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BRAIN UDPGALACTOSE : CERAMIDE GALACTOSYLTRANSFERASE

PURIFICATION OF A CATALYTICALLY ACTIVE PROTEIN OBTAINED AFTER PROTEOLYTIC DIGESTION

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Summary

A procedure for the purification of UDPgalactose—2-hydroxyacylsphingosine galactosyltransferase (EC 2.4.1.45) including detergent extraction, ion-excharge chromatography and proteolytic digestion was developed. The active fraction obtained by this procedure had about 100 times higher specific activity than microsomes. Enzymic activity resisted destruction by pronase treatment at 4°C. Agarose gel chromatography indicated the presence of an enzyme-phospholipid-detergent complex with a molecular weight between 400 000 and 500 000. Intact phospholipids seemed to be required for full enzymic activity as evidenced by the drastic loss of activity upon treatment with phospholipase A or C.

Introduction

Ceramide galactosyltransferase (UDPgalactose—2-hydroxyacylsphingosine galactosyltransferase; EC 2.4.1.45) ** catalyses the synthesis of cerebroside from ceramide and UDPgalactose [1,2]. We have previously solubilized ceramide galactosyltransferase from rat brain microsomes with the non-ionic detergent, Cemulsol NPT 12 [3] and partially purified the enzyme after removing the greater part of detergent by solvent extraction. The solubilized preparation contained mainly large aggregates of lipids and proteins, so that attempts at further fractionation proved mostly unsuccessful. New findings have led us to undertake an additional approach based on the following: (1) by keeping a relatively low detergent concentration during the subsequent steps the enzyme can be puri-

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** The Enzyme Commission number (EC 2.4.1.62), which was erroneously given in the preceeding paper [3] does not correspond to the reaction catalyzed by the enzyme which is the subject of our study, i.e. the transfer of the galactosyl group from UDP galactose to the ceramide-containing α-hydroxy fatty acids.

fied by the use of various ion-exchange adsorbents, (2) ceramide galactosyltransferase activity resists under controlled conditions, the action of proteolytic enzymes which digest most of the proteins in the crude enzyme preparation. By combining procedures previously described for solubilization, including detergent extraction and solvent treatment, with ion-exchange chromatography and proteolytic digestion, about a 100-fold purification of ceramide galactosyltransferase was obtained. In the present report the purification procedure and properties of the purified enzyme are described.

Materials and Methods

Pronase and phospholipase A (isolated from *Vipera russelli* venom) were obtained from Koch-Light Laboratories (Colnbrook, England); phospholipase C from Sigma Chemical Co. (St. Louis, Mo., U.S.A.); appoferritin(horse) and γ-globulin(human) from Schwarz and Mann (Orangeburg, N.Y., U.S.A.); catalase (beef) from Boehringer (Mannheim, Germany). Pre-swollen DEAE-cellulose (Whatman DE-52) and CM-cellulose (Whatman CM-32) were from Balston Ltd. (Maidstone, England); DEAE-Sephadex and Sepharose 6B from Pharmacia Fine Chemicals (Uppsala, Sweden). The sources of ceramide, phosphatidylcholine, Cemulsol NPT-12 and UDP[^{14}C]galactose have been given before [3,4]. Organic solvents were redistilled before use. Other chemicals were of the highest purity commercially available.

Ceramide galactosyltransferase was assayed using ceramide containing hydroxy fatty acids and radioactive UDPgalactose as substrates, in the presence of phosphatidylcholine, as described in a previous publication [3]. A unit of activity is defined as nmol of galactose incorporated per h.

The reaction product was identified as cerebroside containing hydroxy fatty acids by autoradiography of thin-layer chromatograms (System A, ref. 5).

Since the presence of 2-mercaptoethanol and Cemulsol NPT-12 in different fractions interfered with the Lowry method [6] for protein determination, protein content was determined by the following procedure. Proteins were precipitated from samples (50—200 μl) by addition of 2 ml of 10% trichloroacetic acid and centrifugation. Protein determination was then carried out in the presence of sodium dodecyl sulphate by the modified Lowry procedure as described by Wang and Smith [7]. The above procedure was tested with samples of soluble proteins (bovine serum albumin) and membrane proteins (microsomes). In both cases the results were in good accordance with those obtained using the original Lowry method.

Phospholipid phosphorus was determined as described earlier [3]. The ferrous sulphate method [8] was used for cholesterol determination.

Polyacrylamide gel electrophoresis was carried out in the presence of 0.1% sodium dodecyl sulphate [9]. The samples were partially delipidated by extracting with ether/ethanol (4 : 1, v/v) [10]. Dried residues were dissolved in 0.1 M Tris · HCl buffer, containing 0.5% sodium dodecyl sulphate, 1% (v/v) 2-mercaptoethanol and 5% glycerol and incubated for 1 h at 37°C. Gels were stained with 0.25% Coomassie blue in 50% (v/v) methanol containing 10% (v/v) acetic acid and then destained with 5% (v/v) methanol containing 7.5% (v/v) acetic acid.

The microsomal fraction was prepared from brains of 15—20-day-old rats as described in the previous paper [3].

Results

Purification procedure

Unless otherwise stated all operations were carried out at 4°C and different fractions stored in an ice bath at 0°C.

Step 1: Extraction. The microsomal pellet from 100 brains was suspended by homogenization in 0.1 M potassium phosphate buffer, pH 7.6, containing 0.2% (v/v) 2-mercaptoethanol and 2 mM EDTA, to make a final volume of 90 ml. The protein content of this suspension was approx. 20 mg/ml. A 5% (v/v) solution of Cemulsol NPT-12 in the same buffer was slowly added with stirring until the final concentration of detergent was 1% (v/v). After stirring for 30 min the suspension was diluted with an equal volume of cold redistilled water and centrifuged at 100 000 × *g* for 1 h. The supernatant fluid (fraction I) was carefully withdrawn and the pellet discarded.

Step 2: DEAE-cellulose treatment. DE-52 cellulose (100 g) was equilibrated with 50 mM potassium phosphate buffer, pH 7.6, containing 0.25% (v/v) Cemulsol NPT-12, 0.1% (v/v) 2-mercaptoethanol and 1 mM EDTA. The resin suspension was divided in half, and each portion was transferred into a 250-ml centrifuge bottle and allowed to stand for 30 min. After this period, the volume of settled resin was about 80 ml. and the supernatant buffer was removed. To each bottle one half of the detergent extract (fraction I) was added and the mixture stirred mechanically for 15 min. The resin was sedimented by low speed centrifugation for 5 min and the supernatant fluid recovered by aspiration. The residue in each bottle was washed with 20 ml of the buffer and the washings combined with the supernatant fluid. The combined solution was filtered through a plug of glass wool to remove the particles of resin and dialyzed against redistilled water (2 × 5 l) overnight (fraction II).

Step 3: Solvent treatment. Fraction II was freeze-dried and the residue suspended in 60 ml of acetone precooled at −20°C. After stirring the suspension for 2 min and centrifuging for 5 min at −10°C, acetone was removed by decantation, and the extraction was repeated. The residue was then washed with 60 ml of cold benzene and freeze-dried to remove benzene. The white powder (fraction III, about 1.2 g) can be stored in a desiccator at −20°C for several months.

Step 4: Pronase treatment. The powder from step 3 was suspended by homogenization in 50 volumes of 0.1 M potassium phosphate buffer, pH 7.6, containing 0.1% (v/v) 2-mercaptoethanol and 1 mM EDTA. The protein concentration of this suspension was about 6 mg/ml. 15-ml aliquots were sonicated in an ice/water bath for 2 min (MSE sonicator; probe 9.5 mm diameter; intensity setting at 75% of maximum). To the sonicated material, a 1% (w/v) pronase solution in water was added to make the final pronase concentration of 0.2 mg/ml. The mixture was gently stirred for 16 h and assayed immediately for protein and enzymic activity (fraction IV).

Step 5: $(NH_4)_2SO_4$ precipitation. Fraction IV was clarified by centrifugation at 26 000 × *g* for 15 min. The clear solution was vigorously stirred and solid

$(NH_4)_2SO_4$ gradually added to a saturation of 40%. Stirring was continued for 15 min and the liquid centrifuged at 26 000 × *g* for 15 min. The precipitated material formed a layer on the top of the liquid. The underlying liquid was carefully siphoned off and the precipitate which adhered to the wall of the centrifuge tube dissolved in a minimum volume of 50 mM Tris · HCl buffer, pH 8.0, containing 0.25% (v/v) Cemulsol NPT-12, 0.1% (v/v) 2-mercaptoethanol and 1 mM EDTA. The solution was dialyzed against two changes (500 ml each) of the same buffer for 2 h. The dialyzed solution was slightly opalescent and had a protein concentration of about 2 mg/ml (fraction V).

Step 6: DEAE-Sephadex chromatography. DEAE-Sephadex was equilibrated with the buffer used in step 5 and poured to form a column of dimensions 1.6 cm × 30 cm. One-third of fraction V (about 20 mg of protein) was applied, and its elution was carried out with the starting buffer until the concentration of protein being eluted dropped to a constant low level (Fig. 1). The adsorbed inactive material was then displaced with 0.5 M Tris · HCl buffer, pH 8.0, containing 0.25% (v/v) Cemulsol NPT-12, 0.1% (v/v) 2-mercaptoethanol and 1 mM EDTA. Fractions having the highest specific activity were pooled (fraction VI). Usually, two or three batches obtained after DEAE-Sephadex chromatography were pooled in order to obtain enough material for further purification.

Step 7: CM-cellulose treatment. CM-32 cellulose (8 g) was equilibrated with 20 mM Tris · HCl buffer, pH 7.6, containing 0.25% (v/v) Cemulsol NPT-12, 0.1% (v/v) 2-mercaptoethanol and 1 mM EDTA. The resin was allowed to settle in a 100-ml centrifuge tube. The volume of the settled resin was about 40 ml, and the supernatant buffer was removed. Fraction VI was dialyzed against the above buffer and mixed with the resin. After stirring for 15 min and low speed centrifugation, the supernatant was recovered by aspiration; the resin was wash-

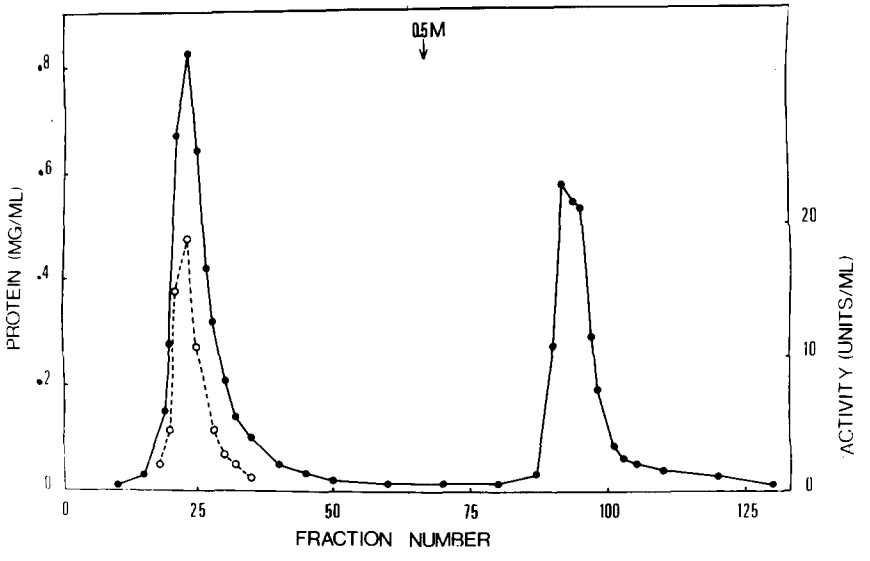

Fig. 1. DEAE-Sephadex column chromatography. Fraction V (about 20 mg of protein) was applied to the column (1.6 × 30 cm). Fractions of 1.5 ml were recovered at a flow rate of 20 ml per h. Other details are presented in the text. ●———●, protein; ○- - - - -○, activity.

TABLE I

PURIFICATION OF RAT BRAIN CERAMIDE GALACTOSYLTRANSFERASE

The enzyme assay and definition of the unit of activity were as described in Materials and Methods. Three batches of DEAE-Sephadex fraction were pooled for further purification.

Fraction	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (-fold)
Microsomes	89	1833	1518	0.83	100	1
I: Detergent extraction	197	1156	1022	0.88	67.3	1.1
II: DEAE-cellulose	321	436	959	2.2	63.2	2.6
III: Solvent extraction	61	357	937	2.6	61.7	3.1
IV: Pronase treatment	62	127	840	6.6	55.3	8
V: $(NH_4)_2SO_4$ precipitation	25	61.6	703	11.4	46.3	14
VI: DEAE-Sephadex	45	18.1	570	31.5	37.5	38
VII: CM-Cellulose	55	2.2	192	87.3	12.6	105

ed with 10 ml of the buffer and the washing combined with the supernatant liquid (fraction VII).

The over-all purification of ceramide galactosyltransferase from microsomes was 105-fold with a yield of 13% (Table I). The specific activities of fraction VII varied from 77 to 108 units per mg of protein.

Stability

The enzyme seems to be quite stable in membrane-bound form. An unfrozen microsomal suspension in phosphate buffer could be stored for 15 days without apparent loss of activity; after 30 days, about 80% of the original activity was still preserved. Freeze-dried powder obtained after solvent extraction could be stored at −20°C for several months without an appreciable loss of enzymic activity. The solubilized enzyme is much more labile. Fraction V lost about 50% of activity after 4 days at 0°C. The purified preparation obtained after DEAE-Sephadex step (fraction VI) lost its activity even more rapidly under the same conditions: about 50% loss was observed after 1 day. Glycerol appears to have a stabilizing effect on the ceramide galactosyltransferase activity. Fraction V was stored in 50% glycerol at −20°C for 30 days without a loss of the enzymic activity; under the same conditions fraction VI kept about 63% of the original activity.

Effect of proteolytic enzymes

Under the conditions used in this study ceramide galactosyltransferase resisted treatment with pronase. After 16 h at 4°C, pronase digested about 64% of total proteins while about 90% of the enzyme activity was preserved. Similar results have been obtained with trypsin (Neskovic, N., unpublished data).

In order to evaluate the possibility that pronase treatment decreased the molecular weight of the enzyme, a sample of the pronase-treated enzyme (fraction V) was chromatographed on a Sepharose 6B column. A control sample of

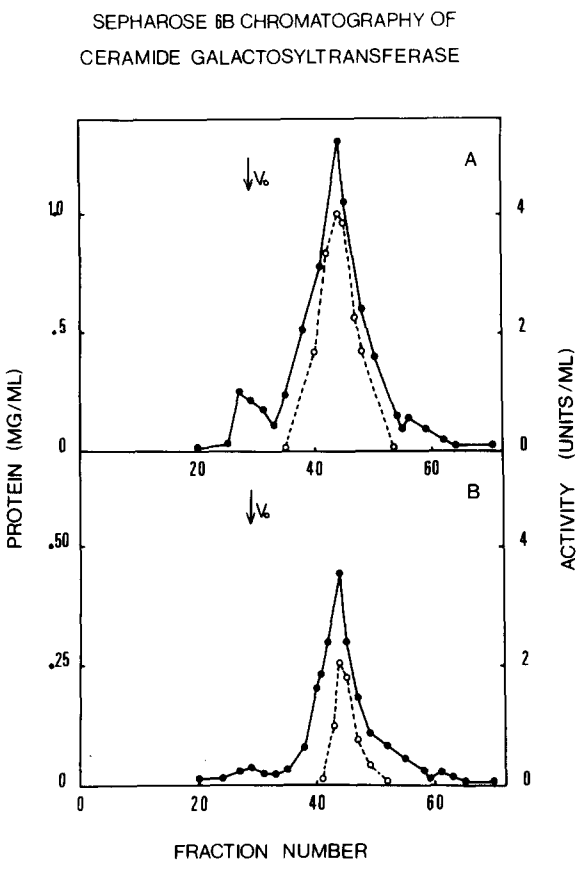

Fig. 2. Sepharose 6B chromatography. Sepharose 6B column (2.6 × 18 cm) was equilibrated with the eluant buffer containing 50 mM Tris · HCl buffer (pH 8.0), 0.5% (v/v) Cemulsol NPT-12, 0.1% (v/v) 2-mercaptoethanol and 1 mM EDTA. The samples of ceramide galactosyltransferase were applied: A, the enzyme purified as described for fraction V (see the text) except that the pronase treatment was omitted; B, fraction V (5.4 mg of protein). Fractions of 1.5 ml were recovered at a flow rate of 13 ml/h. Apoferritin (mol. wt. 480 000), catalase (mol. wt. 240 000) and γ-globulin (mol. wt. 160 000) were used as calibrating proteins. Other details are presented in the text. ●———●, protein; ○- - - - -○, activity.

the solubilized enzyme, obtained when pronase digestion was omitted, was chromatographed under the same conditions. The results of this experiment (Fig. 2) did not reveal a noticeable difference in the elution patterns of the two enzyme preparations. In both cases the apparent molecular weight was in the range of 400 000—500 000.

Phospholipid content

The determination of phospholipids in different fractions indicated that the phospholipid/protein ratio increased during the purification of ceramide galactosyltransferase: 0.68, 1.70, 3.3, 4.9 and 10.0 mg of phospholipid per mg of protein was found in fractions III, IV, V, VI and VII, respectively. Phospholipids were virtually the only lipid components of the purified enzyme fractions. Cholesterol represented less than 2% of total lipids in fraction V; in fractions VI and VII no cholesterol was detected.

In an attempt to elucidate the significance of phospholipids for the enzyme activity and the nature of the eventual phospholipid-enzyme interaction, two techniques for phospholipid removal were examined. The conditions used were similar to those used by Duttera et al. [11] in a study of the effects of phospholipase A and C on the microsomal membrane. Albumin was added to prevent the eventual inhibitory effects of the products of phospholipase A action. The results presented in Table II show that phospholipase A treatment produced almost complete inactivation of ceramide galactosyltransferase, while phospholipase C produced about 56% loss of the original activity. Addition of albumin had no effect on the enzyme inactivation after phospholipase A treatment.

Gel chromatography in the presence of detergents is frequently used as a relatively mild technique for the removal of phospholipids from membrane proteins and lipoproteins [12,13]. Upon Sepharose 6B chromatography in the presence of 0.5% (v/v) Cemulsol NPT-12, more than 50% of total phospholipid phosphorus of the solubilized enzyme preparation (fraction V) was eluted in the peak which coincided with the peak for ceramide galactosyltransferase activity (Fig. 3). A higher detergent concentration may be needed to dissociate the lipid-protein complexes [13]. However, under our conditions it was difficult to check this possibility since the enzyme rapidly lost its activity in the presence of high detergent concentration.

Disc gel electrophoresis

The electrophoretic patterns on sodium dodecyl sulphate gels of the enzyme preparations at different stages of purification are shown in Fig. 4. A large number of protein bands located in the upper part of 15% gels were present in the initial stages of purification (not shown on the figure) and after solvent extraction (fraction III). After pronase treatment and $(NH_4)_2SO_4$ precipitation, the number of the slow moving bands was greatly reduced, and the fast moving

TABLE II

EFFECT OF PHOSPHOLIPASE A AND C ON CERAMIDE GALACTOSYLTRANSFERASE

Fraction III (5.7 mg of protein/ml) in 50 mM Tris · HCl buffer, pH 8.0, was used as the source of ceramide galactosyltransferase. For the phospholipase A treatment incubation mixture (1 ml) contained: phospholipase A, 20 μg; Tris · HCl buffer (pH 8.0), 25 mM; $CaCl_2$, 0.5 mM; fraction III, 2.8 mg of protein. Albumin (15 mg) was added where indicated. For the phospholipase C treatment incubation mixture (1 ml) contained: phospholipase C, 250 μg; Tris · HCl buffer (pH 8.0); 25 mM; $CaCl_2$, 0.5 mM. A control incubation without phospholipases was run under the same conditions. After the incubation for 1 h at 23°C, aliquots were taken and tested for ceramide galactosyltransferase activity as described in Materials and Methods.

Treatment	Specific activity (units/mg)		Decrease (%)	
	Expt. I	Expt. II	Expt. I	Expt. II
Control	1.33	1.38	—	—
Phospholipase A	0.11	0.09	92	93
Phospholipase A + albumin	0.07	0.10	95	93
Phospholipase C	0.62	0.56	53	59

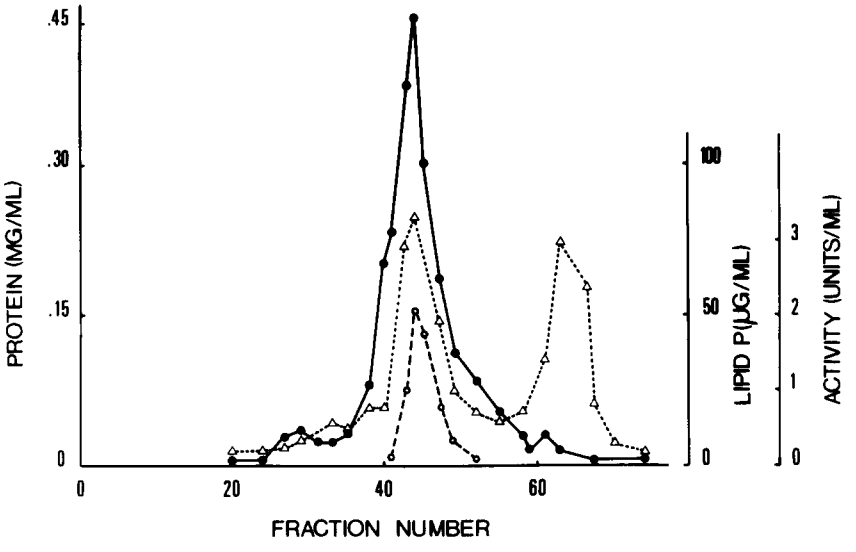

Fig. 3. Separation of proteins and phospholipids from the purified ceramide galactosyltransferase on a Sepharose 6B column in the presence of Cemulsol NPT-12. Fraction V (5.4 mg of protein) was applied. The column and elution conditions were the same as in Fig. 2. Other details are presented in the text. ●———●, protein; △ - - - - - - △, lipid phosphorus; ○ - - - - - - ○, activity.

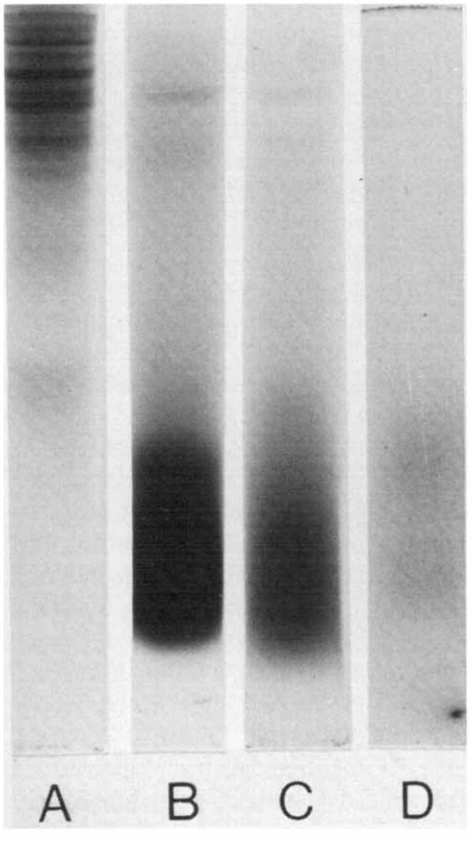

Fig. 4. Sodium dodecyl sulphate gel electrophoresis of ceramide galactosyltransferase at various stages of purification. The amounts of protein applied (approximately): A, fraction III, 30 μg; B, fraction V, 50 μg; C, fraction VI, 30 μg; D, fraction VII, 20 μg. Electrophoresis was carried out in 15% gels and protein bands stained with Coomassie blue as described in Materials and Methods.

bands appeared (fraction V). The fast moving material accumulated in the following stages of purification (fractions VI and VII). In the final step only two low molecular weight components were discernible. A rough estimation indicated molecular weights between 10 000 and 25 000 for these two components.

Discussion

The principal obstacle in the purification of ceramide galactosyltransferase was the lipoprotein nature of the material obtained after the extraction with Cemulsol NPT-12. In a previous study [3] we have shown that lipids and detergent could be partially removed from this material after lyophilization and solvent extraction. However, the "soluble" fraction obtained after this treatment contained presumably the lipoprotein complexes of large size, and the enzyme was excluded in the void volume of a Sepharose 4B column. The inadequacy of different means, such as solvent extraction and sonication, to disaggregate these complexes, made it necessary to carry out the subsequent steps in the presence of detergent. The enzyme could be purified by the use of DEAE-cellulose, CM-cellulose and DEAE-Sephadex, provided that a low (0.25%, v/v) concentration of Cemulsol NPT-12 was maintained in the eluting buffers.

As an additional attempt to release the enzyme from aggregation which seems to occur in the material solubilized with detergent, pronase digestion was employed. The treatment with proteolytic enzymes is known to release some enzymes from membranes [14], and this technique has been used in enzyme purification [15]. Under the conditions used in the present study, ceramide galactosyltransferase activity resisted pronase which digested a major part of the proteins and thus provided an important step in the purification of the enzyme.

On the other hand, the treatment with pronase did not seem to release ceramide galactosyltransferase from the lipoprotein complexes. During the purification which followed the pronase digestion (steps V—VII), the enzyme was found to coincide with the bulk of phospholipids and the phospholipid/protein ratio increased steadily in the corresponding fractions.

The significance of the high phospholipid content of the purified enzyme is open to speculation. The purified preparation was not fully characterized, and it is difficult to define the nature of the enzyme-phospholipid interaction. In material solubilized by detergents, different associations of lipid, protein and detergent may form [16]. The most purified fraction obtained in this work contained as much as 10 mg of phospholipids per mg of protein. It is unlikely that such a high phospholipid content is required for ceramide galactosyltransferase activity. On the other hand, several data presented in this work suggest that at least a part of phospholipids are involved in the enzymic activity. Attempts to remove phospholipids by different means either failed to separate these compounds from the enzyme (Sepharose 6B chromatography) or resulted in a loss of enzymic activity (phospholipase A and C treatment). Previously published results showing the stimulation of membrane-bound [17] and solubilized [3] ceramide galactosyltransferase by exogenous phospholipids also suggest a phospholipid dependence for this enzyme. However, a definitive con-

clusion should be based on the ease of reactivation of the lipid-depleted enzyme upon adding phospholipids.

The use of pronase digestion in the purification of ceramide galactosyltransferase raised an important question, i.e. whether the size of the enzyme molecule decreased as a result of the proteolytic cleavage. The results of agarose gel chromatography did not reveal significant differences between the apparent molecular weights of the pronase-treated and the untreated enzyme. However, it should be noted that the enzyme was eluted from the agarose gel column probably as an enzyme-lipid-detergent complex of high molecular weight and that under such conditions the eventual conversion of the enzyme molecule to a smaller form might not affect its chromatographic mobility. On the other hand, sodium dodecyl sulphate gel electrophoresis showed the presence of low molecular weight polypeptides in the purified enzyme preparation suggesting that the enzyme molecule was split into small fragments, some of which contain the active site. Another possibility is that the phospholipid phase provide the interactions which keep the polypeptide fragments in a spatial relationship allowing the active conformation of the enzyme. Further structural and kinetic studies of the isolated active protein should clarify these questions.

Acknowledgements

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