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EVIDENCE FOR A CYTOSOLIC PRECURSOR OF CHICK EMBRYO LIVER MITOCHONDRIAL δ-AMINOLEVULINATE SYNTHASE

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Following the recent demonstration [Borthwick, I.A., Srivastava, G., Brooker, J.D., May, B.K. and Elliott, W.H. (1982) Eur. J. Biochem. in press] that chick embryo liver mitochondrial δ-aminolevulinate synthase has a minimum molecular weight of 68,000 (rather than the hitherto accepted value of 49,000), we have shown that the primary translation product of δ-aminolevulinate synthase mRNA is a protein of molecular weight 74,000. This protein has for the first time been shown to occur in the cytosol fraction of drug-treated chick embryo livers. This form does not occur in mitochondria nor does the smaller mitochondrial form occur in the cytosol. It is concluded that the 74,000 molecular weight protein is a precursor which is processed during transport into the mitochondria. *In vivo* labelling experiments are consistent with this conclusion.

Evidence exists that proteins destined for transport into mitochondria are synthesized on free polysomes and traverse the membrane of the organelle post-translationally. The majority of such proteins are made as larger precursors and are processed during or after translation (1, 2).

δ-Aminolevulinate synthase [EC 2.3.1.37] is functionally located inside mitochondria where it catalyses the first step of the heme biosynthetic pathway. Its level in chick embryo liver mitochondria can be increased about 500 fold by porphyrinogenic chemicals but activity is undetectable (by colorimetric assay) in the liver cytosol (3). In contrast to the chick embryo a significant amount of active δ-aminolevulinate

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synthase is found in the cytosol of drug-treated adult chickens (4) and rats (5). It has been postulated that the cytosol enzyme of rat liver is *en route* to a mitochondrial location (6) but no direct proof of this is available.

Recently we purified, for the first time, native δ-aminolevulinate synthase from drug-treated chick embryo liver mitochondria and showed that the protein had a minimum molecular weight of 68,000 (7) rather than the accepted value of 49,000 (8). In the present study we have established that the enzyme is made as a precursor of molecular weight 74,000 and provide evidence from pulse-labelling experiments that a cytosol form of the enzyme of this molecular weight is the precursor of mitochondrial enzyme *in vivo*.

MATERIALS AND METHODS

Materials. 2-Allyl-2-isopropylacetamide (AIA) was a gift from Roche, Sydney, Australia. 3,5-Diethoxycarbonyl-1,4-dihydrocollidine (DDC) was purchased from Eastman Organic Chemicals, Rochester, New York.

Drug treatment of chick embryos. White Leghorn chick embryos (18 day old) were treated with a combination of AIA (2 mg) and DDC (4 mg) in 0.1 ml dimethyl sulphoxide (8). Control embryos were treated with 0.1 ml dimethyl sulphoxide alone.

Isolation of RNA from liver and analysis of cell free translation products. Total poly A^+ RNA was isolated using a guanidine-HCl extraction procedure and oligo(dT) cellulose chromatography as described previously (9). Poly A^+ RNA was fractionated on a sucrose/70% formamide density gradient (9), and RNA of size greater than 18S was pooled and translated in a wheat-germ cell free system at 26°C for 60 min with 6.25 μCi of [^{35}S]-methionine (1200 Ci/mmole) per 25 μl. The translation mixture was diluted to 100 μl with water and 20 μl of 10% sodium dodecyl sulphate (SDS) added prior to heating at 100°C for 2 min. To analyse total protein products, 20 μl of the boiled translation mixture was precipitated with 5 volumes of acetone at -20°C and the protein was analysed by SDS-polyacrylamide gel electrophoresis and fluorography. For immunoprecipitation of δ-aminolevulinate synthase, the remainder of the boiled translation mixture (100 μl) was diluted 6 fold with Buffer A (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate), and 10 μl of antiserum added (sufficient to precipitate all δ-aminolevulinate synthase). After incubation overnight at 4°C, 100 μl of a 10% (w/v) suspension of *Staphylococcus aureus* cells was added and incubation continued for 1 h at 4°C. Precipitates were collected by centrifugation and washed three times in Buffer A.

The final pellet was resuspended in 50 µl of gel sample buffer, heated at 100°C for 2 min, centrifuged and the supernatant analysed by SDS-polyacrylamide gel electrophoresis and fluorography.

Homologous cell free translation. Protein synthesis in a chick embryo liver post-mitochondrial supernatant was carried out as previously described (3) using 12 µCi of [^{35}S]-methionine (1200 Ci/mmole) per 100 µl of incubation mixture for 20 min at 30°C. Total products and immunoprecipitates were analysed as described above.

Translation of RNA from free and membrane-bound polysomes. As previously described (9), free and membrane-bound polysomes were prepared and total polysomal RNA isolated by phenol/chloroform extraction. Poly A$^+$ RNA was isolated (9) and translated in a wheat-germ cell free system as described above. Total products and immunoprecipitates were analysed as described above.

Preparation of mitochondrial and cytosol fractions. Each liver was homogenized in 5 ml of buffer containing 0.25 M sucrose, 5 mM Tris-HCl and 0.1 mM pyridoxal phosphate (pH 7.6) containing protease inhibitors at the following concentrations: phenylmethylsulphonyl fluoride (1 mM), 0-phenanthroline (1 mM), chymostatin (10 µg/ml) and leupeptin (10 µg/ml). Mitochondrial and cytosol fractions were prepared by differential centrifugation (7).

Mitochondria were resuspended in 1 ml of buffer containing 5 mM Tris-HCl, 0.1 mM pyridoxal phosphate, 1 mM dithioerythritol (pH 7.4) plus protease inhibitors and freeze-dried. The freeze-dried extract was resuspended in 1 ml of water containing protease inhibitors and centrifuged at 40,000 x g for 60 min. The cytosol fraction was freeze-dried and resuspended in 1 ml of water containing protease inhibitors.

For immunoprecipitation of cytosol and mitochondrial fractions, aliquots (950 µl) were diluted 1:10 with 10 x concentrated Buffer A and 50 µl of antiserum added. The mixture was treated with *S. aureus* cells and immunoprecipitates analysed as above. For analysis of total protein, aliquots (50 µl) of cytosol and mitochondrial fractions were diluted 1:1 with gel loading buffer, heated at 100°C for 2 min and analysed by SDS-polyacrylamide gel electrophoresis and fluorography.

In vivo pulse-labelling. For *in vivo* pulse-labelling, 200 µCi of [^{35}S]-methionine (about 120 Ci/mmole) in 50 µl of 20 mM sodium phosphate buffer (pH 7.6), 150 mM NaCl, was spread over the yolk sac via a small hole in the shell above the air space. For chase experiments, 50 µl of unlabelled 0.1 M methionine was layered in the same way and as well a further 200 µl of unlabelled 0.1 M methionine was injected into the fluid surrounding the chick embryo.

RESULTS AND DISCUSSION

When size fractionated (>18S) poly A$^+$ RNA from drug-treated chick embryo liver was translated *in vitro* in a wheat-germ cell free system, a protein of molecular weight 74,000 was detected immunologically using antibody to homogeneous δ-amino-

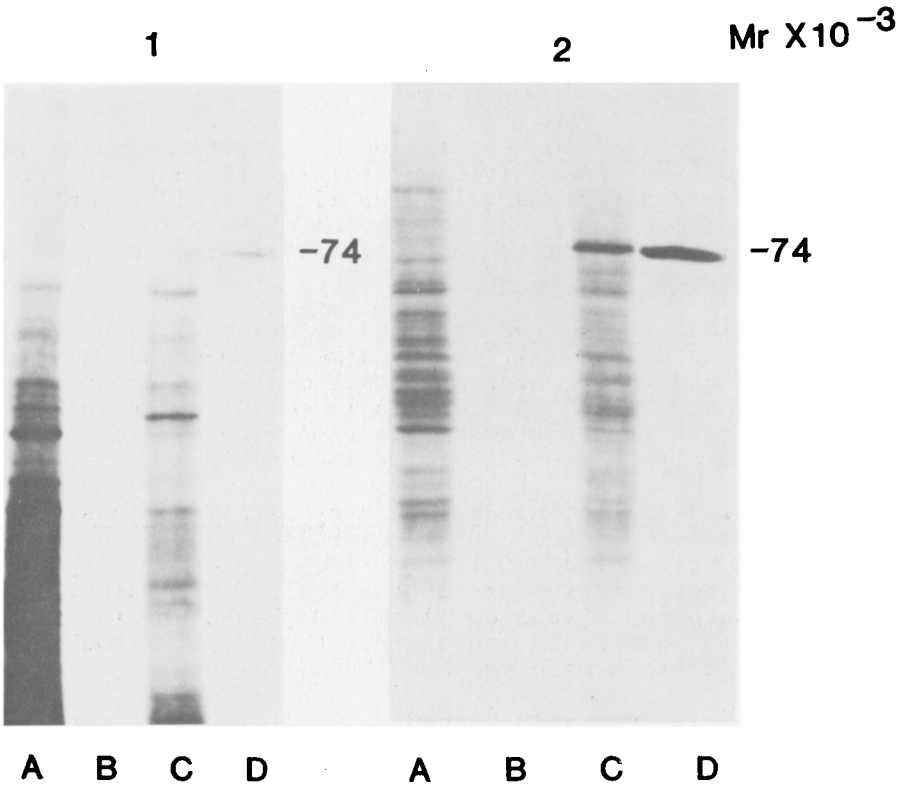

Figure 1. SDS-polyacrylamide gel electrophoretic profiles of total translation products and immunoprecipitated δ-aminolevulinate synthase.
(1) Total products and immunoprecipitates respectively from translation of poly A^+ RNA (>18S) in a wheat-germ cell free system. Control (Lanes A and B) and drug-treated chick embryos (Lanes C and D).
(2) Total products and immunoprecipitates respectively from homologous cell free translation. Control (Lanes A and B) and drug-treated chick embryos (Lanes C and D).

levulinate synthase [Fig. 1 (1)]. The same result was obtained using an homologous system when a post-mitochondrial supernatant from drug-treated chick embryo liver was incubated under conditions supporting protein synthesis [Fig. 1 (2)]. Since chick embryo liver mitochondrial δ-aminolevulinate synthase has a molecular weight of 68,000 (7), these results clearly establish that the enzyme is synthesized as a larger precursor of molecular weight 74,000 which is presumably processed to 68,000 during or after transfer into the mitochondrion.

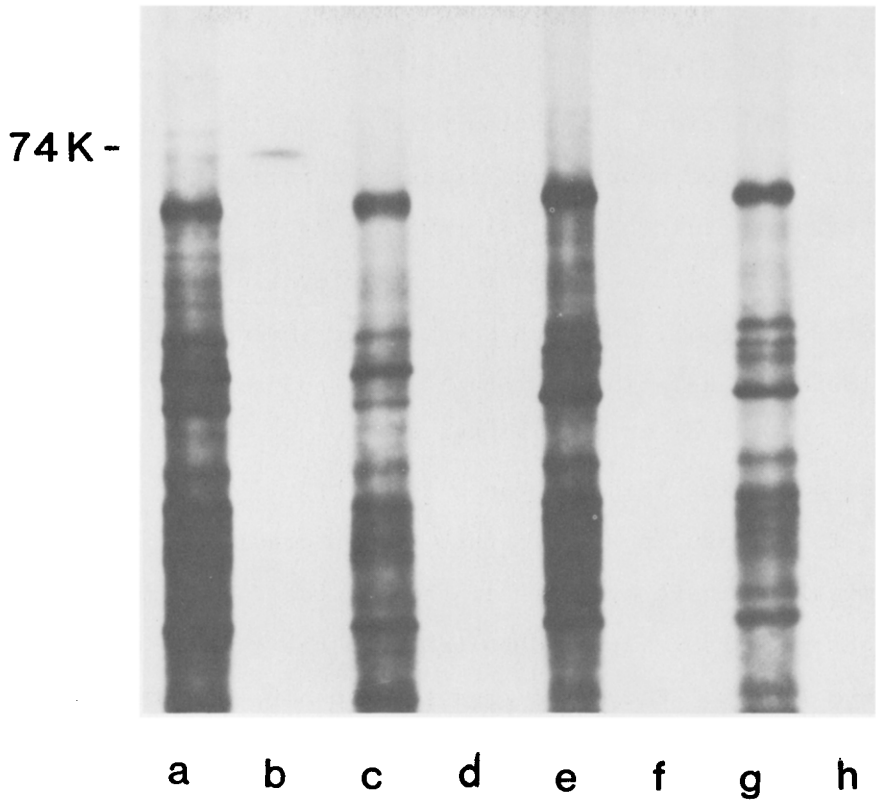

Figure 2. Gel analysis of translation products from free and membrane-bound polysomal RNA.
Total poly A^+ RNA isolated from free and membrane-bound polysomes was translated in a wheat-germ cell free system. Total products and immunoprecipitates respectively of free polysomes (drug-treated, Lanes a and b; control, Lanes e and f) and membrane-bound polysomes (drug-treated, Lanes c and d; control Lanes g and h).

Similar results have been reported by Ades and Harpe (10) who showed that following translation of chick embryo liver mRNA a protein of molecular weight 75,000 was immunoprecipitated by antibody to δ-aminolevulinate synthase. However these results were not unequivocal because the antibody used by these workers was prepared against enzyme which was only 10% pure (as judged by the reported specific activity) and was a degraded form of molecular weight 51,000.

Most mitochondrial proteins made in the cytosol are synthesized on free polyribosomes (1). That the same is true for δ-aminolevulinate synthase is shown in an experiment where

free and membrane-bound polysomes were isolated from drug-treated and control livers and total poly A^+ RNA was prepared from the fractions. When the poly A^+ RNA from free polysomes of drug-treated embryos was translated in a wheat-germ cell free system, a protein of molecular weight 74,000 was immuno-precipitated with antibody to δ-aminolevulinate synthase (Fig. 2, Lane b). No such protein was immunoprecipitated following translation of poly A^+ RNA from control free polysomes (Fig. 2, Lane d) or from either control or drug-treated membrane bound polysomes (Fig. 2, Lanes f and h).

These results suggest that, under conditions of maximal δ-aminolevulinate synthase synthesis, the 74,000 molecular weight precursor may be detectable in the cytosol of chick embryo livers. Enzymatic assays could not, however, detect any significant activity, reflecting either very low levels of enzyme or an inactive precursor. A more definitive method of analysis using pulse-labelling and immunoprecipitation with anti-δ-aminolevulinate synthase antiserum, was carried out.

Chick embryos were induced with drug for 12 h and then pulse-labelled with 200 μCi of [^{35}S]-methionine for 20 min; the cytosol and mitochondrial extract fractions were prepared and immunoprecipitated in the presence of protease inhibitors as described in Materials and Methods. δ-Aminolevulinate synthase protein of molecular weight 74,000 was detected in the cytosol fraction of drug-treated livers but not in the mitochondrial fraction while δ-aminolevulinate synthase of molecular weight 68,000 was present in the mitochondrial fraction but not in the cytosol fraction (Fig. 3, cf. Lanes F and H). In the control (not drug-treated) liver, no cytosol or mitochondrial form of the protein could be detected (Fig. 3, Lanes D and B). Separate experiments showed that the amount of cytosol protein

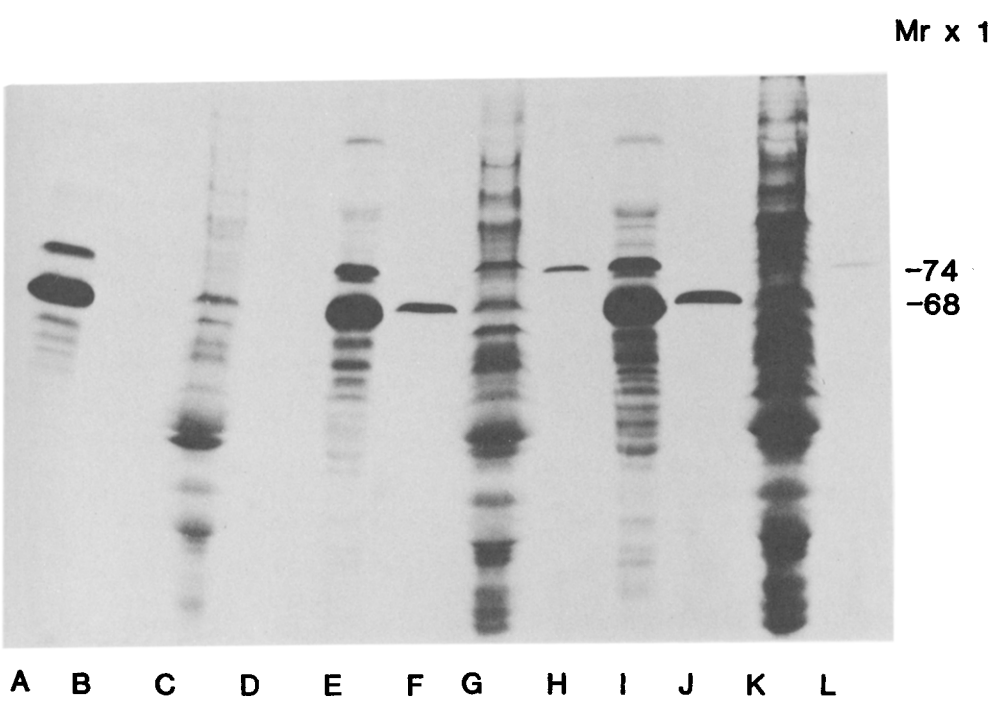

Figure 3. SDS-polyacrylamide gel analysis of total protein and immunoprecipitated δ-aminolevulinate synthase from cytosol and mitochondrial fractions.

Chick embryos drug-treated for 12 h (or controls, not drug-treated) were pulse-labelled for 20 min with [^{35}S]-methionine and cellular fractions prepared (Lanes A - H) or following the pulse-label were chased with unlabelled methionine for 15 min before preparing cellular fractions (Lanes I -L). Total proteins and immunoprecipitates respectively of mitochondria (Lanes A and B) and cytosol (C and D) of control pulse-labelled chick embryos; mitochondria (Lanes E and F) and cytosol (Lanes G and H) of drug-treated pulse-labelled chick embryos. Total proteins and immuno-precipitates respectively of mitochondria (Lanes I and J) and cytosol (K and L) of pulse-labelled and chased drug-treated chick embryos.

did not increase with longer times of drug induction (results not shown).

The fact that in the above *in vivo* experiments the 74,000 molecular weight form of δ-aminolevulinate synthase was detected only in the cytosol and the 68,000 molecular weight form only in the mitochondria clearly support the earlier proposal that the 74,000 molecular weight protein is the *in vivo* precursor of the mitochondrial enzyme and that it is processed during or after transfer into the mitochondrion. This proposal was

supported by separate labelling experiments where drug-treated chick embryos were pulse-labelled with [^{35}S]-methionine for 20 min and then injected with excess unlabelled methionine. After 15 min, cytosolic and mitochondrial fractions were prepared. All fractions were treated with anti-δ-aminolevulinate synthase antibody and analysed by SDS-polyacrylamide gel electrophoresis and fluorography.

The results show that following the chase period the cytosol form (molecular weight 74,000) of δ-aminolevulinate synthase was visibly diminished (Fig. 3, cf. Lanes H and L) while the amount of mitochondrial enzyme (molecular weight 68,000) was increased (Fig. 3, cf. Lanes F and J). This result was consistently observed over several experiments. When the chase-time was increased to 25 min, the cytosol form of δ-aminolevulinate synthase was no longer detected (result not shown). The most likely interpretation of these results is that the newly synthesized cytosol form detected by pulse-labelling studies is *en route* to the mitochondria, and that post-translational processing occurs during or soon after transfer.

It is not yet understood why cytosol δ-aminolevulinate synthase does not accumulate in chick embryo as it does in adult chickens (4) nor is it known whether the precursor in the chick embryo cytosol is enzymatically active. Further studies are in progress to investigate whether the cytosol form accumulating in the adult chick has the same molecular size as that which is detected in the cytosol of drug-treated chick embryos.

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