HYDROCARBON PHASE TRANSITIONS, HETEROGENEOUS LIPID DISTRIBUTIONS AND LIPID–PROTEIN INTERACTIONS IN ERYTHROCYTE MEMBRANES

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1. Introduction

A membrane state transition has been reported to occur in erythrocyte ghosts in the region of 20°C [1,2] which has been tentatively identified as a gel-liquid crystalline phase transition of some portion of the membrane lipids. Such a phase transition would be surprising as the high cholesterol content of the erythrocyte membrane would be expected to eliminate phase transition phenomena if the lipids were homogeneously distributed. Also, the relatively unsaturated fatty acid composition of the erythrocyte lipids (with the exception of sphingomyelin) is inconsistent with such a high transition temperature. Thus the occurrence of a hydrocarbon phase transition would immediately imply a heterogeneous lipid distribution in the plane of the erythrocyte membrane – in particular, a segregation into cholesterol-rich and cholesterol-poor regions. Further, an interaction between the lipids in the cholesterol poor regions and other membrane components would appear to be required in order to account for the elevated hydrocarbon transition temperature.

In this work the nature of the erythrocyte membrane transition and associated conjectures have been investigated employing ^{31}P NMR techniques, which are sensitive to phase transition phenomena and other motional characteristics of membrane phospholipids [3–6]. Particular use is also made of an ether extraction procedure, which has been shown to remove all the cholesterol and ~ 25% of the phospholipids from the human erythrocyte membrane, leaving the $(Na^+ + K^+)$ stimulated and Mg^{2+} dependent ATPase activities unchanged [7]. It is found that whereas phase transition effects are not apparent for normal erythrocyte ghost preparations, a hydrocarbon phase transition in the region of 20°C does occur in the 'tightly bound' phospholipids of the ether extracted ghosts. It is proposed that this phase transition corresponds to the transition observed in normal ghosts, which may then be explained as a hydrocarbon phase transition of phospholipids in cholesterol-poor regions of the membrane. It is further suggested that the elevated transition temperature arises from apolar lipid–protein interactions.

2. Materials and methods

Erythrocyte ghosts were prepared from outdated human bank blood, the cells were washed twice in 3 volumes of isotonic NaCl, the centrifugations being carried out at 2000 × *g* for 10 min. Care was taken to remove the 'buffy coat' between washings. Cells were lysed in 10 volumes of 5 mM Tris-HCl (pH 8.0) held at 0°C. The membrane fragments were subsequently concentrated by centrifugation at 50 000 g for 20 min, and were washed twice in the same buffer. The ghosts were then frozen. Immediately prior to experiments, the ghost preparations were washed once more with 25 mM Tris-HAc (pH 7.2). Samples used for ether extraction were washed in unbuffered distilled water, lyophilized overnight, and subsequently the ether extraction procedure of

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Roelofsen et al. [7,8] was employed. Ether extracted erythrocytes were rehydrated using the 25 mM Tris–HAc (pH 7.2) buffer. Total lipids were extracted from the normal and ether extracted ghosts using the method of Bligh and Dyer [9] with 50 mM EDTA and 100 mM NaCl present in the aqueous phase. The solvent was evaporated and the lipids rehydrated in the 25 mM Tris–HAc (pH 7.2) buffer employing a vortex mixer. Partially hydrated lipids were obtained by placing the dried lipid extract over a saturated NaCl solution (relative humidity 75%) at 50°C for 4 h which resulted in a lipid hydration of 14% H_2O by wt. Pronase digestion of the ether extracted erythrocyte ghosts was carried out according to the procedure of Rottem et al. [10]. Typically, 1 mg pronase was added to 4 ml ether extracted ghosts (containing approximately 50 mg protein) containing 25 mM Tris–HAc buffer (pH 7.2) and the mixture was incubated at 37°C for 1 h. The membranes were then washed twice in the above buffer at 0°C.

Two Fourier Transform ^{31}P NMR spectrometers were employed in this investigation, a high-frequency machine [11] operating at 129 MHz, and a lower-frequency Bruker WH-90 spectrometer operating at 36.4 MHz. Broad band proton decoupling facilities were only available on the 36.4 MHz instrument. Accumulated free induction decays were obtained from up to 50 000 transients with an interpulse time of 0.5 sec.

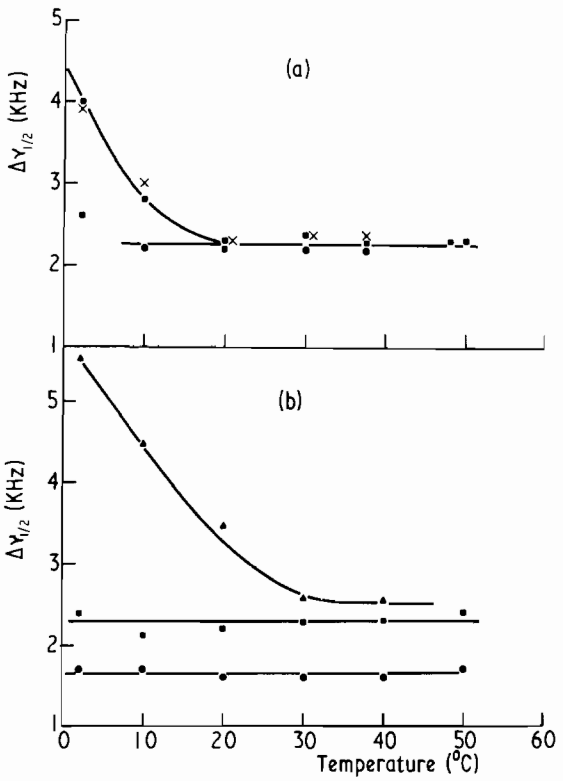

Fig.1. Temperature dependence of $\Delta\gamma\frac{1}{2}$ of the 129 MHz ^{31}P NMR spectra obtained from erythrocytes and derived liposomes (a) ● – erythrocyte ghosts; ■ – erythrocyte ghosts after ether extraction; × – pronase digested ether extracted ghosts (b) ● – liposomes composed of total lipids from erythrocyte ghosts; ■ – liposomes composed of total lipids from ether extracted ghosts; ▲ – partially hydrated (14% by wt) total lipids from ether extracted ghosts. All fully hydrated ghost and derived liposome preparations contain 25 mM Tris–HAC (pH 7.2).

3. Results

The linewidth at half height ($\Delta\gamma\frac{1}{2}$) of the 'solid state' ^{31}P NMR signals (in the absence of proton decoupling) arising from phospholipid liposomes and biological membrane fragments may be employed to detect phase transitions in the constituent phospholipids [4–6]. In particular, $\Delta\gamma\frac{1}{2}$ becomes markedly broader below the phase transition temperature T_c, and is relatively temperature independent above T_c.

As indicated in fig.1a, phase transition effects are not apparent for normal erythrocyte ghost phospholipids, in agreement with previous X-ray and calorimetric studies [12,13]. Phase transition effects are immediately apparent however for the ether extracted erythrocyte ghosts, and, by analogy with previous observations [4,5] the hydrocarbon phase transition occurs in the region of 20°C. As also indicated in fig.1a, equivalent behaviour is obtained for the ether extracted ghosts after pronase digestion of the membrane proteins.

The transition behaviour of aqueous dispersions of the lipids extracted from the normal ghosts and the ether extracted ghosts is illustrated in fig.1b. It may be noted that no phase transition effects are observed for either lipid species when dispersed in an excess of the aqueous buffer. Thus the lipid phase transition observed at 20°C for the ether extracted ghosts is not an intrinsic property of the constituent phospholipids,

but must arise from interactions with other membrane components – presumably proteins. It is interesting to speculate on the nature of this interaction. In particular it has been noted that the hydrocarbon phase transition temperatures of various phosphatidylcholines may be dramatically elevated at low water concentrations ($\lesssim$ 20% by wt) [14]. In order to ascertain whether a similar mechanism promoting tighter lipid packing could be responsible for the elevated transition temperatures of the tightly bound phospholipids of ether extracted ghosts, a hydrated (14% by wt) preparation of these lipids was investigated. As shown in fig.1b, a phase transition in the region of 30°C is then observed.

The similar properties of the phospholipids in the partially hydrated lipid extract and those of the ether extracted ghosts are further illustrated in fig.2. It may be noted that at 37°C the 129 MHz ^{31}P NMR lineshapes obtained from normal ghosts, ether extracted ghosts, and the partially hydrated lipids derived from the ether extracted ghosts are very similar, with a pronounced low field shoulder. This is in strong contrast to the completely hydrated lipids obtained from the ether extracted ghosts (fig.2c), for which the low field shoulder is poorly defined. This lack of definition may arise from a reduction in the effective chemical shift anisotropy ($\Delta\gamma_{CSA}^{EFF}$) [3–5] of the phospholipid

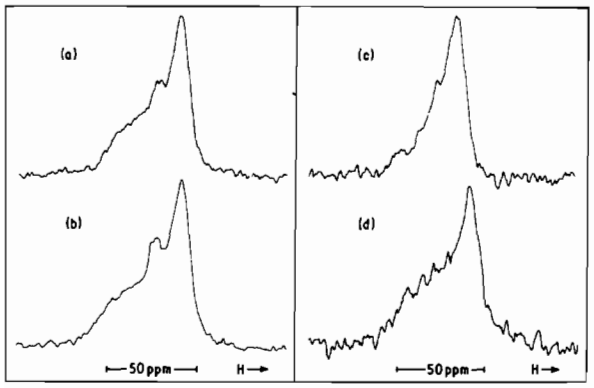

Fig.2. 129 MHz ^{31}P NMR spectra at 37°C in the absence of proton decoupling: (a) normal erythrocyte ghosts; (b) erythrocyte ghosts after ether extraction; (c) liposomes (completely hydrated) of lipids extracted from the ether extracted ghosts; (d) partially hydrated (14% by wt) lipids from ether extracted ghosts. All fully hydrated ghost and liposome preparations contain 25 mM Tris–HAc (pH 7.2).

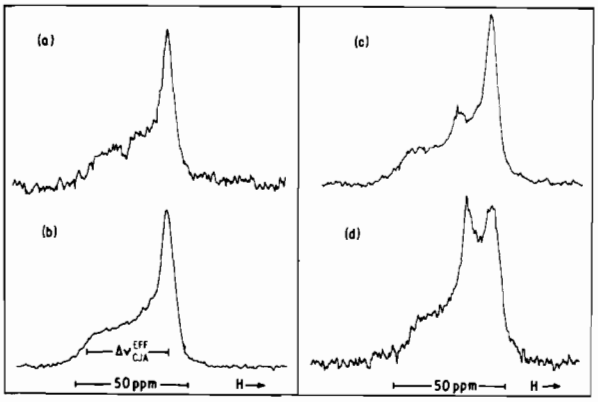

Fig.3. 36.4 MHz ^{31}P NMR spectra at 37°C in the presence of broad band proton decoupling. (a) Normal erythrocyte ghosts; (b) liposomes composed of lipids extracted from erythrocyte ghosts; (c) ether extracted ghosts; (d) liposomes composed of lipids from ether extracted erythrocyte ghosts. All preparations contained 25 mM Tris–HAc (pH 7.2).

phosphorus, which is most accurately measured from spectra obtained in the presence of broad band proton decoupling [5,6]. Such proton decoupled spectra were obtained at 36.4 MHz and are illustrated in fig.3. A decrease of $\Delta\gamma_{CSA}^{EFF}$ (from 45 ppm to 35 ppm) is apparent at 37°C between the phospholipids in the ether extracted ghosts (fig.3c) and the fully hydrated derived liposomes (fig.3d), which is consistent with increased motion in the phospholipid phosphate region in the fully hydrated system. It thus appears that the proteins in the ether extracted ghosts restrict the motion available to the polar headgroups of the tightly bound phospholipids. In order to account for the elevated transition temperature, this must arise from tighter lipid packing induced by membrane proteins with correspondingly increased steric restriction of the headgroup motion.

The remarkably uniform phosphate group motion and conformation of the normal erythrocyte ghost phospholipids at 37°C is illustrated in fig.3a, for which a well defined value of $\Delta\gamma_{CSA}^{EFF}$ = 38 ppm may be obtained. Further, the fully hydrated extracted lipid (fig.3b) exhibits an identical value of $\Delta\gamma_{CSA}^{EFF}$. Thus it may be concluded that the bulk of the phospholipids have very similar phosphate motion and conformation in the normal ghosts and in the derived liposomes. Such observations may reflect the stabilizing role of cholesterol in both systems.

4. Discussion

The hydrocarbon phase transition of the cholesterol depleted ether extracted ghosts as detected by ^{31}P NMR occurs at approximately the same temperature (20°C) as the membrane state transition of normal ghosts as detected by other techniques. Thus it is highly likely that the membrane transition of normal erythrocyte ghosts corresponds to a gel-liquid crystalline phase transition of lipids in cholesterol poor regions of the membrane. Such lipids may correspond to those forming an annulus about integral membrane proteins, as it has been suggested that cholesterol is preferentially excluded from such regions [15,16]. The observation of a discontinuity in glucose transport at 19°C [17] lends further support to such a contention.

The elevated transition temperature of the 'tightly bound' phospholipids of the ether extracted ghosts could conceivably arise from either polar or apolar interactions with surface associated or integral membrane proteins respectively. As indicated in the previous section, these interactions promote tighter lipid packing in both polar and apolar regions. It may be speculated that surface associated proteins which interact electrostatically with the polar region of lipids may reduce the lipid hydration, giving rise to the observed effects. However, such a mechanism would be expected to be sensitive to the pronase digestion procedure, whereas the transition behaviour of ether extracted ghosts is insensitive to such treatment. It is therefore concluded that the 'condensing' proteins are integral membrane components and that the lipid-protein interactions are predominantly apolar.

In summary, the results indicate that erythrocyte membranes contain certain integral membrane proteins which induce tighter packing of the constituent lipids. The specific nature of the apolar lipid–protein interactions concerned is not clear, as many integral membrane proteins investigated do not exhibit such effects [18]. However, the amphipathic polypeptide melittin (which penetrates the hydrocarbon region of phospholipid bilayers) has been shown to produce dramatically higher transition temperature in model membrane systems [19]. It is possible that a similar variety of lipid–protein interactions may be responsible for the effects observed in erythrocyte ghosts.

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