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Studies on the interaction of human erythrocyte band 3 with membrane lipids using deuterium nuclear magnetic resonance and differential scanning calorimetry

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Human erythrocyte band 3, reconstituted into large unilamellar phospholipid vesicles, has been used as a model system for studying the interactions between membrane lipids and large transmembrane glycoproteins. Both ^{2}H-nuclear magnetic resonance (^{2}H-NMR) and differential scanning calorimetric techniques have been used to probe dimyristoylphosphatidylcholine-band 3 interactions over the temperature range 4–32°C. Analysis of ^{2}H-NMR spectra allowed the assignment of liquid crystal, gel phase and two-phase regions for several protein/lipid mole fractions in the range $(1–20) \cdot 10^{-4}$. Sample size was limited by the amount of available glycoprotein and this precluded exact determination of the phase boundaries for this system. The sharp discontinuity in the spectral first moment, M_1, seen at the phase transition of the pure phospholipid is progressively diminished by addition of protein, and at the highest protein concentration the first moment varies smoothly between the two phases. For T greater than 26°C or less than 16°C, the moments are relatively insensitive to protein concentration, while between 20 and 26°C the moments increase with protein concentration up to the boundary of the two-phase region. Beyond this boundary, they remain constant or decrease slightly with increasing amount of protein. A preliminary phase diagram for band 3 in this lipid system is presented, based on ^{2}H-NMR data. Differential scanning calorimetry (DSC) showed that addition of glycoprotein dramatically alters the scan shape and tends to extend the coexistence of two phases to higher temperatures.

Introduction

The interaction of integral membrane proteins with lipids within the lipid bilayer has been a common focal point for membrane biophysics and biochemistry. Changes in the physical properties of the phospholipid matrix have been shown to affect many important functions of membrane proteins and enzymes, including the kinetic parameters of enzymatic catalysis (e.g., Refs. 1 and 2), active and passive transport of ions [3–5] and receptor exposure and binding characteristics [6,7]. Conversely, the presence of integral proteins also alters the properties of the bulk lipid, including DSC scan shape [8], lateral mobility [9], flip-flop rate [10], chain orientational order [11] and

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Abbreviations: NMR, nuclear magnetic resonance; DSC, differential scanning calorimetry; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPC-d_{54}, 1,2-di(perdeuterio)myristoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

bilayer permeability [12]. In addition, many membrane proteins are known to segregate or sequester particular lipid species from the bulk bilayer lipids, leading to the existence of heterogeneous microenvironments [13].

The techniques of ^{2}H-NMR and DSC have been widely used in the study of lipid-protein interactions on a variety of systems, ranging from synthetic amphiphilic polypeptides to small proteins such as cytochrome b_5 and gramicidin A, to large multisubunit complexes such as cytochrome oxidase. Many integral membrane proteins and enzymes are glycosylated and these carbohydrate headgroups play a vital role as receptors for the transduction of biochemical signals across the cell membrane. Recent studies on membrane glycoproteins have shown that they appear to influence a relatively large number of membrane lipids for their size [14–16] and it has been suggested that the carbohydrate headgroup may interact with the surface of the bilayer in addition to the interaction between membrane-spanning peptide domains with the hydrophobic membrane interior [15,16]. Exploring the structural details of these interactions is vital, since they may have important consequences for the molecular behaviour and receptor function of membrane glycoproteins.

The glycoprotein model used in these studies is a subpopulation (the concanavalin A receptor portion) of human erythrocyte band 3, a family of protein which is well-characterized at the molecular level and can be isolated in the milligram quantities necessary for NMR studies. Band 3 is a 95 kDa glycoprotein with a single complex N-linked oligosaccharide chain of around 5–7 kDa, and has been shown to possess at least five membrane-spanning peptide segments [17]. It has been functionally reconstituted into lipid bilayers from the point of view of both ion transport properties and receptor function (see, for example, Refs. 18–21). The band 3 family of proteins acts as receptors for concanavalin A, *Ricinus communis* agglutinin, and other plant lectins, in both the intact erythrocyte membrane and lipid bilayer vesicles [22,23]. DSC studies on the reconstituted glycoprotein in bilayers of DMPC have already indicated that it has dramatic perturbing effects on bilayer lipids [16], which may be related to both the carbohydrate and peptide domains of the band 3 molecule. Our goal in this study was to attempt to evaluate, using both ^{2}H-NMR and DSC, the effects of this large integral glycoprotein on phospholipid orientational order and phase behaviour.

Materials and Methods

The phospholipid 1,2-di(perdeuterio)myristoyl-*sn*-glycero-3-phosphocholine (DMPC-d_{54}) was synthesized according to the procedure described by Gupta et al. [24]. The concanavalin A receptor subpopulation of band 3 was isolated by affinity chromatography on concanavalin A-Sepharose 4B and reconstituted into phospholipid vesicles by a detergent-dialysis technique as previously described [22]. The reconstituted preparations consisted of large unilamellar vesicles (diameter range 0.2–0.4 μm) containing symmetrically incorporated band 3. Tracer levels of di[1-^{14}C]palmitoyl-L-α-phosphatidylcholine (80–120 mCi/mmol, Amersham) were included in the samples and lipid content was determined by liquid-scintillation counting. Protein was assayed by the method of Peterson [25] using bovine serum albumin (Sigma, crystallized and lyophilized) as a standard. Lipid/protein ratios were estimated to be within an uncertainty of $\pm 5\%$. Characterization of these vesicles by electron microscopy, quasi-elastic light scattering and gel-filtration chromatography has been described previously [16,22,23], and has established that the reconstituted vesicles are a homogeneous population following dialysis.

Nuclear magnetic resonance. The NMR samples typically consisted of sedimented reconstituted vesicles containing about 30 mg of phospholipid, dispersed in a total volume of 300–400 μl of 10 mM Hepes buffer (pH 7.4) containing 2.9 g/l NaCl. The NMR experiment for each sample lasted from 48 to 72 h. Thin-layer chromatography on silica gel plates using $CHCl_3/CH_3OH/H_2O/$ 15 M NH_4OH (55 : 35 : 5.6 : 1.6, v/v) was performed on all samples prior to and following the NMR measurements. For each sample, only a single spot containing phosphorus could be detected using Phospray (Supelco).

The ^{2}H-NMR measurements were performed in a superconducting magnet using the quadrupole echo technique at 41.3 MHz. The spectroscopic

methods have been described elsewhere [26]. Because of the possible sensitivity of the spectra to $\pi/2$ pulse length and pulse separation in the echo sequence, these were held fixed at 3.25 and 35 μs, respectively.

The moments of the spectra were routinely calculated at three different spectral widths, with all endpoints well beyond any apparent signal intensity. The variation in M_1 over this range is random and within the statistical error due to spectrum noise (less than $\pm 2\%$ for all spectra). Systematic errors due to finite pulse effects, receiver bandwidth, etc., are of a similar magnitude.

The signal-to-noise ratios in the NMR measurements were limited by the small lipid content attainable and by the presence of salt in the samples, which reduces the quality factor of the probe resonance. For each spectrum, 8000 transients were collected at intervals of 0.5 s. The free induction decays were acquired by slave computer and analysed using an Intel series II (Intel Corp., Santa Clara, CA) microcomputer development system. Each sample was initially equilibrated at 32°C. Temperature was lowered in 2 Cdeg steps until the appearance of a gel-phase component in the spectrum was observed. 1 Cdeg steps were then used until the disappearance of the liquid-crystalline component was noted. At each temperature, approx. 1 h was allowed for the temperature change and equilibration, with an additional hour being taken in the collection of free induction decays. The effective cooling rate was thus between 0.5 and 1 Cdeg per h.

Differential scanning calorimetry. A portion of each NMR sample, containing 5–10 mg of phospholipid, was used for a series of at least three consecutive DSC scans. The nature of the NMR made it impractical to determine the absolute lipid content of the DSC samples and thus no determinations of transition enthalpy were made. Enthalpy changes seen on increasing protein/lipid mole ratios have been reported previously [16]. DSC experiments were performed using a Microcal MC1 differential scanning calorimeter, using a scan rate of 10 Cdeg per h. Data were collected by slave computer and the analysis carried out on the same system as used for the NMR measurements. The correction of the DSC baselines was carried out as described elsewhere [27].

Results and Discussion

Each sample of reconstituted band 3 in bilayers of DMPC-d_{54} was subjected to ^{2}H-NMR analysis over the temperature range 4–32°C. This sample series covered protein mole fractions in the range $1 \cdot 10^{-4}$ to $20 \cdot 10^{-4}$ (lipid/protein weight ratios of approx. 80 : 1 to approx. 4 : 1). Fig. 1 shows the temperature dependence of the resulting ^{2}H-NMR spectra for a sample with protein mole fraction $x_p = 9.75 \cdot 10^{-4}$ (lipid/protein ratio = 8.1 : 1, w/w). For $T \geqslant 24$°C, the spectra are characteristic of liquid-crystal phase lipid. Deuterium atoms at each position on the fatty acid chain contribute a powder pattern characteristic of axially symmetric

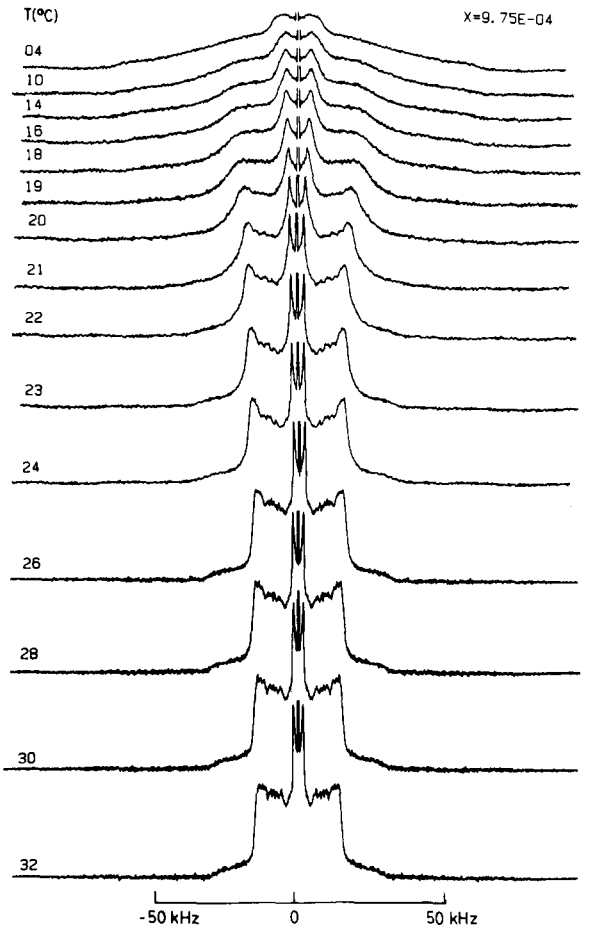


Fig. 1. Temperature dependence of the ^{2}H-NMR spectrum of the protein/lipid mixture at molar protein concentration $x_p = 9.75 \cdot 10^{-4}$. The temperatures at which the spectra were recorded are shown on the left.

motion. As the temperature is decreased from 32 to 24°C the spectra broaden, indicating less effective motional averaging of the quadrupole interaction. For $T \leqslant 19$°C, the ^{2}H-NMR spectra are characteristic of the non-axially symmetric motion within the phospholipid gel phase. Between 19 and 24°C, the spectra appear to be a superposition of gel-like and liquid-crystal-like components, indicating the presence of a two-phase region. Qualitatively similar results were obtained for the other samples in the series with different protein mole fractions, except that the two-phase region occupied different temperature limits.

The average quadrupole splitting of the ^{2}H-NMR spectrum, M_1, can provide useful information about the phase behaviour of protein-deuterated lipid mixtures. The effect of temperature on the spectral first moment, M_1, is shown in Fig. 2. In all samples, including the pure lipid DMPC-d_{54}, there is tendency for M_1 to decrease with increasing temperature. At the phase-transition temperature, the samples with the lowest protein concentrations display a sharp discontinuity in M_1. This is progressively diminished by the addition of protein, and at the highest protein concentrations the first moment varies smoothly between the gel and liquid-crystalline phases.

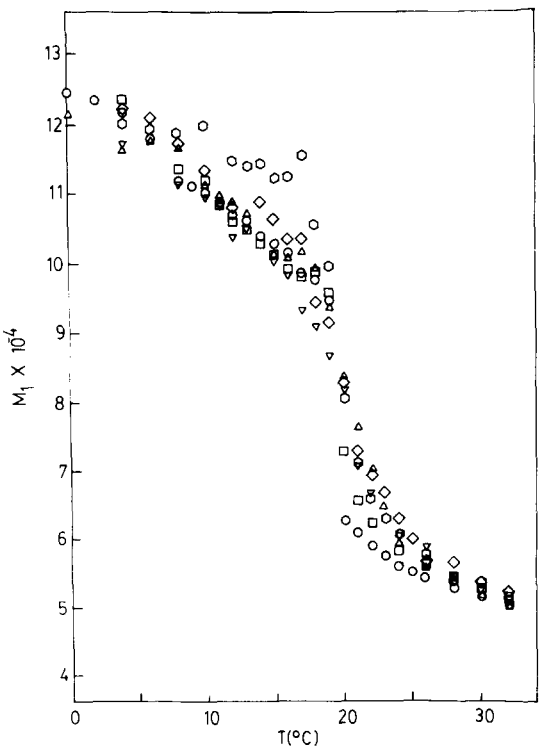


Fig. 2. Temperature dependence of the first moment, M_1, of the ^{2}H-NMR spectrum at various protein concentrations (x_p): ○, pure DMPC-d_{54}; □, $1.09 \cdot 10^{-4}$; ○, $2.42 \cdot 10^{-4}$; △, $6.15 \cdot 10^{-4}$; ◇, $9.75 \cdot 10^{-4}$; ▽, $20.1 \cdot 10^{-4}$.

The behaviour of M_1 as a function of temperature and protein concentration has been investigated in a number of other reconstituted systems. Paddy et al. [28] have looked at the effect of cytochrome *c* oxidase reconstituted in 1-(16, 16,16-trideuteriopalmitoyl)-2-palmitoleoyl-*sn*-glycero-3-phosphocholine. At temperatures well above the phase transition of the pure lipid, they found no dependence of M_1 on protein concentration. Near the gel-to-fluid transition, M_1 was found to decrease linearly with increasing protein concentration. 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'. Bienvenue et al. [29] studied rhodopsin in DMPC bilayers. They observed a fluid-to-gel transition for lipid-to-protein molar ratios, $L/P = 150$ and 50 but not, in the temperature range covered, for $L/P = 30$ or 12. For $L/P = 150$ and 50, the observed spectrum was interpreted as a superposition of a pure lipid gel component and protein-associated component similar to the $L/P = 30$ spectrum. The effect of protein was to lower M_1 over the entire temperature range covered. Davis [30], has argued that the results of Ref. 29 are consistent with the existence of a region of two-phase coexistence in the temperature composition phase diagram. In this case, the two phases are a pure lipid gel phase and a protein-rich fluid phase. At a given temperature within a two-phase region, M_1 should vary linearly with protein concentration. This type of study has been extended by Huschilt et al. [26] to mixtures of synthetic amphiphilic polypeptides with DPPC. A region of gel-liquid crystal coexistence was observed and it was shown that spectra within this region of the phase diagram were superpositions of the spectra at the boundaries of the two-phase region. With increasing polypeptide concentration, M_1 was found to increase slightly in the liquid-crystalline phase and decrease slightly

in the gel phase. In all three of these studies, the effect of the protein on the phase diagram, was to produce a region of two-phase coexistence lying entirely below the transition temperature of the pure lipid.

Figs. 3 and 4 show the band 3 concentration dependence of the ^{2}H-NMR spectra at $T = 19$ and 20°C, respectively. At the lower temperature, the pure lipid exists entirely in the gel state, while at the higher temperature the pure lipid is in the liquid-crystalline phase. In Fig. 3, the samples at $x_p = 1.47 \cdot 10^{-4}$ and $10.8 \cdot 10^{-4}$ are anomalous in showing liquid-crystal-like behaviour to lower temperature than the other samples. As neither high nor low protein concentration exhibits a depressed transition temperature, it is unlikely that

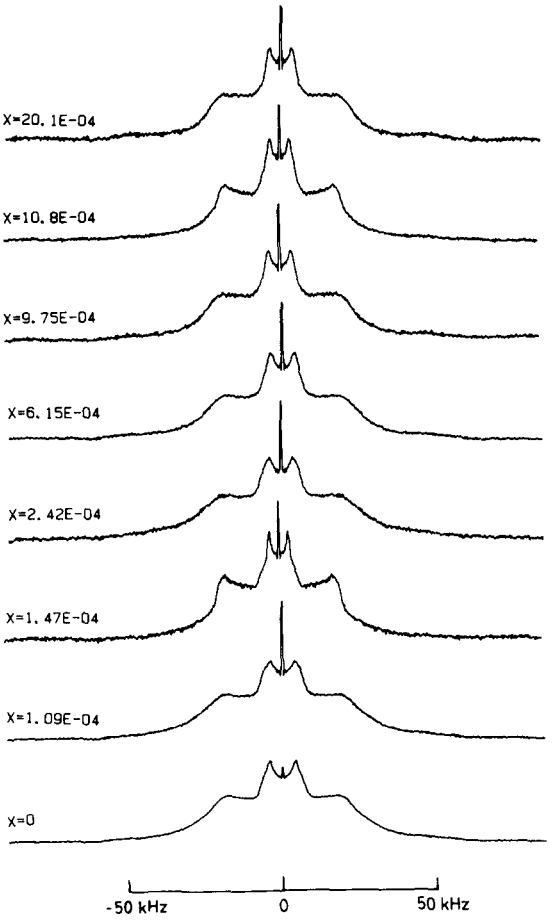


Fig. 3. Concentration dependence of ^{2}H-NMR spectra at $T = 19$°C. Molar protein concentration for each spectrum is shown on the left.

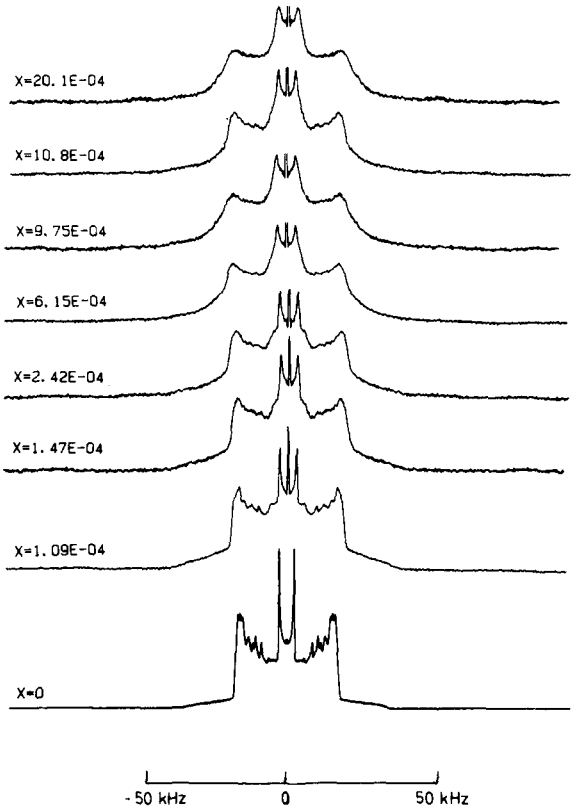


Fig. 4. Concentration dependence of ^{2}H-NMR spectra at $T = 20$°C. Molar protein concentration for each spectrum is shown on the left.

the anomalous behaviour results from an error in concentration determination. Other samples of similar protein concentration were studied by DSC but not by NMR, and since they did not display a depressed transition, the two anomalous samples have accordingly been omitted from further analysis. The most likely explanation for this infrequent anomalous behaviour may be the sensitivity of band 3-lipid samples to factors in their recent history (particularly heating/cooling) of which we are as yet unaware. Fig. 4 displays the coexistence of both gel and liquid-crystal phases at $T = 20$°C for $x_p \geqq 1.5 \cdot 10^{-4}$. Little or no liquid-crystalline phase remains at the highest protein concentration ($x_p = 20.1 \cdot 10^{-4}$). Huschilt et al. [26] have demonstrated the use of spectral subtraction and the lever rule in determining the extent (in concentration terms) of the two-phase coexistence at a given temperature for a synthetic amphiphilic peptide in

phospholipid bilayers. The signal-to-noise ratio of the spectra in the present study were poorer, since sample size was limited by both band 3 availability and the experimental requirements of the reconstitution procedure. The quality of the spectra and the larger uncertainties in sample composition effectively preclude any such analysis in the present work. It is likely, however, that the gel boundary passes in the neighbourhood of $x_p = 20.1 \cdot 10^{-4}$ at $T = 20°C$ and is beyond that concentration for $T = 21$ and 22°C.

It should be noted that no discontinuity in the ^{2}H-NMR parameters is seen over the range of lipid/protein ratios studied, particularly in the region of $x_p = 8.6 \cdot 10^{-4}$ (lipid/protein = approx. 9.2 : 1, w/w). A sharp 'break' is seen in the plot of transition enthalpy ΔH vs. protein/lipid mole ratio at this protein concentration [16], which has been attributed to either protein aggregation or a change in glycosylated headgroup conformation. The ^{2}H-NMR spectra show that if any changes in lipid behaviour occur on passing through this critical protein concentration range, they are either very subtle or ^{2}H-NMR is not sensitive to them.

For $T \geqslant 26°C$ and $T \leqslant 16°C$, the moments are relatively insensitive to band 3 concentration. Between 20 and 26°C, the moments increase with protein concentration up to the boundary of the two-phase region. Beyond this boundary, they remain constant or decrease slightly with increasing protein concentration. The change in slope of M_1 vs. protein concentration seems to indicate entry into a region of two-phase coexistence (data not shown). Unlike the results of Ref. 28, the present results do not indicate a tendency for plots of M_1 vs. protein concentration at different temperatures to intersect at a single value of protein concentration. Given the existence of a two-phase region, such an intersection requires both a vertical phase boundary at the intersection concentration and that M_1 not depend on temperature along that boundary. Neither is true in the present case.

For temperatures above the two-phase region, M_1 increases slightly with increasing protein concentration as seen in Fig. 2. This indicates that the protein partially orders the liquid-crystalline phase, consistent with the results of Ref. 26 on synthetic polypeptide/lipid mixtures but unlike those of Refs. 28 and 29.

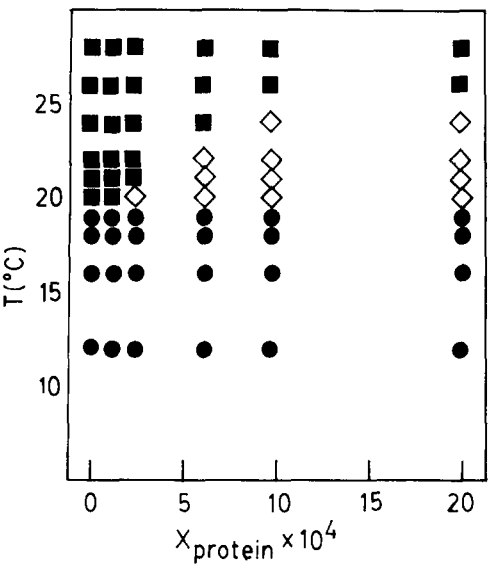
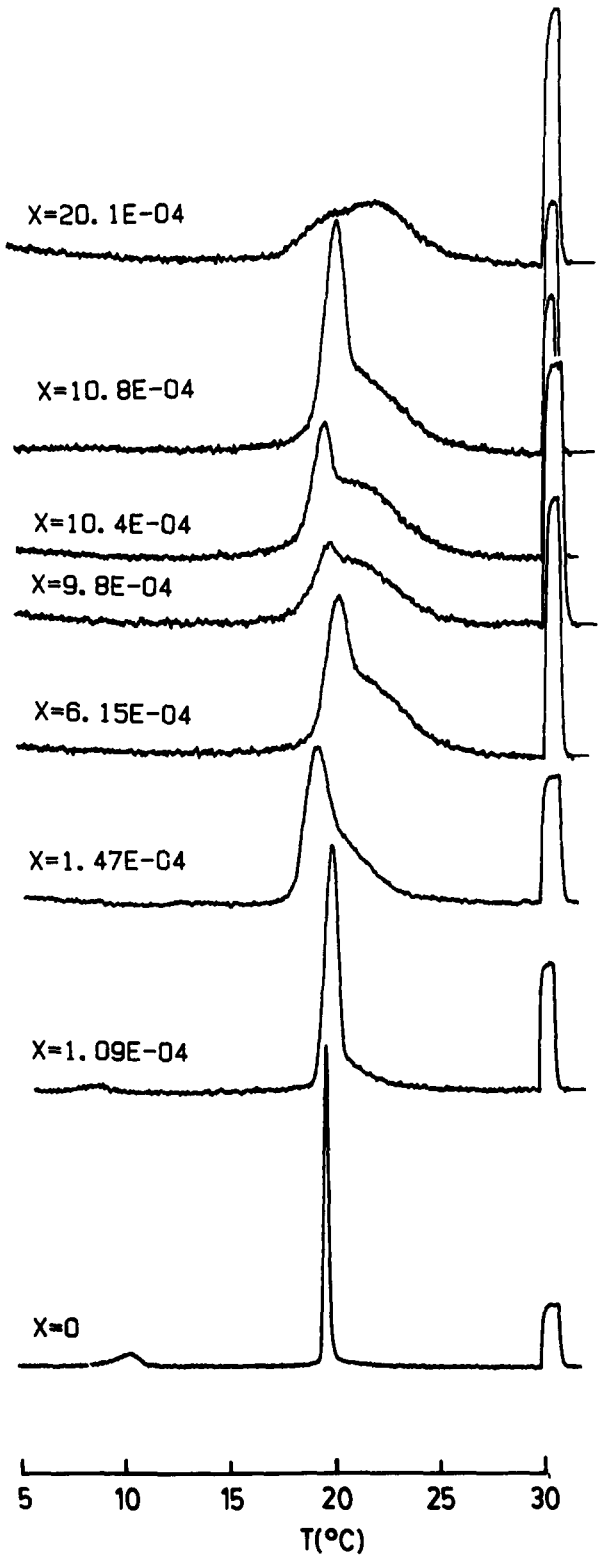


Fig. 5. The data points of Fig. 5 are displayed on a temperature/composition plot. ■, liquid-crystal; ●, gel; ◇, two-phase region.

The existence of a two-phase region is more directly seen in Fig. 5 where we indicate some features of the observed spectra on a plot of temperature vs. composition. Spectra which are unambiguously gel or liquid-crystal are shown as solid circles or squares, respectively. Those which display features of both phases are represented as open diamonds. It is interesting to note that the protein tends to stabilize the gel phase so that the region of two-phase coexistence occurs above the pure lipid transition temperature, in sharp contrast to the behaviour described in Refs. 26, 28 and 29 for cytochrome oxidase, rhodopsin and synthetic peptides, where the region of two-phase coexistence lies below the lipid transition.

Fig. 6 displays the concentration dependence of the DSC scan shape for band 3 reconstituted into DMPC-d_{54} vesicles. The presence of band 3 suppresses the pretransition (seen at 10–11°C in the pure lipid) at the lowest protein concentration, and eliminates it completely as more band 3 is added to the bilayers. These scans demonstrate that the addition of band 3 extends the main phase transition to higher temperatures in a concentration-dependent manner. Fig. 6 also shows that even small amounts of band 3 have a dramatic broadening effect on the shape of the DSC scan.



It should be noted that the three samples with x_p close to $10 \cdot 10^{-4}$ show considerable variation in the sharp component. This may indicate the sensitivity of the scan shape to protein concentration in this neighbourhood or it may indicate that the sharp component is sensitive to sample history. That the latter is a possibility is suggested by the fact that the small sharp component displayed by the sample at $x_p = 9.8 \cdot 10^{-4}$ appeared only after a number of scanning cycles. In the light of the known tendency of band 3 to aggregate [16], it is possible that lateral redistribution of glycoprotein molecules in the plane of the bilayer could account for these observations. For the sample series studied, the upper limit of the phase transition is approx. 24°C and the transition reaches this temperature for all but the lowest protein concentration. In this sense, the DSC results are consistent with the extent of the two-phase region favoured by NMR.

Acknowledgements

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Fig. 6. Concentration dependence of the DSC scan shape. The protein concentration for each scan appears to the left and each scan ends with a calibration pulse. Scans were run at a speed of 10 Cdeg/h. Baselines were flattened as described in Ref. 27.

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