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BIOGENESIS OF MITOCHONDRIAL PROTEINS
REGULATION OF MATURATION OF DELTA-AMINOLEVULINATE SYNTHASE BY HEMIN

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SUMMARY. The effect of hemin on the biogenesis of delta-aminolevulinate (ALA) synthase was examined in primary cultures of chick embryo hepatocytes. Hemin (0.010 mM) in the culture medium significantly inhibited the induction of ALA synthase activity in hepatocytes exposed to the porphyrogenic agent allylisopropylacetamide. In hepatocytes pulse-labeled with [^{35}S]methionine for 45 min in the presence and absence of hemin, it was determined by immunechemical analyses that hemin blocked the processing of the precursor of ALA synthase.

Liver ALA synthase (EC 2.3.1.3.7) is the rate-limiting enzyme of the pathway of heme synthesis (1). It catalyzes the condensation of glycine with succinyl CoA to yield ALA. The enzyme is located in the mitochondrial matrix (2) but is synthesized on cytoplasmic polysomes (3,4). We have been studying the biogenesis of ALA synthase of embryonic chick liver, a model which has been very useful for defining the regulation of expression of this enzyme (1). We have reported that the enzyme in mitochondria has a molecular weight of 63,000 to 65,000 Da (5,6) and determined from cell-free translation studies that the enzyme is synthesized as a precursor of 75,000 Da (5).

Liver ALA synthase becomes induced by a variety of drugs and steroids (10) and AIA is one of the more potent inducers of the enzyme. In chick embryos, AIA significantly increases the rate of synthesis of liver ALA synthase (7). Studies on hepatocytes indicated that hemin prevents the increase in ALA synthase activity resulting from exposure of cells to AIA, and the effect of hemin appeared, in part, to be due to

Abbreviations: AIA, allylisopropylacetamide; ALA, delta-aminolevulinic acid; BSA, bovine serum albumin; PMSF, phenylmethylsulfonylfluoride; SDS, sodium dodecyl sulfate.

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inhibition of synthesis of the enzyme at a post-transcriptional step (1,4,7). In this paper, I report that hemin causes the accumulation of pre-ALA synthase in hepatocytes maintained in primary cultures. The results of the study have indicated that hemin either blocks the translocation of pre-ALA synthase into mitochondria or that it blocks the processing of the precursor following translocation into the organelles.

EXPERIMENTAL PROCEDURES

Preparation of primary cultures of hepatocytes: Primary cultures of hepatocytes from 15-day-old chick embryos (White Leghorn) were prepared by the procedure of Sassa and Kappas (8). Basically, livers were perfused with Earle's medium free of Ca^{++} and Mg^{++} then removed from the embryos. Twelve livers were pooled, minced, then incubated for 30 min at 35°C with 12 ml of 0.1% collagenase plus 0.1% hyaluronidase in the modified Earle's medium to separate the cells. During the last 10 min of incubation, the suspension of cells was drawn several times in and out of a pasteur pipet to help separate the cells. The cells were collected by centrifugation and suspended in 15 ml of buffer consisting of 0.13 M NH_4Cl, 0.017 M Tris, 0.01 M $KHCO_3$, pH 7.6. The suspension of cells was incubated for 10 min at 35°C to lyse red blood cells. The cells were collected by centrifugation, and the wash with ammonium-containing buffer was repeated. The cells were collected by centrifugation, and the pellet was suspended in 200 ml of modified Ham's F-12 medium (described below) containing 10% fetal calf serum. Aliquots from the cell suspension were delivered into plastic tissue culture dishes, and the cells were maintained at 37°C in a humidified incubator (95% air, 5% CO_2). After 16 h, the medium was replaced with modified Ham's F-12 containing 0.2mg/l of insulin and 0.5 mg/ml of deferoxamine mesylate (CIBA Pharmaceutical Company, Summit, NJ, USA). The cells were incubated for 2 h at 37°C then the medium was replaced with fresh modified Ham's/insulin/deferoxamine medium. AIA was added to the culture medium at this point to a final concentration of 0.075 mg/ml (from a stock solution of 60 mg of AIA per milliliter of ethanol). Where indicated, hemin was added to the culture medium from a stock solution containing 2.5 mg of hemin per milliliter of 0.015 M KOH in 50% ethanol. Modified Ham's F-12 medium was prepared in our laboratory as Ham's F-12 (9) lacking sodium pyruvate and fatty acids and containing twice the concentration of glutamine; the medium also contained per liter 10^5 units of penicillin G, 100 mg of streptomycin, 2 X 10^4 units of nystatin, and 10 ml of Fungizone (Flow Laboratories, McLean, VA, USA).

Determination of ALA synthase activity: Cells in given 10-cm dishes were washed with two aliquots of 4 ml saline at 37°C. The cells in each dish were homogenized in 2 ml of homogenizing buffer (0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, 1 mM EGTA, 0.01 mM pyridoxal phosphate) using a rubber policeman. The cells from each set of culture dishes were pooled and collected by centrifugation. The resulting pellet of cells was suspended in 0.5 ml of homogenizing buffer and the cells were homogenized by mild sonication [one 15 s burst using the microtip of a Sonifier Cell Disruptor (Heat Systems Ultrasonics Inc, Long Island, NY, USA) at a setting of 1]. Aliquots from the cell homogenate were assayed in duplicates for ALA synthase activity using the method of Sassa et al (10). Protein was determined by the method of Lowry et al (11) with bovine serum albumin serving as a standard.

Immunological methods: Antibodies against highly purified ALA synthase (6) were raised in New Zealand white rabbits using standard protocols previously described (5).

To immuneprecipitate ALA synthase, primary cultures of hepatocytes labeled with [^{35}S]methionine were rinsed in saline and scraped in homogenizing buffer using a rubber policeman. In these studies, hepatocytes from three 35-mm dishes were pooled. The cells were collected by centrifugation then resuspended in 0.45 ml of 50 mM Tris-HCl, pH 7.4 at 0°C. The cells were disrupted by sonication for 15 s as described above and brought to 5 mM PMSF immediately. To this homogenate 0.50 ml of buffer consisting of 1 M NaCl, 20 mM Tris-HCl, pH 8.3, 2% Triton X-100 (two fold concentrated buffer A) was added and the suspension was brought immediately to 5 mM PMSF. The cell lysate was centrifuged at 15,000 X g for 10 min at 4°C and the supernatant was saved for analysis. To immuneprecipitate ALA synthase, an aliquot of the cell lysate was mixed with 0.003 ml of the given serum then incubated at 4°C for 3 h. At the end of incubation, 0.350 ml of buffer A containing 0.25 mg of goat anti-rabbit IgG covalently linked to a solid phase (immunobeads obtained from Bio-Rad Laboratories, Richmond, Ca, USA) was added to the lysate, and the suspension was incubated at 4°C for two hours. Following the second incubation, the immunobeads were collected by centrifugation then washed twice with 0.8 ml of buffer A followed by two washes with 0.8 ml of 10 mM Tris-HCl, pH 8.3. Immuneprecipitated material was released from the immunobeads by boiling for 3 min in 0.1 ml of buffer consisting of 5% SDS, 1 mM EDTA, 0.1 M Tris-HCl, pH 6.8, 0.03% bromphenol blue, 20% glycerol, 1% beta-mercaptoethanol. The immunobeads were removed by centrifugation and the supernatant was saved for analysis.

Electrophoretic analyses: Electrophoretic analyses of proteins were carried out on 1.5 mm thick slabs of 10% polyacrylamide gels in the presence of SDS using the system of Laemmli (12). Proteins were stained with Coomassie blue R using standard methods (12). Proteins labeled with [^{35}S] were detected by fluorography (13). The standards used in these analyses and their respective molecular weights were: phosphorylase B, 94,000; BSA, 68,000; ovalbumin, 46,000; carbonic anhydrase, 30,000; and cytochrome *c*, 12,400.

Sources of materials: AIA was a gift from Hoffman-LaRoche, Nutley, NJ, USA. [^{35}S]methionine was purchased from Amersham, Arlington Heights, Il, USA.

RESULTS AND DISCUSSION

Primary cultures of chick embryo hepatocytes were used to examine the effect of hemin on the rate of synthesis of ALA synthase. As has been well established by others (7,8), AIA caused a significant increase in ALA synthase activity in hepatocytes maintained as primary cultures. The activity reached maximum approximately 16 h following exposure of cells to AIA, changing from 0.12 nmol of ALA produced/h/mg cell protein before exposure to AIA to 3.2 nmol/h/mg protein at the end of 16 h. Addition of 0.010 mM hemin to the culture medium of hepatocytes exposed to AIA significantly lowered ALA synthase activity (fig. 1). The effect of hemin

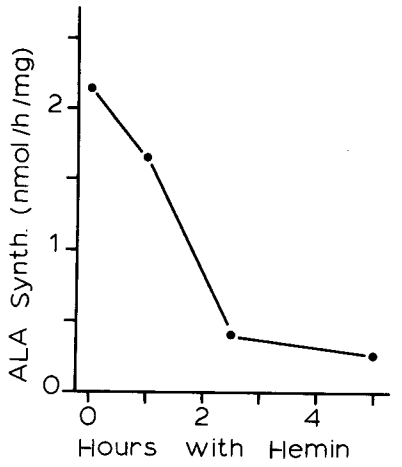

Fig. 1. Effect of hemin on ALA synthase activity in hepatocytes maintained in primary cultures. Primary cultures of hepatocytes were prepared as described in Experimental Procedures. AIA was added to the medium at zero hour, and sixteen hours later the cells were assayed for ALA synthase activity. At the indicated times prior to the assay, hemin (0.010 mM) was added to some of the cultures. Five 10-cm culture dishes (5 mg protein/dish) were pooled for each determination. ALA synthase activity is expressed in nmol of ALA produced per hour per milligram protein.

has been thought to be the result of inhibition of synthesis of the enzyme (7) coupled with the normal relatively rapid turn-over rate of the enzyme in hepatocytes (7). To determine whether hemin affected synthesis of the enzyme, hepatocytes exposed to AIA for 5.5 h were incubated with [^{35}S]methionine in the presence and absence of hemin for 45 min. Following incubation, the cells were lysed and ALA synthase was immuneprecipitated from cell extracts. As shown in fig. 2, hemin did not appear to have a significant effect on overall protein synthesis in hepatocytes (slots 5 and 6). While labeled mature ALA synthase could be recovered from cells treated with AIA (fig.2, slot 3), no labeled mature ALA synthase could be recovered from hepatocytes exposed to hemin. Instead, hemin caused the accumulation of the 75,000 Da pre-ALA synthase in hepatocytes (fig. 2, slot 1)

Most mitochondrial proteins have been found to be synthesized on cytoplasmic ribosomes as larger precursor forms (14). The precursors become processed to their respective mature molecular weights either during or immediately following their transfer into mitochondria (14). The results of this paper indicated that hemin either blocked the transport of pre-

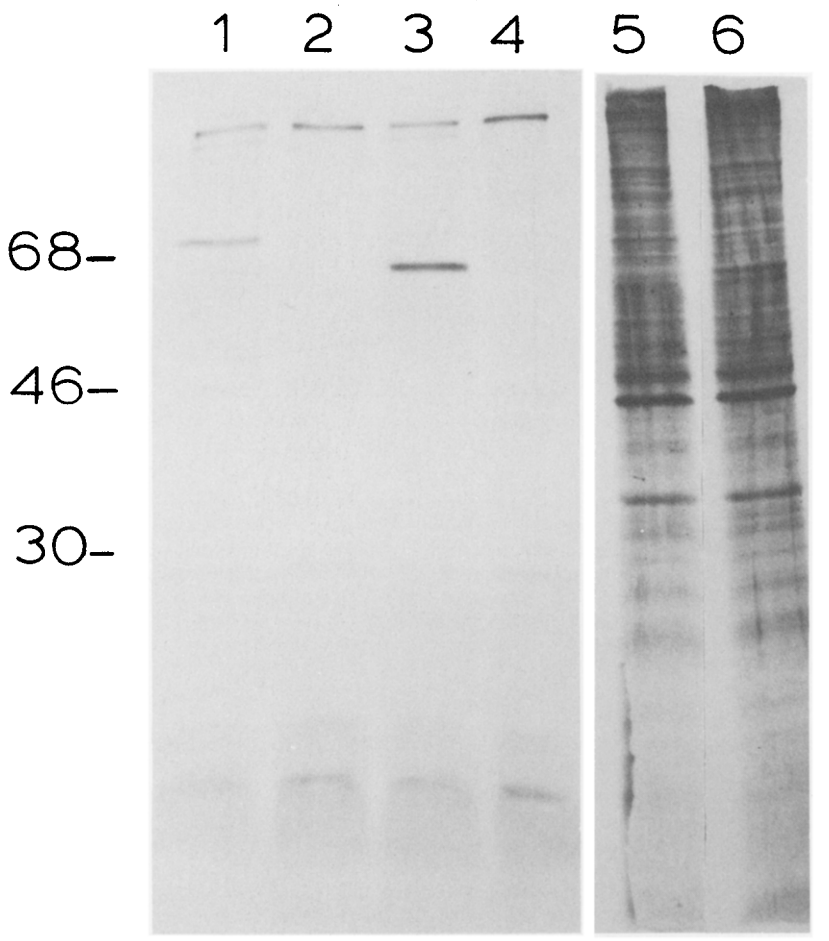

Fig. 2. Effect of hemin on the synthesis of ALA synthase as determined by pulse-labeling. Primary cultures of hepatocytes were incubated in the presence of AIA for 5.5 h under the conditions described in Experimental Procedures. The medium was then replaced with an identical medium containing AIA, but lacking methionine. At that point, hemin (0.010 mM) was added to half the cultures and immediately [^{35}S]methionine was added to all cultures (0.020 mCi/ml). The cells were incubated for 45 min then lysed, and ALA synthase was immunoprecipitated from aliquots of the cell lysates containing 1.5 X 10^6 cpm of [^{35}S]. The immunoprecipitated material was analyzed by polyacrylamide gel electrophoresis in the presence of SDS and detected by fluorography. Slots 1-4 show the compositions of immunoprecipitates obtained from: 1, hemin-treated cells and precipitation with immune serum; 2, hemin-treated cells and precipitation with pre-immune serum; 3, no hemin and precipitation with immune serum; 4, no hemin and precipitation with pre-immune serum. Slots 5 and 6 show the compositions of the cell lysates used for the immune precipitations. Slot 5, lysate from hemin-treated cells; slot 6, lysate from cells without hemin. The migrations of molecular weight markers, expressed in 10^3 X Da, are indicated.

ALA synthase into mitochondria or that it inhibited the processing of this protein following translocation of the protein into the organelles. In addition, densitometer scans of the slots on the fluorograph in fig. 2 indicated that hemin may have also inhibited synthesis of the precursor

or led to its rapid degradation. The results were observed in several experiments (data not shown).

In their studies on rat, Kikuchi and his colleagues (15) demonstrated that hemin injections into AIA-treated animals caused the accumulation of an enzymatically active ALA synthase in the cytosol fraction of liver, and it was suggested that hemin blocked the transfer of ALA synthase into mitochondria from its site of synthesis in the cytoplasm. It can not be established rigorously, however, on the basis of the data obtained by Kikuchi and his collaborators (15) whether the active form detected in the cytosol was in fact pre-ALA synthase or was newly-synthesized enzyme which selectively leaked out of mitochondria due to hemin treatment. These data become even more difficult to evaluate in view of the report that most of their studies on the molecular properties of ALA synthase were carried out on a fragment of the enzyme (16).

In conclusion, the results of this study indicated that hemin regulated the maturation of ALA synthase and established a novel level of regulation of gene expression.

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