Vol. 89, No. 2, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 27, 1979 Pages 580-584

PEROXISOMAL βOXIDATION SYSTEM OF RAT LIVER. COPURIFICATION OF ENOYL-CoA HYDRATASE AND 3-HYDROXYACYL-CoA DEHYDROGENASE

Takashi Osumi and Takashi Hashimoto

Department of Biochemistry, Faculty of Medicine,
Shinshu University, Matsumoto, Nagano 390, Japan

Received June 11,1979

Activity of enoyl-CoA hydratase in rat liver was elevated about 6-fold by the administration of di-(2-ethylhexyl)phthalate, a hepatic peroxisome proliferator. Almost all of the increased activity was the peroxisomal enzyme, which was distinguished by its heat-lability from mitochondrial one. Heat-labile enoyl-CoA hydratase was copurified with peroxisomal 3-hydroxyacyl-CoA dehydrogenase. The purified enzyme corresponded to a peroxisome specific peptide with a molecular weight of 80,000.

Rat liver peroxisomes have a novel fatty acyl-CoA βoxidation system which is markedly increased by the administrations of hypolipidemic agents or di-(2-ethylhexyl)phthalate (1-3). Enzymes involved in the peroxisomal βoxidation system are supposed to be different from those of the mitochondrial one (4,5). The initial step of the peroxisomal system is catalyzed by acyl-CoA oxidase (6). The presence of peroxisomal 3-hydroxyacyl-CoA dehydrogenase, which is different from mitochondrial one, has recently been elucidated (7). The present paper describes that peroxisomal enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase are located in the same protein molecule.

MATERIALS AND METHODS

Materials —— Crotonyl CoA was synthesized from crotonic anhydride and CoA (8). Acetoacetyl-CoA was prepared from diketene and CoA (9). Acetoacetyl pantetheine was synthesized from diketene and pantetheine (10).

Assay Method —— Enoyl-CoA hydratase activity was assayed as described by Steinman and Hill (8). 3-Hydroxyacyl-CoA dehydrogenase activity was assayed using acetoacetyl-CoA or

0006-291X/79/140580-05$01.00/0

acetoacetyl pantetheine as a substrate as described previously (7). When heat-labile and heat-stable enoyl-CoA hydratase activities were assayed, the enzyme preparations were diluted with 50 mM potassium phosphate, pH 7.0 and heated at 57°C for 5 min. When peroxisomal 3-hydroxyacyl-CoA dehydrogenase activity was assayed, the preparations were treated with an antibody against mitochondrial 3-hydroxyacyl-CoA dehydrogenase (7). One unit of enzyme activity is expressed as μmol/min. Protein concentration was determined by the method of Lowry et al. (11).

Preparation of the enzyme —— Male Wistar rats were fed with a diet containing 2% (w/w) di-(2-ethylhexyl)phthalate more than 2 weeks for the induction of peroxisomal fatty acyl-CoA oxidizing system (3). Livers were removed and frozen. The frozen liver (20 g) was thawed and homogenized with 100 ml of 10 mM K_3PO_4 containing 2 mM 2-mercaptoethanol and 0.1% hexamethylphosphoric triamide (all buffers employed in the purification procedure contained these reagents) in a tissue disintegrator (Ultra-turrax). The homogenate was centrifuged at 17,000 x g for 15 min. The supernatant was adjusted to pH 7.0 by addition of 1 M potassium phosphate,pH6.0 and applied on a phosphocellulose column (100 ml bed volume, equilibrated with 50 mM potassium phosphate, pH 7.0). The column was eluted with a linear gradient system of the buffer from 50 to 500 mM in a total volume of 400 ml. The active fractions were pooled and treated with ammonium sulfate and the protein precipitated between 200 and 400 g/l was dissolved in a minimal volume of 50 mM potassium phosphate, pH 7.0. Ammonium sulfate was removed by passing through a Sephadex G-25 column with use of 100 mM potassium phosphate, pH 7.0. The enzyme preparation was diluted by addition of an equal volume of water containing 2 mM 2-mercaptoethanol and 0.1% hexamethylphosphoric triamide, and applied on a CM-cellulose column (25 ml bed volume, equilibrated with 25 mM potassium phosphate, pH 7.0). The column was washed with 25 ml of 50 mM potassium phosphate, pH 7.0 and eluted with a linear gradient system of the buffer from 50 to 200 mM in a total volume of 300 ml. The active fractions were pooled and the enzyme was precipitated by addition of ammonium sulfate (350 g/l).

Anaysis of the enzyme preparation —— The SDS-polyacrylamide gel electrophoresis was performed according to the method of Weber and Osborn (12). Post-nuclear and mitochondrial fractions of rat liver were obtained according to the method of de Duve et al.(13) to demonstrate a peroxisome specific peptide with a molecular weight of 80,000 (14). The following standard proteins were used : γ-globulin, L-chain (23,500), yeast alcohol dehydrogenase (37,000), ovalbumin (43,000), γ-globulin, H-chain (50,000), catalase (60,000), bovine serum albumin (68,000) and phosphrylase a (94,000). Molecular weight of the native enzyme was estimated by gel chromatography using a Sephadex G-100 column. The standard proteins employed were peroxidase (40,000), carnitine acetyltransferase (55,000), malate dehydrogenase (70,000) citrate synthase (100,000) and lactate dehydrogenase (140,000).

RESULTS AND DISCUSSION

Administration of di-(2-ethylhexyl)phthalate to rats caused a striking proliferation of hepatic peroxisomes and an increase of the peroxisomal fatty acid oxidizing activity (3,15). The

Table 1. Purification of enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase.

Step	Total protein	Enoyl-CoA hydratase units x 10^{-3}		3-Hydroxyacyl-CoA dehydrogenase, units		Ratio***
	(mg)	Heat-stable	Heat-labile	Mitochondrial*	Peroxisomal**	
Extract	2250	11.88	69.30	2925	1953	34.5
Phosphocellulose	224	1.46	56.50	890	1613	35.0
1st $(NH_4)_2SO_4$	149	0.10	50.00	632	1560	32.1
CM-Cellulose	85.8	0.05	35.12	20	985	35.7
2nd $(NH_4)_2SO_4$	70.5	0.01	30.20	2.4	885	34.1

* The activities of mitochondrial 3-hydroxyacyl-CoA dehydrogenase towards acetoacetyl pantetheine are listed. ** Mitochondrial 3-hydroxyacyl-CoA dehydrogenase exhibited nearly the same activity toward acetoacetyl-CoA and acetoacetyl pantetheine, but the peroxisomal enzyme was only active toward acetoacetyl-CoA (7). Peroxisomal activities were assayed with use of acetoacetyl-CoA as a substrate after the complete removal of the activity towards acetoacetyl pantetheine by the treatment with an antibody. *** Ratio: Heat-labile enoyl-CoA hydratase/peroxisomal 3-hydroxyacyl-CoA dehydrogenase.

activity of enoyl-CoA hydratase was increased about 6-fold. The activity of the homogenate prepared from the di-(2-ethylhexyl) phthalate group was inactivated to the control level by heating at 57°C within 2 min, whereas the activity of the control group remained unchanged for more than 5 min.

A typical result of the purification of the heat-labile enoyl-CoA hydratase is summarized in Table 1. The specific activity was raised by about 14-fold. The preparation was almost free from the heat-stable activity. The ratio of activities of the heat-labile enoyl-CoA hydratase to peroxisomal 3-hydroxyacyl-CoA dehydrogenase are the same through the purification steps. Both activities were failed to be separated on an isoelectric focusing using Ampholine, pH 3.5-10, calcium phosphate gel-

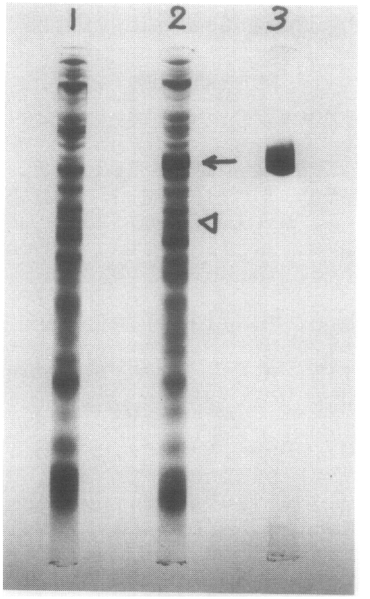

Figure 1. SDS-polyacrylamide gel electrophoresis of post-nuclear fractions of normal and di-(2-ethylhexyl) phthalate-treated rats. Post-nuclear fractions (80 μg protein) and the purified enzyme (10 μg protein) were analyzed on 0.8% SDS-polyacrylamide gels. Electrophoresis was carried out at 8 mA/gel for 5 hr. 1, normal; 2, di-(2-ethylhexyl)phthalate; 3, the enzyme. Arrow indicates the position of 80,000MW polypeptide. Position of catalase is indicated. (◁).

cellulose column chromatography, gel chromatography on Sephadex G-100, Blue Dextran-Sepharose (16) column chromatography using various elution systems. The molecular weight of the native enzyme was estimated to be 70,000 by a Sephadex G-100 column chromatography.

On a SDS-polyacrylamide gel electrophoresis, it was found that the enzyme consisted of a single polypeptide chain with a molecular weight of 77,000. Reddy and Kumar have demonstrated that a peroxisome specific polypeptide with a molecular weight of 80,000 was increased in the liver of rats treated with various hypolipidemic peroxisomal proliferators (14). Fig. 1 indicates that the enzyme corresponds to a peroxisome specific polypeptide. The present study suggests that the peroxisome specific polypeptide is one of main protein components of induced peroxisomes and a multifunctional protein exhibiting enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities.

REFERENCES

1. Lazarow, P.B. and de Duve, C. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 2143-2046.
2. Lazarow, P.B. (1977) Science 197, 580-581.
3. Osumi, T. and Hashimoto, T. (1978) J. Biochem. 83, 1361-1365.
4. Lazarow, P.B. (1978) J. Biol. Chem. 253, 1522-1528.
5. Osumi, T. and Hashimoto, T. (1979) J. Biochem. 35, 131-139.
6. Osumi, T. and Hashimoto, T. (1978) Biochem. Biophys. Res. Commun. 83, 479-485.
7. Osumi, T. and Hashimoto, T. (1979) Biochim. Biophys. Acta, in press.
8. Steinman, H.M. and Hill, R.L. (1975) in Methods in Enzymology (Lowenstein, J.M., ed.) Vol. 35, pp. 136-151, Academic Press, New York.
9. Lynen, F., Henning, U., Bublitz, C., Sörbo, B., and Kröplin-Rueff, L. (1958) Biochem. Z. 330, 269-295.
10. Lynen, F. and Wieland, O. (1955) in Methods in Enzymology (Colowick, S.P. and Kaplan, N.O., eds.) Vol. 1, pp. 566-573, Academic Press, New York.
11. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
12. Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406-4412.
13. de Duve, C., Pressman, B.C., Gianetto, R., Wattiaux, R. and Appelmans, F. (1955) Biochem. J. 60, 604-617.
14. Reddy, J.K. and Kumar, N.S. (1977) Biochem. Biophys. Res. Commun. 77, 824-829.
15. Reddy, J.K. and Moody, D.E., Azarnoff, D.L. and Rao, M.S. (1976) Life Sci. 18, 941-946.
16. Ryan, L.D. and Vestling, C.S. (1974) Arch. Biochem. Biophys. 160, 279-284.