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# Electrostatic Coupling of Spectrin Dimers to Phosphatidylserine Containing Lipid Lamellae†

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ABSTRACT: We studied the interaction of spectrin dimers from human erythrocytes with (bilayer and monolayer) model membranes of mixtures of dimyristoylphosphatidylethanolamine, dimyristoylphosphatidylcholine, and dimyristoylphosphatidylserine (DMPS) by (1) densitometric evaluation of phase transitions and phase boundaries, (2) film balance experiments, and (3) microfluorescence. We demonstrate that spectrin readily adsorbs to mixed bilayers and monolayers even in the presence of small DMPS concentrations (30 mol %) whereas no appreciable interaction with lamellae containing zwitterionic lipids alone is observed. The selectivity of the DMPS/spectrin interaction is established by quantitative evaluation of the shifts of the phase boundaries (liquidus and solidus line) caused by the lipid/protein interaction as a function of the composition of the binary lipid mixtures. Quantitative information about the free energy of the lipid/protein interaction is obtained by computer simulation of the phase diagram of the lipid mixture in the absence or in the presence of a very small molar fraction of the protein and comparison of calculated and measured shifts. A binding energy of about $10^{-17}$ J per spectrin molecule is found. The present perturbation method can be generalized to study selective lipid/protein interaction mechanisms in ternary or higher component mixtures. The present results provide evidence that in addition to the binding to band III, spectrin may also couple directly to the lipid moiety of the inner monolayer of erythrocytes. The spectrin/phosphatidylserine interaction energy is, however, not large enough to account solely for the asymmetric distribution of this lipid in erythrocytes. Since spectrin is flexible, it is expected to be highly folded and can thus act as an entropy spring, the stiffness of which may be controlled by the lipid/protein interaction.

The deformability (Sackmann et al., 1986; Stokke et al., 1986a,b), the microscopic structural and dynamic properties of the erythrocyte membrane (Elgsaeter et al., 1976), and also the cell shape (Branton et al., 1981; Lieber et al., 1984) are determined to a large extent by the coupling of the spectrin/actin network to the lipid/protein bilayer.

It is generally assumed now that the filamentous spectrin dimers are interconnected by self-association and by actin oligomers and that the network formed in this way is primarily coupled to the lipid/protein bilayer by binding of part of the spectrin dimers to the membrane proteins such as to band III via ankyrin and to glycophorin C via band 4.1 (Haest, 1982; Branton et al., 1981). The coupling of the network to the bilayer is, however, rather weak as follows from the findings that the membrane bending elastic modulus is higher by at most about a factor of 5 than the value measured for fluid lipid bilayers (Fricke et al., 1986) and that the bilayer lipid molecules and proteins not coupled to the cytoskeleton exhibit fast lateral diffusion (Schindler et al., 1980; Kapitza & Sackmann, 1980).

The first evidence for an additional mode of coupling, namely, a direct binding of the spectrin filaments into the lipid bilayer moiety by electrostatic binding to the charged phosphatidylserine, was provided by Mombers et al. (1977, 1980).

In the present work, the binding of spectrin to bilayers and monolayers composed of mixtures of (synthetic) zwitterionic lipids (phosphatidylcholine and phosphatidylethanolamine) and phosphatidylserine is studied by densitometry, film balance experiments, and microscopic techniques. Evidence is provided that spectrin interacts selectively with the charged lipid by Coulomb forces whereas evidence for a hydrophobic interaction as postulated by Mombers et al. (1980) could not be found.

A further purpose of the present work is to show that quantitative information about the selectivity of the lipid/protein interaction as well as about protein binding energies is obtained by analyzing the shifts of the phase boundaries of

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the lipid mixtures in terms of regular solution theory. In view of the high flexibility of the spectrin molecule, it is postulated that the adsorbed spectrin filaments are folded like ordinary macromolecules adsorbed to interfaces.

## Materials and Methods

*Materials.* The lipids were commercial products of Avanti Polar Lipids Inc., Birmingham, AL, and were used as purchased.

Spectrin was isolated from human erythrocytes following the procedure of Bennett (1983). The purification of the crude protein fraction was performed by gel chromatography (Sepharose CL-4B) at 4 °C. Small aliquots of the purified spectrin/dimer solution (in the elution buffer) were transferred into plastic vials and stored at 4 °C but not longer than 10 days before use.

The elution buffer contained 100 mM NaCl, 1.0 mM ethylenediaminetetraacetic acid (EDTA), 10 mM $Na_2HPO_4$, 0.2 mM dithiothreitol, and 1.0 mM $NaN_3$ (pH 7.2). Unless otherwise stated, the same buffer was also used for vesicle preparation. The spectrin concentration was determined by following the photometric procedure of Bradford (1976). The purity of the isolated protein was checked by polyacrylamide gel electrophoresis according to the procedure of Morrow and Haigh (1983). In particular, the absence of spectrin tetramers in the dimer fractions was controlled with this technique.

*Mass Densitometry.* The modification of the phase transitions of the pure lipid bilayers and of the phase boundaries of the lipid mixtures as caused by spectrin binding was determined by measuring the mass density of the vesicle suspensions in the absence and in the presence of spectrin as a function of temperature. These experiments were performed with a (DMA 60) densitometer of Paar KG which is equipped with an Apple II microcomputer. The rate of the temperature change was 0.14 °C/min at increasing and decreasing temperature. To avoid denaturation of the spectrin, the highest temperature was 43 °C. In order to account for the thermal expansion of the protein itself, this parameter was measured in a separate experiment and the density vs. temperature plots of the spectrin-containing vesicle suspensions were corrected.

The thin-walled vesicle suspensions are prepared by swelling of a thin layer of the lipid deposited on the wall of a glass flask (by the solvent evaporation technique) in excess buffer: 1.3 mL of the buffer (cf. Materials) is added to 20 mg of lipid. The glass flasks are sealed and kept for 30 min at about 5 °C above the transition temperature of the component with the highest melting point. They are shaken and put in a bath sonifier for several seconds in order to homogenize the vesicle suspension. Prior to measurement, the vesicle dispersion is degassed under vacuum for 30 min and is then transferred to the measuring cell.

For the experiments in the presence of spectrin, a solution of this protein (contained in the same buffer as the lipid) is added to the pre-prepared vesicle suspension, that is, before the degassing procedure. In this case, only 1 mL of buffer is added to the 20 mg of lipid. The spectrin solution is then condensed to a final volume of 0.3 mL. In this way, it is achieved that the total lipid concentration is the same as in the absence of spectrin.

*Monolayer Experiments.* For the monolayer experiments, a homemade film balance of the type described previously (Lösche & Möhwald, 1984) is used. The total volume of the subphase is 240 mL and the total surface area 236 $cm^2$. The area covered by the lipid monolayer is changed by a movable barrier, and the surface tension is measured with the Wilhelmy system. In order to study the spectrin/lipid interaction, the protein is injected into the subphase consisting of the same buffer which was used for the protein elution procedure (but without dithiothreitol and $NaN_3$) before deposition of the lipid monolayer. The lipid monolayer itself is prepared by deposition of $^1/_{25}$th mg of the lipid dissolved in 40 $\mu$L of a 3:1 $CHCl_3/CH_3OH$ mixture in small droplets at different positions of the water surface on one side of the trough. The film balance is equipped with a microfluorescence device consisting of a water immersion objective (Zeiss Achromat 40/0.75W) and a highly sensitive video camera (Hamamatsu). The objective is positioned in the base plate of the Langmuir trough and is focused on the plane of the monolayer by adjustment of the water level (Lösche & Möhwald, 1984). With this device, it is possible to observe the binding of spectrin to the lipid monolayer and the microstructure of the lipid/spectrin layer in a direct way. For that purpose, the spectrin was labeled with fluorescein isothiocyanate following the procedure of Rinderknecht (1962).

## Results

*Densitometric Evaluation of the Interaction of Spectrin with Vesicles.* Quantitative information about the free energy and the selectivity of the lipid/protein interaction is obtained by evaluating (1) the shifts in the phase transition temperatures of pure lipid bilayers and (2) the modifications of the phase boundaries of lipid mixtures. The advantage of such thermodynamic studies is the high sensitivity which enables measurements at very low protein concentrations, thus excluding effects due to the protein/protein interaction or due to protein-induced vesicle instabilities such as bilayer aggregations or bilayer to hexagonal phase transitions.

In the present work, the shifts in the transition temperatures and the phase boundaries were first studied by densitometry. In this technique, the average molar volume, $V$, of the vesicle suspension is measured as a function of temperature. The chain melting transition leads to a jump of the molar volume whereas $V$ increases linearly with temperature both above and below the lipid phase transition (Wilkinson & Nagle, 1979; Schmidt & Knoll, 1986) due to a linear thermal expansion of both the vesicles and the water. It is for that reason that densitometry renders very sensitive determinations of the liquidus and solidus lines of lipid mixtures which are clearly indicated in volume vs. $T$ plots (Schmidt & Knoll, 1986). Typical examples of pure and mixed lipid bilayers are shown below (Figures 2 and 4).

The phase diagrams of the dimyristoylphosphatidylcholine/dimyristoylphosphatidylserine (DMPC/DMPS) mixture as determined by the densitometric technique is shown in Figure 1 together with the theoretical phase diagram which was calculated on the basis of the regular solution theory as described under Discussion. The solidus line is a monotonously ascending function of temperature which strongly suggests that the two components are miscible in the solid state. Further evidence for this comes from accompanying freeze–fracture experiments (not presented) which show a characteristic ripple texture for all mixtures but which do not exhibit the domain structure which is characteristic for phase separation in the solid state [cf. Sackmann et al. (1984)].

Figure 2 shows molar volume vs. temperature plots for vesicle dispersions of pure DMPC and DMPS, respectively, as well as the effect of spectrin on the chain melting transitions. Clearly, spectrin does not affect the DMPC transition to a remarkable extent. For DMPS, however, the jump in $V$ is reduced by about 60%. Simultaneously, the transition is slightly shifted by $\Delta T = +2$ °C. The $T$–$V$ curves have been corrected for the volume change of spectrin alone. It thus

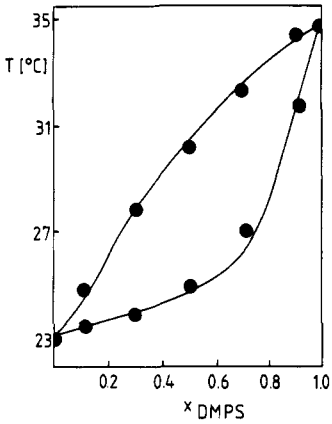


FIGURE 1: Phase diagram of a mixture of DMPC and DMPS. The circles give the positions of the phase boundaries as determined from the molar volume vs. temperature plots following Schmidt and Knoll (1986). The drawn curves were calculated on the basis of the regular solution theory by the computer-fitting procedure described under Discussion. The following values of the transition temperatures and the heats of transition were assumed: DMPC, $T_{m_1}$ = 23 °C; $\Delta H_1$ = 28 kJ $M^{-1}$. DMPS, $T_{m_2}$ = 35 °C; $\Delta H_2$ = 29.2 kJ $M^{-1}$.

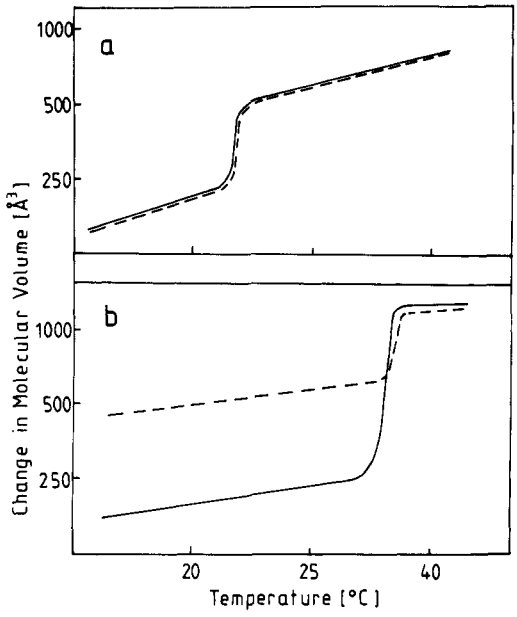


FIGURE 2: Effect of spectrin on the phase transition of bilayer vesicles of pure DMPC (a) and DMPS (b), respectively, in buffer given under Materials and Methods. The drawn solid curves correspond to the absence of spectrin, and the dashed curves correspond to the presence of spectrin. The lipid concentration was 15 mg $mL^{-1}$, and the molar ratio of spectrin to lipid was $r_p = 6 \times 10^{-5}$. The change in the molar volume $V$ is plotted as function of temperature. The curves are corrected for the temperature change of the molar volume of spectrin.

follows from the reduction in the volume jump that the phase transition of 60% of the lipid is suppressed or shifted to temperatures above 50 °C (the upper limit of our measurement). Since the spectrin is added to the vesicle suspension just prior to the measurement, the lipid still exhibiting the transition is attributed to the inner monolayer whereas the transition of the lipid of the outer monolayer is no longer observed. The slight shift of $\Delta T$ = 2 °C can be explained in terms of the coupling of the two opposing monolayers.

The strong binding of spectrin to DMPS vesicles is directly demonstrated in Figure 3 which shows phase-contrast micrographs of giant vesicles of fluid DMPS before and after perforation with spectrin solution (0.7 mg of spectrin/mL). Spectrin causes a transition from a spherical to a polygonal shape. The latter is typical for bilayers in a crystalline state ($L_\beta$ phase). Moreover, the spectrin causes aggregation of the bilayers. This transition caused by the adsorption of spectrin is irreversible at least up to 50 °C (the highest possible temperature).

Figure 4 shows examples of the effect of spectrin on the densitometric curves of mixtures of DMPC and DMPS. It can be clearly seen that the liquidus line is shifted to higher temperatures after addition of spectrin to the vesicle suspension. The shift increases with increasing DMPS concentrations: thus, it is $\Delta T$ = +4 °C for 30 mol % and $\Delta T$ = +6 °C for 50 mol % DMPS. The increase of the high-temperature shift with increasing concentrations of the charged lipid and the absence of any effect of spectrin on the DMPC transition provide strong evidence that the adsorption of spectrin to membranes is dominated by electrostatic forces.

The break of the $V$–$T$ plots at low temperature (corresponding to the solidus line) is apparently not shifted by spectrin. This is, however, due to the fact that the protein was added to the prepared vesicle dispersion so that the inner monolayers are not affected. Thus, the $V$–$T$ curves are superpositions of the undisturbed lipid of the inner and the disturbed lipid of the outer monolayers of the bilayer vesicles. For the same reason, the slope of the $V$–$T$ plots is smaller on the right side than on the left side of the fluid–solid coexistence regime. The position of the solidus line could thus not be determined reliably. On the other side, a decomposition of the lipid into two fractions such as vesicles of pure DMPC and spectrin/DMPS aggregates is avoided by the present technique.

*Interaction of Spectrin with Monolayers.* The interaction of spectrin with monolayers of dimyristoylphosphatidylethanolamine (DMPE) and DMPS and mixtures of the two compounds was studied by film balance and microfluorescence experiments. In Figure 5, the isotherms of DMPE on pure buffer and on a $2 \times 10^{-9}$ M spectrin solution (0.2 mg per 240 mL) are compared. Moreover, the isotherm of the pure spectrin solution is given. The latter exhibits a break at a pressure $\pi_{eq} \approx 6$ mN·$m^{-1}$ above which the protein is squeezed into the bulk solution. The nearly horizontal slope at $\pi > \pi_{eq}$ shows that an equilibrium exists between the protein in the bulk and at the surface. The DMPE monolayer on both the pure buffer and the spectrin solution exhibits the well-known fluid to crystal (or chain melting) transition at the pressure $\pi_m$ (Albrecht et al., 1978). The two isotherms at increasing and decreasing areas exhibit a remarkable difference at low densities: for pure buffer as subphase, the pressure goes to zero at $A \approx 90$ Å$^2$/molecule which corresponds to the transition from the fluid to a two-dimensional foam state (Lösche et al., 1983). In the presence of spectrin, however, the isotherm goes over into a nearly horizontal line at a pressure of about 6 mN·$m^{-1}$ which is about equal to the equilibrium pressure ($\pi_{eq}$) of spectrin (cf. Figure 5). Such behavior is indeed expected if spectrin does not bind to DMPE since a monolayer of the protein coexistent with the lipid monolayer will form if the pressure decreases below $\pi_{eq}$ (Figure 5 insert). The observation that the lipid phase transition exhibits the same sharpness in the presence and absence of spectrin provides further evidence that there exists no specific DMPE/spectrin binding. The small shift in $\pi_m$ (to $\pi_m$*) can be attributed to the change of the surface tension of the water surface due to spectrin or to a different contact angle at the platelet of the Wilhelmi balance.

Figure 6 shows the effect of spectrin on the DMPS monolayer. Pure DMPS exhibits the chain melting transition at 18 mN·$m^{-1}$. In this case, the isotherms are drastically changed

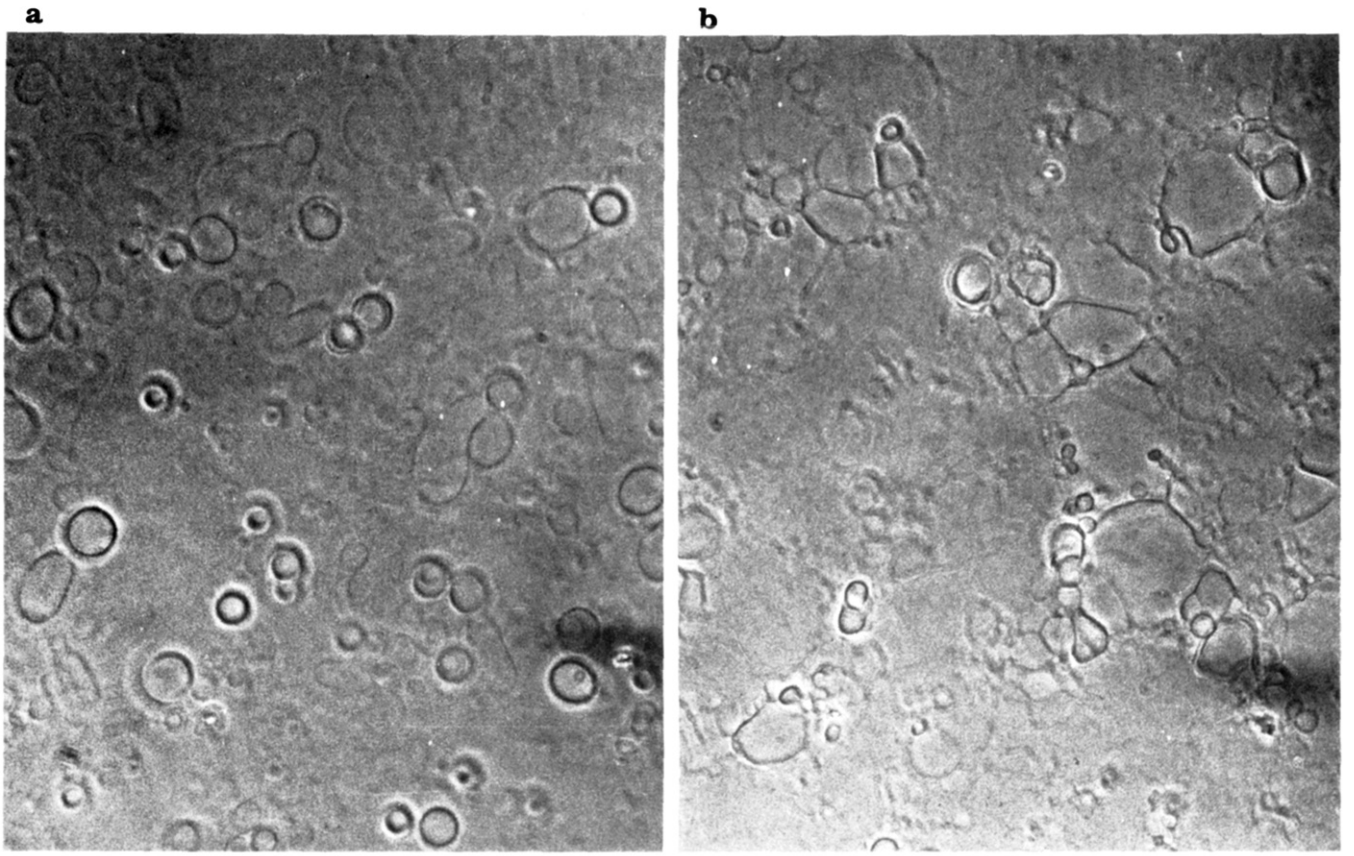


FIGURE 3: Effect of spectrin on the shape of giant vesicles of DMPS. The same vesicle preparation before (a) and after (b) perforation with $2 \times 10^{-7}$ M spectrin solution is shown. Note that the perforation leads to polygonization and aggregation of the vesicles. Temperature, 39 °C.

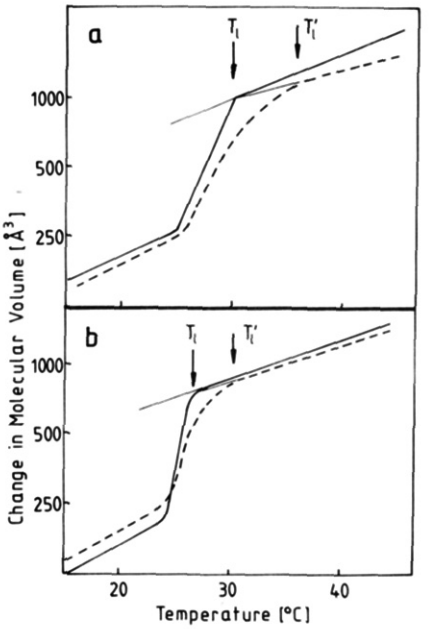


FIGURE 4: Molar volume vs. temperature plots of vesicles of DMPC/DMPS mixtures before (solid curves) and after addition of spectrin (dashed curves): (a) 1:1 mixture of DMPC and DMPS in buffer; (b) 70:30 DMPC/DMPS mixture. The spectrin to lipid molar ratio was again $6 \times 10^{-5}$. The positions $T_l$ of the liquidus and $T_s$ of the solidus lines are determined by the breaks where the $V$–$T$ plots deviate from straight lines characteristic for the completely fluidized and the completely solidified states of the mixture.

by spectrin and exhibit a pronounced hysteresis. Starting at a high pressure (40 mN·m$^{-1}$), the $\pi$–$A$ curve with spectrin decreases very sharply and goes directly over into the horizontal line when the equilibrium pressure $\pi_{eq}$ of spectrin is reached.

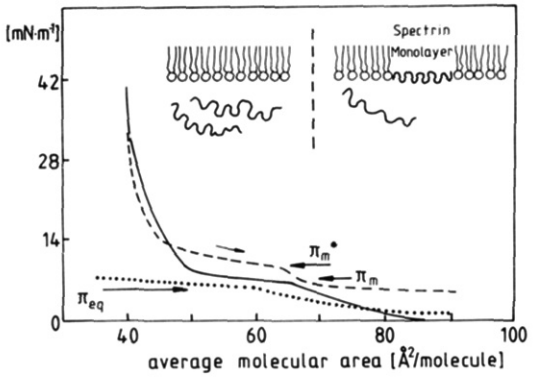


FIGURE 5: Pressure–area diagram of the monolayer of DMPE on pure buffer (solid curve) and on a $2 \times 10^{-9}$ M solution of spectrin in the subphase (dashed curve). The curves are recorded at decreasing pressure. The dotted curve gives the pressure–area diagram of the aqueous solution of spectrin alone. The area per molecule in this case is arbitrary since the surface concentration of the protein is unknown.

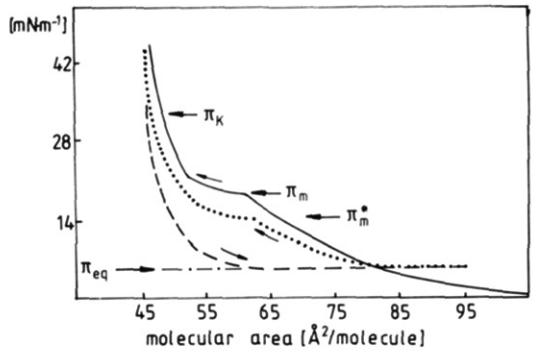


FIGURE 6: Isotherm of the DMPS monolayer on pure buffer (—) and on spectrin solution (same concentration as in Figure 5) taken at increasing (···) and decreasing (- - -) pressure.

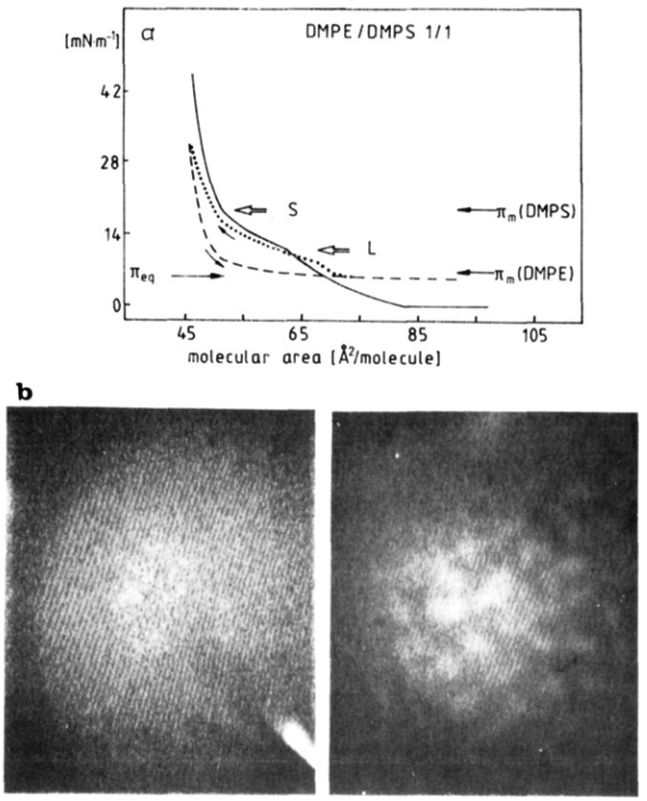


FIGURE 7: (a) Isotherm of a 1:1 DMPE/DMPS mixture on pure buffer (—) and on spectrin solution (as in Figure 6) taken at decreasing (---) and subsequent increasing pressure (···). The thick arrows indicate the liquidus (L) and solidus lines (S), respectively, of the mixture in the absence of spectrin. (b) Fluorescence micrograph of a monolayer of a 1:1 DMPE/DMPS mixture with fluorescence-labeled spectrin in the subphase. (Left side) Image taken at a pressure just below the liquidus line (L) indicated by the thick arrow (L). (Right side) Pressure slightly above S.

The chain melting transition pressure is thus about equal to $\pi_{eq}$ (=6 mN·m$^{-1}$). At increasing pressure, the isotherm resembles more that of DMPS on pure buffer. However, the transition pressure is also decreased by about 5 mN·m$^{-1}$ by the protein. Both the depression of the fluid to solid transition and the strong hysteresis lead to the conclusion that spectrin interacts with DMPS. As is well-known [cf. Sackmann (1983) for references], the Coulomb interaction of the charged lipid head groups leads to a repulsive lateral pressure, $\pi_{el}$, and for that reason, the chain melting transition of a charged lipid occurs at a higher transition pressure, $\pi_m$, than that of a zwitterionic lipid of the same chain length. The electrostatic binding of spectrin to DMPS reduces the repulsive electrostatic pressure $\pi_{el}$, hence, the decrease in $\pi_m$. The strong hysteresis effect provides further evidence for the electrostatic interaction mechanism (Sackmann, 1984).

A similar strong effect of spectrin is observed on mixed monolayers of DMPE and DMPS as is demonstrated in Figure 7 in particular for the isotherm observed at decreasing pressure (---) in Figure 7a]. In this case, the fluid to crystal transition of the mixture on pure buffer occurs over a broad pressure region due to lateral phase separaton. The liquidus and the solidus lines are indicated by the thick arrows (L and S). The liquidus is clearly shifted to lower $\pi$ values, after addition of spectrin to the subphase (corresponding to a higher transition temperature). This shows again that the spectrin interacts selectively with DMPS. The solidus line (S) is also shifted to lower pressures.

Direct evidence for the selective binding of spectrin to DMPS comes from microfluorescence studies of the monolayer using spectrin labeled with fluorescein isothiocyanate. The adsorption of the protein can be observed directly with the water immersion objective inserted into the subphase by focusing on the air/water interface. Figure 7b shows two images of the 1:1 DMPE/DMPS monolayer taken at pressures slightly below the liquidus line L of the fluid–solid coexistence (left side) and slightly above the solidus line S of the mixture (right side of Figure 7b). In the first case, the monolayer exhibits a strong homogeneous fluorescence, showing that the spectrin is adsorbed to the monolayer and is randomly distributed. In the second case, however, the adsorbed protein is organized in patches. Now, it is well-known from many microfluorescence and electron microscope studies (Lösche et al., 1983; Fischer & Sackmann, 1985) that monolayers exhibit a domainlike organization in the coexistence region. The dark patches start to form if the pressure is increased slightly above the solidus S (in Figure 7a). Since DMPE has a lower chain melting transition pressure than DMPS, it follows that spectrin binds only to the charged lipid.

## Discussion

*Mechanism of Spectrin Binding.* Both the bilayer and the monolayer experiments provide strong evidence that the binding of spectrin to phosphatidylserine containing lipid lamellae is primarily electrostatic at least at protein molar fractions smaller than $x_p = 10^{-3}$. It is interesting to note that this protein to lipid ratio compares well with the situation in erythrocytes which contain about $2 \times 10^5$ spectrin dimers (Haest, 1982) and about $2 \times 10^{-5}$ mg of lipid in the inner monolayer (corresponding to $x_p \approx 10^{-3}$).

Concerning the interaction of spectrin with the zwitterionic lipids, the important results are as follows: (1) spectrin does not affect the thermal expansion coefficient or the phase transition of DMPC to a remarkable extent, and (2) the protein does not change the slope of the transition of the DMPE monolayer remarkably. From these findings, we conclude that spectrin does not interact appreciably with lipid layers via the hydrophobic effect or by dipolar interaction with the lipid/water interface.

In contrast, Mombers et al. (1977) found a reduction in the heat of transition of negatively charged lipid layers and concluded that the hydrophobic effect also plays an essential role for the coupling of spectrin to lipid layers. However, this reduction could also be due to the suppression of the transition of the bound lipid as was observed in our densitometric measurements. In addition, those experiments were performed with an actin-containing spectrin fraction. The pronounced spectrin-induced hysteresis of the transition of pure DMPS and DMPS/DMPE mixed monolayers also points to a strong electrostatic binding. Further evidence is provided by the finding that $Ca^{2+}$ displaces spectrin from phosphatidylserine-containing membranes (Mombers et al., 1980) and that the hysteresis of the monolayer transition is less pronounced in the presence of $Ca^{2+}$ (R. Maksymiw, unpublished results).

Bonnet and Begard (1984) concluded from their elegant fluorescence studies that at least the anilinonaphthyl groups of the labeled spectrin penetrate bilayers of egg yolk phosphatidylserine which appears to confirm the conclusion of Mombers et al. (1977). However, since these experiments were performed with sonicated vesicles of pure PS, a destabilization of the bilayers cannot be excluded as is also suggested by the result of Figure 2b. On the other hand, it cannot be excluded by our experiments that the spectrin heterodimers are denatured after adsorption to charged bilayers in such a way that they penetrate into the hydrophobic region.

According to Ungewickell and Gratzer (1978), the dimer–

tetramer equilibrium is a function of temperature, and the question arises whether this affects our interpretation of our data. At the ionic strength of our buffer, the association constant is about $10^5$ $M^{-1}$. Our total spectrin concentration is $10^{-6}$ M; that is, the tetramer to dimer ratio is very small (about 0.1). In addition, the densitometric curves have been corrected for the density of the pure spectrin solution. We therefore rule out the possibility that the equilibrium affects our phase diagram.

The total pressure of charged lipid layers is composed of the intrinsic pressure of the hydrocarbon chains, $\pi_{hc}$, and the repulsive electrostatic pressure, $\pi_{el}$, due to the Coulomb repulsion of the head groups. The adsorption of spectrin leads to a reduction of the electrostatic pressure, so that the transition occurs at a lower pressure, $\pi_m$*.

The order of magnitude of the binding energy ($W_s$) of one spectrin molecule to the lipid layer can be estimated according to

$$W_s = \Delta\pi_s A_s \tag{1}$$

where $A_s$ is the area covered by the protein and $\Delta\pi_s$ is the shift of the transition pressure ($\Delta\pi_s = \pi_m - \pi_m$*; cf. Figure 6). $A_s$ may be estimated from the densitometric curve of Figure 2 which shows that one spectrin molecule interacts with about $10^4$ lipid molecules. Since the area per lipid molecule is $A_L \approx 50$ Å$^2$, it follows $A_s \approx 5 \times 10^5$ Å$^2$. For $\Delta\pi_s \approx 5$ mN·m$^{-1}$, one obtains a binding energy of $W_s = 2 \times 10^{-17}$ J/molecule (or $10^6$ J/mol) of spectrin. The interaction energy per mole of DMPS is then $W_L = 10^{-4} W_s \approx 1$ kJ $M^{-1}$.

Spectrin carries a high negative excess charge. According to Speicher et al. (1983), about 20% of the amino acids (or 800 per dimer) are negatively charged, and 13% are positively (or 500 residues per dimer) charged. In view of this high negative excess charge, the strong binding to DMPS is quite astonishing. There are several possible explanations for this finding: First, binding is mediated by $Ca^{2+}$ bridges. Second, the rather high negative charge density of the filaments (about one-third excess elementary charges per angstrom) could cause a condensation of positive counterions on the filament as is predicted by theory (Manning, 1969). This could lead to a shielding of most of the negative excess charges. Third, a net attractive interaction could result if the protein is folded in such a way that the (unshielded) positive charges are in closer contact with the lipid/water interface than the (unshielded) negative charges. The first possibility can be ruled out since the bivalent ions ($Ca^{2+}$, $Mg^{2+}$) present as impurities were bound by EDTA and since addition of $Ca^{2+}$ reduces the strength of spectrin/membrane coupling (Mombers et al., 1980). We propose the third possibility as the most likely explanation. Because of the exponential decay of the membrane surface potential, an attractive force between spectrin and the negative membrane surface arises if the negative charges are only some 5 Å further away from the surface than the cationic side groups. This can be easily realized since the diameter of the spectrin filament is 28 Å.

*Modification of the Lipid Phase Diagram and Determination of the Selectivity and Energy of the Lipid/Protein Interaction.* In the following, evidence is provided that information about the selectivity and the strength of the lipid/protein interaction can be obtained by analyzing the shifts in the phase boundaries caused by protein binding. For that purpose, the phase diagram of the lipid mixture is first simulated on the basis of the regular solution theory. Then the temperature shifts in the solidus and the liquidus lines are calculated for different values of the free energy of the lipid/protein interaction as a function both of the composition of the lipid mixture and of the protein concentration.

The liquidus and the solidus lines of the binary lipid mixtures itself can be calculated by the well-known standard procedures of the regular solution theory. There exist excellent review articles in the metallurgical literature such as the article by Pelton (1983). Lipid phase diagrams were discussed previously by Lee (1978) on the basis of the athermal solution theory. Denote the fluid $L_\alpha$ phase by $\alpha$ and the solid $L_\beta$ phase by $\beta$. Then $x_1^\alpha$ and $x_2^\alpha$ are the molar fractions of the two components on the liquidus line and $x_1^\beta$ and $x_2^\beta$ the corresponding values along the solidus line so that $x_1^\alpha + x_2^\alpha = 1$ and $x_1^\beta + x_2^\beta = 1$. The transition temperatures of the two components are designated $T_{m_1}$ and $T_{m_2}$, the standard molar enthalpies of fusion (that is, of the $L_\beta \rightarrow L_\alpha$ transition) are $\Delta h_1$ and $\Delta h_2$, and the standard molar entropies of fusion are denoted $\Delta s_1$ and $\Delta s_2$. The Gibbs free energies of the $L_\beta \rightarrow L_\alpha$ transition are then (for $i = 1, 2$)

$$\Delta g^m{}_i = \Delta h_i - T\Delta s_i \tag{2}$$

if $\Delta h_i$ and $\Delta s_i$ are independent of $T$. Since $\Delta s_i = T_{m_i}\Delta h_i$, $\Delta g^m{}_i$ can be expressed as a function of temperature according to

$$\Delta g^m{}_i = \Delta h_i(1 - T/T_{m_i}) \tag{3}$$

It is helpful to remember that $\Delta g^m{}_i$ is equal to the difference between the Gibbs free energies of the pure components in the $\alpha$ and $\beta$ phases: $\Delta g^m{}_i = g_i^\alpha - g_i^\beta$.

In the approximation of the regular solution theory, the deviation from ideality is taken into account by introducing an excess molar enthalpy, $H_{ex}$, whereas the entropy of mixing is assumed to remain ideal. The excess molar Gibbs free energy in the first approximation is given by

$$G_{ex}{}^{\alpha,\beta} = H_{ex}{}^{\alpha,\beta} = W^{\alpha,\beta} x_1 x_2 \tag{4}$$

This approximation appears to hold well for lipid mixtures since the molar areas of the different phospholipid molecules are similar. As is well-known from metallurgy, the thermodynamic properties of many binary alloys can be adequately described by the regular solution theory. This holds especially for the calculation of the boundaries of the fluid/solid coexistence. The partial excess Gibbs free energies as obtained from the expression for $G_{ex}$ in eq 4 are [cf. eq 20 of Pelton (1983)]

$$g_{ex,1}{}^{\alpha,\beta} = W^{\alpha,\beta} x_2{}^2 \qquad g_{ex,2}{}^{\alpha,\beta} = W^{\alpha,\beta} x_1{}^2$$

Following Pelton (1983), the positions of the liquidus ($x_1^\alpha$, $x_2^\alpha$) and the solidus ($x_1^\beta$, $x_2^\beta$) are obtained by solving the following set of (nonlinear) equations:

$$RT \ln\left(\frac{x_1{}^\alpha}{x_1{}^\beta}\right) + \Delta g^m{}_1 + g_{ex,1}{}^\alpha - g_{ex,1}{}^\beta = 0 \tag{5a}$$

$$RT \ln\left(\frac{x_2{}^\alpha}{x_2{}^\beta}\right) + \Delta g^m{}_2 + g_{ex,2}{}^\alpha - g_{ex,2}{}^\beta = 0 \tag{5b}$$

With the use of this procedure, the phase boundaries of the DMPC/DMPS mixture were simulated for the heats of transitions and the transition temperatures given in the figure caption of Figure 1 by varying the values of $W^\alpha$ and $W^\beta$ until best fit was achieved.

This was obtained for

$$W^\alpha = 4.0 \text{ kJ } M^{-1} \qquad W^\beta = 4.6 \text{ kJ } M^{-1}$$

The phase boundaries respond very sensitively to small changes in $W^\alpha$ and $W^\beta$, and the two parameters can be determined to an accuracy of better than 10%. In particular, it must be $W^\alpha$

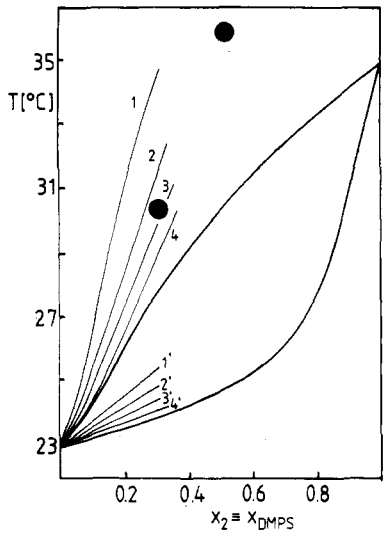


FIGURE 8: Variation of liquidus and solidus lines of DMPC/DMPS mixed bilayers caused by spectrin adsorption as calculated from eq 5a,b for various values of the excess free energy defined in eq 8 (in units of kilojoules). These values are for (1) $\Delta g_{ex,2} = +1.2$, (2) $\Delta g_{ex,2} = +0.4$, (3) $\Delta g_{ex,2} = +0.2$, and (4) $\Delta g_{ex,2} = +0.1$. The thick points give the positions of the liquidus line as obtained from Figure 4.

$< W^{\beta}$ in order to account for the asymmetry of the solidus line.

In order to treat the problem of the modification of the lipid phase diagram caused by the interaction of integral or adsorbed proteins with the lipid layer, it is important to consider the limit of very low protein concentrations (protein molar fractions $x_p \leq 10^{-3}$). In this case, the effect of protein binding can be treated as a perturbation, and the molar fractions of the lipids remain essentially unchanged. The perturbation can be accounted for by introducing an additional excess free energy, $G_{ex}$, which is to a first approximation given by

$$G_{ex} = Qx_p x_2 \tag{6}$$

where $x_p$ is the protein molar fraction with respect to the lipid and $x_2$ the molar fraction of DMPS.

The partial Gibbs free energies for the $\alpha$ and $\beta$ phase, corresponding to eq 6 are

$$g_{p,ex}{}^{\alpha,\beta} = 0 \qquad g_{2,ex}{}^{\alpha,\beta} = Q^{\alpha,\beta}x_p \tag{7}$$

that is, only the second equation (5b) for the determination of the phase boundaries is changed. Formally, the introduction of the selective spectrin/DMPS interaction corresponds to a shift of the chain melting transition to higher temperatures. By comparing experimentally observed shifts of the phase boundaries with the corresponding values calculated by the above procedure, one obtains the partial free interaction energy difference between the fluid ($\alpha$) and the solid ($\beta$) phase.

$$\Delta g_{2,ex} = (Q^{\alpha} - Q^{\beta})x_p \tag{8}$$

Figure 8 shows the changes of the phase diagram as calculated for different values of the partial free energy $\Delta g_{2,ex}$ (in units of kilojoules) for the regime of low DMPS molar fractions. It is seen that only positive values of $\Delta g_{2,ex}$ are in agreement with the experiment which means that the binding energy of spectrin is higher in the solid state (since $Q < 0$).

Comparison of the theoretical and experimental shifts in Figure 8 yields a value of $\Delta g_{2,ex} = +0.2$ kJ for $x_2 = 0.3$, corresponding to 0.6 kJ/mol of lipid. This is a measure for the difference in the free lipid/protein interaction energy (per mole of lipid) between the $L_\alpha$ and $L_\beta$ phases. However, it is expected to be of the same order of magnitude as the interaction energy, for instance, in the fluid $L_\alpha$ phase. It thus follows that the binding energy per mole of DMPS is about 0.6 kJ $M^{-1}$, in good agreement with the value estimated from the monolayer experiment.

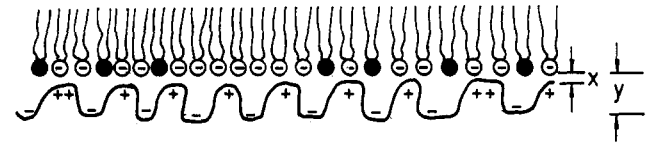


FIGURE 9: Schematic view of folded spectrin molecule attached to the inner monolayer of the membrane by electrostatic forces.

The evaluation of the phase boundary shifts provides information about the selectivity of the lipid/protein interaction in lipid mixtures and yields values of the protein binding energy difference between the two lipid phases. The latter value could be determined more easily by measuring the shift of the phase transition of the pure compound. However, in cases where the stability of the pure bilayer is affected by protein binding such as is the case for the DMPS/spectrin system, the present method is much more reliable.

## CONCLUSION

The present work provides evidence for a second mode of coupling of spectrin to the inner leaflet of the erythrocyte membrane, namely, a direct electrostatic coupling to the lipid bilayer.

On the other side, there is ample experimental evidence that spectrin is a highly flexible molecule [cf. Stokke et al. (1986a,b)]. Moreover, quasi-elastic light-scattering experiments indicate that the spectrin dimer exhibits a globular form with a diameter of 330 nm (Ch. Schmidt, unpublished results). Lemaigre-Dubreuil and Cassoly (1983) found that binding of spectrin to membranes does not affect the mobility of local segments. This strongly suggests that the adsorbed filaments are folded (cf. Figure 9) in the same way as synthetic macromolecules adsorbed to surfaces (de Gennes, 1983). Because of this high flexibility, the spectrin dimers behave as entropic springs. On the basis of this assumption, Stokke et al. (1986a,b) developed a highly intriguing new model of the mechanochemical properties of the erythrocyte membrane. Moreover, they showed that their model of the plasma membrane as an ionic gel (elastomer) can explain the shape transformations of the red blood cell.

The present work suggests that the coupled system of the lipid/protein bilayer and the spectrin/actin meshwork together forms a two-dimensional gel where the lipid plays the role of the solvent (together with the cytoplasm) which can control the swelling properties of the gel.

In the model of Stokke et al. (1986a,b) the membrane bending resistance plays only a role for the smoothing of a cell shape favored by the osmotic tension of the spectrin gel. On the other hand, recent theoretical (Svetina & Zeks, 1983) and model membrane studies (Sackmann et al., 1986) provide evidence that the shape transformations can be understood in terms of the bending elastic properties of the membranes alone and that at a given volume and surface the shape is determined by the chemical-induced bending moment (Evans, 1974) or spontaneous curvature. The coupling of spectrin to the lipid bilayer moiety of the membrane could thus play a decisive role for the ground-state cell shape. This effect could also account for the modulation of cell shapes by variation of the composition of the lipid bilayer which led to the bilayer coupling hypothesis (Sheetz & Singer, 1974).

A further role of the electrostatic interaction between spectrin and the lipid bilayer could be to maintain the asymmetric distribution of phosphatidylserine between the two

monolayers (Haest et al., 1978). The reports concerning the role of spectrin for the maintenance of this asymmetry are still conflicting. While Williamson et al. (1982) conclude from fluorescent probe experiments that an alteration or removal of spectrin leads to a randomization of the lipid asymmetry, Raval and Allan (1984) could not confirm this effect. Seigneuret and Devaux (1984) postulated the necessity of an ATP-driven lipid transport system for the maintenance of lipid asymmetry. Since the binding energy per mole of DMPS (0.6 $kJ \cdot M^{-1}$) is smaller than the thermal energy at room temperature, the spectrin/DMPS interaction cannot account alone for the lipid asymmetry. It is well possible that the membrane potential plays a more important role.

**Registry No.** Dimyristoylphosphatidylserine, 64023-32-1.

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