Acyl Chain Organization and Protein Secondary Structure in Cholesterol-modified Erythrocyte Membranes*

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Fourier transform infrared and Raman spectroscopies are used to study the effects of cholesterol on human erythrocyte membrane acyl chain organization (mobility and conformation) and protein secondary structure. Compared to normal red cell membranes (=0.8 mol of cholesterol/mol of phospholipid) (C/P); acyl chain mobility is greater for the depleted (C/P =0.6) and less for the enriched (C/P =1.2) membranes as monitored by shifts of the IR symmetric methylene C-H stretching band (2852 wave numbers, cm\(^{-1}\)) over the temperature range 5 to 40 °C. There is a continuous first order trend to the IR shifts, but no evidence of a phase change for any of the three cholesterol contents. Raman scattering of C-C stretching vibrations (1065–1130 cm\(^{-1}\)) revealed that acyl chain conformation in the three membrane preparations is in a similar state of high disorder; however, compared to depleted and control membranes, the enriched membrane acyl chains display higher order lattice packing. The a-helical content of membrane proteins is correlated with the relative intensity of the Raman peptide backbone C-C stretching band (940 cm\(^{-1}\)). Spectra of cholesterol-enriched erythrocyte membranes indicate a substantial increase in protein helical structure compared to those of the cholesterol-depleted membranes.

Modification of cholesterol content in human erythrocyte membranes affects various functional and physical properties (1–16). An important question concerning the role of cholesterol in membrane organization is its ability to influence long and short range order. The effects of cholesterol on thermotropic phase transitions in simple lipid bilayers (39) and its role in the formation of protein phase separations in red cell membranes are classic examples of its long range ordering capabilities (4, 9). Several investigations are in agreement that “fluidity” of the red cell membrane increases with temperature increments in a first order manner and that cholesterol enrichment decreases fluidity, while depletion enhances it over a wide range of temperatures (12–17). In contrast, numerous studies of the erythrocyte membrane have found discontinuities in several parameters indicative of phase transitions at temperatures from 18 to 25 °C (18–28), while other reports demonstrate onset temperatures of phase transitions

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EXPERIMENTAL PROCEDURES

Materials—Fresh blood from healthy donors was collected in 10 mM NaEDTA and used within 1 day. All manipulations were at 0–5 °C. Plasma and buffy coat were separated from cells after a 15-min centrifugation at 1500 × g. The cells were washed three times in 10 volumes of 150 mM NaCl, 5 mM Na phosphate (pH 7.5). Egg phosphatidylcholine and cholesterol were purchased from Lipid Products, Nuffield, United Kingdom and NuChek Prep, Elysian, MN, respectively. Both lipids gave a single spot after thin layer chromatography. Water was distilled and deionized. Deuterium oxide (99.8%) was purchased from Aldrich. Salts and solvents were reagent grade from Sigma, Merck, and Mallinckrodt Chemical Works. Defatted serum albumin was obtained from Sigma.

Preparation of Cholesterol-depleted and -enriched Membranes—Modification of erythrocyte membrane content using sonicated liposomes is described by Lange et al. (84). Briefly, cholesterol-egg phosphatidylcholine liposomes with a final C/P of 1.8 to 2.0 were prepared by sonication of mixtures at a concentration of 5 μmol of phospholipid/ml in Buffer A: 150 mM NaCl, 4 mM KCl, 5.5 mM NaHPO₄, 0.8 mM NaH₂PO₄, 1 mM CaCl₂, 10 mM glucose, 0.2% bovine serum albumin, dextrose, penicillin (100 IU/ml) (pH 7.4). Liposomes were prepared by hophosphorylation of the lipids from benzene and sonication under N₂ in Buffer A with a Branson Sonifier in an ice water-cooled cell. The dispersions were centrifuged at 10,000 × g for 20 min to remove titanium particles shed from the probe. For enrichment, 3 volumes of washed erythrocytes were incubated with 6 volumes of liposomes of C/P =2.0 plus 6 volumes of Buffer A in a shaking water bath at 37 °C for 15 h. The mixture contained 30 μmol of liposome phosphatidylcholine/ml of erythrocytes (which contains ≈2.5 μmol of phospholipid). For depletion of cholesterol, 10 μmol of pure phosphatidylcholine was added. The mixture of liposomes and cells was then centrifuged, washed, and resuspended as described for the control experiment.

1 The abbreviations used are: C/P, moles of cholesterol/mol of phospholipid; cm\(^{-1}\), wave number; Tₜ, main phase transition temperature; I, intensity.
phatidylcholine liposomes were present per ml of erythrocytes. In control experiments, erythrocytes were incubated in Buffer A alone.

**Membrane Preparation for Spectroscopy**—At the end of the incubation, the erythrocytes were washed three times in Buffer A, and ghosts were prepared as described (36). For deuterium oxide substitution, the ghosts were equilibrated in 5 mM NaD (pD 7.4) for 1 h at 4 °C before subsequent washings. Ghosts were washed several times in 5 mM NaN, 150 mM NaCl (pH 7.4) and then ultracentrifuged at 350,000 × g for 1 h at 4 °C.

**Fourier Transform Infrared Methods**—Samples were transferred at 4 °C to a Harrick cell equipped with either CaF12 or ZnSe windows. The temperature was controlled with a Haake circulator to ±0.2 °C and monitored with a Digilab copper-constantan thermocouple. Sample thickness (path length) was 50 μm. The samples were allowed to stabilize at each temperature for 15 min before data acquisition. Spectral recording was performed with a Nicolet 7100 Fourier transform infrared spectrometer using parameters described by Cameron et al (36). A liquid nitrogen-cooled HgCdTe or a triglycine sulfate detector was used. Wave number stability was accurate to ±0.01 cm⁻¹. Band positions were determined at peak maxima by a Nicolet computer algorithm using 9 transform points/wave number. At each sample recording, several (4–15) 100-scan averages were co-added to obtain a high signal to noise ratio. Typically, 32,000 data points were used for small numbers of scans and 8000 for larger averages. The results of using the latter is reported here. Band position certainty varied with temperature, see Fig. 2. At 10 °C, 2 standard errors (2 ± S.D.) from a mean of four independent experiments yielded an accuracy of ±0.05 cm⁻¹ while at 35 °C the reproducibility was 0.16 cm⁻¹. These S.E. were approximately the same for the three membrane preparations. Atmospheric background was subtracted from the samples on a one-to-one scale. For water background subtraction, a spectrum of water subtracted. All incubations were for 15 h; the enriched membrane data indicate that the acyl chains have decreased mobility and have rearranged to prevent increasing

**RESULTS AND DISCUSSION**

**Acyl Chain Mobility and Conformation**—Variations in acyl chain mobility and packing can be monitored by the infrared absorption of C–H stretching vibrations of methane residues (37). Asher and Levin (37) reported abrupt shifts (±4.0 wave numbers, cm⁻¹) in IR absorption bands attributed to asymmetric CH₃ stretching vibrations of bilayers of dipalmitoylphosphatidylcholine undergoing an endothermic phase change. A smaller, more sensitive shift occurs at the symmetric methane C–H stretching band, 2850 cm⁻¹. The shift is a cooperative event where heating the bilayer reaches a critical value (Tm) at which the majority of chains simultaneously increase their gauche content (kinked configurations). As cholesterol is added to the bilayer (≥20 mol %), the frequency profile looses the abrupt shift seen at Tm and flattens to a broad trend with increasing temperatures (37). Gauche conformers are formed in a more gradual progression over the temperature profile, and packing which was "hexagonal like" below Tm and unordered above Tm without cholesterol (38) stabilizes in a quasi-hexagonal manner (39). Total gauche content is also reduced over the temperature profile (37). These concepts will be applied in our study of the cholesterol-modified human erythrocyte membrane.

Infrared spectra in the symmetric methylene C–H stretching region are shown in Fig. 1. A–C for cholesterol-enriched, control, and cholesterol-depleted erythrocyte membranes, respectively, at 40 °C. The temperature dependence of the C–H stretching bands for all three membrane preparations are plotted in Fig. 2. The peaks, in per cent transmittance, shift to higher wave numbers with a broad linear trend. Note the subtle shifts in frequency for the enriched membranes. There are no abrupt shifts that would indicate a phase change for any of the three cholesterol contents. The cholesterol-enriched membrane data indicate that the acyl chains have decreased mobility and have rearranged to prevent increasing

![Fig. 1. Infrared methylene C-H symmetric stretching spectra of cholesterol-modified erythrocyte membranes. A, cholesterol-enriched ghosts (C/P =1.2). B, control ghosts (C/P =0.8). C, cholesterol-depleted ghosts (C/P =0.6). The spectra (each is a co-added spectrum of four separate experiments) are of membranes at 40 °C, prepared in 5 mM NaP, 150 mM NaCl (pH 7.4); these are not water subtracted. All incubations were for 15 h; the enriched membranes are from red cells incubated with sonicated lecithin/cholesterol liposomes in Buffer A; control membranes are from red cells incubated only with Buffer A; depleted membranes are from red cells incubated with sonicated lecithin liposomes in Buffer A. Spectral parameters are described under "Experimental Procedures."](image-url)

![Fig. 2. Temperature dependence of infrared methylene C-H symmetric stretching bands of cholesterol-modified erythrocyte membranes in 5 mM NaP, 150 mM NaCl (pH 7.4). A, cholesterol-depleted ghosts (C/P =0.8). B, control ghosts (C/P =0.6). C, cholesterol-enriched ghosts (C/P =1.2). The spectra were solvent subtracted before data reduction. Each point is the mean ± 2 S.D. of four individual experiments. Shifts of cholesterol-depleted and -enriched membranes were significantly different (p < 0.001, paired t test).](image-url)
temperatures from disrupting the packing order. The difference in frequency at 40 °C between the depleted and enriched membrane is \( \approx 0.9 \text{ cm}^{-1} \) (paired \( t \) test, \( p < 0.001 \)). The effects of cholesterol depletion are opposite to those of enrichment. In addition to their greater mobility, the depleted membrane profile in Fig. 2 is more similar to the control profile than to the enriched profile. This probably is due to the fact that the depleted membranes have 25% less cholesterol than the control membranes while the enriched membranes have 50% more cholesterol than the controls. Similar results were obtained for membranes prepared in D_2O, except that the entire C-H stretching absorption region was shifted to lower frequencies. This may be the result of some form of deuterium solvation at the membrane-aqueous interface. The C/P of normal healthy erythrocyte membranes can range from 0.71 to 1.07 (6, 40) with a mean of 0.83 (5). Variations in the vibrational spectra of erythrocyte membranes from one individual to the next and within the same donor over time are expected (55). In this work, all membranes were internally controlled by using blood from the same individual for experimental and control.

Intra-acyl chain carbon-carbon stretching is detected by Raman scattering in the 1000-1200 cm\(^{-1}\) region (41). Relative proportions of all-trans (extended chain)/gauche (kinked chain) configurations in erythrocyte membranes have been indicated by specific bands in this region (42). Using the Raman C-C stretch melting curve of dipalmitoylphosphatidylcholine, Lippert et al. (42) estimated that roughly 55-70% of the lipid in their erythrocyte membrane preparations is in the all-trans configuration. These authors conclude that protein acyl chains contribute only a very weak background to the C-C stretching region and should not interfere with the phospholipid vibrations. Experiments in our laboratory showed that digestion of red cell membrane peripheral and integral proteins with trypsin had no effect on the Raman C-C stretch region (56).

Raman spectra of the acyl chain C-C stretching regions for the three membrane preparations are shown in Fig. 3. The three preparations have spectral contours similar to that of the dipalmitoylphosphatidylcholine dispersions above their gel-liquid crystal phase transition (41, 43). Compared to the depleted and enriched membranes, the control membrane spectral profile in Fig. 3 is more similar to the depleted membranes. Again, the similarity between the spectral profiles is due to the 25% difference in their cholesterol contents relative to the control while the enriched membranes have 50% more cholesterol than controls. The main difference between the spectra in Fig. 3 is the more intense (\( \approx 40 \pm 15\% \), mean \( \pm \) S.E., \( p < 0.001 \), \( t \) test), steeply contoured 1065 cm\(^{-1}\) band of the cholesterol-enriched membranes. Although the 1065 cm\(^{-1}\) band is an all-trans C-C stretch feature, it is reported (44) to primarily reflect differences in acyl chain mobility that arise when the lattice packing structure rearranges as when dipalmitoylphosphatidylcholine bilayers move through the pretransition or main transition. The cholesterol-enriched membrane acyl chains may have either lost some of their gauche conformation, or more likely have rearranged to a higher order packing structure, which is in agreement with the above IR data. The gauche content in the acyl chains of all three membrane preparations appears to be high; however, the enriched membranes display less acyl chain mobility and higher order lattice packing.

**Protein Secondary Structure**—Helical secondary structure is detected by Raman scattering of peptide backbone carbon stretching vibrations at 940 cm\(^{-1}\) (45, 46). Protein helical content has been correlated to the relative intensity of the 940 cm\(^{-1}\) band, \( I_{900}/I_{1004} \) (47, 48). Raman spectra in the protein C-C stretch region for the three membrane preparations are shown in Fig. 4. The \( I_{900}/I_{1004} \) value is increased by 50 ± 15% (\( p < 0.001 \)) and decreased by 31 ± 15% (\( p < 0.001 \)) in cholesterol-enriched and -depleted membranes, respectively, compared with controls. We assume that the 1004 cm\(^{-1}\) bands are unchanged due to our internally controlled preparations and therefore are stable references.

To obtain a rough approximation of protein helicity in the three membrane preparations, their \( I_{900}/I_{1004} \) values are compared in Table 1 to \( I_{900}/I_{1004} \) values of five globular proteins with known \( \alpha \)-helical content. The five proteins were chosen because of their well known primary and secondary structures. They all have approximately 3% Phe which is the amino acid side ring responsible for the magnitude of the reference line 1004 cm\(^{-1}\) (53-56). The correlation coefficient between the estimates of \( \alpha \)-helical content and the \( I_{900}/I_{1004} \) values of the five globular proteins is \( \approx 0.99 \). Since the correlation of \( \alpha \)-helical content and \( I_{900}/I_{1004} \) values is so strong, it seems reasonable to construct a line equation to see how the \( I_{900}/I_{1004} \) values of the membrane preparations compare. Using the data in Table 1 to compute all possible straight lines with the two-point formula, the proteins in the cholesterol-depleted membranes are estimated to have between 30-39 ± 10% \( \alpha \)-helical content (range calculated from the 10 possible equations ± error calculated with the line equations using the ± 15% accuracy of the membrane \( I_{900}/I_{1004} \) values), the control membrane proteins have 44-56% \( \alpha \)-helical content, and the proteins in the cholesterol-enriched membranes have 65-92% \( \alpha \)-helical content. Uncertainties about x-ray, CD, or Raman amide I estimates of the globular protein \( \alpha \)-helical contents (±7%) and reported errors in their \( I_{900}/I_{1004} \) values of ±3 (±2

![Fig. 3. Raman spectra of acyl chain C-C stretching vibrations for cholesterol-modified erythrocyte membranes. Cholesterol-enriched (top), control (middle), and cholesterol-depleted (bottom) erythrocyte membranes in 5 mM NaP, 150 mM NaCl (pH 7.4) at 25 °C. Parameters are described under "Experimental Procedures." These spectra are not smoothed or solvent subtracted. The cholesterol-enriched \( I_{900}/I_{1004} \) value is increased by 40 ± 15% (mean ± S.E., \( p < 0.001 \)) compared to the control and cholesterol-depleted membranes.](image-url)
The estimates of per cent α-helical content in α-chymotrypsin, ribonuclease A, and lysozyme are taken from the circular dichroism (47). The value is the average of a range (50).

The authors report a correlation coefficient of 0.98 between CD estimates and x-ray analysis of Provencher and Glockner (56). The error of these values was found in only two reports and is the average of a range (50).

References—The vibrational spectroscopic evidence leads us to conclude that lipid and protein acyl chains of cholesterol-enriched erythrocyte membranes are sterically less mobile than control or cholesterol-depleted membranes and additionally have their hydrocarbon chains packed in some higher order arrangement than either control or depleted membranes. Although the control membrane acyl chains are less mobile than those of the depleted membranes, they appear to retain a similar lattice packing structure even with a 25% difference in cholesterol content between the two preparations. All three membrane preparations have substantial amounts of the disordered chain configuration, possibly to accommodate a large cholesterol content; however, cholesterol enrichment forces the kinked configurations into higher order lattice packing.

A scheme has been designed to estimate the amount of α-helical structure in the erythrocyte membrane proteins. The cholesterol-enriched erythrocyte membranes display a substantial increase in protein α-helical content relative to the control membranes while proteins in the cholesterol-depleted membranes show significantly less helical structure.

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