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^{2}H-NMR investigation of DMPC/glycophorin bilayers

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Abstract

Deuterium nuclear magnetic resonance spectroscopy was used to investigate the phase equilibria, and the temperature and concentration dependences of the phospholipid hydrocarbon chain order, of mixtures of glycophorin in dimyristoylphosphatidylcholine. In the fluid phase it is found that the protein has only a slight effect on the first moment of the ^{2}H spectrum, which for perdeuterated chains is a direct measure of the average chain orientational order. However, analysis of the rate of change of the first moment with respect to protein concentration, at different temperatures within the fluid phase, shows that at a molar protein concentration of about 0.0295 ± 0.01, the lipid chain order (or M_1) is essentially independent of temperature. At this concentration the chain order is determined by the lipid's interaction with the protein and one can conclude that about 34 (± 12) lipids are required to solvate the protein. At higher lipid concentrations these lipids are freely exchanging, on the NMR time scale, with the other lipids in the bilayer. At glycophorin concentrations below about 1 mol% there is a two-phase coexistence region at temperatures below the pure lipid's chain melting transition. The boundary between the fluid phase and this two-phase region curves downwards (is concave downwards), whereas the boundary between the two-phase region and the gel phase, while naturally occurring at lower temperatures than the upper boundary, is concave upwards. As a consequence the protein partitions preferentially into the fluid phase. This behaviour is similar to that observed in a number of other protein/lipid and peptide/lipid mixtures where it was suggested that those systems may have been close to a critical mixing point and some characteristics of a continuous phase change were noted. Indeed, at glycophorin concentrations near and above 1 mol% there are indications that the phase behaviour becomes more complex, suggesting the presence of significant protein/protein interactions and that this system may be close to a critical point.

Key words: NMR, ^{2}H-; Phase equilibrium; Glycophorin; Lipid–protein interaction

1. Introduction

The interaction of integral membrane proteins with lipids in biological membranes has been under active investigation for over 20 years. Many important functional properties of membrane proteins, including transport, catalysis and ligand binding, are profoundly influenced by the physical properties of the host lipid matrix. Conversely, the phase behaviour and orientational order of the lipid acyl chains may be altered by their interactions with the membrane-spanning regions of integral proteins. In recent years, the experimental tools available to study lipid–protein interactions have grown increasingly more sophisticated, and include techniques such as differential scanning calorimetry (DSC) [1,2], electron paramagnetic resonance (EPR) [3,4], Fourier transform infrared (FTIR) [5,6], and ^{2}H nuclear magnetic resonance (NMR) [7–9] spectroscopy. The use of reconstituted systems consisting of hydrophobic membrane-spanning peptides [10], gramicidin D [1], and integral membrane proteins, such as cytochrome oxidase [11], and Band 3 [12], has allowed a detailed dissection of the consequences of reciprocal lipid–protein interactions.

Abbreviations: NMR, nuclear magnetic resonance; DSC, differential scanning calorimetry; EPR, electron paramagnetic resonance; FTIR, Fourier transform infrared spectroscopy; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPC-d_{54}, 1,2-bis(perdeuteriomyristoyl)-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

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Glycophorin A, a 31 kDa (131 amino acid) sialoglycoprotein of the human erythrocyte membrane, is one of the best characterized integral proteins (for a review, see [13]). It has three distinct structural domains. The N-terminal extracellular domain is highly hydrophilic, and carries 15 *O*-glycosidic oligosaccharide chains, linked to serine and threonine residues, and a single complex *N*-glycosidic oligosaccharide, linked to an asparagine residue. The membrane domain consists of a single transmembrane α-helix, made up of 23 hydrophobic amino acid residues. The C-terminal intracellular domain is rich in charged amino acids, and interacts with the spectrin-actin network of the cytoskeleton via the linker protein Band 4.1.

Although the functions of glycophorin A remain elusive, the glycosylated extracellular domain appears to play a dominant role (for a review, see [14]). The sialylated oligosaccharides in this domain are responsible for the high surface negative charge of the erythrocyte, which prevents unwanted adhesion to other cells and tissues. The oligosaccharides also serve as receptors for parasites, enveloped viruses, and bacteria. In addition, glycophorin A appears to play a structural role in the maintenance of red blood cell shape, via its interaction with the cytoskeleton [15]. The glycosylated region of glycophorin A may also be involved in this process, since membrane deformability arising from such interactions can be modulated by binding of specific ligands to this domain [16]. It has proved possible to visualize glycophorin A directly using rotary shadowing and transmission electron microscopy [17]. The external domain appeared as a cloud-like structure, which was reduced in size after removal of sialic acid. In this respect, it is interesting that other researchers have suggested that the extracellular domain of glycophorin A may exist in two conformations, each of which interacts to a different extent with membrane lipids [18].

Glycophorin A thus provides an interesting and unique model for studies of lipid–protein interactions, and has the additional advantage that it can be purified in milligram quantities and readily reconstituted into defined lipid bilayers. In the present work we report the results of our study of phospholipid hydrocarbon chain orientational order and phase equilibria of glycophorin/1,2-bis(perdeuteriomyristoyl)-*sn*-glycero-3-phosphocholine (DMPC-d_{54}) mixtures. We find that glycophorin has only a small effect on phospholipid order in the fluid bilayer phase, and that there is an extensive two-phase coexistence region at temperatures below the phase transition of the pure lipid. A tentative phase diagram, up to glycophorin concentrations of approximately 1 mol%, is presented and we discuss the implications of the two state conformational model of the external domain of glycophorin. However, our results also raise the question of whether this protein/lipid mixture may be close to a critical mixing point, as has been suggested for mixtures of gramicidin D/DMPC [1] and of synthetic amphiphilic peptides in DPPC [10,19].

2. Materials and methods

2.1. Preparation of perdeuterated phospholipid

The phospholipid DMPC-d_{54} was synthesized in our laboratory following the procedure described by Gupta et al. [20].

2.2. Isolation of glycophorin

Human red blood cell ghosts were prepared from outdated blood bank blood according to the procedure of Dodge et al. [21]. Glycophorin was purified from ghosts by the method of Segrest et al. [22]. Briefly, ghosts were solubilized using sodium deoxycholate, followed by phenol extraction, ethanol precipitation, and subsequent removal of residual detergent on a Sephadex G-150 gel filtration column. Purified glycophorin was dialysed overnight against water, lyophilized, and stored desiccated at −20°C. The final product ran as a homogeneous glycophorin A dimer on SDS-PAGE, using periodic acid-Schiff and Coomassie blue staining procedures.

2.3. Reconstitution of glycophorin into DMPC liposomes

Large multilamellar liposomes containing reconstituted glycophorin were prepared by a modification of the procedure of Ketis and Grant [23]. The desired amount of lipid (10–40 mg) was dissolved in 5 ml of 2-chloroethanol (Aldrich Chemical Co., Milwaukee, WI, USA) in a 100 ml round-bottomed flask. An equal volume of water containing the appropriate amount of glycophorin (5–20 mg) was added dropwise to the lipid solution, and the mixture was stirred gently for 5 min. The solvent was removed on a rotary evaporator at 37°C, to produce a lipid–glycoprotein film on the inside of the flask. The film was dried under vacuum, and the sample hydrated overnight at 42°C with 2 ml of Hepes-buffered saline (20 mM Hepes/0.15 M NaCl, pH 7.4, containing 0.2 g/l sodium azide) in a rotating shaker bath at 120 rpm. Large multilamellar liposomes were separated from smaller vesicles and soluble glycophorin aggregates by differential centrifugation at $15000 \times g$ for 5 min. The liposome pellet was resuspended in 1 ml of Hepes-buffered saline and used directly for NMR experiments.

The amount of reconstituted glycophorin in the liposomes was determined by measurement of sialic acid, as described by Massamiri et al. [24], using both *N*-

acetylneuraminic acid (Sigma Chemical Co., St. Louis, MO, USA) and pure lyophilized glycophorin as standards. Lipid recovery was monitored by the inclusion of tracer amounts of radiolabelled material ([^{14}C]DPPC, 117 mCi/mmol; Amersham Canada, Mississauga, Ont., Canada). After centrifugation, the supernatant and resuspended liposome pellets were individually analyzed for sialic acid content and ^{14}C radioactivity, and the lipid:protein ratio of the liposomes, and the overall recovery of lipid and glycophorin, were estimated.

2.4. ^{2}H-NMR experiments

A typical NMR sample contained about 25 mg of DMPC-d_{54}, dispersed in a total volume of 300–400 μl of 20 mM Hepes-buffered saline. The NMR experiments typically took from 3 to 4 days for each sample. Each sample was initially equilibrated at 40.0°C. The temperature was lowered in 2°C steps until 22.0°C. Then the spectra were observed in 1°C steps until 10.0°C. After that the two degree step was used again. The ^{2}H-NMR spectra were all acquired on a home-built spectrometer operating at 55.26 MHz, corresponding to a magnetic field of 8.5 T. In all cases, the quadrupolar echo technique was employed [24]. The 90° pulses used in the quadrupolar echo sequences were always 3.0 μs, while the repetition time was 0.9 s and the interpulse spacing was 35.0 μs. Typically, 2000 scans were collected at temperatures above 22°C, and 4000 scans were used at temperatures below 22°C. The complex free induction decay (FID) signals were processed with a modified version of the program FT-NMR (Hare Research, Woodinville, WA, USA). The spectra were symmetrized by the method of Davis [7]. Details of the moment calculation are also described in [7].

3. Results

Glycophorin was reconstituted into large multilamellar structures of DMPC-d_{54} by rehydration of a lipid–protein film dried down from a 2-chloroethanol solution. Previous work in our laboratory [26], and by others [27], has shown that incorporation of glycophorin into lipid bilayers by a variety of reconstitution procedures is incomplete. In the case of detergent dialysis, for example, recoveries of glycophorin in the resulting vesicles were very poor (between 3% and 28%, depending on the protein mole fraction) [26,27]. The excess glycophorin is present in the form of water-soluble aggregates of about 52 nm diameter, each consisting of 16–18 protein molecules, as determined by quasi-elastic light scattering and gel filtration chromatography [26]. Failure to remove such structures will result in an incorrect value for the protein mole fraction, and possible errors in interpretation of NMR spectra, or of the results from other experiments such as DSC or EPR. Accordingly, in the present study, reconstituted glycophorin liposomes were carefully separated from soluble aggregates before determination of the recovery of lipid and glycophorin for each sample, and recording of ^{2}H-NMR spectra. The 2-chloroethanol method used for reconstitution led to consistently high recoveries of both phospholipid (56–90%, depending on the initial lipid:protein ratio) and glycophorin (60–84%, depending on the initial lipid:protein ratio). This reconstitution method has been used extensively for lectin binding studies of human erythrocyte glycophorin and Band 3 [23,28], which showed that both glycoproteins retained their receptor function after reconstitution, and were thus likely to be reconstituted in a conformation similar to that existing in intact cells. Glycophorin was observed by electron and fluorescence microscopy to disperse fairly diffusively in the large liposomes, whereas Band 3 tended to aggregate, and was distributed in a highly heterogeneous fashion through the liposome population [28].

The transition temperature from gel phase to liquid-crystalline (fluid) phase for pure DMPC in multilamellar form with excess water or neutral buffer is about 24°C [29]. For DMPC-d_{54} in excess water or neutral buffer, this transition occurs at about 19°C. Fig. 1 shows typical ^{2}H-NMR spectra of DMPC-d_{54} in fluid and gel phases. The fluid phase spectrum (Fig. 1a) covers a frequency range of approximately $\pm$32 kHz about the ^{2}H Larmor frequency while the gel phase spectrum (Fig. 1b) extends out to about $\pm$62 kHz. The flexibility gradient of the hydrocarbon chain results in

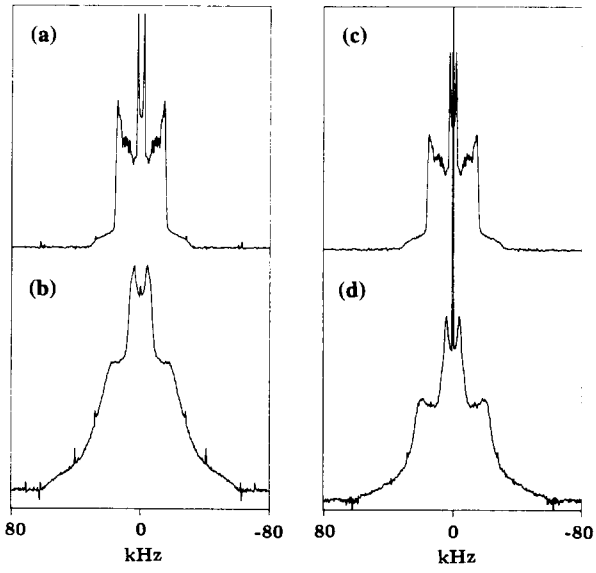

Fig. 1. Comparison of ^{2}H-NMR spectra of DMPC-d_{54} bilayers prepared by mixing lipid in excess buffer (a,b) and by the reconstitution method (c,d). (a) and (c) are at 24°C, within the fluid phase; (b) and (d) are at 17°C, within the gel phase.

different quadrupolar splittings for different chain positions in the fluid phase, the deuterons located near the center of the bilayer having smaller quadrupolar splittings. The ^{2}H-NMR spectra in both fluid and gel phases of DMPC-d_{54} liposomes prepared by the reconstitution method, Fig. 1c and d, are very similar to the spectra of a multilamellar sample formed simply by gently mixing lipids and buffer, shown in Fig. 1a and b.

Spectral moment analysis as applied to ^{2}H-NMR spectra is considered in detail by Davis [7]. The nth moment, M_n, of the spectrum $f(\omega)$ is defined by

$$M_n = \frac{\int_0^\infty d\omega \omega^n f(\omega)}{\int_0^\infty d\omega f(\omega)} \quad (1)$$

Only positive frequencies are considered in order to obtain non-zero even and odd moments from the symmetric ^{2}H spectra. The various moments provide information about the degree and type of orientational averaging in a multiply-labeled system. For example, the first moment is proportional to the average quadrupolar splitting, given by:

$$M_1 \propto \langle S_{CD} \rangle \quad (2)$$

where

$$S_{CD} = \tfrac{1}{2}\overline{(3\cos^2\theta_{CD} - 1)} \quad (3)$$

where θ_{CD} is the angle between the C-D bond and the bilayer normal, and the average is over motions that are fast on the ^{2}H-NMR time scale ($\sim 10^{-6}$ to 10^{-3} s).

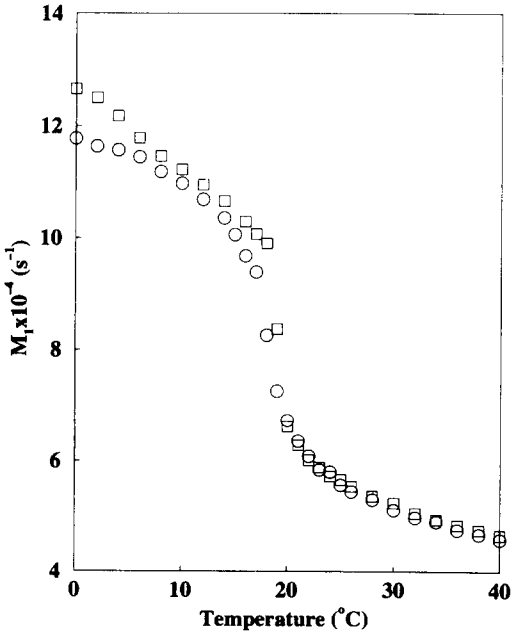

Fig. 2. Temperature dependence of the first moment of the ^{2}H-NMR spectrum from (□) DMPC-d_{54} in excess buffer and (○) DMPC-d_{54} in excess buffer prepared by the reconstitution method.

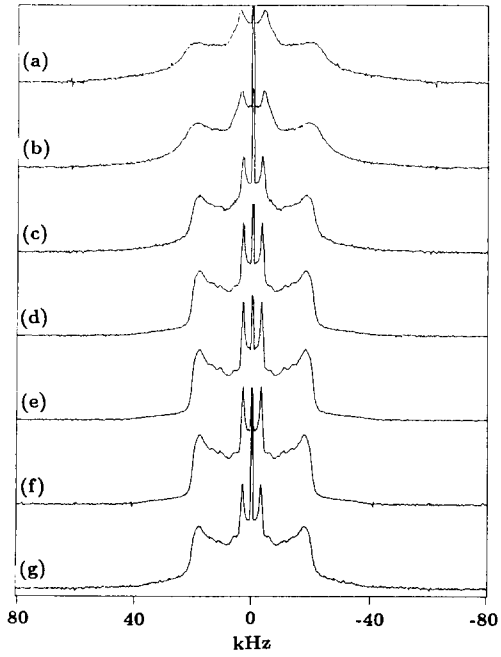

Fig. 3. Concentration dependence of the ^{2}H-NMR spectra of reconstituted DMPC-d_{54}/glycophorin at 17°C. (a) pure DMPC-d_{54}, x_p = 0.0, (b) x_p = 0.0034, (c) x_p = 0.0041, (d) x_p = 0.0065, (e) x_p = 0.0071, (f) x_p = 0.0082, and (g) x_p = 0.0092.

The temperature dependence of the average quadrupolar splitting of the ^{2}H-NMR spectrum, or its first moment M_1, for the two lipid/buffer mixtures formed in these different ways is plotted in Fig. 2. For T = 20°C or above, the M_1 values for both samples are nearly identical. On the other hand, the M_1 values for the multilamellar DMPC-d_{54} sample show a dramatic increase in the phase transition region (from 20 to 18°C) and then a gradual increase of about 8% from 6 to 0°C, probably due to the 'sub-transition' [30]. The M_1 values for the reconstituted DMPC-d_{54} show a more gradual change over the temperature range from 20 to 17°C. It should be noted that the phase transition at about 18°C for the reconstituted DMPC-d_{54} is one degree wider than the transition temperature of the multilamellar DMPC-d_{54}.

Fig. 3 shows the dependence of the ^{2}H-NMR spectra at 17°C on glycophorin concentration. At this temperature, the pure reconstituted DMPC-d_{54} sample is entirely in the gel phase. The sample with DMPC-d_{54} : glycophorin weight ratio of 5.7 : 1 (molar concentration x_p = 0.0041, Fig. 3c) is in a two-phase gel-fluid coexistence region at this temperature. This spectrum has features which are characteristic of both fluid and gel phases, the sharp methyl group resonance near the centre of the spectrum, which is characteristic of the fluid phase, and the broad gently sloping wings, at frequencies beyond about ±30 kHz, typical of the gel phase. This sample is predominantly in the gel phase, but a small fraction of it is in fluid domains. At a

DMPC-d_{54} : glycophorin ratio of 3.6 : 1 ($x_p = 0.0065$, Fig. 3d), the spectrum indicates that the fraction of the sample which is in the fluid phase is considerably larger than it was at $x_p = 0.0041$. This reflects the fact that this sample is located more deeply within the two-phase region; i.e. it is further away from the phase boundary or two-phase line separating the gel phase from the two-phase region. As the glycophorin concentration is increased, the relative area of the fluid phase component of the spectrum increases while that of the gel phase decreases. This behaviour is most clearly evident by close examination of the wings of the spectra (the region beyond about ±30 kHz).

Fig. 4 shows the effect of temperature and glycophorin concentration on the spectral first moment M_1. As expected, M_1 decreases with increasing temperature in all the samples. At the phase transition, the pure lipid DMPC-d_{54} displays a sharp discontinuity in M_1. This sharp transition is progressively smoothed out by the addition of glycophorin. Since M_1 is the average quadrupole splitting of the ^{2}H-NMR spectrum, a change in M_1 corresponds to a change in the average order parameter. Glycophorin is seen to have a strong disordering effect just below the phase transition and a weaker disordering effect just above the pure lipid transition temperature. Fig. 5 plots the first moment values, M_1, of the ^{2}H-NMR spectra vs. glycophorin concentration at several different temperatures, the error bars represent an uncertainty of ±3% in the values of M_1. At 30°C, well above the phase transition

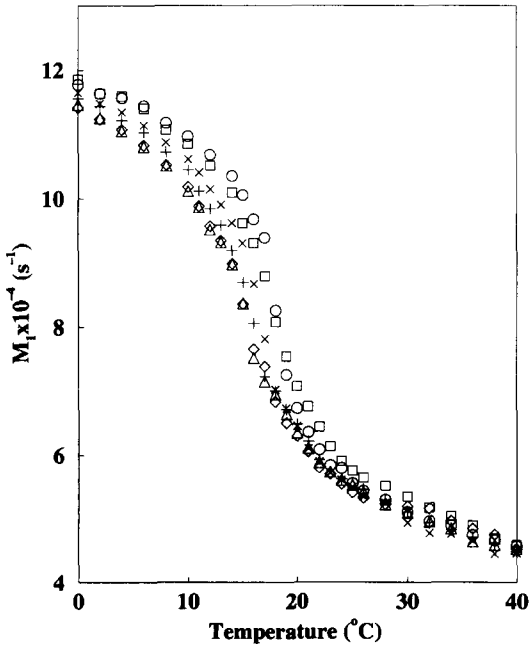

Fig. 4. Temperature dependence of the first moment of the ^{2}H-NMR spectrum at different concentrations of glycophorin; (○) pure DMPC-d_{54}, $x_p = 0.0$, (□) $x_p = 0.0034$, (×) $x_p = 0.0041$, (+) $x_p = 0.0065$, (△) $x_p = 0.0071$, (◇) $x_p = 0.0082$.

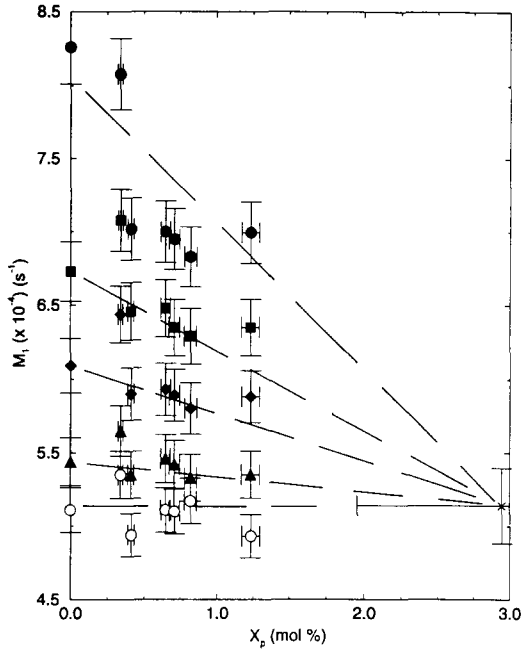

Fig. 5. Protein concentration dependence of the first moment of the ^{2}H-NMR spectrum at various temperatures. The filled circles are the experimental values of M_1 at 18°C, the squares are at 20°C, the diamonds at 22°C, the triangles at 26°C and the open circles at 30°C. The vertical error bars correspond to an uncertainty of 3% in the values of M_1. The horizontal error bars show the 5% uncertainty in sample compositions. The 'star' gives the values of x_0 and M_1^0 obtained by consideration of all the data in the temperature range from 17 to 30°C. The dashed lines are not the result of the linear least squares analysis (see Fig. 8) but are drawn to demonstrate that such an analysis is plausible.

temperature, M_1 is essentially independent of the concentration of glycophorin. At 18°C, just above the phase transition of the pure lipid, M_1 values decrease as the glycophorin concentration increases. Between these extremes the rate of change of M_1 with concentration decreases steadily as the temperature approaches 30°C. Although there is considerable scatter, and only six different glycophorin concentrations covering the range $0 \leq x_p \leq 0.082$ were studied (the data for the sample with $x_p = 1.23$ was not included in this part of the analysis, see below), M_1 varies approximately linearly with protein concentration.

The temperature dependence of the ^{2}H-NMR spectrum of the 3.6 : 1 weight ratio ($x_p = 0.0065$) DMPC-d_{54} : glycophorin sample in excess buffer is shown in Fig. 6. At higher temperatures (17°C and above), the spectra are characteristic of the fluid phase. They contains numerous sharp 90° edges, a narrow methyl group component with a splitting of only a few kHz, and a strong 'plateau' in the variation of quadrupolar splitting with chain position. As the temperature is lowered, within the fluid phase, from 40 to 17°C, there is a significant broadening of the spectrum as seen in the pure lipid (Fig. 1c). At 13°C and below, the spectra

in Fig. 6 are characteristic of the phospholipid gel phase. They have a broader methyl group powder pattern with a splitting of about 10 kHz, while the methylenes exhibit a broad bell-shaped spectrum. Between 14 and 16°C, the spectra are superpositions of two components, one from lipids in gel phase domains and the other from fluid domains. As the temperature is lowered within this two-phase region, the gel phase component of the spectrum increases at the expense of the fluid phase component. The tremendous contrast between the fluid phase spectrum and gel phase spectrum makes it easy to identify the presence, and in some cases even to quantify the amount of each phase under conditions where they coexist. By examining the ^{2}H-NMR spectra, such as those in Fig. 6, it is possible to locate the temperatures at which the phase boundaries occur for each glycophorin concentration studied. For this particular sample (Fig. 6), the boundaries, or the two-phase lines, lie between 16 and 17°C for the upper boundary or 'fluidus' and between 13 and 14°C for the lower boundary or 'solidus'. By varying the sample concentration, this technique of 'nuclear magnetic calorimetry' can be used to map out the phase boundaries in a temperature-composition plot.

Huschilt et al. [10] have demonstrated the use of spectral subtraction and the lever rule in determining the extent (in concentration terms) of two-phase coexistence at a given temperature for a synthetic amphiphilic peptide in phospholipid bilayers. Recently, by combining this method with the results of DSC, Vist and Davis [31] have determined the phase diagram of cholesterol/DPPC and Morrow, et al. [32] have determined the phase diagram of mixtures of DMPC and DSPC. Morrow et al. [12] tried to apply the technique to determine the phase diagram of a reconstituted mixture of human erythrocyte Band 3 in DMPC-d_{54}. However, due to the low signal-to-noise ratio, it was not possible to use the technique in that system. In the present work, we have applied the spectral subtraction technique to the reconstituted glycophorin/DMPC system. A brief overview of the spectral subtraction method will be given here. Details of the technique can be found in [10,31,33].

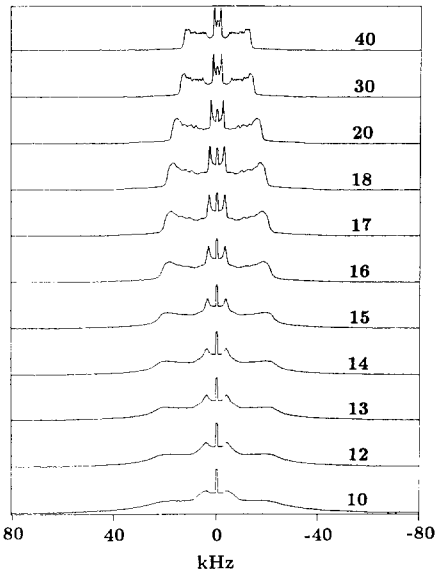

Fig. 6. Temperature dependence of the ^{2}H-NMR spectrum of DMPC-d_{54}/glycophorin liposomes at molar protein concentration $x_p = 0.0065$. The temperature, in degrees Celsius, at which each spectrum was recorded is shown on the right.

Briefly, ^{2}H-NMR spectra $F(x_A,T)$ and $F(x_B,T)$ for two samples with concentrations x_A and x_B ($>x_A$) at temperature T are both superpositions of the same end-point spectra $F_g(T)$ and $F_f(T)$. Therefore, one can subtract a fraction K of the spectrum at x_B, $F(x_B,T)$, from the spectra at x_A, $F(x_A,T)$ to give

$$F(x_A,T) - KF(x_B,T) = (1-K)F_g(T) \quad (4)$$

Similarly, one can subtract a fraction K' of the spectrum at x_A from that at x_B to give,

$$F(x_B,T) - K'F(x_A,T) = (1-K')F_f(T) \quad (5)$$

The values of K and K', where the difference spectra are either pure gel or pure fluid spectra, are used to calculate the protein concentrations at the two end-points as shown below,

$$x_g = \frac{x_A - Kx_B}{1-K}, \quad (6)$$

$$x_f = \frac{x_B - K'x_A}{1-K'} \quad (7)$$

Theoretically, spectra from any two samples, at different concentrations but the same temperature within the two-phase region, can be used to obtain end-point spectra. By performing pairwise subtractions for all possible pairs x_A and x_B, and using Eqs. (6) and (7), we can determine the weighted average value of x_f and x_g. For the ^{2}H-NMR spectra of the reconstituted DMPC-d_{54}/glycophorin system, the subtraction techniques worked well in some spectra, but not in others. For example, at 16°C, with three different concentration pairs, the values of x_f determined by these subtractions are averaged to give $\langle x_f \rangle = 0.0080 \pm 0.0002$. The quoted uncertainty is simply the spread of the end-point concentration values obtained from different pairs. The values of x_g determined by the reverse procedure leading to gel phase end-spectra are averaged in the same way, using Eq. (7), to give, at 16°C, $\langle x_g \rangle = 0.0033 \pm 0.0004$.

Varying sample composition and temperature we obtain the temperature–concentration plots for DMPC-d_{54}/glycophorin as shown in Fig. 7. The open square symbols represent the temperatures at the phase boundaries determined by inspection of the temperature sequences of spectra at each sample concentra-

tion. In determining these temperatures, as the temperature is lowered, the appearance of the broad sloping wings in the spectrum (see for example Fig. 6) signals the appearance of the gel phase and the crossing of the 'liquidus', while the disappearance of the sharp (fluid like) spectral features signals the crossing of the 'solidus'. The vertical error bars on these symbols represent the estimated uncertainty in determining the boundaries from a series of spectra taken at 1 C° intervals, while the horizontal error bars are the uncertainties in the sample concentrations. The difference end-point spectra for some 'workable' pairwise combinations as a function of temperature are used to determine the end-point concentrations. The open circle symbols in the figure are the values of x_f and x_g, obtained by spectral subtraction at temperatures for which there were more than two sample concentrations within the two-phase region. The horizontal error bars on the symbols are the uncertainty in the values of x_f and x_g. There were, however, a number of temperatures and concentrations where the subtraction procedure failed to give reliable end point spectra. This can occur if the exchange rate of lipids between domains of gel and fluid phases is rapid on the ^{2}H-NMR timescale (typically of the order of 10–100 μs for these spectra). Another possible difficulty with the subtraction technique arises if the system is near a critical point. In this case the distinction between the two phases in 'coexistence' becomes less clear as the critical point is approached. This sort of behaviour has been previously suggested for similar model membrane systems [1,10,19,34]. The boundaries shown as the solid curves in Fig. 7 are sketched to include all data with the exception of those for the sample with concentration $x_p = 0.0123$, while the dashed curve is drawn in a fashion to include the results from that sample (see discussion below). As shown by this figure, the protein tends to stabilize the fluid phase so that the region of two-phase coexistence occurs below the pure lipid transition temperature. This is in sharp contrast to the behaviour of DMPC-d_{54}/human erythrocyte Band 3 [12], but is very similar to the behaviour of DPPC/cytochrome-*c* oxidase [11], DMPC/rhodopsin [35], DPPC/gramicidin [1], and synthetic peptides [10], where the regions of two-phase coexistence also lie below the lipid phase transition temperature.

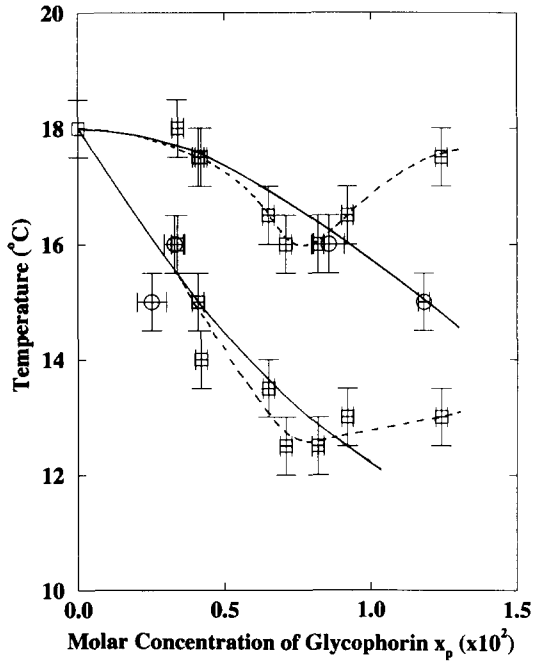

Fig. 7. Phase diagram of the DMPC-d_{54}/glycophorin system. The data points indicated by open square symbols were obtained by visual inspection of a series of spectra of the type shown in Fig. 6. In this case, the horizontal error bars represent the uncertainties in sample concentrations and the vertical error bars the uncertainty in the temperature at which two components can be identified in the spectra. The data points indicated by the open circle symbols were obtained by spectral subtraction, and represent the average values of the end-point concentrations. The horizontal error bars represent the uncertainties in these values. The solid lines are drawn to indicate the tentative two phase boundaries obtained by omitting the sample containing 1.23 mol% glycophorin. The dashed curves, which include the results for this sample, are suggestive of the data of Ruppel et al. [18]. The inflection in the dashed curves may also be suggestive of an azeotropic point near 0.8 mol% glycophorin.

4. Discussion

The phase behavior of the DMPC/glycophorin system is very similar to the behavior found in other systems such as DPPC/cytochrome-*c* oxidase [11], DMPC/rhodopsin [35], DPPC/synthetic peptide [10], and DPPC/gramicidin [1]. For example, for the DPPC/cytochrome-*c* oxidase system, Paddy et al. [11] found no dependence of M_1 on the protein concentration at temperatures well above the phase transition of the pure lipid. Paddy et al. also observed that near the gel to fluid transition, M_1 decreased linearly with increasing protein concentration, in a manner similar to the results shown in Fig. 5. In their case, plots of M_1 versus the protein-to-lipid weight ratio, in this region, were found to intersect at a single value of the weight ratio. They interpreted this as the protein concentration at which there remained no 'free lipid'. Since the first moment is proportional to the average quadrupolar splitting, the change in M_1 reflects the change in the average order parameter of the lipid acyl chains (see Eq. (2)). At temperatures above the phase transition, addition of protein decreased slightly the values of the first moment, i.e. the protein had only a small effect on the average order parameter of the lipid chains in the fluid phase. At low temperatures where the system was probably in a two-phase coexistence region, the first moment decreased as the protein con-

centration was increased. Our results show that the DMPC/glycophorin system behaves in a qualitatively similar fashion.

For DMPC/glycophorin, at temperatures where the entire sample is in the gel phase the first moment is weakly dependent on both temperature and protein concentration. As we cross the two phase region from below, M_1 becomes much more sensitive. Here, the predominant change in M_1 is due to the melting of the gel phase (protein-poor) domains, the small changes in order of the fluid (protein-rich) domains can only be detected in plots like that of Fig. 5, or, more directly, by looking at the end-point spectra obtained by difference spectroscopy. Above the transition temperature for the pure lipid, the plots of M_1 vs. x_p clearly show that, in fluid domains, the temperature dependence of lipid chain order is reduced as the protein concentration is increased.

The slope of M_1 vs. x_p, obtained from a least squares fit at each temperature, is plotted as a function of temperature in Fig. 8. While the M_1 data for the sample with concentration $x_p = 1.23$ mol% is roughly consistent with those for the other concentrations (see Fig. 5), the data for this sample were not used in determining these slopes. The uncertainties in the slopes, determined in the least squares analysis, are given by the error bars in the figure. As the phase transition is approached from above, the magnitude of the slope, which is nominally zero at temperatures above 30°C, increases dramatically, reaching its maximum value within the two phase coexistence region and then dropping back towards zero at lower temperatures. The data for temperatures between 17 and 30°C were used to determine the protein concentration at which the chain order becomes independent of temperature. That is, the point x_0 would be the common intersection of the lines fit to the expression

$$M_1(x_p,T) = M_1(0,T) - \left[\left(M_1(0,T) - M_1^0\right)/x_0\right] \cdot x_p \quad (8)$$

In addition, the data at 30°C were used to determine the value of the first moment at that point, M_1^0. This point is shown as the 'star' in Fig. 5, with $x_0 = 0.0295$ (± 0.01) and $M_1^0 = 5.14$ (± 0.1)$\cdot 10^4$ s^{-1}. The uncertainty in the value of x_0 is determined from the scatter in the values obtained at each temperature (between 17 and 30°C), and the uncertainty in the value of M_1^0 is determined by the the spread in the values of M_1 at 30°C, where it is seen to be approximately independent of protein concentration.

This common point of intersection suggests that about 34 (± 12) lipids are needed to 'solvate' each molecule of glycophorin. This is in contrast to the analysis of the DSC/EPR experiments of Rüppel et al. [18] (see below) where it was concluded that one glycophorin molecule interacted 'directly' with about 100 lipid molecules at $x_p > 0.008$. However, we do not suggest that these 'solvation' lipids are 'tightly bound' or even interacting 'directly' with the protein except that, as indicated by Fig. 5, as the protein concentration is increased a point is reached where the first moment becomes relatively insensitive to temperature. At this protein concentration ($x_p \approx 0.0295$) the lipid chain order is determined by the lipids' interaction with the protein. Below that concentration the data are consistent with a picture where the 'solvation' lipids are freely exchanging on the deuterium NMR time scale with the other lipids in the bilayer.

The effects of glycophorin on the M_1 values of DMPC bilayers in the gel phase are also similar in some respects to the effects of gramicidin [1] and synthetic peptide [10] on these bilayers. Below the pure lipid transition temperature, as the protein or peptide concentration is increased, the M_1 values decrease. However, at temperatures above the lipid phase transition, the M_1 values for DMPC/gramicidin and DMPC/synthetic peptide systems increase as the peptide concentration is increased. This is in contrast to the DMPC/glycophorin system, where the M_1 values decrease slightly or remain constant as the glycophorin concentration is increased. Thus, gramicidin and synthetic peptide have a disordering effect on DMPC below the pure lipid transition temperature and an ordering effect above the transition temperature. In

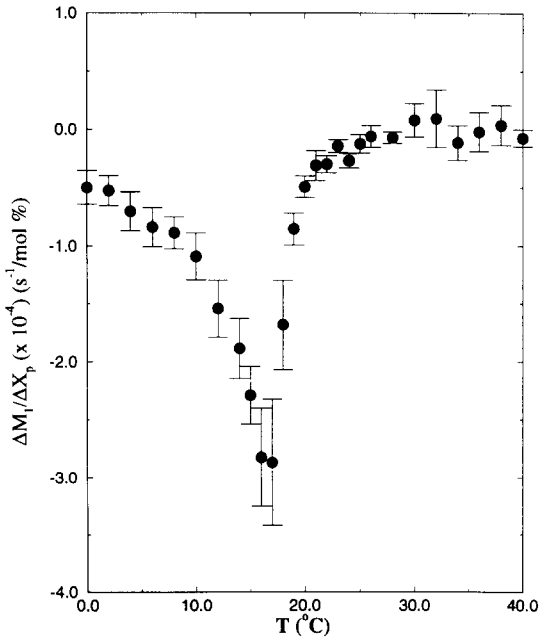

Fig. 8. The slope of M_1 vs. x_p as a function of temperature. The slopes were determined at each temperature by a linear least squares fitting procedure. The error bars indicate the uncertainties in the slope at each temperature. The data for the sample with concentration $x_p = 1.23$ mol% were not included in the analysis.

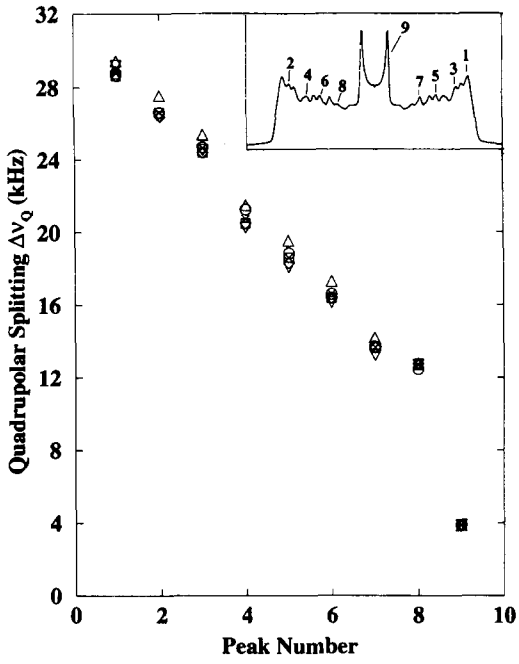

Fig. 9. Quadrupolar splitting, $\Delta\nu_Q$, vs. peak number at 24°C; (△) pure DMPC-d_{54}, $x_p = 0.0$, (○) $x_p = 0.0041$, (□) $x_p = 0.0065$, (×) $x_p = 0.0071$, (▽) $x_p = 0.0082$, (◇) $x_p = 0.0092$. The inset spectrum is the ^{2}H-NMR spectrum of pure DMPC-d_{54} shown in Fig. 1a.

contrast, glycophorin has a disordering effect on the DMPC bilayer in the gel phase and perhaps a slight disordering effect on the lipid chains in the liquid crystalline phase. In addition, Fig. 9 shows the glycophorin concentration dependence of quadrupolar splittings from each individual peak in the spectrum of Fig. 1a (see inset to Fig. 9). At temperatures above the phase transition, almost all of the quadrupolar splittings for DMPC/glycophorin at different protein concentrations are slightly lower than those for the pure DMPC. MacDonald and Pink [36] have predicted the protein concentration dependence of the ^{2}H-NMR splitting, $\Delta\nu_Q$, based on their Monte-Carlo simulation results. They reasoned that the reduced ^{2}H-NMR splitting might reflect the increased lipid hydrocarbon chain disorder resulting from a reduction in effective lateral pressure experienced by lipids under the D conformation of the glycosylated external domains (see below). Accordingly, $\Delta\nu_Q$ should first decrease and then increase as x_p increases from $x_p = 0$. Our results show that not only does the average quadrupolar splitting, M_1, decrease slightly as glycophorin concentration is increased, but also that most of the quadrupolar splittings from different positions on the acyl chain decrease by similar amounts.

Although both glycophorin and human erythrocyte Band 3 are integral membrane proteins, their effects on the DMPC bilayer are very different. In the case of Band 3 [12], the first moment values increased as the protein concentration was increased in the two-phase region. At temperatures above the two-phase region, M_1 increased slightly with increasing protein concentration. This indicated that Band 3 partially ordered the liquid-crystalline phase. Addition of Band 3 to the DMPC bilayer increased the gel to liquid-crystalline phase transition temperature. In this case, the protein tended to stabilize the gel phase so that the region of two-phase coexistence occured above the pure lipid transition temperature. In contrast, the M_1 values for DMPC/glycophorin bilayers decrease slightly as the protein concentration is increased. Addition of glycophorin to DMPC bilayers decreases the DMPC transition temperature so that the phase transition temperatures of all the DMPC/glycophorin mixtures are lower than that of pure DMPC. Therefore glycophorin tends to stabilize the fluid phase.

As shown in the phase diagram in Fig. 7, when the protein concentration is increased from zero along the isotherm at a temperature, T, below the chain melting transition of the pure lipid, T_m, the system is in the gel phase until the two-phase line at concentration $x_g(T)$ is reached. Any further increase in protein concentration results in the conversion of a fraction, $f(T)$, of the system from the gel to the fluid phase. By the lever rule [37,38]

$$f(T) = \frac{x(T) - x_g(T)}{x_f(T) - x_g(T)} \tag{9}$$

is the fluid fraction at concentration x and temperature T, where $x_f(T)$ and $x_g(T)$ are the concentrations at which the isotherm at temperature T intersects the two-phase lines or boundaries of this two-phase region. As the concentration is increased within the two-phase region, the fraction of fluid phase increases, while the gel phase fraction $g(T) = 1 - f(T)$, decreases. Fig. 10a plots the fluid fraction f, as a function of temperature at $x_p = 0.0041$.

The phase diagram in Fig. 7 provides a description of how lipid and protein partition between phases. For example, the mole fraction of protein in the fluid phase, i.e. n_p^f/n_p (the number of moles of protein in fluid phase domain divided by the total number of moles of protein) is given by

$$\frac{n_p^f}{n_p} = \frac{x_f}{x_p} \cdot f \tag{10}$$

where f is the fluid fraction given by Eq. (9). This quantity is also plotted for $x_p = 0.0041$ in Fig. 10b. While the total sample melts relatively slowly as the temperature is raised, the protein enters the fluid phase more rapidly. For example, at 16.0°C, the fluid fraction $f \simeq 0.25$, while $n_p^f/n_p \simeq 54\%$. Only 25% of the total sample is fluid, but 54% of the glycophorin is in those fluid domains. Freeze-fracture electron microscopic studies previously showed that glycophorin

intramembranous particles were preferentially located in fluid regions of the bilayer, in several binary lipid mixtures exhibiting a two-phase separation [39]. In addition, MacDonald noted a greater tendency for reconstitution of glycophorin into fluid, as opposed to solid lipid [40]. Similar results have been reported for DPPC/synthetic peptide by Huschilt et al. [10]. In their studies, the synthetic peptide shows an even more dramatic preference for the fluid domain in the two-phase region. For example, for synthetic peptide 16 at 32.5°C, 11% of the total sample ($f \simeq 0.11$) was fluid, but 50% of the peptide was in the fluid domains.

Finally, the studies of both gramicidin D/DMPC [1] and synthetic amphiphilic peptides in DPPC [10,19] suggested that those systems might be close to a critical point [34] and that the phase boundaries may close onto themselves, forming a closed curve. The results reported here are very similar, and one must consider that the glycophorin/DMPC system may also be close to a critical mixing point.

Several other physical techniques have been used to investigate the DMPC/glycophorin system. Using DSC on a series of samples in the concentration range

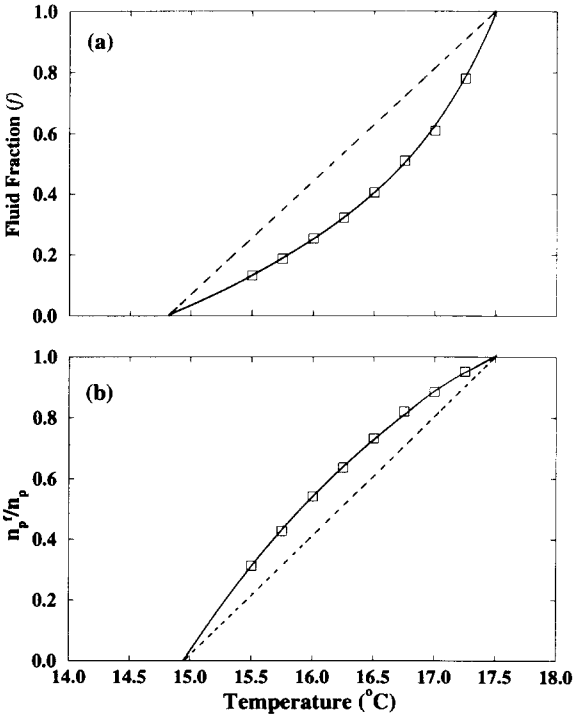

Fig. 10. (a) The fluid fraction, f, as a function of temperature for DMPC/glycophorin bilayers at $x_p = 0.0041$. The dashed line shows the behavior expected if the sample had melted uniformly or linearly as the temperature was increased through the two-phase region. (b) The mole fraction of glycophorin in the fluid phase, n_p^f/n_p, vs. temperature. The dashed curve is what would have been obtained if the sample had melted uniformly or linearly as the temperature was increased through the two-phase region. The solid curves in both (a) and (b) correspond to the solid curves sketched in as phase boundaries in Fig. 7.

between $x_p = 0$ and 0.026, Rüppel et al. [18] found that the maximum width of the heating curve was at $x_p = 0.008$. The results from their spin label EPR study showed that a minimum existed in the phase transition temperature at about 0.8 mol% glycophorin. The order parameters obtained from the EPR study also showed a minimum at $x_p = 0.008$. Based on these results, Rüppel et al. [18] constructed a tentative phase diagram of the DMPC/glycophorin system. A two-conformation model was proposed to explain some features of their results. At glycophorin concentrations below 0.8 mol%, the protein was proposed to adopt a 'pancake' (down, D) conformation, i.e. the glycosylated external domain spreads out at the lipid–water interface. At higher glycophorin concentrations ($x_p > 0.008$), due to increased protein crowding, the external domain is in an extended (up, U) conformation projecting away from the bilayer surface into the aqueous phase. According to these authors [18], in the D conformation one protein molecule interacts with about 300 lipid molecules, while in the U conformation it interacts directly with only about 100 lipid molecules. MacDonald and Pink [36] performed Monte-Carlo simulations using this model, and found that the protein external domain in its D state results in a reduced effective lateral pressure acting on the lipid hydrocarbon chains, relative to that for the U state.

In the DSC experiments of Rüppel et al. [18], as the protein concentration, x_p, was increased from $x_p = 0$, the specific heat curves first broadened to a maximum at $x_p = x_1$, while the transition enthalpy decreased approximately linearly. At this concentration, the surface of the bilayer was considered to be essentially covered with protein external domains in their D states. As x_p was increased further, the specific heat curves narrowed, while the transition enthalpy stayed approximately constant. The two conformation model proposed that in this concentration region, increased protein–protein contact results in an increase in the U population. Finally, as x_p was increased even further, the specific heat peak broadened again, and the transition enthalpy decreased. The simulation results [36] suggested that this behavior may have been the result of the existence of two different lipid environments, one with the unperturbed lateral pressure (U), and the other with a reduced lateral pressure acting on the chains (D). The experimental results presented here do not show any significant changes near lipid/protein ratios of 300 or 100, but do suggest a 'solvation' layer of about 34 lipids/protein. Our phase diagram, Fig. 7, is in rough agreement with their previous results [18,36]. However, due to the difficulty in preparing homogeneous and reproducible glycophorin/DMPC reconstitutions at protein concentrations above about 1 mol% it is hard to know whether one should ignore, for example, our one sample at about 1.23 mol% gly-

cophorin and interpret our phase diagram according to the solid lines drawn in Fig. 7. This phase diagram is then similar to those observed for mixtures of gramicidin A with DMPC [1] and of synthetic amphiphilic peptides in DPPC [10]. If we insist that the sample at 1.23 mol % should be considered of equal importance, then we would obtain quite a different phase diagram, something similar to the dashed lines. This latter picture is very reminiscent of that given by the Monte Carlo simulations of MacDonald and Pink using the two conformation model. On the other hand, these dashed lines are also reminiscent of the behaviour occurring near an azeotropic point, so there may be other means of explaining this result. It should be emphasized that while the two conformation model is quite possible, one should consider other models where protein–protein interactions become significant near 1 mol% glycophorin.

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