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# CORRELATION BETWEEN THE THERMOTROPIC BEHAVIOR OF SPHINGOMYELIN LIPOSOMES AND SPHINGOMYELIN HYDROLYSIS BY SPHINGOMYELINASE OF *STAPHYLOCOCCUS AUREUS*

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## Summary

The hydrolysis of D-erythro beef brain sphingomyelin and D,L-erythro-*N*-palmitoylsphingomyelin dispersed as multilamellar liposomes by sphingomyelinase of *Staphylococcus aureus* is correlated with the thermotropic behavior of the sphingomyelins. In both cases maximal enzymatic hydrolysis was achieved at the beginning of the gel to liquid crystalline phase transition (30°C for beef brain sphingomyelin and 41°C for *N*-palmitoylsphingosine-phosphorylcholine) with much lower activity both below and above these temperatures. The enzymatic activity was depressed in the presence of cholesterol in the bilayer which also depressed the phase transition. The profile of the enzymatic activity is explained by the uniqueness of the lipid molecules arrangement at the phase transition.

Naturally occurring sphingomyelins have a broad gel to liquid crystalline phase transition in the range of 28—40°C [1—4]. When these sphingomyelins are present in a lipid bilayer together with egg phosphatidylcholines, phase separation of regions enriched with sphingomyelins occur (refs. 3, 4 and 5 and Barenholz and Thompson, in preparation). Similar phenomena were observed for synthetic sphingomyelins, although their thermotropic phase transition occurred over a much narrower temperature range [2, 6]. Similar phenomena was observed for membranes enriched in sphingomyelins, such as red blood cells of ruminants (Borchov et al., submitted). High mole ratios of cholesterol to sphingomyelin depress this phase transition [6]. The cholesterol effect is mainly on the hydrophobic region but not on the head group of the sphingomyelin [4]. Bilayers made of the synthetic sphingomyelin were affected by the presence of cholesterol in a similar way (Barenholz et al., in preparation).

However no such direct information is available on the interaction of these two lipid molecules at their interfacial region, which is unique for the sphingolipid in its hydrogen bond capability [22].

The phase separation occurring during the phase transition of lipids in the bilayer has a great influence on processes connected with membrane activities. In bilayers of phospholipids, the phase transition from gel phase to liquid crystalline phase is accompanied by a loss of order in the two dimensional structure and by abrupt increase in membrane fluidity; this is also accompanied by a change in bilayer thickness and density [7]. Various physical studies have shown that during the gel→liquid crystalline phase transition, the hydrocarbon chains of the phospholipid molecules change from being tightly packed to being mobile [7]. In a bilayer near the phase transition, it is energetically possible to have extensive regions in the membrane fluctuating between the gel and liquid-crystalline states. These fluctuations may be of critical importance for a number of membrane activities. Maximum permeability is observed near the phase transition [9, 10] activities of transport systems; other membrane-bound enzymes are also affected by the phase transition [11—15]. The susceptibility of phosphatidylcholines in lipid bilayers to pancreatic phospholipase [16, 25] or β-bungarotoxin [24] are also related to their thermotropic behavior, since activity is observed mainly at the phase transition range. No such relation was observed for bee venom phospholipase $A_2$ [16]. In this communication experiments are carried out to demonstrate that at the onset of the gel→liquid crystalline phase transition where the membrane fluctuations are extensive, hydrolysis of membrane components (sphingomyelin) by enzymes (*Staphylococcus aureus* sphingomyelinase) are also maximal, the structural instabilities of the bilayer may thus favor enzymatic hydrolysis of membrane component in contact.

D-erythro beef brain sphingomyelin was prepared and analyzed as described elsewhere [2]. D,L-erythro-*N*-palmitoylsphingosinephosphorylcholine was a gift of Professor D. Shapiro, the Weizmann Institute, Rehovot, Israel. Cholesterol (J.T. Baker Co.) was further purified by the bromination procedure of Schwenk and Werthessen [17] and by repeating washing in a two-phase solvent system [18]. Multilamellar liposomes were prepared in 50 mM Tris·HCl buffer, pH 7.4, containing 5 mM $MgCl_2$ as described by Bangham et al. [19], at 5°C above the gel to liquid crystalline phase transition. The sphingomyelin concentration was determined by the method of Bartlett [20]. Cholesterol concentration was determined using cholesterol oxidase as described by Moore et al. [21].

The purified enzyme (stored in 50% glycerol at –20°C) was a gift of Dr. S. Yedgar, Department of Biochemistry, School of Medicine, Jerusalem. Gel electrophoresis carried out with the sample showed the presence of two to three protein bands. The assay system in final volume of 0.3 ml contained multilamellar liposomes made of 3 mM of sphingomyelin in 50 mM Tris·HCl, pH 7.4, containing 5 mM $MgCl_2$ and 3.6 μg protein; final glycerol concentration in the incubation mixture was less than 1%. Reactions were terminated after 20 min according to Folch et al. [18] by adding 2 ml of $CHCl_3/CH_3OH$ (2:1, v/v) and 0.2 ml of water. The released phosphorylcholine was determined in aliquots of the upper phase by the method of Bartlett [20]. Control

experiments were carried out through the temperature range of 20—50°C by incubating the substrate in the absence of the enzyme and adding the enzyme after the reaction was terminated. The thermotropic behavior of the sphingomyelin liposomes was studied using fluorescence depolarization of the fluorophore 1,6-diphenylhexatriene [1] and followed the procedures described by Lentz et al. [23]. The thermotropic behavior of the substrate is described as the temperature-dependent anisotropy.

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}}$$

where $I_{\parallel}$ is the fluorescence intensity parallel to and $I_{\perp}$ is the intensity perpendicular to the plane of polarization of the excitation beam [1]. $I_{\parallel}$ and $I_{\perp}$ are corrected for light scattering [23].

The effect of temperature on the reaction rate was studied under the conditions where the enzymatic activity was proportional to the incubation time and to enzyme concentration. Fig. 1, curve A shows the thermotropic behavior of multilamellar liposomes made of beef brain sphingomyelin. The effect of temperature on the enzymatic activity is shown by curve B. In parallel, the thermotropic behavior and the enzymatic activity upon liposomes made of sphingomyelin and cholesterol in a mole ratio of 1.5 : 1 is shown by curves C and D, respectively. The thermotropic behavior of the beef brain sphingomyelin liposomes is characterized by a broad phase transition ranging from 27

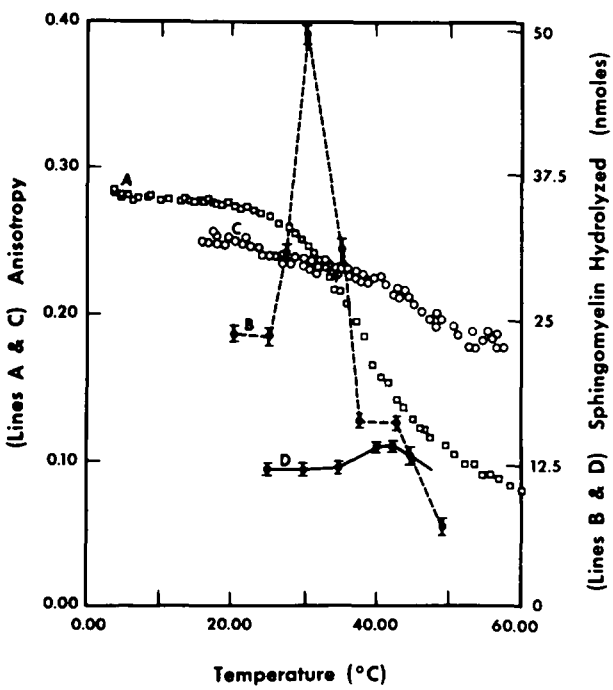


**Fig. 1. Correlation between the thermotropic behavior of multilamellar liposomes made of D-erythro beef brain sphingomyelin and its hydrolysis by sphingomyelinase of *S. aureus*. For details see text. The curves of the enzymatic activity are the average of four different experiments.**

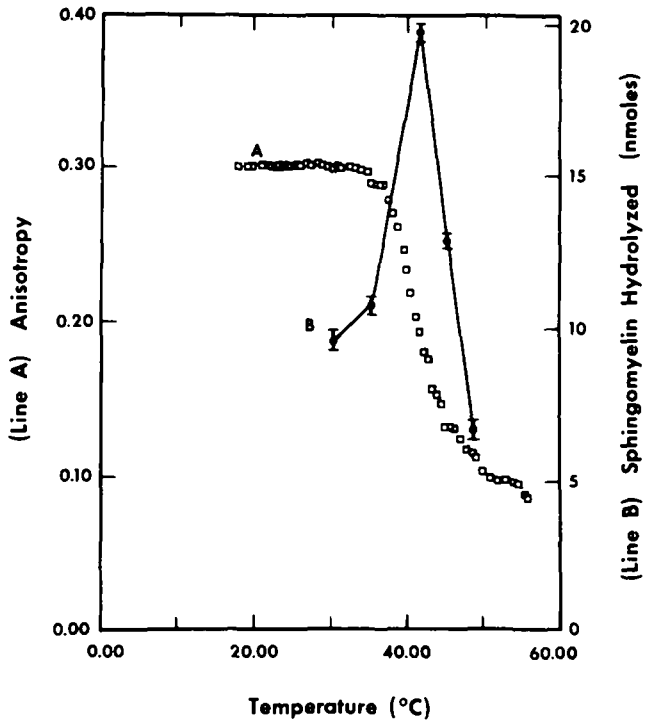


**Fig. 2. Correlation between the thermotropic behavior of multilamellar liposomes made of DL-erythro-*N*-palmitoylsphingosinephosphorylcholine and its hydrolysis by sphingomyelinase of *S. aureus*. For details see text. The curve of the enzymatic activity is an average of four different experiments.**

to 45°C with maximum change started at 30°C. This is in agreement with previous data obtained using fluorescence depolarization, NMR and differential scanning calorimetry [2, 4]. Three endothermic peaks were observed (i.e., 30.4°C, 32.5°C and 37.0°C) using the latter technique which gives the most precise and detailed description of the phase transition [2]. The enzymatic activity show a very clear maximum around 30°C. (Reaction rate at 30°C doubled from the one obtained at 25°C and was eight times the one obtained at 48°C.) This temperature profile of the enzymatic activity is completely abolished by the presence of 40 mol% cholesterol in the mixture (curve D). Under these conditions almost no phase transition of the sphingomyelin can be observed (curve C) and in parallel there is almost no effect of temperature on the enzymatic activity, although residual transition is observed at the range 40—50°C by the diphenylhexatriene and by the enzyme. This residual transition may result from a phase separation among the various species composed the beef brain sphingomyelin. This is suggested by the fact that the effect of cholesterol on various parameters of the thermotropic phase transition is strongly dependent upon the acyl chain of the sphingomyelin (Barenholz et al., unpublished results). This was further tested by performing similar experiments using multilamellar liposomes made of D,L-erythro-*N*-palmitoylsphingosinephosphorylcholine. The thermotropic behavior of this substrate is shown by Fig. 2, curve A, describing the anisotropy as a function of tempera-

ture. Contrary to the broad transition observed for beef brain sphingomyelin a sharp phase transition for D,L-erythro-*N*-palmitoylsphingosinephosphorylcholine is observed centered around 41°C, which is in good agreement with the results obtained by differential scanning calorimetry [2]. It is worth noting that the enzymatic activity show a maximum at this temperature (curve B); only about 50% of the maximal reaction rate was observed at 30°C and less than 25% at 48°C. The combined results demonstrate that the maximal rate of the enzymatic hydrolysis of sphingomyelins in a bilayer catalyzed by *S. aureus* sphingomyelinase is achieved at the temperature corresponding to the thermotropic phase of the bilayer. The enzyme is 2.5 times more active on liposomes made of D-erythro beef brain sphingomyelin than on liposomes made of D,L-erythro-*N*-palmitoylsphingosinephosphorylcholine (0.7 μmol/mg protein per min versus 0.28 μmol/mg protein per min). This difference may be explained by stereospecificity of the enzyme or by some differences in the liposome structure or both.

For each of the two sphingomyelins, maximal reaction rate is invariably obtained at its characteristic temperature corresponding to the thermotropic gel→liquid crystalline phase transition. This, together with the fact that no such temperature effect on enzymatic activity occurred in sphingomyelin liposomes containing 40 mol% of cholesterol (under these conditions almost no thermotropic phase transition can be observed and the residual phase transition is recognized by the enzyme), provides strong evidence that the enzymatic activity is closely related to the physical state of the lipid molecules in the lipid bilayer.

It is worth noting that similar results have been obtained for the sphingomyelinase using monolayers of various sphingomyelins as substrates. For instance, maximal enzymatic activity was obtained at the characteristic surface pressure, at which the monolayer is changing from a condensed to an expanded state. Also, the enzymatic activity was inhibited by the presence of cholesterol in the monolayer (Yedgar et al., in preparation).

These results can best be explained by the coexistence of domains of lipids in the pure gel phase and domains of lipids in the pure liquid crystalline phase, as was suggested for the hydrolysis of phosphatidylcholine bilayers by phospholipases A [16, 24, 25]. This was very well demonstrated by the detailed study [25] where the activity of pig pancreas phospholipase $A_2$ was strongly enhanced by irregularities in the bilayer structure imposed by coexistence of gel and liquid crystalline domains at the phase transition, or obtained by phase separation in phospholipid mixtures which show monotectic behavior. How does phase transition or phase separation in a lipid bilayer enhance the enzymatic activity upon components of the bilayer? There are a few explanations, among them, the highest compressibility at the phase transition [16] or the presence of transient 'holes' at the boundary regions between the solid and liquid crystalline domains. The lipid molecules at these boundary regions may be exchanged rapidly among the solid and liquid crystalline domains and, consequently, the bilayer is less organized over the transition range, while below and above the phase transition there is much more order in the packing of the lipid molecules in the bilayer. This region which is present only in the range of phase transition due to phase separation is probably the

most dynamic in the bilayer. The fluctuations in the bilayer structure are directly related to the size of the border region. This 'disorder' occurring during the phase separation may facilitate penetration or enzyme interaction with the sphingomyelin due to formation of transient 'holes'. This is supported by an increase in bilayer permeability [9, 10, 22]. It is also possible that partial exposure of hydrophobic regions of the bilayer at this boundary is the driving force for the increase in enzyme penetration [24]. According to this model the enzymatic activity will be directly proportional to the size of these boundary regions and may be maximal at the onset of the transition. This hypothesis can be tested only when the domain size will be measurable. Alternatively, the sharp optimum observed for the hydrolysis of beef brain sphingomyelin by the sphingomyelinase which coincides with one of the endothermic peaks observed by differential scanning calorimetry [2] may be explained by a preferential hydrolysis of one or more species of sphingomyelin, as was demonstrated for phospholipase $A_2$ [25]. However, in our case one cannot rule out a certain orientation of the headgroups as the reason for the sharp optimum in the enzymatic activity.

For comparing the activities of phospholipases A and sphingomyelinase upon their respective substrates one has to be aware of the differences between the phosphatidylcholines and the sphingomyelins [1, 2, 4, 22] as well as the differences in the hydrolyzable bond. While the sphingomyelinase is hydrolyzing a phosphate ester bond facing the aqueous medium, the phospholipase A is hydrolyzing an ester bond which is facing the hydrophobic region of the bilayer (although both groups are at the interface between the polar and apolar regions of the lipid bilayer). Therefore, it is to be expected that differences will be found due to penetration of the enzymes which will enable the sphingomyelinase to be active above and below the phase transition having maximum activity in this range, while phospholipase A will be active mainly when enzyme penetration is possible, such as in the phase transition range or due to phase separation [16, 24, 25]. The relation between the thermotropic behavior of the bilayer and *S. aureus* sphingomyelinase activity and a similar behavior observed for phospholipases [16, 24, 25] suggest that the activity of part of the enzymes acting upon lipid substrate organized in bilayer, monolayer, or lipoproteins may be strongly affected by the physical state of the lipid molecules at the molecular level and, therefore, will be controlled by the exact composition of the membrane or the lipoprotein. Therefore, the enzyme activity can serve as a sensitive probe to the physical state of the lipid and as well as giving better insight into lipid-protein interactions.

One must be aware that investigation of the activity of enzymes on lipids arranged in a membrane-like structure requires the correlation of the activity to the thermotropic behavior of the substrate-containing system.

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