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^{31}P and ^{19}F NMR Studies of Glycophorin-Reconstituted Membranes: Preferential Interaction of Glycophorin with Phosphatidylserine

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Summary. Glycophorin A, a major glycoprotein of the erythrocyte membrane, has been incorporated into small unilamellar vesicles composed of a variety of pure and mixed phospholipids. Nuclear spin labels including ^{31}P and ^{19}F have been used at natural abundance or have been synthetically incorporated in lipids to act as probes of lipid-protein interaction. Interactions produce broadening of resonances in several cases and it can be used to demonstrate preferential interaction of certain lipids with glycophorin. ^{31}P and ^{19}F probes show a strong preferential interaction of glycophorin with phosphatidylserine over phosphatidylcholine. There is some evidence that interactions are more pronounced at the inner surface of the bilayer and these results are rationalized in terms of the asymmetric distribution of protein and lipid.

Key Words ^{31}P NMR · ^{19}F NMR · glycophorin reconstitution · phosphatidylcholine · phosphatidylethanolamine · phosphatidylserine

Introduction

A number of integral membrane proteins may have hydrophobic segments of appropriate length to span a lipid bilayer when in an α-helical configuration. A protein which is well characterized, is known to be transbilayer, and has a single such hydrophobic segment would seem an ideal choice for the study of the basic properties of postulated lipid-hydrophobic helix interactions. Glycophorin A (GPA), the major glycoprotein in human erythrocyte membranes, would seem an ideal protein. This protein, with a molecular weight of 31,000, has been purified and its primary structure elucidated [30]. The series of some 23 nonpolar amino acid residues from 73 to 95 [8, 25] are believed to exist as a single transmembrane α-helix in the red blood cell [7].

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An additional interesting aspect of the interaction of glycophorin with membrane lipids is the possibility of preferential interaction with particular lipids. In our previous publication [21] we have found a slightly preferential interaction with phosphatidylethanolamine (PE) over phosphatidylcholine (PC) in the reconstituted system. It is also suggested by Gerritsen et al. [11] that PE in a GPA-lipid mixture is necessary to maintain the ion permeability barrier of GPA-containing vesicles. Moreover, several reports of preferential association of glycophorin with anionic lipids, such as phosphatidylserine (PS) or phosphatidylinositol (PI), also exist [1, 5, 26, 31]. Earlier, Jokinen and Gahmberg [13] showed that external labeling of aminophospholipids in human En (a-) erythrocyte membranes, which lack GPA, is more efficient than in normal membranes.

A number of authors have recognized the potential utility of GPA in studying lipid-protein interactions and have pursued these studies in reconstituted GPA membranes. A procedure we have found particularly advantageous involves solubilization of lipid and protein in sodium cholate followed by rapid removal of cholate on a Sephadex G-100 column [20]. It has produced small unilamellar vesicles identical in size to vesicles prepared by a similar method but without protein. Since morphology of preparations is known to directly affect properties of lipid bilayer systems, the similarity of vesicle sizes facilitates association of changes in properties with the presence of protein. The protein is incorporated asymmetrically with the carbohydrate bearing amino-terminus to the outside, and the protein distribution among vesicles is known from column chromatography and isopycnic centrifugation to be highly uniform [20, 21].

Among the spectroscopic methods for probing lipid properties, nuclear magnetic resonance (NMR), in particular, ^{31}P and ^{19}F NMR, remains a popular tool. In most cases, increases in line widths or decreases in spin-spin or spin-lattice relaxation times are indicative of motional restriction on interaction with protein. In a few cases, chemical shift changes are indicative of interaction. Each of the two nuclei mentioned above offers special advantages. ^{31}P occurs naturally at high abundance in the headgroups of phospholipids. It is a relatively sensitive nucleus yielding high resolution spectra in small unilamellar vesicle preparations. In the case of vesicle membranes containing anionic phospholipids, it is sometimes possible to resolve resonances arising from neutral and charged components, and from inner and outer halves of the bilayer [3]. Information obtained relates most directly to the headgroup region where phosphates reside. This is, however, quite appropriate for studies of preferential interactions which most likely arise at the headgroup level. ^{31}P NMR has been used quite frequently and productively in studies of membrane systems [3, 34, 35].

Although ^{19}F does not occur naturally in membranes, its incorporation as a spectroscopic label offers several advantages. First, the sensitivity of ^{19}F NMR is high, 83% relative to that of ^{1}H NMR. Second, since ^{19}F is not normally present in biological systems, there are no complications due to overlapping resonances. Third, ^{19}F NMR chemical shifts are large and reflect much greater sensitivity to chemical environments. ^{19}F NMR studies on the structure as well as the dynamics of phospholipids in model membranes have been reported, using fluorinated lipids [9, 10, 16]. The ^{19}F nuclei can be incorporated by either chemical synthesis or biosynthesis as a difluoromethylene substituent. The CF_2 group is similar to the CH_2 group in its size, geometry and physical characterization, so it is expected not to greatly perturb the membrane. However, the thermotropic behavior of some tetrafluorinated lipids has been found to be different [29, and references therein]. To minimize the perturbation of the fluorinated lipids, we employ the difluorinated mixed with a large amount of natural lipid in the present study.

Thus, we plan to present evidence for specific interactions of phospholipids with glycophorin using ^{31}P, ^{19}F NMR, and linewidth data. Mixed-lipid membranes with appropriate PS/PC ratios similar to that in erythrocyte membranes are used (for a review, *see* reference [22]). We will first examine the possibility of preferential PS interaction with glycophorin using ^{31}P NMR. Second, we will examine the extent to which preferential interactions may be transmitted to the hydrocarbon region using a ^{19}F-labeled analog of PS, 1-palmitoyl-2-6′,6′-difluoromyristoyl phosphatidylserine.

Materials and Methods

Materials

Glycophorin A was isolated and purified following the procedures of Furthmayr et al. [8]. Methods are detailed in our previous publication [20]. Egg yolk phosphatidylcholine (EYPC) was isolated and purified according to the procedure of Singleton et al. [27]. Phosphatidylethanolamine (PE) was obtained from *E. coli* extracts. Phosphatidylserine (PS) from bovine brain extracts was purchased from Sigma (St. Louis, Mo.) and further purified on a silicic acid column. The fluorinated PC, 1-palmitoyl-2-6′,6′-difluoromyristoyl-*sn*-glycero-3-phosphorylcholine (6′,6′F-PC) was synthesized by the acylation of egg yolk lysolecithin with 6,6-difluoromyristic acid anhydride [23]. The lysolecithin was prepared by the degradation of EYPC using phospholipase A_2 extracted from *Crotalus adamanteus* venom (Sigma) [12] and the acid anhydride was the reaction product of the fluorinated fatty acid with dicyclohexylcarbodiimide (DCC). 1-Palmitoyl-2-6′,6′-difluoromyristoyl-*sn*-glycero-3-phosphorylserine (6′,6′F-PS) was prepared from the corresponding 6′,6′F-PC via transphosphatidylation catalyzed by cabbage phospholipase D (Type I, Sigma) in the presence of excess L-serine (Aldrich, Milwaukee, Wis.) [6]. All the lipids employed were shown to be pure by thin-layer chromatography.

Fluorinated fatty acids were the fluorination product of the corresponding keto-acids made using Fluoreze-M, a MoF_6 reagent supplied by P.C.R. (Gainesville, Fla.). The keto-acids were most commonly synthesized through a Grignard reaction previously described in Gent et al. [9].

Sample Preparation

Glycophorin A was incorporated into vesicles by one of two processes previously described [20]. They are bath sonication and cholate solubilization followed by Sephadex G-100 filtration and dialysis. A typical buffer was 100 mM NaCl, 100 mM tris(hydroxymethyl)aminomethane (Tris), 10 mM (ethylenedinitrilo)tetraacetic acid (EDTA) and 0.02% wt/vol NaN_3 at pH 7.4. In cases of dilute samples, only one-tenth the amounts of Tris and EDTA were used in the buffer. Lipid concentrations in the samples prepared by sonication were usually 2 to 10% wt/vol in 5-mm NMR tubes. Lipid concentrations for cholate-prepared samples were typically 0.3% wt/vol. These more dilute samples were prepared in quantities sufficient for 10-mm NMR tubes. Samples were characterized primarily by Sepharose 2B column chromatography monitoring lipid content of the eluent by phosphate analysis [2] and protein by either Lowry [17] or sialic acid assays [28, 32].

NMR Measurements

^{31}P NMR measurements were carried out on a Varian CFT-20 spectrometer operating at 32.4 MHz in the Fourier transform mode. Ambient temperature in the probe was measured to be 29 ± 1 °C with broad-band noise ^{1}H decoupling. Spectra were normally recorded with 2K data points transforming into a 1K Hz sweep width after an accumulation of approximately 10K scans. Chemical shifts were measured with respect to external 1% H_3PO_4. Line widths were directly measured from the

spectra and reported in Hz as the width at the half-height of the peak. For PS in the PS-PC vesicles, where the PS resonance is not completely resolved, the linewidth was estimated by doubling the distance at half-height from the less-perturbed edge of the PS resonances to a hypothetical vertical line at the PS chemical shift. The errors in the latter measurement were estimated at $\pm 20\%$.

^{19}F NMR studies were carried out on an extensively modified Bruker HFX-90 pulsed Fourier transform spectrometer at a ^{19}F frequency of 84.7 MHz. Modifications unique to the present work included the use of orthogonal transmitter coils for ^{1}H decoupling and addition of selective filters to remove interference between the decoupler and the ^{19}F receiver channel. Samples in 5-mm NMR tubes were examined using a deuterium lock from the solvent for field-frequency stabilization. Efficient signal averaging was achieved by using a 45° pulse and a repetition rate less than 1 sec. Usually 30,000 FID's were accumulated for vesicle samples at concentrations of a few mM nucleus. Typical spectra were recorded with a sweep width of 5K Hz using 4K data points. The temperature of the probe was controlled within ± 1 °C.

Results

^{31}P NMR

Figure 1*a* shows a spectrum of PS/PC 1:4 vesicles prepared by cholate solubilization. A resonance due to PS appears as a weak shoulder ~ 0.5 ppm downfield from the dominant PC resonance. The shoulder is lower in intensity than expected on the basis of the 4:1 PS to PC ratio in the sample. Inner and outer resonances are sometimes resolved in ^{31}P spectra and it is possible that either the inner or outer PS resonance lies under the main peak. Addition of Co^{2+} can be used to broaden and shift outer resonances, while leaving inner resonances unperturbed [3]. Co^{2+} has been added to the sample whose spectrum is shown in Fig. 1*b*. The residual peak at -0.3 ppm suggests that a large part of the shoulder belongs to PS molecules on the outer half of the bilayer. The ratio of PC to PS peak areas under these conditions is approximately 8:1, indicating that there is an excess of PS in the outer half of the bilayer. This is also supported by our ^{1}H NMR measurements (*data not shown*), in which the distribution of PC across the bilayer can be estimated via the addition of Pr^{3+} to the exterior of the vesicle.

Glycophorin A has been incorporated into the PS/PC 1:4 vesicles by both sonication and cholate solubilization methods. Normally, there is no detectable phosphorus resonance in the GPA spectrum prior to reconstitution. Spectral changes on addition of GPA in samples prepared by cholate solubilization are illustrated in Fig. 2. Spectra exhibit broadening of PS and, to a much lesser extent, of PC. When Co^{2+} is added to the samples to remove outer resonances, the inner PC peak shows no broadening at a lipid-to-GPA ratio of 500:1, whereas the inner PS peak is undetectable presumably because of extensive broadening. The line broadening of the entire PS and PC resonances in the absence of Co^{2+} can be plotted as a function of GPA incorporated as shown in Fig. 3. It is noted that below the lipid/GPA molar ratio of 300:1, the PS resonance has become undetectable and its

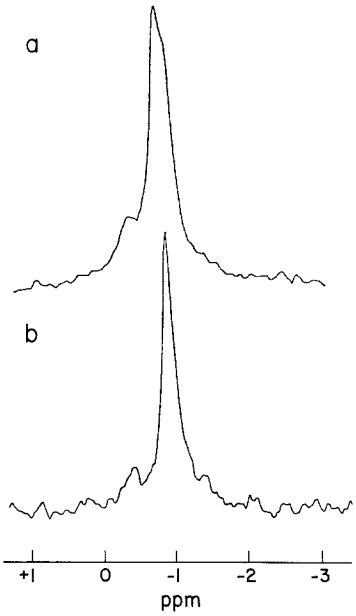

Fig. 1. The ^{1}H-decoupled ^{31}P NMR spectra of cholate-prepared PS/PC 1:4 vesicles. (*a*) In the absence of Co^{2+}. (*b*) In the presence of external Co^{2+}

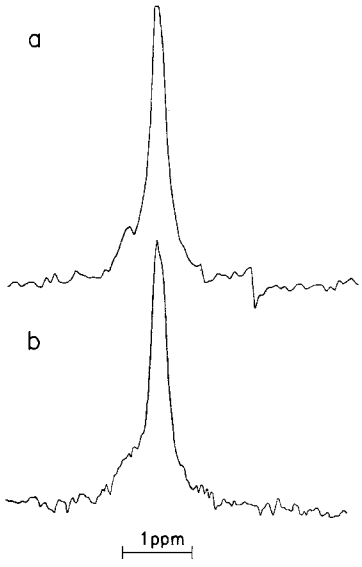

Fig. 2. The ^{1}H-decoupled ^{31}P NMR spectra of cholate-prepared PS/PC 1:4 vesicles. (*a*) In the absence of glycophorin. (*b*) In the presence of glycophorin incorporated at a lipid-to-protein mole ratio of 500:1

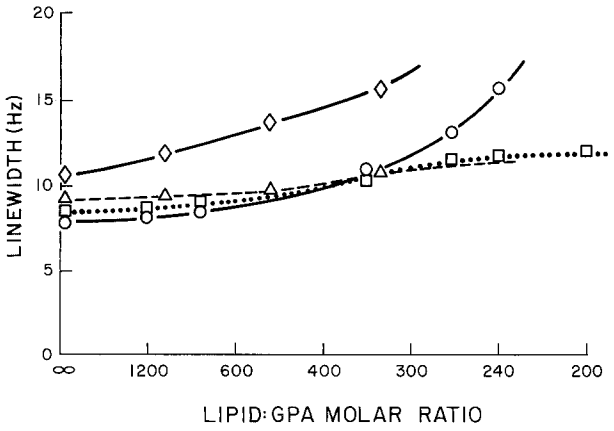

Fig. 3. Linewidth measurements as a function of GPA contents in the PS/PC 1:4 vesicles at 29 °C. Line widths are measured for both cholate reconstitution, i.e., PS (◇) and PC (△), and sonication reconstitution, i.e., PS (○) and PC (□)

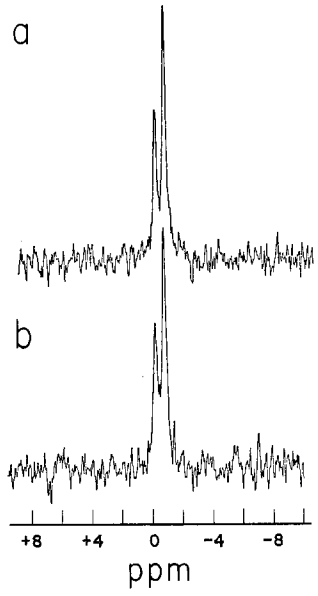

Fig. 4. The ^{1}H-decoupled ^{31}P NMR spectra of cholate-prepared PE/PC 1:2 vesicles. (*a*) In the absence of glycophorin. (*b*) In the presence of glycophorin incorporated at a lipid-to-protein mole ratio of 80:1

line width impossible to measure. Up to this mole ratio, the line width of the PS line increases in a nearly linear fashion, suggesting that PS molecules are in rapid exchange among lipid and protein sites, and that not all protein sites have been saturated. If we assume a high binding constant, this would imply binding sites for no more than 60 lipid molecules.

^{31}P NMR spectra of PE/PC 1:2 vesicles prepared by cholate solubilization were also investigated as shown in Fig. 4. The PE resonance situated at −0.15 ppm is well resolved from the PC

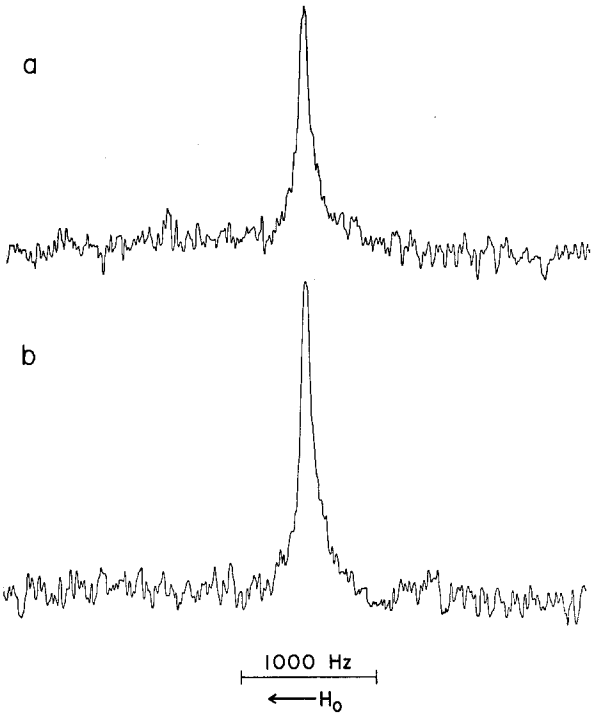

Fig. 5. Broad-band noise ^{1}H-decoupled ^{19}F NMR spectra of cholate-prepared 6′,6′F-PC/EYPC 1:3 vesicles at 25 °C. (*a*) In the absence of glycophorin. (*b*) In the presence of glycophorin incorporated at a lipid-to-protein mole ratio of 240:1

resonance and line widths of each can be measured in the presence of glycophorin. At a total lipid-to-GPA ratio of 300:1, no broadening is observed. In Fig. 4*b* very slight but detectable broadening is noted for the sample with a lipid-to-protein ratio of 80:1. At a lipid-to-protein ratio of 60:1, both are broadened; 20% for PC and 60% for PE. At 20:1, both are broadened 80%. Our spin-lattice relaxation data (*unpublished*) also reveal a slight rate increase for PE in the protein-containing PE/PC 1:2 vesicles. Clearly, neither lipid interacts as strongly as PS, but there may be a slight preference for interaction of GPA with PE over PC. A similar finding has been obtained from ^{13}C NMR studies in the previous publication [21].

^{19}F NMR

Since a pronounced preferential interaction of GPA with PS over PC and PE has been indicated at the headgroup level, it is of some interest to examine interactions further down in the hydrocarbon layer. ^{19}F substituted at the 6′ carbon of one of the esterified fatty acids in a phospholipid provides a suitable probe for such interactions. Figure 5 shows the ^{1}H-decoupled spectra of cholate-prepared 6′,6′F-PC/egg yolk PC (EYPC) 1:3 vesi-

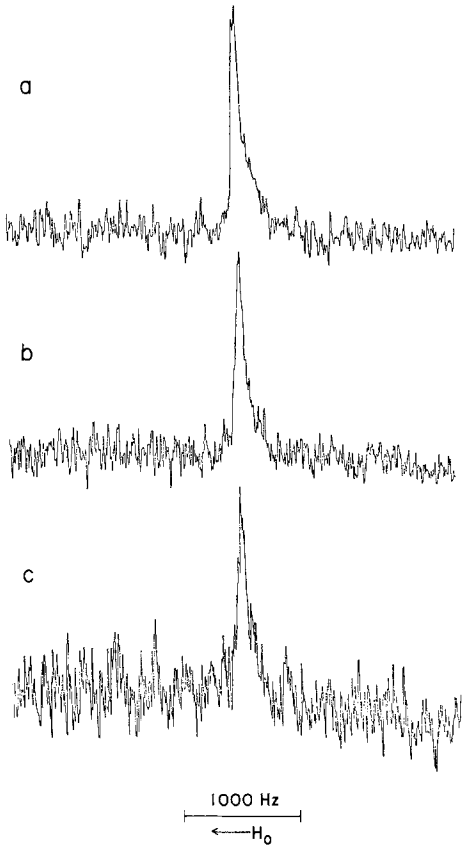

Fig. 6. ^{19}F NMR spectra of cholate-prepared 6′,6′F-PS/EYPC 1:5 vesicles at 25 °C. (*a*) In the absence of glycophorin. (*b*) In the presence of glycophorin incorporated at a lipid-to-protein mole ratio of 400:1. (*c*) In the presence of glycophorin at lipid-to-protein 160:1

cles at 25 °C. At a lipid/GPA ratio of 240:1, the line width increases from 90 Hz to 105 Hz showing approximately 15% broadening, a value consistent with ^1H NMR line broadening previously observed on PC vesicles containing GPA [20]. When unlabeled PS is incorporated to a one-sixth portion in this sample, the line broadening of the fluorine resonance from labeled PC molecules is almost undetectable at the same GPA level (*data not shown*), suggesting PS competes for interaction sites on GPA.

Figure 6 presents spectra recorded at 84.7 MHz and 25 °C using a system in which PS molecules are labeled 6′,6′F-PS in EYPC at a 1:5 ratio. The appearance of the ^{19}F signal in the non-GPA 6′,6′F-PS/PC vesicle prepared by cholate solubilization is different from those of fluorinated PC. A shoulder downfield of the major resonance is apparent even in the absence of glycophorin (*see* Fig. 6*a*). It is not due to a contribution from any large multilayer structures in the sample, since a homogeneous size distribution for the vesicle results from the cholate reconstitution procedure. It can be interpreted as PS at the inner as opposed to the outer half of the bilayer. The intensity of the downfield shoulder is estimated to be 15%, a value consistent with the inner PS intensity as determined in the present ^{31}P NMR study of the asymmetry of PS distribution across the bilayer. The downfield chemical shift for the inner PS is also consistent with the assignment for fluorinated PC made by Longmuir and Dahlquist [16]. As the GPA content in the vesicle increases, instead of broadening the entire resonance as in the case for 6′,6′F-PC, the line becomes less asymmetric with a gradual decrease in the downfield shoulder intensity (*see* Fig. 6*b* and *c*). Using the number of scans, signal/noise and $\Delta\nu_{1/2}$ in the spectra obtained, estimations of the apparent loss of intensity are $10\pm10\%$ for the 400:1 lipid/GPA sample and $40\pm20\%$ for the 160:1 sample shown in Fig. 6*b* and *c*, respectively. The shoulder visible in 6*a* is probably being broadened to a point where detection is impossible. Thus, interactions between GPA and PC and PS in general may not be as different at the hydrocarbon level as they are at the headgroup level. For the bulk of PS, presumably that fraction in the outer half of the bilayer, interactions of GPA with PC and PS are very similar. We can, however, interpret our data as evidence for a stronger preferential interaction with PS on the inner half of the bilayer.

Discussion

This work is designated to demonstrate any specific interaction of glycophorin with phospholipids by first establishing an appropriate model system. Information on the interaction can then be obtained by mainly linewidth and chemical shift data in normal Fourier transformed NMR spectra. To demonstrate the postulated PS-GPA interaction in human erythrocyte membranes [4, 31], one would like to have a model system with a large excess of PS at the inside surface of the vesicle. Yet, in addition to the salt concentration and pH in solution, the composition of mixed lipids ought to affect the distribution of a component lipid in the vesicle. There have been reports presenting different views on the asymmetry of certain phospholipids in the vesicle [14, 15, 18, 19]. However, the methods used might have led to different conclusions. Our finding that at low mole ratios PS resides preferentially at the outer surface of the bilayer vesicle is due probably to the charge effect. This is in good agreement with Massari's [18], but not necessarily contradictory to the observation of

Berden et al. [3] in which a much higher PS content was employed in the sonicated PS-PC sample.

We have also used ^{31}P NMR and ^{19}F NMR to provide evidence for a range of specificities of interaction of glycophorin with different lipids in reconstituted unilamellar vesicles. Such specificities are not unexpected and have been proposed by a number of authors [1, 5, 16, 31]. Previous evidence has, however, been based on chemical modification and co-isolation studies which can be interpreted in other ways. Broadening of NMR lines in homogeneous systems is generally interpreted as arising from motional restriction and thus provides more direct evidence of protein-lipid association. The strongest evidence of preferential association comes from the broadening of the resolvable PS ^{31}P resonance in PS-PC mixtures. At least a portion of this resonance could be assigned to PS on the inner half of the vesicle bilayer and thus its broadening supports the notion that GPA is incorporated in a transbilayer fashion.

However, our ^{31}P NMR results do not show the type of line broadening, or loss of intensity obtained by Yeagle et al. [24, 33, 35]. It may probably be attributed to the different procedures in the reconstitution of GPA. As a result, much larger structures are formed in Yeagle's preparation, and the protein may not be in the same state with respect to surrounding lipids as in our preparation. A thorough investigation of the effects of organic solvent and cholate employed in the two reconstitutions will hopefully provide a satisfactory explanation.

Our ^{19}F data also support a preferential interaction with PS, not only at the headgroup level but at the hydrocarbon chain level. The origin of the asymmetry observed in Fig. 6, however, warrents some discussion. An alternative interpretation is that it is reminiscent of phase immiscibility for the saturated fluorinated PS and the unsaturated EYPC. At this mole ratio of F-PS/EYPC 1:5, the lipids would possibly be segregated into PS-enriched and PS-deficient phase. In ^{19}F NMR spectra, the PS-enriched phase would give rise to the main peak. However, since the phase transition temperatures for both fluorinated lipids, F-PC and F-PS, are much higher than that of EYPC, this main peak would appear broader than the minor peak due to the PS-deficient phase. Evidently this is not observed in our data. Therefore, we consider the interpretation more likely that the asymmetry could be attributed to the distribution of PS across the bilayer. Drawing an analogy with data showing resolution of fluorinated PC molecules in the inner and outer halves of bilayers obtained by Longmuir and Dahlquist [16], the portion broadened in the presence of GPA may be associated with PS in the inner half of the bilayer. We know that GPA is incorporated asymmetrically [20] and this finding, together with the ^{31}P NMR data, would provide supportive evidence for the postulate of Bretscher [4] that the positively charged Arg and Lys enriched C-terminus of GPA may interact through electrostatic forces with the negatively charged PS on the cytoplasmic side of the erythrocyte membrane.

Although our PS-PC system has been found with excess PS in the outer half of the bilayer, which is in contrast to the situation in the erythrocyte membrane, the interaction of PS with the C-terminus of glycophorin exists in our system. The origin of this specificity is probably electrostatic forces. Moreover, there has been no indication that the protein alters the distribution of PS across the membrane in this system. Hence, there must be other factors in the erythrocyte membrane to produce the asymmetric distribution, with most PS on the cytoplasmic side of the membrane. Interactions of PS with other proteins or lipids in the erythrocyte membrane remain vast possibilities.

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