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Interaction Between Glycophorin and Phospholipids in Recombined Systems

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Both the MN-glycoprotein from human erythrocytes and the hydrophobic fragment from the protein isolated with trypsin treatment, T(is), have been recombined with egg phosphatidylcholine in bilayers at various phospholipid/protein ratios. In order to investigate the effect of the protein on the phospholipid headgroups, ^{31}P nuclear magnetic resonance spectra were obtained with the MN-glycoprotein recombined with egg phosphatidylcholine, which revealed two classes of phospholipid environments, one immobilized and one not immobilized. Electron spin resonance (ESR) of fatty acid methyl ester spin labels provided supporting evidence.

Computer analysis of the ESR spectra indicate that 4–5 moles of phospholipid are immobilized per mole of protein over a wide range of lipid-to-protein ratios. The immobilization of the phospholipids appears mediated by both the polar headgroups and the hydrocarbon tails of the phospholipid.

Key words: boundary lipid, ^{31}PNMR, spin labels, glycophorin

Glycophorin, the major sialoglycoprotein of human erythrocyte membranes, is an integral membrane protein which spans the erythrocyte membrane [1, 2]. The NH_2-terminal region of the glycoprotein contains all the carbohydrate and is exposed to the extracellular milieu [3]. The COOH-terminal region of the molecule is enriched in hydrophilic residues such as aspartic acid, glutamic acid, lysine, and arginine, as compared to the entire molecule [3], and is exposed on the cytoplasmic surface of the membrane [1, 2]. A portion containing 23 amino acid residues connects these two regions and is contained within a tryptic peptide T(is) which is insoluble in aqueous solution [4, 5]. Amino acid

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analysis and subsequent sequencing showed that this peptide has a high content of nonpolar amino acids [3–5]. The hydrophobicity of this intermediate region of the protein allows it to interact favorably with the hydrocarbon chains of phospholipids as it spans the bilayer.

By the use of electron spin resonance (ESR) spin labels it has been shown that the integral membrane proteins cytochrome b_5 [6], cytochrome oxidase [7], myelin basic protein [8], and the Ca^{++}ATPase [9, 10] immobilize a portion of the lipid in the membrane, called boundary lipid, by contact between the hydrophobic portion of the membrane protein and the hydrocarbon chains of the lipids. Boundary lipid most likely constitutes one or more layers of affected lipid around the protein and may regulate its interaction with the membrane as a whole.

To our knowledge, a detailed structural study of the interactions between phospholipids and protein in a membrane has not been published for any purified membrane protein from the red cell. The purpose of this study is to demonstrate that glycophorin immobilizes lipid when recombined with phospholipid in model membrane systems, and that the immobilization is mediated by the polar headgroups as well as the hydrocarbon chains of the membrane phospholipids.

MATERIALS AND METHODS

Egg phosphatidylcholine was obtained in benzene from Avanti Biochemicals; it exhibited only a single spot on thin layer chromatographic analysis (65:25:4, chloroform, methanol, water) when about 1 mg sample was applied. Phospholipid concentrations were determined using the method of Bartlett [11]. The C-12 spin label, methyl 12-(4′,4′-dimethyloxazolidinyl-N-oxyl) stearate, was synthesized [12].

Glycophorin was isolated from red blood cell ghosts by the Lithium 3,5-diiodosalicylate extraction method of Marchesi and Andrews [13]. T(is) was obtained by trypsin digestion of glycophorin and solubilized in 2,2,2-trifluoroethanol. Its concentration was determined by amino acid analysis on a Beckman 121-M analyzer with computing integration after hydrolysis for 24 h at 110°C in 6 N HCl.

T(is) was associated with egg phosphatidylcholine by first mixing the phospholipid and protein in 2,2,2-trifluoroethanol and, after evaporation of the solvent, hydration with an excess of 100 mM NaCl, 10 mM EDTA, H_2O. For the ESR spin label experiments, the spin label in ethanol was added to the mixture of phospholipid and protein before evaporation of the organic solvent.

Glycophorin was associated with egg phosphatidylcholine by the following procedure. Anhydrous protein and phospholipid were solvated for 3h in 2-chloroethanol and subsequently evaporated under vacuum for three days. The dry mixture was then hydrated in 4 mM histidine, 150 mM NaCl, pH 7.2, overnight. This suspension was used directly for ^{31}P nuclear magnetic resonance (NMR) measurements; for ESR experiments, the spin label in ethanol was dried in a test tube and the aqueous suspension of protein and phospholipid was subsequently incubated in the tube for an hour or more before obtaining ESR spectra. Sucrose density gradient centrifugation indicated that the largest stable protein-to-lipid ratio was 1:20; that ratio was therefore used for both the ESR and NMR experiments with intact glycophorin.

ESR spectra were obtained with a Varian E-109 ESR spectrometer, at 9.07 GHz using a variable temperature controller to maintain the temperature at 23 ± 1°C. Instrument settings were chosen to avoid instrument-induced line broadening or saturation. The ESR spectra for several preparations of the T(is)/egg phosphatidylcholine samples were digitized

in a PDP 11/VO3 computer system. Spectral subtraction and numerical integration of spectra were performed using programs developed on the computer (S.E. O'Conner and C.M. Grisham, to be published).

NMR spectra were obtained at 40.48 MHz for ^{31}P on a JEOL PS100/EC100 Fourier transform spectrometer at 23°C, except where noted. ^{31}P NMR spectra were obtained with gated broadband proton decoupling to remove the ^{31}P [^{1}H] nuclear Overhauser effect [14] and to reduce the dipolar contributions to the linewidth [19] so that the magnitude of the effective chemical shift anisotropy could be measured. The decoupler was gated "on" during aquisition to effect proton decoupling and "off" the remainder of the time to prevent buildup of the nuclear Overhauser effect. We used 90° pulses with a repetition rate five times the spin lattice relaxation time of unsonicated egg phosphatidylcholine aqueous dispersions (about 1.5 s [20]). Intensities of spectra were evaluated by cutting and weighing photocopies of the spectra.

RESULTS

T(is)

The hydrophobic peptide obtained from glycophorin by trypsin treatment was associated successfully with egg phosphatidylcholine at initial protein-to-lipid mole ratios of 1:5, 1:10, 1:20, and 1:50. An attempt to associate T(is) with egg phosphatidylcholine at a protein-to-lipid ratio of 1:2.5 left some unassociated protein as a precipitate, so that ratio was not used for the magnetic resonance experiments. Egg phosphatidylcholine was used because it resembles the lipid content of the red cell membrane more closely than a synthetic, saturated phospholipid species, yet can be obtained as a pure phosphatidylcholine.

Methyl esters of fatty acids spin-labeled at various positions produce ESR spectra diagnostic of the motional order of the region of the membrane near their location [15]. The ESR spectra of the C-12 spin label used here, incorporated into the unsonicated bilayers of an egg phosphatidylcholine dispersion at a concentration of 1 mole %, appears in Fig. 1. It is similar to spectra obtained by others [15], reflecting the fluidity of a phospholipid bilayer well above its phase transition temperature. The phospholipid concentration was 5 mg/ml in this and each of the following spin label experiments.

ESR spectra of the 12-doxyl methyl stearate spin label incorporated in T(is)/egg phosphatidylcholine dispersions are shown in Fig. 1. Spectra B–F were taken at the indicated protein-to-lipid mole ratios. Each of the spectra B–E shows that it is composite spectrum consisting of a sum of immobilized and nonimmobilized lipid components. Spectrum B was obtained from the sample containing the largest percentage of immobilized lipid and hence was used to obtain the spectrum of purely immobilized lipid spin label. This spectrum is shown at the top of Fig. 1. Spectrum A′ was synthesized by subtraction of an appropriate amount of the fluid bilayer spectrum (F at the bottom of the fig.) from spectrum B. Spectrum A′ is highly anisotropic and is indicative of lipid spin label in a very immobilized environment. This anisotropic spectrum clearly demonstrates the immobilization of lipids surrounding the protein.

To obtain an estimate of the number of lipids immobilized by the protein in these experiments, the contribution to the total spectral intensity arising from the immobilized lipids was calculated. Spectrum A′ was subtracted from each of spectra B–E until the resulting difference spectrum was indistinguishable from spectrum F. Since spectrum F was taken from a sample containing no protein, it is characteristic of spin labels in fluid bilayers.

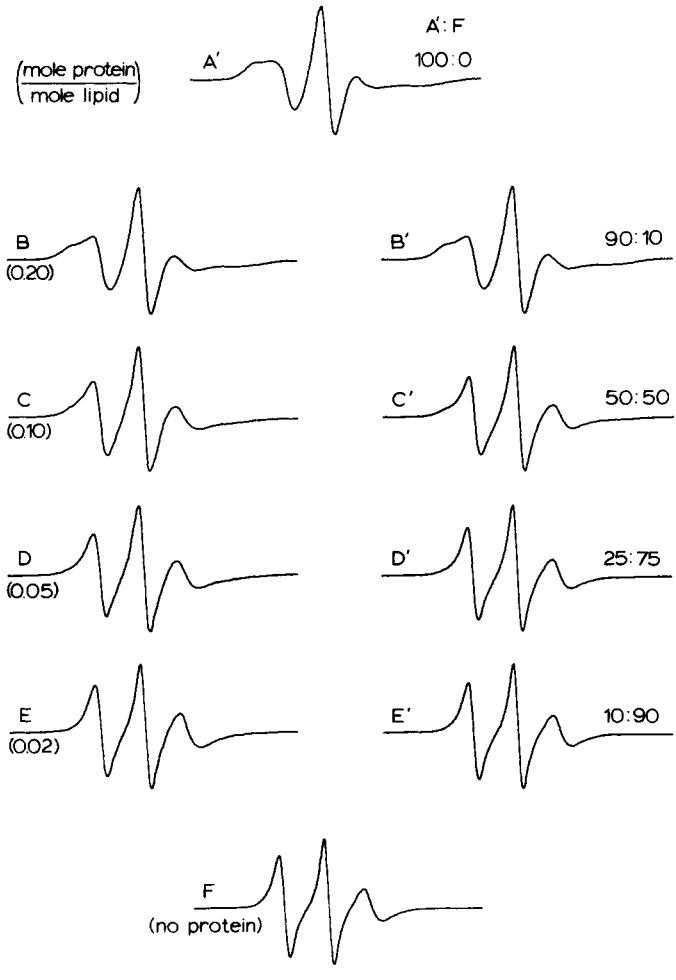

Fig. 1. ESR spectra of 12-doxyl stearic acid incorporated into T(is)/egg phosphatidylcholine dispersions in 100 mM NaCl, 10 mM EDTA solutions, with varying amounts of protein and lipids. Spectra B–F are experimental spectra taken at the indicated protein-to-lipid mole ratios. The simulated spectra, indicated by primed letters, are obtained by spectral additions of spectra A′ and F, using the weighted ratios shown. Spectrum A′ was obtained from spectrum B by appropriate subtraction of the fluid component (spectrum F). The experimental spectra were taken at 23 ± 1°C and 100 G scan range.

Integration of the original spectrum and the difference spectrum gives absorption curves from which the percentage of fluid spin label component can be obtained from the ratios of the areas under the absorption curves. The spectrum of the spin label diffused in T(is) associated with egg phosphatidylcholine at a protein-to-lipid mole ratio of 0.20 shows that 87% of the intensity of its ESR spectrum arises from immobilized spin label. Similar analysis of the other spectra gave percentages of lipid residing in an immobilized environment of 43%, 24%, and 8.1% for the sample containing protein-to-lipid mole ratios of 1:10, 1:20, and 1:50, respectively. As shown in Table I, these results suggest that 4–5 lipids are immobilized by the protein in these experiments.

TABLE I. Quantitative Analysis* of ESR Spectra of 12-Doxyl Methyl Stearate in T(is)-Egg Phosphatidylcholine Dispersions

Sample (lipid/protein mole ratio)	Percentage of total spectral intensity due to bound label	Number of moles of bound lipid per mole of T(is)
5:1	86.9%	4.4
10:1	43.8%	4.4
20:1	23.9%	4.8
50:1	8.1%	4.1

*ESR spectra were resolved into immobilized and nonimmobilized components as shown in Fig. 1 and then integrated to yield absorption and integral mode spectra, which were analyzed as described in the text.

Further support of the values obtained for the number of lipids immobilized by the protein is given by the close correspondence between the experimental spectra and the spectra synthesized by addition of spectrum A and spectrum F. In the right-hand column of Fig. 1 can be seen the spectra obtained by summation of the immobilized and non-immobilized spectra for varying amounts of each. The weighting factors used in the spectral simulation are shown in the figure. These spectra strongly suggest that the T(is) protein under the conditions of the experiment immobilizes 4–5 lipids per protein monomer. It is also interesting to note that the same number of lipids are immobilized irrespective of the amount of lipid present. The results shown here seem to indicate that there are two environments for the lipids in the presence of the protein. A third spectral component, which was detected in the course of the data analysis, can be seen in the sample containing a protein-to-lipid ratio of 1:10. This third component exhibits an ESR spectrum which is intermediate between the highly immobilized and nonimmobilized lipid spectra, and accounts for less than 10% of the total intensity of spectrum C.

Successful incorporation of T(is) into the bilayers was indicated by the appearance of an immobilized component in the ESR spectrum. Since T(is) is insoluble in water, the perturbation of the bilayer as deep as the location of the C-12 spin label indicates incorporation into the bilayer. Furthermore, density gradient centrifugation at all ratios used in the ESR experiments, with representative material prepared in the identical manner, produced a single band. The density corresponded to the initial ratio of protein to phospholipid recombined, which indicated a stable association of the two components. No significant amount of material appeared at the bottom of the gradient. Freeze-fracture electron microscopy of recombinants revealed bumps on one of the fracture faces.

Glycophorin

The glycophorin/egg phosphatidylcholine complexes were not stable at as high a protein-to-lipid ratio as T(is)/egg phosphatidylcholine complexes. The ESR spectrum of a 1:20 (glycophorin/egg phosphatidylcholine) mixture, the highest ratio obtainable as a stable complex, was essentially the same as for T(is) at the same protein-to-lipid ratio.

It is of interest to know not only whether the hydrocarbon chains of the phospholipids are immobilized by interaction with this integral membrane protein, but also whether the phospholipid headgroups are immobilized by interaction with glycophorin. Recently it was found that the B protein in human low-density lipoprotein immobilized some of the phospholipid of the particle, probably by interaction with the headgroups [16]. This

immobilization was detected because it caused, most likely, a large increase in the proton dipolar contributions to the linewidth, leading to phospholipid ^{31}P resonance linewidths so broad that they did not contribute to the high resolution resonance. In an analogous manner, the interactions between phospholipid headgroups and glycophorin was studied.

For these experiments, glycophorin was associated with egg phosphatidylcholine at an initial protein-to-lipid ratio of 1:20. After sucrose density gradient centrifugation of a representative sample prepared in the same fashion as for the magnetic resonance experiments, Lowry and phosphate analyses indicated that a stable lipid/protein complex was formed. A single band was observed at the density expected for a 1:20 mole ratio of glycophorin to phospholipid. No significant amount of material appeared at the bottom of the gradient. Further evidence of the incorporation of glycophorin into bilayers of phosphatidylcholine is seen in the freeze-fracture electron micrograph of Fig. 2, where bumps are seen on one of the fracture faces of the liposomes.

The ^{31}P NMR spectrum of an unsonicated egg phosphatidylcholine dispersion containing glycophorin at a 1:20 initial protein-to-lipid mole ratio appears in Fig. 3. Also in Fig. 3 is the ^{31}P NMR spectrum of an unsonicated egg phosphatidylcholine dispersion, containing the same amount of phospholipid as the glycophorin-phosphatidylcholine sample. These spectra are similar in shape to ^{31}P NMR spectra obtained from the erythrocyte membrane [17], vesicular stomatitus viral coat membrane [18], and unsonicated aqueous dispersions of pure phospholipids [19]. These spectra were obtained with broadband proton decoupling, so the lineshape is dominated by the chemical shift anisotropy of the phosphorus, which can be related to the motional order of the phosphate region of the phospholipid [19]. Furthermore, the proton decoupler was gated to remove the ^{31}P [^{1}H] nuclear Overhauser effect (about a 40% enhancement of signal intensity in unsonicated egg phosphatidylcholine [20]), and the spectra were obtained with 90° pulses separated by five T_1 of the phosphorus nuclei, so that the intensities would be directly proportional to the amount of phospholipid present. These spectra were obtained twice, from independent samples.

Two important pieces of information are contained in these spectra. First, the intensity of the glycophorin-containing sample is 25–30% less than the sample without the protein. The missing phospholipid must be in an immobilized environment such that the dipolar and/or chemical-shift anisotropy contributions to the lineshape broaden it sufficiently so that it cannot be seen under the conditions of these experiments. This corresponds to five to six phospholipids per glycophorin whose headgroups are immobilized by the protein.

Second, the effective chemical-shift anisotropy is reduced from 45 ± 1 ppm in the pure egg phosphatidylcholine sample to an apparent 36 ± 1 ppm in the glycophorin-containing membrane. The decrease in the apparent chemical-shift anisotropy may indicate a decrease in phospholipid headgroup order (or orientation) and/or a change in organization of those phospholipids not immobilized by the protein.

DISCUSSION

Phospholipids in unsonicated aqueous dispersions produce anisotropic ^{31}P resonances. When the phospholipids are in bilayer form, these ^{31}P resonances generally resemble the spectrum of egg phosphatidylcholine in Fig. 3 (for a review of these phenomena, see Seelig [23]. This spectrum results from partial motional averaging of the ^{31}P chemical-shift tensor by rapid axial motion of the phospholipid perpendicular to the bilayer surface.

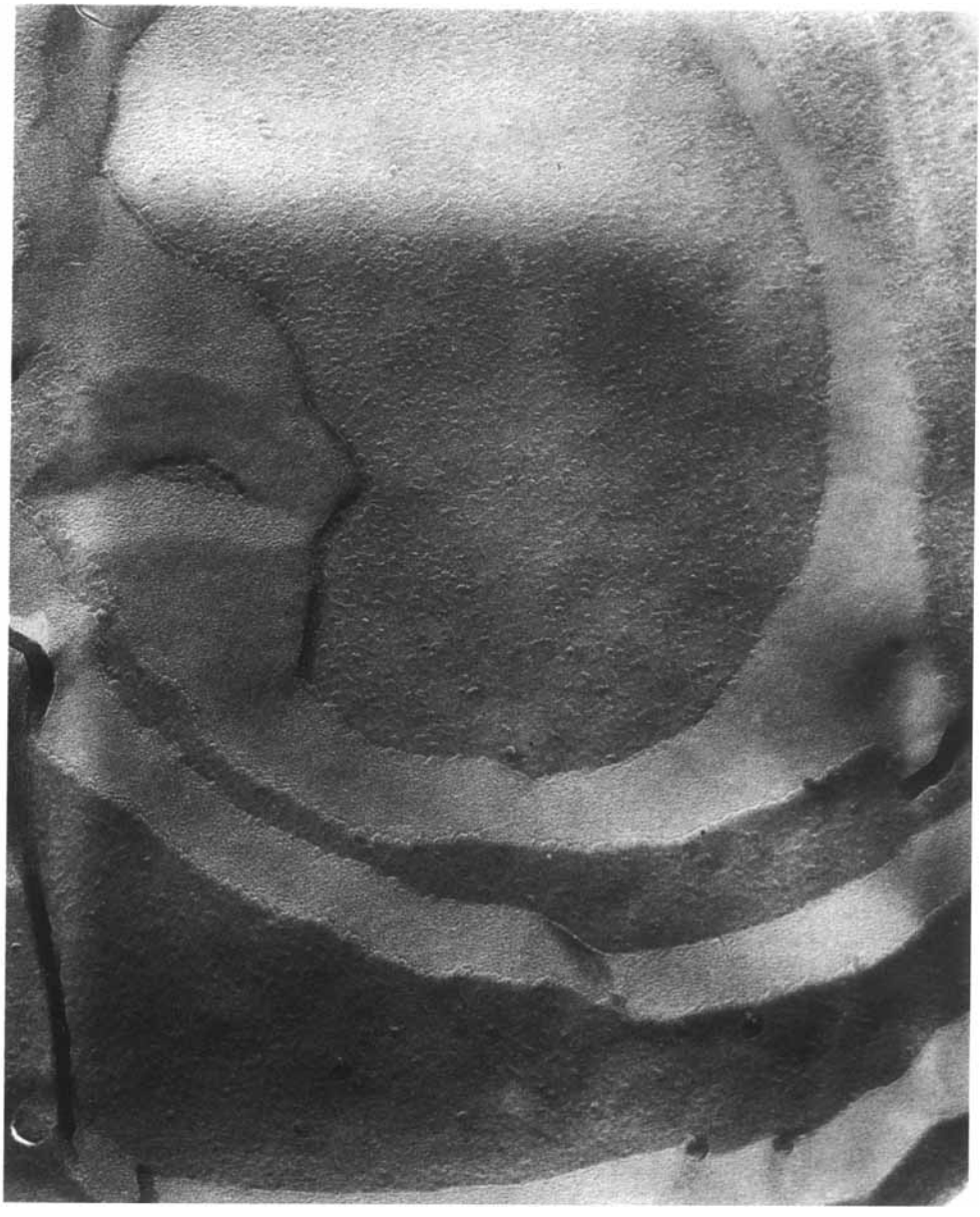

Fig. 2. Freeze-fracture electron micrograph of a 1:50 glycophorin/phosphatidylcholine recombinant at a magnification of 86,000 ×.

The chemical shift tensor describes the three-dimensional behavior of the chemical shift of the phosphate ^{31}P nucleus. The shielding of the nucleus, which determines the chemical shift, is dependent upon the direction from which the nucleus is approached. Because this is a three-dimensional dependence, the chemical shift is described by a tensor, and it is anisotropic because the shielding around the phosphorus is anisotropic. Thus, in a crystal where the molecule containing a phosphorus does not rotate rapidly, the observed chemical shift is a function of the angle of the crystal with the applied magnetic field [22].

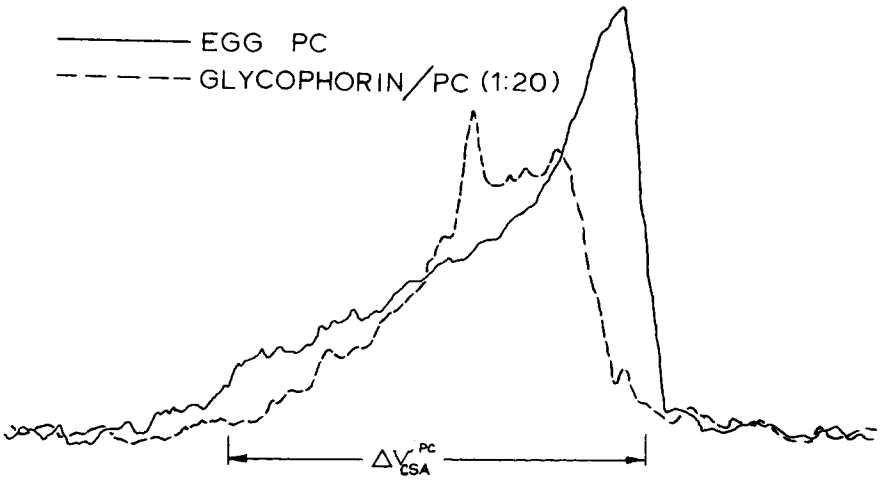

Fig. 3. ^{31}P NMR spectra of unsonicated egg phosphatidylcholine dispersions containing glycophorin. – – –) glycophorin/egg phosphatidylcholine 1:20 (20 mg egg phosphatidylcholine); ——) egg phosphatidylcholine (20 mg). Spectra were obtained with gated broadband proton decoupling. The effective chemical-shift anisotropy for egg phosphatidylcholine is 45 ± 1 ppm (or about 1800 Hz at this field strength) and for the glycophorin-containing sample is 36 ± 1 ppm (or about 1450 Hz at this field strength). Total spectral width is 5,000 Hz; 3,170 scans were obtained with a repetition rate of 7 s; 4,000 data points were collected, with 90° pulses (20 μsec).

When the molecules are randomly oriented, a powder pattern results. For a powder from dry phospholipids the chemical shift is spread over the full range of the chemical-shift tensor with the intensity at any one point proportional to the probability of an orientation in the random powder giving rise to that chemical shift. Discontinuities and shoulders in the spectrum provide the principle values of the chemical-shift tensor.

For phospholipids in randomly oriented bilayers in an aqueous environment rapid axial motion of the phospholipids perpendicular to the bilayer surface (and movement of the motional axis) averages two of the elements of the chemical-shift tensor, producing an apparent axially symmetric spectrum of considerably less breadth than the phospholipid powders. Analysis of ^{31}P NMR spectra of these systems, such as the spectra in Fig. 3, has led to information on motional ordering of the phospholipid head groups [19] and to a description of headgroup orientation in agreement with other, independent approaches [23, 24].

When phospholipids are not in a bilayer, different motional averaging may occur which leads to different spectral shapes. Some phosphatidylethanolamines form a hexagonal phase where additional motional averaging can occur by diffusion of the phospholipids around the cylinders in that phase. The ^{31}P spectra of these hexagonal systems show the opposite sign of the chemical-shift anisotropy and one-half the magnitude of a bilayer system. Similar results have been obtained for some sphingomyelins [25] and for the lipids of the bovine rod outer segment membranes [26].

With this background it is possible to examine the ^{31}P NMR spectra obtained with glycophorin recombined with phosphatidylcholine and obtain some useful information. From the intensity analysis it appears that a portion of the phospholipid is immobilized by the presence of the protein. Approximately five to six lipids appear to be immobilized per glycophorin molecule. This finding is supported by the ESR measurements with the

hyrophobic portion of the protein. The identification of immobilized lipid as a separate entity appears justified, because exchange with bulk lipid is slow enough that the spectrum of the bulk lipid is little affected by it. Since these two measurements sense two different portions of the membrane (Fig. 4), apparently the phospholipids interact with the protein through their hydrocarbon tails and their polar headgroups.

These results would be expected to have ramifications for the structure of the erythrocyte cell membrane in which glycophorin is found. Presumably glycophorin would immobilize phospholipid in the erythrocyte membrane just as in the recombined system. Recent work has suggested that there may be preferential interactions between glycophorin and negatively charged phospholipids [21]. Since it appears that glycophorin does bind some phospholipid tightly, and if there is a preferential interaction with negatively charged phospholipids, glycophorin might exert some influence on phospholipid asymmetry of the erythrocyte membrane.

The effects of glycophorin do not appear to be confined to the immediate region of the protein. Figure 3 shows that the ^{31}P resonances of the glycophorin-containing membranes are somewhat less broad than for egg phosphatidylcholine without glycophorin. This result may be interpreted in two different ways. First, the shapes of the two spectra are similar and the lesser breadth of the glycophorin-containing sample may reflect a decrease in the ordering of the headgroups of phospholipids not immobilized by the protein [19]. Alternatively, the spectral difference may reflect a change in organization of the phospholipids not immediately adjacent to the protein. Conversion to a hexagonal phase produces a dramatic change in spectral shape as mentioned earlier [27]. While a hexagonal phase does not appear to dominate here, since the sign of the anisotropy has not changed, a modification of the bilayer structure found in pure phosphatidylcholine systems by the glycophorin might lead to the result observed here. Either interpretation suggests a disorganization of bilayer structure of the phospholipid not immediately adjacent to the protein by the high concentration of glycophorin. Such a result is not altogether surprising, since the protein is no longer in a sea of lipid but rather constitutes a significant portion of the membrane area itself. It is not clear at this point whether glycophorin exhibits long-

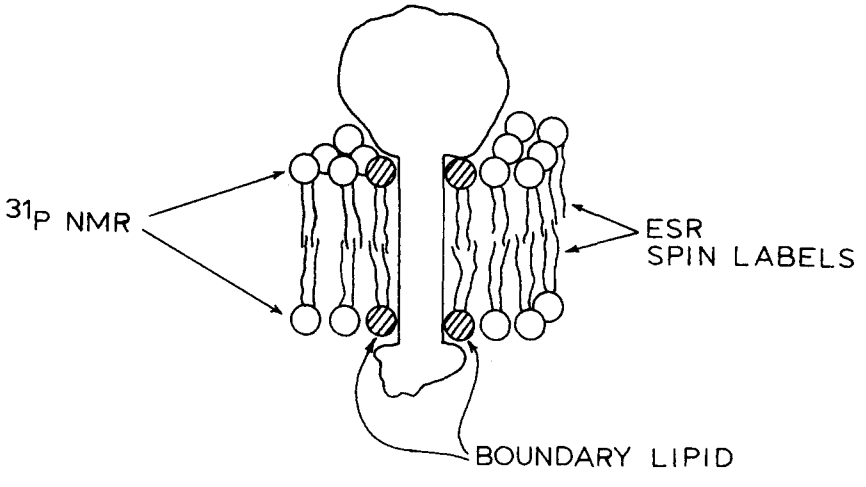

Fig. 4. Schematic of glycophorin in phospholipid bilayer, showing sampling areas of the NMR and ESR measurements.

range effects on the lipids of the erythrocyte membrane of any importance, or whether this effect is merely induced by the close proximity of glycophorin molecules in this recombined preparation. In this regard, it is interesting that rhodopsin at similar protein-to-phospholipid ratios produces no such disordering effect on the phospholipids [26].

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