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Influence of metal ions and a local anesthetic on the conformation of the choline group of phosphatidylcholine bilayers studied by Raman spectroscopy

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A Raman band assigned to the 'totally' symmetric stretching vibration of the choline C-N bonds is relatively strong and sensitive to the conformation of the choline backbone (Akutsu, H. (1981) Biochemistry 20, 7359–7366). By monitoring this Raman band, the influence of Eu^{3+}, La^{3+}, Ca^{2+} and a local anesthetic, dibucaine, on the conformation of the choline group was examined for the bilayers of dipalmitoylphosphatidylcholine and those of deuterated one at the choline methyl group ($-N(C^2H_3)_3$). NMR lanthanide-shift studies proposed that the interaction with metal ions induces a conformational change from the *gauche* to the *trans* form in the O-C-C-N^+ backbone of the choline group. However, present Raman work clearly showed that neither metal ions nor anesthetics induce such a conformational change. Therefore, a structural change in the polar group detected by ^{2}H-NMR on addition of metal ions should not include a significant conformational change in the choline group as well. Deuterated phosphatidylcholine used here was proved to be more suitable for the direct detection of the amount of the *trans* conformation by Raman spectroscopy than the nondeuterated one. The spectra of the deuterated compound in the gel and liquid-crystalline states confirmed that the *trans* conformation of the choline group does not appear at all in both states.

Introduction

The polar groups of a lipid bilayer form an interface between the hydrophobic region of the inner part of the membrane and the aqueous phase surrounding the membrane. Therefore, they would play an important role in the interaction between the membrane and other molecules. Especially, it is well-known that the polar group is the interaction site of charged molecules. Such interactions would change the physicochemical properties of the lipid bilayers and end up with the modification of the biological functions of the membrane. The elucidation of the molecular mechanism of such interactions is quite important to understand the properties of the biomembranes. Thus, the problem of charged molecules interacting with lipid bilayers has attracted much attention and a variety of methods have been employed [1]. The phosphatidylcholine bilayer is one of the most extensively investigated phospholipid membranes because phosphatidylcholine is the major phospholipid in higher organisms along with phosphatidylethanolamine. It was shown by ^{2}H-NMR [2–5], ^{1}H- and ^{31}P-NMR [6–8] and ^{14}N-NMR [9] that a structural change in the polar head-group of phosphatidylcholine was induced by the addition of either metal ion or local anesthetic to the aqueous phase. As a step to clarify the molecular mechanism of such interactions, the influence of

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charged molecules on the conformation of the choline group was investigated by the use of Raman spectroscopy in this work.

It is now established on the basis of Raman spectroscopical results [10] that the backbone of the choline group (O-C-C-N^+) mostly takes on the *gauche* conformation around the C-C bond in the bilayers in the absence of charged ions. The same was inferred from structural analyses by ^{1}H- and ^{31}P-NMR of the polar head-group of phosphatidylcholine monomers, its micelles and model compounds [8,11]. In the presence of trivalent lanthanide ions, however, it was argued on the basis of the analysis of pseudo contact shifts and relaxations induced by lanthanide ions that a structural change from the *gauche* to the *trans* conformation in the choline and phosphodiester groups took place [6,7]. A similar structural change was claimed for the polar head-group of lysophosphatidylcholine micelles as well [12].

The assignment of the Raman bands due to the stretching vibrations of the choline C-N bonds [10] has shown that the Raman band assigned to the 'totally' symmetric stretching vibration of the C-N bonds (ν_1) is relatively strong and sensitive to the choline conformation (the word 'totally' was used because of the local C_{3v} symmetry of the quarternary ammonium of the choline group. The quotation marks will be removed hereafter for simplicity). The *gauche* conformation of the O-C-C-N^+ backbone gives a band in the region from 710 to 720 cm^{-1}, while the *trans* conformation does at about 770 cm^{-1}. Therefore, the band can be used to examine the structural change in the choline group induced by lanthanide ions. The effect of the europium ion was examined in this work. The quantity of the *trans* conformation would be determined more accurately by employing phosphatidylcholine deuterated at the choline methyl groups because the key bands are expected to appear at isolated positions [10]. The effects of other metal ions and dibucaine were investigated on this substance. It has become clear that neither metal ion nor local anesthetic induces a significant conformational change in the choline group.

Materials and Methods

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) was purchased from Sigma Chemical Co. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine deuterated at the choline methyl groups (DPPC-$N(C^2H_3)_3$) was synthesized according to a modified method of the reported one [13], which was developed by Dr. J. Seelig. A mixture of 0.2 g 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine, 1 g trideuteriomethyl iodide (C^2H_3I), 1 g $KHCO_3$ and 12.5 ml methanol/chloroform (1 : 1) was incubated at 40°C for 24 h under stirring where almost 100% of phosphatidylethanolamine was converted to phosphatidylcholine. Deuterated phosphatidylcholine was purified by treatment with mixed ion-exchange resin, followed by silicic acid column chromatography. The yield of deuterated DPPC recrystallized from ethyl methyl ketone was about 100 mg. Purity was checked by silicic acid thin-layer chromatography. A part of sample used was kindly donated by Dr. J. Seelig. $EuCl_3 \cdot 6H_2O$, $LaCl_3 \cdot 7H_2O$ and $CaCl_2 \cdot 6H_2O$ were purchased from Wako Pure Chemical Industries, and dibucaine was obtained from Sigma Chemical Co.

The sample for measurements of Raman spectroscopy was prepared as follows. 10 mg of phospholipid was dispersed in 0.1 ml of either distilled water, a 0.1 M $EuCl_3$ solution (pH 5.3), a 0.5 M $EuCl_3$ solution (pH 5.0), a 2.0 M $EuCl_3$ solution (pH 4.9), a 3.0 M $CaCl_2$ solution (pH 6.2), a 2.0 M $LaCl_3$ solution (pH 3.7) or a 75.5 mM dibucaine solution (pH 6.3) at a temperature higher than the phase-transition temperature. Although pH of the $LaCl_3$ solution is quite low, it was impossible to go to a higher pH without precipitation at this concentration. The properties of phosphatidylcholine bilayers at this pH would not be different from those at neutral pH judging from the surface-area curve of DPPC [14]. The lipid dispersion was centrifuged by a Tomy Seiko refrigerated centrifuge MR-15A at 12000 rpm for 30 min. The pellet was transferred to a capillary tube and it was sealed. Raman spectra were taken with a JEOL JRS-400 D laser Raman spectrometer with excitation of an argon ion laser line at 488.0 nm. The power of the laser beam was 200–300 mW. The slitwidth was 250–300 μm. The frequency calibration was done using calibrated Raman lines of indene. The temperature of a sample was controlled by a copper sample holder, which is in direct contact with thermopanels connected to a

controller, Model DR-610B from Komatsu Electronics. The temperature of the sample holder was monitored by a thermistor. The temperature difference between the sample and the sample holder was less than 1 K judging from the phase-transition temperature of DPPC bilayers observed by Raman spectroscopy. Raman spectral data were processed by a NEC PC-9801E personal computer and data-processing programs developed by Dr. H. Sugeta.

Results

Lanthanide ions were used in the previous NMR analyses which suggested a structural change in the choline group. Therefore, europium ions have been employed to see their effect on the Raman spectrum of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) bilayers. The Raman spectra of DPPC from 600 to 1400 cm^{-1} are presented in Fig. 1A and B for the gel and liquid-crystalline states, respectively. The state of DPPC bilayers can be identified from the Raman bands at 1064 and 1128 cm^{-1} which were assigned to the C-C stretching vibration mode of the hydrocarbon chain backbone in the *trans* conformation [15]. The Raman bands associated with the choline conformation appear in the region from 700 to 800 cm^{-1} [10]. As pointed out in our previous paper, a strong Raman band at 717 cm^{-1} in the absence of Eu^{3+} (A(1) and B(1)) shows that most of the choline groups take on the *gauche* conformation in DPPC bilayers in the liquid-crystalline state as well as in the gel state. Although there is a weak band at around 770 cm^{-1}, it is difficult to say whether it comes from the choline group in the *trans* conformation or from other chemical groups of DPPC. Anyhow, if a structural change from the *gauche* to the *trans* conformation is induced in the choline group by the interaction with the europium ions, the Raman band at 717 cm^{-1} is expected to be weakened on addition of the europium ions to the aqueous phase. Up to 2.0 M $EuCl_3$, however, no drastic change was detected in the intensity of the Raman band at 717 cm^{-1} not only in the gel state but also in the liquid-crystalline state (in Fig. 1). To present it quantitatively, the Raman band at 1300 cm^{-1} which was assigned to the CH_2 twisting vibration of hydrocarbon chains [16], was taken as an internal reference. The band is sensitive to the state of the hydrocarbon chains but is expected to be relatively insensitive to the confor-

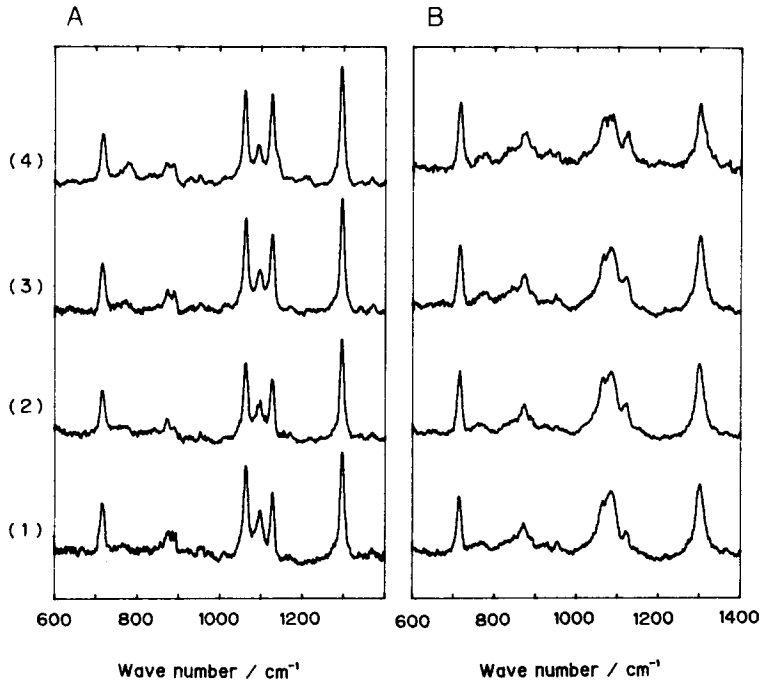

Fig. 1. Raman spectra of DPPC bilayers in the absence and presence of $EuCl_3$. (A) In the gel state; (B) in the liquid-crystalline state. (1) Distilled water (A, 25°C; B, 60°C); (2) 0.1 M $EuCl_3$ (A, 25°C; B, 60°C); (3) 0.5 M $EuCl_3$ (A, 25°C; B, 70°C) and (4) 2.0 M $EuCl_3$ (A, 25°C; B, 70°C).

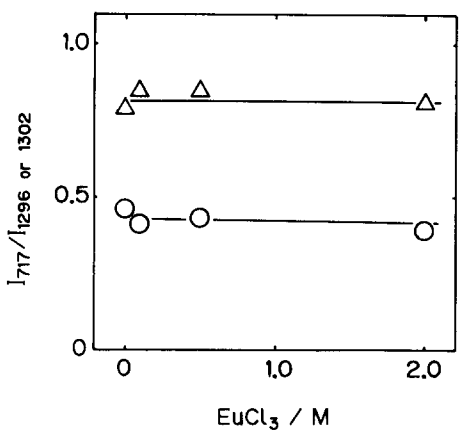

Fig. 2. Intensity ratio of the band at 717 cm^{-1} to that at 1296 (gel) or 1302 (liquid-crystalline) cm^{-1} as a function of $EuCl_3$ concentration. ○, In the gel state; △, in the liquid-crystalline state.

mation of the polar head-group. The ratio of the band at 717 cm^{-1} to that at 1300 cm^{-1} is shown in Fig. 2 as a function of the europium ion concentration. No change is seen within the experimental errors both in the gel and liquid-crystalline states up to 2.0 M $EuCl_3$. Judging from ^{2}H-NMR studies the structural change induced by La^{3+} is almost saturated at 2.0 M $LaCl_3$ [3]. ^{14}N-NMR studies showed that the efficiencies of La^{3+} and Eu^{3+} in the interaction with the polar group are similar to each other [9]. Therefore, the structural change induced by Eu^{3+} is expected to have been saturated at 2.0 M $EuCl_3$. Accordingly, the result in Fig. 2 strongly suggests that the interaction between the polar head-group and europium ion does not induce such a structural change as to the *trans* conformation in the choline group.

To elucidate the nature of the structural change detected by ^{2}H-NMR, the effects of cations which had been used in the ^{2}H-NMR studies [3,4] were further examined. For that purpose, the method mentioned above is not adequate since the band of internal reference was obscured by the Raman bands of an anesthetic molecule used. Furthermore, if the bands both due to the *trans* and *gauche* conformations are detected as isolated ones, they can provide more quantitative and solid information. The Raman band of the totally symmetric stretching vibration of the C-N bonds of the choline iodide deuterated at the methyl groups ($HO\text{-}CH_2\text{-}CH_2\text{-}N(C^2H_3)_3$) appears at 672 and 708 cm^{-1} in the *gauche* and *trans* conformations, respectively [10]. Since there are no other bands in the regions mentioned above in the spectrum of DPPC, these two bands should be adequate for the determination of the amount of each conformation in the DPPC bilayers.

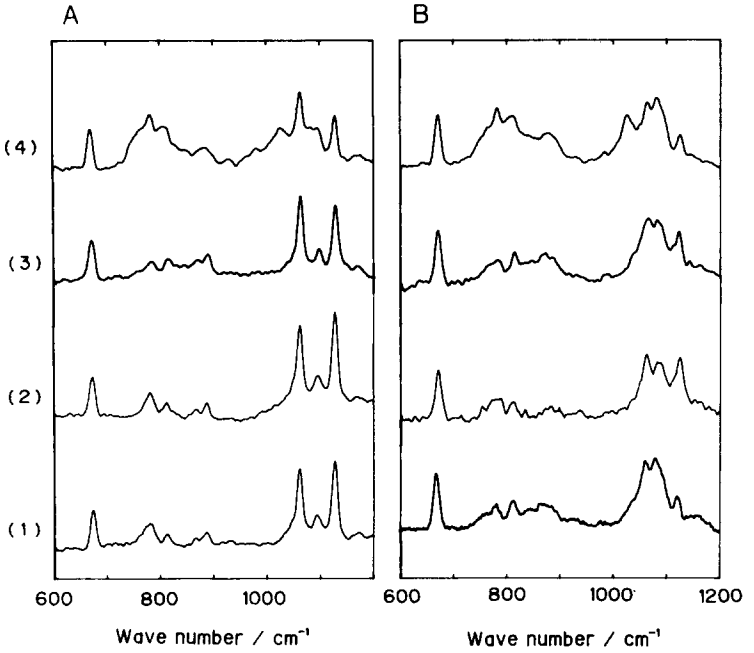

Fig. 3. Raman spectra of DPPC-$N(C^2H_3)_3$ bilayers in the absence and presence of metal ions or local anesthetic. (A) In the gel state; (B) in the liquid-crystalline state. (1) Distilled water (A, 10°C; B, 50°C); (2) 2.0 M $LaCl_3$ (A, 25°C; B, 70°); (3) 3.0 M $CaCl_2$ (A, 25°C; B, 70°C) and (4) 75.5 mM dibucaine (A, 20°C; B, 45°C).

The Raman spectra of the bilayers of DPPC deuterated at the choline methyl groups (DPPC-$N(C^2H_3)_3$) in the gel and liquid-crystalline states are displayed for the region from 600 to 1200 cm^{-1} in A(1) and B(1) of Fig. 3, respectively. In the former, strong bands at 1064 and 1128 cm^{-1} due to the C-C stretching vibrations of the hydrocarbon chains in the *trans* conformation evidence the gel state of the bilayers. A rather strong band at 672 cm^{-1} can be assigned to the C-N totally symmetric stretching vibration in the gauche conformation because the Raman band was observed at the same position in the spectrum of choline-$N(C^2H_3)_3$ iodide. Thus, the C-N totally symmetric stretching band for the *trans* conformation is expected to appear at around 708 cm^{-1} in the spectrum of DPPC-$N(C^2H_3)_3$ as well. Neither the spectrum A(1) nor B(1) of Fig. 3 displays a band in the region of interest. Now we can definitely say that the *trans* conformation of the choline group is not present at all both in the gel and liquid-crystalline states of DPPC bilayers.

The effects of 2.0 M $LaCl_3$, 3.0 M $CaCl_2$ and 75.5 mM dibucaine were examined in Fig. 3 (2), (3) and (4), respectively, for the gel and liquid-crystalline states. Judging from ^{2}H-NMR results [3,4], these concentrations are high enough to almost saturate the cation binding to the polar head-group. However, there is no sign of the trans band in any spectrum of Fig. 3. Additional Raman bands in the spectra of Fig. 3A(4) and B(4) come from dibucaine (the spectrum of dibucaine was measured as well but is not shown here). Now it can be concluded that the structural change in the polar head-group detected by ^{2}H-NMR does not include such a drastic change in the choline group as that from the *gauche* to the *trans* conformation.

Discussion

It is verified in this work that the Raman band due to the totally symmetric stretching vibration of the choline C-N bonds is a powerful tool to investigate the conformation of the choline group in lipid bilayers. Especially, phosphatidylcholine deuterated at the choline methyl groups is shown to be very useful, because the totally symmetric stretching bands both for the *gauche* and *trans* conformations do not overlap with other bands. It gives explicit evidence on generation of the *trans* conformation of the choline group.

On the basis of this principle, it is now established that all choline groups in the DPPC bilayers take on the *gauche* conformation in the liquid-crystalline state as well as in the gel state. Furthermore, neither metal ions nor local anesthetics can induced a change to the *trans* conformation through the interaction with the polar head-group. This conclusion does not preclude the possibility that such interaction induces a small change in the dihedral angle of the O-C-C-N^+ backbone. Bush et al. observed a shift from 711 cm^{-1} to 717 cm^{-1} on addition of water molecules to anhydrous DPPC at the ratio of 2 : 1 [17]. This shift could be associated with a change in the dihedral angle. Although such a large shift was not observed in our case, a small shift cannot be ruled out because of its broad bandwidth and low resolution.

There were arguments that the *gauche* conformation of the O-C-C-N^+ system would be stabilized by the electrostatic interaction between the oxygen and the positively charged nitrogen [18,19] and that the electrostatic repulsion between the cation and the positively charged nitrogen would induce a structural change in the choline group [12]. The results mentioned above, however, showed that any electrostatic interaction between cations and the polar head-groups does not induce a structural change from the *gauche* to the *trans* conformation in the choline group. The crystal structure of choline phosphate calcium chloride tetrahydrate [20] is consistent with our results. Despite the presence of the highly charged calcium and chloride ion matrix, the O-C-C-N^+ backbone assumes the *gauche* conformation in this structure. These facts confirm the idea that the *gauche* conformation of the O-C-C-N^+ backbone is very stable in comparison with its *trans* conformation. Furthermore, they suggest that the bond interactions rather than the through-space electrostatic interactions are responsible for the stabilization of the *gauche* conformation. Thus, the major reason for the stabilization of the *gauche* conformation should be ascribed to a more general mechanism which causes the so-called *gauche* effect in many structurally similar molecules, both organic and inorganic, containing pairs of electronegative atoms or highly polar bonds [3,21]. INDO-SCF-

MO calculations showed that the *gauche* conformation is stabilized by vicinal interactions between orbitals of bond and anti-bond types [22].

^{2}H-NMR studies showed that the quadrupole splittings at the α (O-C^{2}H$_2$-CH$_2$-N$^+$) and β (O-CH$_2$-C^{2}H$_2$-N$^+$) positions decrease and increase, respectively, on addition of either metal ion or local anesthetic [3–5]. These changes led to the conclusion that metal ions and local anesthetics induce a structural change in the polar head-group and the mode of interaction is similar for all cations. Present work clearly showed that the induced structural change does not include a conformational change in the choline group which contradicts the conclusion of the NMR lanthanide shift studies. On the basis of ^{2}H-NMR results the effect of metal ions on the glycerol backbone was found very small [3]. The chemical shift anisotropy of phosphorus, on the other hand, changes from -45 to -49 ppm on addition of 0.35 M $CaCl_2$ [3]. Although this fact indicates a change taking place in the dihedral angle $\alpha 2$ ((glycerol-)C-O-P-O), the change should not be significant because the chemical shift anisotropy is very sensitive to the structural parameters [23]. Thus, the contributions of the dihedral angles $\alpha 3$ (O-P-O-C(-choline)) and $\alpha 4$ (P-O-C-C(-N$^+$)) would be more important in elucidation of the change of the quadrupole splittings in the ^{2}H-NMR spectra of the choline group. Such change would shift the position of the positive charge layer of the quarternary ammonium ion relative to the negative ion layer of the phosphate group, leading to a modification of the electrostatic nature of the membrane surface.

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