Raman and Fourier Transform Infrared Spectroscopic Studies of the Interaction between Glycophorin and Dimyristoylphosphatidylcholine†

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ABSTRACT: Glycophorin from the human erythrocyte membrane has been isolated in pure form and reconstituted into large unilamellar vesicles with 1,2-dimyristoyl-3-*sn*-phosphatidylcholine at lipid/protein mole ratios ranging from 50:1 to 200:1. The effect of protein on the phospholipid phase transition has been monitored by Raman and Fourier transform infrared spectroscopy and differential scanning calorimetry. No evidence for an immobilized higher melting lipid component is observed. The gel to liquid-crystalline phase transition is significantly broadened and shifted to lower temperatures as the proportion of protein is increased, while the pretransition is abolished. At all temperatures, the mobility of the acyl chains is increased by the addition of protein while interchain lateral interactions are disrupted. However, there is no evidence for a significant change in the conformational order at low temperatures (~5 °C) or in the liquid-crystalline phase.

Physical studies of lipid–protein complexes have led to various conclusions concerning the physical state and conformation of phospholipid molecules in the immediate vicinity of membrane protein. A common consequence of protein incorporation into a bilayer is that the calorimetric transition is broadened and generally shifted to lower temperatures (Petri et al., 1980; Gomez-Fernandex et al., 1980). Of particular interest is the question of whether there is an immobilized class of lipid immediately adjacent to the protein, with such lipid generally being referred to as boundary lipid.

The evidence at present is inconclusive and somewhat contradictory. For example, electron spin resonance (ESR)[1] studies of 1,2-dimyristoyl-3-*sn*-phosphatidylcholine (DMPC)–cytochrome oxidase complexes (Jost et al., 1973; Knowles et al., 1979) have led to the conclusion that a distinct boundary layer exists in the liquid-crystalline phase at 32 °C. Deuterium NMR studies of the same complex (Dahlquist et al., 1977) demonstrated two splittings in the signal from the terminal methyl groups of the acyl chains at low temperatures in the gel phase.

However, recent ^{2}H NMR investigations (Oldfield et al., 1978; Kang et al., 1979a,b) have led to different conclusions and shed doubt on the existence of an unique boundary layer. Oldfield and co-workers have investigated several integral membrane proteins (including cytochrome oxidase) reconstituted with specifically deuterated phosphatidylcholines. They showed that protein increases the disorder of the lipid hydrocarbon chains and prevents them from cocrystallizing into the gel phase at temperatures below the gel–liquid-crystal phase transition (T_m) of the phospholipid. Above T_m, a disordering of the lipid in the presence of protein was noted. No evidence was obtained for a particular immobilized lipid class.

The same authors also suggest that the results may differ according to the particular protein incorporated in the membrane. This contention is supported by the results from other studies. Using spin probes attached to rhodopsin, Davoust et al. (1979) have found no indication of boundary layer lipid at physiological temperatures but observed a signal suggestive of immobilized lipids at lower temperatures. Finally, Levin and coworkers have found two transitions in DMPC–mellitin complexes (Lavialle et al., 1980) but only one in lipid–myelin proteolipid apoprotein complexes (Lavialle & Levin, 1980). In the latter paper, they also reported differences related to the degree of saturation of the lipid component.

Generally, the different results obtained by applying different spectroscopic techniques in these and many other examples in the literature have been related to the different time constants of the methods (see, for example, Oldfield et al., 1978; Kang et al., 1979a,b; Knowles et al., 1979). However, it has also been suggested that the spin probe may cause the probe phospholipid molecules to interact with the protein in a fashion different from that of ordinary membrane lipids which lack the bulky polar group (Taylor & Smith, 1980; Oldfield et al., 1978).

The current work involves a vibrational spectroscopic study of the temperature dependence of DMPC–glycophorin complexes. The protein is a well-characterized transmembrane protein, can be isolated in reasonable quantities (Marchesi & Andrews, 1971), and can be reconstituted into both gel and liquid-crystalline phases (MacDonald & MacDonald, 1975), while it exhibits a preference for the latter (MacDonald, 1980).

Previous physical studies of glycophorin–lipid interaction have been reported. Brulet & McConnell (1976), using ^{13}C NMR spectroscopy, showed that perturbed lipid in glycophorin/DPPC complexes had increased motional freedom compared with the pure lipid gel state at temperatures $T < T_m$ for DPPC. Initial Raman spectroscopic studies (Taraschi & Mendelsohn, 1980) of the glycophorin/DPPC system demonstrated that about 135 lipid molecules in the vicinity of the protein were unable to undergo a cooperative melting event. Instead, they gradually formed gauche rotamers noncooperatively at temperatures well below T_m for DPPC.

Materials and Methods

Phospholipids. 1,2-Dimyristoyl-3-*sn*-phosphatidylcholine was purchased from Sigma Chemical Co. and its purity checked by TLC with a $CHCl_3$/MeOH/H_2O (65:25:4 v/v/v) solvent system. Development of the chromatogram with I_2 vapor showed one spot at the appropriate R_f. Calorimetric scans showed main transition and pretransition characteristics

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[1] Abbreviations used: DMPC, 1,2-dimyristoyl-3-*sn*-phosphatidylcholine; ESR, electron spin resonance; NMR, nuclear magnetic resonance; DPPC, 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; T_m, gel–liquid-crystalline acyl chain melting phase transition temperature; T_p, pretransition.

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in agreement with literature values (Mabrey & Sturtevant, 1976).

Isolation of Glycophorin. Freshly outdated human blood was obtained from the local blood bank and used without regard to type. Hemoglobin-free erythrocyte ghosts were prepared by hypotonic lysis of the packed red cells according to Dodge et al. (1963). Glycophorin was extracted from the lyophilized ghosts by using the procedure of Marchesi & Andrews (1971). Purification and dilipidation of the crude protein preparation was a modification of the methods of Hamaguchi & Cleve (1972) and van Zoelen et al. (1978). The lyophilized protein was suspended in 10 mM Tris/0.1 mM EDTA, pH 7.4, to which 9 volumes of $CHCl_3$/MeOH (2:1) were added. The mixture was stirred at room temperature for 30 min and centrifuged at 2000 rpm for 10 min to separate phases. Ethanol (9 volumes) was added to the collected aqueous layer and the clear solution stirred 15 min at room temperature. Addition of 0.1 volume of a 1.0 M Tris/10 mM EDTA, pH 7.4, solution resulted in precipitation of the glycoprotein. After centrifugation, the protein was redissolved in distilled water, dialyzed against several changes of distilled water, and lyophilized.

Formation of Lipid–Protein Complexes. Glycophorin was incorporated into DMPC vesicles according to the procedure of MacDonald & MacDonald (1975). Reconstitution at desired lipid/protein ratios was accomplished by the simultaneous mixing of measured amounts of lipid and protein in CHCl/MeOH/H_2O (6.5:2.5:0.4 v/v/v). Solvent was removed by N_2 drying, and traces were eliminated by evacuation under reduced pressure for 2 h. The lipid–protein film was rehydrated in 4.0 mL of 1.25 M NaCl/0.01 M Tris, pH 7.5 (ρ = 1.05). At this density, the lipid–protein complexes sediment during centrifugation at 20000*g* for 1 h at 4 °C, but free lipid remains in the supernatant. Lipid phosphorus was determined by the method of Chen et al. (1956), while glycophorin was determined as sialic acid by the method of Warren (1959). Reconstitution of lipid–protein complexes in D_2O was accomplished as described above except that the rehydration buffer was 1.26 M NaCl/0.01 M Tris in D_2O, pD 7.5.

Differential Scanning Calorimetry. Calorimetric experiments were performed on a Du Pont 990 calorimeter. Lipid–protein complexes were transferred to sealable aluminium sample pans. Each sample was scanned 3 times at 2 °C/min between 5 and 35 °C. Transition temperatures were reproducible to ±1 °C.

Raman Spectroscopy. The Raman apparatus consists of a Jarrell-Ash 3/4 m double monochromator, Spectra-Physics Model 164 Ar^+ laser, and RCA C31034 photomultiplier. Photon counting detection is accomplished with a Spex Industries DPC-2 counter. The photon counter output is processed by a Spex Industries "Scamp" minicomputer, which controls data acquisition and reduction. Typical spectral conditions used were the following: excitation frequency, 5145 Å; laser power, 150 mW; residence time, 0.5 s/cm^{-1}; number of scans varied from 5–10 for high lipid–protein ratios to 20–30 for low ratios; frequencies are calibrated by using atomic emission lines from a neon lamp. Frequency accuracy is estimated at ±1–2 cm^{-1} for sharp features.

Lipid–protein complexes were injected into "Kimex" melting point capillaries (approximately 1 mm in diameter) and examined in the transverse mode. Samples were packed in a hematocrit centrifuge prior to Raman spectroscopic examination. The final concentration of material in the laser beam is estimated at 60 mg/mL. Temperature control was achieved in a cell similar to that described by Thomas & Barylski (1970). Temperature calibration has been described elsewhere (Mendelsohn & Koch, 1980) and is accurate to ±1 °C.

Polyacrylamide Gel Electrophoresis. The purity of the isolated glycophorin was checked by using two continuous systems of electrophoresis. The method of Weber & Osborn (1969) used 10% acrylamide with an electrophoretic buffer of 0.1% $NaDodSO_4$/0.1 M sodium phosphate, pH 7.2. Scanning optical densitometry of these gels revealed more than 97% of the stain to be localized in a single band, consistent with a previous report (Silverberg & Marchesi, 1978). The method of Fairbanks et al. (1971) used 5.6% acrylamide in a buffer of 0.04 M Tris/0.02 M sodium acetate/0.002 M EDTA/1% $NaDodSO_4$, pH 7.4. Gels run using this system showed a more heterogeneous pattern than those run using the first system. The pattern obtained was consistent with that obtained by Furthmayr et al. (1975). The discrepancy between the two patterns has been suggested to be caused by a monomer–multimer reassociation, the extent of which depends on the ionic strength of the running buffer (Silverberg & Marchesi, 1978). All gels were stained for carbohydrate by using the periodic acid–Schiff stain; gels run concurrently and stained for protein with Coomassie blue gave identical patterns.

Fourier Transform Infrared Spectroscopy. Samples were prepared for infrared spectroscopy by using methods described in detail elsewhere (Cameron et al., 1979). A typical preparation included a 50-μm-thick lipid–protein sample prepared in a Hg–Pb amalgam cell with CaF_2 windows. Spectra were recorded on a Digilab FTS-11 Fourier transform infrared spectrometer equipped with a Hg CdTe detector. Five hundred interferograms were collected and coadded with a maximum optical retardation of 0.5 cm, apodized with a triangular function, and Fourier transformed with one level of zero filling to yield a resolution of 2.0 cm^{-1} and digitization every 1 cm^{-1}.

Temperatures were controlled by a thermostated EtOH/H_2O mixture flowed through a hollow cell mount and monitored by a copper–constantan thermocouple placed against the edge of the cell window (Cameron & Charette, 1981; Cameron & Jones, 1981).

Frequencies were determined by computing the center of gravity (Kauppinen et al., 1978) of the topmost 3 (CH_2 stretching) and 7 (C=O stretching) data points. The band widths were determined relative to a straight base line interpolated from 2880 to 2820 cm^{-1}, following the subtraction of the gently sloping H_2O or D_2O background absorption bands.

Electron Microscopy. Negative-stain electron microscopy was performed on a Philips EM 200 operating at 80 kV with an initial magnification of 12000–44000×. Vesicles in 0.01 M Tris/1.26 M NaCl, pH 7.5, were placed on a Formvar-filmed and heavily carbon-coated grid for 5 min before the excess was blotted off. Phosphotungstic acid (1%) was added for 5 min and the preparation air-dried before examination.

Results

Biochemical Characterization of Complexes. The reconstitution procedure of MacDonald & MacDonald (1975) leads to the formation of unilamelar vesicles with a size ranging from 0.1 to 0.15 μm. At lipid–protein ratios of 400:1 and below, there was no sign of multilamellar structures. At higher levels of lipid, however, such structures occur. All spectroscopic experiments in the current work were performed on samples containing the unilamellar species. A typical electron micrograph is shown in Figure 1.

The orientation of glycophorin in the vesicles was monitored via chemical determination of the sialic acid residues following treatment of the vesicles with trypsin. Approximately symmetric reconstitution was observed with 55–60% of the sialic

Table I: Comparison of Raman, Fourier Transform IR, and Calorimetric Melting Temperatures for DMPC–Glycophorin Complexes

lipid:protein mole ratio	Raman transition temp (°C)		Fourier transform IR transition temp (°C)		DSC transition temp (°C)	
	T_p	T_m	T_p	T_m	T_p	T_m
DMPC	16.5	23.5	14.5	23.9	14.6	24.6
200:1	*a*	18–22	*a*	20.9–24.5	*a*	23.3
100:1	*a*	15–27	*a*	19.7–23.6	*a*	19.1
50:1	*a*	13–26	*a*	10–25	*a*	*a*

[a] Transition not observed.

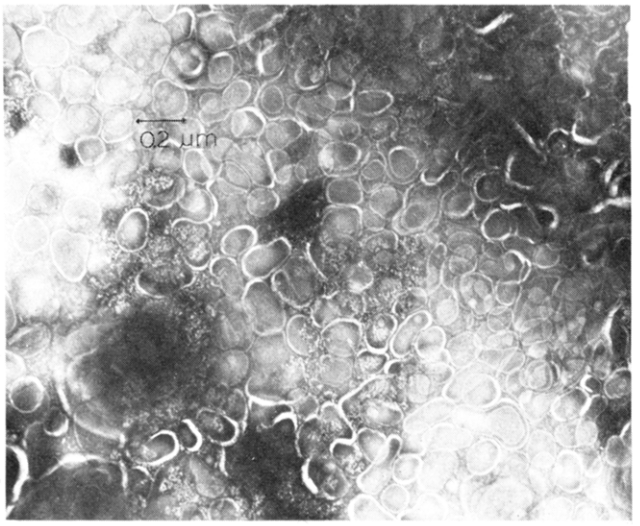

FIGURE 1: Typical electron micrograph of DMPC–glycophorin complexes. The sample had a lipid:protein mole ratio of 100:1. Magnification is 30 625× The size distribution (1000–1500-Å diameter) is typical of all preparations used in the current work.

acid residues pointed exterior to the vesicle. Previous studies demonstrated slightly more asymmetry with up to 75% of the sialic acid residues exterior (van Zoelen et al., 1978).

Calorimetric Studies. Calorimetric data for pure DMPC and DMPC–glycophorin complexes at various mole ratios are summarized in Table I. With increasing amounts of protein, the main transition is shifted to slightly lower temperatures and is significantly broadened. In addition, the pretransition is abolished for all lipid–protein complexes, while no transition can be observed for the 50:1 sample with the Du Pont 990 calorimeter.

Raman Spectroscopy. Raman spectra were monitored as a function of temperature in the C–C stretching (1000–1200 cm^{-1}) and C–H stretching (2800–3000 cm^{-1}) regions, both of which are essentially free from interfering protein bands. The modes giving rise to the bands in the 1000–1200 cm^{-1} region are highly intramolecularly coupled and, hence, sensitive to chain geometry. The band at 1130 cm^{-1} arises from all-trans segments in the acyl chains. Recently, Pink et al. (1980) have discussed quantitative aspects of the relationship between the Raman intensity of the 1130 cm^{-1} band and the average number of gauche rotamers in the chains, while Snyder et al. (1980, 1981) have examined its behavior in acyl systems used as the basis of interpretation of lipid spectra. It would appear that the intensity is intramolecularly coupled and responds in a highly nonlinear way to the introduction of gauche conformers. Nonetheless, it can be used for monitoring phase changes and for qualitatively comparing conformational order between systems.

Typical spectra for the C–C stretching region are shown in Figure 2 for the 200:1 DMPC–glycophorin complex. The sensitivity of the 1130 cm^{-1} peak height and intensity to temperature variations is readily apparent from this figure. In

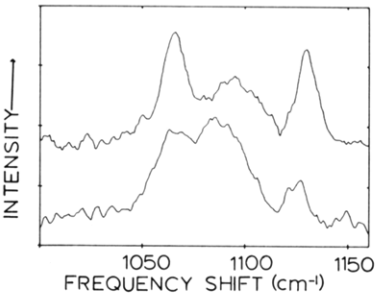

FIGURE 2: Typical Raman spectra of the 1025–1150-cm^{-1} region (C–C stretching modes) for a 200:1 DMPC–glycophorin complex: (top spectrum) 5 °C; (bottom spectrum) 25 °C. Spectral conditions are as given under Materials and Methods.

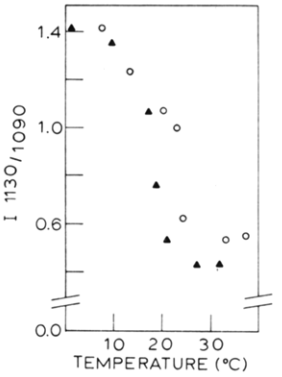

FIGURE 3: Temperature-induced variation in the C–C stretching region for DMPC in multibilayer vesicles (○) and reconstituted with glycophorin in 200:1 complexes (▲). The ordinate scale is the ratio of peak heights of the bands near 1130 and 1090 cm^{-1}.

Figure 3, the peak height of the 1130 cm^{-1} band is plotted relative to that of the band near ~1090 cm^{-1}, which results in part from chain segments containing gauche rotamers. Plots for the control system (DMPC in multilamellar vesicles) show abrupt decreases at points corresponding to the calorimetric transition temperature (24 °C). It is noted that sonicated unilamellar vesicles show essentially the same temperature-induced variations in the C–C intensity parameters as do the large multilamellar vesicles, indicating similar degrees of conformational order in the two cases (Taraschi & Mendelsohn, 1979). The plot obtained from the spectra of the 200:1 DMPC–glycophorin complex shows a broadened transition, with the temperature of the midpoint being reduced relative to that of pure DMPC. At low temperatures, the absolute values of the parameters in Figure 3 are within experimental error, suggesting the same complement and distribution of trans bonds. Further evidence for ordered structures at low temperatures in the 200:1 complex comes from the frequency of the C–C stretching band. At 5 °C, the value of 1130 cm^{-1} is appropriate for an ordered chain. The frequency is reduced upon formation of gauche rotamers so that at 30 °C the peak maximum is at about 1126 cm^{-1} in all systems.

Typical data for the C–H stretching region of the Raman spectra of the 100:1 and 50:1 DMPC–glycophorin complexes

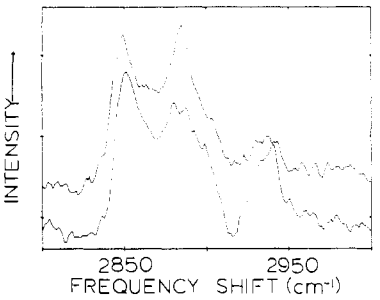

FIGURE 4: Typical Raman spectra for the C–H stretching region for DMPC–glycophorin complexes: (top) 100:1 mole ratio 9 °C; (bottom) 50:1 mole ratio 8 °C. Spectral conditions are as given under Materials and Methods. Note the decreased intensity at 2880 cm^{-1} as the protein content is increased.

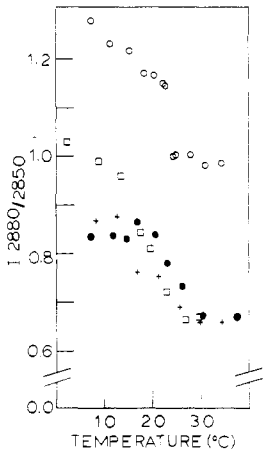

FIGURE 5: Temperature dependence of I 2880/2850 for DMPC multilamellar vesicles (○), DMPC sonicated unilamellar vesicles (●), 100:1 DMPC/glycophorin complexes (□), and 50:1 DMPC/glycophorin complexes (+).

are shown in Figure 4. The spectral parameter used to characterize the C–H stretching bands is the ratio of the peak height of the antisymmetric methylene stretching band of the acyl chains near 2880 cm^{-1} to that of the symmetric methylene stretching band near 2850 cm^{-1}. The temperature dependence of I 2880/2850 for multilamellar and unilamellar DMPC vesicles as well as 100:1 and 50:1 DMPC–glycophorin complexes is shown in Figure 5. The phase transition for the multilamellar suspensions occurs over a narrow range of temperatures centered at ~24 °C and is complete within about 0.5 °C. The transition is characterized by a rapid decrease in I 2880/2850 at T_m. For sonicated vesicles, the transition is broadened and occurs over a 10 °C range. In addition, relative to those of the multilamellar DMPC, the absolute values of the intensity parameter are greatly reduced at all temperatures in the unilamellar vesicles.

The incorporation of protein is seen to result in increased broadening of the DMPC transition relative to that of the multilamellar vesicles and, in the gel phase, a progressive reduction in the absolute value of this parameter as the lipid/protein mole ratio is decreased from 100:1 to 50:1. We also note that in the liquid-crystalline phase the parameter is of the order of that found in the unilamellar vesicles (Figure 5).

The C–H stretching region responds to variation in a variety of structural and dynamic parameters of the phospholipid acyl chains. Through isotopic dilution experiments (Gaber & Peticolas, 1977), it has been demonstrated that the height of the 2880 cm^{-1} band is sensitive to a static factor, intermolecular coupling. More recently, Snyder et al. (1981) have shown that the peak height of this band is also sensitive to the rate of rotation of an all-trans segment about its long axis; that is,

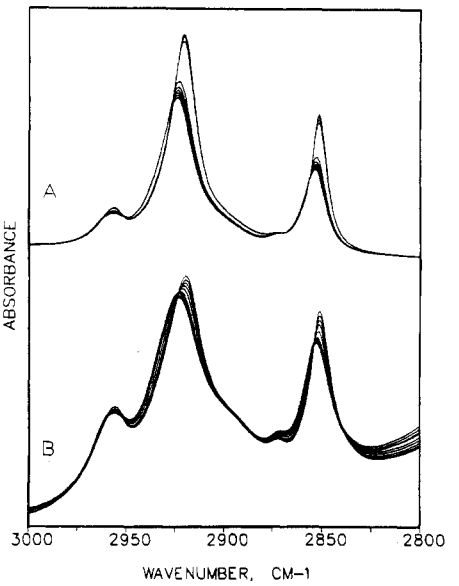

FIGURE 6: Temperature dependence of the C–H stretching bands of the infrared spectra of pure DMPC multibilayers (A) and 50:1 DMPC–glycophorin unilamellar vesicles (B). Spectra decrease in peak height with increasing temperature and are plotted in intervals of ~3.5 °C over the range 5–40 °C.

it also depends on a dynamic factor, the mobility. In addition, the peak height is reduced by the introduction of gauche rotamers, as evidenced by the large changes during the transition.

When the values prior to the onset of the transition are considered, the 1130-cm^{-1} parameters indicate the same high level of conformational order in all systems. Consequently, the reductions in I 2880/2850 at low temperature must reflect a reduction in the interchain vibrational coupling and an increase in the mobility of the acyl chain. It is clear that this effect is promoted by the incorporation of protein, although only in the 50:1 DMPC–glycophorin complex is the effect as large as that in the sonicated vesicles of DMPC. In the latter system, the large reduction in the uniformity of the acyl chain packing, but not the conformational order, has been attributed to the small diameter of the vesicles (Huang & Mason, 1978). It should, however, be kept in mind that the DMPC–glycophorin vesicles have a 5–6-fold larger diameter. Consequently, the effects of curvature are minimal, and in many ways, the data are better compared to those obtained with the large multilamellar vesicles which have similar curvatures. The primary result from Figures 4 and 5 is that the lipids in the presence of protein are not present in a well-packed array even at low temperature where they are highly ordered.

Infrared Spectroscopy. Acyl Chain Modes. The temperature dependencies of the C–H stretching regions of the infrared spectra of multilamellar DMPC and the 50:1 DMPC–glycophorin complex are shown in Figure 6. Five spectral features are evident in each case. The asymmetric and symmetric C–H stretching modes of the terminal methyl groups result in the bands at 2956 and 2872 cm^{-1}, respectively (Cameron et al., 1980). The antisymmetric and symmetric C–H stretching bands of the methylene groups are observed at 2920 and 2850 cm^{-1}, respectively, and there is a broad Fermi resonance band centered at about 2900 cm^{-1}.

The close similarity of two sets of spectra in Figure 6 shows that there is little interference from the underlying protein band at about 2978 cm^{-1} (Parker, 1971). The figures also illustrate that variations in temperature result in significant changes in line width and position during the gel to liquid-crystalline phase transition, with the changes being abrupt in the spectra of the DMPC multibilayers but occurring over a wide temperature

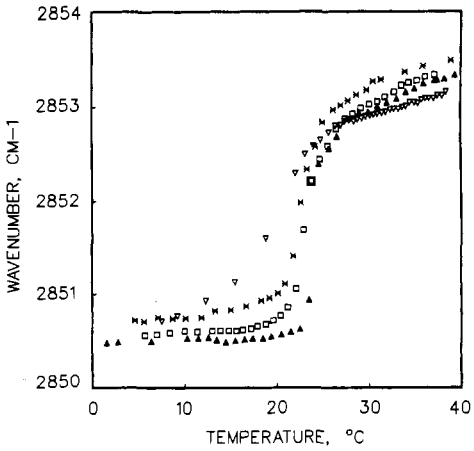

FIGURE 7: Plots of frequency vs. temperature for the symmetric CH_2 stretching band in the infrared spectra of DMPC multibilayers (▲) and DMPC–glycophorin complexes [(□) 200:1, (*) 100:1 and (▽), 50:1].

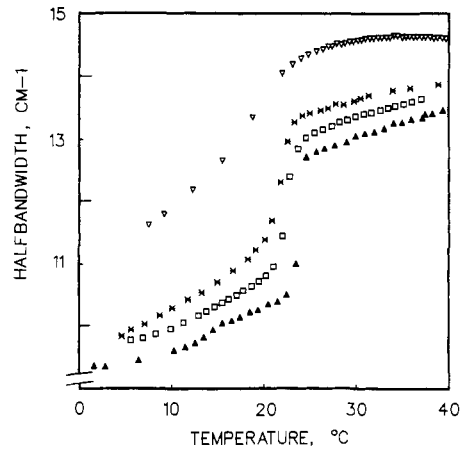

FIGURE 8: Plots of half-bandwidth vs. temperature for the symmetric CH_2 stretching band in the infrared spectra of DMPC multibilayers (▲) and DMPC–glycophorin complexes [(□) 200:1, (*) 100:1, and (▽) 50:1).

range in the spectra of the 50:1 complex. These variations reflect the sensitivity of particular spectral parameters to variations in phospholipid conformation and mobility. The CH_2 stretching frequencies are primarily sensitive to the degree of conformational disorder, increasing in response to the introduction of gauche rotamers. The line widths broaden as a result of increases in the rates and amplitudes of the motions of the methylene groups. Hence, in addition to responding to gauche rotations, the bandwidths also reflect changes in the rates and amplitudes of the torsional and librational motions about the long axes of the chains, that is, the acyl chain mobility.

The temperature dependencies of the infrared frequencies and bandwidths of DMPC and the DMPC–glycophorin complexes are shown in Figures 7 and 8, respectively. In the gel phase, the acyl chains of DMPC are highly ordered, with the gauche population immediately before the transition to the liquid-crystalline phase being concurrently estimated at one or less per chain (Pink et al., 1980; Snyder et al., 1981). The only change evident for pure DMPC above the pretransition T_p is an increase in the acyl chain mobility (as evidenced by the increase in bandwidth; Figure 8) reflecting the reduced interchain interactions characteristic of the hexagonal packing between T_p and T_m (Cameron et al., 1980). At T_m, there are abrupt increases in frequency and bandwidth reflecting the introduction of gauche rotamers; above T_m, further increases are observed, which indicate a progressive reduction in conformational order as the temperature is raised in the liquid-crystalline phase (Seelig & Seelig, 1974).

The incorporation of protein results in a broadening of and a reduction in the temperature of the midpoint of the main transition. Transition ranges as monitored by IR and Raman spectroscopy are listed in Table I.

On a more detailed level, the coincidence, at 5 °C, of the frequencies in the spectra of the DMPC–glycophorin complexes with that of the multilamellar DMPC demonstrates that at this temperature, the incorporated protein results in little or no conformational disorder in the lipids. It does, however, result in increased acyl chain mobility, as evidenced by the progressive increase in bandwidth with increasing protein concentration (Figure 8). This observation is in keeping with the markedly decreased I 2880/2850 ratios in the Raman spectra of the complexes.

As the temperature is raised, the differences between the complexes become marked. In the spectra of the 200:1 and 100:1 complexes, some noncooperative melting in the range 15–22 °C is indicated by the increase in frequency, with the effect being more pronounced with increasing protein concentration. A similar increase is observed in the bandwidths of the complexes, indicating a decrease in the lateral interactions and an increase in the mobility. However, both complexes still exhibit well-defined gel to liquid-crystal phase transitions. The dramatic effects of high protein levels on the DMPC phase behavior are evident in the data obtained with the 50:1 complex. Under these conditions, the transition is broadened to the point where it takes place over an ~15 °C range (10–25 °C) with a midpoint shifted to a significantly lower temperature (19.5 °C) compared to that of pure DMPC (24.5 °C).

In the liquid-crystalline phase, the symmetric CH_2 stretching bands of all samples exhibit essentially monotonic increases in frequency and bandwidth with increasing temperature, indicating a continuous increase in the conformational disorder. There is no indication of any second transition which might be associated with boundary lipid. The frequencies are essentially the same, indicating that the conformational disorder is not affected to any significant degree by the incorporation of proteins. However, at any given temperature, the bandwidth progressively increases with increasing protein concentration. This indicates an increased mobility of the acyl chains in the liquid-crystalline phase resulting from the presence of protein.

Head-Group and Protein Bands. A broad band is observed near 1740 cm^{-1} resulting from the C=O stretching modes of the myristoyl ester groups. It is comprised of two principle components, which have recently been assigned (Bush et al., 1980) to the carbonyl stretching modes of the *sn*-1 (1741 cm^{-1}) and *sn*-2 (1721 cm^{-1}) acyl chains in DPPC.

The temperature dependence of this band in the spectra of multilamellar DMPC and the 50:1 DMPC–glycophorin complex is shown in Figure 9. The DMPC spectra fall into three groups, those characteristic of the gel phase below T_p, those characteristic of the gel phase between T_p and T_m, and those characteristic of the liquid-crystalline phase, with the spectrum changing abruptly at each transition.

The C=O stretching spectrum of the 50:1 DMPC–glycophorin complex at the lowest temperature (~5 °C) resembles that of the hexagonally packed DMPC. This was confirmed by measuring bandwidths and the positions of the peak maxima, both of which were in close agreement between the two systems.

When the temperature is increased, in the band contour progressively changes toward that observed in the liquid-

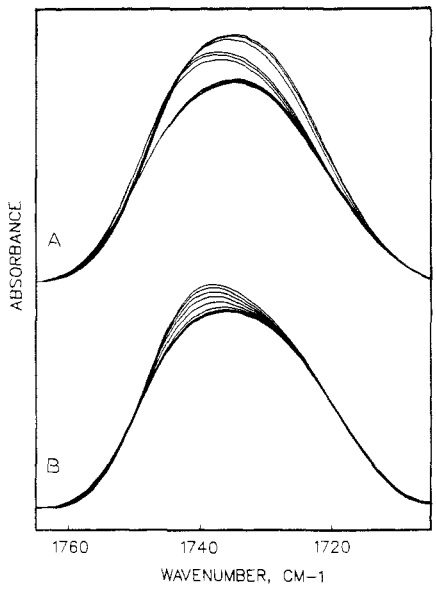

FIGURE 9: C=O stretching bands in the infrared spectra of DMPC multibilayers (A) and the 50:1 DMPC–glycophorin complex (B). A sloping base line from 1765 to 1705 cm^{-1} has been subtracted from each spectrum in order to permit direct comparison. Spectra decrease in peak height with increasing temperature and are plotted in the range 5–40 °C in approximately 3.5 °C intervals.

crystalline phase of pure DMPC. Above 25 °C, the bandwidths and peak maxima are once again identical with those of DMPC at the same temperature.

The above changes appear to solely reflect the changes in the acyl chain packing and conformational order, with the changes in the spectrum of the DMPC–glycophorin complex merely being an expanded version of the changes observed in pure DMPC and reflecting the noncooperative nature of the melting in the complex. There is no evidence of a specific interaction of the carbonyl groups with the protein. These data may be compared with those observed in lipid–cholesterol systems, where large changes in band contours and absolute values of other spectral parameters are observed (Umemura et al., 1980).

Finally, a number of other bands resulting from the protein (1650–1550 cm^{-1}), the phosphate (~1240 and 1100 cm^{-1}), and choline (~970 cm^{-1}) groups were also monitored. Since no significant variations were observed with temperature or protein concentration, the data are not shown.

Discussion

Prior to detailed discussion of the data, the following question must be addressed: what effect would two distinct populations of phospholipid molecules have on the vibrational spectrum? The coexistence of an ordered boundary population along with a disordered bulk phase should result in the appearance of a discrete set of bands for each population (or conformer), provided that the rate of exchange between populations is less than the time scale of the vibrational spectroscopic experiment (10^{-13} s). In practice, the spectral parameters used to identify each conformer are similar and, hence, experimentally difficult to resolve. However, if the observed contour is a composite, it will respond to changes in one or both of the component bands. Thus, if there were two distinct melting phenomena occurring in separate temperature ranges, two distinct inflections should be evidenced in the spectral parameters for a given band. Examples of how sensitive a monitor such as an infrared spectral parameter can be are found in studies of natural phosphatidylethanolamines where two transitions, a gel to liquid-crystalline and liquid-crystalline to inverted hexagonal phase transition, are observed (Mantsch et al., 1981). On the other hand, if most of the lipid were immobilized, we should expect to observe an increase in the temperature of transition.

Similarly considerations apply to the Raman data. In particular, the Raman intensity at 1130 cm^{-1} is characteristic of long trans segments of acyl chains. Consequently, a second melting phenomena should be evidenced by a further reduction in the intensity of this band at a temperature above T_m for pure lipid.

With regard to the question of boundary lipid, the conclusion from this study is that there is no evidence for immobilized lipid of the type reported by Lavialle et al. (1980), that is, immobilized to the extent that it undergoes a discrete transition to the liquid-crystalline phase at a higher temperature than the bulk lipid. Several experimental data point to this conclusion:

(i) The calorimetric data show a progressive broadening and shift to lower temperature of the DMPC endotherm as the amount of protein is increased and show no evidence of a second transition. These data imply that bulk lipid, at least, is disordered in the presence of protein.

(ii) The infrared data permit a more detailed examination of the phase transition without loss of the signal-to-noise ratio as the proportion of protein is increased. In none of the extremely detailed curves (Figures 7 and 8) is there evidence of a second transition. In particular, with the 50:1 DMPC–glycophorin complex, some 30 spectra were collected by using a temperature increment of 0.5 °C in the range 25–40 °C. The data show only one broad transition, with a midpoint lowered relative to that of the other systems. This can only be construed as indicating an increase in the mobility as a result of protein addition.

(iii) As shown in Figure 6, at all temperatures, the bandwidth of the symmetric CH_2 stretching band increases as the proportion of protein is increased. This indicates that the increased acyl chain mobility of DMPC is directly linked to the addition of protein. This is opposite to the behavior expected if protein were to immobilize, that is, decrease the mobility of, the acyl chains.

Apart from the question of boundary lipid, the data do provide evidence concerning the average conformation and packing characteristics of the phospholipid molecules in the presence of protein. The Raman data for the 1130 cm^{-1} band (Figure 3) and the infrared data for the symmetric CH_2 stretching frequencies (Figure 7) indicate that at 5 °C the conformational order is high in all systems, with the acyl chains being predominantly trans. However, the lateral interactions are progressively reduced and the acyl chain mobility increased relative to that in pure multilamellar DMPC, as evidenced by the I 2880/2850 Raman parameter (Figure 5) and the increased CH_2 stretching bandwidths, indicating that protein perturbs the acyl chain packing in its immediate environment.

The transition itself is progressively broadened and shifted to lower temperatures as more protein is incorporated, indicating the induction of noncooperative melting in the regions where the packing is perturbed. This is characteristic of lipid–protein samples. An even more dramatic example is observed in the DPPC–glycophorin system where the midpoint of the transition is at 18 °C (Taraschi & Mendelsohn, 1980), compared with 42 °C for pure lipid.

In the liquid-crystalline phase, the protein incorporation results in little change in the conformational order. It does, however, substantially increase the mobility of the acyl chains. Finally, there is no evidence of any strong interaction between the protein and the lipid head groups.

A comparison of the current data with previous studies of glycophorin–lipid complexes indicates agreement with the work of Brulet & McConnell (1976), who found evidence for perturbed lipids at temperatures below that of the main transition. Romans et al. (1979) concluded that there was immobilized lipid present in the liquid-crystalline phase of DMPC–glycophorin complexes, in apparent conflict with our data. Since they worked with highly unsaturated egg phosphatidylcholine at much lower lipid–protein mole ratios (5:1 to 50:1), the data may not be directly comparable. However, they did conclude that there was a "disorganization" of the bilayer structure, in accord with our observation of an increase in the mobility due to protein incorporation.

With regard to comparisons with studies of other lipid–protein complexes, the observation of a broadened transition and increased acyl chain mobility in the gel phase is in general concurrence with most results. Most such studies have also concluded that in the liquid-crystalline phase the conformational disorder is substantially increased by protein, an effect not observed in this study. However, many of the changes in parameters monitored, particularly in ESR and deuterium NMR studies, can be accounted for simply in terms of increased acyl chain mobility. Another point, which makes us hesitate to attempt detailed comparisons with other systems, is the evidence for different interactions in different lipid–protein systems (Lavaille & Levin, 1980; Lavaille et al., 1980).

Theoretical considerations suggest that the state of order in the lipid in the immediate vicinity of membrane protein is determined by the nature of the surface presented by protein to the lipid. Owicki & McConnell (1979) have shown that depending on lipid order at the interface, transition temperatures can be increased or decreased. In addition, a broadening and decreased amplitude of the phase transition are expected regardless of the direction of the transition temperature alteration. Pink & Chapman (1979) have suggested that proteins have significant probabilities for being adjacent to one another and so have called into question for the concept of an unbroken "annulus" of lipid. They have described a situation of "ii b" where protein either binds disordered lipids or presents an inhomogeneous surface to the lipid molecules. Under these conditions, the gel phase of the lipid becomes fluidized, while the fluid phase can either become more rigid or become more fluid, depending upon the strength of the interactions. This would appear to be pertinent to our observation of constant conformational order but increased mobility in both gel and liquid-crystalline phases of the lipid–protein complexes.

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Effect of Basic Compounds on the Polymerization of Clathrin†

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ABSTRACT: The effects of several divalent cations, various polybasic amines, and lysozyme on the rate of polymerization of 8S clathrin to the 300S coat structure have been evaluated by turbidimetric procedures. Ca^{2+} and Mn^{2+} strongly enhance the rate of polymerization. Only spermine among the naturally occurring polybasic amines had an important effect. Of the several basic proteins evaluated, only lysozyme stimulated the rate of polymerization. Some of these substances were able to increase the rate sufficiently so that polymerization occurred at physiological pH values. Without these compounds, clathrin will only polymerize at pH values of 6.8 or less.

The coated pit regions of plasma membranes have been reported to be the site of endocytosis of lipoproteins (Goldstein et al., 1979) and glycoproteins (Wall et al., 1980), as well as peptide (Schlessinger et al., 1978; Maxfield et al., 1978; Fitzgerald et al., 1980; Salisbury et al., 1980) and nonpeptide (Cheng et al., 1980) hormones. Coated vesicles have been shown to be involved in the secretion of glycoproteins (Rothman et al., 1980) and in membrane exchange processes (Heuser & Reese, 1973). The coated pit regions of membranes and coated vesicles are readily identifiable by electron microscopy since they appear as very characteristic polygonal structures (Kanaseki & Kadota, 1969; Heuser, 1980). The coat protein which is responsible for this appearance has been isolated by Pearse and called clathrin (Pearse, 1975, 1976, 1978). Purified preparations of clathrin have been shown to be capable in the absence of membrane of re-forming the coat structure by self-association (Pearse, 1978; Schook et al., 1979; Keen et al., 1979; Woodward & Roth, 1978, 1979; Nandi et al., 1980; Pretorius et al., 1981). The polygonal coat structure (devoid of membrane) will be referred to as baskets.

Although the endocytotic process resulting in the pinching off of coated pits into vesicles is now well documented, very little is known of the cellular regulation of this process. It is possible that regulation could be controlled either by modifying the structure of clathrin (or other proteins) in coated pits or by interfering with the recycling of clathrin from its soluble, cytoplasmic form to its network structure in pit regions of the membrane. It has been postulated by Kanaseki & Kadota (1969) that the formation of curvature in the coats of pit regions results from the introduction of pentagons into the hexagonal structure of coated pits. Thus, a rearrangement of clathrin interactions could lead to the formation of coated vesicles from pits. Heuser (1980) has presented electron microscopical evidence that this type of geometrical switch occurs in coated pit regions of membranes.

We have reported that the rate of polymerization of protomeric clathrin, i.e., 8 S, to form baskets, i.e., 300 S, can be conveniently followed by light scatter or absorption measurements (Van Jaarsveld et al., 1981). In this paper, we present the results obtained with three types of basic molecules, (a) divalent bases (cations), (b) polybasic amines, and (c) a basic protein, which accelerate the rate of clathrin polymerization.

Materials and Methods

Materials

$CaCl_2$, $MgCl_2$, and $MnCl_2$, and ammonium acetate were certified grade and supplied by Fisher Scientific Co. Putrescine, cadaverine, dansylcadaverine, spermidine, and spermine were purchased from Sigma Chemical Co. and used without further purification. Lysozyme was obtained from Miles Laboratories. It was dialyzed to remove salt and lyophilized.

Methods

Preparation of Clathrin. Bovine brains were used for preparing clathrin by a procedure described previously (Nandi et al., 1980) which is a mofidication of the method of Schook et al. (1979). The 8S protomer of clathrin is obtained by extracting a crude vesicle preparation with 0.05 M Tris (pH 8.0) followed by ammonium sulfate precipitation (30%) and gel chromatography on Sepharose 4B-Cl in 0.02 M Tris, 0.25 M NaCl, and 5 mM NaN_3 (pH 8.0). Three distinct peaks are obtained in the gel chromatographic separation. The major band (peak II) contains 8S clathrin. This peak was analyzed routinely by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Pretorius et al., 1981) in order to check the uniformity of the polypeptide composition of the 8S clarthrin preparation. In all cases, about 80–90% of the total protein migrated with the mobility of clathrin.

Measurement of Polymerization Rates. In order to follow the rate of polymerization of clathrin, we have used light scatter measurements as described previously (Van Jaarsveld et al., 1981). A Brice Phoenix Universal light scatter pho-

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