochelatase. Limited proteolysis of precursor ferrochelatase by trypsin generated a protein of M_r 40000, the size of mature ferrochelatase (Fig. 1, lane 5), as well as a protein with M_r of about 38000. Digestion of both mature and precursor forms of ferrochelatase with *S. aureus* V8 proteinase as described by Cleveland *et al.* (1977) yield similar patterns, indicating some sequence homology (Fig. 2). Major protein bands are similar for both forms of the enzyme, with additional bands seen with the precursor form.

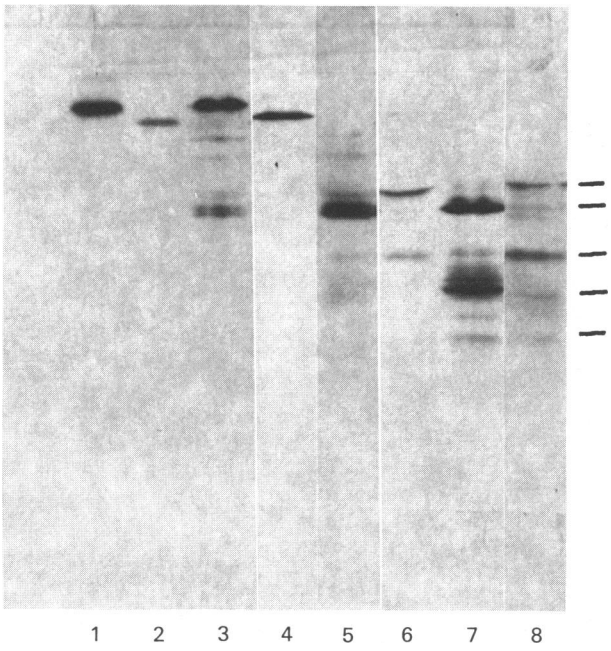

Fig. 2. Fluorogram of Cleveland-digestion peptide map of mouse ferrochelatase

Lanes 1, 3, 5, 7, translation immunoprecipitates; lanes 2, 4, 6, 8, MEL-cell immunoprecipitates; lanes 1, 2, no proteinase; lanes 3, 4, 0.02 μg of proteinase; lanes 5, 6, 0.2 μg of proteinase; lanes 7, 8, 2 μg of proteinase. Experimental details are given in the Materials and methods section. The marks in the right-hand margin indicate the most intense bands seen after digestion.

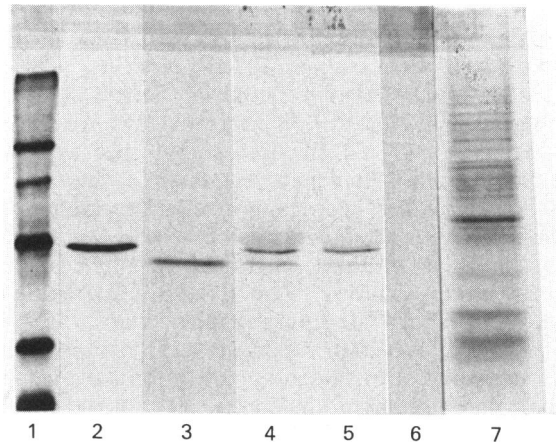

Fig. 3. Flourogram of SDS/polyacrylamide gel of precursor and processed forms of synthesized mouse ferrochelatase *in vitro*

Lane 1, ^{14}C-labelled protein standards; lane 2, immunoprecipitate of cell-free translation of mixture; lane 3, MEL-cell immunoprecipitate; lane 4, immunoprecipitation of translation mixture after processing by mitochondria; lane 5, inhibition of mitochondrial processing as described in the text; lane 6, control immunoprecipitate of cell-free translation mixture, with non-immune serum; lane 7, acetone precipitation of mouse-liver-RNA-directed translation assay.

Processing of precursor ferrochelatase by isolated mitochondria

To demostrate the relationship between the mature ferrochelatase and precursor ferrochelatase, translation assays were processed by mouse liver mitochondria. After translation, 25 μl of mitochondrial preparation (approx. 20 mg of protein/ml) was added to the translation mixture. As demonstrated in Fig. 3, lane 4, mouse liver mitochondria were able to process precursor ferrochelatase to a protein of M_r corresponding to mature ferrochelatase. Processing of precursor ferrochelatase by mitochondria was inhibited in the presence of CCCP, phenanthroline and EDTA (Fig. 3, lane 5). Addition of CCCP or phenanthroline individually also inhibited processing, and this was not reversed by addition of ATP (results not shown). Quantification of the data presented in Fig. 3 show that CCCP, phenanthroline and EDTA caused 90% inhibition of processing. These data indicate that ferrochelatase, like many mitochondrial proteins, is translocated in an energy-requiring step and processed by a metalloproteinase.

DISCUSSION

The enzymes of the haem-biosynthetic pathway in eukaryotic cells are organized in a unique fashion. The first step is catalysed by the enzyme 5-aminolaevulinate

synthase, which is found in the mitochondrial matrix; the next three steps are catalysed by cytoplasmic proteins, and the last three steps are catalysed by enzymes associated with the mitochondrial inner membrane (Kappas *et al.*, 1983). All of these enzymes are encoded in the nucleus. Of all the mitochondrially associated enzymes, 5-aminolaevulenate synthase is the only one whose synthesis has been examined in any detail. This synthase has been shown to be synthesized in the cytoplasm as a larger precursor that contains an *N*-terminal leader sequence, and it is translocated post-translationally into the mitochondrial matrix (Hayashi *et al.*, 1983). It has been reported that its transcription, translation and translocation are inhibited by high concentrations of haem, the end product of the pathway (Srivastava *et al.*, 1982; Yamauchi *et al.*, 1980). Before the present study, there had been no report about the synthesis and membrane insertion of the terminal membrane-bound enzyme ferrochelatase. The low abundance of ferrochelatase in cells, its poor immunogenicity, and the presence of a blocked *N*-terminus had slowed work on this enzyme.

Above, we have identified ferrochelatase that was synthesized both *in vitro* and *in vivo*. The synthesis *in vitro* was carried out with RNA isolated from mouse liver, whereas the synthesis *in vivo* was in growing MEL cells. Interestingly, when we attempted translation *in vitro* of RNA isolated from differentiating MEL cells, we were unable to detect significant amounts of ferrochelatase. This is in spite of the fact that the cells could be shown to be actively synthesized ferrochelatase. The explanation for the inability to produce ferrochelatase from MEL-cell RNA is unknown at present.

The product of cell-free translation was a molecule of M_r 43000. This is 3000 larger than mouse ferrochelatase synthesized *in vivo*. Incubation of the ferrochelatase synthesized *in vitro* with isolated mouse liver mitochondria resulted in the proteolytic conversion of the precursor form into the mature-sized enzyme. Such an observation is common to most mitochondrial-membrane proteins that are nuclear-encoded. So, too, is the inhibition of processing by both CCCP, which is an uncoupler that is known to dissipate the membrane potential, and the metal chelator phenanthroline. In these regards ferrochelatase appears to be similar in its biosynthesis and assembly to other mitochondrial-inner-membrane proteins in that it requires an electrogenic membrane potential for translocation. Added ATP had no effect on uptake and processing of CCCP-treated mitochondra.

In the genetic disorder protoporphyria, ferrochelatase activity is decreased to about 20% of normal (Bloomer, 1980). In cattle the disorder is genetic recessive (Bloomer *et al.*, 1982) and appears to be attibutable to production of normal amounts of a modified , less active, form of the enzyme (Bloomer *et al.*, 1987). In man, however, protoporphyria is a dominantly inherited disease, and at present there are no data available to demonstrate clearly the biochemical basis of the disorder. To explain the unusually low activity of ferrochelatase in protoporphyric cells, a variety of hypotheses have been proposed, including suggestions of multiple subunits and/or synthesis of an unstable enzyme. However, since ferrochelatase is a monomer of M_r 40000 (Dailey & Fleming, 1983), other alternatives must be proposed. Possibilities that remain to be examined are that the enzyme in protoporphyria is defective in its regulation or assembly into the membrane. Presence of a defective non-processible precursor form of ferrochelatase in these cells might block processing of the normal precursor forms of the enzyme, and thereby lead to a greater than 50% loss of activity. This model should be testable once the normal and defective human enzymes and mRNAs have been isolated.

The demonstration of a precursor and a processed mature form of normal mouse ferrochelatase is a first step towards examining the biochemical basis of protoporphyria as well as defining the precise nature of the membrane assembly of the terminal enzymes of the haem-biosynthetic pathway. Since both ferrochelatase and protoporphyrinogen oxidase, the penultimate enzyme of the pathway, are bound to the mitochondrial inner membrane and are synthesized in the cytosol, the possibility exists that they are co-ordinately synthesized and translocated. Preliminary studies in this laboratory, however, suggest that during haematopoiesis in MEL cells the two proteins may not by co-ordinately regulated (S. R. Karr & H. A. Dailey, unpublished work), and therefore may be inserted into the mitochondrial inner membrane at different times.

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