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EFFECT OF GLYCOPHORIN INCORPORATION ON THE PHYSICO-CHEMICAL PROPERTIES OF PHOSPHOLIPID BILAYERS

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Summary

1. The thermotropic behaviour of phospholipid molecules in reconstituted glycophorin-containing vesicles has been investigated by means of differential scanning calorimetry. Each glycophorin molecule is able to perturb the properties of 80—100 phospholipid molecules in such a way that these lipid molecules no longer participate in the cooperative gel to liquid-crystalline phase transition. This number of perturbed phospholipid molecules was discovered to be independent of the lipid charge.

2. By means of freeze-facture electron microscopy it could be demonstrated that glycophorin is not excluded from the solid lipid phase upon cooling the lipids below their gel to liquid-crystalline phase transition temperature. In mixtures of phosphatidylcholines which show solid-solid immiscibility, glycophorin is preferentially associated with the lower-melting lipid component upon phase separation, as could be demonstrated by both differential scanning calorimetry and freeze-fracture electron mircoscopy.

3. The effect of glycophorin on the mobility of phospholipids has been investigated by means of ^{31}P NMR. Glycophorin, incorporated into sonicated vesicles of dioleoylphosphatidic acid, is able to immobilize nine lipid molecules very strongly in their phosphate region. Evidence for an electrostatic interaction between the protein and this negatively charged phospholipid has been presented.

4. The presence of glycophorin causes discontinuities in the lipid bilayer. This results in higher susceptibility of the bilayer towards attack by lipolytic enzymes and in enhanced membrane permeability.

Introduction

Lipids and proteins are the main constituents of biological membranes. One of the major concerns in membrane biology is to understand how lipids and

proteins interact with each other and how such interaction affects the properties of both lipids and proteins in the bilayer. One way to tackle this problem is to investigate the properties of recombinants of purified membrane proteins and well characterized phospholipids. In a first approach the activity of membrane-bound enzymes as a function of their lipid environment can be investigated [1]. A second approach is to investigate how a protein, incorporated into a lipid bilayer, affects the properties of the lipid molecules in its environment. A protein well suited for this type of study is glycophorin, the major sialoglycoprotein of the human erythrocyte membrane [2]. Due to its high solubility in aqueous solutions, it can be purified from the red cell membrane and reconstituted into lipid bilayers without the use of detergents (details are given in ref. 3). The protein contains a hydrophobic region by which it spans the membrane, and since the total amino acid sequence of the protein has been solved [4], its interaction with lipids can be investigated on a molecular level. In this paper some of the physico-chemical properties of phospholipid molecules in the immediate environment of glycophorin in reconstituted systems will be investigated by means of differential scanning calorimetry, freeze-fracture electron microscopy, ^{31}P NMR, phospholipase A_2 attack and permeability measurements;

Materials and Methods

Materials

Ghosts of human erythrocytes were prepared according to the method of Dodge et al. [5]. Glycophorin was extracted from the membrane according to Marchesi and Andrews [6], treated with chloroform/methanol and delipidated by ethanol precipitation as described previously [3]. The purified protein was labeled with tritium as described before [3]. Phospholipase A_2 from pig pancreas was a gift from Dr. A.J. Slotboom from this laboratory. 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (14 : 0/14 : 0-phosphatidylcholine), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (16 : 0/16 : 0-phosphatidylcholine), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (18 : 1_c/18 : 1_c-phosphatidylcholine), 1,2-dipalmitoleoyl-*sn*-glycero-3-phosphocholine (16 : 1_c/16 : 1_c-phosphatidylcholine) and 1-stearoyl,2-oleoyl-*sn*-glycero-3-phosphocholine (18 : 0/18 : 1_c-phosphatidylcholine) were synthesized as described before [7]. 1,2-Di(1-[^{14}C]-oleoyl)-*sn*-glycero-3-phosphocholine [^{14}C]18 : 1_c/18 : 1_c-phosphatidylcholine) was prepared from glycero-3-phosphocholine and [1-^{14}C]oleic acid (Radiochemical Centre, Amersham, U.K.) as described by Warner and Benson [8]. 1,2 Dioleoylphosphatidic acid and 1,2 di([1-^{14}C]-oleoyl)phosphatidic acid were prepared from the corresponding phosphatidylcholines according to Davidson and Long [9]. 1,2 Dimyristoyl-*sn*-glycero-3-phosphoglycerol (14 : 0/14 : 0-phosphatidylglycerol) and 1,2 dimyristoyl-*sn*-glycero-3-phosphoserine (14 : 0/14 : 0-phosphatidylserine) were prepared as described by Comfurius and Zwaal [10]. Beef brain phosphatidylserine was purified according to Sanders [11]. Trypsin was obtained from Serva (Heidelberg, G.F.R.), Sephadex-G 50 coarse and Sepharose-4 B from Pharmacia (Uppsala, Sweden), ^2H_2O from Merck, Sharp and Dohme (Montreal, Canada) and ^3H_2O from the Radiochemical Centre (Amersham, U.K.).

Methods

Phosphorus was determined according to a modification [12] of the procedure of Fiske-Subbarow. Sialic acid was determined according to Warren [13]. Radioactivity was counted using a Packard Tricarb Liquid scintillation spectrometer, according to standard procedures. Large, unilamellar glycophorin-containing vesicles were prepared according the MacDonald and MacDonald [14]. Glycophorin and lipids were dried together from a chloroform/methanol/water (150 : 75 : 1) solution, and the lipid-protein film was suspended in a 100 mM NaCl/10 mM Tris-HCl buffer, pH 7.4 (unless stated otherwise). Vesicles were pelleted by centrifugation at 125 000 $\times g$ for 1 h at 4°C (details are given in ref. 3). Protein incorporation in the vesicles was determined from the sialic acid to phosphate ratio in the pellet, using a value of 2.1 μmol sialic acid/mg protein (as determined according to Lowry et al. [15]) and a molecular weight for the protein part of glycophorin of 12 500 [4].

Sonication of lipids was performed using a Branson tip sonifier, at power setting 4, for a duration of 3 min at 0°C. Metal particles from the probe were removed by centrifugation at 30 000 $\times g$ for 20 min at 4°C. This procedure resulted in the formation of small vesicles, as was checked by Sepharose-4 B column chromatography. Differential scanning calorimetry was performed on a Perkin-Elmer 2 calorimeter, using a scanning rate of 5°C/min [16]. ^{31}P NMR measurements were done on a Bruker WH-90 spectrometer at a frequency of 36.4 MHz as described in ref. 17. Free induction decays were accumulated with a 1.7-s interpulse time using 90° pulses under conditions of broad band proton decoupling (4 W). Signal intensities were determined by computer integration relative to an external triphenylphosphine standard, which was calibrated with a phosphate solution of known concentration under conditions of gated decoupling with $5T_1$ waiting times between the pulses. Comparison of the signal intensity observed with the employed 1.7 s interpulse time under conditions of broad band decoupling and that observed under conditions of gated decoupling with $5T_1$ waiting times between the pulses, showed differences of less than 4% for sonicated vesicles of phosphatidic acid. Freeze-fracture electron mircoscopy was performed as described in ref. 18. Outleakage of K^+ was measured continuously using a potassium selective electrode as described in ref. 19. Outleakage of glucose was measured enzymatically as described in ref. 20. Absorbance changes were measured using a Varian Techtron double beam spectrophotometer (model 635).

Results

Freeze-fracture electron microscopy and differential scanning calorimetry on glycophorin-containing vesicles prepared from a single phospholipid species

Reconstitution of glycophorin and phospholipids according to the method of MacDonald and MacDonald [14] results in the formation of large unilamellar vesicles, with a diameter of 100—500 nm [3]. It has been demonstrated that glycophorin spans the lipid bilayer of this reconstituted system and that it is predominantly oriented with its sugar residues to the outside of the vesicles [3]. By means of freeze-fracture electron microscopy very small intramembranous particles (4 nm diameter) were observed, representing aggre-

gates of at least two and perhaps even four molecules of glycophorin [3]. Unlike most membrane proteins [21], glycophorin is not excluded from the solid lipid phase, when the lipids are cooled to temperatures below their gel to liquid-crystalline phase transition temperature. This is demonstrated in Fig. 1A, which shows a random distribution of intramembranous particles in glycophorin-containing vesicles of 14 : 0/14 : 0-phosphatidylcholine in the solid phase. However, the amplitude of the so-called band pattern, which is characteristic for this phospholipid in the solid state [21], is strongly reduced by the incorporation of the protein. These results are in agreement with a note in a paper of Kleemann et al. [22].

An important method in studies of lipid-protein interaction is an investigation of the effect of proteins on the gel to liquid-crystalline phase transition of phospholipids [23]. Calorimetric data can be used to divide membrane proteins into three groups, depending on whether the interaction with lipids is purely electrostatic, a combination of electrostatic and hydrophobic forces or mainly hydrophobic [24]. The effect of glycophorin incorporation on the gel to liquid-crystalline phase transition of 14 : 0/14 : 0-phosphatidylcholine, 14 : 0/14 : 0-phosphatidylglycerol and 14 : 0/14 : 0-phosphatidylserine has been studied by means of differential scanning calorimetry in order to determine the stoichiometry of the lipid-protein interaction. Examples of the thermograms recorded during these experiments are shown in Fig. 2. As can be seen, the introduction of glycophorin into bilayers of 14 : 0/14 : 0-phosphatidylcholine has hardly any influence on the shape of the calorimetric peak, in agreement with observations of MacDonald and MacDonald [14]. Only a slight broadening of the peak could be observed, while the temperature at the onset of the phase transition was never lowered more than 1°C. No pretransition could be detected at protein to lipid ratios larger than 1 : 500. In bilayers of the negatively charged phospholipids 14 : 0/14 : 0-phosphatidylglycerol and 14 : 0/14 : 0-phosphatidylserine, significant differences with respect to the shape of the calorimetric peaks and to the temperature of the onset of the phase transition were also not observed upon incorporation of the protein (see Fig. 2). The enthalpy change of the phase transition (ΔH), however, turned out to depend strongly on the protein content of the bilayer. Fig. 3 shows that for each of these three lipids the ΔH value is reduced linearly with the protein to lipid molar ratio under the conditions used. No such reduction in ΔH was observed when lipids were suspended in a glycophorin-containing solution, indicating that under those conditions no incorporation of the protein into the bilayer occurs.

According to the criteria of Papahadjopoulos et al. [24], the observed phenomena are characteristic for a purely hydrophobic interaction between the protein and the lipid molecules. It has been suggested, however, that besides hydrophobic interactions, glycophorin and negatively charged phospholipids also interact electrostatically via the negative charge of the polar head group of the lipid molecules and a number of positively charged amino acids on the protein molecule [25,26]. Obviously this electrostatic interaction does not result in a shift of the phase transition temperature of these negatively charged phospholipids. This observation combined with the gradual decrease in the ΔH value with increasing protein-to-lipid ratio strongly suggests that each glyco-

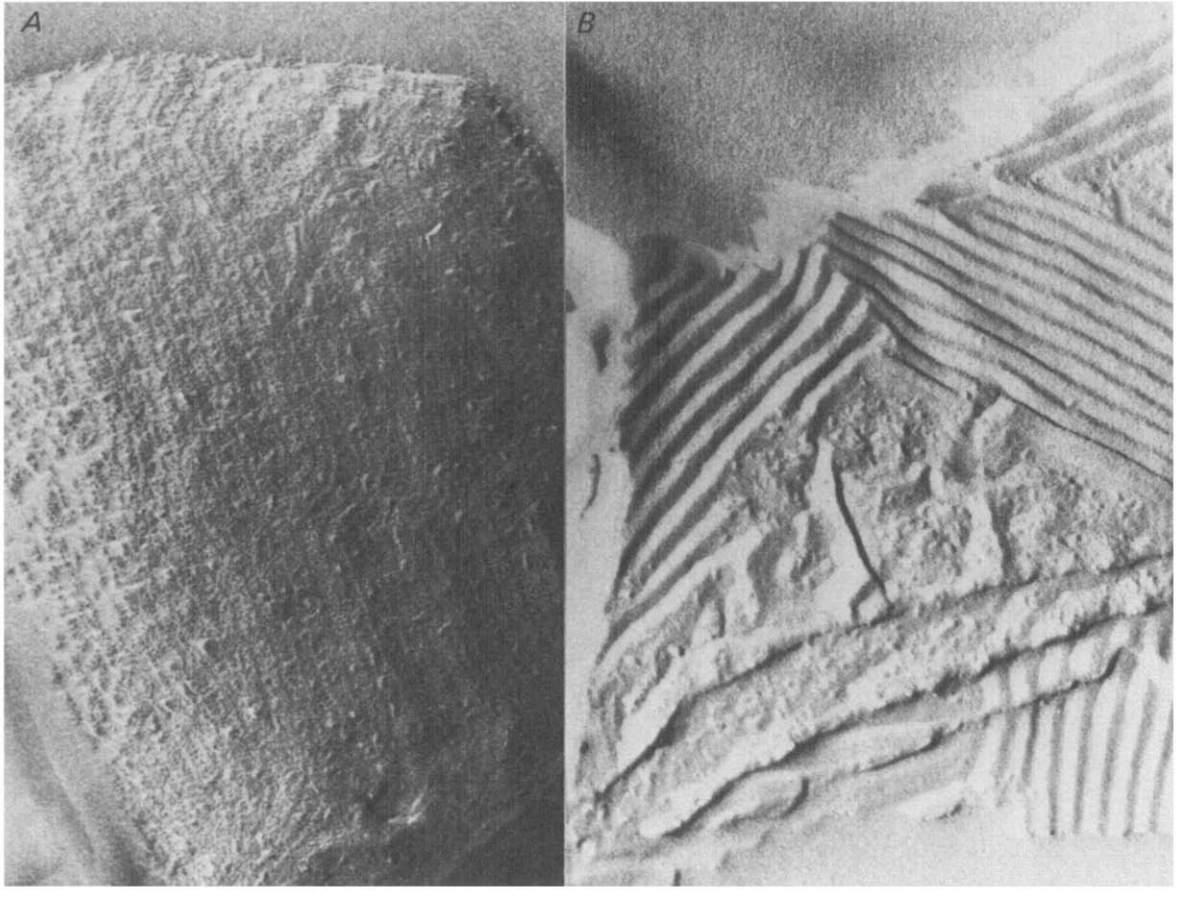

Fig. 1. Freeze-fracture electron micrographs of A. Glycophorin-containing vesicles of 14 : 0/14 : 0-phosphatidylcholine (1 : 400 molar ratio) Sample was quenched from 4°C. Magnification, ×127 500. B. Glycophorin-containing vesicles of an equimolar mixture of 18 : 0/18 : 1_c-phosphatidylcholine and 16 : 0/16 : 0-phosphatidylcholine (1 : 400 molar ratio). Sample was quenched from 20°C. Magnification, ×127 500.

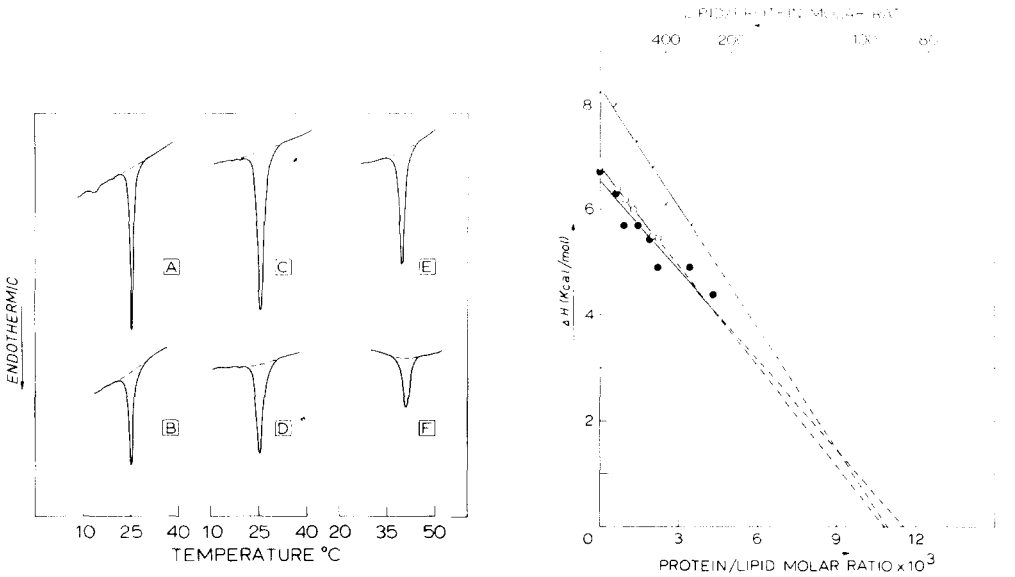

Fig. 2. Thermotropic behaviour of large glycophorin-containing vesicles, compared with multilamellar liposomes. A. 14 : 0/14 : 0-phosphatidylcholine; B. 14 : 0/14 : 0-phosphatidylcholine + glycophorin (380 : 1 molar ratio); C. 14 : 0/14 : 0-phosphatidylglycerol; D. 14 : 0/14 : 0-phosphatidylglycerol + glycophorin (400 : 1 molar ratio); E. 14 : 0/14 : 0-phosphatidylserine; F. 14 : 0/14 : 0-phosphatidylserine + glycophorin (310 : 1 molar ratio). Vesicles were prepared in 100 mM NaCl/10 mM Tris-HCl, pH 7.4, and pelleted by centrifugation. In all cases heating scans were recorded.

Fig. 3. Enthalpy change of the gel to liquid-crystalline phase transition as a function of the glycophorin-to-lipid molar ratio. After scanning, the samples were suspended in a 0.5% Triton X-100 solution and assayed for phosphorus and sialic acid. Experimental error in each point 5—10%. Number of lipid molecules withdrawn from the cooperative phase transition as calculated by linear regression: ●, 14 : 0/14 : 0-phosphatidylcholine, 86 ± 10; ○, 14 : 0/14 : 0-phosphatidylglycerol, 93 ± 4; X, 14 : 0/14 : 0-phosphatidylserine, 92 ± 6.

phorin molecule is able to prevent a number of lipid molecules from participating in the cooperative lipid phase transition. As a results only the gel to liquid-crystalline phase transition of those lipid molecules that are not involved in an interaction with the protein are visualized in the calorimetric experiments. The observation that the shape of the peaks is hardly affected by the incorporation of the protein, indicates that the properties of the free lipid molecules remain essentially unaffected by the presence of the protein-lipid complexes. Assuming that each glycophorin molecule is able to withdraw N lipid molecules from the cooperative phase transition, the measured ΔH value will be given by:

$$\Delta H = \Delta H_0 (1 - NP/L)$$

In this equation ΔH is the measured enthalpy change of the gel to liquid-crystalline phase transition, expressed in kcal/mol, ΔH_0, the value for the pure lipid system and P/L the molar protein-to-lipid ratio in the bilayer. From the slope of the plot in Fig. 3 or by extrapolation to $\Delta H = 0$, values of N between 80 and 100 are obtained for each of the three lipids used in this study (see legend Fig. 3). In this respect it is worth mentioning that in the presence of a large excess of protein, a maximum of one glycophorin molecule per 80 lipid

molecules can be incorporated into the lipid bilayer by this method of reconstitution [13]. Together, these two results suggest that each glycophorin molecule influences a lipid environment of 80—100 lipid molecules. The number of lipid molecules withdrawn from the cooperative phase transition by glycophorin is very high when compared with other proteins which, according to the criteria of Papahadjopoulos et al. [24], are comparable with glycophorin in the nature of their lipid-protein interaction. A value of 15 lipid molecules per protein molecule has been reported for lipophilin [27], ten for melittin [28] and six for gramicidin [29]. For these proteins this roughly implies that one layer of phospholipid molecules surrounding the protein molecule is not participating in the cooperative phase transition [29]. Assuming a diameter of 1 nm for the membrane-spanning region of glycophorin, however, this would mean that glycophorin is able to withdraw three layers of surrounding phospholipid molecules in each monolayer from the cooperative phase transition. It is not clear at the moment, how such a process should be visualized. The actual situation is more complicated by the fact that glycophorin is slightly aggregated in this reconstituted system [3]. The linearity observed in Fig. 3 indicates that each protein molecule complexes 80—100 lipid molecules, independent on the protein to lipid ratio in the bilayer. This linearity was observed up to a molar protein to lipid ratio of 1 : 250, a condition under which 30—40% of the lipid molecules are perturbed by protein. It is difficult to study the calorimetric behaviour of bilayers with a higher protein-to-lipid ratio, since this would require excesses of glycophorin [3]. In addition, the results obtained with vesicles with high protein-to-lipid ratios were not very reproducible, probably due to heterogeneity in the structures.

The fact that the bulk lipids are not perturbed by the presence of the lipid-protein complexes, does not rule out the possibility that exchange between bulk and complexed lipid occurs. For example, cholesterol behaves calorimetrically in a way that is comparable to hydrophobic membrane proteins; it removes two phospholipid molecules from the cooperative phase transition without markedly affecting the cooperativity of the free lipid molecules, even up to complexing 60% of the lipid molecules [30]. It is believed, however, that cholesterol-complexed and free phospholipid molecules rapidly exchange with each other [31]. The freeze-fracture electron microscopy data showed, that the amplitude of the so-called band pattern, which is seen in solid bilayers of 14 : 0/14 : 0-phosphatidylcholine, is strongly reduced upon incorporation of glycophorin. In light of the recent theories of Janiak et al. [32,33] this fits well with the observation that under these conditions no pretransition is observed in the calorimetric experiments. In this respect glycophorin behaves very much like lipophilin [27] and cholesterol [34]. There could well be a correlation between the observation that the lipid-protein complexes do not perturb the properties of the free lipid molecules and the observation that the distribution of these complexes in the plane of the membrane is similar in both the gel and liquid-crystalline state.

Freeze-fracture electron microscopy and differential scanning calorimetry on glycophorin-containing vesicles prepared from mixtures of phospholipids

Since the interaction of glycophorin with lipids can be detected by a reduc-

tion of the ΔH value of the lipid phase transition, differential scanning calorimetry can be used for studying preferential interactions in mixtures of lipids which show solid-solid immiscibility. Fig. 4 shows that in an equimolar mixture of 16 : 0/16 : 0-phosphatidylcholine and 16 : 1_c/16 : 1_c-phosphatidylcholine, glycophorin reduces the ΔH value only of 16 : 1_c/16 : 1_c-phosphatidylcholine, indicating that during phase separation the protein is preferentially associated with the lower-melting component *. In this respect glycophorin resembles cholesterol [35] and gramicidin [29]. The preferential interaction of glycophorin with the lower-melting component could also be demonstrated by means of freeze-fracture electron microscopy of a mixture of 16 : 0/16 : 0-phosphatidylcholine and 18 : 0/18 : 1_c-phosphatidylcholine, when quenched at a temperature between the two calorimetric peaks (see Fig. 1B). The protein molecules are located in the fluid lipid domains and are excluded from the solid regions which are indicated by the band pattern, which is in agreement with the observation of Grant and McConnell [36]. It can be concluded that differential scanning calorimetry can be used for determining the number of lipid molecules that are perturbed by protein molecules, and under specific conditions, together with freeze-fracture electron microscopy, for studying preferential lipid-protein interactions. However, these techniques give no information about the physicochemical properties of the perturbed lipid molecules.

Immobilization of phospholipids by glycophorin as detected by ^{31}P NMR

In order to study the physicochemical properties of phospholipids in the immediate environment of glycophorin, recombinants with very high protein-to-lipid ratios are required. Using the reconstitution method of MacDonald and MacDonald [14], a maximum of one protein molecule per 80 lipid molecules can be incorporated and this requires a large excess of protein [3]. Recombinants of glycophorin and lipids can also be obtained by cosonication of lipids and glycophorin in an aqueous solution. Fig. 5 shows the elution pattern of a cosonicated mixture of beef brain phosphatidylserine and glycophorin on a Sepharose-4 B column. Almost all lipid molecules chromatograph identically as sonicated lipid vesicles of beef brain phosphatidylserine. Depending on the protein-to-lipid ratio, 50—90% of the glycophorin comigrates with these vesicles, while the remainder behaves similar to free glycophorin in solution and gives a peak which contains negligible amounts of lipid. As will be demonstrated, recombinants with a very high protein-to-lipid ratio can be obtained in this way. It should be realized that the structure of these recombinants is not very well defined. In spite of the fact that they chromatograph like sonicated vesicles, it is not known whether the lipids are arranged in a bilayer, how the protein molecules are incorporated in these structures or whether the state of aggregation of the protein molecules changes with a variation of the lipid-to-protein ratio. However, this is a problem that all recombinants with high protein-to-lipid ratios have in common.

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* No such specificity was observed in a phase-separating mixture of 12 : 0/12 : 0-phosphatidylcholine and 14 : 0/14 : 0-phosphatidylserine, since the ΔH value of both peaks was found to be reduced in the presence of the protein. Thus there appears to be a competition between the fluid phosphatidylcholine species and the solid phosphatidylserine species in their interaction with glycophorin.

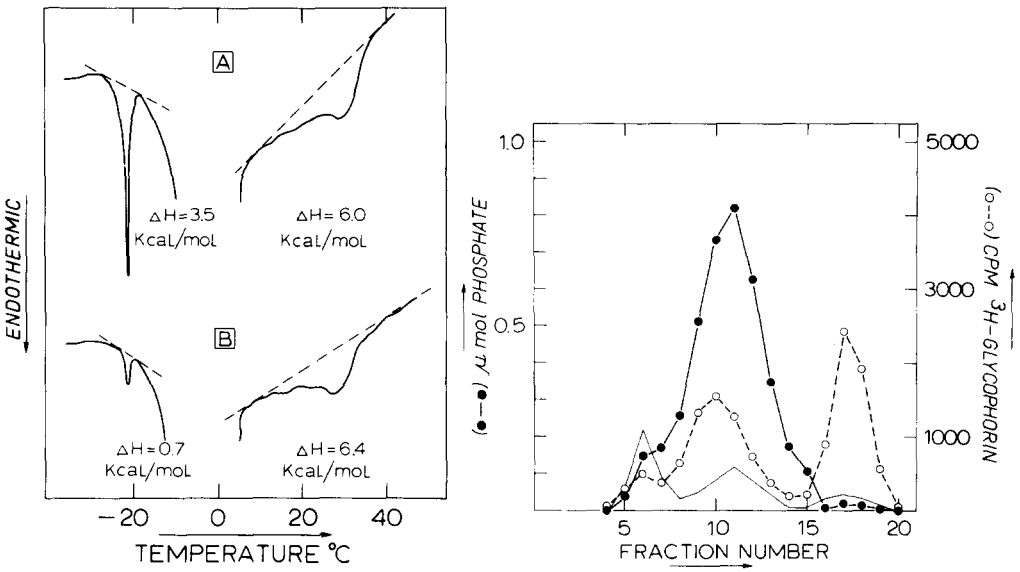


Fig. 4. Thermotropic behaviour of an equimolar mixture of 16 : 0/16 : 0-phosphatidylcholine and 16 : 1_c/16 : 1_c-phosphatidylcholine without (A) or with (B) glycophorin (250 : 1 molar ratio). Cryoprotectants were not used. Before scanning samples were stored for 30 min at −40°C in order to avoid undercooling.

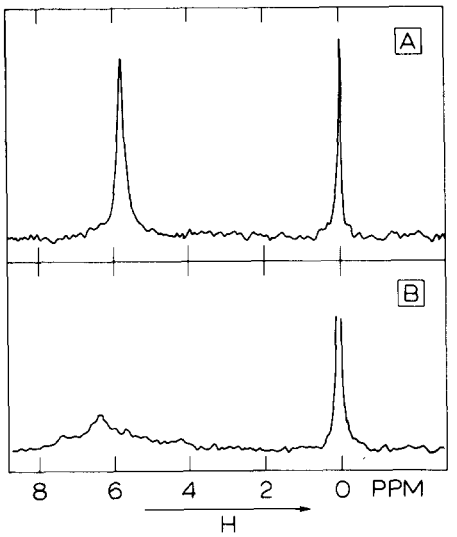
Fig. 5. Sepharose-4 B column (12 × 2 cm) chromatography in 100 mM NaCl, 10 mM Tris-HCl buffer, pH 7.4. ^{3}H-labeled glycophorin (95 cpm/μg protein) was added to 5.0 μmol beef brain phosphatidylserine in a 1 : 300 molar ratio. After sonication for 3 min, the mixture was put on the column and fractions of 1.8 ml were samples. The amounts of phosphate (●———●) and [^{3}H]glycophorin (o- - - - -o), and also the absorbance at 280 nm (———) are shown. Recovery of lipid was 79% and of protein was 72%. Average protein to lipid ratio in the vesicle peak is 1 : 550.

High resolution ^{31}P NMR spectra can be obtained from small sonicated lipid vesicles [17]. Large lipid structures in a bilayer arrangement give rise to very broad resonances [37] because of their low tumbling rate, and these signals are too broad for observation under the NMR conditions used in recording a vesicle spectrum. The ^{31}P NMR signal arising from lipid molecules which are strongly restricted in their motion due to interactions with proteins can also be too broad for detection in a vesicle spectrum as was recently demonstrated for low density lipoproteins [38]. The effect of glycophorin on the mobility of phospholipid molecules will be investigated by measuring the fraction of the lipid molecules that contributes to the ^{31}P NMR spectrum as a function of the protein-to-lipid ratio in the cosonicated recombinants.

The glycophorin molecules obtained by treatment of the red cell membrane with lithium diiodosalicylate [6] still contain 10—15 lipid molecules [25]. These lipid-protein complexes chromatograph of a Sepharose-4 B column identically as delipidated glycophorin in solution, indicating that they are arranged in lipoprotein-like structures. However, ^{31}P NMR was not able to detect any lipid resonances from these structures. Since these lipoprotein-like structures will have a smaller size than sonicated vesicles, as can be judged from their chromatographic behaviour, this must mean that the lipid molecules in these complexes are strongly immobilized by the protein. After addition of 1%

sodium dodecyl sulphate, which results in a dissociation of the lipid-protein complexes, all lipid molecules were visible in the spectrum; a major resonance was revealed with a chemical shift corresponding to that of phosphatidylserine, in agreement with the chemical analyses [25]. The delipidated protein which has been used throughout the experiments, still contains two phosphate residues per molecule [3], due to the presence of a single phosphatidylinositol-phosphate molecule [39]. Armitage et al. [40] were able to detect these phosphate residues by means of ^{31}P NMR in a detergent-containing buffer. Our preparations of glycophorin after sonication gave a small NMR signal, detectable only by computer integration. Corrections for these signals were made during the following experiments.

Glycophorin, containing a trace of ^{3}H-labeled protein, was dissolved in a $^{2}H_2O$-containing buffer consisting of 100 mM NaCl, 10 mM Tris, 0.2 mM EDTA, HCl, p^{2}H 7.0. This protein solution was added to a dried film of [^{14}C]-dioleoylphosphatidic acid, and the lipid molecules were brought into suspension. This lipid was chosen because it has a pK for protonation which is close to the pH used in this study [41]. Therefore, the chemical shift of the phosphatidic acid resonances will strongly depend on the presence of charged groups around the phosphate region of the phospholipid molecules. The mixture was sonicated, while carefully adjusting the p^{2}H value, and subsequently centrifuged as described in Materials and Methods. After recording the ^{31}P NMR spectrum aliquots were taken for liquid scintillation counting in order to determine protein and lipid concentration, and for Sepharose-4 B column chromatography in order to determine the percentages of protein and lipid present in the vesicle and free protein fraction, respectively. Some of the ^{31}P NMR spectra recorded during these experiments are shown in Fig. 6. Sonicated vesicles of dioleoylphosphatidic acid (Fig. 6A) gave a single sharp resonance with a chemical shift of 5.77 ppm. Based on the calibration of the external standard it could be shown that 96% of the phosphorus atoms contributed to this vesicle spectrum. The incorporation of glycophorin into this system (Fig. 6B) resulted in a broadening of the resonance and a shift to lower field. In addition it was observed that a significant percentage of the phosphorus atoms did not contribute to the spectra of these glycophorin-lipid recombinants. Since only a negligible amount of lipid was found to be present in larger structures (chromatographing in the void volume of the Sepharose-4 B column), this must mean that the protein strongly immobilizes a number of lipid molecules in their phosphate region. Fig. 7A shows the percentage of phosphorus atoms contributing to the spectrum as a function of the glycophorin-to-lipid ratio, relative to the value obtained for the phosphatidic acid vesicles without protein. Fig. 7B shows the number of lipid molecules per molecule of protein not visible in the spectrum, for different protein-to-lipid ratios. Almost all lipid molecules are immobilized in their phosphate region at very low lipid-to-protein ratios, but at higher ratios an almost constant value of eight lipid molecules were immobilized per protein molecule. Column chromatography showed that 92—97% of the lipid molecules and 74—83% of the protein molecules were present in the vesicle structures. It is known from the above results that lipid molecules in the lipoprotein-like structures give no contribution to the spectrum. One can calculate from the lipid to protein ratios in the vesicles, that in these structures an average of nine



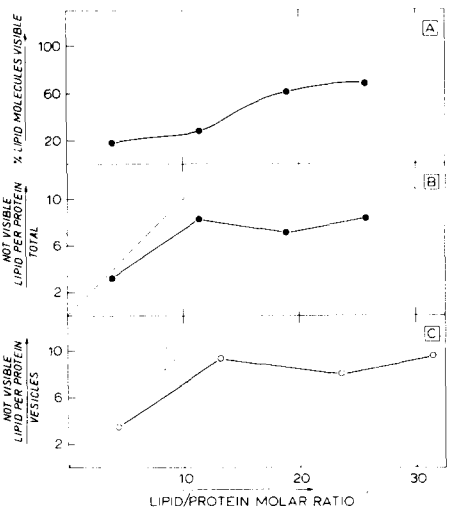


Fig. 6. 36.4 MHz ^{31}P NMR spectra: A: sonicated vesicles of dioleoylphosphatidic acid. 6 mM lipid; 2000 transients recorded; B: sonicated recombinants of dioleoylphosphatidic acid and glycophorin (19 : 1 molar ratio) 3.5 mM lipid; 30 000 transients recorded. Triphenylphosphine was used as external standard; chemical shifts are reported in ppm downfield from this standard. Experiments were carried out in a 100 mM NaCl, 10 mM Tris, 0.2 mM EDTA-HCl buffer p^2H 7.0, at 25° C.

Fig. 7. Immobilization of dioleoylphosphatidic acid by glycophorin in sonicated recombinants. Data were calculated from the amounts of lipid and protein in the recombinants and the intensity of the phospholipid resonances in the ^{31}P NMR spectrum as measured by computer integration. A. Percentage of phosphate atoms that contribute to the ^{31}P NMR spectrum as a function of the phospholipid-to-protein ratio, relative to that in sonicated vesicles of phosphatidic acid without protein (dashed line). B. Number of lipid molecules invisible in the NMR spectrum per protein molecule in the sonicated recombinants as a function of the lipid-to-protein molar ratio. The dashed line represents the situation in which all lipid molecules are immobilized. C. Number of lipid molecules invisible in the NMR spectrum per protein molecule in the vesicle structures as a function of the lipid-to-protein molar ratio in the vesicles. The dashed line represents the situation in which all lipid molecules in the vesicles are immobilized.

lipid molecules per protein molecule is immobilized, as shown in Fig. 7C. It is tempting to speculate that a correlation exists between these nine lipid molecules, immobilized by glycophorin, and the 10—12 lipid molecules which are required to surround the protein molecule with one layer of lipids in the bilayer. However, it should be realized that a much better characterization of the recombinants is needed to prove this statement.

Strongly immobilized lipid molecules are not visible in the NMR spectrum, therefore the resonances seen in the spectrum must have originated from the non- or less-immobilized lipid molecules. No sharp peak could be observed at low lipid-to-protein ratios and the presence of lipid signals was detectable only by computer integration. A molar lipid-to-protein ratio of 19 : 1 causes a broad resonance with a chemical shift 0.59 ppm downfield to that of phosphatidic acid in protein-free vesicles, as shown in Fig. 6B. No sharp resonance is seen at this latter position, which indicates that in these glycophorin-phosphatidic acid recombinants most of the lipid molecules are in a chemical environment which is different from that in protein-free vesicles. By increasing the lipid-to-protein ratio, this downfield shift decreased gradually and the resonance became more narrow. The observed line broadening can be explained either by a partial im-

mobilization of the phosphate groups of the lipid molecules by the protein, or by the occurrence of chemical exchange processes. This latter possibility is favoured by the fact that a concomitant shift of the resonance position is observed, although a combination of both factors is also possible. No sharpening of the resonance was observed after tryptic digestion of the recombinants, indicating that the line-broadening does not result from a decrease in the tumbling rate of the vesicles which could arise from a locally higher viscosity due to the presence of the large sugar moieties of the protein. The most likely explanation for the above phenomena is that the strongly immobilized lipid molecules are in a different chemical environment than the non-immobilized lipid molecules, corresponding to higher chemical shift. Assuming that immobilized and non-immobilized lipid molecules can exchange rapidly on the NMR timescale, all lipid molecules will be in a chemical environment intermediate between the two situations, depending on the ratio of immobilized and non-immobilized lipids present. It is known that the phosphate group of phosphatidic acid has a pK for protonation of approx. 8.0 [41], which is close to the pH used inthis study, and that protonation of a phosphate group results in an upfield shift [42]. If the immobilized phosphate groups of the lipid molecules have a resonance position at lower field than the non-immobilized molecules at the same pH, this implies that the pK for phosphate protonation is lower for the immobilized than for the non-immobilized lipids. Such a decrease in pK value can be explained by an interaction between the negatively charged phosphate groups of the lipids and positively charged groups on the protein molecules [42]. From the amino acid sequence of the protein [4] it is known that the C-terminal region of the protein contains four positively charged amino acids next to the hydrophobic region of the protein. The observed downfield shift in the resonance position of phosphatidic acid upon incorporation of glycophorin therefore provides strong support to the hypothesis that glycophorin and negatively charged phospholipid molecules interact with each other by a combination of hydrophobic and electrostatic forces [25,26]. The exact nature of this electrostatic interaction is subject to further investigation.

Some effects of the incorporation of glycophorin on the properties of the lipid bilayer

Proteins in the membrane will interrupt the continuous two-dimensional structure of the lipid bilayer. Both on the border between perturbed and bulk lipids and on the border between lipid and protein, discontinuities in the bilayer must exist. The presence of these discontinuities could be demonstrated by the susceptibility of the bilayer for phospholipase attack. Unsonicated liposomes of 14 : 0/14 : 0-phosphatidylcholine can not be hydrolyzed by pancreatic phospholipase A_2, neither above nor below the gel to liquid-crystalline phase transition temperature [43], since the packing of the phospholipid molecules is too tight for the enzyme to penetrate [44]. At the phase transition temperature (23.5°C), however, the enzyme can penetrate the bilayer at the discontinuities that exist on the border between solid and liquid lipid regions, resulting in a rapid lipid hydrolysis [43]. This result was confirmed and is illustrated in Fig. 8B. Fig. 8A shows that large glycophorin-containing vesicles of 14 : 0/14 : 0-phosphatidylcholine can be attacked by phospholipase A_2 above

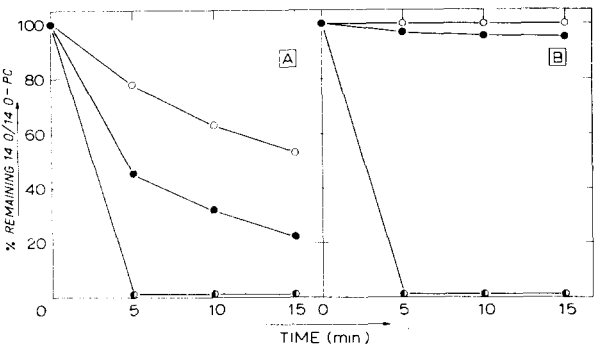


Fig. 8. Timecourse for the hydrolysis of 14 : 0/14 : 0-phosphatidylcholine by pancreatic phospholipase A_2 at 4°C (○), 23.5°C (◐) and 37°C (●). A: glycophorin-containing vesicles (1 : 400 molar ratio); B: multilamellar liposomes (see ref. 43). Vesicles were prepared in 100 mM Tris-HCl, pH 7.2, containing 1 mM $CaCl_2$. For each incubation 0.85 μmol lipid and 30 μg enzyme was used. The reaction was stopped by the addition of 0.2 M EDTA, followed by lipid extraction, separation of phosphatidylcholine and lysophosphatidylcholine by means of thin-layer chromatography and phosphorus analysis (see ref. 43).

(37°C), at (23.5°C) and below (4°C) the lipid phase transition temperature. A complete lipid hydrolysis was obtained under all conditions after prolonged incubation. This demonstrates that the presence of glycophorin causes discontinuities in the lipid bilayer.

Unsonicated glycophorin-containing vesicles of 18 : 1_c/18 : 1_c-phosphatidylcholine are almost as efficient as multilamellar liposomes in trapping glucose and potassium ions. This is demonstrated in Table I, which shows a value of 2.1—2.3 μl/μmol lipid for the trapped water volume of these structures. Taking into account the fact that these protein-containing vesicles are uni-

TABLE I

Permeability of large glycophorin-containing vesicles of 18 : 1_c/18 : 1_c-phosphatidylcholine (DOPC-glyc.) and multilamellar liposomes of 18 : 1_c/18 : 1_c-phosphatidylcholine containing 4 mol% egg phosphatidic acid (DOPC) for glucose and potassium. For measurements of glucose leakage, glycophorin-containing vesicles (1 : 400 molar ratio) were prepared in 300 mM glucose, 10 mM Tris-HCl, pH 7.4, and centrifuged at 10 000 × *g* for 10 min at 4°C in order to remove structures without protein (ref. 3). 1 ml of the supernatant was applied to a Sephadex-G 50 coarse column (30 × 1 cm) which was equilibrated with 150 mM NaCl, 10 mM Tris-HCl, pH 7.4. Elution of the vesicles was recorded continuously by following the absorbance at 280 nm. Vesicle-containing fractions were assayed for glucose [20] and phosphorus. Trapped glucose was determined by the addition of 2% Triton X-200. For potassium leakage, vesicles were prepared in 150 mM KCl/10 mM Tris-HCl, pH 7.4, and run over the Sephadex G-50 column equilibrated with 150 mM choline chloride/10 mM Tris-HCl, pH 7.4. The glycophorin-containing vesicles were centrifuged three times at 125 000 × *g* for 60 min at 4°C in order to measure the effect of high-speed centrifugation on the trapped amount of potassium.

	Trapped water volume (μl/μmol lipid)	Percent leakage in 10 min at 25°C
Glucose		
DOPC	3.9	3.9
DOPC-glyc.	2.3	6.8
Potassium		
DOPC	2;9	4.9
DOPC-glyc.	2.1	8.6
DOPC-glyc. after centrifugation	5.1	n.d.

lamellar, it would require a spherical volume with a diameter of approx. 100 nm to obtain this trapped volume of water (compare data in ref. 45). After centrifugation of these glycophorin-containing vesicles at 125 000 $\times g$ for 60 min at 4°C, however, a more than two-fold increase in the trapped volume of water was measured, indicating that during the high-speed centrifugation fusion of the structures to larger, unilamellar vesicles must have occurred. In this respect it is worth mentioning that freeze-fracture electron microscopy reveals vesicles with sizes ranging from 100—500 nm diameter after high-speed centrifugation [3].

Table I also shows that the rate of efflux of potassium ions and glucose, expressed as a percentage per unit of time, is higher in case of the glycophorin-containing vesicles than in multilamellar liposomes. It is difficult to compare the actual numbers directly because of differences in geometry between the two structures, but is demonstrates that the protein-containing bilayers are certainly capable of retaining solutes. It is known that the conductance of black lipid membranes is significantly increased upon the incorporation of glycophorin [46]. The introduction of the hydrophobic tryptic peptide of glycophorin into a lipid bilayer also results in an enhanced membrane permeability [47]. Furthermore it has been demonstrated by means of ^{13}C NMR [48] that the unsonicated glycophorin-containing vesicles of 18 : 1_c/18 : 1_c-phosphatidylcholine are permeable to trivalent cations like Dy^{3+}, while protein-free bilayers are not. All these data indicate that the permeability of lipid bilayers is strongly enhanced by the incorporation of glycophorin, and it seems most likely that this is due to the formation of discontinuities in the bilayer, since an enhancement of membrane permeability is also observed when solid and fluid lipids coexist in the plane of the membrane [49]. The data of this paper show, however, that the integrity of the lipid bilayer is certainly not fully lost upon incorporation of glycophorin, which is also illustrated by the fact that these structures are impermeable to enzymes [3]. Recently it has been demonstrated (Gerritsen, W.J., unpublished) that upon incorporation of 10 mol% of 18 : 1_c/18 : 1_c-phosphatidylethanolamine or 1—18 : 1_c-lysophosphatidylcholine into unsonicated glycophorin-containing vesicles of 18 : 1_c/18 : 1_c-phosphatidylcholine, these structures are no longer permeable to trivalent cations. This indicates that the nature of the discontinuities in the bilayer, and therefore the permeability characteristics of these vesicles are a function of the membrane lipid composition.

Discussion and conclusions

Glycophorin, incorporated into a lipid bilayer, is able to perturb the properties of 80—100 lipid molecules in such a way that these lipids do not participate in the cooperative gel to liquid-crystalline phase transition. Preliminary results from electron spin resonance measurements have shown that these perturbed phospholipid molecules undergo a very broad, non-cooperative phase transition (ranging between 8 and 28°C in case of 14 : 0/14 : 0-phosphatidylcholine), which cannot be detected by the calorimetric experiments. Only a relatively small number of nine phospholipid molecules are strongly immobilized in their phosphate region due to the presence of glycophorin. Probably,

this immobilization results from a combination of electrostatic and hydrophobic forces between protein and lipid molecules. Most likely, a rapid exchange between the immobilized and the perturbed lipid molecules takes place. In this respect it is worth mentioning that according to recent theoretical considerations [50] the number of protein-perturbed phospholipid molecules will be optimal at the lipid phase transition temperature. This may be an explanation for the relatively high number obtained with differential scanning calorimetry.

The physicochemical properties of phospholipid molecules in the immediate environment of proteins can be different from those of the bulk phospholipids, as predicted by the models of Marčelja [51] and Israelachvili [52]. The nature of the discontinuities in the bilayer, which will result from this, will strongly depend on the capability of the phospholipid molecules to accommodate the protein molecules [52]. It seems reasonable to assume that these discontinuities will be most pronounced when the protein is reconstituted with only one phospholipid species. The presence of these discontinuities is reflected by the high susceptibility of the bilayer towards attack by lipolytic enzymes. The possibility cannot be excluded, however, that the relatively high rate of lipid hydrolysis of large glycophorin-containing vesicles of 14 : 0/14 : 0-phosphatidylcholine by phospholipase A_2 at 4°C has resulted from a small fraction of non-cooperative melting lipid molecules which is still fluid at this temperature. The discontinuities in the bilayer give also rise to an enhanced membrane permeability, and probably also cause the enhancement of the rate of transbilayer movement of phospholipid molecules in sonicated glycophorin-containing vesicles [48,53].

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