Biochimica et Biophysica Acta, 980 (1989) 37–41
Elsevier

BBA 74350

Hydrogen bonding between anhydrous cholesterol and phosphatidylcholines: an infrared spectroscopic study *

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(Received 3 October 1988)

Key words: Hydrogen bonding; Cholesterol; Phosphatidylcholine; Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy performed with a high pressure diamond anvil cell was used to study hydrogen bonding between anhydrous phosphatidylcholines and cholesterol at the molar ratio 4:1. The hydroxyl group of cholesterol which acts as a proton donor, engages in strong hydrogen bonding to the *sn*-2 chain carbonyl group of DMPC, DPPC and HPPC and in weak hydrogen bonding to the phosphate group of all these phospholipids. No evidence of hydrogen bonding between cholesterol and the *sn*-1 chain carbonyl group of DMPC and DPPC was found. From a comparison of the relative hydrogen-bond strengths between cholesterol or water and the *sn*-2 chain carbonyl and phosphate groups of all these phospholipids, it is predicted that in aqueous dispersions of cholesterol containing phospholipids, the hydrogen bond of cholesterol to the phosphate group would be replaced by that of water, while the hydrogen bond of cholesterol to the *sn*-2 chain carbonyl group would remain intact.

Introduction

Cholesterol is the main sterol found in animal cell membranes. While its biological role is not yet completely understood, it is clear that cholesterol has an ordering effect on membranes which influences such phenomena as membrane permeability, conformation of membrane-bound proteins, and membrane stability [1]. However, the molecular basis of cholesterol–lipid interaction, which is important in understanding these effects, has not been unequivocally established.

The possibility of interaction between the polar portions of cholesterol and phospholipids was first suggested by studies which showed that sterol-like behavior, namely ordering, condensation and reduction of permeability of phospholipid systems, was limited to those sterols possessing not only the ring system and a side chain at carbon-17, but also a 3β-hydroxyl group [2–6]. However, subsequent studies have failed to agree on the existence or nature of such a polar interaction.

Early evidence indicated that a hydrogen bond exists between the hydroxyl group of cholesterol and the phosphate group of phospholipids [7,8]. However, the presence of such a hydrogen bond was refuted by subsequent ^{13}C-NMR [9] and ^{31}P-NMR [10] studies, and measurement of the condensing effect of cholesterol on natural and synthetic phospholipids with different polar moieties found no evidence of any hydrogen bonding [11].

A second possible site of hydrogen bonding with cholesterol is the carbonyl group of ester-containing lipids. X-ray and neutron diffraction data [12] showed that the hydroxyl group of cholesterol is aligned with the ester carbonyl groups of phosphatidylcholine (PC) at the hydrocarbon/water interface in PC/cholesterol bilayers, and both the *sn*-1 [9–13] and the *sn*-2 [14] chain carbonyl groups of 1,2-diacyl phospholipids have been suggested to hydrogen bond with cholesterol. Raman and infrared spectroscopic investigations [15–17], however, as well as studies of membrane permeability [18,19] have found no evidence of hydrogen bonding of cholesterol with a phospholipid carbonyl group. Yet, most of these investigations were carried out in an aqueous medium, and therefore interference from water molecules, which have been shown to form hydrogen bonds with the *sn*-2 C = O group and the PO_2^- group of phospholipids [20], could explain the inconsistent con-

* Issued as NRCC publication No. 28182.

Abbreviations: PC, phosphatidylcholine; FTIR, Fourier transform infrared spectroscopy; DMPC, L-1,2-dimyristoylphosphatidylcholine; DPPC, L-1,2-dipalmitoylphosphatidylcholine; DHPC, L-1,2-dihexadecylphosphatidylcholine; HPPC, L-1-hexadecyl-2-palmitoylphosphatidylcholine.

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clusions regarding cholesterol–phospholipid interactions.

The aim of this study was to investigate the possibility of hydrogen bonding between cholesterol and the carbonyl and/or phosphate groups of anhydrous phospholipids by means of Fourier transform infrared (FTIR) spectroscopy. This technique has already been used successfully in detecting hydrogen bonding between water and the carbonyl groups in the model lipid triacetin [21], as well as between neighbouring hydroxyl and carbonyl groups in the glycerol lipid 1,2-dipalmitoylglycerol [22]; more recently, high pressure FTIR was used to determine the water binding sites of 1,2-diacyl phospholipids [20]. The present work examines anhydrous systems in order to distinguish the effects of cholesterol on the spectra of phospholipids from those of water. It is expected that by comparing the relative hydrogen bond strength between cholesterol and the phospholipids with that between water and the phospholipid [20], one can predict the competition between cholesterol and water for the hydrogen bond interactions with phospholipids in an aqueous medium.

Experimental

Materials. Samples of L-1,2-dimyristoylphosphatidylcholine (DMPC) and L-1,2-dipalmitoylphosphatidylcholine (DPPC) were obtained from Avanti Polar-Lipids, Inc. The L-1,2-dihexadecylphosphatidylcholine (DHPC) sample was from Fluka Chemical Co. and cholesterol from Sigma Chemical Co. L-1-hexadecyl-2-palmitoylphosphatidylcholine (HPPC) was synthesized by Dr. R. Berchtold of Biochemisches Labor, Switzerland. All lipid samples were recrystallized from chloroform/acetone, while cholesterol was recrystallized from acetone alone. Prior to being used all lipids were lyophilized for 48 hours. Cholesterol/lipid mixtures were prepared by co-dissolving the solid components in chloroform, drying the solution with nitrogen gas, then lyophilizing for 48 hours. The sample was placed in a hole of 0.37 mm diameter in a 0.23 mm thick stainless steel gasket mounted on a diamond anvil cell, along with KRS-5 and powdered α-quartz [23]. In order to remove the last water molecules, the diamond anvil cell containing the sample was placed in the infrared spectrophotometer and purged with dry nitrogen for at least 70 hours [20]. The resulting anhydrous sample was then sealed by closing up the diamond anvils. Evidence that the sample was indeed anhydrous came from the infrared intensity of the water OH stretching band relative to that of the lipid CH stretching bands before, during and after purging.

Spectra and data reduction. Infrared spectra were measured on a Bomem Model DA 3.02 Fourier transform spectrophotometer with a liquid nitrogen cooled mercury cadmium telluride detector. A sodium chloride lens system was used to condense the infrared beam onto the sample in the diamond anvil cell. Spectra were recorded at different pressures from atmospheric pressure up to 25 kbar; each spectrum was composed of 512 co-added scans, at a spectral resolution of 4 cm^{-1}.

Powdered α-quartz placed in the sample hole along with the sample served as an internal pressure calibrant with pressures being determined from the shift in frequency of the 695 cm^{-1} infrared phonon band of the α-quartz [23]; Fourier domain derivation [24] and Fourier self-deconvolution [25] techniques were used to separate instrumentally unresolvable bands. The frequencies of the C = O, O-H and PO_2^- stretching bands were obtained from third-power derivative spectra using the breakpoints indicated in the figure captions.

Results and Discussion

In order to study the interaction of cholesterol with phosphatidylcholines (PC) three discrete spectral regions, the O-H, C = O and asymmetric PO_2^- stretching regions of the infrared spectra of anhydrous PC/cholesterol mixtures were investigated. The molar ratio of phospholipid to cholesterol was 4 : 1.

1. The cholesterol O-H stretching region (3100–3600 cm^{-1})

Fig. 1 shows the OH stretching bands (ν_{OH}) for cholesterol alone and for mixtures of cholesterol with DPPC, DMPC, HPPC and DHPC. The spectrum of anhydrous cholesterol (Fig. 1A) contains two overlapping bands, at 3363 and 3451 cm^{-1}, which arise from two sets of vibrationally inequivalent hydroxyl groups

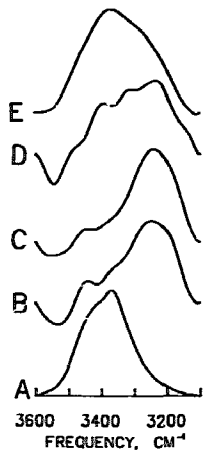

Fig. 1. Hydroxyl bands in the OH stretching region of the infrared spectra of anhydrous cholesterol (A) and of mixtures of anhydrous cholesterol with solid DPPC (B), DMPC (C), HPPC (D) and DHPC (E). All cholesterol/lipid samples contained 20 mol% cholesterol.

within the eight-molecule unit cell of anhydrous cholesterol [26]. Since the OH stretching frequency of a free, non-hydrogen bonded hydroxyl group is above 3600 cm^{-1} [22], and hydrogen bonding is known to lower the stretching frequency of the hydroxyl group [22,27], these two bands clearly represent cholesterol hydroxyl groups hydrogen bonded among themselves. These cholesterol bands can also be found at similar frequencies in the spectra of the cholesterol/PC mixtures shown in Fig. 1B, 1C and 1D, though they are much weaker. However, in the latter spectra a new strong band appears at lower frequency which must be assigned to the cholesterol hydroxyl groups hydrogen bonded to the lipid.

Thus, the strongest band in the ν_{OH} region of DMPC/cholesterol, (Fig. 1B) DPPC/cholesterol (Fig. 1C) and HPPC/cholesterol (Fig. 1D) represents lipid-bound cholesterol ν_{OH} vibrations. The two higher frequency ν_{OH} bands, due to self-associated cholesterol, are present but are much less intense, suggesting that in these systems the majority of cholesterol is hydrogen bonded to the lipid.

On the other hand, in the DHPC/cholesterol mixture (Fig. 1E), the most intense band is at 3382 cm^{-1} and represents self-associated cholesterol, while the band due to the lipid-bound OH groups is less intense. Since the double-bonded oxygen of the phosphate group is the only possible proton acceptor in DHPC, the presence of the 3269 cm^{-1} band provides clear evidence for cholesterol-phosphate group hydrogen bonding. Moreover, since the exchange of ester linkages in DPPC for ether linkages in DHPC leads to a decrease in the intensity of the lipid-bound ν_{OH} band, these results suggest that one or both carbonyl groups in DPPC also provide a site of hydrogen bonding.

The strong lipid-bound ν_{OH} band in Figs. 1B, C and D consists of two components; this becomes clearly evident from deconvolution of this band in the DPPC/cholesterol mixture, as illustrated in Fig. 2. The frequency component at 3268 cm^{-1} is attributed to the phosphate-bound cholesterol OH stretch and corresponds to the 3269 cm^{-1} band in the DHPC/cholesterol spectrum; the frequency component at 3184 cm^{-1} is attributed to the carbonyl-bound cholesterol OH stretch, which is supported by the shift in the carbonyl stretching frequency (vide infra).

The pressure dependence of the two component bands in the DPPC/cholesterol mixture is shown in Fig. 3. The two ν_{OH} frequencies do not change appreciably with increasing pressure. In hydrogen bonded systems, as the pressure is increased, the increased intermolecular repulsion results in bond compression and an increase in the stretching frequency of those functional groups [20]. However, an increase in pressure also strengthens the hydrogen bonds, causing an elongation of the chemical bonds within a hydrogen-bonded functional group and thus a decrease in the corresponding stretching frequency [22]. The balance between these effects of pressure on a hydrogen-bonded functional group is reflected in the plateau in the frequency versus pressure graph of the ν_{OH} cholesterol bands in Fig. 3.

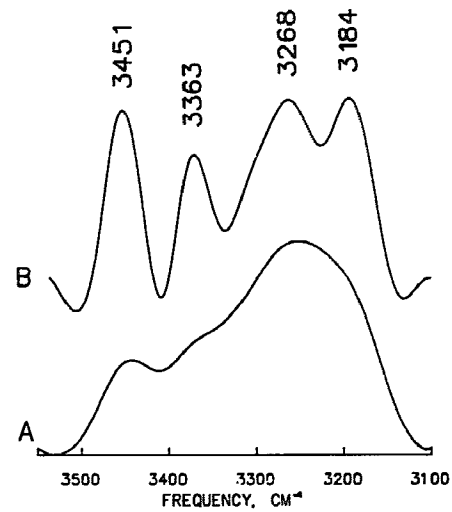

Fig. 2. Original (bottom) and deconvolved (top) infrared spectra in the OH stretching region of a DPPC/cholesterol mixture containing 20 mol% cholesterol. Deconvolution was carried out with a Lorentzian of halfwidth 100 cm^{-1}, using a k factor of 2 (for details see Ref. 25).

2. The lipid C = O stretching region (1700–1800 cm^{-1})

The carbonyl stretching region of 1,2-diacyl phospholipids consists of a broad band contour which upon deconvolution is found to contain at least two overlapping components; the high-frequency component bands originate from the stretching vibration of the *sn*-1 C = O

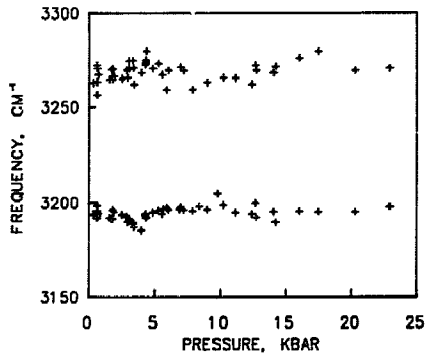

Fig. 3. Pressure dependence of the OH stretching frequency of cholesterol hydrogen bonded to phosphate groups (upper band) and to the *sn*-2 carbonyl groups (lower band) of anhydrous DPPC. Frequencies were obtained from third-power derivative spectra, using a breakpoint of 0.06 in the Fourier domain.

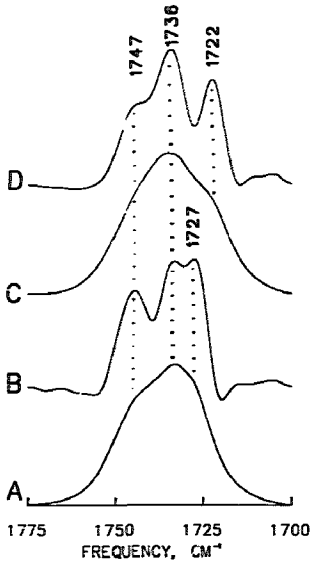

Fig. 4. Original (A) and deconvolved (B) infrared spectra in the C = O stretching region of solid DMPC containing two molecules of crystallization water, and original (C) and deconvolved (D) infrared spectra of an anhydrous DMPC/cholesterol mixture containing 20 mol% cholesterol. Deconvolution was performed with a Lorentzian of halfwidth 15 cm^{-1}, using a k factor of 2.3.

group while the low-frequency component bands originate from the *sn*-2 C = O group [20]; the difference in frequency of these two vibrational modes is attributed to the inequivalence of the conformation at the two acyl linkages produced by a rotation of the *sn*-2 chain about the C_1-C_2 bond. In the spectrum of anhydrous DMPC these two $\nu_{C=O}$ bands are located at 1747 and 1736 cm^{-1}. A third band, at 1727 cm^{-1}, in the spectrum of hydrated DMPC and in solid DMPC containing two molecules of crystallization water (Figs. 4A and 4B) represents the hydrogen-bonded *sn*-2 C = O group [20]. Similarly, three bands can be resolved from the $\nu_{C=O}$ band contour of anhydrous DMPC/cholesterol (Figs. 4C and 4D). The bands at 1747 and 1736 cm^{-1} represent the free *sn*-1 and *sn*-2 C = O stretching vibrations, respectively, while the band at 1722 cm^{-1} represents a C = O group hydrogen bonded to cholesterol. The spectrum of anhydrous DPPC/cholesterol (not shown) also has three bands, at 1746, 1735 and 1721 cm^{-1}.

Evidence that it is the *sn*-2 and not the *sn*-1 carbonyl group which hydrogen bonds with cholesterol in 1,2-diacyl phospholipids comes from the infrared spectrum of HPPC. HPPC is a synthetic phospholipid with only one acyl linkage, at the *sn*-2 position. As was shown earlier [20], the $\nu_{C=O}$ region of anhydrous HPPC contains a single, symmetric band at 1736 cm^{-1}, while hydrated HPPC shows an additional band at 1723 cm^{-1} which represents the stretching vibration of the *sn*-2 C = O group hydrogen-bonded to water. Similarly, the $\nu_{C=O}$ region of anhydrous HPPC/cholesterol (not shown) contains an asymmetric band which on deconvolution yields two component bands at 1736 and 1719 cm^{-1}. If the site of hydrogen bonding in 1,2-diacyl phosphatidylcholines were the *sn*-1 C = O group, one would not expect to find a second band in the spectrum of anhydrous HPPC/cholesterol; thus, the band at 1719 cm^{-1} can only represent hydrogen bonded *sn*-2 C = O groups. These results confirm those of a recent monolayer compression study which concluded that no hydrogen bond exists between cholesterol and the *sn*-1 C = O group of phospholipids [28].

Another point of interest is the difference between the stretching frequency of the *sn*-2 C = O group when hydrogen-bonded to water or to cholesterol. For the three lipids investigated DMPC, DPPC and HPPC, the free *sn*-2 C = O stretching frequency shifts from 1736 cm^{-1} to 1722, 1721 and 1719 cm^{-1}, respectively, on addition of cholesterol, whereas on hydration it shifts to 1727, 1728 and 1723 cm^{-1}, respectively, [20]. Since for a stronger hydrogen bond the C = O stretching frequency shifts to a lower frequency [20–22], despite the slightly different environment one can conclude that the hydrogen bond formed between cholesterol and the *sn*-2 C = O group of these phospholipids is stronger than the hydrogen bond formed between the same C = O group and water. We therefore predict that water will not replace cholesterol in hydrogen bonding to the *sn*-2 C = O group in hydrated phosphatidylcholine/cholesterol systems.

3. The lipid PO_2^- stretching region (1000–1300 cm^{-1})

Infrared bands characteristic of the phosphate group in phospholipids occur in the region 1000–1300 cm^{-1} (single bond P-O and double bond P = O stretching bands). Because of the complexity of the 1000–1200 cm^{-1} region, the effect of addition of cholesterol is most clearly observed by monitoring the change in the asymmetric PO_2^- stretching band ($\nu_{as}PO_2^-$) in the region 1220–1270 cm^{-1} [29,30]. Fig. 5 compares the $\nu_{as}PO_2^-$ band of DPPC/cholesterol with that of pure DPPC at ambient pressure. In our sample of anhydrous DPPC, this band is at 1262 cm^{-1}, which is 17 cm^{-1} higher than the value previously reported [29]; this is due to the vigorous purging used (see Experimental) that reduces the water content of the sample to almost nil, as shown by the absence of a ν_{H_2O} signal in the region 3000–3600 cm^{-1}. Addition of cholesterol, however, causes a shift of the $\nu_{as}PO_2^-$ frequency of DPPC from 1262 to 1256 cm^{-1}. This shift is a result of hydrogen bonding between the phosphate group of DPPC and the hydroxyl group of cholesterol, and is small compared with the shift of 40 cm^{-1} produced by

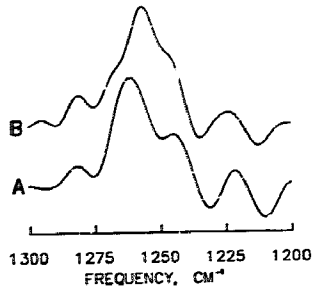

Fig. 5. Infrared spectra in the region of the asymmetric PO_2^- stretching band of anhydrous DPPC (A) and of a DPPC/cholesterol mixture containing 20 mol% cholesterol (B); the spectra are deconvolved with a Lorentzian of halfwidth 15 cm^{-1} and a resolution enhancement factor of 1.5 (see Ref. 25).

hydration of DPPC [20]. These results suggest that the hydrogen bond formed by the phosphate group of DPPC with cholesterol is weaker than that formed with water, and that water will replace cholesterol in bonding to the phosphate group on hydration of DPPC/cholesterol.

Similar results were obtained from mixtures of cholesterol with DMPC, DHPC or HPPC. Addition of cholesterol caused the $\nu_{as}PO_2^-$ band to shift from 1261 to 1258 cm^{-1} in DMPC, from 1259 to 1251 cm^{-1} in DHPC, and from 1266 to 1262 cm^{-1} in HPPC. In all cases, the shift produced by hydrogen bonding with cholesterol was small compared to the shift on hydration [20].

Addition of cholesterol was found to have little effect on the pressure dependence of the $\nu_{as}PO_2^-$ band of DPPC, DMPC or DHPC, suggesting that the cholesterol-phosphate hydrogen bond is weak.

Conclusions

Changes in the infrared spectra of anhydrous DPPC, DMPC, DHPC and HPPC in the OH, C = O and PO_2^- stretching regions on addition of cholesterol (molar ratio 4:1) reveal that:

(i) A hydrogen bond does form between the hydroxyl group of cholesterol and the *sn*-2 chain carbonyl group of DPPC, DMPC and HPCC. This bond is stronger than the water - *sn*-2 C = O hydrogen bond described in Ref. 20. Thus, we predict that water will not replace cholesterol in hydrogen bonding to the *sn*-2 C = O group on hydration of these phosphatidylcholine/cholesterol systems.

(ii) No evidence of hydrogen bonding between cholesterol and the *sn*-1 chain carbonyl group of any of the phospholipids studied was found.

(iii) A weak hydrogen bond does form between the hydroxyl group of cholesterol and the phosphate group of the anhydrous phosphatidylcholines studied. This bond is weaker than the corresponding hydrogen bond with water (see Ref. 20) and thus water will replace cholesterol in hydrogen bonding to the phosphate group of these phosphatidylcholines.

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