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On the Interactions of Lipids and Proteins in the Red Blood Cell Membrane*

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Abstract. The effects of temperature and of the action of a purified phospholipase C enzyme preparation on human red blood cell membranes has been investigated by chemical analyses, circular dichroism, and proton magnetic resonance measurements. The results indicate that a substantial fraction of the phospholipids and the proteins of the membranes can change structure independently of one another, suggesting a mosaic pattern for the organization of the lipids and proteins in membranes.

The application of new or improved physical techniques to membrane systems has recently yielded some useful information about their structure. For example, optical rotatory dispersion and circular dichroism (CD) measurements[1, 2] in the peptide bond absorption band have shown that a substantial fraction of the protein is in the α-helical conformation in several different membranes. Proton magnetic resonance (pmr) spectra of intact red blood cell membranes under physiological conditions show very broad resonances,[3] suggesting that the lipid and protein constituents of the membranes are relatively immobile. Electron spin resonance studies have yielded information about the mobility of spin-labeled adducts within membranes.[4]

Only limited information is available at present concerning the interactions between, and the organization of, the lipids and proteins of membranes. One approach to this problem is to perturb intact membranes in different well-defined ways, and by the use of a variety of techniques to determine the effects of the perturbations on the lipid and protein components. Phospholipase C is an enzyme that specifically catalyzes the hydrolysis of phospholipids to diglycerides and water-soluble phosphorylated amines.[5] Lenard and Singer[6] showed that treatment of red blood cell membranes with phospholipase C released about 70 per cent of the phosphorylated amines of the phospholipids, but had no significant effect on the CD spectrum of the protein portion of the membrane. These observations have now been extended to include a coordinated CD and pmr study of the effect of phospholipase C action on red blood cell membranes. The results indicate that the conformation of a substantial fraction of the membrane protein is not strongly dependent upon the physical state of the bulk of the membrane phospholipids.

Materials and Methods. Human red blood cell membranes were prepared from fresh blood by the procedure of Dodge, Mitchell, and Hanahan.[7] The phospholipase C experiments shown in this paper were performed with a purified enzyme from *B. cereus* prepared and generously donated by Dr. A. C. Ottolenghi.[8] Several preliminary experiments were performed with a crude phospholipase C prepared from *Cl. perfringens* (obtained from Worthington Co., Freehold, N. J.) which had been heated for 10 min at 60°C and freed of precipitated protein. The red blood cell membranes were transferred by centrifugation and resuspension (three times) into phosphate-free buffers for the phospholipase C digestions: 5 mM Tris, 5 mM KCl, pH 7.3, in the case of the purified enzyme, and 5 mM Tris, 2 mM $CaCl_2$, and 2 mM KCl, pH 7.3, for the crude enzyme. Approximately 1 enzyme unit was added per 2.5 mg of membrane protein, and the mixtures were incubated at 37°C for 20 min. Ten volumes of cold buffer were then added and the membranes were collected by centrifugation and washed with buffer. After removal of a portion of the sample for CD and analytical measurements, the remainder was transferred by five cycles of centrifugation at 50,000 rpm and resuspension into 7 mM Na phosphate in D_2O, apparent pD 8.1. The final pellet was used for pmr, CD, and analytical measurements. Control samples were used through all operations in parallel except that no phospholipase C was added.

CD spectra were obtained with the J-10 modification of the Durrum-Jasco ORD/UV/CD-5 instrument in 0.5 mm cells. The effect of temperature was observed in 1.0 mm water-jacketed cells; samples were kept at each temperature for 20 min before observation. The absorbance did not exceed 2.0 for any measurements. A mean residue weight of 114 was assumed for calculations of the ellipticity. Proton magnetic resonance spectra were made using a Varian HR-220 spectrometer equipped with a Varian C-1024 time-averaging computer. A capillary filled with a calibrated acetone-D_2O mixture containing $MnSO_4$ provided a standard for area measurements. All chemical shifts were referred to the methyl protons of 2,2-dimethyl-2-silapentane-5-sulfonate (DSS).

Analyses were performed as follows: for protein content, the method of Lowry *et al.*[9] was used, calibrated by Kjeldahl *N* determinations; for total phosphorus, the method of Bartlett[10] was used; for fatty acid esters, the method of Snyder and Stephens[11] was used with an incubation time of 30 min at 75°C; for cholesterol, the method of Abell *et al.*[12] was used.

Results. The purified phospholipase C from *B. cereus* released 62 per cent of the total phosphorus of the red blood cell membrane in the experiment whose results are shown in Figures 1–3. Ottolenghi and Bowman[13] report that 61–68 per cent of the membrane phosphorus is released by a similar enzyme preparation. With the crude enzyme from *Cl. perfringens*, 60–75 per cent of the membrane phosphorus was released in different experiments. The cholesterol content of the membranes was 0.22 and 0.23 mg/mg protein before and after treatment with the purified enzyme, respectively. The fatty-acid ester content of the membranes before and after enzyme treatment was close to 2.3 μEq/mg protein. No protein was released from the membranes by the enzyme treatment.

The original CD spectra from 190 to 240 nm of the membranes in phosphate-buffered H_2O at 26°C before and after treatment with the purified enzyme are reproduced in Figure 1; they are essentially identical. The value of θ at 223 nm was $-16{,}000$ degrees cm^2 decimole^{-1}. The absorbances of the two samples were also closely similar (Fig. 1). No significant differences between treated and untreated samples were observed in D_2O or H_2O. In experiments with the crude enzyme, however, the CD spectra exhibited some small differences; for the treated sample, the minimum at 223 nm was shifted to 224 nm, the ratio of θ at the minimum near 208 nm to that near 223 nm was smaller, and θ at the maxi-

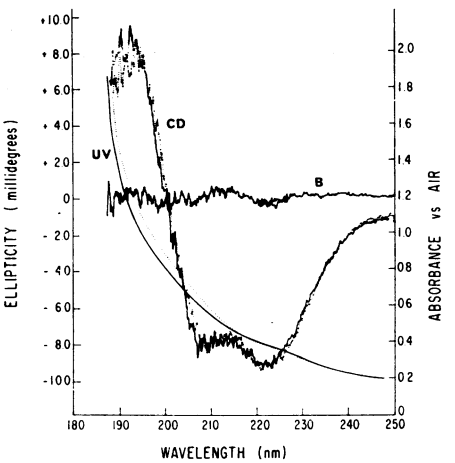

FIG. 1.—Tracings of the original CD and absorbance (UV) spectra of human red blood cell membranes before (*solid lines*) and after (*dotted lines*) treatment with phospholipase C. The protein concentrations of the two samples were the same. B is the baseline for the CD spectra. The solvent is Tris-buffered H_2O at 26°C.

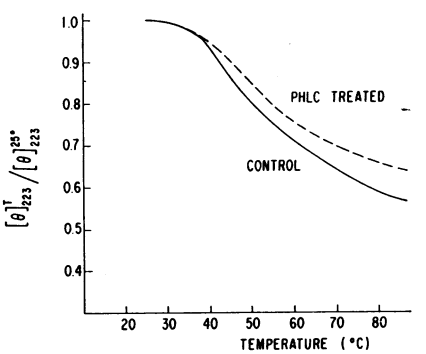

FIG. 2.—The effect of temperature on the value of θ at 223 nm of the phospholipase C- (PHLC) treated and untreated red blood cell membranes in phosphate-buffered D_2O.

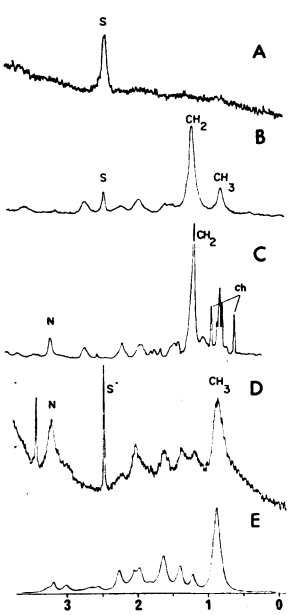

FIG. 3.—Proton magnetic resonance spectra in the region of the methylene and methyl proton resonances, in phosphate-buffered D_2O unless otherwise indicated. (*A*) untreated red blood cell membranes at 18°C; (*B*) phospholipase C-treated membranes at 18°C; (*C*) $CHCl_3$-extracted lipids from untreated membranes, in $CDCl_3$ at 18°C: (*D*) untreated membranes at 82°C and (*E*) computed membrane protein spectrum (see text). The abscissa gives the chemical shifts in ppm relative to the methyl protons of DSS. The designations are: S, standard; N, choline methyl protons; and ch, cholesterol methyl protons.

mum near 193 nm was smaller. The treated membranes also showed a uniformly larger absorbance than the untreated from 195 to 240 nm.

The effect of increasing temperature upon the CD spectra of the enzyme-treated and untreated membranes was to compress the curves to the axis $\theta = 0$ without appreciable distortion of shape; the value of θ at the minimum near

223 nm therefore serves as an index of the temperature effect. The ratio of θ_{223} at temperature T to that at 26° is plotted in Figure 2. Again, the enzyme-treated and untreated membranes showed similar behavior as a function of temperature.

The pmr spectrum of intact red blood cell membranes at 18°C shows very little detail[3] (Fig. 3*A*). However, after phospholipase C treatment, a well-defined spectrum was observed (Fig. 3*B*), with a pronounced methylene proton resonance at 1.26 ppm. This spectrum closely resembles that of a solution of lipids extracted from the intact red blood cells (Fig. 3*C*), except that the treated-membrane spectrum shows a much diminished peak at 3.25 ppm for the choline methyl protons (which were largely removed from the membranes by the phospholipase C treatment); and an absence of peaks at 0.72 and 0.98 ppm arising from cholesterol protons.[14]

When untreated red blood cell membranes were subjected to increased temperature, more detail appeared in the pmr spectrum than was observed at 18°C. Up to about 40°C, three small peaks at 0.90 (due to methyl protons), 2.06 (probably due to acetamido protons of neuraminic acid residues), and 3.25 ppm (due to choline methyl protons) were observed. At about 40°C, there was an abrupt increase in the methyl proton peak area, and five new resonances at 1.22, 1.41, 1.64, 2.26, and 3.02 ppm arose. As the temperature was increased, the areas under these peaks increased, but the line width (20–40 Hz) did not change appreciably. At temperatures above 70°C, the resonances at 1.22 and 1.41 ppm began to coalesce because of the appearance of a broad resonance centered between them. The pmr spectrum of untreated membranes elevated to 82°C is shown in Figure 3*D*.

Discussion. In an earlier study,[6] phospholipase C treatment of red blood cell membranes was shown not to affect significantly the CD spectrum of the protein portion of the membrane. In the present experiments, this work has been confirmed and extended in several important respects. In the first place, a more highly purified phospholipase C preparation[8] was used. Secondly, more accurate CD measurements were made with the modified Durrum-Jasco instrument than were previously possible; this has allowed a finer analysis of the effect of the enzyme treatment on the membrane proteins to be made. Thirdly, and most significantly, new information about the physical state of the lipids has been obtained by pmr measurements on the same samples that were analyzed by CD.

The chemical effects of phospholipase C treatment of the red blood cell membrane were essentially the same for the purified and crude enzymes: a maximum of 60 to 70 per cent of the phosphorylated amines was released, but no cholesterol or diglycerides were lost from the membranes. The gross physical effects on the membranes were also similar for the purified and crude enzymes. The membranes remained intact, but somewhat shrunken in size, and contained some nodules. The latter have been observed by Ottolenghi and Bowman,[13] and they have suggested that they contain the diglycerides formed by the enzyme action on the phospholipids.

The CD spectrum of the membrane, reflecting the average conformation of the proteins in the membrane, was not detectably changed after treatment with the purified phospholipase C (Fig. 1). This is in contrast to the small changes

we observed after treatment with a crude phospholipase C, and to the similar changes reported by Gordon *et al.*[15] with another preparation of the crude enzyme. The small CD changes we have observed with the crude enzyme are correlated with an increase in absorbance, probably due to increased light scattering, of the treated sample. These light scattering changes are most likely responsible for the small CD changes observed; such artifacts have been proposed by Urry and Ji,[16] and have been examined in detail for membrane systems.[17] In any event, the fact that no CD or absorbance change was produced by treatment of the membranes with the purified enzyme indicates that no detectable protein conformation changes accompany the release of the phosphorylated amines from the lipids of the intact membranes.

The effects of temperature on the CD spectra of the treated and untreated membranes were very similar (Fig. 2). The value of θ at 223 nm largely reflects the right-handed α-helical content of the membrane protein. If the values of θ at 223 nm for a polypeptide chain in the random coil and α-helical conformations are taken as -1000 and $-36{,}000$, respectively, then the protein of the untreated membrane changed from 43 to 23 per cent helix when the temperature was raised from 26 to 85°. Certainly no profound destabilization of the membrane protein resulted from the phospholipase C treatment; if anything, the protein in the treated sample was slightly more stable to increasing temperature.

On the other hand, the pmr spectra show that the physical state of the fatty-acid chains of the phospholipids in the membranes was greatly changed by the phospholipase C treatment. Prior to treatment, line broadening was so pronounced that very little spectral detail was observed with the intact membranes at 18°C (Fig. 3*A*).[3] (The spectra that have been obtained with sonicated red cell membranes, or with intact membranes at temperatures above 60°C,[18, 19] are of uncertain relevance to the native state of the membranes.) After enzymic release of the phosphorylated amines, however, a fairly sharp resonance appeared which accounts for about 75 per cent of the methylene protons present in the fatty acid chains of the membrane phospholipids. This indicates that after the enzyme treatment about three-quarters of the fatty acid chains became much more mobile than in the unmodified membrane, i.e., "melted."[20] The phospholipase C experiments therefore demonstrate that the chemical structure and physical state of a large fraction of the phospholipids of the red blood cell membrane can be radically altered with no effect detectable by CD on the average conformation of the membrane proteins. This conclusion is valid whether or not the diglycerides formed are redistributed into the membrane nodules which are observed in the light microscope.[13]

When untreated red blood cell membranes were examined at elevated temperatures by pmr, a very different spectrum was observed (Fig. 3*D*). This spectrum is dominated by a resonance due to methyl protons at 0.90 ppm, and there is no indication of the methylene proton peak from the lipid fatty-acid chains. These results suggested that the protein portion of the membrane might be primarily responsible for the pmr spectrum at elevated temperatures. The amino acid composition of the total proteins of human red blood cell membranes is known.[21] Using the chemical shifts of the protons from the different amino acid residues in the random coil conformation,[22] we have computed the spectrum

expected for the membrane proteins in the region of the methylene and methyl protons. (CD results in Figure 2 indicate that the random coil is the predominant conformation of the proteins at 82°.) This computed spectrum is shown in Figure 3*E*, and is remarkably similar to the observed spectrum in Figure 3*D*. The observed spectrum has a pronounced choline methyl proton peak at 3.25 ppm, and probably has contributions to the peak at 2.06 ppm from the acetamido protons of neuraminic acid residues, that should be taken into account in the comparison with the computed spectrum. On the basis of this spectral assignment, the observed intensity of the methyl proton peak corresponds to 20 to 40 per cent of the total methyl protons of the membrane proteins. Furthermore, as discussed above, the CD results indicate that there is a change of about 20 per cent of the protein from α-helix to random coil conformations in the temperature interval from 25 to 82°. From these considerations, we conclude that a substantial fraction of the proteins of untreated membranes change physical state with increasing temperature, but that the fatty acid chains of the intact phospholipids do not "melt" under these circumstances.

In the experiments involving phospholipase C, the chemical structure and physical state of about three-fourths of the phospholipids were radically altered, with no detectable effect on the gross physical state of the protein; conversely, in the experiments with untreated membranes raised to high temperatures, the physical state of as much as two-fifths of the protein was altered, with no indication of a change in the state of the lipid fatty-acid chains. It is therefore reasonable to conclude that a substantial fraction of the phospholipids and the proteins of the red cell membrane can change structure independently of one another.

While this work was in progress, differential calorimetry experiments with mycoplasma membranes (which contain no cholesterol) were reported,[23] which showed that the bulk of the lipids in the intact membranes, and the same lipids isolated and dispersed in aqueous suspensions, melted at about the same characteristic temperature. These authors concluded therefore that the bulk of the lipids in the intact membrane were in the form of bilayers. Interestingly enough, however, only about 75 per cent of the lipids in the intact membrane appeared to melt at the characteristic temperature; the remainder was unaccounted for. It is intriguing that in several quite different types of experiments with different systems a similar fraction of lipids undergoes alteration: about 60 to 70 per cent of the lipid phosphorus of several different types of membrane is hydrolyzable by phospholipase C;[6, 13, 15, 24] about 75 per cent of the methylene protons of the lipids are melted after phospholipase C treatment of red blood cell membranes; and about 75 per cent of the lipids in intact mycoplasma membranes melt at a characteristic temperature. Although other explanations can be adduced for these observations, it is possible that about 25 to 30 per cent of the phospholipids are in a physical state different from the remainder of the lipids, perhaps involved in a more tightly coupled interaction with the membrane proteins. To our knowledge, however, there is as yet no satisfactory direct evidence for such a differentiation of phospholipids in membranes.

All of the results discussed in this paper, and the properties of membranes in general, are consistent with a segregation of a large part of the l pids and proteins of membranes, suggesting a mosaic pattern for the organization of the lipids

and proteins in membranes. In this scheme, globular protein molecules, perhaps noncovalently bound to about one-quarter of the phospholipids, are interspersed in a matrix consisting of the remaining lipids in a form similar to that of a discontinuous bilayer (Fig. 4). This schematic model is a somewhat more detailed version of the one previously given by Lenard and Singer,[2] in which all ionic and polar groups of the phospholipids and the ionic residues of the proteins are in contact with the bulk aqueous phase; the helical portions of the proteins are in the internal hydrophobic portion of the membrane; and the interactions holding the membrane components together are primarily hydrophobic interactions.[25, 26] A detailed discussion of this schematic model, its variegation to account for the complexity of membrane proteins, and its relation to membrane function, is in preparation.[27] Structures referred to as mosaic have been proposed in the past (cf. ref. 28), but these have been very different in detail.

As noted before,[6] the phospholipase C experiments indicate (*a*) that the polar heads of the phospholipids in the intact membrane must be accessible to (i.e., come into atomic contact with) the active site of the enzyme, and (*b*) that electrostatic interactions with the phospholipid polar heads cannot be the dominant factor determining the conformation of the membrane proteins. These facts are explained by the model in Figure 4, but not by models in which the protein is spread out over the membrane surfaces covering the phospholipid polar heads (cf. Fig. 2 in ref. 23, and Fig. 8 in ref. 29).

It is worth noting that the choline methyl protons of intact membranes become mobile at temperatures where methylene protons of the fatty-acid chains are still relatively immobile (Fig. 3*D*), suggesting that the choline groups are able to rotate more freely than the nonpolar portions of the phospholipids in the membranes. This fact can also be rationalized by the model in Figure 4, in which the choline moieties are at the exterior surfaces of the membrane and may rotate freely in the surface plane.

A mosaic model such as is represented in Figure 4 may also explain the discrete structures seen in freeze-etching experiments with membrane systems, and which have been interpreted to reside within the membrane interior.[29]

Finally, the pmr results bear on the interaction of the cholesterol and the phospholipids in the red blood cell membrane. These two classes of lipids are

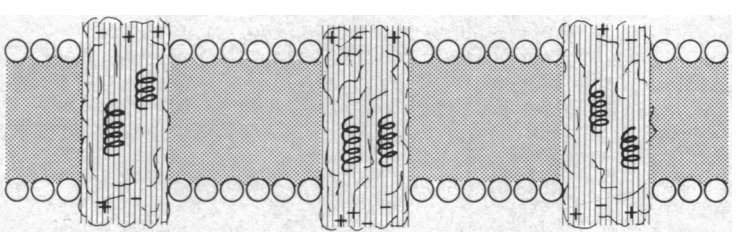

FIG. 4.—A highly schematic representation of a section of a mosaic model for the organization of lipids and proteins in cell membranes. The protein is represented by the lined structure, largely in the random coil conformation, with helical portions of the protein internal to the membrane, and the + and − ionic residues at the external faces. The balls represent the polar and ionic groups of the phospholipids in contact with water, and the stippled region the fatty acid chains, cholesterol, and other hydrophobic structures. Possible differentiation of 25–30% of the phospholipids as more tightly bound to the protein, as discussed in the text, is not explicitly shown in the figure.

present in close to equimolar proportions in the membranes. The cholesterol interacts with the phospholipids and is thought to be responsible for the immobilization ("freezing") of the fatty-acid chains of the phospholipids,[30, 31] but the nature of the interaction is still unclear.[32, 33] Our results suggest an important role for the ionic heads of the phospholipids in this interaction, for they are required to maintain the "frozen" state of the fatty acid chains of the lipids in the presence of cholesterol. This role might be a direct one, involving the interaction of the ionic heads of the phospholipids with the OH group of cholesterol,[32] or a less direct one, involving the maintenance of the appropriate phospholipid lattice into which the cholesterol may intercalate.

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