UDP-Galactose:ceramide galactosyltransferase of rat central-nervous-system myelin

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The properties of ceramide galactosyltransferase associated with myelin and microsomal fractions of rat brain were studied. The enzyme from both the fractions had similar properties during development and synthesized the same molecular species of the product cerebroside. The results suggested that during myelination the turnover rate of enzyme protein is altered instead of regulatory modulation of the enzyme protein.

The last step in the biosynthesis of galactosylceramides (cerebrosides) is catalysed by the enzyme UDP-galactose:ceramide galactosyltransferase (UDP-galactose:2-hydroxyacylsphingosine galactosyltransferase, EC 2.4.1.45). In brain the activity of this enzyme is primarily localized in the microsomal fraction (Morell & Radin, 1969; Morell *et al.*, 1970). However, a significant amount of activity is also associated with myelin membrane fraction (Neskovic *et al.*, 1973; Costantino-Ceccarini & Suzuki, 1975). We have shown that during development the specific activity of the enzyme changed markedly in myelin and microsomal membranes of rat brain (Koul *et al.*, 1980). The specific activity of the microsomal UDP-galactose:ceramide galactosyltransferase reached a maximum when the animals were about 20 days old and then declined. By contrast, the specific activity of the enzyme in the myelin membrane was maximal at 16 days, and it was three to four times higher than that of the microsomal membranes at that age. Of the various complex-lipid-synthesizing enzymes studied *de novo*, only UDP-galactose:ceramide galactosyltransferase was associated with myelin membrane. It was postulated that the high activity of the enzyme in myelin at the onset of the myelination period may have some role in the assembly process of myelin membrane.

The properties of the UDP-galactose:ceramide galactosyltransferase from brain microsomal fraction have been extensively studied (Shah, 1971; Basu *et al.*, 1971; Brenkert & Radin, 1972). However, little information is available regarding the characteristics of the myelin-associated enzyme. In

this report, we compare the kinetic and other properties of the enzyme from both these sub-cellular fractions, especially during development. Part of this work was presented previously (Koul & Jungalwala, 1980).

Experimental

Materials

UDP-[¹⁴C]galactose (sp. radioactivity 200 mCi/mmol) was from New England Nuclear, Boston, MA, U.S.A. Hydroxy-fatty-acid-containing ceramide (bovine) and cerebroside were purchased from Applied Science Labs., State College, PA, U.S.A. H.p.l.c.-grade solvents were from Burdick and Jackson Labs., Muskegon, MI, U.S.A. The sources of other chemicals and materials have been described previously (Koul *et al.*, 1980).

Methods

The myelin and microsomal membranes were isolated from male Sprague-Dawley rat brain essentially as described previously (Koul *et al.*, 1980). The assessment of the purity of the isolated myelin has been previously described and is based on the activity of the marker enzymes (Ruenwongsa *et al.*, 1979; Koul *et al.*, 1980). UDP-galactose:ceramide galactosyltransferase was assayed with UDP-[¹⁴C]galactose as the donor and hydroxy-fatty-acid-containing ceramide as the acceptor as previously described (Koul *et al.*, 1980).

Delipidation and reconstitution of myelin and microsomal membranes. The lipids from freeze-dried myelin and microsomal membranes (100 µg of protein) were extracted three times with 1 ml of benzene at room temperature after sonication. The extracted lipids were removed by centrifugation. The

Abbreviation used: h.p.l.c., high-pressure liquid chromatography.

residue was used as the delipidated enzyme source after drying under a stream of N_2 . For reconstitution, similarly extracted lipids from equivalent amounts of the membrane proteins were added to the delipidated membranes and the mixture sonicated and dried under N_2 .

Characterization of molecular species of cerebroside. The incubation for the synthesis of [^{14}C]cerebrosides from hydroxy-fatty-acid-containing ceramide by the UDP-galactose:ceramide galactosyltransferase from myelin or microsomal membranes was done as described previously (Koul *et al.*, 1980), except that the time of the incubation was for 2 h and no non-radioactive UDP-galactose was used to dilute the radioactive UDP-[^{14}C]galactose. The contents of 10–15 incubated tubes were pooled to obtain [^{14}C]cerebrosides having about 1×10^5 d.p.m. The lipid extract of the incubation mixture was chromatographed on a silicic acid column to separate [^{14}C]cerebroside from the rest of the lipids (Hayes & Jungalwala, 1976). The cerebroside fraction was perbenzoylated and the [^{14}C]-labelled-hydroxy-fatty-acid-containing cerebroside was separated from non-hydroxy-fatty-acid-containing cerebroside by h.p.l.c. (Jungalwala *et al.*, 1977). More than 93% of the injected radioactivity was recovered as hydroxy-fatty-acid-containing cerebroside. The molecular species of the perbenzoylated hydroxy-fatty-acid-containing cerebroside was resolved by reversed-phase h.p.l.c. on a Nucleosil- C_{18} ($5\mu m$) (Macherey–Nagel, Düren, Germany) column ($30 cm \times 3.9 mm$ internal diam.) with methanol pumped at a flow rate of 2 ml/min. The detection was at 230 nm. Individual molecular species resolved on the column were collected in counting vials, dried and the radioactivity was determined in a Packard liquid-scintillation counter with a toluene-based scintillant. In a separate experiment the individual molecular species of [^{14}C]-labelled-hydroxy-fatty-acid-containing cerebroside collected from the reversed-phase column were debenzoylated by mild alkaline hydrolysis (Kishimoto & Hoshi, 1972), followed by anhydrous acid hydrolysis and g.l.c. analysis of the recovered non-esterified fatty acid methyl esters (Kishimoto & Hoshi, 1972).

Results

Enzyme characterization

The properties of the UDP-galactose:ceramide galactosyltransferase of the cerebral myelin and microsomal fraction were compared to determine if both the particulate fractions contained the same enzyme. The apparent K_m with hydroxy-fatty-acid-containing ceramide as the substrate for the microsomal enzyme from forebrain of 16 day-old rats was 0.44 mM, whereas that for the myelin enzyme was

0.54 mM. These values were not significantly different. However, the V_{max} for the myelin enzyme at this age was twice that of the microsomal fraction. The enzyme from both the fractions was inhibited with higher concentrations of the hydroxy-fatty-acid-containing ceramide.

The effect of the variation of pH on the activity of the enzyme was studied. There was a sharp increase in the activity of the enzyme between pH 7.2 and 7.8 for the microsomal fraction of the cerebral hemisphere from 18 day-old animals. However, the optimum pH was the same for the enzyme from both the fractions. A broad pH optimum around 7.4–7.8 was observed for these membranes from 14 day-old animals. The effect of varying the concentration of Mn^{2+} and Mg^{2+} on the enzyme activity was studied. The activity of the enzyme from both the fractions was stimulated about 2-fold with 5 mM- Mn^{2+} . At higher concentrations than 5 mM there was a decline in activity so that at 30 mM- Mn^{2+} , activity was about 70% of the maximum. There was a 1.5–2-fold increase in the activity of the enzyme from both membranes with Mg^{2+} up to 20 mM. There was no striking difference in the requirement of metal ions for the enzyme from both the membranes.

The maximal activity of the enzyme from both the membrane fractions from 16 day-old animals was at 45°C. The rate of activation of the enzyme from both the fractions with temperature was the same. The Arrhenius plot also showed that the changes in the energy of activation with temperature for the enzyme from both the fractions were parallel, although the specific activity of the myelin enzyme was higher than that of the microsomal enzyme. Parallel curves for the activation energy were obtained with the enzyme from myelin and microsomal fractions from brain of animals at 14, 19, 20 and 30 days.

The results of the heat-inactivation studies in aqueous medium and in benzene showed that both myelin and microsomal UDP-galactose:ceramide galactosyltransferase was inactivated at the same rate. Of the original activity 90% was lost within the first 5 min of heating at 50°C in aqueous medium. The rate of decline in the activity of the enzyme from both the fractions was also similar when the temperature of pre-incubation was varied between 20 and 60°C for a fixed time of 2 min. In benzene, 50% of the normal activity was lost when the particles were pre-incubated at 70°C for 10 min. Surprisingly, more than 30% of the UDP-galactose:ceramide galactosyltransferase activity could still be detected in both the membranes even though the particles were heated previously at 80°C for 10 min.

Delipidation and reconstitution

The myelin and microsomal membranes from 16

day-old rat cerebral hemispheres, having a UDP-galactose:ceramide galactosyltransferase specific activity of 8.2 and 1.9 nmol/mg per h respectively, were extracted with benzene. The activity of the UDP-galactose:ceramide galactosyltransferase in the microsomal and myelin fraction after extraction was about 58% and 43% of the original respectively. When the extracted lipids from the respective membranes were quantitatively added back, the activity of the enzyme was restored within the normal value range. When equivalent amounts of extracted lipids from myelin (based on protein) were added to the delipidated microsomal preparation, the microsomal UDP-galactose:ceramide galactosyltransferase activity was restored only to its original value. Similarly, when equivalent amounts of lipids from the microsomal fraction were added to the delipidated myelin, 82% of the original myelin UDP-galactose:ceramide galactosyltransferase activity was obtained. Thus the source of the lipid did not significantly affect the extent of restoration of the enzymic activity.

The fatty-acid species of hydroxy-fatty-acid-containing cerebroside synthesized by myelin and microsomal UDP-galactose:ceramide galactosyltransferase

The fatty-acid species of hydroxy-fatty-acid-containing cerebroside synthesized during the incubation by the enzyme from both the microsomal and myelin membranes were determined by h.p.l.c. The reversed-phase h.p.l.c. separation of various molecular species of the acceptor bovine brain perbenzoylated hydroxy-fatty-acid-containing ceramide and that of the rat brain myelin and microsomal perbenzoylated hydroxy-fatty-acid-containing cerebroside is shown in Fig. 1. The distribution of the radioactivity in the various molecular species of hydroxy-fatty-acid-containing cerebroside is given in Table 1. The percentage distribution of radioactivity incorporated into various molecular species of cerebroside was similar, both in the case of microsomal and myelin membranes. The major incorporation was in cerebroside with $C_{18:0}$, $C_{22:0}$ + $C_{24:1}$ and $C_{24:0}$ hydroxy-fatty-acid species. The percentage distribution of radioactivity was also similar for both the membranes either isolated from 16 or 20 day-old animals. The percentage distribution of radioactivity into various molecular species of cerebroside generally followed the percentage amount of the ceramide available in the reaction mixture, except in the case of $C_{22:0}$ + $C_{24:1}$ and $C_{24:0}$ fatty-acid containing species. Although hydroxy-fatty-acid-containing ceramide acceptor contained about 13% of the $C_{22:0}$ + $C_{24:1}$ hydroxy-fatty-acid species, the percentage incorporation was 34–37%, whereas $C_{24:0}$ hydroxy-fatty-acid species of the ceramide was 26% of the total, but the

Fig. 1. Reversed-phase h.p.l.c. analysis of perbenzoylated hydroxy-fatty-acid-containing ceramide (bovine brain) and cerebroside from rat brain myelin and microsomal membranes

The separation was on a Nucleosil-5- C_{18} column with methanol as the solvent pumped at 2 ml/min. Perbenzoylated hydroxy-fatty-acid-containing ceramide ($5 \mu\text{g}$ dissolved in carbon tetrachloride) and myelin and microsomal cerebroside (55 and $1.5 \mu\text{g}$ respectively) were injected. The recorder sensitivity was set at 0.1, 1.0 and 0.04 absorption unit full scale for the ceramide myelin and microsomal cerebroside respectively. The major fatty acid of the molecular species resolved is indicated near each peak. The major long-chain base of these lipids was C_{18} -sphinganine. However, $C_{18:0}$ hydroxy-fatty-acid-containing ceramide also had C_{18} -sphinganine (DiH_2) as the long-chain base. (a) Hydroxy-fatty-acid-containing ceramide; (b) hydroxy-fatty-acid-containing cerebroside (myelin fraction); (c) hydroxy-fatty-acid-containing cerebroside (microsomal fraction).

percentage incorporation into this species was only about 14%. Of the total, maximum incorporation was in the long-chain ($>C_{22:0}$)-hydroxy-fatty-acid-containing cerebroside. These fatty-acid-containing cerebroside also dominate in the amount of the total

Table 1. Incorporation of UDP-[¹⁴C]galactose into various molecular species of myelin and microsomal cerebroside synthesized *in vitro*

Myelin and microsomal membranes from 20 day-old animals were incubated with UDP-[¹⁴C]galactose and acceptor hydroxy-fatty-acid-containing ceramide as described in the text. The product, hydroxy-fatty-acid-containing [¹⁴C]cerebroside was isolated and resolved into various molecular species by reversed-phase h.p.l.c. as in Fig. 1. The incorporation of radioactivity into various molecular species of the cerebroside is presented as d.p.m./nmol and as a percentage of the total recovered after h.p.l.c. Percentage composition of molecular species of the acceptor hydroxy-fatty-acid-containing ceramide (bovine) and myelin and microsomal cerebroside is given. The values are averages of two separate determinations.

Hydroxy-fatty-acid species	Myelin cerebroside				Microsomal cerebroside			
	Amount (mole %)	Radioactivity		Ceramide (mole %)	Amount (mole %)	Radioactivity		Ceramide (mole %)
		(d.p.m./nmol)	(% of total)			(d.p.m./nmol)	(% of total)	
C _{18:0}	1.1	2234 (42.0)†	20.7	25.2	3.9	26259 (31.4)	19.1	25.2
C _{18:0} (D.H.S.)*	0.3	2134 (40.2)	4.5	5.3	1.3	27439 (33.0)	6.7	5.3
C _{20:0} , C _{22:1}	3.5	177 (3.3)	5.1	3.1	4.5	6003 (7.2)	4.9	3.1
C _{23:1}	0.8	324 (6.2)	2.1	2.2	3.8	4375 (5.2)	2.8	2.2
C _{22:0} , C _{24:1}	34.1	130 (2.5)	36.9	13.2	29.0	6126 (7.3)	33.3	13.2
C _{23:0} , C _{25:1}	11.7	107 (1.9)	10.5	10.1	13.3	4766 (5.7)	11.6	10.1
C _{24:0}	37.8	46 (0.8)	14.5	26.7	30.7	2451 (2.9)	14.0	26.7
C _{24:0} (D.H.S.), C _{25:0}	8.7	52 (0.9)	3.8	7.5	11.3	2863 (3.4)	5.5	7.5
C _{26:0}	1.9	92 (1.7)	1.4	3.5	3.7	2838 (3.3)	1.6	3.5

* D.H.S. indicates sphinganine containing lipid; others have sphingenine as a long-chain base.

† The numbers in parentheses indicate the relative percentage of specific radioactivity.

cerebroside of these membranes. Surprisingly, although the C_{18:0} hydroxy-fatty-acid-containing species of cerebroside is only a minor component of rat brain myelin and microsomal hydroxy-fatty-acid-containing cerebroside, about 20–25% of the total incorporation was in this species. For this reason the specific radioactivity (d.p.m./nmol) of the C_{18:0} hydroxy-fatty-acid-containing cerebroside was highest and the specific radioactivity generally decreased with increasing chain length of the fatty-acid species (Table 1).

Discussion

It is now generally accepted that the UDP-galactose:ceramide galactosyltransferase associated with myelin could not be due to contamination by the microsomal, mitochondrial or axolemma-enriched fractions (Neskovic *et al.*, 1973; Costantino-Ceccarini & Suzuki, 1975; Costantino-Ceccarini *et al.*, 1979; Koul *et al.*, 1980). The enzymic characteristics of the UDP-galactose:ceramide galactosyltransferase associated with myelin membranes was compared with that of the microsomal membranes. The apparent *K_m* values of the enzyme with respect to hydroxy-fatty-acid-containing ceramide for both the membranes were similar, whereas the *V_{max}* varied according to the age of the animals. The pH optimum and the metal-ion requirement of the enzyme associated with myelin

and microsomal membranes were the same, although some variation was observed. The enzyme from both the membranes had maximal activity at 45°C. The considerably higher activity observed at 45°C than at 37°C could be due to better accessibility of the substrate ceramide to the enzyme and/or could be due to an increased activation energy of the enzyme protein at higher temperature. A parallel rate of activation energy of the enzyme with temperature for both the membranes was observed. At higher temperatures than 45°C the activity was decreased, possibly due to inactivation of the enzyme. The inactivation curves for the enzyme associated with both the membranes were not significantly different. Based on these observations it appears that the enzyme from both the membranes have similar characteristics and thus may be the same. We have also compared some of the properties of the enzyme such as activation energy from both the membranes as a function of age of the animals (O. Koul & F. B. Jungalwala, unpublished work) and no significant differences were observed, except the changes in specific activity reported. This would suggest that during development, the total amount of the active enzyme protein changes in the myelin and microsomal membranes instead of the enzyme protein structure being modified with age. However, definite conclusions regarding this point should await the purification of the enzyme from both the membranes.

It has been shown previously that the activity

of UDP-galactose:ceramide galactosyltransferase could be modulated by addition of exogenous lipids (Costantino-Ceccarini & Suzuki, 1975). Siegrist *et al.* (1976) have shown that microsomal cerebroside sulphotransferase, which shows a definite developmental activity pattern, could be modulated by partial delipidation of microsomal fraction and addition of the extracted lipids in an age-dependent manner. However, our results on partial delipidation and reconstitution of myelin and microsomal membranes showed that UDP-galactose:ceramide galactosyltransferase activity was not significantly modulated by the composition of specific membrane lipids added back for reconstitution. Thus higher UDP-galactose:ceramide galactosyltransferase activity observed in the myelin membranes compared with that of the microsomal membranes of 16 day-old rats could not be accounted for by the different composition of membrane lipids. These results also suggest that the characteristic development profile of the myelin and microsomal galactosyltransferase was not due to modulation of the enzyme by the changing lipid composition of the membranes during the myelination period.

Reversed-phase h.p.l.c. methods developed in our laboratory (Jungalwala *et al.*, 1977) have been employed for the analysis of molecular species of cerebroside biosynthesized *in vitro*. The percentage incorporation of [^{14}C]galactose from UDP-[^{14}C]galactose into various molecular species of cerebroside was not significantly different between myelin and microsomal membranes or between these membranes isolated from either 16 or 20 day-old animals. The enzyme from both the membranes and of both age groups synthesized similar fatty-acid-containing cerebroside. These results are consistent with the notion that UDP-galactose:ceramide galactosyltransferase associated with both the

myelin and microsomal membranes is similar and that instead of regulatory modulation of the enzyme protein with development during myelination, it is more likely that the turnover rate of enzyme protein is altered.

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