Activation of 3-Hydroxy-3-methylglutaryl-Coenzyme A Reductase in Homogenates of Developing Rat Brain

By William A. MALTESE and Joseph J. VOLPE

Departments of Pediatrics and Neurology and Neurosurgery (Neurology), Washington University School of Medicine, St. Louis, MO 63110, U.S.A.

(Received 19 March 1979)

The specific activity of 3-hydroxy-3-methylglutaryl-CoA reductase increases when homogenates of developing rat brain are incubated at 37°C or kept on ice. This increase is completely blocked by the addition of F^- to the homogenization medium and the assay mixture. The capacity for activation of the reductase is greatest during the early postnatal period and declines as brain maturation proceeds. The data suggest that catalytic modification of the reductase may play a role in the regulation of cholesterol synthesis in the developing brain.

Little is known about the regulation of cholesterol biosynthesis in the developing brain, although the importance of this sterol as a constituent of myelin as well as of axonal, dendritic and intracellular membranes is generally recognized. The specific activity of HMG-CoA reductase (EC 1.1.1.34), a major rate-limiting enzyme in the cholesterol-biosynthetic pathway in a variety of tissues [see Volpe (1977) for review], undergoes significant developmental changes in the microsomal fraction of mouse and rat brain (Kandutsch & Saucier, 1969; Sudjic & Booth, 1976; Aragon *et al.*, 1978). However, nothing is known about the mechanisms underlying these changes.

It is well established that under appropriate conditions the hepatic HMG-CoA reductase can undergo catalytic modification (activation or inactivation) *in vitro* (Beg *et al.*, 1973; Berndt & Gaumert, 1974; Berndt *et al.*, 1976; Nordstrom *et al.*, 1977). Of particular interest is the observation that much of the reductase in rat liver may be converted from an inactive into an active form during isolation of the microsomal fraction (Nordstrom *et al.*, 1977). This suggests that the reductase activity *in vivo* can increase severalfold without an increase in enzyme synthesis.

In the present study we have sought to determine whether activation of HMG-CoA reductase occurs in homogenates of developing rat brain. Our results indicate that the reductase can undergo a striking degree of activation during the early postnatal period, but that this capacity is lost as brain maturation progresses.

Abbreviation used: HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A.

Experimental

Materials

DL-3-Hydroxy-3-methyl[glutaryl-3- ^{14}C]glutaryl-CoA (49.5 mCi/mmol), DL-3-hydroxy-3-[methyl- 3H]methylglutaryl CoA (5.6 Ci/mmol) and DL-[2- ^{14}C]mevalonic acid (dibenzoylthylenediamine salt; 46 mCi/mmol) were purchased from New England Nuclear, Boston, MA, U.S.A. Radioactive HMG-CoA was diluted to 10 mCi/mmol with unlabelled HMG-CoA. NADPH, HMG-CoA, mevalonolactone, bovine serum albumin and dithiothreitol were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Silica-gel G-HY (0.2 mm $\times$ 20 cm $\times$ 20 cm) t.l.c. plates (Brinkman Instruments, Westbury, NY, U.S.A.), 3a70 scintillation fluid (Research Products International, Elk Grove, IL, U.S.A.) and AG1-X8 resin (200–400 mesh; formate; Bio-Rad, Richmond, CA, U.S.A.) were obtained from the designated sources.

Animals

All rats were of the Sprague–Dawley strain and were maintained on a standard diet of Purina Rat Chow. Lighting conditions consisted of 12 h of light from 08:00 h to 20:00 h, and 12 h of darkness from 20:00 h to 08:00 h. Suckling animals were left with their mothers until immediately before use and all animals were killed at the same time of day (09:00 h). Rats referred to as adults weighed approx. 300 g.

Preparation of homogenates and microsomal fractions

Brains were homogenized in 9 vol. of cold 0.32 M-sucrose / 5 mM-dithiothreitol / 30 mM-EDTA / 40 mM-

potassium phosphate, pH 7.5, in a Potter-Elvehjem glass/Teflon tissue grinder with a motor-driven pestle. When indicated, 50mM-NaF was included in the homogenization buffer. Microsomal fractions were isolated by differential centrifugation (Gray & Whittaker, 1962). Washed microsomal fractions were prepared by gently homogenizing the initial 100000g pellet in 2 vol. of homogenization buffer and centrifuging the suspension a second time at 100000g for 60 min.

Protein determination

Protein was precipitated with 10% (w/v) trichloroacetic acid, dissolved in 2M-NaOH, and assayed by a microbiuret method (Munkres & Richards, 1965), with bovine serum albumin as a standard.

Enzyme assays

The reaction mixture used routinely for the assay of HMG-CoA reductase in brain homogenates and microsomal fractions consisted of 40mM-potassium phosphate, pH 7.5, 30mM-EDTA, 5mM-dithiothreitol, 2.5mM-NADPH, 75 μ M-[³H]HMG-CoA and 200 μ g of protein in a total volume of 200 μ l. Under these conditions the formation of mevalonate was linear up to 60 min at concentrations of protein ranging from 0.25 to 2.0mg/ml. The optimal conditions for the reductase assay were the same, regardless of the age of the rats from which brains were obtained. When indicated, the reaction mixture was modified to contain 50mM-NaF. Reaction blanks contained all components, except that the protein was boiled for 10 min. In experiments aimed at determining the effects of preincubation or the presence of F⁻ on the activity of HMG-CoA reductase in crude homogenates, blanks containing active protein, but no NADPH, were also run. The reaction was started by addition of the HMG-CoA and was terminated after 60 min by addition of 20 μ l of 5M-HCl. [¹⁴C]Mevalonic acid (approx. 2000 d.p.m.) was added as an internal standard and lactonization was allowed to proceed at 37°C for 45 min. Mevalonolactone was isolated from the reaction mixture by an ion-exchange procedure on AG1-X8 resin, and radioactivity was measured in a liquid-scintillation spectrometer (Volpe & Hennessy, 1977). When mevalonolactone was isolated by t.l.c., conditions were essentially the same as those used by Shapiro *et al.* (1969).

The reaction mixture used for the assay of HMG-CoA lyase was identical with that used for assay of HMG-CoA reductase, except that NADPH was omitted and [¹⁴C]HMG-CoA was used instead of [³H]HMG-CoA. The reaction was started by addition of the HMG-CoA and was terminated by the addition of 1.5 ml of 6M-HCl. Lyase activity was determined by measuring the amount of non-volatile [¹⁴C]HMG-CoA converted to volatile [¹⁴C]acetoacetate (Goodwin & Margolis, 1976).

Results

When homogenates of brains from 10-day-old rats were incubated at 37°C, the specific activity of HMG-CoA reductase underwent a substantial increase (Fig. 1). This effect was maximal after 2 h and declined with longer incubation times, possibly because of protein denaturation or the action of proteinases. An increase in activity was also observed when the homogenates were kept on ice; however, the degree of activation was not as great as that obtained at 37°C. Inclusion of 50mM-NaF in the homogenization medium and in the assay mixture completely blocked the activation of the reductase. When in a separate experiment 50mM-NaCl was substituted for NaF, the reductase activity measured after a 2 h preincubation at 37°C was equal to 92% of that in activated control homogenates. Thus the inhibition of reductase activation observed in Fig. 1 appears to be related specifically to the presence of F⁻.

Crude homogenates rather than microsomal fractions were used for enzyme assays because of observations that much of the HMG-CoA reductase and NADPH-cytochrome *c* reductase (microsomal marker) activity in the brain is removed from homogenates by low-speed centrifugation (Sudjic & Booth, 1976; Aragon *et al.*, 1978; Maltese & Volpe, 1979). In order to preclude the possibility that the increased reductase activity measured in preincubated homogenates was due to increased conversion of the [³H]HMG-CoA into products other than mevalono-

Fig. 1. Activation of HMG-CoA reductase as a function of time of incubation of homogenates before assay

Brains from 10-day-old rats (littermates) were homogenized either in the presence (four brains) or absence (four brains) of 50mM-NaF. Half of each homogenate was incubated at 37°C and the remainder was left on ice. At the designated times samples were removed and assayed in duplicate for HMG-CoA reductase (see the Experimental section). ■, 37°C, -NaF; □, 37°C, +NaF; ●, ice, -NaF; ○, ice, +NaF. Values are means of duplicate determinations, and standard deviations were less than 10% of the mean values.

lactone, which did not bind to AG1-X8 resin, representative assays were performed with the use of t.l.c. to isolate the lactone. This method is generally accepted as a means for isolating radiochemically pure mevalonolactone from HMG-CoA reductase assay mixtures (Shapiro *et al.*, 1969). The specific activities for HMG-CoA reductase (in activated and non-activated homogenates), calculated on the basis of product isolated by t.l.c., were identical ($\pm 10\%$) with those obtained when the product was isolated by the ion-exchange method. Moreover, when samples of the resin eluate were subjected to t.l.c., the ratio of $^3\text{H}/^{14}\text{C}$ in the spot that co-migrated with authentic mevalonolactone was the same as that in the unchromatographed eluate. Thus the radiochemical purity of the product isolated by the ion-exchange method is at least as great as that of the mevalonolactone isolated by t.l.c. Finally, when NADPH was omitted from the assay mixture the radioactivity recovered in the product was always the same as that measured in the blanks containing boiled protein. This ruled out the possibility that a radioactive product formed by an NADPH-independent reaction was contributing to the enzyme activity being measured.

Samples of homogenate assayed for HMG-CoA lyase under the same conditions as were used to assay the reductase did not contain detectable lyase activity. The presence of EDTA in the reaction mixture appears to suppress the activity of the lyase, which requires bivalent cations for activity (Stegink & Coon, 1968). Thus the increase in HMG-CoA reductase activity does not appear to result from an inhibition of HMG-CoA lyase, which can compete for available substrate. Although a more precise definition of the mechanism responsible for the activation must await further study, the fact that the increase in reductase activity can be completely blocked by F^- , a phosphatase inhibitor, raises the possibility that an activator protein similar to that characterized in the liver (Nordstrom *et al.*, 1977) may also exist in the rat brain during the postnatal period.

To determine whether activation of the reductase required only microsomal constituents, we assayed the enzyme in washed and unwashed microsomal fractions prepared by differential centrifugation (Table 1). Although some activation of HMG-CoA reductase was obtained in unwashed microsomal fractions prepared from preincubated homogenates, the specific activity of the reductase in washed microsomal fractions was the same regardless of whether the assay mixture contained F^- . Attempts to activate the reductase by resuspending washed microsomal fractions, prepared in the presence of NaF, in buffer without NaF were unsuccessful. Nor were we able to activate the reductase in washed microsomal fractions by incubating them with the

Table 1. *HMG-CoA reductase specific activity in microsomal fractions of 10-day-old rat brains assayed in the presence and absence of 50 mM-NaF*

Washed or unwashed microsomal fractions were prepared from homogenates of 10-day-old rat brains which had been incubated for 2 h, either in the presence or absence of 50 mM-NaF. The microsomal fractions (approx. 1.0 mg of protein) were resuspended in 300 μl of homogenization medium with no addition ($-\text{NaF}$), with 50 mM-NaF ($+\text{NaF}$), or in 300 μl of 100000g supernatant solution (HSS) without NaF. Microsomal suspensions then were preincubated at 37°C for 1 h and assayed for HMG-CoA reductase activity (see the Experimental section). Specific activities are expressed as pmol of mevalonate formed/min per mg of protein and represent means of duplicate determinations which did not vary by more than 10%.

Homogenate	Microsomal fraction	Enzyme specific activity	
		+NaF	-NaF
+NaF	Washed	87.9	90.6
-NaF	Washed	85.2	89.8
-NaF	Unwashed	113.0	217.0
-NaF	Washed+HSS	—	70.2

100000g supernatant solution. Thus the data suggest that at least one component necessary for the activation of the reductase is loosely associated with the microsomal fraction and can be easily lost or inactivated. Nevertheless, it remains entirely possible that other components present in the cytosol are also involved.

The degree to which the reductase could be activated in homogenates was not uniform throughout the period of postnatal brain development. Rats from litters of several different ages were killed, and the activity of the reductase in brain homogenates was determined after preincubation in the presence or absence of 50 mM-NaF (Fig. 2a). Two major points are apparent. First, the extent to which the reductase was activated in the absence of NaF showed a decline between 10 and 21 days. Second, in both the presence and absence of NaF, the specific activity of the enzyme exhibited a similar developmental profile, i.e. an overall decline from 4 days until adulthood, with a marked decrease in specific activity occurring between 10 and 15 days. However, because there was a greater capacity for activation of the reductase in the homogenates from younger animals, the changes in specific activity that accompany brain maturation were accentuated in the absence of NaF.

In view of the dramatic increases in brain protein and wet weight (primarily due to the accumulation of myelin) that occur during the postnatal period, and the importance of assaying brain HMG-CoA reductase in crude homogenates, expression of enzymic activity on a per brain rather than a per mg

Fig. 2. Activation of HMG-CoA reductase as a function of postnatal age

Brains were obtained from rats of each designated age and were homogenized either in the presence or absence of 50mM-NaF. All homogenates were incubated at 37°C for 2h and were assayed for HMG-CoA reductase. (a) Specific activities in the presence (○) or absence (●) of F⁻ are shown as function of age. (b) The results are expressed as total units of enzyme activity per brain. All assays were carried out in duplicate, and each point represents the mean (±s.d.) of separate determinations performed on three different homogenates. Each homogenate contained one or two brains. One unit of enzyme activity equals 1 nmol of mevalonate formed/min.

of protein basis might provide a more meaningful assessment of the developmental changes of this enzyme (Fig. 2b). Thus, although the specific activity of the reductase declined after 4 days (Fig. 2a), the total activity per brain increased markedly, as one might expect on the basis of the rise in the cholesterol content of the brain during this period (Wells & Dittmer, 1967; Cuzner & Davison, 1968). It is also apparent that when reductase activity is expressed per brain, two different developmental profiles are generated, depending upon whether NaF is present during homogenization and assay.

Discussion

The demonstration of a capacity for activation of HMG-CoA reductase in brain homogenates has important implications for the interpretation of developmental studies of this enzyme. Thus, although it remains to be determined to what extent this type of activation represents a physiological mechanism, the data suggest for the first time that regulation of the reductase at the level of catalytic efficiency may

be of particular importance in the developing brain. This suggestion is compatible with the fact that the period during which the capacity for activation of the reductase is greatest (4–10 days) correlates well with the period during which sterol biosynthesis from acetate has been shown to be most active in the developing mouse brain (Kandutsch & Saucier, 1969). The presence of a phosphatase capable of modifying the catalytic efficiency of HMG-CoA reductase in the brain during the critical period of growth and early myelination might provide an important mechanism whereby reductase activity can be increased rapidly without a change in the rate of synthesis and/or degradation of the enzyme. Future studies along these lines should yield important new insights into the regulation of sterol synthesis in the developing brain.

J. J. V. is a recipient of a Research Career Development Award 1K4 HD-70608 from the National Institutes of Health. This investigation was supported by grants 1 R01 HD-07464 and 5-T 32-NS 07027-03 from the National Institutes of Health. We gratefully acknowledge the excellent technical assistance of Ms. Sharon Place.

References

- Aragon, M. C., Gimenez, C., Valdivieso, F. & Mayor, F. (1978) *J. Neurochem.* **30**, 649–650
- Beg, Z. H., Allmann, D. W. & Gibson, D. M. (1973) *Biochem. Biophys. Res. Commun.* **54**, 1362–1369
- Berndt, J. & Gaumert, R. (1974) *Hoppe-Seyler's Z. Physiol. Chem.* **355**, 905–910
- Berndt, J., Hegardt, F., Bove, J., Gaumert, R., Still, J. & Cardo, M.-T. (1976) *Hoppe-Seyler's Z. Physiol. Chem.* **357**, 1277–1282
- Cuzner, M. L. & Davison, A. N. (1968) *Biochem. J.* **106**, 29–34
- Goodwin, C. D. & Margolis, S. (1976) *J. Lipid Res.* **17**, 297–303
- Gray, E. G. & Whittaker, V. P. (1962) *J. Anat.* **96**, 79–89
- Kandutsch, A. A. & Saucier, S. E. (1969) *Arch. Biochem. Biophys.* **135**, 201–208
- Maltese, W. A. & Volpe, J. J. (1979) *J. Neurochem.* in the press
- Munkres, K. O. & Richards, F. M. (1965) *Arch. Biochem. Biophys.* **109**, 466–479
- Nordstrom, J. L., Rodwell, V. W. & Mitschelen, J. J. (1977) *J. Biol. Chem.* **252**, 8924–8934
- Shapiro, D. J., Imblum, R. L. & Rodwell, V. W. (1969) *Anal. Biochem.* **31**, 383–390
- Stegink, L. D. & Coon, M. J. (1968) *J. Biol. Chem.* **243**, 5272–5279
- Sudjic, M. M. & Booth, R. (1976) *Biochem. J.* **154**, 559–560
- Volpe, J. J. (1977) in *Diabetes, Obesity and Vascular Disease: Metabolic and Molecular Interrelationships* (Katzen, H. M. & Mahler, R. J., eds.), pp. 37–125, Halsted Press, New York
- Volpe, J. J. & Hennessy, S. W. (1977) *Biochim. Biophys. Acta* **486**, 408–420
- Wells, M. A. & Dittmer, J. C. (1967) *Biochemistry* **6**, 3169–3175