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CALORIMETRIC STUDIES ON THE INTERACTION OF GANGLIOSIDES WITH PHOSPHOLIPIDS AND MYELIN BASIC PROTEIN

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Differential scanning calorimetry was employed to investigate the interaction of G_{M1} gangliosides with phospholipids (phosphatidylethanolamine, phosphatidylserine or phosphatidylcholine). It was found that G_{M1} is completely miscible with phosphatidylethanolamine; however, the interaction with phosphatidylserine is minimal. Addition of excess Ca^{2+} to the interaction products of G_{M1} with phosphatidylcholine or phosphatidylethanolamine did not induce phase separation. The influence of myelin basic protein on the thermotropic behaviour of G_{M1} was also studied. It was found that basic protein has a very strong perturbing effect on G_{M1} micelles.

Introduction

The sialoglycosphingolipids (gangliosides) have elicited much interest in recent years, especially as typical membrane constituents in the brain, though their functional role in the central nervous system is far from being understood [1]. In extraneural tissue gangliosides have been implicated in various surface recognition and physiological processes [2–7]. However, as cell membranes are abundant in phospholipids, the various membraneous functions of gangliosides may be dependent on their interaction with these membrane lipids. As a continuation of our previous studies we undertook to investigate the interaction of gangliosides with phospholipids by employing the technique of differential scanning calorimetry (DSC). Recently we have shown that the monosialoganglioside G_{M1} is completely miscible with egg phosphatidylcholine, as only one transition peak is obtained in the differential scanning calorimetry thermograms [8]. This approach was extended and the interaction of G_{M1} with phosphatidylethanolamine or with phosphatidylserine is reported here. As calcium causes phase separation in mixtures of negatively charged lipids with zwitterionic ones, the influence of calcium ions on the interaction of G_{M1} with phospholipids was also investigated.

Another example for a possible intramembraneous interaction of gangliosides with a proteinous entity is of relevance to the case of myelin. The encephalitogenic basic protein is a dominant component in myelin, where it constitutes about 40% of myelin proteins. Fidelio et al. [10] found that myelin basic protein penetrates monolayers of gangliosides, and it was also reported by Moore [11] that basic protein binds to micelles of G_{M1}. Fong et al. [12] showed that peripheral myelin contains gangliosides and basic protein in approximately equimolar amounts, which lead Moore [11] to postulate that gangliosides might bind

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Abbreviations: DSC, differential scanning calorimetry; PC, phosphatidylcholine,; PE, phosphatidylethanolamine; PS, phosphatidylserine.

specifically to basic protein in the myelin membrane. Mullin et al. [13] have demonstrated that myelin basic protein interacts with the myelin specific ganglioside G_{M4} entrapped in liposomes. To further our understanding of the interaction between these glycolipids and the proteinous structures in the myelin sheath, we investigated the interaction of myelin basic protein with G_{M1} by employing differential scanning calorimetry.

Part of this work was presented at a poster session of the International Symposium on Thermodynamics of Proteins and Biological Membranes, Granada, Spain, May 1983.

Materials and Methods

Ganglioside preparations were isolated from bovine brain by chloroform/methanol extraction, followed by phase partition, alkaline methanolysis, DEAE-Sephadex, DEAE-Sepharose and Iatrobeads column chromatography [14,15]. In the present study the ganglioside G_{M1} (galactosyl-*N*-acetylgalactosaminyl-(*N*-acetylneuraminyl)-galactosylglucosylceramide) was employed. The purity of the ganglioside was assessed by thin-layer chromatography on high performance Silica gel coated plates.

Egg phosphatidylcholine grade I, egg phosphatidylethanolamine grade I and phosphatidylserine from bovine spinal cord (monosodium salt) grade I (all the phospholipids are in chloroform/methanol (2 : 1, v/v) were purchased from Lipid Products, South Nutfield, U.K. Basic protein was separated from bovine myelin according to Hirshfeld et al. [16]. All the chemicals and solvents were of analytical grade.

The gangliosides were dissolved in chloroform/methanol (1 : 1, v/v) and appropriate volumes of phospholipid solutions were added. The samples were mixed, the solvents were driven off by a stream of nitrogen and kept under high vacuum for 3 h. Subsequently 1.5–2 mg G_{M1} or G_{M1}-phospholipid mixtures were weighed directly into the aluminum pans, and excess of a mixture of ethylene glycol/salt (about 10 mg, 1 : 1, v/v) was added. The salt solution used was $1.5 \cdot 10^{-1}$ M NaCl in 10^{-2} M Tris-HCl buffer (pH 7.3). For the experiments with calcium ions, a concentrated solution of $CaCl_2$ was added to the salt solution to obtain the Ca^{2+}: G_{M1} molar ratios as indicated. The use of ethylene glycol enabled us to start scanning below 0°C without interference by melting water.

To prepare the interaction products of G_{M1} with basic protein, ganglioside (about 1 mg) was weighed into an aluminum pan, and about 8 mg of water or salt solution (without ethylene glycol) was added. After dispersion of the ganglioside in the solution, basic protein was added and the pan was reweighed. The pan was sealed, vortexed for about 2 min and incubated at 37°C for 2 h. Subsequently the scanning was started at +1°C. The ganglioside without protein was treated similarly.

The calorimetric measurements were performed on a Du Pont 990 Differential Scanning Calorimeter equipped with cell base II. The calibrated mode was used with a scan rate of 5 deg. C/min. In all the experiments the samples were rescanned several times: usually after the first heating scan the thermotropic profile became constant. In the experiments in which basic protein was present, no difference could be detected between the first and the subsequent scans.

Results and Discussion

Interaction of G_{M1} with phosphatidylethanolamine

The thermograms showing the interaction of G_{M1} with phosphatidylethanolamine (PE) as well as the thermograms of the pure interactants are presented in Fig. 1. As seen from the figure, the glycolipid-phospholipid interaction brings about a progressive decrease in the temperature of the middle of the peak (T_m) of the ganglioside in addition to shortening its range of melting. The downward shift of the melting temperature is even more pronounced in Fig. 2a, where T_m is drawn as a function of the molar fraction of G_{M1}. The decrease in T_m of G_{M1} shifting towards T_m of PE and the appearance of one peak only (up to quite high ratios of PE/G_{M1}) in the thermograms, shows that these two lipids are completely miscible. The difference in the T_m values of G_{M1} and of PE is about 20 deg. C. Moreover, the maximum in the PE heat flow is at a temperature where the heat flow of G_{M1} is still low, implying that in the absence of interaction, the appearance of two separate peaks should be expected. At high PE/G_{M1}

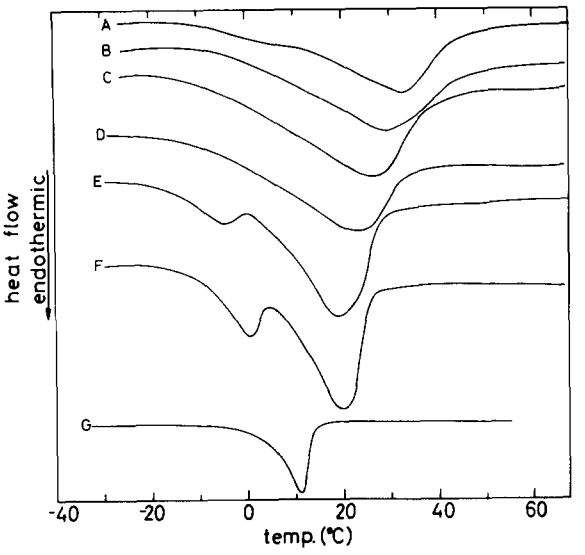

Fig. 1. Thermograms of G_{M1} ganglioside, egg PE and their interaction products. x-molar fraction of gangliosides: A, 1.0; B, 0.89; C, 0.69; D, 0.54; E, 0.30; F, 0.21; G, egg PE only. Scan rate 5 deg. C/min; sensitivity 0.02 mcal·s^{-1}·inch^{-1} except for curve G with a sensitivity 0.1 mcal·s^{-1}·inch^{-1}.

ratios (molar fraction of G_{M1} approx. 0.3, Fig. 1E), a second peak at lower temperatures appears and T_m of the main peak remains almost constant. At these PE/G_{M1} ratios, a phase separation probably occurs yielding a complex of PE/G_{M1} and 'impure' PE containing some G_{M1}. The melting temperature of the second peak is lower than that of the pure PE and shifts upward towards the T_m of the phospholipid as the molar fraction of PE increases (Fig. 1F).

Gangliosides in aqueous solutions form micelles with very low critical micelle concentration [17], whereas PE in water forms open lamellar structures due to the small area of the headgroup relative to the area of the hydrocarbon chains [18]. Interaction between gangliosides and PE leads to the formation of more stable structures, probably vesicles.

Fig. 2b presents the enthalpy of melting of the interaction products between the ganglioside G_{M1} and PE. The solid line in the figure is a theoretical line, whereas the points represent experimental values. The enthalpy values presented in Fig. 2b were calculated from the thermograms in Fig. 1 and additional thermograms (not shown), and from

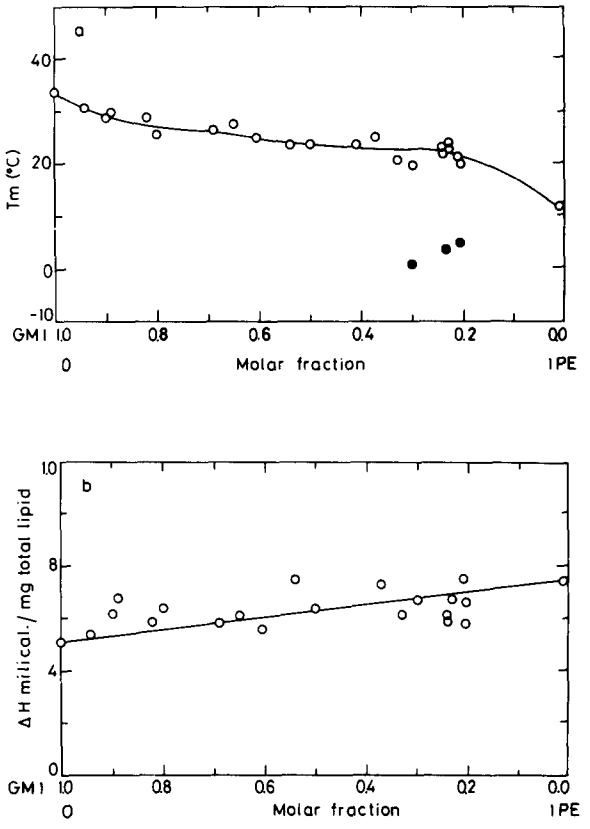

Fig. 2. The temperature of melting (at the middle of the peak) (a) and the enthalpy of melting (b) as a function of the molar fraction of the components. (a) ○, T_m of the main peak; ●, T_m of the low temperature peak. (b) ○. Experimental points; ———, theoretical line.

the known amount of total lipid in the pan. In the cases where two peaks were obtained, the total enthalpy over the two peaks was calculated. Due to difficulties in defining the base line, the spread of points is quite large. No apparent maximum in the enthalpy was detected as in the case of phosphatidylcholine-G_{M1} interactions [8]. Based on the almost constant T_m values at G_{M1} molar fractions 0.3 and 0.2, and on the appearance of the peak of free PE at these ratios, it can be concluded that about three molecules of PE interact with one molecule of G_{M1}.

Interaction of G_{M1} with phosphatidylserine

Phosphatidylserine (PS) is another important membrane lipid so it was of interest to investigate its interaction with gangliosides. PS has one nega-

tive charge at neutral pH and its high melting temperature (T_m approx. 21°C) is due to hydrogen bonding. Due to this high temperature of melting, the peak of PS is within the melting range of G_{M1}, implying that even in the absence of mixing or interaction between these two components the appearance of a separate PS peak is not expected. In this case the only indication of interaction could be the shift of T_m of the G_{M1} peak. In the case of the interaction of G_{M1} with egg phosphatidylcholine [8] the difference in the melting temperature of the two lipids is about 47 deg. C and the shift in T_m of G_{M1} at a molar fraction of 0.3 is about 25 deg. C. In the case of G_{M1}-PE interaction, the difference in the mid-point melting temperature of the pure interactants is about 23 deg. C, causing a downward shift in T_m (at G_{M1} molar fraction = 0.3) of about 15 deg. C. The difference in melting temperatures (T_m) of PS and G_{M1} is only about 14 deg. C, and by the same reasoning as above the interaction between the two lipids should cause a downward shift of T_m of G_{M1} of about 7 deg. C (for an interaction product at G_{M1} molar fraction = 0.3). Fig. 3 shows the thermograms of PS (3D), G_{M1} (3A) and of their interaction products at G_{M1} molar fractions 0.61 and 0.34 (3B and 3C, respectively). In the case of the higher molar fraction the shift in the T_m is very small, and even at lower molar fraction the shift in T_m is at most 2.5 deg. C. These data suggest that intermixing of G_{M1} with PS is very limited. A possible cause for this behaviour is the electrostatic repulsion of the two negatively charged components, and this repulsion overcomes any tendency for interhydrogen bonding. No attempt was made to abolish the electrostatic repulsion by adding Ca^{2+} to neutralize the negative charge, as it is known that Ca^{2+} binds strongly to PS converting the lipid into a crystalline state with a shift of the melting temperature to very high values [19]. However, by employing ESR, Sharom and Grant [20] found that in the Ca^{2}-mediated ganglioside-PS interaction, immobilization of the headgroups and clustering are taking place.

Interaction of G_{M1} with phosphatidylcholine or phosphatidylethanolamine in the presence of calcium ions

Gangliosides, like other negatively charged lipids, bind calcium ions. This interaction was

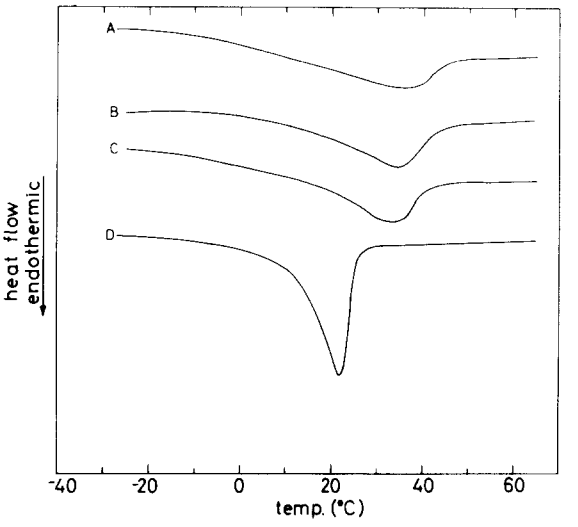

Fig. 3. Thermograms of G_{M1} ganglioside, PS and their interaction products. x - molar fraction of ganglioside: A, 1.0; B, 0.61; C, 0.34; D, 0 (PS only). Scan rate 5 deg. C/min; sensitivity 0.04 mcal·s^{-1}·inch^{-1}.

demonstrated by surface potential measurements in monolayers [21], by $^{45}Ca^{2+}$ binding in ganglioside micelles [22], by ESR measurements [20] and by using a highly sensitive calcium electrode [23]. Felgner et al. [23] have claimed that calcium binding occurs only in phospholipid-ganglioside vesicles with very little binding to pure ganglioside micelles.

As Ca^{2+} is involved in many biological processes [24] and as calcium ions are able to induce phase separation in mixtures of PC with acidic phospholipids (phosphatidic acid or PS) [25,26] it was of interest to test whether calcium ions could induce phase separation in mixtures of G_{M1} with either PC or PE, thus producing domains with different permeability properties. DSC is a useful techique for detecting phase separation. In the case of two lipids that mix ideally, only one peak is detected in the thermograms; however, if phase separation is induced by some external agent (e.g., Ca^{2+} or basic protein) a shift in the melting temperature will occur towards the temperature of the separating free component [27]. Since Ca^{2+} binds to gangliosides, the effect of this ion on the thermotropic behaviour of G_{M1} was investigated. No difference in the T_m or in the enthalpy of melting (ΔH) of G_{M1} was detected in the presence of Ca^{2+} in excess (molar ratio Ca^{2+}/G_{M1} 8 : 1) as

compared to T_m and ΔH of G_{M1} in the absence of Ca^{2+} (not shown). This finding is in keeping with the data of Felgner et al. [23] who showed very little binding of Ca^{2+} to pure ganglioside micelles and strong binding to gangliosides incorporated into phospholipid vesicles. The latter finding prompted us to study the possible phase separation in phospholipid-ganglioside mixtures by Ca^{2+}. The experiments were performed at various molar fractions of G_{M1} to phospholipid and at different ratios of Ca^{2+}/G_{M1}. Phase separation induced by calcium ions was not detectable even when the ganglioside was interacted with an excess of PE and Ca^{2+} (x G_{M1} 0.24; molar ratio Ca^{2+}/G_{M1} 8 : 1), as the thermotropic profiles (not shown) in the presence and in the absence of calcium were identical. In the case of interaction between G_{M1} and PC (x G_{M1} 0.24) only a very small upward shift in the presence of Ca^{2+} (at molar ratio of $Ca^{2+}/G_{M1} = 9:1$) was detected. In the case of phase separation, a downward shift would be expected towards T_m of the component that does not bind calcium (PC). From these experiments it can be concluded that Ca^{2+} does not induce phase separation in G_{M1}-phospholipid mixtures.

It has previously been reported [28] that addition of Ca^{2+} to G_{M1}-PC vesicles causes lateral phase separation in the vesicle membrane. This effect was detected only when an ESR probe which locates near the surface region of the lipid bilayer was used, but was not found with a probe located in the interior of the bilayer. As the differential scanning calorimetry technique gives an overall effect, the data reported in this paper are not in contradiction with those obtained by Bertoli et al.

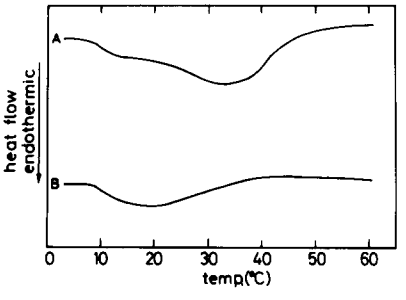

Fig. 4. Thermograms of gangliosides interacting with basic protein: A, G_{M1} only; B, G_{M1} + 45% basic protein (w/w). Scan rate 5 deg. C/min; sensitivity 0.01 mcal·s^{-1}·$inch^{-1}$.

[28]. With respect to phase separation induced by Ca^{2+}, it is noteworthy that calcium ions induce phase separation only in mixtures of some acidic lipids (PS, phosphatidic acid [25,26]) with the zwitterionic ones but do not cause phase separation in the case of mixtures of zwitterionic lipids with phosphatidylinositol [29] or phosphatidylglycerol [30]. A probable prerequisite for phase separation is a very strong binding of Ca^{2+} to the negatively charged group located on a small polar headgroup and weak interaction between the mixed lipids. The negatively charged sialic acid is located on a rather bulky head group (five sugar residues), so the steric hindrance is probably responsible for the lack of phase separation induced by calcium ions.

Interaction of G_{M1} with myelin basic protein

Interaction of myelin basic protein with lipids has been investigated extensively (see, for example Refs. 27 and 31). The protein interacts preferentially with negatively charged lipids. The initial electrostatic interaction is followed by the penetration of the hydrophobic segments of the protein into the lipid bilayer. Such interaction causes a perturbation of the lipid bilayer as seen by the change of the thermotropic profile (decrease of T_m with decrease of ΔH in certain cases). However, the interaction between basic protein and the zwitterionic lipid sphingomyelin, another important component in the brain membrane, was also reported recently [32].

Fig. 4 shows the effect of basic protein on the thermograms of G_{M1}. It is apparent from the figure that the effect of the protein on the thermotropic profile of G_{M1} is very strong. Basic protein at a concentration of 45% (w/w) causes a significant downward shift of the melting temperature, and the range and the enthalpy of melting (Fig. 4B). At this concentration of the protein the decrease in the enthalpy is about 70%, depending on the concentration of the basic protein. The same results were obtained when the experiment was performed in water or in salt solution as a dispersing medium. The significant effect of myelin basic protein on the thermotropic profile of G_{M1} shown in Fig. 4 is evident at a molar ratio of 1 : 11 (basic protein/G_{M1}). However, when the weight ratio of the added basic protein is decreased to 15% (equivalent to a molar ratio of approx. 1 : 60), its

effect on the melting temperature as well as on the range and enthalpy of melting of G_{M1} is completely abolished. The strong perturbing effect of the protein on G_{M1} micelles proves that basic protein has a pronounced affinity for that glycolipid which might prove relevant to the physiological interaction and functional expression of these two components in myelin.

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