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THE OPTICAL ACTIVITY OF D-ERYTHRO-SPHINGOMYELIN AND ITS CONTRIBUTION TO THE CIRCULAR DICHROISM OF SPHINGOMYELIN-CONTAINING SYSTEMS

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SUMMARY

Circular dichroism studies on bovine brain sphingomyelin show the presence of a strong negative cotton effect below 200 nm, the position and magnitude of which depend on the physical state of the lipid. This cotton effect is thought to arise from the π-π^* transition of the amide group in the sphingomyelin backbone. The sphingomyelin contribution to the observed ellipticity of membranes and lipoprotein complexes depends on the mol fraction of amide groups present as sphingomyelin: this contribution is calculated to be less than 2 % in the case of serum high density lipoprotein and the order of 20 % below 200 nm in the case of the erythrocyte ghost membrane. Due to the similarity of the CD spectrum of sphingomyelin to that of a random coil polypeptide, use of uncorrected ellipticity data is expected to lead to an overestimate of the random coil content of proteins in systems containing a high sphingomyelin content.

INTRODUCTION

Sphingolipids differ from other lipid classes in that they contain an amide group; this is an inherently symmetric chromophore possessing a plane of symmetry and therefore optically inactive. Extensive studies of small molecules, polypeptides and proteins have amply demonstrated that, when this chromophore is placed in an asymmetric environment, a strong induced optical activity results. Amide-containing sphingomyelin molecules occur in nature as the stereochemically distinct D-erythro conformer [1], which contains within its molecular framework asymmetric carbon atoms. Hence, one finds the same potential for amide-associated, optically active absorption bands in sphingolipids as is present in proteins. To evaluate the magnitude of this effect we have studied the CD of sphingomyelin both in the form of single bilayer vesicles, where the lipid is arranged in an ordered structure [2] and as a micellar dispersion. One expects the amide group of sphingomyelin to be located intermediate

between the ionic head group and the hydrophobic side chains whether the sphingomyelin is in a micelle, inverted micelle or vesicle structure, where it may serve as a probe for molecular interactions in this region.

MATERIALS AND METHODS

Lipid purification and vesicle preparation

Egg yolk phosphatidylcholine and bovine brain sphingomyelin were prepared and purified on alumina and silicic acid columns as described elsewhere [3]: both were chromatographically pure. The fatty acid side-chain compositions of these lipids is also given elsewhere (ref. 3 and Barenholz, Y. and Thompson, T. E., unpublished). Dicetylphosphate of high purity was obtained from Sigma and used without further purification. For the preparation of mixed vesicles, the lipids were first dissolved in chloroform/methanol (2 : 1, v/v) at the desired molar ratio, then evaporated to dryness under N_2 and dispersed in either 10 mM KCl or 10 mM KF to yield a final concentration of 0.5 mM total phospholipid. The dispersions were then subjected to high intensity ultrasonic irradiation for 3–8 min (depending on the phospholipid concentration) by a J17A Branson 300 W sonifier, at 20 °C under N_2. Vesicles were formed using a 0.5-inch probe immersed in 5 ml of solution contained in a 17-mm diameter test tube: 40 % of the full power output of the sonifier was employed. The clear suspensions were centrifuged for 60 min at 100 000 $\times g$ to remove large structures. Under these conditions 90–95 % of the lipid was found in the supernatant. Based on the ratio of the choline *N*-methyl protons in the two surfaces of the bilayers (ref. 5 and Barenholz, Y. and Thompson, T. E., unpublished) more than 97 % of the lipid was in the form of single bilayer vesicles. Micelles were formed by dissolving dry sphingomyelin in either trifluoroethanol or 20 % methanol solution: these solvents were the only ones of a number tried that had the combined properties of a low absorbance below 200 nm and a good enough solvent capacity for sphingomyelin, so as to allow CD measurements to be made in this wavelength range.

Circular dichroism measurements

Ellipticities were calculated using the formula $[\theta] = \theta \cdot \mathrm{MRW}/lc10$, where θ is observed ellipticity, MRW is the mean residue weight or the molecular weight in the case of sphingomyelin, l is the pathlength in cm, and c is the concentration in g/ml. $[\theta^0]$ is the maximum ellipticity at the trough position, λ_0. CD measurements were carried out on a Cary 60 spectropolarimeter equipped with a 6001 CD attachment, using cylindrical cells of pathlength of 0.1 or 1 mm. Spectra were taken at 27 °C. All samples employed had absorbances less than two in the range in which data were collected. The reported CD data represent the result of at least two scans through the region of interest. The molar concentration of sphingomyelin was determined directly by phosphate analysis [5].

RESULTS AND DISCUSSION

A large negative cotton effect below 200 nm attributable to the π-π^* transition of the amide group of sphingomyelin has been observed (Fig. 1). The shape of the CD spectrum is qualitatively similar to that of a random coil polypeptide [6]. How-

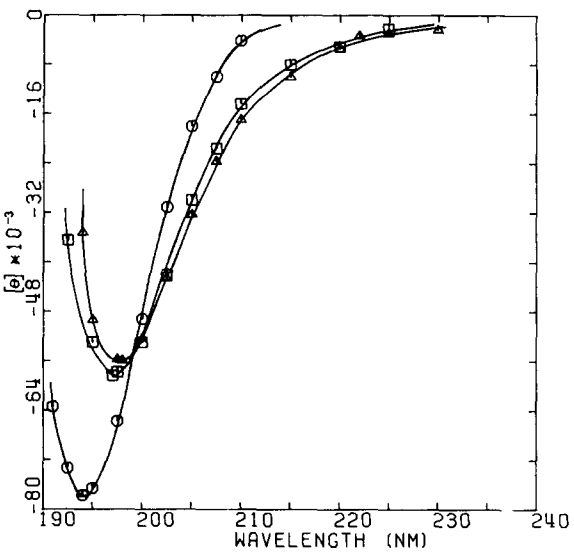

Fig. 1. The CD spectra of: sphingomyelin vesicles (△), sphingomyelin in 20 % methanol (□), sphingomyelin in trifluoroethanol (◇). Samples were prepared as described in the legend of Table I.

TABLE I

MOLECULAR ELLIPTICITY OF SPHINGOMYELIN

Sample	Physical state	$[\theta^\circ]$ degree · cm^{-1} · $dmol^{-1}$	λ_0 (nm)
Sphingomyelin	vesicles	−56 000	198
Sphingomyelin/phosphatidylcholine (50/50)	vesicles	−54 000	198.5
Sphingomyelin/dicetylphosphate (100/5)	vesicles	−51 900	197.5
Sphingomyelin/phosphatidylcholine/ Dicetylphosphate (50/50/5)	vesicles	−53 900	197.5
Sphingomyelin	micelles in 20 % methanol	−58 400	197
Sphingomyelin	micelles in trifluoroethanol	−77 700	194

ever, the magnitude of the sphingomyelin molecular ellipticity at 198 nm is about 1.5 times that of the mean residue ellipticity of a random coil polypeptide at this wavelength. The effect of introducing an additional phospholipid into the vesicle bilayer and a negative potential in the vesicle surface on the optical activity of sphingomyelin was determined by making CD measurements on vesicles formed from the following mixtures: sphingomyelin/phosphatidylcholine (50/50), sphingomyelin/

dicetylphosphate (100/5) and sphingomyelin/phosphatidylcholine/dicetylphosphate (50/50/5). These measurements, summarized in Table I, demonstrate that the observed CD spectrum is only weakly affected by the presence of either a negative surface potential or another phospholipid in the vesicle bilayer wall.

The observed CD spectra depend on the physical state of the sphingomyelin. The dependence of the position and amplitude of the negative trough in the sphingomyelin CD spectrum on the physical state of the sphingomyelin in solution is shown in Fig. 1. The trough position is at 194 nm for sphingomyelin in trifluoroethanol, with a magnitude of −77 700: while in the form of vesicles the trough position is shifted to 198 nm and the magnitude is diminished to −56 000. The CD spectrum of sphingomyelin in 20 % methanol resembles most closely that observed for the vesicles. Although no unique explanation is presently available for the differences observed in the CD spectrum of sphingomyelin in trifluoroethanol and vesicle form, turbidity measurements and analytical ultracentrifuge measurements suggest that both the scattering and the state of aggregation are much higher for the vesicles than for the trifluoroethanol micellar form. Sphingomyelin in 20 % methanol, which has a CD spectrum closely resembling the vesicles, also shows scattering properties which are similar to the vesicle form. These observations suggest that the source of the difference between the vesicle and micellar dispersion spectra is the type of optical artifact observed in natural membrane systems and attributable to the particulate nature of the vesicle. These effects have been treated by various authors with respect to membrane systems and their source seems to be the differential scattering of left and right handed circularly polarized light by the membrane particles [7–9]. Since the size of the sphingomyelin vesicles is well defined [2], it may be possible to assess the contribution of the scattering artifact in this system. Hence it remains to be seen if the amplitude and trough position are unique to the vesicle structure or the result of optical artifacts.

It is of interest to note that the CD spectrum of the random coil form of a phosphoprotein, phosvitin [10], although weaker in magnitude, bears a very close resemblance to the CD spectrum of sphingomyelin vesicles. Both sphingomyelin and phosvitin lack the weak positive cotton effect at 217 nm, which has been associated with random coil structures. This similarity may indicate that the phosphate group plays a major role in generating the local asymmetry in the neighborhood of the amide group in both phosvitin and sphingomyelin. A comparison of sphingomyelin amide ellipticity and poly-L-lysine amide ellipticity is shown in Table II.

Unlike phosphatidylcholine, which has a weak positive cotton effect around 210 nm (Litman, B. J., unpublished), sphingomyelin exhibits a strong negative cotton effect below 200 nm. The observation of this cotton effect in sphingomyelin, in addition to being of interest in its own right, is of importance with respect to the use of CD as a tool for the study of biological membranes, i.e. one cannot assume that the lipid background contribution to the CD spectrum of either biological membranes or lipoproteins is negligible. Only after a determination of the lipid content of the system in question can an estimation of the lipid contribution be made. We have employed the known composition of serum high density lipoprotein [11], the human erythrocyte ghost membrane [12], and a reconstituted apo-high density lipoprotein-sphingomyelin complex [13] to estimate the range of the contribution of sphingomyelin to the observed CD spectra of biological membranes and lipoprotein complexes:

TABLE II

A COMPARISON OF AMIDE GROUP ELLIPTICITY IN SPHINGOMYELIN AND IN VARIOUS POLYPEPTIDE SECONDARY CONFIGURATIONS

Sample	Physical state	$[\theta]_{197}$	$[\theta]_{208}$	$[\theta]_{222}$
Sphingomyelin	vesicles	−55 800	−23 700	− 3 350
Sphingomyelin	micelles in trifluoroethanol	−68 100	− 9 900	0.0
Sphingomyelin	micelles in 20 % methanol	−58 400	−21 500	− 3 040
Poly-L-lysine*	α-helix	+44 300	−32 600	−35 700
	β-structure	+ 30 000	− 4 700	−13 800
	random coil	−41 900	− 3 400	− 3 900

* Values of Greenfield and Fasman [6].

the mol fractions of sphingomyelin amide groups in these membranes are 0.008, 0.022 and 0.26, respectively.

The human erythrocyte ghost membrane phospholipid contains 25 % sphingomyelin by weight: the contribution of this lipid to the circular dichroism at 208 and 222 nm, the two wavelengths generally employed to estimate the helical content, is less than 4 % relative to a protein which is 40 % α-helix (Table III). Hence estimates of helical content employing uncorrected ellipticities at these wavelengths would show little error resulting from the sphingomyelin contribution. However, the calculated contribution of sphingomyelin at the trough position of the vesicle form, 198 nm, is 17 % (Table III). Although data taken above 200 nm will not be in error due to the lipid contribution, any calculations made employing data below 200 nm will be in serious error and will be expected to result in a large overestimation of the amount of random coil present in the erythrocyte membrane protein. Circular dichroism spectra of erythrocyte ghosts have been employed to evaluate the ability of various scattering theories to explain the red-shifted spectra commonly observed in studies on biological membranes [7, 8, 15]: to what degree the lipid contribution complicates these calculations remains to be determined.

Analysis of the CD spectra of native high density lipoprotein indicates that the protein is highly ordered, containing approx. 60–70 % α-helical structure [16]. Evidence obtained from a variety of physical studies on high density lipoprotein has resulted in the formulation of several models for the relative arrangement of the various components of native high density lipoprotein [17–19]. A characteristic common to almost all of these models is the arrangement of the sphingomyelin in a monolayer or micelle-like packing in the surface of the high density lipoprotein particle. It therefore seems appropriate to employ the sphingomyelin micelle CD data in these calculations. As can be seen from Table III, the sphingomyelin contribution to the CD spectrum of native high density lipoprotein is negligible at all wavelengths considered, being less than one percent of the observed ellipticity.

In contrast to the low mol fraction of sphingomyelin in native high density lipoprotein, the reconstituted apo-high density lipoprotein-sonicated sphingomyelin

TABLE III

THE CONTRIBUTION OF SPHINGOMYELIN TO THE CIRCULAR DICHROISM SPECTRUM OF ERYTHROCYTE GHOST MEMBRANES AND SERUM HIGH DENSITY LIPOPROTEIN

The mol fraction of sphingomyelin amide and protein amide was calculated on the basis of published composition data for the human erythrocyte ghost membrane [12], native high density lipoprotein [11] and the reconstituted apo-high density lipoprotein-sphingomyelin complex [13], using an average molecular weight of 775 for sphingomyelin (Barenholz, Y. and Thompson, T. E., unpublished) and a mean residue weight of 115 for high density lipoprotein [14].

Sample	Contributing component	$[\theta]_{197}$	$[\theta]_{208}$	$[\theta]_{222}$	Mol fraction of amide groups present as sphingomyelin	Mol fraction of amide groups present as protein
Erythrocyte ghost membrane*					0.022	0.978
	protein	−7300	−14 750	−11 680		
	sphingomyelin**	−1230	− 520	− 70		
Native high density lipoprotein***			−24 100	−25 800	0.008	0.992
	sphingomyelin†	− 550	− 80	0.0		
	sphingomyelin††	− 470	− 170	− 30		
Reconstituted apo-high density lipoprotein-sphingomyelin complex†††			−29 300	−24 600	0.26	0.74
	sphingomyelin**		6 200	− 870		

* Since the erythrocyte ghost membrane CD spectra contains a scattering artifact, which increases with decreasing wavelength, the protein contribution for this membrane was estimated by taking a linear combination of the poly-L-lysine ellipticity data made up of 40 % α-helix and 60 % random coil: this represents the current estimates of the secondary structural features of the erythrocyte ghost membrane protein [7].

** Calculation based on the CD data for sphingomyelin vesicles.

*** Ellipticity data of Lux et al. [14].

† Calculation based on the CD data for sphingomyelin micelles in trifluoroethanol.

†† Calculation based on the CD data for sphingomyelin micelles in 20 % methanol.

††† Ellipticity data of Assmann and Brewer [13].

complex, whose CD spectrum we are concerned with here, contains 0.26 mol fraction of its amide groups as sphingomyelin. Since sonicated sphingomyelin was employed as an interactant in forming the reconstituted lipoprotein complex, we have employed the sphingomyelin vesicle ellipticity data to calculate the contribution of the lipid to the observed CD spectrum. On this basis, the calculated lipid contribution is 4 % at 222 nm and 21 % at 208 nm. The helical content of the apo-high density lipoprotein in the reconstituted lipoprotein complex, estimated from the observed ellipticity data at 222 nm, is 72 %, whereas a value of 89 % is obtained using the data at 208 nm. If ellipticities which are corrected for the sphingomyelin contribution are used to

estimate the helical content, a value of 70% is obtained at 222 nm and a value of 68% at 208 nm. This demonstrates that good agreement can be obtained at these two wavelengths if proper corrections are made for the lipid background contribution.

The application of corrections for the lipid background is not a straight forward process. We have seen that the CD spectrum of sphingomyelin is dependent on the physical state of the lipid. In biological membranes, the interaction of sphingomyelin with the protein components may introduce new features into the CD spectrum. The seriousness of this effect will depend on the fraction of lipid interacting with the protein and the presence of strong interactions of sphingomyelin amide groups with the protein chromophores. Interaction of sphingomyelin with the solvent or other lipid components may also complicate the interpretation of the CD data. In the most favorable case, the presence of such effects may serve as a tool in the study of protein-lipid, lipid-lipid and solvent-lipid interactions.

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