Biochimica et Biophysica Acta, 685 (1982) 153–161
Elsevier Biomedical Press

BBA 71080

EFFECT OF GLYCOPHORIN ON LIPID POLYMORPHISM

A ^{31}P-NMR STUDY

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(Received August 18th, 1981)

Key words: ^{31}P-NMR; Glycophorin; Lipid polymorphism

(1) The effect of glycophorin, a major intrinsic glycoprotein of the human erythrocyte membrane, on lipid polymorphism has been investigated by ^{31}P-NMR (at 36.4 MHz) and by freeze-fracture electron microscopy. (2) Incorporation of glycophorin into vesicles of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) results in the formation of unilamellar vesicles (1000–5000 Å diameter) which exhibit ^{31}P-NMR bilayer spectra over a wide range of temperature. A reduction in the chemical shift anisotropy ($\Delta\sigma_{csa}^{eff}$) and an increase in spectral linewidth in comparison to dioleoylphosphatidylcholine liposomes may suggest a decrease in phospholipid headgroup order. (3) 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), in the presence of excess water, undergoes a bilayer to hexagonal (H_{II}) phospholipid arrangement as the temperature is increased above 0°C. Incorporation of glycophorin into this system stabilizes the bilayer configuration, prohibiting the formation of the H_{II} phase. (4) Cosonication of glycophorin with DOPE in aqueous solution (pH 7.4) produces small, stable unilamellar vesicles (300–1000 Å diameter), unlike DOPE alone which is unstable and precipitates from solution. (5) The current study demonstrates the bilayer stabilizing capacity of an intrinsic membrane protein, glycophorin, most likely by means of a strong hydrophobic interaction between the membrane spanning portion of glycophorin and the hydrophobic region of the phospholipid.

Introduction

Several recent investigations suggest that individual lipid species isolated from biological membranes assume either a bilayer or hexagonal (H_{II}) arrangement upon hydration [1]. It has been demonstrated that certain factors such as temperature, lipid composition, pH and divalent cations can modulate the bilayer/non-bilayer preference of lipids [1]. Since non-bilayer forming lipid has been implicated as a possible key component in fundamental membrane phenomenon such as transbilayer transport and fusion, it is essential that they be more fully characterized under conditions which most closely mimic the in vivo environment.

The nature of lipid-protein interactions in biological membranes is a topic of considerable current interest in light of frequent observations that membrane-bound enyme activity is markedly dependent on the physical state and chemical structure of phospholipid in contact with protein [2–5]. Accordingly, several physical techniques have been applied in order to elucidate the molecular nature of the lipid-protein interaction, which plays an important role in membrane organization [4,6–9].

Abbreviations: DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; $\Delta\sigma_{csa}^{eff}$, effective phosphorus-31 chemical shift anisotropy; $\Delta\nu_{1/2}$, linewidth at half maximum spectral intensity.

0005-2736/82/0000-0000/$02.75

Little is known, however, about the ability of proteins to modulate the structure of the phospholipid matrix in systems containing lipids which prefer non-bilayer organization. In the isolated bovine rod outer segment membrane, the phospholipids were shown to be almost exclusively organized in a bilayer, whereas the hydrated total lipid extract at 37°C consisted of the hexagonal (H_{II}) phase, the lamellar phase and 'lipidic particles'. Accordingly, a bilayer stabilizing capacity of rhodopsin was suggested in the photoreceptor membrane [10]. Poly(L-lysine), a polypeptide model of an extrinsic membrane protein, was shown to inhibit the ability of Ca^{2+} to induce the hexagonal (H_{II}) phase in cardiolipin [11]. Cytochrome *c*, a highly basic protein, can specifically induce the hexagonal (H_{II}) phase and possibly an inverted micellar structure of part of the phospholipids in cardiolipin-containing model membranes [12]. Stier et al. [13] have observed that vesicles prepared by reconstitution of NADPH-cytochrome *P*-450 reductase and cytochrome *P*-450 with egg PC and egg PE result in ^{31}P-NMR spectra dominated by the contribution of isotropic motional averaging. To date, these are the only studies reported for non-bilayer forming lipid and protein interactions. Thus, in an attempt to gain more insight regarding the possible effects of protein on the structure of membrane lipids, we investigate the effect of glycophorin on the phase behaviour of two phospholipids, one (DOPE) which prefers the H_{II} phase at physiological temperature, the other (DOPC) preferring the bilayer phase.

Glycophorin, the major intrinsic glycoprotein of the erythrocyte membrane, has been chosen for this study since it can be isolated in a pure form in large quantities, it is biochemically well characterized [14] and has been the subject of many physicochemical investigations when reconstituted with phospholipid [9,15–20].

The effects of glycophorin on lipid polymorphism as studied by ^{31}P-NMR and freeze-fracture electron microscopy are reported.

Materials and Methods

Phospholipids. 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine ($18:1_c/18:1_c$-phosphatidyl-ethanolamine) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine ($18:1_c/18:1_c$-phosphatidylcholine) were synthesized according to established methods [21,22].

Preparation of glycophorin. Ghosts were prepared according to Parpart [23]. Glycophorin was isolated and purified from ghosts according to a modification of the method described by Verpoorte [24]. 1 volume of ghosts, 0.2 volumes of cold 1.0 M Tris, pH 8.0 and 1.2 volumes of cold *n*-butanol were stirred for 30 min at 0°C. The resulting suspension was centrifuged at $8000 \times g$ for 10 min and the lower aqueous phase collected with a syringe. The aqueous phase, which contained a small amount of butanol was dialyzed against 10 mM Tris pH 8.0 at room temperature for 2 h. The aqueous phase is extracted with an equal volume of 50% phenol, centrifuged and the phenol poor upper aqueous phase is dialyzed for 3 days at 4°C against 1 mM Tris, pH 8.0. To 1 volume of the dialyzed phase is added 0.2 vol. of 1.0 M Tris, pH 8.0. This volume is mixed with 2.1 volumes of 95% ethanol (−20°C) and left at 0°C for 2 h. The precipitate is removed by centrifugation at $8000 \times g$ for 15 min. The clear supernatant (1 volume) which is 60% ethanol is mixed with an equal volume of cold (−20°C) acetone. After 30 min, the glycophorin precipitate is collected by centrifugation at $5000 \times g$ for 10 min, redissolved in 1 mM Tris-HCl, pH 7.4 and dialyzed overnight at 4°C against distilled water. The dialyzed glycophorin is freeze-dried and stored at −20°C. Disc gel electrophoresis was carried out according to Fairbanks [25]. The purified glycophorin showed two bands on SDS-polyacrylamide gels stained with Coomassie blue and with periodate-Schiff reagent (PAS I and PAS II). Glycophorin prepared by this method contained $\leq 0.03\%$ phosphorus as determined according to a modification of the procedure of Fiske-Subba Row [26], and 2.1 μmol sialic acid/mg protein using the method of Warren [27].

Reconstitution of glycophorin with phospholipid. The reconstitution of DOPC and DOPE was performed by the method according to MacDonald and MacDonald [16]. Glycophorin (50–70 mg) was dissolved in 1–2 ml of 1 mM Tris-HCl, pH 7.4 and added to a solution of $CHCl_3$/MeOH in the proportion 3 : 1.5 : 0.02 ($CHCl_3$: MeOH : H_2O, v/v). Organic solvents were removed by rotary

evaporation under water aspirator vacuum (bath temperature approx. 15°C for DOPC, 0–4°C for DOPE). Final solvent traces were removed under oil pump vacuum overnight. The dry-lipid protein film was hydrated with 100 mM NaCl, 0.01 M Tris-HCl, pH 7.4 (0°C for DOPE). It was noted that the glycophorin-DOPE sample dispersed much more readily when buffer was added than a pure DOPE sample which usually requires shaking with glass beads for long periods of time. The dispersed material was centrifuged two times at $10000 \times g$ for 10 min to remove large structures containing little or no protein. Glycophorin containing vesicles were pelleted by centrifugation at $125000 \times g$ for 60 min at 4°C. Protein incorporation was measured by determination of the sialic acid to phosphate ratio in the pellet using a value of 2.1 μmol sialic acid/mg protein and a molecular weight of 12500 for the protein part of glycophorin. Freeze-fracture electron microscopy was performed as described previously [28].

Sonicated vesicles were prepared by cosonication of approx. 25 μmol of DOPE and 50 mg of glycophorin in 1.5 ml of 100 mM NaCl, 0.01 M Tris-HCl, using a Branson tip sonicator (power setting 5) for eight 30 s intervals. The resulting opaque solution was centrifuged for 20 min at $30000 \times g$ at 4°C to remove metal particles from the probe.

K^+ permeability. DOPE-glycophorin vesicles were prepared in 150 mM KCl, 20 mM Tris-HCl, pH 7.4 and passed over a Sephadex G-50 column equilibrated with 150 mM choline chloride, 20 mM Tris-HCl, pH 7.4 to remove external K^+. Efflux of K^+ was measured using a potassium selective electrode as previously described [29].

Orientation of sialic acid. In order to establish the orientation of the sugar residues in the DOPE-glycophorin vesicles, either neuraminidase (50 U/mg glycophorin) or trypsin (5%,w/w glycophorin) were added, the samples held at 37°C for 1 h and centrifuged at $125000 \times g$ for 60 min at 4°C. Previous studies have demonstrated that trypsin splits glycophorin into four major peptides and that the peptide containing the hydrophobic region of the protein and a small part of the N-terminal region without sugar residues remains incorporated in the membrane upon incubation with trypsin [30]. Comparison of the amount of sialic acid present in solution before and after centrifugation gives insight into the protein orientation in the membrane. Following enzymatic treatment, the samples were analyzed by ^{31}P-NMR and electron microscopy to investigate any structural reorganization which might have occurred.

^{31}P-NMR measurements. Spectra shown were obtained on a Bruker WH-90 Fourier transform spectrometer at 36.4 MHz.

DOPE-glycophorin vesicles. All spectra were obtained in the presence of broad band proton decoupling (20 W). Accumulated free induction decays were obtained from up to 200000 transients, employing a delay time of 83 μs, an interpulse time of 0.17 s and a 45° radiofrequency pulse. An exponential multiplication corresponding to 50 Hz line broadening was applied to all free induction decays. Approximately 1 ml of 100 mM NaCl, 25 mM Tris-HCl, 0.2 mM EDTA, pH 7.4 was added to the pelleted lipid-protein vesicles and 0.2 ml of the ^2H_2O analog of the buffer, resulting in a final volume of approx. 1.3–1.4 ml. Lipid concentrations were of the order of 20 μmol/ml. DOPC-glycophorin spectra were obtained from up to 40000 transients under spectral conditions identical to those described above. Chemical shift anisotropies were estimated by measuring the chemical shift difference between the isotropic component at 0 ppm and the upfield bilayer component. This value represents 1/3 of the total chemical shift anisotropy.

Sonicated vesicles. Spectra were obtained in the presence of broad band decoupling (4 W). Accumulated free induction decays were obtained from 500 transients, employing a delay time of 83 μs, interpulse time 1.7 s and employing 90° radiofrequency pulses. An exponential multiplication corresponding to 2 Hz line broadening was applied to all free induction decays. Spectra were recorded in the absence and presence of neodymium (5 mM); triphenylphosphine was used as an internal standard.

Results

Lipid polymorphism

The use of ^{31}P-NMR to detect lipid polymorphism is well documented (for review, see Ref. 1 and references therein). Three major ^{31}P-NMR

lineshapes may be observed when individual lipid species from biological membranes are hydrated. The first type, a 'bilayer' spectrum, results from partial averaging of the chemical shift anisotropy by rapid rotation of the molecules along their long axis [21,31–33]. Under conditions employing proton decoupling, the resulting broad spectrum is characterized by a low-field shoulder and high-field peak separated by $\Delta\sigma_{csa}^{eff} \simeq 40$ ppm. Handshaken liposomes (radius ≥ 2000 Å) of phosphatidylcholine, saturated phosphatidylethanolamine, phosphatidylserine, phosphatidylglycerol and sphingomyelin exhibit this type of ^{31}P-NMR spectra when in the liquid-crystalline phase. Lipids in the hexagonal (H_{II}) phase, such as unsaturated phosphatidylethanolamine [31] and cardiolipin in the presence of Ca^{2+} [34] exhibit lineshapes with a high-field shoulder and a low-field peak where $\Delta\sigma_{csa}^{eff} \simeq 20$ ppm. This lineshape results since lipids in the H_{II} phase experience additional motional averaging compared to large liposomal structures due to lateral diffusion around the aqueous channels (20 Å) of the hexagonal 'tubes'. Lastly, lipids in inverted micellar configurations and sonicated vesicles allow effectively isotropic motion to occur as lateral diffusion results in averaging over all orientations resulting in a narrow, symmetric ^{31}P-NMR spectrum [1].

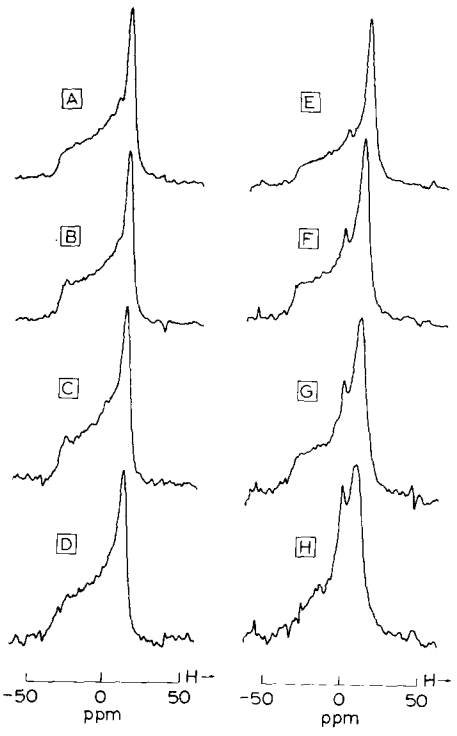

Fig. 1. ^{31}P-NMR spectra of DOPC in the absence (A–D, 0, 20, 40 and 60°C) and presence of glycophorin (E–H, 0, 20, 40 and 60°C) at 36.4 MHz as a function of temperature.

Reconstitution of glycophorin with phospholipid

Glycophorin reconstituted with DOPC by the method of MacDonald and MacDonald [16] produces unilamellar vesicles (1000–5000 Å) which have been biochemically well characterized [17]. Introduction of glycophorin into DOPC vesicles gives rise to intramembranous particles (40–50 Å diameter) as observed by freeze-fracture electron microscopy. Phospholipid/protein ratios can be varied by mixing the appropriate amount of each component. Vesicles used in this study were prepared at a 300:1 molar lipid/protein ratio since this closely mimics the situation in the erythrocyte membrane.

The ^{31}P-NMR spectra obtained from aqueous dispersions of DOPC at various temperatures (0–60°C) have the characteristic asymmetry and chemical shift anisotropy associated with phospholipids in the bilayer phase. Spectra resulting from unsonicated vesicles with glycophorin incorporated at a 300:1 lipid to protein mole ratio are shown in Fig. 1 (E–H). The majority of phospholipid remains in a bilayer organization over the entire temperature range measured in this experiment (0–60°C). As the temperature is increased above 0°C, a sharp spectral component appears at 0 ppm. The intensity of this peak becomes more pronounced at higher temperature (Fig. 1, G,H). This component most likely arises from either increased lipid lateral diffusion in the smallest glycophorin-containing vesicles in the preparation or an increased disordering of the phospholipid headgroup region by the protein. This spectral behaviour is completely reversible upon cooling, with a spectrum identical to that in Fig. 1E obtained after cooling the sample to 0°C (spectra not shown).

The chemical shift anisotropy and the ^{31}P-NMR spectral linewidth (full width at half spectral height) have been commonly used to characterize the local order of the phosphate region of lipid

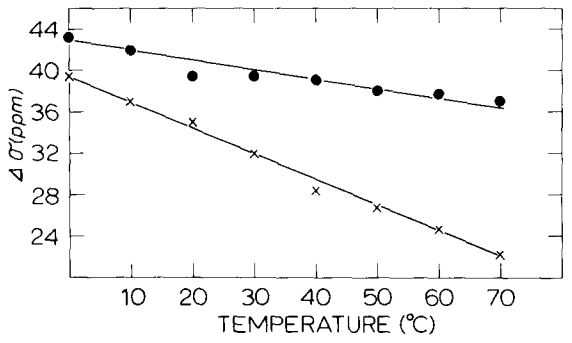

Fig. 2. Temperature dependence of $\Delta\sigma$ for DOPC liposomes (●) and DOPC-glycophorin vesicles (×).

molecules in bilayers (for review, see Ref. 35). A plot of the variation in the chemical shift anisotropy as a function of temperature for DOPC liposomes (●) and DOPC-glycophorin vesicles (×) is presented in Fig. 2. It is observed that the effective chemical shift anisotropy is reduced in the glycophorin-containing vesicles as compared to the DOPC liposomal system. Additionally, an increase in the linewidth of the spectra with increasing temperature is detected in the DOPC-glycophorin vesicles which is not observed in the pure liposomal system (compare Figs. 1C and 1G). It should be noted, however, that the apparent increase in the width of the high-field component of the bilayer spectra may be due to overlap of the high-field component with the isotropic signal and may not necessarily reflect phospholipid disordering by the protein.

Reconstitution of glycophorin with DOPE, which prefers the hexagonal (H_{II}) arrangement above 0°C results in the formation of small, unilamellar vesicles (300–1500 Å diameter) with ill-defined intramembranous particles as revealed by freeze-fracture electron microscopy (Fig. 3A), indicating the bilayer stabilizing capacity of glycophorin. It should be noted that a small fraction of hexagonal (H_{II}) phase lipid is found in addition to the vesicles in this preparation. Results obtained from extensive freeze-fracture studies on numerous other DOPE-glycophorin samples (data not shown) reveal that hexagonal (H_{II}) phase lipid is totally absent from or present in extremely minute quantity in the vesicle preparations. In Fig. 3A, a 'MacDonald' vesicle is shown adjacent to a small area of DOPE in the hexagonal (H_{II}) phase whose fracture face is composed of long, parallel lines which have a 'ribbed' appearance. Hexagonal phase lipid present in the preparation comprises an extremely small fraction of the entire lipid population and is most likely due to incomplete separation of the supernatant (containing the vesicles) from the pelleted material after centrifugation at $10000 \times g$ or from physically connected phases (Fig. 3A). Final phospholipid/protein ratios were always 20:1–30:1 in the DOPE vesicles regardless of the ratio present in the dry film prior to hydration with buffer. Treatment of the vesicles with trypsin or neuraminidase, as described in Methods revealed that 95 ± 5% of the sialic acid residues of glycophorin are oriented towards the outside of the vesicle. It has been previously reported that 75–80% of the sugar residues are directed to the outside of DOPC vesicles containing glycophorin [17].

Permeability experiments performed on the DOPE-glycophorin vesicles prepared in 150 mM KCl/20 mM Tris-HCl, pH 7.4, yielded trapped K^+ values (2.5 μl/μmol phospholipid) in good agreement with values calculated for 800–1000 Å unilamellar phospholipid vesicles. A slow leak of approx. 5% of the trapped K^+ was detected after 15 min. Glycophorin-containing DOPC vesicles, which are larger and have less protein incorporated that the DOPE-glycophorin vesicles have trap values of 2.1 μl/μmol phospholipid and approx. 8.6% of the K^+ leaks out of the vesicle in 10 min [18]. This value is low for the size of the vesicles (1000–5000 Å) in the DOPC-glycophorin preparation indicating a high K^+ leak for the majority of the vesicles.

The ^{31}P-NMR spectrum of an aqueous dispersion of DOPE is shown in Fig. 4A. The resulting spectrum, having the characteristic asymmetry and chemical shift anisotropy of lipid in the hexagonal (H_{II}) phase is in good agreement with previous studies [21].

The temperature induced variation in the ^{31}P-NMR spectral lineshape of unilamellar vesicles of DOPE and glycophorin (20:1 lipid/protein mole ratio, 300–1500 Å diameter) is shown in Fig. 4B–F. In the presence of glycophorin at 0°C, a considerable portion of the phospholipid (approx. 40–50%) is in a phase undergoing isotropic motion and the

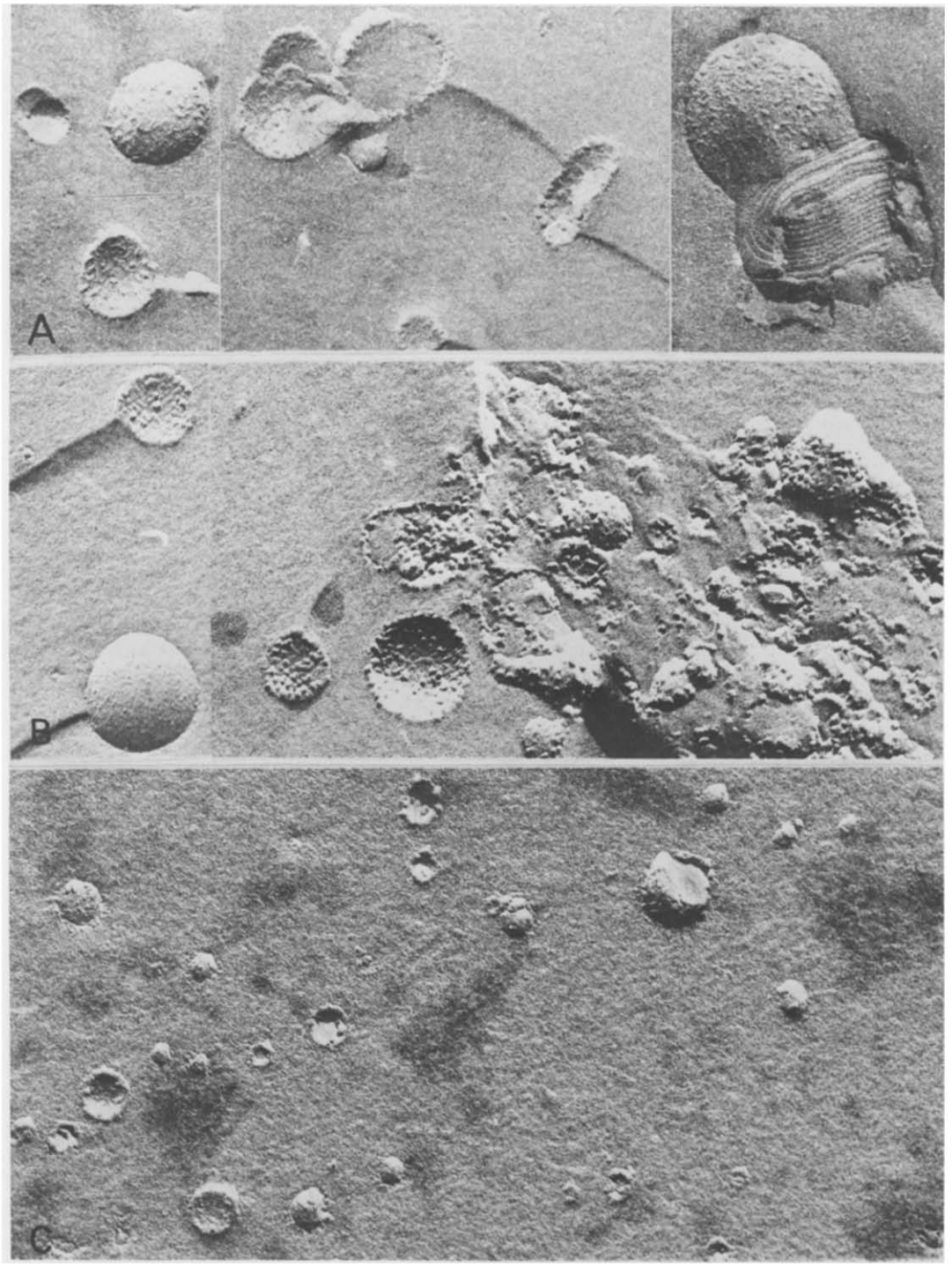

Fig. 3. Freeze-fracturing of (A) DOPE-glycophorin vesicles, and a DOPE-glycophorin vesicle next to DOPE in the hexagonal (H_{II}) phase, (B) DOPE-glycophorin vesicles after treatment with trypsin, (C) sonicated vesicles of DOPE-glycophorin. Final magnification, 100000×.

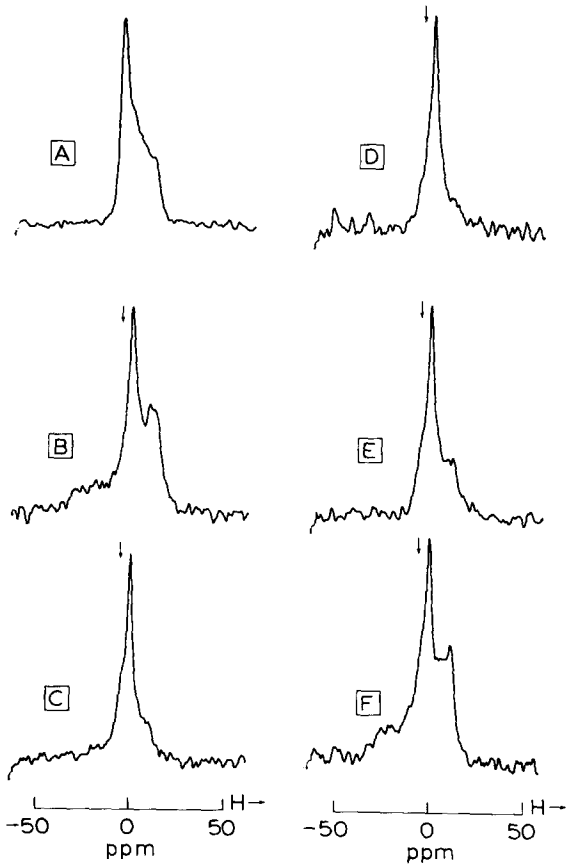

Fig. 4. ^{31}P-NMR spectra of DOPE in the absence (A, 0°C) and presence of glycophorin (B–F, 0, 25, 50, 25, 0°C) at 36.4 MHz. Spectra E and F were obtained after cooling of the sample from 50°C. Arrows indicate the position of the main spectral component of phospholipid in the hexagonal (H_{II}) phase.

remaining lipid is arranged in a bilayer configuration. In comparison, DOPE liposomes are arranged in a hexagonal (H_{II}) phase at this temperature (Fig. 4A). Increasing the temperature to 25°C results in nearly 90% of the lipid being present in an isotropic averaging phase. Further temperature increase to 50°C (Fig. 4D) reveals nearly 100% of the lipid population in an isotropic phase. It is important that the narrow, symmetric component present at 0 ppm in Fig. 4B–F is not confused with the main spectral feature of the hexagonal (H_{II}) phase which occurs approx. 6.7 ppm downfield from the isotropic component. Spectra 4B–F also lack the characteristic asymmetry and chemical shift anisotropy of lipid arranged in the H_{II} phase. For clarity, arrows marking the position of the main spectral component of the H_{II} phase are shown in spectra B–F. Cooling of the sample to 0°C (Fig. 4F) results in a ^{31}P-NMR spectra nearly identical to that shown in Fig. 4B. Thus, the combination of the ^{31}P-NMR and electron microscopic data clearly demonstrates the bilayer stabilizing capacity of glycophorin over a wide range of temperature. The stability is a long term one as samples checked weeks after initial investigation reveal spectra virtually identical to those originally obtained.

In order to investigate the involvement of the sugar residues of glycophorin in the stabilization of DOPE bilayer structure, vesicles were treated with either trypsin of neuraminidase as described in Methods. Removal of sialic acid from the vesicles had no effect on the structural integrity of the vesicles as monitored by ^{31}P-NMR (spectra not shown). Freeze-fracture electron microscopy revealed small, unilamellar vesicles with intramembranous particles (70–100 Å) larger than those seen in the non-treated vesicles (compare Fig. 3A and B). In addition, some clumps or aggregates of vesicles were observed after removal of the sugar residues (Fig. 3B).

Cosonication of DOPE and glycophorin

In order to further examine the influence of glycophorin on DOPE structure, we attempted to reconstitute DOPE and glycophorin by means of cosonication. A 25 : 1 molar ratio was chosen since this was the prefered interaction observed in the vesicles prepared by the MacDonald and MacDonald method. Cosonication of an aqueous dispersion of DOPE and glycophorin results in the formation of small, stable, unilamellar vesicles (300–1000 Å diameter, Fig. 3), with ill-defined protein particles as revealed by freeze-fracture electron microscopy. This was a rather remarkable result in that attempts to produce sonicated vesicles of phosphatidylethanolamine have been unsuccessful [36–38] except at low ionic strength and high pH (9.2, Ref. 39). These vesicles (25 : 1 lipid/protein ratio) when examined by ^{31}P-NMR, exhibit a linewidth of approx. 18 Hz which is somewhat broader than that reported for sonicated vesicles of pure phosphatidylcholine [40]. The addition of neodymium chloride (3 mM) to small, unilamellar vesicles shifts the ^{31}P-NMR resonance arising from

the outside of the spherical bilayer vesicle downfield from the inner vesicle membrane [41]. Addition of neodymium to the glycophorin-DOPE vesicles reveals that the majority of the vesicles are impermeable to the shift reagent (spectra not shown) with 20% of the inner resonance visible. The obtained value for the percentage of lipids in the inner monolayer is somewhat low compared to sonicated phosphatidylcholine vesicles, suggesting that part of the small, high protein containing structures in the preparation are permeable to the shift reagent.

Discussion

^{31}P-NMR spectra of vesicles comprised of DOPC and glycophorin are characterized by a decrease in the chemical shift anisotropy and an increase in spectral linewidth in contrast to the situation found for DOPC liposomes where these parameters are found to be invariant. Previously, these parameters have been utilized to gain information on the local order of the phosphate region of lipid molecules in the bilayer [35]. Romans et al. [19] have observed that the chemical shift anisotropy was reduced from 45 ± 1 ppm in egg phosphatidylcholine to 36 ± 1 ppm in egg phosphatidylcholine vesicles containing glycophorin. The authors suggested that the decrease in the chemical shift anisotropy reflected a decrease in phospholipid headgroup order. Recently however, Burnell et al. [42] have studied the temperature dependence of $\Delta\sigma_{csa}^{eff}$ and $\Delta\nu_{1/2}$ for a variety of phospholipid vesicle sizes. Their results demonstrated that the temperature dependence of D_{diff} (which is the major source of isotropic averaging) yielded values for both $\Delta\sigma$ and $\Delta\nu_{1/2}$ which were temperature dependent. Consequently, the reduction in the effective chemical shift anisotropy at all temperatures in the DOPC-glycophorin system as compared to DOPC liposomes may possibly result from the smaller size of the reconstituted system rather than a decrease in phospholipid order. Both possibilities must be considered in the analysis of this system.

The ^{31}P-NMR and freeze-fracture electron microscopic results presented in this study clearly demonstrate the ability of glycophorin, an intrinsic membrane protein, to stabilize lipid bilayer organization. This stabilization most likely is mediated by both the polar headgroups and hydrocarbon chains of the phospholipid. Reconstitution of glycophorin with DOPE, a phospholipid which prefers the hexagonal (H_{II}) phase above 0°C, produces small, unilamellar vesicles, which are stable over a wide variety of temperatures. 90–95% of the sialic acid residues on glycophorin are oriented toward the outside of the membrane. This outer orientation undoubtedly reflects the tendency to minimize charge repulsion and steric hindrance of the carbohydrate moieties of glycophorin in the small protein containing vesicles. Removal of sialic acid residues with trypsin has no effect on the structural integrity of the vesicles, however larger protein particles were visualized by freeze-fracture electron microscopy after trypsin treatment. Removal of the sugar residues eliminates the charge repulsion, thereby allowing the possibility for protein aggregation to occur. Evidence of vesicle aggregation is seen in the freeze-fracture micrographs.

The high affinity of glycophorin for DOPE is reflected by the high amount of protein incorporation (20:1 lipid/protein mole ratio) in the vesicles compared to reconstitution studies involving phosphatidylcholine and glycophorin [17]. K^+ efflux studies reveal that the vesicles have a more efficient K^+ trap and slower leak than that observed for DOPC-glycophorin vesicles [17]. Further evidence that DOPE may have a somewhat special interaction with glycophorin is obtained from the recent observation that reconstituted DOPC-glycophorin membranes require the addition of DOPE to render them impermeable to shift reagents [43]. Additionally, cosonication of glycophorin and DOPE results in small, stable, unilamellar vesicles whereas sonication of pure DOPE results in unstable vesicles which precipitate from solution. On the basis of previous studies which demonstrated that cylindrical molecules (phosphatidylcholine, sphingomyelin, phosphatidylserine) stabilized bilayer structure and in accordance with the results obtained in the current study it is suggested that the hydrophobic region of glycophorin must be in a rigid, cylindrical form for the observed stabilization to occur. Treatment of the erythrocyte membrane which exists in a bilayer as detected by ^{31}P-NMR with various phospholipases produces products which form non

-bilayer phases yet the membrane itself still remains in a bilayer structure [44]. It may be suggested, therefore, that membrane proteins such as glycophorin may play a role in maintaining bilayer organization. The current study, clearly demonstrating the bilayer stabilizing capacity of glycophorin, provides some evidence for this interpretation. Further studies involving more complex lipid systems in the presence of protein and cholesterol are necessary to gain better insight into membrane organization. Studies along these lines are currently in progress.

Acknowledgement

We thank J. Leunissen-Bijvelt for carrying out some of the freeze-fracture experiments.

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