Solubilization and Localization of Cholesteryl Oleate in Egg Phosphatidylycholine Vesicles

A CARBON 13 NMR STUDY*

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Co-sonicated mixtures of egg phosphatidylycholine and small amounts (<4%, w/w) of [carbonyl-13C]cholesteryl oleate have been studied by 13C NMR spectroscopy at 50.3 MHz. The carbonyl chemical shift from cholesteryl oleate solubilized in vesicles was 1 ppm downfield from the carbonyl chemical shift of cholesteryl oleate present in a separate oil phase. The maximum solubility of the steroid in vesicles determined by chemical analysis of purified vesicles was 1.8 weight % (~2 mol %), in close agreement with the maximum solubility estimated from NMR peak intensity measurements (1.4 weight %). The downfield shift indicates hydrogen bonding of solvent (H2O) molecules with the cholesteryl oleate carbonyl group, suggesting that vesicle-solubilized cholesteryl oleate molecules are located in the phospholipid bilayer with the carbonyl group close to the aqueous interface and the sterol ring and fatty acyl chain approximately parallel to the fatty acyl chains of the phospholipid. Such a folded conformation and localization of the carbonyl group at the aqueous interface may facilitate interactions of sterol esters with cholesteryl ester transfer proteins and cholesterol esterase.

In plasma, cholesterol is carried in lipoproteins mainly in an esterified form. This cholesteryl ester enters cells through the receptor-mediated pathway(s) (1) and is then hydrolyzed in the lysosome to free cholesterol. Cellular free cholesterol regulates the synthesis of cholesterol and certain lipoprotein receptors and is re-esterified into cholesteryl ester. This cholesterol ester enters cells through the receptor-mediated pathway(s) (1) and then is hydrolyzed by cholesterol esterases. (This cholesterol ester is a nonperturbing probe which can be used to study molecular environment as well as molecular motions.)

MATERIALS AND METHODS

Egg yolk PC was obtained from Lipid Products, Nutley, England; 99% [1-13C]oleic acid was from Koz Isotopes, Inc., Cambridge, MA, and cholesteryl oleate was from Nu Chek Prep Inc., Elysian, MN. Purity of the lipids (>98%) was verified by thin layer chromatography using the solvent system chloroform/methanol/water/acetic acid (65:25:4:1) and Silica Gel G plates and by 13C NMR spectroscopy. 90% [carbonyl-13C]cholesteryl oleate was synthesized by first converting the 90% [1-13C] oleic acid to its anhydride (24) followed by acylation of cholesteryl (25). The cholesteryl oleate was purified in a silicic acid column and the purity was verified by thin layer chromatography (25). The 13C NMR spectrum of the 13C-enriched product was consistent with the expected structure and showed a single carbonyl resonance at signal-to-noise ratios of >50:1.

Vesicles were prepared by co-sonication of the appropriate proportions of lipids in 1.8 ml of 0.075 M (0.56%, w/v) aqueous KCl for 30-60 min as described for triolein-PC vesicles (23). Unless noted otherwise, the internal sample temperature was regulated at -35 °C. The sample compositions are given as percentage of CE by weight of total lipid. Selected samples were fractionated by ultracentrifugation for 4 h at 140,000 × g and 15 °C. The density (ρ = 1.094) of the 0.36% KCl solution allowed clear separation of the oil phase cholesteryl oleate.

Samples were analyzed for purity and composition following NMR studies. NO (<1%) unesterified fatty acid or lysocholesterol was detected by thin layer chromatography. The CE concentration was determined by the method of Rudel and Morris (26) and the PC concentration by a modified Bartlett method (27).

Fourier transform NMR spectra were obtained at 50.3 MHz with a Bruker WP 200 spectrometer system using a 10-mm 1H probe as detailed previously (23). Chemical shifts (δ), line widths (ω1/2), and peak areas were measured as before (23). Spin lattice relaxation times (T1) were measured by a fast inversion recovery method (28) and calculated using a three-parameter fitting routine (29). NOE was measured as the ratio of integrated intensities with broad band decoupling and with inverse gated decoupling (maximum NOE = 3.0) by the technique described by Opella et al. (30).

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RESULTS

Solvation effects on the \(^{13}\)C chemical shift of the carbonyl resonance were investigated in organic solvents of differing polarity. Neat cholesteryl oleate exhibits a carbonyl resonance at 171.15 ppm; \(\delta\) values increase with increasing solvent polarity (171.25 ppm in heptane, 172.35 in CD\(_3\)OD, 173.32 ppm in CDCl\(_3\), and 174.56 ppm in 1:1 CD\(_3\)OD/CDCl\(_3\)). CE and water are mutually immiscible, and the \(\delta\) of the \(^{13}\)C carbonyl resonance of cholesteryl oleate in the presence of H\(_2\)O at 52 °C is identical with that for neat cholesteryl oleate.

The spectrum of a sonicated 1.0% \([\text{carbonyl-}\(^{13}\)C]\)-cholesteryl oleate, 99.0% egg PC mixture is shown in Fig. 1. This spectrum is identical (\(\delta\) and \(\nu_{1/2}\) values, relative intensities) to sonicated PC with no added CE, except for the peak at 172.06 ppm in the carbonyl region, which is attributable to the CE carbonyl carbon. The peaks at 173.90 and 173.61 ppm are from PC carbonyl groups on the outside (designated \(P_o\)) and inside (designated \(P_i\)) of the bilayer, respectively (31). The \(\delta\) of the CE carbonyl is ~1 ppm downfield from the value for unhydrated CE carbonyl carbon (neat CE) and reflects carbonyl groups which are hydrogen-bonded with water molecules at the aqueous interface of the bilayer surface (see “Discussion”).

\(T_1\) and NOE values for carbonyl resonances were measured in spectra of sonicated 1% CE, 99% PC and 2% CE, 98% PC. The \(T_1\) values for the \(P_o\) and \(P_i\) resonances were 2.2 ± 0.1 and 2.1 ± 0.1 s, respectively. The NOE was 1.8 ± 0.1 for the \(P_i\) resonance, which was similar to but could not be measured accurately. The \(T_1\) of the CE carbonyl resonance was 1.3 ± 0.1 and the NOE was 1.6 ± 0.2.

Using the NOE results, a theoretical carbonyl peak area ratio of CE/PC can be calculated (23). Based on the \(T_1\) data, the pulse interval employed in these studies (8.0 s) gave equilibrium intensities for all carbonyl peaks. The peak area ratio from the spectrum in Fig. 1 (0.47) is in good agreement with the theoretical value (0.45). This formulation can also be used to calculate the CE/PC ratio in the vesicle from the peak area measurement (see below).

Samples were prepared with different initial amounts of cholesteryl oleate (0.50, 1.0, 2.0, 3.0, and 4.0%) under otherwise identical conditions to determine the concentration dependence of the CE carbonyl peak intensity, \(\delta\), and \(\nu_{1/2}\). Samples containing >1% CE showed progressively larger amounts of uniform turbidity after sonication. After the low speed centrifugation step to remove titanium fragments, these samples had a small amount of turbid floating material which was resuspended prior to NMR analysis. Fig. 2 shows the carbonyl region of the \(^{13}\)C NMR spectra for four samples with different initial concentrations of CE. All spectra (obtained under identical conditions) were identical except for the region shown. The peak area of the CE carbonyl resonance relative to the area of the PC carbonyl resonances increased with increasing percentage of CE in the starting mixture up to a maximum value in the 2% spectrum and did not increase with higher initial amounts of CE. The carbonyl \(\delta\) for these samples and for other preparations was the same (± 0.1 ppm) at different concentrations; the \(\nu_{1/2}\) of the resonance was 10 ± 3 Hz (median ± range).

Following initial NMR analysis, samples containing >1% CE were fractionated by ultracentrifugation to obtain homogeneously sized unilamellar vesicles (32). Each sample yielded a small pellet, a thin band of floating turbid material (which was removed by pipetting), and a large clear zone containing vesicles. \(^{13}\)C NMR spectra obtained for the clear zones were identical (see below) with corresponding spectra of the unfractionated samples except for a small uniform reduction of all peak intensities. Thus, the CE/PC carbonyl peak area ratio did not change following centrifugation.

Chemical analysis of the fractionated NMR samples showed a maximum incorporation of 1.6 ± 0.2% CE into the vesicle fraction, based on data for the 2, 3, and 4% samples. The CE/PC carbonyl peak area ratio reached an average maximum of 0.61 ± 0.07, based on the NMR spectra of unfractionated samples containing saturating amounts of CE (2-4%). Using this average CE/PC carbonyl peak area ratio, calculation of the composition of the vesicles gave 1.4% CE, 98.6% PC.

A second 4.0% CE sample was prepared by sonicating the lipids above the crystal → liquid transition of cholesteryl oleate.
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Fig. 3. Carbonyl region of the $^{13}$C NMR spectrum at 52 °C of sonicated 4.0% [cholesteryl-$^{13}$C]cholesteryl oleate, 90% egg PC before (A) and after (B) fractionation by ultracentrifugation. Peaks are designated as in Fig. 1; peak at 171.9 ppm is from surface CE and that at 171.35 ppm from oil CE.

oleate (3) at an internal sample temperature of 52 °C. After low speed centrifugation at 52 °C, the sample was transported (without lowering the temperature) to the NMR probe equilibrated at 52 °C. The carbonyl region of the $^{13}$C NMR spectrum of this sample, with the turbid phase resuspended, is shown in Fig. 3A. The spectrum differs from the 4% spectrum in Fig. 2 in that it contains an additional narrow peak ($v_{1/2} = 3$ Hz) at 171.35 ppm, close to the $\delta$ of neat CE (171.15 ppm). This peak will be designated as "oil" CE. The hydrated ("surface") CE/PC peak area ratio was slightly higher than that for the sample prepared at 35 °C; the incorporation measured chemically was also ~30% larger. After cooling to 38 °C (9 °C below the liquid → liquid crystalline transition of cholesteryl oleate; Ref. 3) the peak was no longer detectable and the remaining spectrum was unchanged (spectra not shown). The sample was then fractionated at 15 °C by ultracentrifugation to float up the excess CE, and the resulting clear zone containing vesicles was re-examined by NMR at 52 °C (Fig. 3B). The oil CE peak was not present in this spectrum and there was a small decrease in signal-to-noise ratios of all resonances. Otherwise, the spectrum was very similar to that for the starting material, in particular, the CE/PC carbonyl peak area ratio was not significantly (<10%) different in the two spectra.

Finally, a 4.0% CE sample was prepared below the transition temperature of pure cholesteryl oleate. The carbonyl region of the $^{13}$C NMR spectrum showed a single peak at 172.0 ppm for CE as in Fig. 2. When the sample was heated to 54 °C (after dispersing the cloudy material by agitation) and examined by NMR, an additional peak appeared at 171.35 ppm (as in Fig. 3A) from the melted oil phase CE. After cooling to 38 °C, the oil CE peak disappeared, as above.

To determine whether CE in vesicles could crystalize, a 0.5% CE sample was stored for 24 h at 4 °C after initial NMR analysis. A spectrum obtained at 38 °C (12 °C below the melting point of crystalline cholesteryl oleate) was identical with the original, indicating that the CE in the vesicles was still liquid.

**DISCUSSION**

The chemical shift of the cholesteryl oleate carbonyl carbon has a strong solvent dependence, showing a downfield shift with increasing hydrogen-bonding capacity of the solvent molecule(s). This solvent-induced deshielding effect has been documented for the $^{13}$C carbonyl resonance of organic molecules (33), phospholipids (31, 32), and triglycerides (23). The $\delta$ values of the CE carbonyl in neat form and in CDCl$_3$ solution are the same as the values reported by Sears et al. (35), who suggested that the observed $\delta$ difference was a solvent-dependent effect.

$^{13}$C NMR spectra of sonicated CE/egg PC mixtures exhibit, in addition to the PC carbonyl resonances, a carbonyl resonance at 172.0 ± 0.1 ppm, almost 1.0 ppm downfield from the carbonyl resonance of neat cholesteryl oleate, indicating an interaction between solvent (water) molecules and CE carbonyl groups. The CE molecules are therefore located in the vesicle with the carbonyl group in close proximity to the aqueous surface of the bilayer. The $\delta$ difference between unhydrated (neat) CE and hydrated CE in the vesicle is very similar to that found for the β carbonyl group of triolein (23). Since the chemical shift difference between neat CE and CE in CDCl$_3$ (2.2 ppm) was larger than the corresponding difference for triolein (1.3 ppm), the fractional hydration of CE carbonyls may be somewhat smaller than that of β carbons of triolein (fractional hydration = -0.5; Ref. 23).

Cholesteryl oleate was chosen for this study not only because of its major biological importance but also because its stable state is crystalline at 38 °C. Neat cholesteryl oleate melts from the crystalline phase at 50.5 °C; on cooling, it forms two metastable liquid crystalline phases, the cholesteric phase at 47 °C and the smectic phase at 42 °C, from which crystallization occurs (3). Natural abundance $^{13}$C NMR studies of neat cholesteryl oleate have demonstrated that resonances from steroid ring carbons and the carbonyl carbon are too broad to detect in the liquid crystalline and crystalline states (7). Therefore, only cholesteryl oleate which is solubilized in vesicles would be expected to produce an observable carbonyl peak at 38 °C, and heating to 4°C or 51 °C would be necessary to observe CE in an oil phase. Our experimental results are in accord with these predictions. At 38 °C, only one CE carbonyl peak was observed (downfield from neat CE). The intensity (and $v_{1/2}$) of this peak was not affected by increasing the temperature to 54 °C or by prolonged incubation at 4°C. The maximum solubility of cholesteryl oleate in vesicles estimated from NMR peak intensity ratios (1.4%) was in good agreement with the maximum solubility determined chemically (1.6%). Thus, all the CE present in the vesicle is surface-oriented CE, and this CE does not exhibit phase transitions characteristic of cholesteryl oleate, in accord with expectations based on previous studies (8). When ≥2% CE was present in the starting compositions, excess CE partitioned into a turbid phase which could be separated by centrifugation. The $^{13}$C NMR spectrum of an unfractionated 4% CE sample showed only one CE peak at 38 °C; an additional peak at the $\delta$ characteristic of the carbonyl resonance of neat CE was observed at 52 °C; this peak broadened beyond detection when the temperature was decreased to 38 °C. Thus, the phase behavior of the oil phase CE is similar to that for neat cholesteryl oleate.

Cholesteryl oleate solubilized in the vesicle must be present in the PC monolayer with the fatty acyl chain folded over the steroid ring, since the carbonyl group is proximal to the aqueous interface and since the ring and fatty acyl chain are too apolar to be extended into the aqueous medium. The folded conformation of CE (illustrated schematically in the Fig. 2 inset) has been previously suggested from x-ray diffraction (6), electron spin resonance (19), and $^1$H magnetic

3The exact localization of the cholesteryl oleate carbonyl relative to the phospholipid carbonyl groups cannot be determined from our results, and the schematic drawing is intended to indicate the proposed conformation and to suggest only an approximate location.

4Introduction of a nitroxide group close to the CE carbonyl for spin labeling could increase the probability of the folded conformation.
resonance (20, 21) studies; however, none of these studies provided evidence for the localization of the CE carbonyl group at the aqueous interface.

The T$_1$/T$_2$, NOE, and T$_R$ results, which can be related to molecular motions, provide indirect evidence supporting the conformation proposed above. The motions of the CE carbonyl may be different from the PC carbons or surface-located triglyceride carbonyls (23) because of the steric hindrance of the steroid ring. The motions of oil phase and surface-located CE carbonyl groups will also differ. The average conformation of a CE molecule in the isotropic liquid is probably an extended one, with the chain projecting away from the sterol ring (36); molecular motions will occur in all spatial directions, though at different rates (7). Motions of surface CE will be highly anisotropic, with preferred motion along the long molecular axis and a much slower isotropic component from vesicle tumbling. The T$_1$/T$_2$ is larger, and the T$_R$ and NOE values are smaller, for the surface CE carbonyl resonance than for PC and surface triglyceride carbonyl resonances (23), consistent with a slower and/or more anisotropic reorientation of the CE carbonyl. The T$_1$/T$_2$ and NOE values for the surface CE carbonyl resonance are similar to the values for neat CE near the liquid $\rightarrow$ liquid-crystalline transition temperature, where motions are anisotropic (7); however, the T$_R$ is shorter for the surface CE resonance, possibly indicating that the long rotational axis is different in the two cases and that motion along the long axis is more restricted in the case of surface-located CE.

Our finding that CE is present in a PC bilayer with the CE carbonyl group at the aqueous interface has important implications for metabolism of the sterol esters. The CE molecule will be available for hydrolysis, with the second substrate, H$_2$O, present at the hydrolytic site. Thus, CE hydrolyases can act at the aqueous interface, and penetration of the enzyme deep into the hydrocarbon interior should not be necessary. Rapid hydrolysis of cholesteryl oleate in egg PC vesicles containing 1.5 weight % CE has been demonstrated using rat liver (37) and rabbit aortic (38) cholesterol esterases. In addition, CE molecules will be available for transfer to other particles, which occurs via carrier proteins because of the extremely low water solubility of CE (38-41).

The maximum incorporation of cholesteryl oleate into egg PC vesicles is ~2 mol % (~1.5 weight %) as measured by both chemical and NMR means. A range of solubilities (0.2 to 0.6 mol %) has been reported previously for various CE in phospholipid vesicles and liposomes (4, 6, 8, 19-21). The maximum CE solubility may depend on the particular CE, the type of phospholipid dispersion, and the temperature at which samples are prepared (6, 8). Our present methodology allows the quantitative determination of surface-located CE in a phospholipid bilayer and a clear distinction between such CE and CE present in an oil phase.

The maximum amount of CE as a surface component is slightly lower than that of triolein in egg PC vesicles (2.5 mol %; Ref. 23). Such surface-oriented nonpolar lipid molecules may be present in plasma lipoproteins and certain cell membranes and may be a substrate for many biochemical reactions.

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