Effect of Spectrin on Structure Properties of Lipid Bilayers Formed from Mixtures of Phospholipids. Fluorescence and Microcalorimetric Studies

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Abstract. Effect of spectrin from human erythrocytes on structure properties of lipid bilayers formed from a mixture of phosphatidylethanolamine/phosphatidylserine (PE/PS) and/or phosphatidylethanolamine/phosphatidylcholine (PE/PC) was studied with the use of fluorescence and microcalorimetric methods. Spectrin did not affect the order parameter of lipids in PE/PS vesicles. However, spectrin binding to liposomes did influence temperature, half-width and enthalpy of phase transitions in mixtures of dimyristoylphosphatidylethanolamine (DMPE) and dimyristoylphosphatidylcholine (DMPC), and this effect was dependent on DMPE to DMPC weight ratio. A change in miscibility of the components in the presence of spectrin was observed and it might be due to spectrin-PE interactions.

Key words: Spectrin — Lipid-protein interaction — Order parameter — Lipid phase transitions

Introduction

Spectrin, the major component of membrane skeleton, is bound within the membrane as a result of interactions with other membrane proteins (Benett and Brantom 1977; Goodman et al. 1988). However, direct binding of spectrin with lipids is also possible (Mombers et al. 1979; Mombers et al. 1980). Preceding studies have shown a particularly strong interaction of spectrin with vesicles formed from a mixture of phosphatidylethanolamine and phosphatidylserine (PE/PS) or phosphatidylethanolamine (PE) alone (Sikorski et al. 1987). By labelling the spectrin molecule with the isoindole fluorescence probe, we could demonstrate that spectrin binding with PE/PS affects the conformation of the protein and its thermal stability (Michalak et al. 1990). Maksimiv et al. (1987) have shown, that in binding of spectrin with lipid bilayer the major role is played by electrostatic interactions

between charged groups of the protein and the charged surface of the membrane. Specific electrostatic interactions were detected between spectrin dimers and monolayers and/or PS bilayers (Bonnet and Begard 1984; Cohen et al. 1986). However, some controversy exists if spectrin associates with bilayers at their surface only, or if it can penetrate lipid moiety to some extent. So far, it is not clear what is the role of PE in the interactions of spectrin with membranes. The purpose of the present studies was the estimation of the effects of spectrin binding on order parameter S and on thermotropic properties of lipid bilayers formed from mixtures of two phospholipids, PE/PS and PE/PC.

The order parameter of lipid bilayers was calculated from measurements of fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH). The thermotropic properties of model systems were studied using differential scanning calorimetry (DSC).

Materials and Methods

Materials: Erythrocyte ghosts were isolated from human blood according to Dodge et al. (1963). Spectrin dimers were extracted from erythrocyte ghosts at 37 °C for 30 min with 0.3 mmol/l phosphate buffer (pH 7.2) containing 0.1 mmol/l EDTA, 13 μmol/l phenylmethylsulphonylfluoride. The concentrated extract was purified on a Sepharose Cl-4B column (55 x 1.6 cm) equilibrated with 12.5 mmol/l borate buffer (pH 8.0) containing 0.14 mol/l NaCl, 0.1 mmol/l 2-mercaptoethanol, 0.1 mmol/l EDTA. The purity of spectrin was tested by 0.1 % SDS (5.6%) - polyacrylamide gel electrophoresis, and no traces of other protein were observed. Protein concentration was determined according to Mejbaum-Katzenellenbogen (1955). Spectrin was used within two days after isolation.

Phosphatidylserine (PS) and phosphatidylethanolamine (PE) from bovine brain used in fluorescence studies were purchased from Koch-Light Labs. The purity of the lipids was checked by thin-layer chromatography on silica gel plates. 1,6-Diphenyl-1,3,5-hexatriene (DPH) was purchased from Sigma. Other chemicals used in fluorescence experiments were of analytical grade.

Synthetic phospholipids used in microcalorimetric studies: dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE) were purchased from Calbiochem. The lipids were used without further purification.

Vesicles preparation for fluorescence measurements: Borate buffer (pH 8.0) was added to evaporated mixture of PE/PS (weight ratio PE:PS = 60:40) and the suspension of phospholipids was shaken for 40 min; then, the lipids were centrifuged at $12,500 \times g$ for 20 min. The concentration of lipids was estimated with the method of Bartlett (1959). PE/PS vesicles obtained in this way were labelled with DPH. DPH solution in tetrahydrofuran (2×10^{-3} mol/l) was mixed with the buffer to give a final DPH concentration of 2×10^{-6} mol/l; then, equal volumes of DPH and phospholipid vesicle suspension were mixed together. The molar ratio of DPH to phospholipids was approximately 100. The lipids were incubated with DPH for 30 min before addition of the protein solution. The molar ratio of lipid to protein was approximately 700.

Fluorescence measurements: For fluorescence studies a Perkin-Elmer MPF-3L spectrofluorimeter was used. The probe fluorescence was excited at the wavelength $\lambda_{ex} = 360$ nm. Temperature measurements were made with the use of a temperature controlling device and sample temperature was measured with a platinum resistance sensor. Fluorescence polarization was measured with a polarization accessory.

The degree of ordering of a lipid bilayer can be estimated from DPH fluorescence measurements. The degree of polarization P is calculated from intensities of fluorescence emission for the analyzer oriented in parallel ($I_{\parallel}$) and perpendicularly ($I_{\perp}$) to the excitation light polarity:

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \tag{1}$$

Fluorescence anisotropy (r_s) in conditions of "steady state" is given by:

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

Initially, fluorescence anisotropy measurements were interpreted only in terms of "microviscosity" (Shinitzky and Barenholz 1978) described by the rate of rotational diffusion of the probe. According to this interpretation (Hildenbrand and Nicolaou 1979; van Blittersvijk et al. 1981), r_s contains a static component r_∞ and a dynamic one, r_f. The latter component (r_f) is related to the time of rotational relaxation of the fluorophore; this in turn is proportional to "microviscosity" (Shinitzky and Barenholz 1978). On the other hand, r_∞ is proportional to squared order parameter of lipid chains. The relationship between r_s and r_∞ was used to calculate the order parameter S_{DPH} for various isolated biological membranes (van Blittersvijk et al. 1981). The order parameter can be calculated from r_s measurements provided $r_s > 0.1$.

Microcalorimetric studies: The PE:PC weight ratio was varied and was 25:75, 50:50, and 75:25. The solution of mixed lipids in chloroform was first dried under nitrogen, and then evaporated in a vacuum evaporator for about 2 hours. Then, 1 ml Tris/EDTA buffer (Tris 10^{-2} mol/l, EDTA 10^{-3} mol/l, pH 7.5) was added and the suspension was sonnicated for 5 min. Next, 1 ml of spectrin solution was added and the suspension was incubated for additional 30 min at 37 °C. Then, the samples were centrifuged at $19,000 \times g$ for 1 hour. Protein concentration was estimated in the supernatant collected after the centrifugation. The pellet was removed to the sample pans and scanned. Microcalorimetric measurements were made with a UNIPAN microcalorimeter type 600 equipped with a thermostat modified in our laboratory. The scanning rate was 1 °C/min, the area under transition peaks was estimated by the planimetric method. The error of phase transition temperature determination was 0.1 °C, except for very broad transitions (such as that illustrated in Fig. 3*f*) when this error was approximately 0.5 °C. For each of the DMPE/DMPC/spectrin combinations two separate samples were prepared, and each sample was scanned twice to check the reproducibility of transitions.

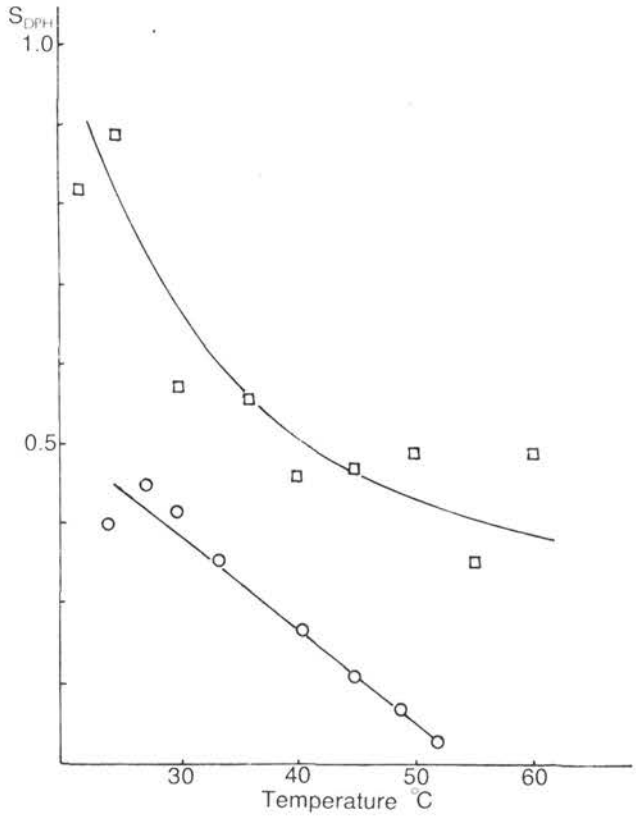

Figure 1. Order parameters S_{DPH} for human erythrocyte ghosts (–□–) and PE/PS vesicles (– o –) in dependence on temperature, λ_{ex} = 360 nm.

Results

The effect of spectrin binding on the ordering of hydrocarbon chains in lipid bilayers in PE/PS vesicles was investigated over a range of temperatures. The order parameter S_{DPH} was calculated from values of DPH fluorescence anisotropy. Fig. 1 illustrates the changes of order parameter S_{DPH} for PE/PS vesicles in dependence on temperature, and it is compared with the changes of S_{DPH} for human erythrocyte ghosts. Over the range of temperatures studied (20–60°C), the degree of ordering of hydrocarbon chains in PE/PS vesicles is much lower than that in the

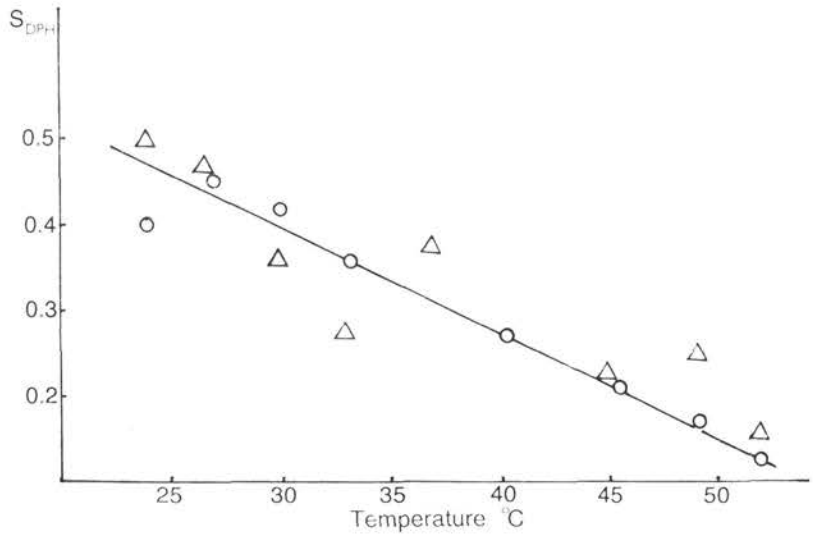

Figure 2. Effect of spectrin binding with lipid membranes on order parameter S_{DPH} for PE/PS vesicles at different temperatures. λ_{ex} = 360 nm, lipid concentration 5×10^{-5} mol/l, DPH concentration 5×10^{-7} mol/l, lipid to protein ratio 700; circles - PE/PS vesicles, triangles - PE/PS vesicles in the presence of spectrin.

erythrocyte ghosts. Addition of spectrin to the vesicle suspension did not markedly influence the order parameter of the lipid chains up to approximately 40 °C (Fig. 2).

Fig. 3 shows examples of thermograms obtained for vesicles of different lipid composition and for vesicles incubated with spectrin (protein concentration 290 μg/ml). The thermograms cannot be compared directly because of the different lipid contents of the samples. However, it is obvious that phase transitions of vesicles incubated with spectrin are broader than those observed for pure lipids. The following parameters were determined from the thermograms obtained for the mixtures studied: phase transition temperature (T_m) and change of enthalpy of the systems studied during transition (ΔH). The values of these parameters were plotted against DMPE concentration in the samples (Figs. 4*a* and 4*b*, mixtures of lipids alone and those incubated with spectrin, respectively). As it is seen from Fig. 4*a*, in both cases the temperature of phase transition depends on molar ratio of DMPE to DMPC. For vesicles incubated with spectrin, T_m grew with the increasing DMPE concentration in the mixture faster than it did for lipids without spectrin. Thus, for the lowest phosphatidylethanolamine content tested, i.e. 25:75 (w/w), the temperature of phase transition was about 4.5 °C lower for samples incubated with spectrin than for similar mixtures without the protein. For samples containing

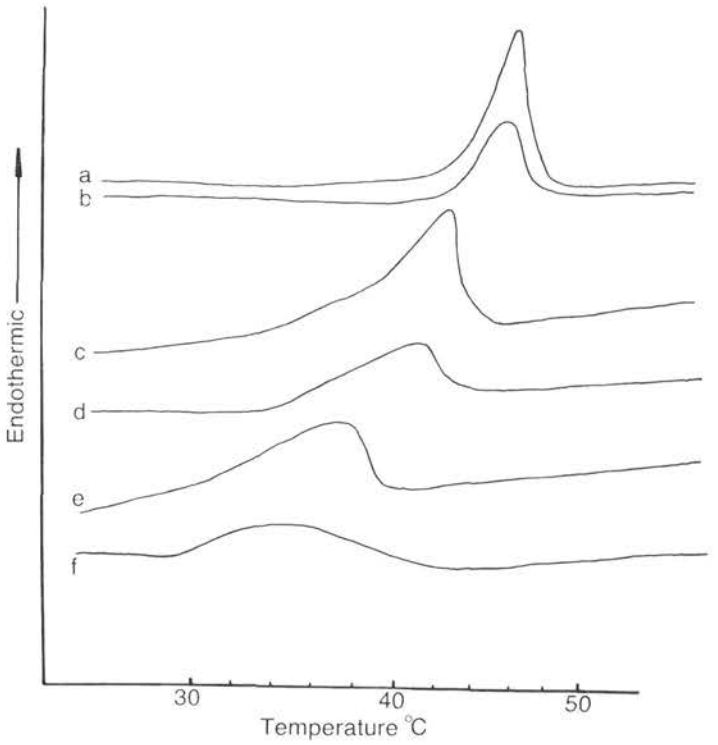

Figure 3. Examples of thermograms obtained in microcalorimetric experiments for lipid vesicles containing different weight ratios of DMPE and DMPC, in the presence and absence of spectrin. *a,b* – PE:PC = 75:25; *c,d* – PE:PC = 50:50; *e,f* – PE:PC = 25:75; *a,c,e* - in absence of spectrin; *b,d,f* - in presence of spectrin.

75:25 DMPE:DMPC (w/w), T_m was slightly higher in the presence of the protein than for pure lipids.

Transition enthalpies obtained for vesicles incubated with spectrin were lower for all DMPE/DMPC ratios studied than those in the absence of the protein. Particularly great differences were observed in comparison with samples containing 75:25 DMPE:DMPC (w/w) (see Fig. 4*b*), although a decrease of ΔH was recorded for pure lipids (without spectrin) as well.

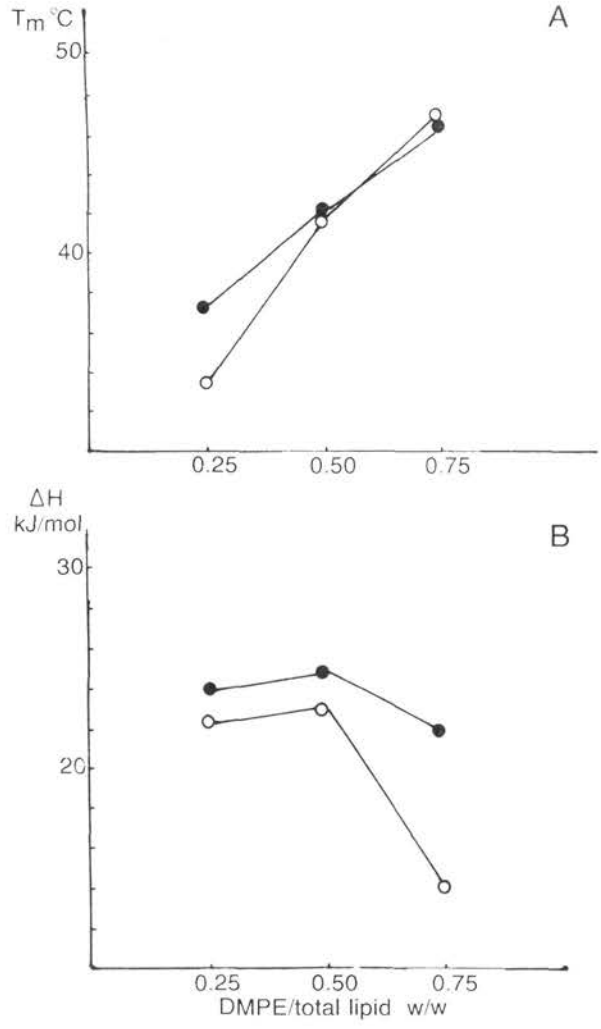

Figure 4. Effects of spectrin on phase transition parameters; vesicles of different PE:PC weight ratios. *A* - transition temperature vs DMPE content; *B* - transition enthalpy vs DMPE content. (•) - DMPE:DMPC mixtures, (○) - DMPE:DMPC mixtures in the presence of spectrin.

Discussion

So far, several reports have appeared concerning the type of interactions in spectrin-lipid bilayer model systems. However, it has reminded unclear whether spectrin can penetrate lipid bilayers to some extent, or whether only polar fragments of protein interact with the membrane surface.

From our present results it is evident that spectrin does not affect the order parameter of hydrocarbon chains in lipid membranes. This would suggest a typically superficial association of spectrin with the lipid bilayers.

Maksimiv et al. (1987) have shown that spectrin can interact selectively with charged lipids and that coulomb forces are involved in these interactions. Hydrophobic interactions have been excluded. The interactions observed are too weak to maintain an asymmetric distribution of PS between the inner and the outer layer of the erythrocyte membrane.

As revealed by ^{2}H and ^{31}P nuclear magnetic resonance studies (Bitbol et al. 1989) spectrin does not affect the conformation of the polar head group of phosphatidylserine.

In spite of the existence of some hydrophobic domains in the spectrin molecule (Calvert et al. 1980) our results also support the occurrence of purely superficial interactions of spectrin with lipid membrane. This interpretation is confirmed by the observation that in the presence of Ca^{+2} ions spectrin, negatively charged at pH higher than 5.6 (isoelectric point), is adsorbed on the surface of negatively charged bilayers particularly easily (Mombers et al. 1980). It has also been suggested that upon spectrin-lipid interaction, positively charged fragments of the protein can face towards negatively charged membrane surface (Maksimiv et al. 1987; Tilley et al. 1986).

A two-component model system formed from lipids with different polar head groups (as are mixtures of DMPE/DMPC used for the microcalorimetric studies) is an example of a system with non-ideal mixing of the components (Chapman et al. 1974). Solidus line of the phase diagrams for such systems have a segment, within which the phase transition temperature shows only slight changes in dependence on the component proportions. This phenomenon is observed for low contents of the component with a higher gel-liquid crystalline transition temperature. The next segment of solidus line is characterized by a fast increase of T_m in dependence on the content of the higher-melting component (Lee 1977; Blume 1980). So, from the phase diagrams obtained for two different non-ideally mixing lipids it may be concluded that when lipid mixing is worst, the first of the segments is longer and the other has a steeper course. Another phenomenon associated with non-ideal miscibility of lipids is an increase of the half-width of transition peaks of such mixtures in comparison with the half-widths of transition of well-mixing systems (Lee 1977). Our results concerning phase transition temperatures of DMPE/DMPC

mixtures correspond to the solidus line of phase diagram obtained by van Dijk et al. (1977). An increase of the slope of the solidus line and an increase of the half-width of phase transition are observed for DMPE/DMPC vesicles incubated with spectrin (Fig. 4*a*); it may suggest that the presence of spectrin in the mixtures decreases the miscibility of the components. Probably, the only reason for a non-ideal PE/PC mixing is a difference in the structures of polar head groups between these lipids. Thus, the changes of T_m observed may be due to differences in interactions or packing of head groups of lipids in the presence of spectrin in the mixtures. Studies of Mombers et al. (1980) who used monolayer technique suggested that there are no interactions between spectrin and phosphatidylcholine. At the same time, our earlier observations revealed interactions between spectrin and phosphatidylethanolamine (Sikorski et al. 1987). It may well be that the change of miscibility in the system studied (DMPE/DMPC) results from an interaction between spectrin and DMPE. An additional support for this view may be derived from the observation that the enthalpy of phase transition markedly decreases for mixtures containing 75:25 (w/w) DMPE. It may be suggested that, in the high content, DMPE determines the magnitude of transition enthalpy. The enthalpy was much lower than for the same mixtures in absence of spectrin (see Fig. 4*b*).

Only few reports are available so far, concerning the influence of spectrin on the ordering of hydrocarbon chains in model systems. Contrary to the results obtained in this work using the fluorescence method, Sikorski and Jezierski (1986) observed a slight drop in order parameter for PE/PS systems (from 0.683 to 0.605) as determined by the EPR technique.

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