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AFFINITY OF SPHINGOMYELIN IN MYELIN PROTEOLIPID OF BOVINE BRAIN WHITE MATTER

BY

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ABSTRACT

A preparation of proteolipid, the principal component of the myelin sheath, was obtained from the bovine brain white matter with satisfactory reproducibility. Much of the lipid portion, containing most of the component sphingomyelin, was removed by precipitation with a mixture of ethanol and ether, and the precipitate fraction exhibited a preferential affinity for sphingomyelin. Since phosphatidylcholine, which did not show so much combining affinity for the precipitate fraction, exhibited an enhanced interaction by means of hydrogenation of the fatty chains, it is assumed that the affinity of sphingomyelin depends on the hydrophobic region of the molecule. Sphingomyelin is considered to interact with a complex formed by the ionic linkage between the basic protein and acidic phospholipid, which is obtained by further removal of the lipid portion from the precipitate fraction by dialysis.

INTRODUCTION

Although the brain proteolipid, the principal component of the myelin sheath, has been studied by many workers[1–8], few detailed information has been available on the lipid-protein and lipid-lipid interaction involved in proteolipid. We have previously isolated a preparation of proteolipid from the bovine brain white matter with satisfactory reproducibility[9]. The preparation was found to be rich in sphingomyelin and separated into the lipid portion containing most of the amount of sphingomyelin and the residual portion by precipitation with a mixture of ethanol and ether. The residual portion, composed of almost equal amounts of lipid and protein, still retains the solubility in a mixture of chloroform-methanol and exhibits a remarkable combining affinity for springomyelin.

In the present paper, the structural effect of sphingomyelin on the specific interaction in the proteolipid was studied and the nature of the interaction of lipid and protein was discussed.

MATERIALS AND METHODS

Materials: All chemicals were of reagent grade and the solvents were distilled before use. A sample of phosphatidylcholine was prepared from the bovine brain and purified by silicic acid-column chromatography[10]. Fatty acyl chain of phosphatidylcholine was saturated by catalytic hydrogenation with palladium-charcoal in hydrogen flow. Preparations of sphingomyelin were isolated from the bovine brain, rat liver and spleen of a patient with Niemann-Pick disease[11] and purified by mild alkaline hydrolysis to remove the gly-

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Table 1. Phospholipid Composition of Proteolipid and Its Fraction

	Proteolipid (%)	Ethanol-ether precitate (%)
Phosphatidylcholine	6.4	5.0
Phosphatidylethanolamine	5.2	6.0
Phosphatidylserine	19.3	45.0
Phosphatidylinositol	2.6	4.5
Choline-plasmalogen	0.8	
Ethanolamine-plasmalogen	3.4	4.0
Serine-plasmalogen	0.8	
Sphingomyelin	53.2	26.9
Glycerylether phospholipid	2.4	1.5
Unidentified	7.2	3.1

cerophosphatides, followed by silicic acid-column chromatography according to the method of Sweely[12]. Each sample of the individual lipid gave a single spot on a thin layer chromatogram[13]. Proteolipid was prepared from the bovine brain white matter and purified by precipitation from the chloroform-methanol solution with acetone followed by ether. The preparation is readily soluble in chloroform-methanol but insoluble in chloroform or methanol alone as well as acetone, ether, and ethanol. The phospholipid composition is given in Table I, showing that sphingomyelin occupies more than half of the total bound phospholipid, with phosphatidylserine coming next. To a chloroform-methanol (1:1) solution of the proteolipid, eight volumes of cold ethanol-ether (3:1) were gently added to form two layers. The fibrous precipitate, nearly corresponding to half of the amount of the proteolipid, formed after 48 hours. By this treatment, about 70% of phospholipid and 80% of glycolipid of the original proteolipid were removed but the precipitate still retains the solubility in a mixture of chloroform and methanol. The phospholipid composition of the precipitate exhibits a marked decrease of sphingomyelin and concomitant increase of phosphatidylserine (Table 1), suggesting that the ethanol-ether precipitation resulted in the specific release of sphingomyelin from the component phospholipid.

Combining affinity: The combining affinity of lipid and the ethanol-ether precipitate fraction was estimated by the procedure of fluff formation. When the fraction dissolved in chloroform-methanol (2:1) was mixed with lipid dissolved in the same solvent and the system was gradually brought to equilibrium with an excess of water, the lipid combined with the fraction was recovered in a fluff formed at the interface of the upper aqueous layer and the lower chloroform layer. The lipid portion which did not interact remained in the chloroform layer. The experimental details were described in the previous paper[9].

Analytical methods: The phosphorus content was measured by the method of Fiske and Subbarow[14], or according to Rouser *et al.*[15] when the amount of phosphorus was small. The fatty acids and sphingosine bases were converted to methyl ester and trimethylsilyl derivative, respectively, and analyzed by gas-liquid chromatography according to the method of Kawamura nd Taketomi[16].

Results and Discussion

Table 2 shows the result of the experiment on the combining affinity of phos-

Table 2. Affinity of Ethanol-Ether Precipitate for Phospholipids[1]

	Added phospholipid		
	PE (564 μg P)	PC (564 μg P)	Sph (549 μg P)
Phosphorus recovered in			
Fluff (μg)	378 (41.4%)	511 (55.9%)	763 (85.2%)
Chloroform layer (μg)	467 (51.2%)	391 (42.9%)	31 (3.5%)
Total recovery (μg)	845 (92.6%)	902 (98.8%)	794 (88.7%)

1) The ethanol-ether precipitate fraction (348 μg of P) dissolved in 50 m*l* of chloroform-methanol (2 : 1) was mixed with the individual phospholipids dissolved in 50 m*l* of the same solvent. Fluff formation was carried out as descibed in the text and the phosphorus recovered in the fluff and the chloroform layer were measured. PE: phosphatidylethanolamine; PC: phosphatidylcholine; Sph: sphingomyelin.

Table 3. Combining Affinity of Hydrogenated Phosphatidylcholine[1]

	Control[2]	Added phospholipid		
		PC (652 μg P)	PC (482 μg P)	Sph (603 μg P)
Phosphorus (μg) recovered in				
Fluff	312	475	695	856
Chloroform layer	10	468 (71.8%)	131 (27.2%)	24 (4.0%)[3]
Total recovery (%)	93.3	94.6	99.9	92.8

1): The combining affinity was estimated by fluff formation as described in the text. The experiment was performed with the ethanol-ether precipitate fraction (345 μg of P) and the individual phospholipid.
2): The control experiment was run without the added phospholipid.
3): Indicates the ratio to the added phospholipid.

pholipids to the ethanol-ether precipitate fraction of the proteolipid. In the case with phosphatidylethanolamine and phosphatidylcholine, large amounts of phosphorus were found in the chloroform layer, nearly corresponding to the amounts of phosphorus of the phospholipid added initially. With sphingomyelin, however, only a small amount of phosphorus was found in the chloroform layer and most of it was recovered in the interfacial fluff. These findings would reveal that the ethanol-ether precipitate fraction exhibits a very preferential affinity for sphingomyelin, while no significant interaction was recognized with phosphatidylethanolamine and phosphatidylcholine.

When the structure of phosphatidylcholine is compared with that of sphingomyeline, the ionic moiety is identical while phosphatidylcholine retains the fatty acid of commonly shorter and more unsaturated carbon chain than sphingomyelin. Thus another experiment was run with hydrogenerated phosphatidylcholine. As shown in Table 3, the phosphorus content recovered in chloroform layer, in the case using hydrogenated specimen as an additive, accounted for only 27% of the added lipid, whereas 72% of the added lipid did not show any interaction in the control experiment with the native phosphatidylcholine. This result clearly demonstrated that the combining affinity of phosphatidylcholine increased markedly by the hydrogenation of the fatty acyl moiety,

Table 4. Principal Fatty Acid of Shingomyelin Used in Combining Experiment

Chain length	Sphingomyelin isolated from		
	Bovine brain	Rat liver	Niemann-Pick spleen
		Mole %	
16 : 0	4.6	35.4	46.8
18 : 0	30.9	5.8	15.1
20 : 0	0.5	1.9	5.8
22 : 0	2.3	10.1	10.7
23 : 0	2.0	9.1	2.1
24 : 0		23.5	5.0
24 : 1	55.9	14.3	13.2
Saturated fatty acid	40.5	85.7	86.8

though the extent did not attain that of sphingomyelin.

The combining experiment was next carried out with several preparations of sphingomyelin isolated from various sources: bovine brain, rat liver, and spleen of a patient with Niemann-Pick disease. The long chain base of the preparations showed a similar pattern consisting mostly of C_{18}-sphingosine, but the preparation from the bovine brain contained a predominant amount of monoenoic acid of long chain compared with the other preparations (Table 4). As shown in Table 5, a significant difference in the affinity was not observed among the preparations of sphingomyelin in spite of the low content of saturated fatty acid in the preparation from the bovine brain. From these results it is revealed that the combining affinity of phospholipid for the ethanol-ether precipitate fraction correlates largely with hydrophobicity. The difference in the combining affinity between sphingomyelin and hydrogenated phosphatidylcholine would be still due to their hydrophobicity and the prominent affinity of the brain sphingomyelin would be due to the high hydrophobicity still retained in the monoenoic acid of the very long carbon chain. Many observations on the binding of lipid to the membranous proteins support indeed the concept that at least two sites of the lipid molecule interact with protein, *i.e.* the ionic region and the nonpolar hydrocarbon moiety[17–20]. Thus, it seems reasonable to assume that the combination of the ethanol-ether precipitate fraction and sphingomyelin is mediated by both the ionic and hydrophobic interactions.

In the previous paper[9], it was shown that the ethanol-ether precipitate is composed of phospholipid, cerebroside and protein in the ratio of 1:1:2 and dialysis of the fraction against chloroform-methanol-water resulted in the further removal of half of the phospholipid and most of the cerebroside[21]. In the undialyzable fraction, protein occupies more than 80% and the phospholipid is composed mostly of

Table 5. Affinity of Sphingomyelins Isolated from Various Sources[1]

	Added sphingomyelin		
	Bovine brain (405 μg P)	Rat liver (405 μg P)	Niemann-Pick spleen (420 μg P)
Phosphorus (μg) recovered in			
Fluff	641	661	698
Chloroform layer	35 (8.5%)	20 (4.9%)	20 (4.8%)[2]
Total recovery (%)	97.5	98.3	101.4

1) : The experiment was performed with the ethanol-ether precipitate fraction (288 μg of P).
2) : Indicates the ratio to the added sphingomyelin.

acidic phospholipid. This acidic phospholipid-protein complex was found to be still able to exhibit a combining affinity for sphingomyelin. However, the resulting complex appeared as a thin film instead of a bulky fluff and showed a poor solubility in chloroform-methanol (2:1) compared with the fluff formed in the experiment performed in the presence of cerebroside. Addition of cerebroside to the system of combining experiment with the undialyzable fraction did not affect the amount of phosphorus remaining in the chloroform layer. These findings suggest that cerebroside does not affect directly the affinity of sphingomyelin for the undialyzable fraction but is closely related with the stability of the combination product. The acidic phospholipid in the undialyzable fraction has been deduced to be combined with the basic amino acids in the protein in a stoichiometric ratio by electrostatic affinity[21]. Therefore, it is assumed that sphingomyelin interacts with the acidic phospholipid-protein complex and that the binding affinity of sphingomyelin depends on the hydrophobic region rather than on the ionic moiety of the molecule.

Since a low affinity of sphingomyelin for proteolipid protein has been recently demonstrated[8], the fundamental structure of proteolipid would be the lipid-protein complex formed by the electrostatic interaction of protein and acidic phospholipid, as pointed out previously[21], and the complex would exhibit a preferential affinity for sphingomyelin characterized by the high hydrophobic properties.

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