Vol. 105, No. 2, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 30, 1982 Pages 639-646

CELL-FREE SYNTHESIS OF THE ENZYMES OF PEROXISOMAL β-OXIDATION

Shuichi Furuta, Takashi Hashimoto, Satoshi Miura*, Masataka Mori*, and Masamiti Tatibana*

Department of Biochemistry, Shinshu University School of Medicine, Matsumoto, Nagano 390, and *Department of Biochemistry, Chiba University School of Medicine, Inohana, Chiba 280, Japan

Received January 20, 1982

Three enzymes of peroxisomal β-oxidation of rat liver were synthesized in a cell-free protein-synthesizing system derived from rabbit reticulocyte lysate. The in vitro products of acyl-CoA oxidase and enoyl-CoA hydratase-3-hydroxyacyl-CoA dehydrogenase multifunctional protein were similar in size to or slightly larger than the subunit of the respective mature enzymes. The in vitro product of peroxisomal 3-ketoacyl-CoA thiolase was about 3,000 daltons larger than the mature subunit. The hepatic levels of translatable mRNAs coding for these three enzymes were about 10 times higher in rats fed a di(2-ethylhexyl)phthalate-containing diet than in control animals.

Fatty acid β-oxidation system in rat liver peroxisomes consists of peroxisome-specific enzymes which are different from those of the mitochondrial β-oxidation system with respect to molecular and catalytic properties. The peroxisomal system consists of acyl-CoA oxidase, a multifunctional protein exhibiting activities of enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase, and peroxisomal 3-ketoacyl-CoA thiolase.

The activity of the peroxisomal system is greatly increased when rats are fed with a diet containing peroxisome proliferators, such as various hypolipidemic drugs or a widely used plasticizer, DEHP. All component enzymes of peroxisomal β-oxidation in rat liver are markedly induced concomitantly by treatment with DEHP (1). The purified preparation of acyl-CoA oxidase consists of three subunits. The largest subunit with M_r=75,500 (A subunit) has been

Abbreviations : DEHP, di(2-ethylhexyl)phthalate; SDS, sodium dodecyl sulfate.

0006-291X/82/060639-08$01.00/0

suggested to be cleaved into B subunit (M_r=50,100) and C subunit (M_r=19,000) in the cell (2). Enoyl-CoA hydratase-3-hydroxyacyl-CoA dehydrogenase multifunctional protein is composed of one polypeptide with a moleuclar weight of 77,000 (3). Peroxisomal 3-ketoacyl-CoA thiolase consists of two polypeptides with an identical size (M_r= 40,000) (4). Biochemical studies on biogenesis of peroxisomes have been studied with special reference to catalase (EC 1.11.1.6) (5,6). The cell-free synthesis of rat liver catalase (7,8) and urate oxidase (EC 1.7.3.3) (8) with use of mRNA from rat liver has been reported. The mobilities of translation products of these enzymes were identical with those of the mature enzymes.

In the present study, we describe the cell-free synthesis of the three enzyme proteins of rat liver peroxisomal β-oxidation. Apparent molecular sizes of the primary translation products of acyl-CoA oxidase and enoyl-CoA hydratase-3-hydroxyacyl-CoA dehydrogenase multifunctional protein were nearly the same as those of the subunits of the corresponding mature enzymes but that of peroxisomal 3-ketoacyl-CoA thiolase was larger than the subunit of the mature enzyme.

MATERIALS AND METHODS

Purification of Enzymes —— Acyl-CoA oxidase (2), enoyl-CoA hydratase-3-hydroxyacyl-CoA dehydrogenase multifunctional protein (3), and peroxisomal 3-ketoacyl-CoA thiolase (9) were purified from rat liver as described previously.

Preparation of Antibodies —— Antibodies against rat liver enzymes of peroxisomal β-oxidation were raised in rabbits and purified by three successive ammonium sulfate precipitation at 33% saturation.

Isolation of RNA —— Male Wistar rats weighing about 200 g were fed ad libitum a diet with or without 2% (w/w) DEHP for 10 days. Total hepatic RNA was isolated by the SDS-phenol method as described previously (10).

Cell-Free Protein Synthesis —— Total RNA of rat liver was translated in a nuclease-treated rabbit reticulocyte lysate with [^{35}S]methionine (400 μCi/ml) as described previously (11).

Experiment with Liver Slices —— Liver slices were prepared from a rat fed a diet containing 2% (w/w) DEHP for 3 days and incubated with [^{35}S]methionine (150 μCi/ml) as described previously (12).

Immunoprecipitation —— Cell-free translation products and the mature enzymes synthesized in the liver slices were immunoprecipitated using antibodies against the respective enzymes and Staphylococcus aureus cells as described previously (11).

Other Methods —— SDS-polyacrylamide gel electrophoresis (13) and fluorography (14) were performed by the cited methods. The diphenyloxazole-impregnated and dried gel strips were cut out and used for measurement of radioactivity (11). Trichloroacetic acid-insoluble radioactivity was determined for measurement of the translation capacity of total RNA (15).

Materials —— Microbial protease inhibitors (antipain, leupeptin, chymostatin, and pepstatin) were purchased from the Peptide Institute (Osaka, Japan). [^{35}S]methionine (>1000 Ci/mmol) was from New England Nuclear.

RESULTS AND DISCUSSION

Total RNA of rat liver was translated in a rabbit reticulocyte lysate system in the presence of [^{35}S]methionine, and translation products of individual enzymes of the peroxisomal β-oxidation system were immunoprecipitated with corresponding antibodies and S. aureus cells. The immunoprecipitates were subjected to SDS-polyacrylamide slab gel electrophoresis and radioactive bands were detected by fluorography. The purified preparation of acyl-CoA oxidase gave three polypeptide bands in SDS-gel electrophoresis (Fig. 1 A, lane 3). As described below, incorporation of [^{35}S]methionine into the immunopreipitate was very low in the control (Fig. 1 A, lane 1) compared to the DEHP group (Fig. 1 A, lane 2). The fluorogram of the DEHP group, however, shows that the translation product of acyl-CoA oxidase migrates to nearly the same position as that of the largest subunit of the purified enzyme. The data substantiate our previous proposal that the largest polypeptide (A subunit) of acyl-CoA oxidase is cleaved into B and C subunits in the cell (2).

The molecular size of the translation product of enoyl-CoA hydratase-3-hydroxyacyl-CoA dehydrogenase multifunctional protein was nearly the same as that of the purified preparation (Fig. 1 B, lanes 2 and 3).

The in vitro product immunoprecipitated by anti-(peroxisomal 3-ketoacyl-CoA thiolase)immunoglobulin migrated at a slower rate than that of the purified enzyme (Fig. 1 C, lanes 2 and 3). The molecular weight of this putative precursor is 43,000 daltons which is about 3,000 daltons larger than the subunit of the enzyme.

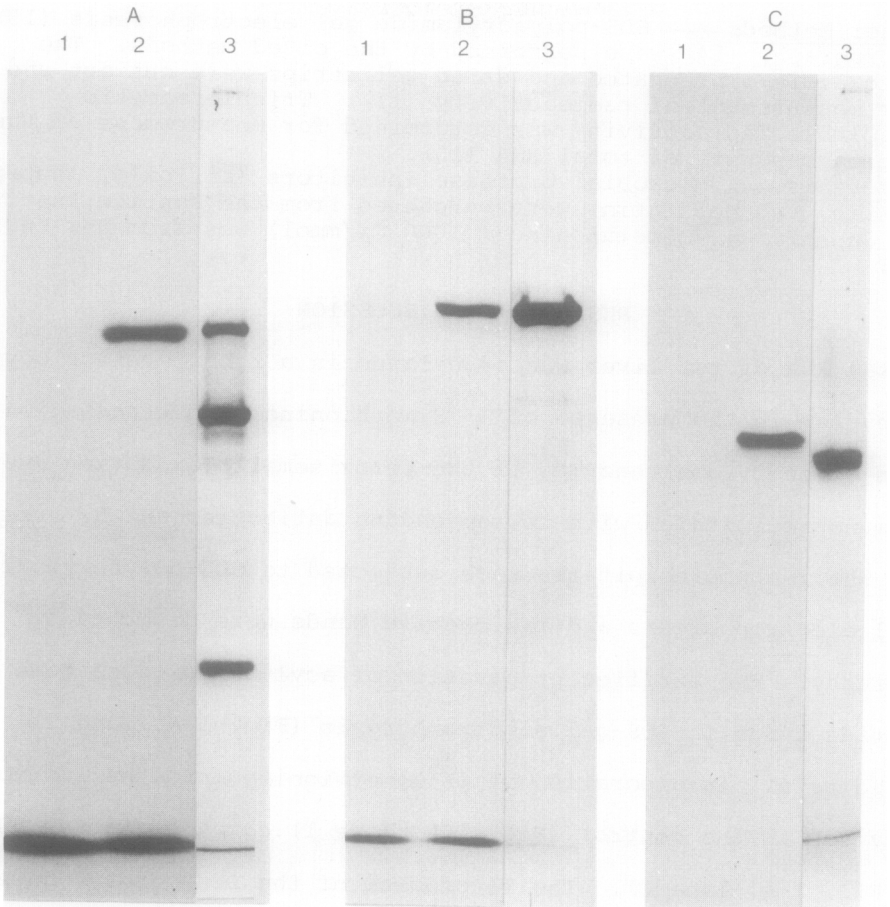

Fig. 1. Synthesis of the enzymes of peroxisomal β-oxidation in a rabbit reticulocyte protein-synthesizing system. A, acyl-CoA oxidase; B, enoyl-CoA hydratase-3-hydroxyacyl-CoA dehydrogenase multifunctional protein; C, peroxisomal 3-ketoacyl-CoA thiolase. Lanes 1 and 2, the fluorograms of the immunoprecipitates from the *in vitro* products with liver mRNAs of the control (lane 1) and the DEHP-fed rats (lane 2). Lane 3, the electropherograms of the purified enzyme preparations for comparison (stained with Coomasie Blue R-250). Electrophoresis was carried out on 7.5% (A, B) and 10% polyacrylamide gel.

When electrophoresis was carried out for a longer period, it was found that the *in vitro* products of acyl-CoA oxidase and enoyl-CoA hydratase-3-hydroxyacyl-CoA dehydrogenase multifunctional protein migrated at a slightly slower rate than those of the subunits of the purified preparations (data not shown). To compare the molecular weight of the *in vitro* products with those of subunits of the mature enzymes in a greater detail, the mature enzymes labeled in liver slices were used. SDS-gel electrophoresis was performed for additional 1.5 h

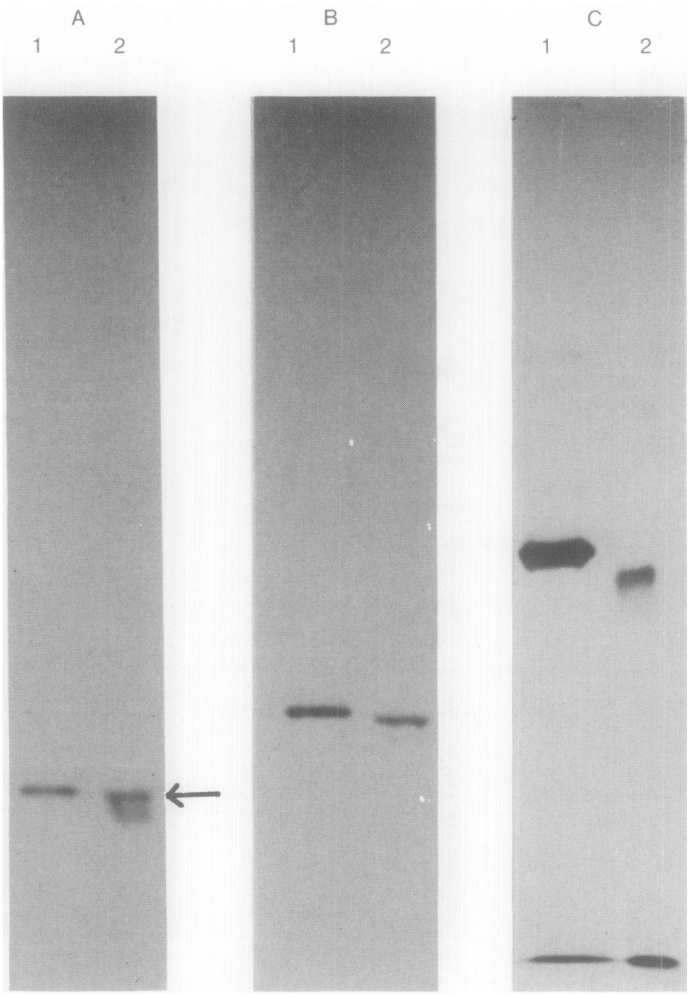

Fig. 2. Comparison of the molecular weights of the in vitro products with those of subunits of the mature enzymes. A, immunoprecipitation with anti-(acyl-CoA oxidase)immunoglobulin; B, immunoprecipitation with anti-(enoyl-CoA hydratase-3-hydroxyacyl-CoA dehydrogenase multifunctional protein)immunoglobulin; C, immunoprecipitation with anti-(peroxisomal 3-ketoacyl-CoA thiolase)immunoglobulin. Lane 1, the immunoprecipitate from the in vitro products with liver mRNA of the DEHP-fed rat. Lane 2, liver slices (about 50 mg wet weight) prepared from a DEHP-fed rat were incubated with [^{35}S]methionine for 2 h and labeled enzymes were extracted and immunoprecipitated as described under "MATERIALS AND METHODS" (mature enzyme). The immunoprecipitates were electrophoresed and fluorographed. Electrophoresis was carried out on 7.5% (A, B) and 10% (C) polyacrylamide gel for 5.5 h (A, B) and 3 h (C), respectively. An arrow shows a position of the largest subunit (M_r=75,500) of acyl-CoA oxidase.

after the tracking dye had reached the bottom of the gel (5.5 h in total) (Fig. 2, A and B). The in vitro product of acyl-CoA oxidase migrated at a rate slightly lower than that of the mature enzyme (Fig. 2, A).

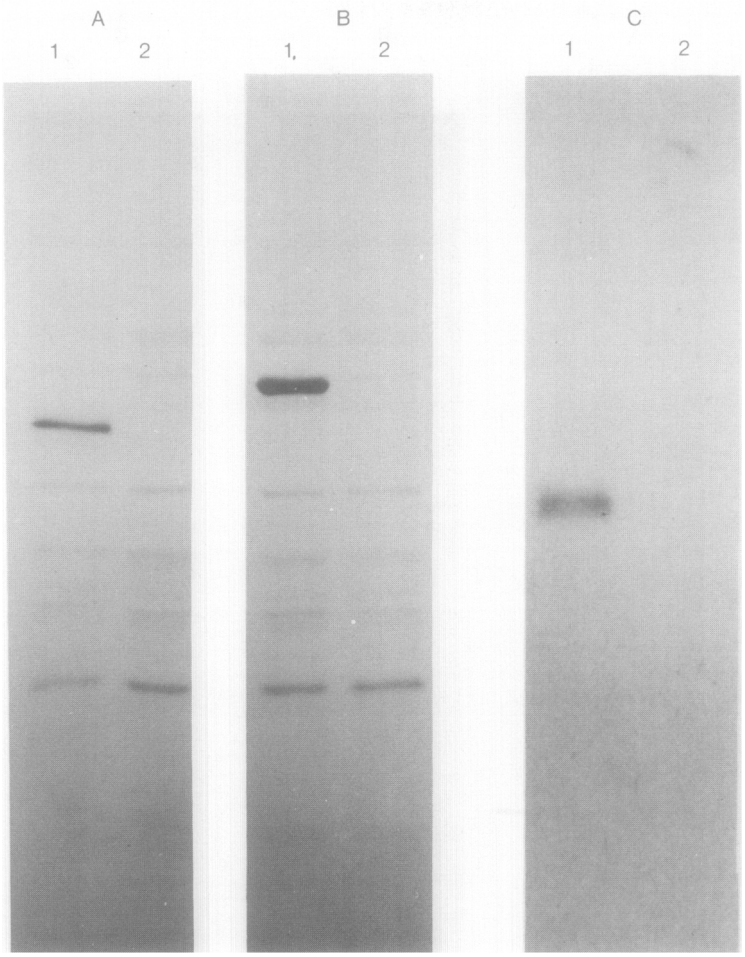

Fig. 3. Competition of the translation products with the purified enzymes for the corresponding antibodies. A, acyl-CoA oxidase; B, enoyl-CoA hydratase-3-hydroxyacyl-CoA dehydrogenase multifunctional protein; C, peroxisomal 3-ketoacyl-CoA thiolase. Immunoprecipitates with 60 μg of antibody in the absence (lane 1) or presence of 10 μg (lane 2) of the purified enzymes were electrophoresed and fluorographed. Electrophoresis was carried out on 7.5% (A, B) and 10% (C) polyacrylamide gel.

The *in vitro* product of enoyl-CoA hydratase-3-hydroxyacyl-CoA dehydrogenase multifunctional protein migrated at a rate slightly but definitely lower than the mature enzyme (Fig. 2, B). The mobility of the *in vitro* product of peroxisomal 3-ketoacyl-CoA thiolase was lowe than the labeled mature enzyme (Fig. 2, C) and this mature form comigrated with the subunit of the purified enzyme.

To exclude the possibility that the *in vitro* products are artifacts, immunoprecipitation was performed with a limited amount of

antibody in the presence of an excess amount of the purified enzyme. Fig. 3 shows that the fluorographic band was displaced by addition of 10 μg of the enzyme.

Lazarow and his coworker have reported that the *in vitro* translation product of rat liver catalase migrates on electrophoresis at the same rate as that of the mature enzyme (16), and that catalase synthesized *in vitro* contains structural information directing the transport into peroxisomes without the need for proteolytic processing (7). In contrast to catalase, the *in vitro* translation product of peroxisomal 3-ketoacyl-CoA thiolase was 3,000 daltons larger than the mature subunit. The finding suggests that the peroxisomal 3-ketoacyl-CoA synthesized *in vitro* possesses an extra peptide and that the translation product is readily converted to the mature enzyme in the cell. The translation products of acyl-CoA oxidase and enoyl-CoA hydratase-3-hydroxyacyl-CoA dehydrogenase multifunctional protein are seemed to be slightly larger than the mature enzyme forms. Therefore, it may be possible that synthesis and maturation of the individual enzymes of the peroxisomal β-oxidation system are different from those of catalase and urate oxidase.

Incorporation of [^{35}S]methionine into individual enzymes programmed with total hepatic RNA of a DEHP-fed rat was measured by cutting and counting the strips of the respective bands. Radioactivities were about 0.5%, 0.5%, and 0.1% of that of the total translation products for acyl-CoA oxidase, enoyl-CoA hydratase-3-hydroxyacyl-CoA dehydrogenase multifunctional protein, and peroxisomal 3-ketoacyl-CoA thiolase, respectively. Incorporation of [^{35}S]methionine into total translation products with total RNA of the control rat was 60% of that of the DEHP group in this experiment. Fluorography (Fig. 1, lanes 1) indicates that the synthesis of individual enzymes *in virto* with rat liver mRNA of the control is very low compared with that with mRNA from DEHP-treated rats. The rates of synthesis of all three enzyme proteins with mRNA from

DEHP-treated rats in the cell-free system relative to that of the contro were 8- to 12-fold (data not shown). Kinetic studies of the induction of these enzyme activities by administration of DEHP to rats and the deduction by its withdrawal suggests that the synthesis rates of these enzymes are increased about 10-fold after administration of the peroxisome proliferator (1). In a preliminary experiment (17), incorporation of [^{3}H]leucine into individual enzymes 2 h after the injection into rats was determined. Incorporation of radioactivities into these enzymes was increased 10- to 20-fold after DEHP administration. It is suggested that administration of DEHP causes the marked increase of mRNAs coding for individual enzymes of the peroxisomal β-oxidation system.

ACKNOWLEDGEMENTS

This work was supported in part by grants from the Ministry of Education, Science and Culture of Japan and a Naito Foundation Research Grant for 1980.

REFERENCES

1. Miyazawa, S., Furuta, S., Osumi, T., and Hashimoto, T. (1980) Biochim. Biophys. Acta 630, 367-374.
2. Osumi, T., Hashimoto, T., and Ui, N. (1980) J. Biochem. 87, 1735-1746.
3. Furuta, S., Miyazawa, S., Osumi, T., Hashimoto, T., and Ui, N. (1980) J. Biochem. 88, 1059-1070.
4. Miyazawa, S., Furuta, S., Osumi, T., Hashimoto, T., and Ui, N. (1980) J. Biochem. 90, 511-519.
5. Lazarow, P. B. and de Duve, C. (1973) J. Cell Biol. 59, 491-506.
6. Lazarow, P. B. and de Duve, C. (1973) J. Cell Biol. 59, 507-524.
7. Robbi, M., and Lazarow, P. B. (1978) Proc. Natl. Acad. Sci. USA 75, 4244-4248.
8. Goldman, B. M., and Blobel, G. (1978) Proc. Natl. Acad. Sci. USA 75, 5066-5070.
9. Miyazawa, S., Osumi, T., and Hashimoto, T. (1980) Eur. J. Biochem. 103, 589-596.
10. Mori, M., Miura, S., Tatibana, M., and Cohen, P. P. (1979) Proc. Natl. Acad. Sci. USA 76, 5071-5075.
11. Mori, M., Miura, S., Tatibana, M., and Cohen, P. P. (1981) J. Biol. Chem. 256, 4127-4132.
12. Morita, T., Mori, M., Tatibana, M., and Cohen, P. P. (1981) Biochem. Biophys. Res. Commun. 99, 623-629.
13. Laemmli, U. K. (1970) Nature 227, 680-685.
14. Bonner, W. M., and Laskey, R. A. (1974) Eur. J. Biochem. 91, 179-191.
15. Roberts, B. E., and Paterson, B. M. (1973) Proc. Natl. Acad. Sci. USA 70, 2330-2334.
16. Lazarow, P. B. (1980) Ann. NY Acad. Sci. 343, 293-303.
17. Furuta, S., Miyazawa, S., and Hashimoto, T. (1980) Proc. Japanese Conference Biochem. Lipid (in Japanese) 22, 431-433.