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INHIBITION BY HEMIN OF IN VITRO TRANSLOCATION OF CHICKEN LIVER δ-AMINOLEVULINATE SYNTHASE INTO MITOCHONDRIA

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SUMMARY The precursor form of chicken liver δ-aminolevulinate synthase was synthesized in a reticulocyte lysate cell-free translation system and then incubated with the homologous liver mitochondria. The precursor enzyme was incorporated into the mitochondria with an attendant processing to the mature enzyme. In this in vitro experimental system, both the transport and processing of the enzyme were significantly inhibited by the addition of hemin as low as about 3 μM. This provides further support to the view, which had been derived from the studies in vivo, that inhibition by hemin of the translocation of δ-aminolevulinate synthase into mitochondria could be one of the regulatory mechanisms for heme biosynthesis in the liver cells.

Liver δ-aminolevulinate synthase (EC 2.3.1.37) is a rate-limiting enzyme of the heme biosynthetic pathway (1). This enzyme is synthesized on cytoplasmic free ribosomes as a larger precursor protein and transferred post-translationally into the mitochondrial matrix with an attendant processing to the mature enzyme (2-4). Studies with chemically induced porphyria animals revealed that synthesis of δ-aminolevulinate synthase in the liver is subject to feedback control by heme at both the transcriptional (5-7) and translational (8-13) levels. Moreover, our previous studies in vivo indicated that heme inhibits the transfer of the enzyme from cytosol to mitochondria, thus leading to an increased accumulation of the precursor enzyme in the liver cytosol (13-15). Recently Ades also reported that treatment of cultured chick embryo liver cells with hemin blocked the processing of the precursor of δ-aminolevulinate synthase (16). δ-Aminolevulinate synthase is probably the only case for which the occurrence of regulation of intracellular translocation has been clearly demonstrated.

Recently, we have reported that chicken liver δ-aminolevulinate synthase is synthesized in a cell-free translation system as a polypeptide with a molecular weight of about 73,000, while the mature enzyme in mitochondria has a subunit molecular weight of about 65,000 (4). The precursor accumulating in

Abbreviations: CCCP, carbonyl cyanide m-chlorophenylhydrazone; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.

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the cytosol has apparently the same size as that of the enzyme synthesized in vitro (unpublished). In the present study we have demonstrated that δ-aminolevulinate synthase synthesized in a reticulocyte lysate cell-free translation system was imported into mitochondria and concomitantly converted to the size of the mature enzyme when incubated with isolated mitochondria, and that both import and processing of the precursor enzyme were blocked by the addition of hemin to the in vitro transfer system.

MATERIALS AND METHODS

Materials: L-[4,5-^{3}H]leucine (130 Ci/mmol) and [^{14}C]methylated proteins were obtained from Amersham International Ltd.; hemin, from Sigma Chemical Co.; human serum albumin, from Miles Laboratories, Inc.; microbial protease inhibitors (antipain, chymostatin, elastatinal, leupeptin and pepstatin), from Peptide Institute, Osaka.

Drug Treatment of Chickens: For the induction of liver δ-aminolevulinate synthase, white Leghorn chickens weighing about 100 g were treated with allylisopropylacetamide and 3,5-diethoxycarbonyl-1,4-dihydrocollidine in a similar way to that described previously (4).

Preparation of Polysomes: Total liver polysomes were prepared from drug-treated chickens as described previously (4).

Preparation of Mitochondria and Cytosol Fraction: The liver was removed from drug-untreated chickens and homogenized in 9 volumes of 220 mM mannitol containing 70 mM sucrose and 2 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate (Hepes)-KOH buffer (pH 7.4) (medium A). The homogenate was centrifuged at 650 x g for 10 min, and the supernatant was centrifuged again at 5,000 x g for 10 min to obtain a mitochondrial pellet. The mitochondria were washed once with 5 volumes of medium A and resuspended in the same medium. For the preparation of the cytosol fraction, the liver was homogenized in 2 volumes of medium A, and the homogenate was centrifuged successively at 20,000 x g for 15 min and at 160,000 x g for 90 min. The resulting supernatant was used as the cytosol fraction.

Cell-Free Synthesis of δ-Aminolevulinate Synthase: The rabbit reticulocyte lysate used was the supernatant after the centrifugation at 150,000 x g for 90 min. The reaction mixture contained, in a final volume of 100 µl, 20 mM Hepes-KOH buffer (pH 7.5), 100 mM KCl, 2 mM magnesium acetate, 2 mM dithiothreitol, 0.6 mM spermidine, 1 mM ATP, 0.2 mM GTP, 8 mM creatine phosphate, 50 µg of creatine kinase, 25 µM each of 19 amino acids except leucine, 10 to 20 µCi of [^{3}H]leucine, 0.1 mM pyridoxal phosphate, 10 µg of bovine liver tRNA, 1 to 2 A_{260} units of polysomes and 50 µl of reticulocyte lysate. The mixture was incubated at 30°C for 40 min; at the end of incubation mannitol, sucrose, Hepes-KOH buffer (pH 7.4) and cycloheximide were added to final concentrations of 220 mM, 70 mM, 10 mM and 100 µg/ml, respectively.

Incubation of δ-Aminolevulinate Synthase Synthesized In Vitro with Mitochondria: The translation mixture containing the precursor labeled with [^{3}H]-leucine was incubated with chicken liver mitochondria (3-4 mg of protein/ml) for 1 h at 25°C in a volume of 0.3 ml. The incubation mixture was also supplemented with human serum albumin (3 mg/ml) and chicken liver cytosol (3 mg of protein/ml) to support the transfer reaction of the precursor, since preliminary experiments indicated that the efficiency of the enzyme transfer was considerably higher when these components were added. Reaction was stopped by chilling at 0°C, and the mitochondria were separated from the supernatant by centrifugation at 20,000 x g for 10 min. A portion (50-100 µl) of the supernatant was mixed with 10 volumes of 0.05 M Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl, 1% Triton X-100, 5 mM EDTA, 0.1 mM pyridoxal phosphate, 5 mM leucine, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 50 µg/ml each of five microbial protease inhibitors (medium B) and subjected to immunoprecipitation. The mitochondrial pellet was suspended in 0.5 ml of medium A containing 100 µg/ml of trypsin and incubated for 30 min at 0°C, being followed

by the addition of 1 mM PMSF and 1 mg/ml leupeptin. The mitochondria were collected and washed once by centrifugation with medium A containing 1 mM PMSF and 1 mg/ml leupeptin. When the trypsin treatment was omitted, the mitochondria were washed twice with medium A containing the same protease inhibitors as above. The washed mitochondria were solubilized in 0.5 ml of medium B, followed by centrifugation to remove insoluble materials, and used as the mitochondrial extract for immunoprecipitation. About 5 mM hemin solution was prepared in ethylene glycol (17) and diluted with medium A to an appropriate concentration immediately before use. Hemin concentration was determined by the pyridine hemochrome method (18).

Immunoprecipitation: The sample was incubated with about 50 μg of anti-chicken liver δ-aminolevulinate synthase IgG prepared as described previously (4), then added with 20 mg dry weight of Protein A-Sepharose CL-4B (Pharmacia Fine Chemicals) and gently shaken for 2 h at 0°C. Protein A-Sepharose was collected by centrifugation and washed 4 times with medium B and once with 0.05 M Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl and protease inhibitors as in medium B. The gel was suspended in 150 μl of 0.125 M Tris-HCl buffer (pH 6.8) containing 2% sodium dodecyl sulfate (SDS), 5% 2-mercaptoethanol and 20% glycerol, and heated for 5 min in a boiling-water bath. The supernatant was analyzed by SDS-10% polyacrylamide gel electrophoresis.

Analytical Methods: SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli (19). Fluorography was carried out by using EN^{3}HANCE (New England Nuclear). Radioactivity in sliced gels was counted as described previously (7). Protein concentration in the mitochondria and cytosol fractions was determined by the biuret method (20).

RESULTS

Transfer of δ-Aminolevulinate Synthase into Isolated Mitochondria: A reticulocyto lysate system supplemented with chicken liver polysomes was allowed to incorporate [^{3}H]leucine into proteins and then was incubated with mitochondria for 60 min at 25°C in the presence of cycloheximide. Then the mitochondria and the supernatant were separated by centrifugation and analyzed for δ-aminolevulinate synthase by immunoprecipitation and SDS-polyacrylamide gel electrophoresis. As shown in Fig. 1A, the mitochondria after the incubation contained two major components (lane 2); the first one had the same molecular weight as that of the precursor form of δ-aminolevulinate synthase recovered from the supernatant (Mr = about 73,000) (lane 1), and the second one coincided in molecular weight with the mature enzyme in the mitochondria (Mr = about 65,000) (lane 4). No apparent component corresponding to the mature enzyme was observed in the supernatant (lane 1). When these mitochondria were incubated with trypsin at 0°C for 30 min before lysis, the second component was largely resistant to proteolysis, while most of the first component disappeared (lane 3). These findings indicate that the precursor δ-aminolevulinate synthase was incorporated *in vitro* into a mitochondrial site, probably into the matrix, where the enzyme was no longer easily accessible to the added protease, and that the precursor was converted to the mature enzyme by a proteolytic processing during its transfer into mitochondria. The precursor recovered from the mitochondria (lane 2) seems to have been bound mostly to the outer surface of mitochondria.

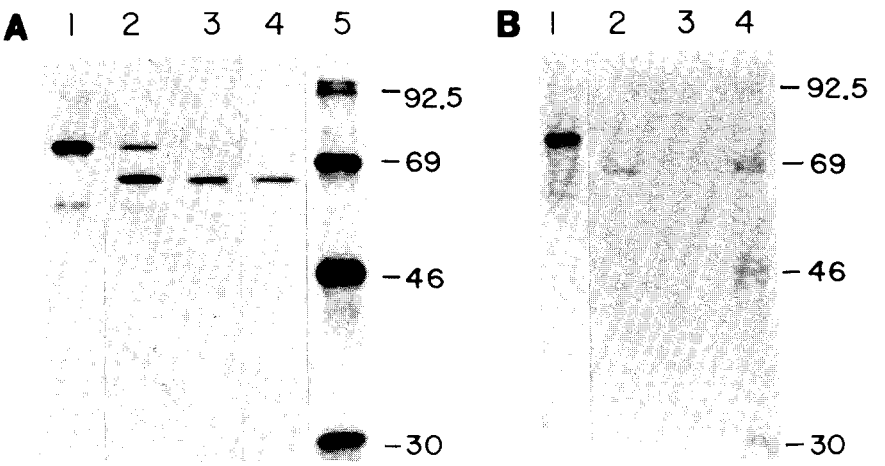

Fig. 1. Transfer into isolated mitochondria of the δ-aminolevulinate synthase synthesized in a cell-free translation system. Conditions for protein synthesis and for the transfer reaction were as described in the Methods section except for CCCP; CCCP, when added, was 10 μM. After the incubation for translocation, the mitochondria were separated from the supernatant. δ-Aminolevulinate synthases were immunoprecipitated from the supernatant and the mitochondrial extract, and analyzed by SDS-polyacrylamide slab gel electrophoresis and fluorography. A. Lane 1, supernatant; lane 2, mitochondria, without trypsin treatment; lane 3, mitochondria, with trypsin treatment; lane 4, mitochondrial enzyme labeled in vivo as described previously (4); lane 5, [^{14}C]methylated Mr marker proteins: phosphorylase b (92,500) bovine serum albumin (69,000), ovalbumin (46,000) and carbonic anhydrase (30,000). B. Lane 1, supernatant; lane 2, mitochondria, without CCCP; lane 3, mitochondria, with CCCP; lane 4, [^{14}C]methylated Mr marker proteins. Numbers to the right of the figures represent Mr x 10^{-3}.

Fig. 1B shows that the transfer of δ-aminolevulinate synthase into mitochondria in vitro was blocked by the addition of 10 μM carbonyl cyanide m-chlorophenyl hydrazone (CCCP), an uncoupler of oxidative phosphorylation. This would indicate that the enzyme was translocated into mitochondria in this reconstituted system by some mechanism involving a certain energized state of the mitochondrial membrane, as has been proposed for mitochondrial proteins which have to cross the inner membrane (21).

Inhibition by Hemin of In Vitro Transfer of δ-Aminolevulinate Synthase into Mitochondria: The in vitro enzyme transfer reaction as described above was carried out in the presence of various concentrations of externally added hemin. After the incubation, the mitochondria were separated, treated with trypsin, and δ-aminolevulinate synthase was isolated from the mitochondrial extract by immunoprecipitation and was analyzed by gel electrophoresis.

As shown in Fig. 2A, both the transfer of δ-aminolevulinate synthase into mitochondria and the concomitant processing to the mature enzyme were markedly

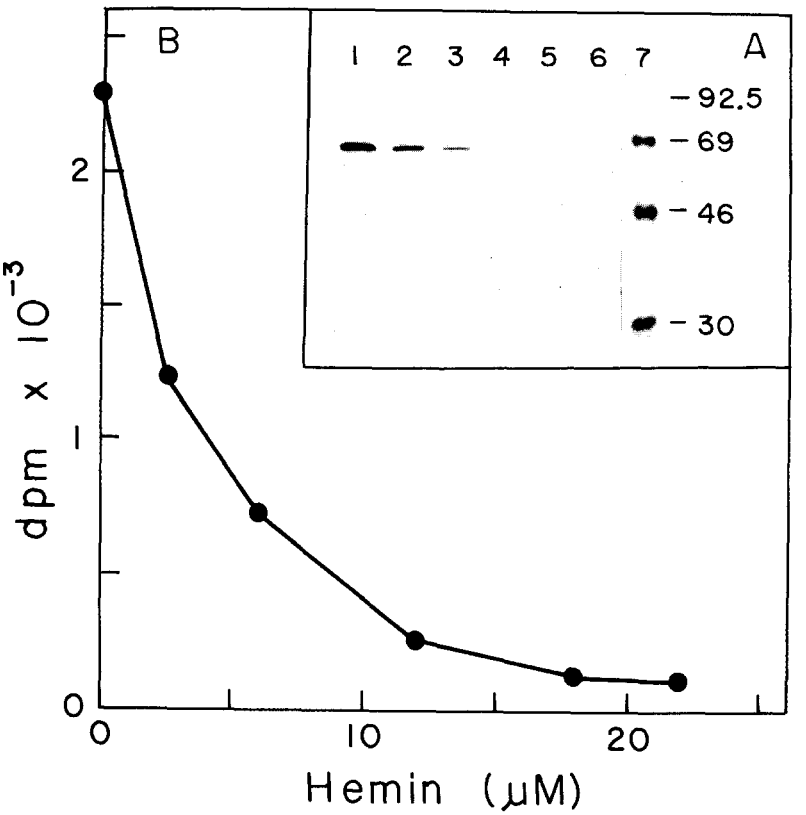

Fig. 2. Inhibition by hemin of the transfer of δ-aminolevulinate synthase into mitochondria in vitro. Transfer in vitro of δ-aminolevulinate synthase synthesized in reticulocyte lysate to isolated mitochondria was performed in the presence of various concentrations of hemin added. Immunoprecipitates obtained from the trypsin-treated mitochondria were analyzed by SDS-polyacrylamide slab gel electrophoresis visualized by fluorography (A) and by SDS-polyacrylamide rod gel electrophoresis followed by counting of the radioactivity in sliced gels (B). A. Concentrations of hemin added were as follows (μM); lane 1, o; lane 2, 2.5; lane 3, 6; lane 4, 12.5; lane 5, 18; lane 6, 25. Lane 7, [^{14}C]methylated Mr marker proteins. Numbers to the right of the figure represent Mr x 10^{-3}. B. Sum of the radioactivity in the three 65,000-dalton peak fractions is plotted against the final concentration of hemin added.

inhibited by the addition of hemin. In an experiment we analyzed δ-aminolevulinate synthase isolated from the trypsin-untreated mitochondria and found that the band of the mature enzyme on the slab gel was also significantly reduced in the hemin-added system while the band of the precursor form of the enzyme was not appreciably influenced by hemin (data not shown).

In the experiment shown in Fig. 2B, the same samples as in Fig. 2A were analyzed for radioactivity in the 65,000-dalton fractions on the rod gel which correspond to the mature enzyme. The translocation of δ-aminolevulinate synthase into mitochondria, under the conditions employed, was reduced to about

50% by the addition of about 3 μM hemin and was almost completely inhibited by 25 μM hemin.

DISCUSSION

The present study demonstrated that the precursor form of δ-aminolevulinate synthase is post-translationally transferred into mitochondria with concomitant processing of the enzyme protein and that the transfer of the enzyme protein is actually inhibited by hemin. The effective concentration of hemin added to the *in vitro* transfer system, however, was much higher than that of the so-called "regulatory" heme or free heme in the liver cell which has been assumed to be about 10^{-8} M (7, 13, 22) and which has been supposed to be adequate for the regulation of intracellular translocation of δ-aminolevulinate synthase in the liver cell (13). The requirement of higher concentrations of hemin for the *in vitro* translocation is probably due, at least in part, to nonspecific binding of hemin to large amounts of proteins in the incubation mixture, especially human serum albumin added (about 3 mg/ml), thus reducing the effective concentration of hemin as the inhibitor. In fact, as mentioned in the Methods section, in the reaction system without hemin added, the transfer as well as the processing of the precursor enzyme was considerably smaller when the incubation mixture was not added with either one or both of human serum albumin and the liver cytosol, although the effect of these additions may not be solely accounted for by their heme-binding capacities. It is interesting in this connection to note that Kd of human serum albumin for hemin was also reported to be about 10^{-8} M (23). At any rate, it seems reasonable to assume that the same mechanism may be operating in both *in vitro* and *in vivo* systems in the hemin inhibition of the translocation of δ-aminolevulinate synthase.

Post-translational transfer of extramitochondrial precursors into mitochondria can be inhibited when an electrochemical potential across the inner membrane is dissipated by uncouplers, ionophores or inhibitors of electron transport (21, 24, 25). In addition, Miura *et al.* reported that basic proteins such as protamines and histones strongly inhibited the precursor processing of ornithine transcarbamylase, suggesting that the basic proteins may compete with the precursor for interaction with mitochondria (26). Among these inhibitors, hemin is unique in that it is the end-product of the biosynthetic pathway and it blocks the intracellular translocation of the rate-limiting enzyme in the biosynthetic pathway in a feedback manner. One possibility to account for the observed hemin effect is that hemin binds or interacts specifically with the precursor form of the enzyme so as to interfere with the interaction of the precursor protein with the component(s) of the transport machinery. Recently it was demonstrated in this laboratory that hemin also inhibits the synthesis of δ-aminolevulinate synthase directed by rat liver polysomes in a reticulocyte lysate cell-free translation system, and

the data obtained suggested that a specific interaction of hemin with nascent peptide chains of the enzyme might be important in the inhibition (27).

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