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A LASER RAMAN SPECTROSCOPIC INVESTIGATION OF PHOSPHOLIPID AND PROTEIN CONFIGURATIONS IN HEMOGLOBIN-FREE ERYTHROCYTE GHOSTS

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SUMMARY

Configurations of both the protein and lipid components of human red blood cell ghosts are examined by laser Raman spectroscopy. Protein configuration is estimated from bands observed in the Amide III region at 1240–1267 cm^{-1} in water and the Amide I′ region at 1630–1670 cm^{-1} observed in ^2H_2O. The protein fraction appears to contain 40–55 % α-helix with little β-configuration. The hydrophobic side chains of the phospholipid component, as interpreted from the 1060–1130 cm^{-1} C-C stretching region, are estimated to contain 55–65 % all-*trans* rigid configuration. These estimates are within the limits set by other physical techniques.

INTRODUCTION

Numerous physical studies of the structure of cell membranes have been made in the past decade, with a picture gradually evolving of a relatively fluid lipid bilayer containing areas of protein embedded in this lipid matrix. For example, infrared spectroscopy [1–3] as well as optical rotatory dispersion and circular dichroism [4–6] have been particularly important in supporting the suggestion that membrane protein is highly α-helical. In the former, however, membrane preparations must be dried and associated changes in protein structure may occur. Results from optical rotation experiments are rather difficult to interpret unambiguously because of red shifts associated with light scattering by the membrane preparations [4–6].

With respect to the lipid component of the cell membrane, electron paramagnetic resonance spin-label techniques [7–9] have been particularly helpful in providing a picture of the phospholipid portion of the membrane as a fluid bilayer whose mobility may be limited by the presence of protein [10]. The electron paramagnetic resonance technique however, involves the introduction of a molecular spin probe, a foreign material which has the potential of perturbing the membrane.

Laser Raman spectroscopy has recently been applied to the study of the configurations of proteins [11, 12], polypeptides [13, 14], phospholipids [15–18], and membrane-active antibiotics [19, 20], and provides a potential method for examining both protein and phospholipid configurations of membrane preparations in

aqueous suspension. An initial report [21] of the Raman spectrum of hemoglobin-free erythrocyte ghosts showed only a weak, poorly resolved spectrum of lipid on a very high luminescence background.

We report improved Raman spectra of hemoglobin-free human erytrocyte ghosts in water and ^2H_2O which are interpreted in terms of a protein fraction containing 40-55 % α-helix and a lipid fraction containing predominantly rigid hydrocarbon side chains. The protein and lipid portions of the Raman spectrum are well separated and relatively free from interference.

EXPERIMENTAL

The Raman spectrometer is a SPEX 1403 half-meter double monochrometer with a Spectra-Physics 164 Argon ion laser typically operated at 400 mW at 488 nm. A Baird-Atomic interference filter is placed before the sample to remove plasma lines. The detection system is an EMI 9502 SA uncooled photomultiplier coupled to a Keithley picoammeter. Typical dark current is 3×10^{-9} A. The samples are sealed in glass melting-point capillaries and held horizontally in a thermostated brass block whose temperature is controlled by a circulating water bath. Temperature control in the laser beam is within one degree as determined by comparing melting points of hydrocarbons with those measured by Raman spectroscopy. The laser beam enters the sample vertically; the scattered Raman light is collected at right angles to both the laser axis and the sample-tube axis.

Outdated whole human blood cells were generously donated by the Red Cross Blood Bank. Hemoglobin-free erythrocyte ghosts were prepared by the method of Dodge et al. [22] except that 10 washes of 20 mosM phosphate buffer (pH 7.4] were used to remove all traces of fluorescent material. This large number of washes was necessary to reduce luminescence to a manageable level so that Raman spectra could be obtained. The ghosts were rinsed three times with 20 vol. of distilled water or ^2H_2O to remove interfering phosphate ion, and then centrifuged at $20\,000 \times g$ for 40 min. Small (10–20 μl) portions of the pellet were sealed in melting-point capillaries and maintained at 4 °C for use as Raman samples.

The Raman spectra obtained from these preparations are shown in Figs 1–3. The background luminescence and the water spectrum, 5–8 times the Raman intensity of the ghost samples have been suppressed by the detection system. The samples contained approx. 1–3 % ghosts as estimated by comparing the ghost peak heights with the Raman band of water at 1645 cm^{-1} or the ^2H_2O band at 1210 cm^{-1}.

RESULTS AND DISCUSSION

All bands in the Raman spectra (Figs 1–3) of human erythrocyte ghosts are tentatively assigned to either phospholipid or protein in Table I, except the bands at 1160 cm^{-1} and 1527 cm^{-1} in Fig. 1, which are discussed below. No bands were observed that can be assigned to cholesterol which makes up approx. 24 % of the total lipid in human erythrocyte ghosts [23]. However, cholesterol exhibits a Raman spectrum in which bands either coincide with those of phospholipid, or are much weaker than those of, for example, dipalmitoyllecithin in 1:1 mixtures [15]. In addition, no bands could be assigned to polysaccharide, present as about 7 % ghost weight [24].

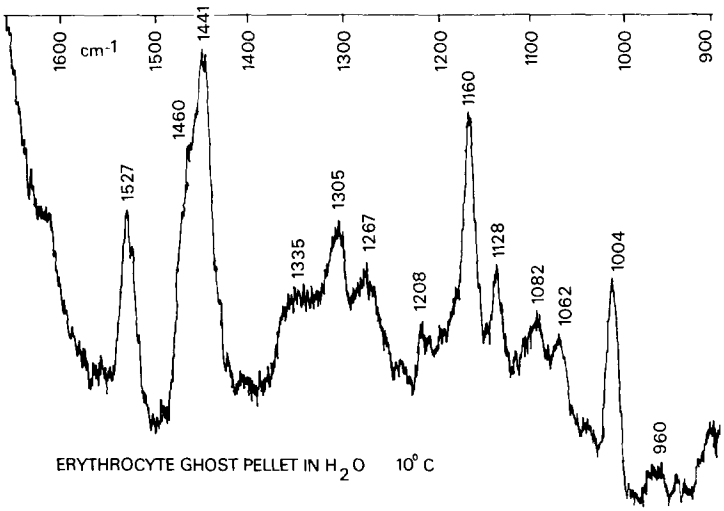

Fig. 1. Laser Raman spectrum of human erythrocyte ghosts in aqueous suspension at 10 °C, showing the intense bands at 1160 cm^{-1} and 1527 cm^{-1} which were observed in approximately half the ghost samples prepared as outlined. 600 mW 488.0-nm incident radiation; resolution, 5 cm^{-1}; scan rate, 0.1 cm^{-1}/s wavelength accuracy, ± 5 cm^{-1}; sensitivity, 10^{-8} A full scale.

Raman spectra obtained from several separate preparations of ghosts from different donors were identical except in two regards. First, the intensities of bands assigned below to protein were different in different preparations relative to those assigned to phospholipid, indicating that the extensive washing required to remove fluorescence from the ghosts also removed some protein. The removal of protein, particularly spectrin, by washing with solutions of low ionic strength has been reported previously [25]. Thus the conclusions reached here refer only to spectrin-depleted ghosts.

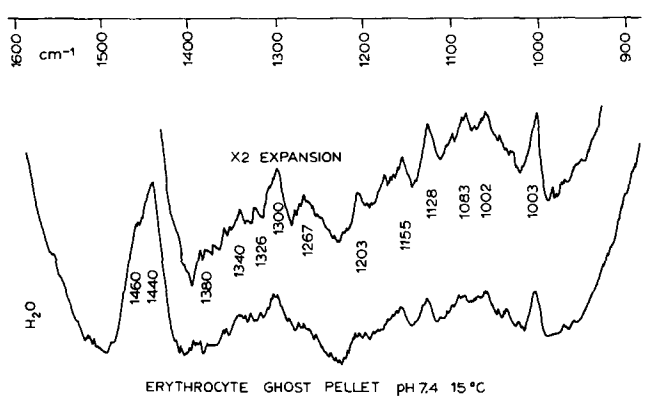

Fig. 2. Laser Raman spectrum of human erythrocyte ghosts in aqueous suspension at 15 °C, representative of approximately half the ghost samples prepared as outlined and which do not show the 1160 cm^{-1} and 1527 cm^{-1} bands of unknown origin. 300 mW of 488.0-nm incident radiation; resolution, 10 cm^{-1}; scan rate, 0.5 cm^{-1}/s; wavelength accuracy, ± 5 cm^{-1}; sensitivity, 10^{-8} A full scale in the bottom spectrum. The faster scan rate and lower resolution were the result of a recorder chart-drive malfunction.

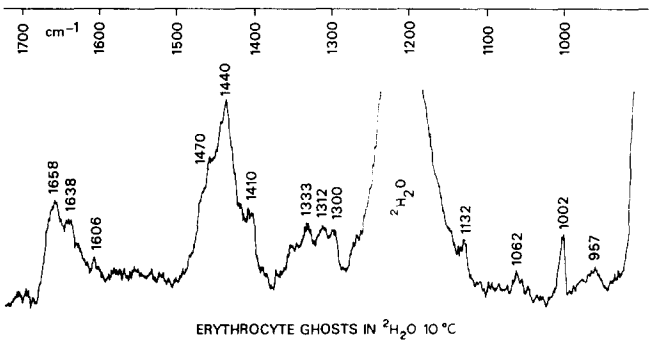

Fig. 3. Laser Raman spectra of human erythrocyte ghosts suspended in ^2H_2O at 10 °C. Conditions for the spectrum are the same as for Fig. 1.

TABLE I

TENTATIVE ASSIGNMENTS OF THE OBSERVED RAMAN SPECTRA OF HEMOGLOBIN-FREE ERYTHROCYTE GHOSTS IN WATER AND ^2H_2O SUSPENSION

Numbers in parentheses are intensities relative to the 1004-cm^{-1} band taken as 10. Relative intensities for the aqueous suspension are taken from Fig. 1. The frequencies are accurate to $\pm 5\ cm^{-1}$.

Frequency (cm^{-1})		Tentative assignment	
Water	^2H_2O	Protein	Phospholipid
960 (2)	957 (4)	C-C stretch	
1004 (10)	1002 (10)	Trp and Phe ring mode	
1062 (5)	1062 (5)		C-C stretch of *trans* chain
1083 (5)			O-P-O and C-C stretch of random chain
1109 (3)		C-C and C-N stretch	C-C stretch of bound *trans* chain
1128 (7)	1132 (6)		C-C stretch of *trans* chain
1160 (14)		Unidentified	
1208 (4)		Tyr and Phe	
1267 (6)		Amide III of α-form and C-H twist	
1305 (9)	1300 (8)		$C-H_2$ twist of long alkyl chain
1312 (6 sh)	1312 (8)		
1326 (4)	1333 (8)	$C-H_2$ twist	
1340 (4)	1340 (6)		
1410 (6 sh)	1410 (12 sh)		
1440 (18)	1440 (30)	$C-H_2$ and $C-H_3$ bend	$C-H_2$ and $C-H_3$ bend
1460 (12 sh)	1470 (20 sh)		
1527 (10)		Unidentified	
	1606 (6)	Tyr and Phe	
	1638 (13)	Amide I′ of α-form	
	1658 (16)	Amide I′ of β- and random form	

Second, in roughly half of the spectra obtained from different donors, large, relatively sharp Raman bands were observed at 1160 cm^{-1} and 1527 cm^{-1}. Figs 1 and 2 are representative of spectra obtained with and without, respectively, these bands present. These are not grating ghosts or emission lines, but are of unknown origin. In view of the intensity and lack of reproducibility of these bands, we suspect that they may be due to resonance enhanced Raman bands from a very minor component which is washed from the ghosts in some experiments.

It is important to draw attention to the fact that the major structural features assigned to phospholipid and protein are retained in Figs 1 and 2. This implies a general continuity of protein and phospholipid configuration which is carried through the extensive washings. Phospholipid structural information is contained primarily in the 1062–1130 cm^{-1} C-C stretching region in H_2O, while evidence of the protein secondary structure is found in the Amide III region at 1230–1300 cm^{-1} in water and the Amide I′ region at 1620–1680 cm^{-1} in ^2H_2O. The CH_2 regions are complicated by contributions from both protein and phospholipid and so are difficult to interpret.

Phospholipid

A number of Raman examinations of phospholipid [15–18] have assigned sharp bands at 1062–1066 cm^{-1} and 1120–1133 cm^{-1} to the C-C stretching vibrations of the all-*trans* rigid configuration of hydrocarbon side chains in phospholipids. Below the gel-liquid crystal transition temperature of phospholipids, a weak band at 1089 cm^{-1} occurs which has been assigned to the O-P-O symmetric stretch of the phosphate group[16], while a sharp band at 1097–1120 cm^{-1} occurs in unsaturated fatty acids and phospholipids and has been assigned to the C-C stretch of the all-*trans* C-C segment between glycerol and the unsaturated double bond. This segment has been referred to as the "bound" chain [16].

On the other hand, fluid lipid above the gel-liquid crystal transition temperature is characterized by the appearance of an intense, broad band at 1085 cm^{-1}, as well as the disappearance of the 1065 and 1120–1132 cm^{-1} all-*trans* bands [15]. This intense, broad band at 1085 cm^{-1} has been tentatively assigned to the C-C stretching vibration of a fluid, random orientation of the side chains [15, 16]. The presence of sharp bands at 1063 and 1128 cm^{-1} in Figs 1 and 2 strongly suggests the existence of considerable amounts of rigid, all-*trans* side chains at the measurement temperatures. By comparison with the melting curve of dipalmitoyllecithin [15], we estimate that roughly 55–70 % of the lipid in human erythrocyte ghosts is in the all-*trans* configuration. In this region of the spectrum, proteins such as lysozyme, ribonuclease [12], and bovine serum albumin (Lippert, J. L., unpublished) show only a very weak background which should not interfere to an appreciable extent with the phospholipid vibrations.

The Raman spectra contrast with infrared measurements [2] of the 720-cm^{-1} band which present a view of the erythrocyte membrane as containing little *trans* configuration. The Raman experiment agrees with electron paragmagnetic resonance spin-label measurements which show that erythrocyte membrane lipids are more rigid than myelin or synthetic vesicles [7]. Although our rather large estimate of rigid configuration may be partially a result of the low temperatures used in our measurements, we feel that our estimate may not be unreasonable at slightly higher

body temperature, since 30–45 % fluid lipid content should be enough to support the sorts of molecular movement observed in cell membranes [26, 27].

We have not observed the intense, broad band at 1110 cm^{-1} reported as the major feature of the Raman spectrum of hemoglobin-free erythrocyte ghosts by Bulkin [21]. That spectrum was obtained from a suspension in phosphate buffer which has a strong band at approx. 1090 cm^{-1} below pH 7, which might be the origin of the anomaly.

Protein

The Raman spectra of model polypeptides and proteins [11–14] have been compared to the spectra of the ghost membranes in order to infer the secondary structure of the protein component. The Raman spectrum of α-helical poly-L-lysine [13] is characterized by lack of Amide III band at 1240 cm^{-1} in water, and by an Amide I′ band at 1632 cm^{-1} in ^2H_2O. Poly-L-lysine in the β-configuration shows a very intense Amide III band at 1240 cm^{-1}, as well as a sharp, intense Amide I′ band at 1660 cm^{-1} in ^2H_2O[13]. The random configuration of poly-L-lysine shows bands at 1243 cm^{-1} in water and 1658 cm^{-1} in ^2H_2O ,which are approximately half as intense as those in the β-configuration, and considerably broader [13]. Similar bands are observed in other polypeptides and proteins in those configurations. In addition α-helix in water is often accompanied by a weak band at 1267 cm^{-1} and enhanced intensity at 1312 cm^{-1} [28].

Thus, it would appear that α-helix can be readily distinguished from β-configuration and random form in the Raman spectrum, but relative amounts of the latter two are somewhat more difficult to estimate. This is contrasted to the infrared, where β-form is readily distinguished from combined α-helix and random configuration [1, 2]. In the erythrocyte ghosts examined here, (Figs 1–3) high α-helix content is marked by the weak band at 1267 cm^{-1} and the absence of an Amide III band at 1240-1243 cm^{-1} in water, which is an indicator of β- and random configurations, and by the band at 1638 cm^{-1} in ^2H_2O. We estimate that at least 40–55 % of the protein is in the α-helical form. The rest is probably random configuration, with no evidence of appreciable β-structure (probably less than 10 %). This estimate is made by comparing the peak heights at 1240 cm^{-1} in water, and 1632 cm^{-1} and 1659 cm^{-1} in ^2H_2O relative to the 1004 cm^{-1} trytophan-phenylalanine ring mode, to those of specific configurations of poly-L-lysine. This estimate is within the range of infrared and optical rotation determinations. An identical procedure, the subject of another paper, (Lippert, J. L. and Tyminski, D., unpublished) has been used by us to estimate protein configurations within 15 % of those determined by X-ray analysis.

There is evidence [3] that ^2H_2O can change membrane structure and integrity. However, in these experiments, the absence of a band at 1240 cm^{-1} in the Amide III region in water and the appearance of an Amide I′ band at 1632 cm^{-1} in ^2H_2O consistently suggests large amounts of α-helix in both solvents.

As mentioned above, the experiments outlined above are appropriate only to spectrin-depleted ghosts. However, the circular dichroism spectrum of spectrin indicates a very highly α-helical protein [25]. Thus it would appear that the high α-helical protein content measured in these experiments is a general property of most membrane proteins.

These observations extend the configurational understanding of the components

of the erythrocyte membrane. Laser Raman spectroscopy allows the examination of both protein and phospholipid configurations of membranes without introducing potential experimental perturbations. We have chosen for these experiments a membrane which, before preparation, contains large amounts of colored material in order to show that even in this extremely difficult experimental situation, much configurational information can be obtained. The implications of the configurational information obtained here and elsewhere remain largely open questions, but laser Raman spectroscopy may prove a powerful tool to examine those implications.

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