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A DIFFERENCE INFRARED SPECTROSCOPIC STUDY OF GRAMICIDIN A, ALAMETHICIN AND BACTERIORHODOPSIN IN PERDEUTERATED DIMYRISTOYLPHOSPHATIDYLCHOLINE

DAVID C. LEE, AZIZ A. DURRANI and DENNIS CHAPMAN

Department of Biochemistry and Chemistry, Royal Free Hospital School of Medicine (University of London), Rowland Hill Street, London NW3 2PF (U.K.)

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Difference infrared spectroscopy has been used to study the way in which the intrinsic molecules gramicidin A, alamethicin and bacteriorhodopsin perturb their environment when present within a lipid bilayer structure. Dimyristoylphosphatidylcholine containing perdeuterated chains has been used to enable the lipid chain C–^{2}H stretching absorption band to be separated from the C–H bands arising from the intrinsic polypeptide or protein. The C–^{2}H stretching bands of the phospholipid are sensitive to two different types of chain conformation. The C–^{2}H stretching frequency provides information about the static order of the lipid chains, whilst the half-maximum bandwidth provides a measure of chain librational and torsional motion. From the measurements it is concluded that: (1) Above the lipid phase transition temperature t_c, low concentrations of either gramicidin A or alamethicin cause a small decrease in lipid chain *gauche* isomers whilst bacteriorhodopsin in the lipid bilayer has no effect. At higher concentrations each intrinsic molecule causes an increase to occur in lipid chain *gauche* isomers. (2) The lipid acyl chain motion, as deduced from the bandwidths is increased by the presence of a low concentration of gramicidin A within the lipid bilayer. The presence of the other intrinsic molecules studied have little effect. A higher concentration of alamethicin causes a decrease in chain motion whilst gramicidin A and bacteriorhodopsin have no effect. (3) Below t_c each of the intrinsic molecules when present in the lipid bilayer causes an increase in *gauche* isomers to occur as well as an increase in the lipid chain motion. A broadening of the lipid phase transition occurs as the concentration of the polypeptide increases.

Introduction

The interactions which may exist between membrane lipids and intrinsic proteins and the degree to which intrinsic proteins can perturb a lipid bilayer structure have been the subject of much discussion and many studies [1]. Various spectroscopic probe techniques have been used, e.g. fluorescence, electron spin resonance and nuclear magnetic resonance probes. The degree to which electron spin resonance and fluorescent probes in particular are able to mimic and at the same time to perturb the lipid environment is, however, uncertain. Non-perturbing techniques such as deuterium or phosphorus NMR spectroscopy are more reliable. Deuterium magnetic resonance studies have shown that the effect of intrinsic proteins on the lipid chain conformation (above the main lipid

Abbreviations: DMPC-d_{54}, 1,2-diperdeuteriomyristoyl-*sn*-glycero-3-phosphocholine; NMR, nuclear magnetic resonance; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid: t_c, onset temperature of the gel to liquid-crystalline phase transition.

transition temperature, t_c) differs from that observed with cholesterol [2,3].

Difference infrared spectroscopy and Fourier transform infrared spectroscopy provide new powerful non-perturbing techniques operating on an entirely different time scale than NMR spectroscopy for studying the conformation of membrane lipids and intrinsic proteins. This paper concerns the study of lipid-polypeptide and lipid-protein interactions and uses difference infrared spectroscopy to analyse them.

The proportion of *gauche* to *trans* conformer and therefore the static order of lipid acyl chains can be measured by shifts in the methylene C–H asymmetric and symmetric stretching frequencies [4–7]. Fourier transform infrared spectroscopy has been used to monitor lipid and intrinsic protein conformational changes in reconstituted systems [8–10]. The view that intrinsic proteins and gramicidin A differ from cholesterol in their interaction with membrane lipids has been supported by Cortijo et al. [6,7] using difference infrared spectroscopy.

The presence of high intrinsic protein concentrations within the lipid bilayer structure introduces considerable amino acid side chain contribution to the C–H bands. High frequency shoulders on the C–H stretching bands were indeed attributed by these workers to the high levels of intrinsic protein present.

For this study we have therefore synthesised dimyristoylphosphatidylcholine-d_{54} in which the acyl chain hydrogen atoms are completely substituted by deuterium atoms. Gramicidin A, alamethicin and bacteriorhodopsin have been reconstituted in this lipid, enabling us to study acyl chain C–^{2}H stretching frequencies without interference from amino acid side chains at high protein concentration. The advantages of using perdeuterated or specifically deuterated phospholipid derivatives in infrared spectroscopy of biological membranes and reconstituted systems has been demonstrated by Mantsch et al. [8,10,11].

Difference infrared spectroscopy can also be used to monitor changes in protein conformation [7]. The structure of the reconstructed bacteriorhodopsin protein has been studied above and below the main lipid phase transition t_c of the deuterated lipid. Difference spectra were generated and used to study conformational changes in the protein structure.

Materials and Methods

Myristic acid-d_{27} was purchased from Larodan Chemicals (Sweden). Dimyristoylphosphatidylcholine-d_{54} was synthesised according to Johnston et al. [12]. Its purity was checked by thin-layer chromatography and differential scanning calorimetry. Gramicidin A was purchased from Sigma Chemical Company (Poole, U.K.). Alamethicin was purchased from Centre for Applied Microbiology and Research (Porton, U.K.). The polypeptides were used without further purification. *Halobacterium halobium* was grown on a complex medium described by Oesterhelt and Hartman [13] and purple membrane purified according to the method of Oesterhelt and Stoeckenius [14]. Two purification steps were carried out in sucrose gradients.

The buffer used for infrared spectroscopic measurements was 10 mM Hepes, 50 mM sucrose, 1 M KCl dissolved in H_2O or ^2H_2O and adjusted to pH 7.0 or pH 6.6, respectively, at 20°C. It will be referred to from now on as the standard buffer.

DMPC-d_{54}/ gramicidin A or DMPC-d_{54}/ alamethicin liposomes were prepared by taking stock solutions containing the required amount of lecithin and polypeptide in benzene/methanol (95 : 5, v/v), mixing and freeze-drying for 6 h. The dry powder formed was dispersed in the standard buffer at 30°C for 30 min with vortexing. Final lipid concentrations were between 15 and 30 mg/ml standard buffer.

Reconstitution of bacteriorhodopsin was carried out as described previously [15]. Lipid analysis was performed by gas-liquid chromatography as in Ref. 16. Protein content was analysed by the method of Lowry et al. [17]. Infrared spectroscopic measurements wre carried out as in Ref. 7. Ten scans were computer averaged in the region 2250 cm^{-1}–2050 cm^{-1} using a Perkin-Elmer 3600 Data Station linked to a Perkin-Elmer IR681 Spectrometer. For protein studies the scans were averaged over the region 1800 cm^{-1} to 1500 cm^{-1}. In all cases the maximum noise suppression of the instrument was used together with the wide slit-width to reduce scanning time. The band maxi-

mum frequencies could be measured with an error of (± 0.25 cm^{-1}). Spectra were not smoothed prior to band measurement.

Details of the Data Station software routines used in spectra analysis are given in Ref. 7.

Results

Fig. 1 shows the difference infrared spectra of DMPC-d_{54} in the region of the C–^{2}H stretching frequencies after subtraction of the standard ^2H_2O buffer spectrum. The bands observed can be assigned as C^2H_3 asymmetric stretching at 2212 cm^{-1} (shoulder), C^2H_2 asymmetric stretching at 2191 cm^{-1}, C^2H_3 symmetric stretching at 2153 (weak) and 2070 cm^{-1} (shoulder) and C^2H_2 symmetric stretching at 2088 cm^{-1} in agreement with Ref. 11. As previously observed for C^2H_2 bands the band maximum frequencies and half-maximum bandwidths of the C^2H_2 bands increase sharply as the temperature of the sample increases through the gel to liquid-crystalline phase transition (Figs. 1 and 2). Plots show the variation of C^2H_2 symmetric band parameters; in all cases measurement of C^2H_2 asymmetric band parameters yielded similar data. Our infrared data show that the main gel to liquid-crystalline phase transition of DMPC-d_{54} occurs at 20.5°C, in agreement with our calorimetric measurements.

The effect on the phase transition of cholesterol incorporation into the liposomes (at 1 : 1 lipid/protein molar ratio) is shown in Fig. 2. The plot of C^2H_2 symmetric band frequency (Fig. 2a) confirms previous observations [3,7] that below t_c cholesterol causes an increase in the number of *gauche* conformers while above t_c there is a de-

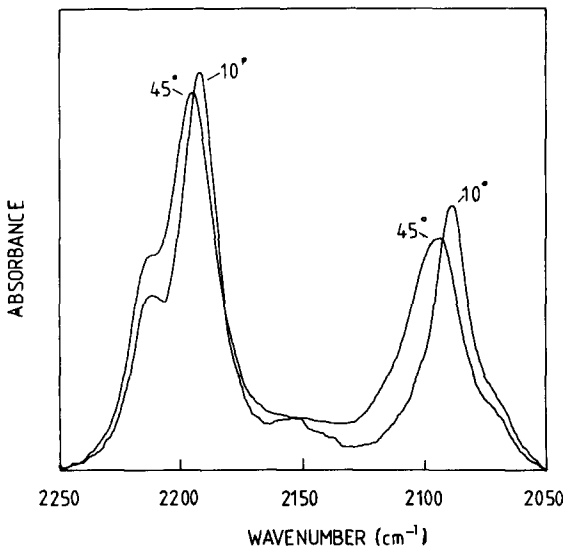

Fig. 1. Difference infrared spectra of DMPC-d_{54} dispersed in the ^2H_2O buffer minus the buffer at 10°C and 45°C. Abex 40.

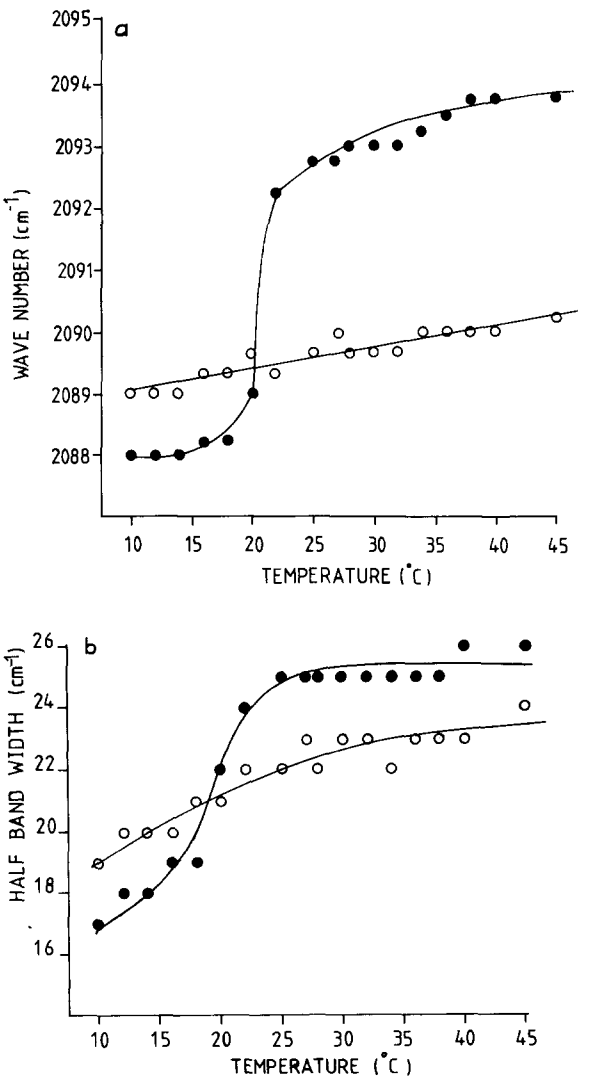

Fig. 2. (a) Temperature dependence of the maximum wavenumber of the C^2H_2 symmetric stretching vibrations in (●) DMPC-d_{54} and (○) DMPC-d_{54}/cholesterol 1 : 1 molar ratio. (b) Temperature dependence of the C^2H_2 symmetric stretching vibration bandwidth at half-maximum intensity as in (a).

crease in these isomers compared with the pure lipid. The C^2H_2 symmetric bandwidth, measured as full width at half-maximum intensity, varies with temperature as shown in Fig. 2b. This parameter reflects acyl chain effects which do not involve an increase in *gauche* conformers such as increases in librational and torsional motion [18]. The introduction of high levels of cholesterol into the bilayers increases such motion below t_c and decreases motional freedom above t_c (Fig. 2b).

Fig. 3 shows the effect incorporation of gramicidin A into the bilayers has on the C^2H_2 symmetric stretching frequency and bandwidth. Below the main lipid phase transition temperature the presence of gramicidin A causes an increase in *gauche* conformers within the lipid acyl chains (Fig. 3a). However, above t_c both the 50 : 1 and 5 : 1 lipid/polypeptide ratios have similar amounts of *gauche* isomers to that of the pure lipid. There is some indication that just above t_c there is a decrease in *gauche* conformers at a 50 : 1 ratio and an increase in these conformers at a 5 : 1 ratio. (Experimental error is ± 0.25 cm^{-1} under our conditions). This effect is removed on heating above 35°C. Bandwidth measurements (Fig. 3b) indicate that below t_c gramicidin A causes an increase in the motional freedom of the lipid acyl chains. Motional freedom is increased as the concentration of the polypeptide is increased to a lipid/polypeptide ratio of 5 : 1. When above t_c and at a lipid/polypeptide ratio of 50 : 1, gramicidin A causes a slight increase in the motional freedom of the lipid acyl chains. As the polypeptide concentration is increased to a 5 : 1 lipid/polypeptide ratio, acyl chain motional freedom becomes similar to the pure lipid.

The measured band parameters confirm previous observations of C–H stretching frequencies [7]: that incorporation of cholesterol or low levels of gramicidin A decreases the phase co-operativity of the main transition while the t_c value is unaltered. At higher levels of gramicidin A there is a decrease in t_c in addition to the decrease in phase co-operativity. The effect of alamethicin on the C^2H_2 symmetric band parameters is shown in Fig. 4. Alamethicin has similar properties to gramicidin A in its perturbation of the lipid bilayer. Above t_c there is a decrease in the proportion of *gauche* conformers at 50 : 1 lipid/polypeptide compared

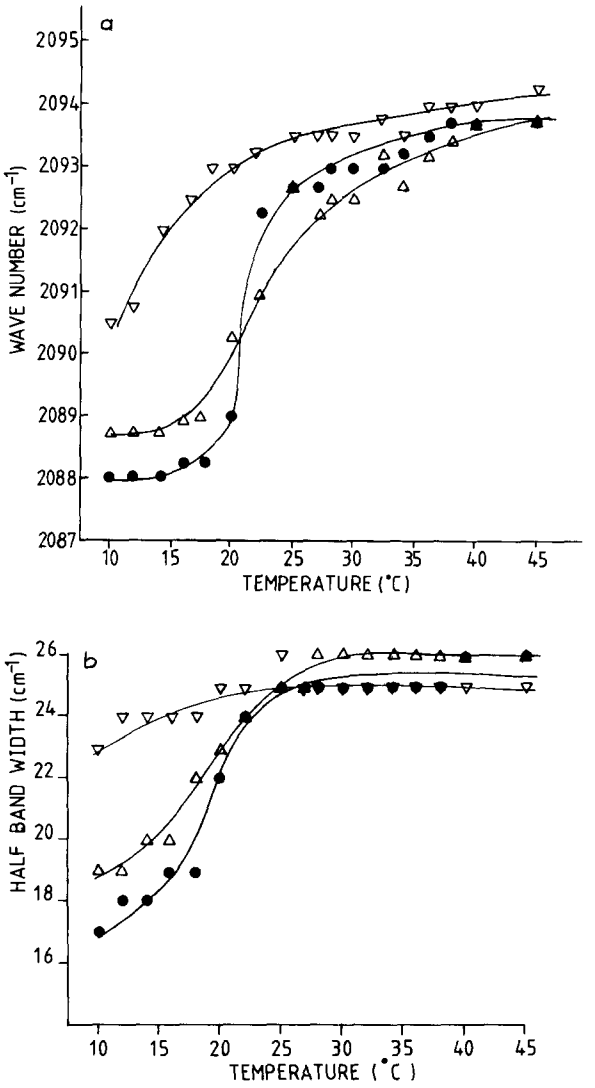

Fig. 3. (a) Temperature dependence of the maximum wavenumber of the C^2H_2 symmetric stretching vibrations in (△) DMPC-d_{54}/gramicidin A 50 : 1 and (▽) DMPC-d_{54}/gramicidin A 5 : 1 compared with the pure lipid (●). (b) Temperature dependence of the C^2H_2 symmetric stretching vibration bandwidth at half-maximum intensity as in (a).

with pure lipid, while at 5 : 1 molar ratio the proportion of *gauche* conformers is markedly increased. Below t_c there is a large increase in the proportion of *gauche* conformers for the 5 : 1 molar ratio. At a lipid polypeptide ratio of 50 : 1 any differences in the proportion of *gauche* isomers are within experimental error. Incorporation of alamethicin into the bilayers at 50 : 1 lipid/protein

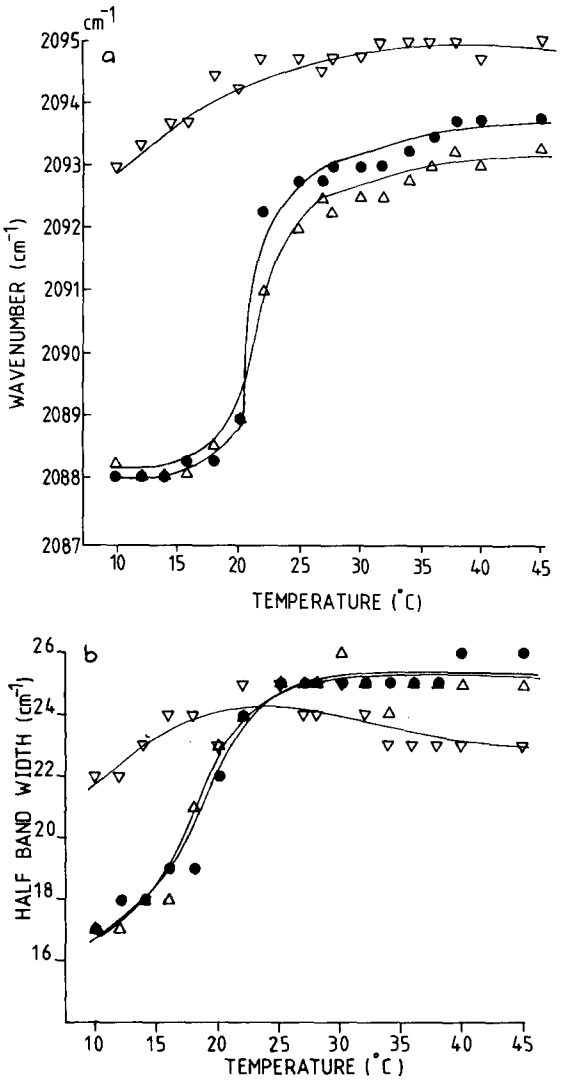

Fig. 4. (a) Temperature dependence of the maximum wavenumber of the C^2H_2 symmetric vibrations in (△) DMPC-d_{54}/alamethicin 50:1 and (▽) DMPC-d_{54}/alamethicin 5:1 compared with the pure lipid (●). (b) Temperature dependence of the C^2H_2 symmetric stretching vibration bandwidth at half-maximum intensity as in (a).

molar ratio has little effect on t_c and the phase co-operativity is only slightly reduced. Fig. 4b shows the effect of alamethicin on the C^2H_2 symmetric bandwidth of DMPC-d_{54}. For the 50:1 lipid/polypeptide ratio there is no difference from the pure lipid system. However, at a 5:1 ratio there is an increase in vibrational motion of the chains below t_c and a decrease in such motion above t_c, compared with the pure lipid.

The effects observed upon incorporation of the intrinsic protein bacteriorhodopsin into the lipid bilayer are shown in Fig. 5 (these experiments were performed in standard H_2O buffer). Fig. 5a shows that below t_c the proportion of *gauche* conformers is increased compared with the pure lipid for each protein concentration. Above t_c there is little or no effect at a lipid/protein ratio of 102:1.

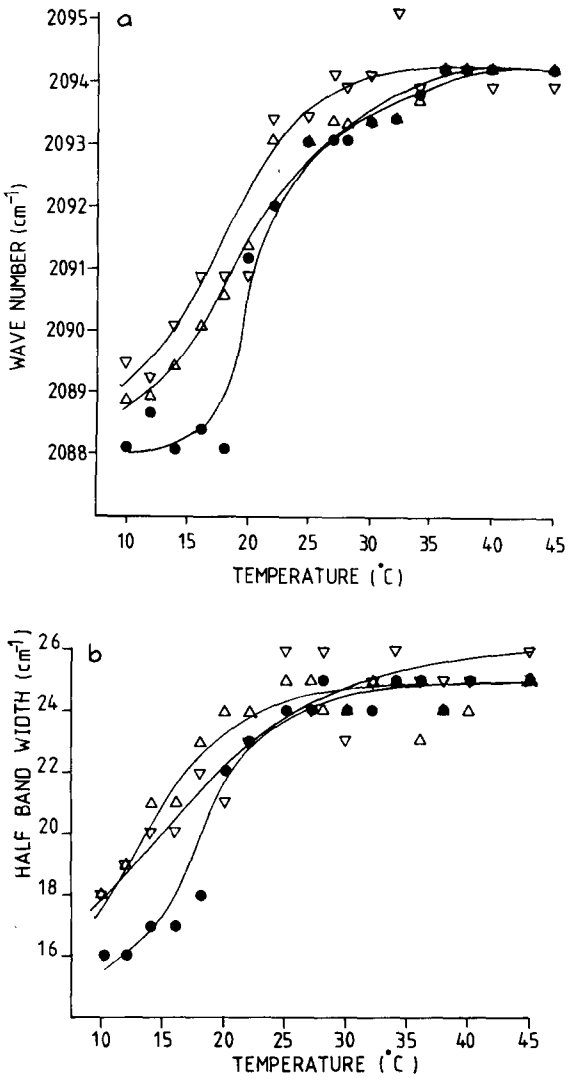

Fig. 5. (a) Temperature dependence of the maximum wavenumber of the C^2H_2 symmetric stretching vibrations in (△) DMPC-d_{54}/bacteriorhodopsin 102:1 and (▽) DMPC-d_{54}/bacteriorhodopsin 22:1 compared with the pure lipid (●). (b) Temperature dependence of the C^2H_2 symmetric stretching vibration bandwidth at half-maximum intensity as in (a).

At 22 : 1 the differences are small but there is some evidence for a slight increase in the proportion of *gauche* conformers just above t_c. There is an increase in vibrational motion of the chains for each lipid : protein ratio compared with pure lipid below t_c (Fig. 5b). However, above t_c the difference is not significant. The presence of bacteriorhodopsin makes the main lipid phase transition less sharp and reduces the t_c value at a lipid/protein ratio of 22 : 1.

A difference spectrum obtained by subtracting a spectrum of DMPC-d_{54}/ bacteriorhodopsin 102 : 1 at 16°C from a spectrum of the same sample at 36°C is shown in Fig. 6. The difference spectrum is obtained in the frequency range where the absorption bands corresponding to the lipid C=O stretching, amide I and amide II bands occur. Positive bands indicate an increase in intensity of a particular band at that frequency. The major positive bands seen occur at 1683 cm^{-1} and 1616 cm^{-1} on the high and low frequency side of the protein amide I band. Major negative bands occur at 1700 cm^{-1} and 1636 cm^{-1}. Noise levels are $\pm$ 0.001 absorbance units. The results suggest that a protein conformational change may be occurring when the lipid is heated above the main lipid phase transition temperature t_c of 20.5°C. A difference infrared spectroscopic study of the purple membrane over this temperature range does not show any similar spectral change.

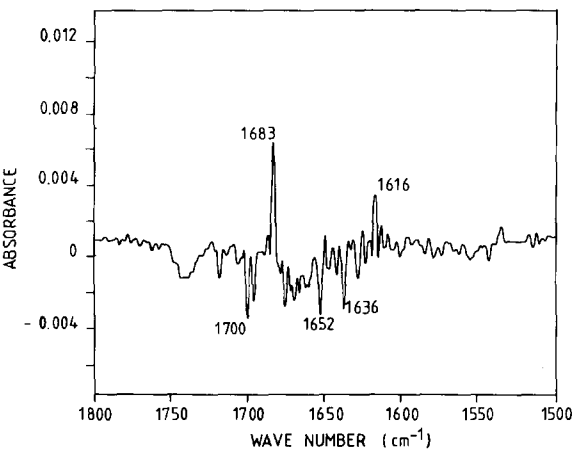

Fig. 6. Difference infrared spectrum of DMPC-d_{54}/ bacteriorhodopsin 102 : 1 at 36°C minus the spectrum obtained at 16°C. Difference spectra were obtained by subtracting standard buffer reference spectra at 36°C and 16°C from the sample spectra.

Discussion

Our observations of an abrupt shift in the asymmetric and symmetric stretching frequencies as a result of the endothermic lipid phase transition are in agreement with previous studies of C–H [6–8] and C–^{2}H bands [10,11,18]. The increase in frequency which occurs above the lipid phase transition temperature is attributable to an increase in the proportion of *gauche* conformers in the lipid acyl chains [5,7]. Incorporation of cholesterol into the lipid bilayer at a 1 : 1 molar ratio results in a large increase in the number of *gauche* conformers below t_c and a large decrease in the number above t_c such that the phase transition is effectively removed as in Fig. 2. This demonstrates the sensitivity of the C–^{2}H stretching frequency to the static order of the acyl chains. The C–^{2}H stretching half bandwidth is sensitive to the degree of librational and torsional motion of the lipid acyl chains [18]. We are therefore able to study the effects of high concentrations of intrinsic proteins on the static and dynamic aspects of the lipid acyl chain conformation.

Below the lipid transition temperature t_c, gramicidin A, alamethicin and bacteriorhodopsin act in a manner similar to cholesterol, i.e. there is an increase in the proportion of *gauche* conformers and an increase in librational motion of the acyl chains. This is consistent with the conclusion that all these intrinsic molecules affect the crystalline packing of the lipid thereby allowing some disorder of chains and *gauche* isomers to occur.

Above the lipid phase transition temperature t_c, our results with gramicidin A confirm previous deductions [7] that this polypeptide produced an increase in chain order at low concentration but we also observe an increase in chain disordr with respect to the pure lipid at higher concentrations.

This ordering and disordering effect is similar to that observed by ^{2}H-NMR spectroscopy [19]. Our infrared spectroscopic studies show that a similar effect is observed with the intrinsic polypeptide, alamethicin (Fig. 4a). A decrease in the number of *gauche* conformers above t_c (i.e. chain ordering), is followed by an increase in *gauche*

isomers at the higher polypeptide concentration (5 : 1). In the case of the intrinsic protein, bacteriorhodopsin (Fig. 5a), there is no evidence for an increase in lipid chain order at a high lipid/protein ratio of 102 : 1. When the concentration of protein is increased (lipid/protein 22 : 1) there is an increase in chain disorder a few degrees above t_c.

Rice and Oldfield [19] explain their data on gramicidin by suggesting that the lipid chains adjacent to the polypeptide are constrained in a twisted configuration within the crevices of the molecular surface. They propose that this gramicidin-lipid complex then presents a smooth cholesterol-like surface to the remainder of the lipids which it then orders. Pink et al. [20] give a different interpretation of the same NMR data. They consider that three populations of lipid can occur within the bilayer and these can vary with the protein concentration: (a) those not adjacent to any intrinsic protein: 'free' lipid; (b) those adjacent to a protein; (c) those touched or trapped between two or three proteins. These authors conclude that the NMR data are satisfied by the varying populations of these different lipid environments where the methyl groups of adjacent lipids are slightly more statically ordered than those of free lipids, and where the methyl groups of 'trapped' lipids are more statically disordered than those of the 'free' lipid.

A question of importance for interpreting the effects of the intrinsic polypeptides on the lipid chain order is to what extent is the bilayer structure retained when large amounts of polypeptide are present. Recent studies by Van Echtfeld et al. [21] using ^{31}P-NMR on gramicidin A/lipid/water systems suggest that gramicidin A may induce the hexagonal H_{II} phase with some lipid/water systems. This effect is thought to occur with dielaidoylphosphatidylethanolamine and dioleoylphosphatidylethanolamine at ratios of 1 : 200 and higher. It also occurs with dioleoylphosphatidylcholine when present in molar ratios of 1 : 25 or higher. This raises the possibility that high concentrations of gramicidin A in lipid/water systems consisting of L-DMPC or L-DPPC may also tend towards a hexagonal rather than a lamellar structure. These authors however indicate that L-DPPC remains in a lamellar structure in the presence of gramicidin A whilst distearoyl and the C_{22} phosphatidylcholine show an H_{II} phase. The available X-ray evidence on L-DMPC and L-DPPC with gramicidin A incorporated in the lipid bilayers show well-defined lamellar X-ray diffraction patterns with these systems.

Alamethicin is a polypeptide antibiotic which has been shown to induce voltage dependent conductance in black lipid membranes. Infrared attenuated total reflectance spectroscopy has been used to examine the interaction between alamethicin and dipalmitoylphosphatidylcholine [22]. These workers concluded that in the absence of an electric field alamethicin spans the lipid bilayer. It was further been demonstrated that voltage dependent gating involves conformational changes of inserted alamethicin molecules [23]. McIntosh et al. [24], using freeze-fracture and X-ray diffraction techniques, have shown that alamethicin increases the aqueous space between multilamellar vesicles and at the same time perturbs the hydrocarbon core of the bilayers in both the gel and lipid-crystalline state. No detailed studies have been made using deuterium NMR spectroscopy of lipid chain perturbation caused by alamethicin.

The infrared results presented here suggest that alamethicin is similar to gramicidin A in its perturbation of the bilayer. Below t_c a high concentration of alamethicin causes a decrease in chain static order and an increase in librational motion. Above t_c alamethicin produces chain ordering and then disordering as the concentration of this intrinsic molecule is increased.

Bacteriorhodopsin incorporated into lipid bilayers has been studied by a number of workers [25,26]. Various physical techniques e.g. calorimetry indicate that only at very high concentrations of the protein is the lipid phase transition broadened whilst the mid point transition temperature remains virtually unchanged [25]. It is known that below the lipid phase transition the bacteriorhodopsin monomers aggregate into a crystalline array [25]. The difference infrared spectra in the 1800–1500 cm^{-1} range suggest that a conformational change in bacteriorhodopsin occurs as a result of the lipid phase transition. Further studies are underway to define the exact nature of this change. (It is important to note that difference

spectra in this region are particularly sensitive to water vapour absorption bands.) Rothschild and co-workers [27,28] have used Fourier transform difference infrared spectroscopy of the purple membrane to detect alterations in both the retinylidene chromophore and the bacteriorhodopsin molecule during the photocycle. The infrared spectral changes which we detect in our reconstituted system do not correspond with those detected by Rothschild and co-workers.

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