MULTIPLE CYTOSOLIC FORMS OF HEPATIC β-HYDROXY-β-METHYGLUTARYL CoA SYNTHASE: POSSIBLE REGULATORY ROLE IN CHOLESTEROL SYNTHESIS

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SUMMARY:

Two cytosolic forms (I and II) of HMG CoA synthase have been obtained in homogeneous form from chicken liver. The two enzymes have distinctly different electrophoretic and chromatographic properties; synthase I is composed of a single polypeptide chain of about 52,000 daltons, whereas, two identical or similar polypeptide chains of about 55,000 daltons comprise synthase II. On the basis of their cytoplasmic localization and the fact that cholesterol feeding has a negative feed-back effect on cytosolic synthase activity, it appears that one or both of these enzymes carry out the committed step in hepatic cholesterol synthesis.

Acetoacetyl CoA and β-hydroxy-β-methylglutaryl CoA (HMG CoA) are the first two intermediates of both cholesterol synthesis and acetoacetate formation in liver (1,2). For this reason many investigators have held the view (1-7) that HMG CoA is a branch-point in these pathways, thus HMG CoA reductase has been considered the first step unique to cholesterogenesis and, therefore, the primary control point. The observation (2) that hepatic reductase activity fluctuates in response to alterations in physiological state which affect cholesterol synthesis is consistent with this hypothesis. Inasmuch as ketogenesis, via the HMG CoA cycle, is primarily a mitochondrial process (1,8,9), while cholesterol synthesis from HMG CoA occurs in the cytoplasm (1,2), transport of this precursor from the mitochondrion to the cytoplasm would be required for cholesterol synthesis. Recent investigations in our laboratory reveal that substantial acetoacetyl CoA thiolase (10) and HMG CoA synthase (this communication) activities are

present in the cytoplasmic cell fraction of avian liver. These findings suggest that the cytoplasmic thiolase and synthase may in fact initiate hepatic cholesterol synthesis and function independently from their mitochondrial counterparts which participate in ketogenesis. The role of the cytoplasmic synthase in hepatic cholesterol synthesis is further supported by the finding that the activity of this enzyme is markedly depressed by cholesterol feeding.

Multiple forms of HMG CoA synthase can be detected by ion-exchange chromatography of either the cytosolic fraction[1] of avian liver or of preparations having undergone preliminary fractionation with ammonium sulfate and calcium phosphate gel. Two distinct synthase activities are found in the partially purified fraction by chromatography on phosphocellulose (Fig.1); similar results have been obtained with the unfractionated cytoplasmic fraction. Synthase II is eluted in the "break-through" volume at low ionic strength, whereas synthase I is eluted at higher ionic strength. Both enzymes exhibit reciprocal behaviour upon subsequent anion-exchange chromatography on DEAE cellulose, i.e. synthase I being eluted in the "break-through" volume at low ionic strength and synthase II at high ionic strength. Further purification of each synthase by chromatography on hydroxylapatite and then by gel filtration on Sephadex G-200, leads to enzyme preparations which appear homogeneous and have distinctly different electrophoretic

[1]The cytoplasmic fraction was virtually free of the contaminating mitochondrial marker enzymes, glutamate dehydrogenase and citrate synthase. It was necessary to dialyze the cytoplasmic and cytosolic fractions overnight against 20 mM potassium phosphate buffer, pH 7, to inactivate traces of HMG CoA lyase prior to the assay of HMG CoA synthase. Of the total cytoplasmic HMG CoA synthase activity (about 0.2 μmole of HMG CoA formed per min per g wet weight of liver) greater than 90% was cytosolic. Two synthase activities were found in the crude cytosolic fraction which exhibited chromatographic behaviours on DEAE- and phosphocellulose columns identical to those of purified synthases I and II described below. It was not possible to accurately assess total mitochondrial synthase activity due to the high HMG CoA lyase activity of this cell fraction.

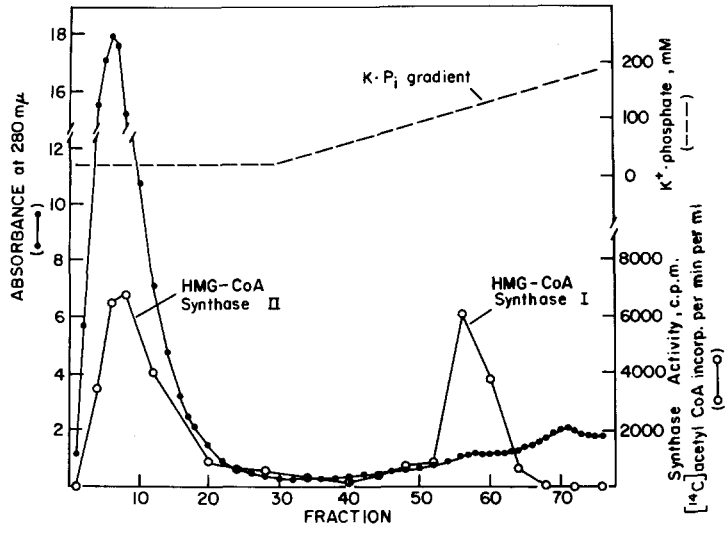

Fig. 1. Resolution of HMG CoA synthases I and II by chromatography on phosphocellulose. After preliminary fractionation of 4 kg of chicken liver high-speed supernatant fraction with ammonium sulfate (between 30% and 45% saturation), adsorption on and elution from calcium phosphate gel (11) and dialysis against 20 mM potassium phosphate buffer, pH 7.0, containing 0.1 mM EDTA and 5 mM β-mercaptoethanol, the synthase preparation (19.3 g of protein in 500 ml) was applied to a 9 x 80 cm phosphocellulose column. 3.5 l of the same buffer was passed through the column followed by a 4 l linear potassium phosphate gradient (pH 7; 20-200 mM) also containing EDTA and β-mercaptoethanol. Each fraction (70 ml) was assayed for HMG CoA synthase activity using homogeneous β-ketothiolase (10) to generate acetoacetyl CoA. The reaction mixture contained 20 mM Tris·Cl buffer, pH 8.4, 0.03 mM EDTA, 0.15 mM [1-^{14}C] acetyl CoA (1.1 x 10^5 c.p.m. per μmole), 0.7 unit of thiolase, and an aliquot of column eluate in a volume of 0.3 ml. Following a 10 min incubation at 37° radioactivity incorporated into HMG CoA was determined as [^{14}C] activity not volatile when 0.1 ml of reaction mixture was taken to dryness in 0.2 ml of 6N HCl at 95° and is expressed in the Fig. as c.p.m. incorporated per ml of column eluate. The non-volatile [^{14}C] activity was determined to be HMG CoA in two chromatographic systems.

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and molecular properties.

Polyacrylamide gel electrophoresis (Fig. 2) shows that each synthase gives rise to a single stained protein band which is exactly coincident with enzymatic activity. Using 7.5% acrylamide gels and Tris-glycine buffer at pH 8.9, synthase I has an R_m (mobility relative to the tracking dye) of 0.37; in contrast, synthase II has an unusually high mobility, R_m = 0.91, which indicates that it is a particularly acidic protein and/or is of low molecular weight. That the former is the case is indicated by its

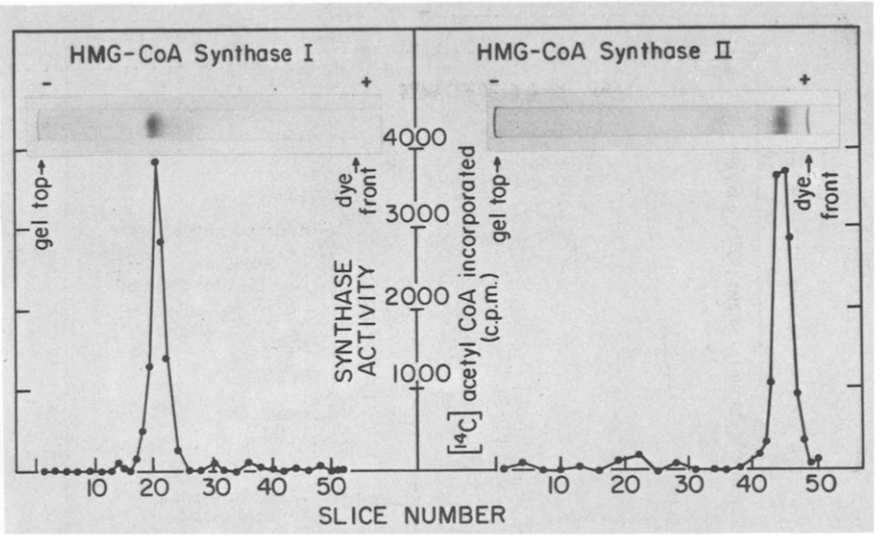

Fig. 2. Polyacrylamide gel electrophoretic patterns of purified HMG CoA synthases I and II. Approximately 30 μg of synthase I and 20 μg of synthase II were applied to separate paired gels containing 7.5% acrylamide. After electrophoresis in 50 mM Tris-glycine buffer, pH 8.9, for 2 hours at a constant current of 4 mA, one gel containing each enzyme was stained and its companion gel sliced into approximately 50 uniform segments for synthase assays. Synthase I activity was located by incubating each slice overnight at 25° with 0.2 ml of a reaction mixture containing 50 mM Tris-Cl buffer, pH 7.2, 0.1 mM dithiothreitol, 0.1 mM EDTA, 0.2 mM [1-^{14}C] acetyl CoA and 0.1 mM acetoacetyl CoA. Synthase II was extracted from each slice by incubation in 0.2 ml of 25 mM Tris-Cl$^-$ buffer, pH 7.2, containing 0.1 mM EDTA, and 0.5 mM dithiothreitol for 20 hours at 4°; assays were conducted using the reaction mixture described above. [^{14}C] activity incorporated in HMG CoA was determined as described in the legend of Fig. 1.

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behaviour in the gel filtration experiments with Sephadex G-200 summarized in Fig. 3A. Using avidin labeled with [^{14}C] biotin as an internal marker, it is evident that synthase II has a considerably larger Stokes radius than synthase I. The elution volume of synthase II was not altered when the run was carried out at the same pH (pH 8.9, Tris-glycine buffer) as that employed in the acrylamide gel electrophoresis experiments. A comparison with a series of protein markers indicates that synthases I and II have approximate molecular weights of 50,000 and 100,000, respectively. The molecular weight of synthase II as determined by sedimentation equilibrium (15) was found to be 94,000 $\pm$ 3,000 which is in good agreement with that obtained by gel filtration.

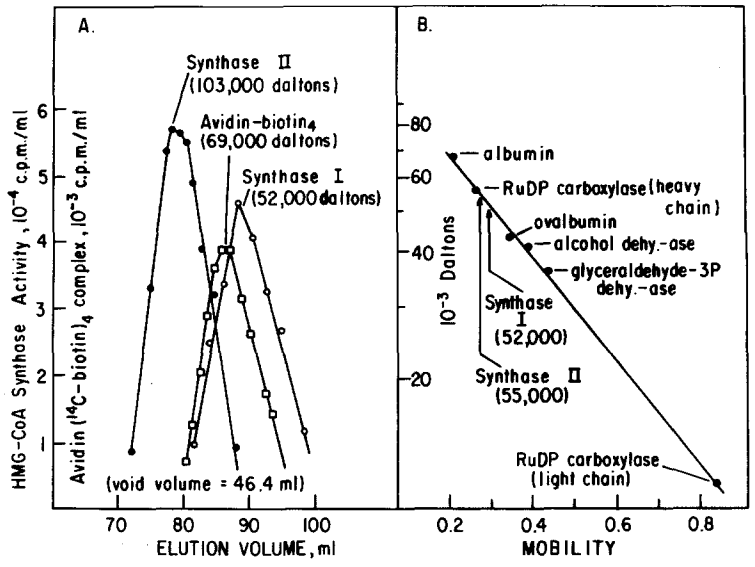

Fig. 3. Comparison of the molecular weights of native synthases (A) and their subunits (B). In 3A each synthase (I, 0.5 unit and II, 0.2 unit) was subjected to gel filtration on a Sephadex G-200 column (132 ml, 1.5 x 75 cm) using avidin-[^{14}C] biotin complex (0.24 mg, 4 x 10^4 c.p.m.) as an internal marker. The eluting buffer was 20 mM potassium phosphate, pH 7, which also contained 0.1 mM EDTA and 0.1 M KCl. Synthase assays were carried out for 6 min as described in the legend of Fig. 1; synthase activity (ordinate) is expressed as c.p.m. of [^{14}C] acetyl CoA (1.1 x 10^5 c.p.m. per μmole) incorporated into HMG CoA during the 6-min assay per ml of eluate. Catalase, lactate dehydrogenase, hexokinase and avidin-biotin complex were used to calibrate the column and the method of Andrews et al. (12) was used to calculate the molecular weights of the synthases. In 3B 1% sodium dodecylsulfate-10% acrylamide gels and protein samples were prepared and electrophoresis conducted according to Weber and Osborn (13). RuDP carboxylase refers to ribulose-1, 5-diphosphate carboxylase (14).

Subunit weight analysis by sodium dodecylsulfate-gel electrophoresis shows that each enzyme gives rise to a single stained protein band, synthase I corresponding to 52,000 daltons and synthase II to 55,000 daltons (Fig. 3B). Repetitive runs consistently showed that the subunit of synthase II was slightly larger than that of synthase I. From these data and the molecular weights of the native enzymes, it can be concluded that synthase I is composed of a single polypeptide chain of 52,000 daltons, while synthase II has two identical or similar, polypeptide chains of about 55,000 daltons. No interconversion of the

Table I

Summary of kinetic parameters of HMG CoA synthases

	Synthase I	Synthase II
pH optimum	9.2-9.5	9.0-9.4
K_m (acetoacetyl CoA)*	<2.5 μM	<2.5 μM
K_m (acetyl CoA)*	0.29 mM	0.31 mM
V_{max} (μmoles/min/mg)	1.3	0.83

*under standard assay conditions: 50 mM Tris-Cl buffer, pH 8.0, 0.1 mM acetoacetyl CoA, and 0.3 mM acetyl CoA; 37° for 5 min.

two synthases could be demonstrated upon repetitive ion exchange chromatography or electrophoresis.

Despite their distinctive molecular and electrophoretic characteristics, the two HMG CoA synthases appear to have similar kinetic properties. As is evident from the results summarized in Table I, the pH optima, K_m values, and maximal velocities of the two enzymes are indistinguishable. It is interesting and perhaps significant that the K_m for acetoacetyl-CoA is extremely low, that is, < 2.5 μM. In view of the unfavorable equilibrium of the thiolase-catalyzed formation of acetoacetyl-CoA ($K_{eq} = 1.6 \times 10^{-5}$ at pH 7) (16) and the consequent low cytoplasmic concentration of this intermediate, the low K_m of the synthases for acetoacetyl CoA is most likely of kinetic advantage in HMG CoA biosynthesis. Furthermore, the combined actions of the cytoplasmic thiolase (also obtained in homogeneous form in our laboratory) and synthase rapidly convert acetyl CoA to HMG CoA in a thermodynamically-favorable manner; at equilibrium (pH 8.0, 30°), 70% of the acetyl CoA added is converted to HMG CoA. It is evident, therefore, that one or both of these enzymes must be regulated. Preliminary investigations indicate that the cytosolic HMG CoA synthase activity is markedly depressed by cholesterol feeding. Eight week old

cockerels (4 per group) were fed a cholesterol-low commercial diet containing 10% oleic acid; one group was fed this basal diet and another the basal diet plus 2% cholesterol. Studies in Siperstein's laboratory (17) have shown that under these conditions, cholesterol feeding suppresses hepatic cholesterogenesis from acetate. In the present experiments, feeding the cholesterol-containing diet for one week led to a depression of hepatic cytosolic synthase activity to a level 18% $\pm$ 2% that of control values (3 ± 0.3 milliunits of synthase per mg of cytosolic protein). Cytosolic acetoacetyl CoA thiolase activity is not responsive to feeding cholesterol under these conditions. Whether both synthases I and II are subject to feed-back regulation by cholesterol and the mechanism by which this control is exerted are currently under investigation.

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References:

1. Bucher, N.L.R., Overath, P., and Lynen, F., Biochim. Biophys. Acta, 40, 491 (1960).
2. Siperstein, M.D., Curr. Topics Cell. Regulation, 2, 65 (1970).
3. Gould, R.G., and Popjak, G., Biochem J., 66, 51P (1957).
4. Siperstein, M.D., and Guest, M.J., J. Clin. Invest., 39, 642, (1960).
5. Linn, T.C., J. Biol. Chem., 242, 990 (1967).
6. Siperstein, M.D., and Fagan, V.M., J. Biol. Chem., 241, 602, (1966).
7. White, L.W., and Rudney, H., Biochemistry, 9, 2725 (1970).
8. Greville, G.D., and Tubbs, P,K., Essays in Biochem., 4, 171 (1968).
9. Garland, P.B., In The Biochemical Roles of Citrate, Biochem. Soc. Symp. No. 27, p. 41, Ed. by T.W. Goodwin, Academic Press New York (1968).
10. Sugiyama, T., Clinkenbeard, K., Moss, J., and Lane, M.D., Federation Proc., 31, 475 (1972).
11. Gregolin, C., Ryder, E., and Lane, M.D., J. Biol. Chem., 243, 4227 (1968).
12. Andrews, P., Methods Biochem. Anal., 18, 1 (1970).
13. Weber, K. and Osborn, M., J. Biol. Chem., 244, 4406 (1969).
14. Rutner, A., Biochem. Biophys. Res. Communs., 39, 923 (1970).
15. Roark, D.E., and Yphantis, D.A., Ann. New York Acad. Sci., 164, 245, (1969).
16. Hartmann, G. and Lynen, F., In The Enzymes, 2nd ed., vol. 5, pp. 381-386, Ed. by Boyer, P.D., Lardy, H. and Myrbäck, K., Academic Press, New York (1961).
17. Sakakida, H., Shediac, C.C., and Siperstein, M.D., J. Clin. Invest., 42, 1521 (1963).