In Figure 4, which shows the ^{1}H-NMR spectrum of [Co (dmedba)(L-pro)], the methyl protons at the α-carbon atom are shown 1.0 ppm as a triplet and the methyl protons at the N-methyl protons are shown as a two singlet at 2.4, 2.6 ppm. If the complex has the *uns-cis* configuration, the methyl protons at the α-carbon atom would have shown two butyrato arms are no longer equivalent in the *uns-cis* geometry. We also confirm the stereoisomer of the *s-cis*-[Co(dmedba)(L-pro)] complexes from the CD spectra in Figure 2.

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NMR Studies of Lipid–Protein Interaction in Apolipoprotein B / Phosphatidylcholine Recombinants

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^{31}P{^{1}H} nuclear Overhauser effects (NOEs) have been obtained for complexes formed between apolipoprotein B (apo B) and dipalmytoylphosphatidylcholine (DPPC) vesicles. NOE measurements have been conducted with broad-band irradiation of the entire ^{1}H spectrum in order to identify the proton source of the NOE. In a unilamellar vesicle formed spontaneously upon mixing aqueous suspensions of long-chain phospholipid with small amount of short-chain lecithin, the maximum NOE occurs at the N-methyl proton resonance position of the choline moiety. With addition of cholesterol to vesicles, the position of the NOE maximum shifts further away from the choline methyl frequency. For the ternary apo B-vesicle-cholesterol complex, the position of the maximum NOE lies halfway between those in vesicles with and without cholesterol.

Introduction

The low-density lipoprotein (LDL) of human plasma plays a central role in the control of cholesterol transport and in the pathogenesis of atherosclerosis[1,2]. The overall structure of the LDL is that of a spherical particle with a neutral lipid core surrounded by phospholipids and apolipoprotein B (apo B)[3,4]. Phospholipid polar head-group behavior of the LDL has only recently been explored in any detail due to the lack of adequate probing method. NMR has proven to be a useful nonperturbing probe of the head-group region[5-9]. For Phosphatidylcholine, sphingomyelin bilayer, and LDL Yeagle *et al.*[5,6] reported a ^{31}P{^{1}H} nuclear Overhauser effect (NOE) profile with a maximum at the choline methyl ^{1}H frequency. Addition of cholesterol apparently disrupted the interactions, causing the NOE maximum to shift toward ^{1}H frequencies of methylene groups on either side of the phosphate. This study reports ^{1}H frequency dependence of ^{31}P NOEs and ^{1}H NMR chemical shift data for complexes formed between apolipoprotein B and small unilamellar vesicles with cholesterol. The object of our NMR studies is to provide deeper understanding of lipid-protein interactions as they occur in this model system.

Experimental

Materials. Egg phosphatidylcholine and phosphatidylethanolammine were obtained from Calbiochem; Dipalmitoylphosphatidylcholine and diheptanoylphosphatidylcholine was purchased from Sigma. Cholesterol was purchased from General Biochemical and the phospholipid purity was monitored by thin-layer chromatography.

Preparation of small Unilamellar Vesicles. Vesicles were usually prepared by cosolubilizing both long-chain and short-chain phospholipids in $CHCl_3$, removing organic solvent under a stream of N_2, and then evacuating all remaining traces of solvent at low pressure for at least 12h. Samples were hydrated in the appropriate solvent containing 0.15M NaCl, bath sonicated for 1 min (this aided in dispersal of material on the side of the tube and did not provide sufficient power to form unilamellar vesicles of pure long-chain phospholipid), and equilibrated at room temperature for 6–8h. The pH was adjusted if necessary to be within the range of 6.5–7.5[10].

Isolation of Lipoprotein and Apolipoprotein Fractions. Plasma was obtained from fasting normal volunteers by plasmapheresis. The plasma was collected in 0.01% disodium EDTA, pH 7.5 and used within 24 h for the isolation of lipoprotein fractions. LDL were isolated by ultracentrigugal flotation between KBr densities 1.019 and 1.063. All fractions were washed twice by ultracentrifugation at the respective densities used for isolation. After the final wash, LDL was desalted by dialysis for 72 h against 100 volumes of 0.01% disodium EDTA, pH 8. Prior to NMR analysis, samples were dialyzed against 0.05M Tris, 0.15M NaCl, and 0.02% NaN_3 in D_2O, pH 8.0.

The LDL samples were delipidized with ethanol–ether (3:1, v/v) under N_2. The ether was purified and redistilled before use. The delipidization procedure and solubilization of Apo-B protein were carried out according to the method of Lee *et al.*[11] with minor modifications.

Polyacrylamide Gel Electrophoresis in SDS. The Apo-B protein in 6M urea and in ammonium acetate were analyzed by 3.3% polyacrylamide gel electrophoresis in SDS according to the method of Fairbanks *et al.*[12]. About 10 μg of protein was applied onto each gel.

Formation and Isolation of Protein/Lipid Complexes. A solution of apo-B in 0.01M Tris, 0.15M NaCl, and 0.02% NaN_3, pH 8.0, was added to vesicles (~15 mg/m*l*) at the appropriate initial molar ratio desired and incubated at 24 °C for 24h. Following incubation, a 1-m*l* aliquot of the mixture, contining no less than 10 mg of vesicles, was applied to a 1.2 × 95 cm Sepharose 4B column and eluted at room temperature with H_2O buffer. Fractions of 1 m*l* were collected at a flow rate of 4 m*l*/h and utilized in subsequent experiment within 24h. Samples eluted with the H_2O buffer were dialyzed against the D_2O buffer prior to NMR measurements.

NMR Spectroscopy. ^{31}P NMR spectra at 120 MHz were obtained on a Bruker 300 spectrometer equipped with a Aspect 3000 data system. Spin-lattice relaxation times (T_1's) and NOEs were determined at 25 °C. Selective NOEs were obtained with a single-frequency proton irradiation, where the methyl and methylene portions of the ^1H spectrum were sampled (randomly) in 5-Hz increments. The power level was adjusted to decouple the protons of trimethyl phosphate in D_2O within 5 Hz of the observed. ^1H frequency. This produces a large signal enhancement but not a very selective ^1H frequency profile (as judged by the width of the NOE distribution). The irradiation power was then set to a level at which peak enhancement was about 75–85% of the maximum value. The spectrometer itself was operating in the normal proton mode with the broad-band ^{31}P deconpling on at all times. All proton NMR spectra were measured with a

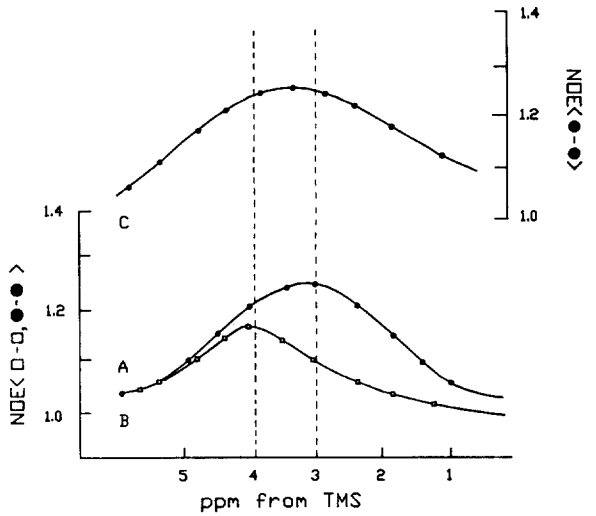

Figure 1. (A) (●) ^{31}P NOE as a function of proton decoupling frequency for small unilamellar vesicles of diheptanoyl-PC/dipalmitoyl-PC (5 mM: 20 mM). The scale for the proton decoupler is in ppm from tetramethylsilane. (B) (□) Small unilamellar vesicles containing 30 mol% cholesterol. (C) (○) Complexes formed between Apo B and vesicles with cholesterol.

Bruker AM 300 spectrometer. A 5-mm probe incorporated with air flow temperature-controlled unit was used through the experiment. A 45° pulse of 8 μs and a repetition rate of 2s were used to sample the signal. Under these conditions, no saturation of the resonances was observed. A 0.4-Hz line broadening was applied to each FID prior to the Fourier transform.

Results and Discussion

Figure 1A shows the NOE frequency dependence for small unilamellar vesicles formed spontaneously upon mixing aqueous suspensions of long-chain phospholipid with small amount of short-chain lecithin.

For phospholipid systems without cholesterol, a maximum is observed at the frequency corresponding to the N-methyl protons of the choline moiety. Several other PC samples give similar results (data not shown). As with all other such experiments, a single smooth curve is drawn through the points, since the inherent resolution of the experiment does not allow fine structure such as more than one maximum in the curve to be observed. Addition of high cholesterol leads to a shift in the maximum of the plot from the N-methyl protons to the methylene protons (Figure 1B). The location of cholesterol in LDL is a problem important to the structure of the particle as a whole. Free cholesterol does not mix well with cholesterol esters and triglycerided[13], and because of its polar hydroxyl group the cholesterol most likely resides in the surface monolayer of lipid and protein surrounding the cholesterol ester core. One question which could be asked is whether the cholesterol is distributed throughout the phospholipid monolayer, or associated with the protein component. For the ternary apo B-vesicle-cholesterol complex, the position of the maximum NOE lies halfway those in vesicles with and without cholesterol (Figure 1C).

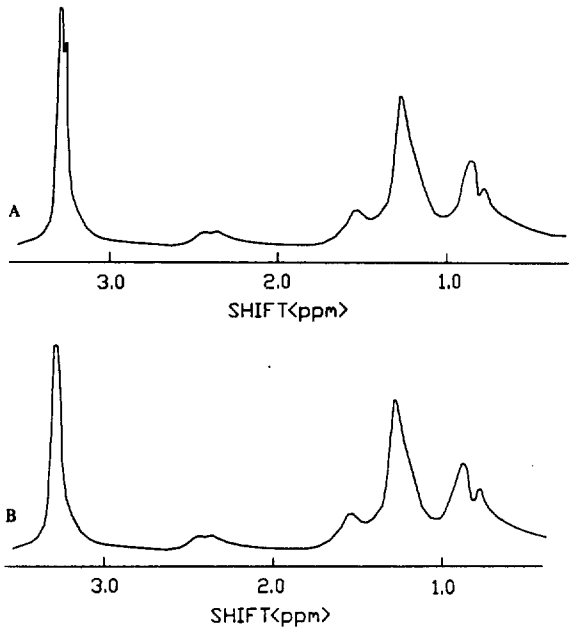

Figure 2. (A) 300-MHz ^{1}H NMR spectrum of diheptanolyl-PC ldipalmitoyl-PC (5 mM : 20 mM) at 30 °C. (B) The vesicle/Apo B complex formed at a molar ratio of 50.

Although the inherent resolution of the experiment does not show fine structure, the position of the maximum shifts clearly toward N-methyl protons. This result indicates that the free cholesterol is not located among the phospholipids, but with the protein as suggested by Yeagle *et al.*[6].

In order to obtain more information for the environment of vesicle in the presence of protein ^{1}H NMR study was performed. In a small unilamellar vesicle which is used in this investigation, two choline N-methyl proton resonances are observed, corresponding to lipids residing in the outer and inner monolayer, respectively (Figure 2A). When apo B is incubated with vesicle, the complex formed displays a ^{1}H NMR spectrum qualitatively very similar to that of vesicle alone, with the notable exception of the phosphatidylcholine N-methyl resonance which is now a single peak (Figure 2B). Addition of the shit reagent results in a complete shift of the new N-methyl resonance (data not shown), suggesting that all the choline head groups are now exposed to the bulk solvent as in the case of high density lipoprotein (HDL)[8].

Conclusion

From the results of ^{31}P{^{1}H} NOE studies for complexes formed between apo B and small unilamellar vesicles with cholesterol, cholesterol is located in the surface of the LDL particle and might be associated with protein. But the validity of this model system might be questioned because vesicles consist of a bilayer and LDL apparently a monolayer of phospholipid. The exact molecular mechanism of interactions between protein and lipid in LDL is still unknown and presently under investigation.

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