Vol. 115, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1983 Pages 225-231

TRANSLATIONAL INHIBITION BY HEME OF THE SYNTHESIS OF HEPATIC δ-AMINOLEVULINATE SYNTHASE IN A CELL-FREE SYSTEM

Masayuki Yamamoto, Norio Hayashi, and Goro Kikuchi

Department of Biochemistry, Tohoku University School of Medicine, Sendai 980, JAPAN

Received July 15, 1983

SUMMARY. Synthesis of δ-aminolevulinate synthase in a rabbit reticulocyte lysate system directed by total polysomes from the liver of allylisopropylacetamide-treated rats was studied with the combined use of [^{3}H]leucine and a specific rabbit antibody. The protein synthesis observed in the cell-free system employed represented mainly the peptide chain elongation and its termination rather than the net synthesis involving initiation. Synthesis of δ-aminolevulinate synthase in this cell-free system was inhibited progressively with the increased addition of hemin; the synthesis was reduced to about 40% by about 30 μM hemin. Synthesis of total protein, however, was not significantly affected by the addition of hemin. The data obtained suggest that heme inhibits a peptide chain elongation step in the synthesis of δ-aminolevulinate synthase.

Regulation of heme biosynthesis in animals has been explored through the study of experimental porphyria which can be easily induced by the administration of porphyrinogenic drugs such as allylisopropylacetamide (AIA) (1). In hepatic porphyria animals the amount of δ-aminolevulinate (ALA) synthase [EC 2.3.1.37], which is the first and rate-limiting enzyme of the heme biosynthetic pathway, is greatly increased in liver mitochondria, and under these conditions a considerable amount of ALA synthase accumulates in the liver cytosol fraction. The enzyme accumulating in the liver cytosol fraction has been shown to be a precursor in transit to the mitochondria (2, 3).

Several lines of evidence suggest that heme inhibits the synthesis of ALA synthase at both transcriptional (4-6) and translational (7-12) steps. In addition, heme also inhibits the transfer of ALA synthase from the cytosol into mitochondria in the liver (2, 3, 12). Thus, currently ALA synthase in the liver is thought to be under strict feedback regulation by heme at three sites. Among these regulations by heme, the translational control of synthesis of ALA synthase was first suggested through the kinetic studies of the decrease in the activity of ALA synthase which was observed in cultured chick embryo liver cell systems after the addition of either hemin or inhibitors of protein synthesis

The abbreviations used are: AIA, allylisopropylacetamide; ALA, δ-aminolevulinate; SDS, sodium dodecyl sulfate; S_{150}, the supernatant of the lysed cell suspension centrifuged at 150,000 x g for 90 min.

0006-291X/83 $1.50

(7-10). Subsequently the study was extended to mammalian systems, and we have obtained evidence that the translational control by heme also exists in the rat liver (11, 12).

To further clarify the mechanism of translational control of the ALA synthase synthesis by heme, we have examined the effect of the addition of hemin on cell-free synthesis of rat liver ALA synthase in a rabbit reticulocyte lysate system. The results obtained in the present study suggest that heme inhibits specifically the synthesis of ALA synthase principally at an elongation step of protein synthesis.

EXPERIMENTAL PROCEDURES

Conditioning of Rats and Isolation of Ribosomes. Male Std: Wistar strain rats weighing about 150 g were injected subcutaneously with two doses of AIA (30 mg/100 g body weight/dose) at a 12 h interval and killed by decapitation 15 h after the first injection of AIA. Total polysomes were prepared in the same way as described previously (6). The polysome pellets finally obtained were suspended to give a final concentration of 180-250 A_{260} units/ml.

Polysome-Directed Cell-Free Protein Synthesis in a Rabbit Reticulocyte Lysate System. The rabbit reticulocyte lysate used in this study was the supernatant of the lysed cell suspension centrifuged at 150,000 x g for 90 min (S_{150}). Cell-free protein synthesis was carried out at 30°C using 1 A_{260} unit of polysomes in a final volume of 100 µl, which contained 20 mM potassium 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonate 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 µCi of L-[4,5-^{3}H]leucine (130 Ci/mmol; Amersham International, Amersham), 10 µg of calf liver tRNA (Boehringer Mannheim GmbH, Mannheim), and 50 µl of reticulocyte lysate S_{150}. The incubation mixture also contained 50 µg/ml each of antipain, chymostatin, elastatinal, leupeptin and pepstatin (the Peptide Institute Inc., Osaka). Hemin was dissolved to give 1.6 mM solution and the hemin solution contained 100 mM potassium phosphate buffer (pH 7.5) and 20 mM NaCl. The concentration of hemin was determined by the pyridine hemochrome method (13).

Determination of Radioactivity Incorporated into ALA Synthase and Total Protein. Syntheses of ALA synthase and total proteins were estimated by analyzing the radioactivity incorporated. Cell-free protein synthesis was stopped by chilling, followed by the addition of two volumes of a solution containing 50 mM Tris-HCl buffer (pH 7.5), 150 mM NaCl, 5 mM leucine, 5 mM EDTA, 1% Triton X-100, 0.1 mM pyridoxal phosphate and 40 µg/ml each of microbial protease inhibitors. A portion (5-10 µl) of the mixture was used for the determination of radioactivity incorporated into total protein. Rat liver ALA synthase synthesized was isolated from an aliquot (280 µl) of the mixture by immunoprecipitation using 35 µg of a specific antibody (IgG) raised in rabbit (14) and 25 mg of Protein A-Sepharose CL-4B (Pharmacia Fine Chemicals AB, Uppsala). Protein A-Sepharose was collected by centrifugation and washed six times in 50 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl, 5 mM leucine and 5 mM EDTA. Immunoprecipitated materials were released from Protein A-Sepharose by boiling for 5 min in 100 µl of a sample buffer consisting of 4% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol, 10 mM Tris-HCl buffer (pH 6.8) and 20% glycerol. Protein A-Sepharose was removed by centrifugation and resulting supernatant was analyzed by SDS-polyacrylamide gel electrophoresis.

Electrophoretic analyses of proteins were carried out by either SDS-10% polyacrylamide slab gel electrophoresis (15) or SDS-7.5% polyacrylamide rod gel electrophoresis (16). The slab gel electrophoresis was followed by fluorography performed by use of EN^{3}HANCE (New England Nuclear, Boston). The

rod gel was sliced, solubilized and counted as described previously (6). Synthesis of ALA synthase was quantitated in terms of the sum of radioactivity recovered in three peak fractions of gel slices after appropriate subtraction of background.

RESULTS

Cell-free protein synthesis was performed using total polysomes isolated from AIA-treated rat liver and a rabbit reticulocyte lysate S_{150} with various concentrations of hemin added. After 45 min of incubation, the ALA synthase synthesized was immunoprecipitated using a specific antibody. Immunoprecipitated materials were analyzed by SDS-polyacrylamide rod gel electrophoresis followed by subsequent counting of the sliced gels. As shown in Fig. 1A, synthesis of ALA synthase decreased with increased addition of hemin; the addition of about 30 μM hemin brought about almost 60% reduction of the synthesis of ALA synthase, while synthesis of total protein was not significantly influenced by the addition of hemin. Immunoprecipitated materials were also analyzed by SDS-polyacrylamide slab gel electrophoresis followed by fluorography. As shown in Fig. 1B, the density of the major band corresponding to ALA synthase with a molecular weight of about 77,000 decreased with the increased addition of hemin. Previously we reported that the major radioactive product immunoprecipitated from the cell-free protein synthesizing system had a molecular weight of about 75,000 when analyzed by SDS-polyacrylamide rod gel electrophoresis (6). The difference in apparent molecular weight of ALA synthase in the present and previous studies may be due to differences in analytical procedures used. Also, a minor band corresponding to a molecular weight of about 51,000 was detected in both control and hemin-added systems and the density of this band also decreased with the increased addition of hemin. This band would represent a product of partial proteolysis of the major product; it has been shown repeatedly that ALA synthase is easily degraded to this size of product (6, 17, 18). However, there was no apparent indication of the accumulation of intermediary polypeptides of smaller size in the hemin-added system, as can be seen from the comparison of lane 1 and lane 5 in Fig. 1B.

To further characterize the specificity of the effect of hemin on the synthesis of ALA synthase, the total cell-free translation products were subjected to SDS-polyacrylamide slab gel electrophoresis. As shown in Fig. 1C, the band corresponding to ALA synthase (arrow a) was greatly diminished in the hemin-added system, and there were also some differences in the electrophoretic pattern of the product synthesized in the control and the hemin-added systems, for instance, as indicated by arrow b in the figure. Except for those peptides, the majority of translational products appeared to be synthesized with comparable efficiencies in both control and hemin-added systems. These re-

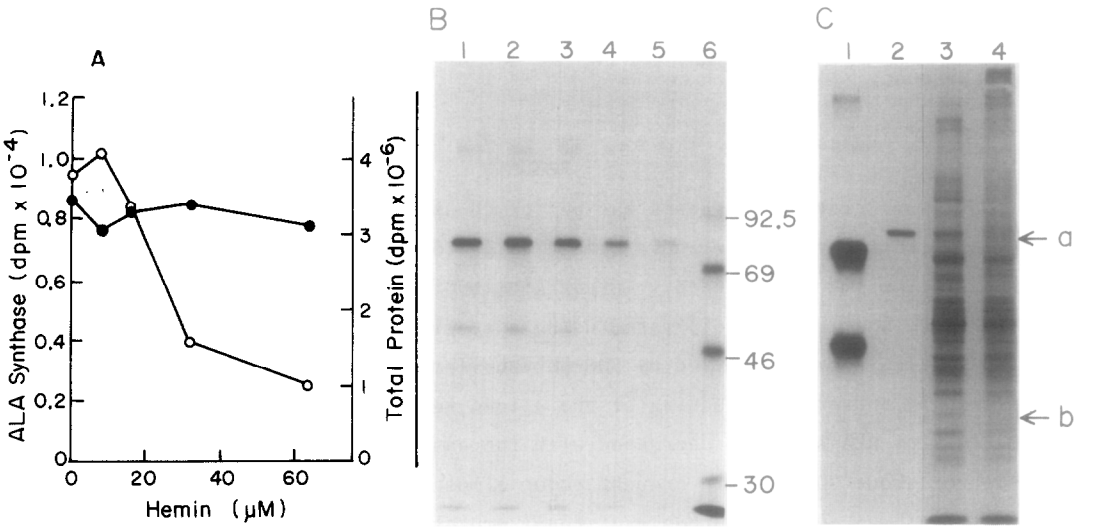

Fig. 1. Effects of hemin on polysome-directed cell-free syntheses of ALA synthase and total protein. Total liver polysomes were incubated with L-[4,5-^{3}H]leucine for 45 min (A, B) or 20 min (C) at 30°C in a translation system containing rabbit reticulocyte lysate S_{150}. Various concentrations of hemin were added to the incubation mixture. (A) ALA synthase synthesized was collected by immunoprecipitation and analyzed by electrophoresis on SDS-polyacrylamide rod gels and subsequent counting of radioactivity in the sliced gels. Radioactivity incorporated into total protein was determined by a glass fiber disc method. o, ALA synthase; •, total protein. (B) ALA synthase synthesized was collected by immunoprecipitation and analyzed by SDS-polyacrylamide slab gel electrophoresis followed by fluorography. Lane 1, immunoprecipitates from control translation system without hemin added; lanes 2 to 5, immunoprecipitates from translation systems to which hemin was added at concentrations of 8 (lane 2), 16 (lane 3), 32 (lane 4), and 64 (lane 5) μM. The following [^{14}C]-methylated marker proteins (Amersham International, Amersham) were used (lane 6): phosphorylase b (92,500), bovine serum albumin (69,000), ovalbumin (46,000), and carbonic anhydrase (30,000). Numbers to the right of lane 6 indicate Mr x 10^{-3}. (C) Total cell-free translation products in the control system and the hemin-added system were analyzed by SDS-polyacrylamide slab gel electrophoresis and fluorography. Lane 1, [^{14}C]methylated bovine serum albumin and ovalbumin used as molecular weight markers; lane 2, ALA synthase immunoprecipitated from the control system; lane 3, total translation products in the control system; lane 4, total translation products in the hemin (64 μM)-added system. Arrow a, the band corresponding to ALA synthase; arrow b, a band which was significantly diminished in the hemin-added system.

sults suggest that hemin specifically, if not exclusively, inhibits the synthesis of ALA synthase in this system.

Time courses of synthesis of total protein under various conditions are shown in Fig. 2. Synthesis of total protein reached a plateau after about 20 min of incubation and synthesis of total protein was not significantly affected by the addition of 64 μM hemin. When the reaction mixture contained 100 μM aurintricarboxylic acid, an inhibitor of initiation of mRNA translation (19), the protein synthesis proceeded as rapidly as in the control system for about

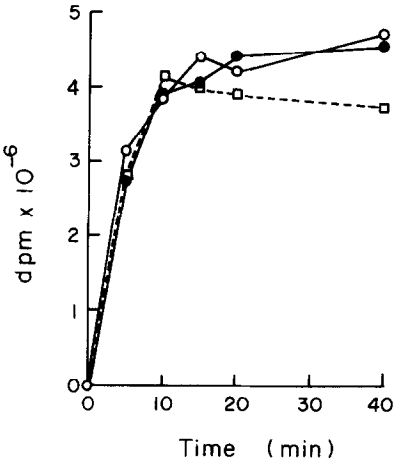

Fig. 2. Time courses of the synthesis of total protein in the cell-free system in the presence and absence of hemin or aurintricarboxylic acid. Radioactivity incorporated into total proteins was determined as described in Fig. 1A. o, control; •, with 64 μM hemin; □, with 100 μM aurintricarboxylic acid.

10 min, but then the synthesis completely ceased; the proportion of the aurintricarboxylic acid-sensitive protein synthesis was less than 20%. The data suggest that the protein synthesis observed in the present cell-free system represents mainly the elongation of pre-formed nascent polypeptides and its termination rather than the net protein synthesis involving initiation.

Time courses of synthesis of ALA synthase with and without hemin (64 μM) were examined (Fig. 3). In both cases synthesis of ALA synthase continued to proceed for about 20 min, but the rate of synthesis as well as the final yield of ALA synthase were significantly reduced in the hemin-added system. When

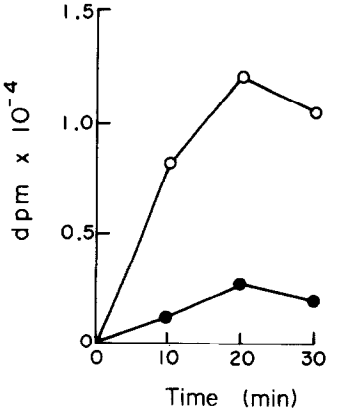

Fig. 3. Time courses of the synthesis of ALA synthase in the cell-free system in the presence and absence of 64 μM hemin. ALA synthase synthesized was collected and analyzed as described in Fig. 1A. o, control without hemin; •, with hemin.

the incubation was continued for more than 20 min, however, the amount of radioactivity recovered in ALA synthase (77,000 dalton fraction) was rather diminished. This is probably due to degradation of the once-formed enzyme protein by endogenous proteases in the reticulocyte lysate. In an independent experiment, we incubated a set of two identical reaction mixtures without hemin for 30 min, and added hemin (final 64 μM) to one of the mixtures at the end of incubation. Essentially the same amount of radioactivity was recovered in ALA synthase in both systems, excluding the possibility that heme might affect in some way the immunoreactivity of the newly synthesized ALA synthase. In another independent experiment we carried out the reaction in the presence of 100 μM aurintricarboxylic acid, but the yield of ALA synthase was not appreciably reduced as compared with that in the control system. All these data support the view that the liver polysome-supported synthesis of ALA synthase in the rabbit reticulocyte lysate system is inhibited by heme mainly at a step of peptide chain elongation.

DISCUSSION

It is well known that the mRNA translation for general protein synthesis in the cell-free system is controled by hemin (20) and by double-stranded RNA (21). In addition to these general control of mRNA translation, there are a number of cases in which a specific control of translation of individual mRNAs has been demonstrated in the cell-free translation system. For example, the cell-free synthesis of procollagen is inhibited specifically by its N-terminal extension peptides (22). The translation of yeast catalase mRNA is accelerated specifically by the addition of hemin to a cell-free system containing the lysate of heme-deficient mutant yeast cells (23). The present study demonstrates that the translation of ALA synthase-mRNA in the rabbit reticulocyte lysate system is specifically inhibited by the addition of hemin. To our knowledge this is the first example that the specific translational control of synthesis of a rate-limiting enzyme by the end product of the biosynthetic pathway has been demonstrated in the cell-free system.

The results obtained in the present study suggest that heme inhibits a peptide chain elongation step of the synthesis of ALA synthase. This hemin effect might involve a specific interaction of heme with nascent peptide chains of ALA synthase on polysomes. In the hemin-added system, there was no apparent indication of the accumulation of nascent peptides of smaller size when analyzed by SDS-polyacrylamide slab gel electrophoresis. One possibility to account for these observations is that heme interacts with nascent peptide chains which are not matured yet to form immune-complex with the ALA synthase-specific antibody and in this way heme effectively inhibits the elongation of the ALA synthase peptide. The present study, however, does not exclude the possibility that heme might also interfere more or less with the initiation

step of the synthesis of ALA synthase. Further investigations are required to clarify the detailed mechanism of the translational inhibition by heme of the synthesis of ALA synthase.

We reported in the previous paper that intravenous administration of hemin to AIA-pretreated rats resulted in a dose-dependent and time-dependent decrease of the ability of liver polysomes that direct the synthesis of ALA synthase in vitro in a reticulocyte lysate system, suggesting that the level of mRNA coding for ALA synthase in the liver cytosol was reduced by the administration of hemin (6). We interpreted this as representing the "transcriptional" regulation of the synthesis of ALA synthase. Now we may assume that synthesis of ALA synthase in the liver is regulated by heme, the end product of the biosynthetic pathway, at both transcriptional and translational levels.

ACKNOWLEDGMENTS. We are grateful to the Nippon Roche Company, Tokyo, for generous supply of allylisopropylacetamide. This work was supported in part by the Ministry of Education, Science and Culture, Japan (Grants in Aid for Scientific Research 56480421 and 5657009), the Ministry of Health and Welfare, Japan (the Research Grant for the Intractable Diseases), and the Yamanouchi Foundation for Metabolic Studies, Japan.

REFERENCES

1. Granick, S., and Beale, S.I. (1978) Adv. Enzymol. 46, 33-203.
2. Hayashi, N., Kurashima, Y., and Kikuchi, G. (1972) Arch. Biochem. Biophys. 148, 10-21.
3. Yamauchi, K., Hayashi, N., and Kikuchi, G. (1980) J. Biol. Chem. 255, 1746-1751.
4. Whiting, M.J. (1976) Biochem. J. 158, 391-400.
5. Srivastava, G., Brooker, J.D., May, B.K., and Elliott, W.H. (1980) Biochem. J. 188, 781-788.
6. Yamamoto, M., Hayashi, N., and Kikuchi, G. (1982) Biochem. Biophys. Res. Commun. 105, 985-990.
7. Sassa, S., and Granick, S. (1970) Proc. Natl. Acad. Sci. USA 67, 517-522.
8. Strand, L.J., Manning, J., and Marver, H.S. (1972) J. Biol. Chem. 247, 2820-2827.
9. Tyrrell, D.L.J., and Marks, G.S. (1972) Biochem. Pharmacol. 21, 2077-2093.
10. Tomita, Y., Ohashi, A., and Kikuchi, G. (1974) J. Biochem. 75, 1007-1015.
11. Hayashi, N., Terasawa, M., Yamauchi, K., and Kikuchi, G. (1980) J. Biochem. 88, 1537-1543.
12. Yamamoto, M., Hayashi, N., and Kikuchi, G. (1981) Arch. Biochem. Biophys. 209, 451-459.
13. Fuhrhop, J.-H., and Smith, K.M. (1975) in Porphyrins and Metalloporphyrins (Smith, K.M., ed.), pp. 804-807, Elsevier/North-Holland, Amsterdam.
14. Nakakuki, M., Yamauchi, K., Hayashi, N., and Kikuchi, G. (1980) J. Biol. Chem. 255, 1738-1745.
15. Laemmli, U.K. (1970) Nature 227, 680-685.
16. Weber, K., and Osborn, M. (1969) J. Biol. Chem. 244, 4406-4412.
17. Ades, I.Z., and Harpe, K.G. (1981) J. Biol. Chem. 256, 9329-9333.
18. Srivastava, G., Borthwick, I.A., Brooker, J.D., May, B.K., and Elliott, W.H. (1982) Biochem. Biophys. Res. Commun. 109, 305-312.
19. Stewalt, M.L., Grollman, A.P., and Haung, M-T. (1971) Proc. Natl. Acad. Sci. USA 68, 97-101.
20. Ranu, R.S., and London, I.M. (1979) Methods Enzymol. 60, 459-484.
21. Das, H.K., Das, A., Ghosh-Dastidar, P., Ralston, R.O., Yaghmai, B., Roy, R., and Gupta, N.K. (1981) J. Biol. Chem. 256, 6491-6495.
22. Paglia, L., Wilczek, J., Diaz de Leon, L., Martin, G.R., Hörlein, D., and Müller, P. (1979) Biochemistry 18, 5030-5034.
23. Hamilton, B., Hofbauer, R., and Ruis, H. (1982) Proc. Natl. Acad. Sci. USA 79, 7609-7613.