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Glycophorin-Induced Cholesterol–Phospholipid Domains in Dimyristoylphosphatidylcholine Bilayer Vesicles†

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ABSTRACT: Glycophorin has been incorporated into unilamellar cholesterol-containing dimyristoylphosphatidylcholine vesicles that were reconstituted by the freeze and thaw technique. Evidence was obtained for a protein-induced structural reorganization of these mixed membranes. By differential scanning calorimetry, we were able to construct a phase diagram for the phospholipid/cholesterol mixture consisting of a liquid-ordered, a solid-ordered, and a liquid-disordered phase. Glycophorin at low molar fractions ($X_G < 3 \times 10^{-3}$) increases the relative amount of lipid in the liquid-ordered phase, which is interpreted as an enrichment of cholesterol in the vicinity of the protein. The formation of such steroid-enriched domains could be demonstrated directly by electron paramagnetic resonance using a spin-labeled cholesterol analogue. A drastic increase of the spin–spin interaction of the labeled steroid was observed in the presence of glycophorin.

Cholesterol is a major component of all eucaryotic membranes, where this ubiquitous amphiphilic sterol amounts up to a mole fraction of $X_{Ch} = 0.5$ with respect to the total lipid. Its interaction with phospholipids has been the subject of numerous studies leading to a large number of different and sometimes controversial models that try to explain the thermodynamic properties of cholesterol-containing membranes. However, different experimental techniques appear to yield different results for the miscibility of phospholipids and cholesterol or for their association stoichiometry. Suggestions have been given for the construction of phase diagrams with phase boundaries at $X_{Ch} = 0.2$ (Shimshick & McConnell, 1973; Copeland & McConnell 1980; Melchior et al., 1980), at $X_{Ch} = 0.33$ (Engelman & Rothmann, 1972; Gershfeld, 1978; Lentz et al., 1980), at $X_{Ch} = 0.4$ (Kroon et al., 1975), and at $X_{Ch} = 0.5$ (Engelman & Rothmann, 1972). Phase boundaries at

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X_{Ch} = 0.08, 0.24, and 0.435 were observed for the L_{β}'-phase of DMPC with small-angle neutron scattering by Knoll et al. (1984).

There are numerous attempts to interpret the experimental data in terms of the formation of stoichiometric complexes with cholesterol-to-phospholipid ratios of 1:21, 1:7, and 1:3 (Gershfield, 1978; Cadenhead & Müller-Landau, 1984). The molecular interaction, however, that gives rise to the cholesterol clustering has remained obscure (Presti & Chan, 1982). Hydrogen bonding (Brockerhoff, 1974), van der Waals interactions (Yeagle et al., 1977), and molecular length (Suckling et al., 1979) have all been proposed as the essential features for a sterol–lipid interaction.

By a lattice model, Caillé et al. (1980) could explain the drop of the phase-transition temperature and of the transition enthalpy observed by Mabrey et al. (1978) and Ladbrooke et al. (1968), as well as the large increase of the phase-transition width (Hinz et al., 1978) and the phase separation into cholesterol-rich domains (Ipsen et al., 1987). By the proposal of a simple path model for the lateral diffusion in cholesterol–phospholipid monolayers, Slater and Caillé (1982) extended this model by a two-dimensional percolation theory to explain the stepwise increase of the diffusion constant near a cholesterol mole fraction of X_{Ch} = 0.2 (Rubenstein et al., 1979).

Ipsen et al. (1987) combined the results of different techniques and postulated a model for the phase equilibria in the phosphatidylcholine–cholesterol system. Calorimetry (Ladbrooke et al., 1968; Mabrey et al., 1978; Estep et al., 1978), magnetic resonance spectroscopy (Recktenwald & McConnell, 1981; Presti & Chan, 1982; Presti et al., 1982; Kusumi et al., 1986); fluorescence depolarization (Alecio et al., 1985; Smutzer & Yeagle, 1985); infrared and Raman spectroscopy (Lippert & Peticolas, 1971; Levin et al., 1985; O'Leary & Levin, 1986; Rooney et al., 1986), and neutron and X-ray scattering as well as electron microscopy (Copeland & McConnell, 1980; Hui & He, 1983; Knoll et al., 1985) and micromechanics (Evans & Needham, 1986) have been considered. They all agree that addition of cholesterol to phosphatidylcholine membranes leads to a conformationally higher ordered state of the lipid fatty acid chains. However, within a given phase, the molecular translational and rotational motions are only slightly affected by cholesterol (Rubenstein et al., 1979).

Some evidence has been published for a cholesterol–protein interaction within lipid bilayer membranes. Gordon et al. (1983) discussed a model based on a lipid phase separation in human platelet and rat liver plasma membranes into cholesterol-rich and cholesterol-poor domains at physiological temperatures. In addition, the formation of cholesterol-rich domains in human erythrocyte plasma membranes may be due to a protective effect of cold-induced hypertonic lysis (Gordon & Mobley, 1984). The authors suggested a significant restriction of the lipid lateral mobility by membrane proteins. Using fluorescence probes, Golan et al. (1984) could show that the lateral mobility of phospholipid and cholesterol was affected by the protein–lipid interaction. In general, cholesterol was found to influence the molecular organization of phospholipids in erythrocyte membranes (Manevich et al., 1985). As shown by electron microscopy with the technique of gold-labeling, components of the erythrocyte membrane tend to form moving domains. The motion was related to a lipid phase separation in the bilayer membrane (He & Hui, 1985).

Klugermann et al. (1984) suggested that the conformation of the band 3 protein of human erythrocyte membranes is influenced by the level of cholesterol within the bilayer. Schubert and Boss (1982) postulated that the band 3 protein possesses a high-affinity steroid-binding site that modulates the anion transport. Very recently we reported a strong interaction between cholesterol and glycophorin isolated from the erythrocyte membranes (Tampé et al., 1989b). As shown by an EPR[1] study, cholesterol seems to be separated from the bulk lipid phase, thus forming a stable protein–cholesterol domain in the surrounding fluid bilayer membrane. With DSC measurements, we now analyzed the phase properties of phosphatidylcholine–cholesterol membranes in the absence and in the presence of glycophorin. Good agreement between EPR data and DSC measurements was found with both showing the glycophorin-induced formation of cholesterol-enriched domains.

Experimental Procedures

Materials

Dimyristoylphosphatidylcholine and cholesterol were obtained from Fluka (Neu-Ulm, FRG). Spin-labeled cholestane (3-doxylcholestane) was from Aldrich (Steinheim, FRG). Glycophorin was isolated and purified from human erythrocyte membranes as described earlier (Tampé et al., 1989a). The purity of the isolated protein was checked by SDS–polyacrylamide gel electrophoresis using silver and PAS stains, by protein determination (Lowry et al., 1951; Peterson, 1977), and by sialic acid determination using the resorcinol method of Svennerholm (1957). Lipids were quantified by phosphate analysis according to Ammon and Hinsberg (1966). Trypsin from bovine pancreas and neuraminidase from *Clostridium perfringens* were from Boehringer (Mannheim, FRG).

Methods

Reconstitution. Glycophorin-containing vesicles were prepared by successive freezing and thawing of sonicated samples (Strauss, 1983). Dried lipid films containing the spectroscopic probes and/or cholesterol were sonicated in a 10 mM aqueous Tris-HCl buffer (pH 7.2) in the presence of glycophorin with a Branson sonicator (2 min, 20 mW of power) until a clear suspension was formed. For the EPR measurements the lipid concentration was 1 mg/mL. For the DSC scans, lipid concentrations up to 10 mg/mL were used, especially at higher cholesterol molar fractions. The obtained suspensions of small unilamellar vesicles were frozen from 4 °C to −20 °C in a refrigerator and thawed again at a temperature above the lipid phase-transition temperature. This cycle was repeated three times yielding fused vesicles with an average diameter of 75 nm as was determined by electron microscopy. The obtained vesicles were freed from surface-adsorbed glycophorin by repeated centrifugation and resuspension. Before EPR or DSC measurements, the vesicle suspensions were incubated for 1 h at 40 °C. The pelleted vesicles were assayed for phosphate and sialic acid. Samples used for DSC were analyzed after the temperature scan. The analytical values were corrected for the remaining intermediate volume between the vesicles as described earlier (Tampé et al., 1989a).

Enzymatic Digestion. Cleavage of sialic acids and proteinase digestion of lipid–protein sediments were performed by 0.1 mg of neuraminidase within 30 min or by 50 μg of trypsin within 5 min, both at 37 °C and in buffered aqueous

[1] Abbreviations: DMPC, 1,2-dimyristoylphosphatidylcholine; SL, spin label; Cholestane-SL, 3-doxylcholestane; DSC, differential scanning calorimetry; EPR, electron paramagnetic resonance; X_G, vesicular molar fraction of glycophorin; X_{Ch}, molar fraction of cholesterol; $X_{Ch\text{-}SL}$, molar fraction of spin-labeled cholestane; ΔH, phase-transiton enthalpy; $\Delta H_{rel,lo}$, relative transition enthalpy of the liquid-ordered phase; C_p, heat capacity.

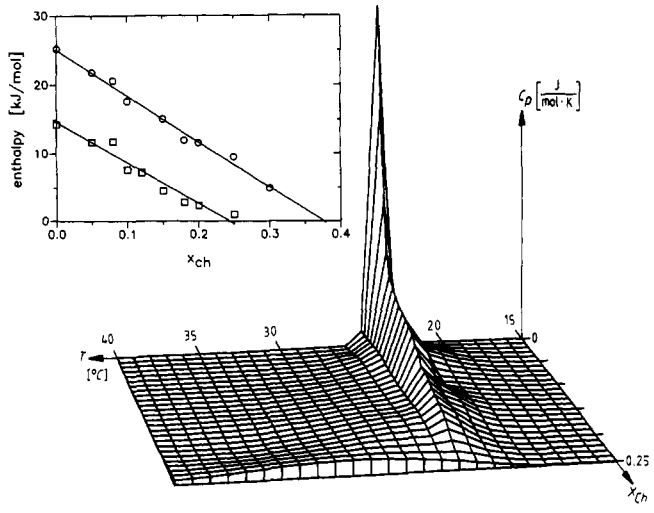

FIGURE 1: Three-dimensional low-resolution presentation of the DSC phase-transition curves obtained from large unilamellar DMPC/cholesterol bilayer vesicles as function of cholesterol content. The insert shows the linear decrease of the enthalpy ΔH_0 of the complete transition, (○) and of the cooperative transitions $\Delta H_1 + H_2$ (□). Values are given in Table I.

solution (10 mM Tris-HCl, pH 7.2).

DSC Measurements. For DSC measurements we used an AT-PC-controlled Microcal MC-2 scanning calorimeter with an adiabatic differential power scanning twin system. One of the two chambers was filled with the sample; the other was filled with the corresponding buffer solution. The volumes of the vesicle suspension were 1.5 mL with lipid concentrations up to 10 mg/mL. The buffer system was 10 mM Tris-HCL (pH 7.2). A scan rate of 30 °C/h was used in the temperature range between 5 and 60 °C. The phase-transition curves were analyzed with a deconvolution program after base-line correction. Phase-transition enthalpies are given with respect to the analytically determined phosphate content. Phase-transition temperatures were taken as the on- and offset temperature of a calorimetric peak obtained by linear extrapolation and correction as proposed by Mabrey and Sturtevant (1976).

EPR Measurements. EPR measurements were performed with a computer-controlled Varian E4 spectrometer. Samples were transferred into quartz tubes (0.8-mm diameter) and were centrifuged at 13000*g*. The supernatant was discarded. Closed samples were embedded in a second tube filled with silicon oil that could be thermostated by a heated nitrogen stream. The temperature control had an accuracy of ±0.1 °C. Spectral recording, data analysis, and presentation of the spectra were performed by an AT-PC.

Results

DSC Measurements. In order to understand the influence of glycophorin on the organization of DMPC–cholesterol membranes, we first reinvestigated the protein-free membranes with differential scanning calorimetry. Large unilamellar vesicles containing cholesterol mole fractions up to $X_{Ch} = 0.25$ were prepared by the freeze and thaw technique in 10 mM Tris-HCl buffer (pH 7.2). Figure 1 summarizes DSC scans between 15 and 40 °C with a low-temperature resolution of 1 °C. The cholesterol-induced suppression of the main phase transition around 24 °C is clearly visible. An additional broad and uncooperative phase transition with a maximum around 30 °C was observed at an increasing mole fraction of cholesterol. The insert of Figure 1 shows the linear decrease of the enthalpy of the main phase transition around 24 °C with an increasing cholesterol content. The enthalpy of this cooperative

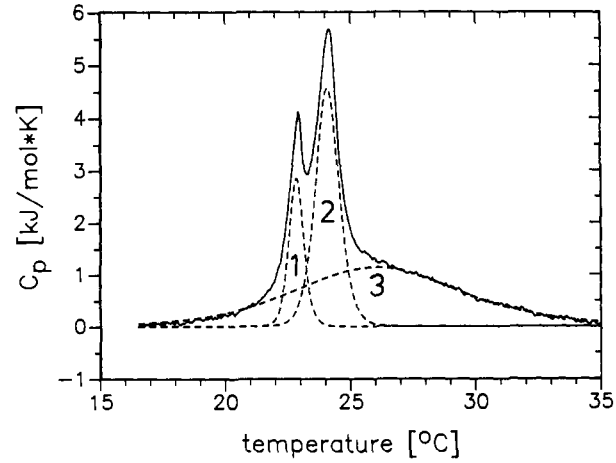

FIGURE 2: Experimental high-resolution phase-transition curve of DMPC/cholesterol vesicles with $X_{Ch} = 0.12$. Deconvolution has been performed to obtain the corresponding simulated curves (broken lines). The complete phase transition exhibits two transitions with high cooperativity (1 and 2) and with transition temperatures close to the phase-transition temperature of pure DMPC membranes. An additional uncooperative transition (3) shifted to higher temperatures has been observed but only at cholesterol contents of $X_{Ch} > 0.08$.

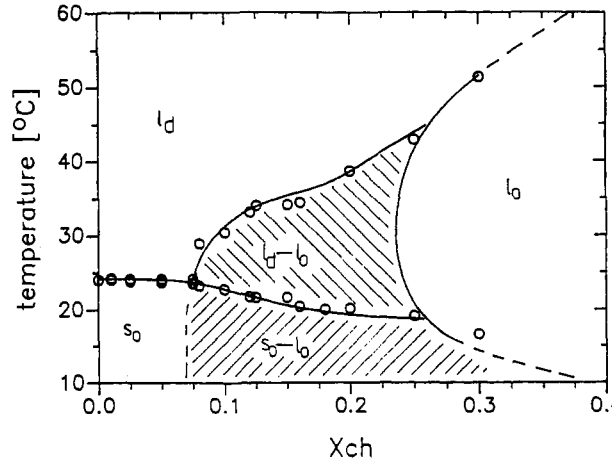

FIGURE 3: Phase diagram for DMPC/cholesterol membranes obtained from DSC-scans. Abbreviations: l_o, liquid-ordered phase; l_d, liquid-disordered phase; s_o, solid-ordered phase; s_o–l_o, two-phase region; l_d–l_o, two-phase region. The assignment of the lipid phase has been adapted to a phase diagram of DPPC/cholesterol mixtures presented by Ipsen et al. (1987).

phase transition may be extrapolated to zero at $X_{Ch} = 0.25$. The complete transition including the broad peak is suppressed at $X_{Ch} = 0.38$. The standard deviation of five different enthalpy determinations was ±2.5 kJ/mol, including the error of the phosphate analysis performed after the scans.

Figure 2 shows a more detailed phase-transition curve of DMPC–cholesterol vesicles with $X_{Ch} = 0.12$. The complete melting process is deconvoluted into three transitions. In the presence of cholesterol, two highly cooperative transitions are found to be close to the phase transition of the pure lipid at $T = 23.9$ °C, whereas a third broad and uncooperative transition was observed at cholesterol mole fractions exceeding $X_{Ch} = 0.08$. The separation of the calorimetric scan into two cooperative transitions in DMPC–cholesterol vesicles occurs in a cholesterol mole fraction range between $X_{Ch} = 0.05$ and 0.2. This separation is not visible in Figure 1 due to the low resolution (1 °C of this pretransition. The presentation of the freeze and thaw DMPC–vesicles was already suppressed at $X_{Ch} = 0.03$ and has not been considered in this study.

From a series of phase-transition curves of cholesterol-containing DMPC vesicles, we are able to construct the phase

Table I: Enthalpy Values for the Deconvoluted Experimental DSC Scans (e.g., Figure 2) as a Function of Membrane Cholesterol Content[a]

X_{Ch}	ΔH_o (kJ/mol)	ΔH_1 (kJ/mol)	ΔH_2 (kJ/mol)	ΔH_3 (kJ/mol)	$\Delta H_{rel,lo}$
0.00	25.1	14.3	0.0	10.8	0.00
0.05	21.8	7.6	4.1	10.1	0.06
0.08	20.6	4.1	7.7	8.8	0.00
0.10	17.6	2.6	5.0	10.0	0.26
0.12	17.5	2.3	4.9	10.3	0.28
0.15	15.1	0.9	3.6	10.6	0.47
0.18	12.0	1.1	1.5	9.4	0.61
0.20	11.6	2.3	0.0	9.3	0.65
0.25	9.5	1.0	0.0	8.5	0.81
0.30	4.9	0.0	0.0	4.9	1.00

[a] ΔH_o is the enthalpy of the complete transition ($\Delta H_o = \Delta H_1 + \Delta H_2 + \Delta H_3$). The given data are average values obtained from five measurements with a standard deviation of ±2.5 kJ/mol. $\Delta H_{rel,lo}$ is the relative transition enthalpy obtained from the transition of the l_o phase (ΔH_3) with respect to ΔH_o ($\Delta H_{rel,lo} = \Delta H_3/\Delta H_o$). $\Delta H_{rel,lo}$ is normalized to 0 and 1 for pure DMPC membranes and membranes with a cholesterol content of $X_{Ch} = 0.3$, respectively.

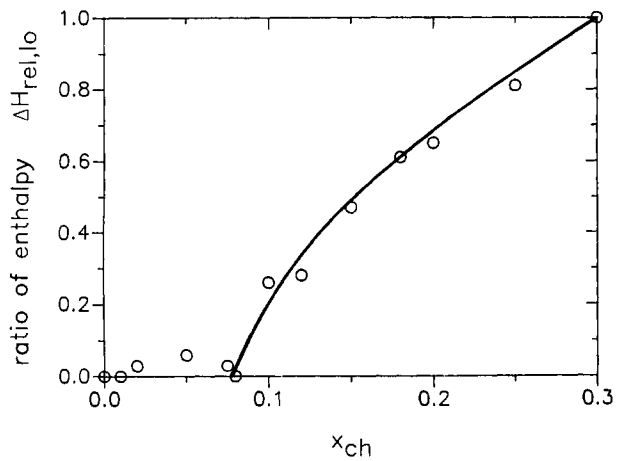

FIGURE 4: Relative enthalpy fraction $\Delta H_{rel,lo}$ of the uncooperative transition marked ΔH_3 in Figure 1 as a function of the mole fraction of cholesterol X_{Ch} for DMPC/cholesterol membranes. Values for $\Delta H_{rel,lo}$ were obtained from the DSC transition curves ($\Delta H_{rel,lo} = \Delta H_3/\Delta H_o$), where ΔH_o is the enthalpy of the complete transition. Values for $H_{rel,lo}$ are normalized between 0 and 1 for pure DMPC membranes and membranes containing cholesterol in a mole fraction of $X_{Ch} = 0.3$. The l_o phase starts to form progressively at $X_{Ch} = 0.08$.

diagram shown in Figure 3. The onset and completion temperatures of the phase transitions were determined by linear extrapolation of the calorimetric peaks and were corrected for the contribution of the finite width of the transition curve of the pure lipid according to the formalism of Mabrey and Sturtevant (1976). In support of earlier results obtained by Ipsen et al. (1987) for mixed DPPC–cholesterol membranes, we define a solid-ordered (s_o), a liquid-disordered (l_d), and a liquid-ordered (l_o) phase also for DMPC–cholesterol membranes. Up to $X_{Ch} = 0.08$, the cooperative transition correlates with the change from the solid-ordered to the liquid-disordered phase. The transition temperature corresponds to the one of the pure DMPC membrane. A two-phase transition region that includes the l_o phase exists within a cholesterol mole fraction range from $X_{Ch} = 0.08$ to 0.25. This was concluded from the appearance of the broad uncooperative phase transition (marked H_3 in Figure 2) at higher temperatures.

With a deconvolution program, we analyzed the DSC scans at different cholesterol contents and determined the enthalpy of the individual transitions. Values for ΔH_1, ΔH_2, and ΔH_3 marked in Figure 2 are given in Table I. ΔH_o is the enthalpy of the complete transition ($\Delta H_o = \Delta H_1 + \Delta H_2 + \Delta H_3$), and

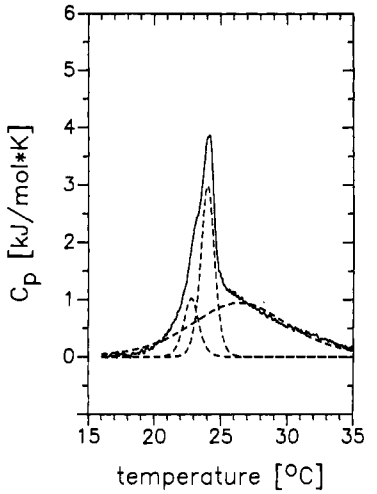

FIGURE 5: DSC phase-transition curve of DMPC/cholesterol membranes with a cholesterol content of $X_{Ch} = 0.12$ and with a mole fraction of membraneous glycophorin of $X_G = 7 \times 10^{-4}$.

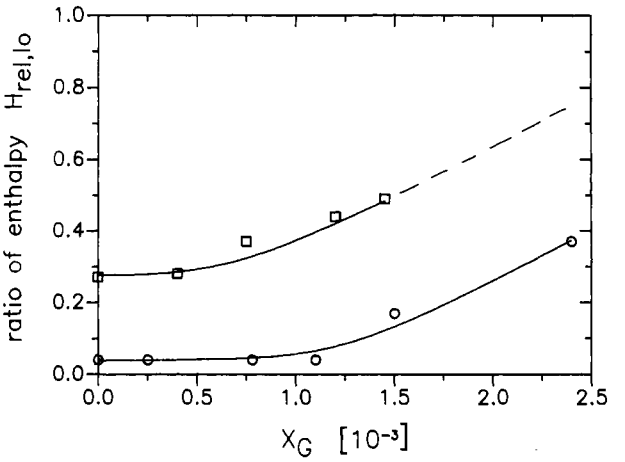

FIGURE 6: Relative ratio $\Delta H_{rel,lo}$ of the enthalpy of the uncooperative transition denoted H_3 in Figure 2 as a function of glycophorin content. Results for two membrane preparations with cholesterol contents of $X_{Ch} = 0.05$ (○) and $X_{Ch} = 0.12$ (□) are shown.

$\Delta H_{rel,lo}$ is the relative enthalpy of lipid in the l_o phase. Values obtained from the ratio of $\Delta H_3/\Delta H_o$ are normalized to 0 for $X_{Ch} = 0$ and to 1 for $X_{Ch} = 0.3$. Thus the relative fraction of the l_o phase is 0 in a pure DMPC membranes and 1 at a cholesterol mole fraction of $X_{Ch} = 0.3$. This fraction $H_{rel,lo}$ is given as function of the cholesterol content in Figure 4. Up to a cholesterol mole fraction of $X_{Ch} = 0.08$, cholesterol distributes homogeneously in the DMPC membrane. The l_o phase only appeared above this critical mole fraction of cholesterol.

With this detailed information on the phase properties of the large unilamellar DMPC–cholesterol vesicles, we were able to investigate the influence of the membrane-bound protein glycophorin on the organization of DMPC–cholesterol vesicles. Two characteristic constant cholesterol mole fractions ($X_{Ch} = 0.05$ and 0.12) were chosen, the lower being in the region of a homogeneous cholesterol distribution, whereas the higher one is in the region where the l_o phase is already formed.

Figure 5 reproduces a standardized transition curve again with a cholesterol fraction of $X_{Ch} = 0.12$ but with $X_G = 7 \times 10^{-4}$ as the mole fraction of glycophorin. The broad uncooperative phase transition is consecutively increased at the expense of the cooperative transitions with increasing glycophorin content. Figure 6 summarizes the calculated $\Delta H_{rel,lo}$ values for membranes with cholesterol mole fractions of X_{Ch}

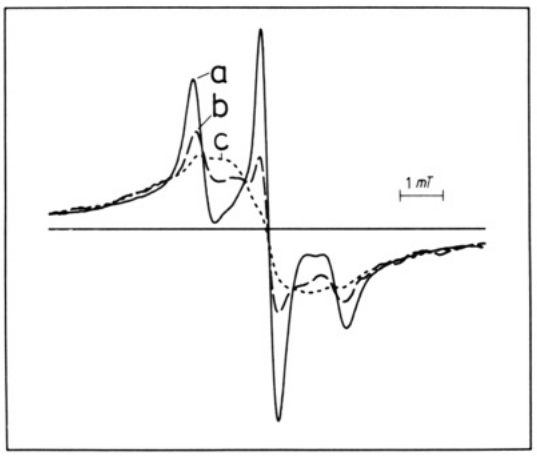

FIGURE 7: EPR spectra of spin-labeled cholestane in large unilamellar DMPC bilayer vesicles containing different amounts of glycophorin. The mole fraction of the cholestane label is $X_{Ch\text{-}SL} = 0.05$. The spectra were taken at 36 °C. The mole fractions of membrane bound glycophorin are (a) $X_G = 0$, (b) $X_G = 9 \times 10^{-4}$, and (c) $X_G = 3 \times 10^{-3}$. The intensity of spectrum a is reduced by a factor of two.

= 0.05 and $X_{Ch} = 0.12$ with increasing glycophorin content up to $X_G = 2.5 \times 10^{-3}$. In a homogeneous mixture of phospholipid and cholesterol ($X_{Ch} = 0.05$, lower curve) glycophorin increases the relative enthalpy ratio $\Delta H_{rel,lo}$. A lateral phase separation occurs by a formation of the l_o phase. At $X_{Ch} = 0.12$ (upper curve), where the membrane already contains a considerable fraction of the l_o phase, the enthalpy ratio $\Delta H_{rel,lo}$ is further increased with increasing glycophorin content. Note that the strong influence on the structural organization of a cholesterol-containing membrane occurs at very low glycophorin mole fractions of $X_G < 2.5 \times 10^{-3}$.

EPR Measurements. The DSC measurements yield information on the phase behavior of the complete membrane system. By EPR spectroscopy, we investigated the structural organization of the steroid in DMPC membranes in the presence of glycophorin by the use of a spin-labeled cholestane. Typical EPR spectra of this probe incorporated into large unilamellar bilayer vesicles ($X_{Ch\text{-}SL} = 0.05$) are given in Figure 7 with increasing glycophorin content at a temperature above the phase-transition temperature of the pure lipid. The very low protein mole fraction in the range of $X_G = 1.7 \times 10^{-3}$ (curve b) already causes a strong line broadening. The three-line spectrum is superimposed on a broad one-line spectrum. This broadened component is solely present at a glycophorin mole fraction of $X_G = 3.6 \times 10^{-3}$ (curve c). The progressively changing shape of the EPR spectrum could be explained by a drastically increased local concentration of the spin-labeled cholestane in the presence of glycophorin that then causes a line broadening due to the increased spin–spin exchange and dipole–dipole interactions. These results fit the interpretation that glycophorin induces a phase separation. The steroid is locally concentrated, thus forming the l_o phase that in the absence of protein is only observed at higher cholesterol content.

The formation of cholestane-SL-enriched domains could be reversed by tryptic protein digestion. If trypsin is added to the preformed vesicles, it could only cleave the protein segments that protrude to the outside of the vesicle. After this procedure, the broadened EPR spectrum of DMPC vesicles containing $X_{Ch} = 0.05$ cholestane-SL and $X_G = 1.2 \times 10^{-4}$ glycophorin becomes partially restructured (Figure 8). Application of a short ultrasonication pulse that allows the trypsin to enter the vesicles causes the reappearance of a three-line spectrum that is identical with the one obtained in the absence

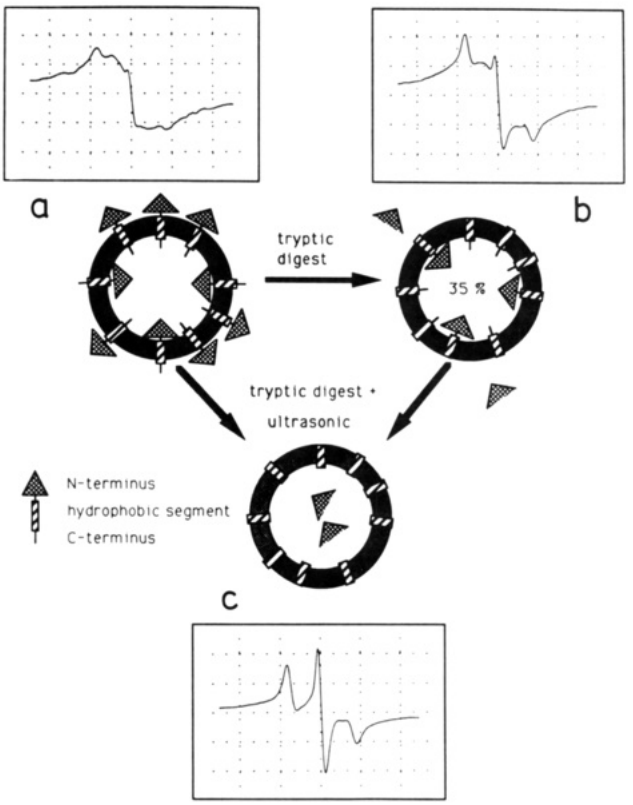

FIGURE 8: The effect of tryptic digestion on the EPR spectra of unilamellar DMPC bilayer vesicles containing the cholestane spin label ($X_{Ch\text{-}SL} = 0.05$) and glycophorin ($X_G = 1.2 \times 10^{-3}$). Spectra were taken at $T = 36$ °C. After the reconstitution, 35% of the N termini were oriented to the inside of the vesicles and were thus protected from digestion. Shown are (a) the initial spectrum, (b) the spectrum after incubation of the vesicles with a trypsin solution, where only external protein fragments were cleaved, and (c) the spectrum after tryptic digestion and treatment with ultrasonic pulses. External as well as internal protein fragments are cleaved.

of glycophorin. Sonication in absence of trypsin did not affect the spectral shape.

Discussion

Cholesterol may be an important factor in the control of lipid–protein as well as protein–protein interactions in biological membranes. However, despite a wealth of experimental data, this long-standing problem is not yet understood even in simple reconstituted phospholipid bilayer membranes. The aim of the present paper is to elucidate the interaction between cholesterol and the erythrocyte membrane protein glycophorin. In a preceding EPR study (Tampé et al., 1989b), we were able to report the segregation of cholesterol in the presence of glycophorin in dimyristoylphosphatidylcholine vesicles by the use of a spin-labeled cholesterol analogue. We now investigated the phase behavior of mixed DMPC–cholesterol membranes in the absence and presence of glycophorin by differential scanning calorimetry. Again the formation or the enhancement of cholesterol-enriched domains was observed in the presence of glycophorin.

Phase properties of cholesterol-containing membranes have already been investigated by many authors using different techniques. Calorimetric measurements were performed with DPPC–cholesterol membranes (Ladbrooke et al., 1986; Hinz & Sturtevant, 1972; Mabrey & Sturtevant, 1976; Mabrey et al., 1978; Estep et al., 1978). Multilamellar hydrated lipid films were used, in contrast to our experiments that are based on better defined large unilamellar DMPC–cholesterol vesicles obtained by the freeze and thaw technique. At cholesterol

mole fractions above $X_{Ch} = 0.02$, we observed two highly cooperative phase transitions with transition temperatures separated by a maximum of 1 °C. This has also been observed by Mabrey et al. (1978) in multilamellar vesicles. The authors also could not explain their observation. At $X_{Ch} > 0.08$, an additional new phase with a noncooperative transition was formed. At $X_{Ch} > 0.25$, we could only observe this broad transition.

The phase diagram established by our experiments corresponds to the one of the cholesterol–DPPC system obtained by Ipsen et al. (1987). The highly cooperative phase transitions with phase-transition temperatures close to the one of the pure lipid are considered to be the change from the gel-like, solid-ordered (s_o) to the fluid-like, liquid-disordered (l_d) phase. The broad uncooperative transition then corresponds to the change from the liquid-ordered to the liquid-disordered phase. The classification of the broad transition is not yet clear (Estep et al., 1984). This transition might directly correlate to the melting of the liquid-ordered phase, but it may as well be related to a boundary lipid bordering this phase. Because of the low melting enthalpy of the lipid in the l_o phase, this transition may hardly be detected. However, from deconvolution of the DSC scans, it was possible to determine the fraction of lipid in this liquid-ordered phase (Table I).

The structural organization of the l_o phase was described by NMR spectroscopy (Presti & Chan, 1982; Presti et al., 1982) as well as by Raman and IR spectroscopy (O'Leary & Levin, 1986; Rooney et al., 1986). Due to better steric adaption, cholesterol and phospholipid molecules are joined together and a cholesterol-rich domain is formed. The fatty acid chains of the phospholipids undergo a transition into the highly ordered all-trans conformation. Consequently, a hindered mobility of the lipid chains was observed. However, an increased free volume causes an enhanced mobility of the hydrophilic lipid head groups.

Our DSC data clearly show that glycophorin incorporated into DMPC–cholesterol membranes increases the relative amount of lipid in the l_o phase. The fraction of lipid with the noncooperative transition increases with increasing glycophorin content. The l_o phase extends at the expense of the lipid phase exhibiting the cooperative transitions. At a low cholesterol content, where a homogeneous DMPC–cholesterol mixture exists, glycophorin induces a lateral phase separation, thus forming the l_o phase. At a higher cholesterol content ($X_{Ch} > 0.08$), glycophorin increases the fraction of lipid in the l_o phase, which in these DMPC–cholesterol mixtures is already present in the absence of glycophorin. Recently, we showed that cholesterol facilitates the reconstitution of glycophorin into DMPC bilayer membranes (Tampé et al., 1989b). With EPR-spectroscopy, we were able to directly demonstrate a specific cholesterol–glycophorin interaction (Tampé et al., 1989b). Cholestane-SL, a spin-labeled sterol analogue, was used for this study; it is a well-suited substitute for cholesterol due to its unchanged cross-sectional area and due to the preserved polarity. It is therefore expected to provide information about cholesterol-rich regions of the bilayer into which phospholipid spin probes may not even enter (Presti & Chan, 1982). After incorporation of glycophorin into DMPC/cholestane-SL membranes, the three-line EPR spectrum was converted into a broad one-line spectrum due to a strongly increased spin-exchange interaction. This means that the probe molecules are concentrated in a steroid-enriched lipid domain, since a glycophorin-induced increase of the diffusional rate in the fluid phase is excluded by earlier photobleaching experiments (Kapitzka et al., 1984).

Most interestingly, this effect could be reversed by tryptic digestion of the hydrophilic protein residues. Cleavage of the protein segments on the outside of the vesicles reduced the spin-exchange interaction, as was determined by spectral subtraction, which gives the fraction of segregated spin-label (Tampé et al., 1989b). Sialic acid determination after enzymatic digestion with trypsin or neuraminidase revealed that after reconstitution by the freeze and thaw method about 35% of the glycophorin N termini were oriented to the inside of the vesicles and were therefore protected against digestion. Calculating the fraction of segregated cholesterol from EPR-spectra, Tampé et al. (1989b) found that the value obtained after trypic digestion corresponds to that of a membrane with a 65%-reduced glycophorin content. The clustering was completely reversed if tryptic digestion was applied on both sides of the vesicle, leaving in the bilayer membrane the pure hydrophobic protein segments that are known to aggregate spontaneously (Furthmayer & Marchesi, 1976). Cleavage of the sialic acid residues with neuraminidase alone did not affect the EPR spectra.

It was concluded that the voluminous N terminus prevents self-aggregation of the proteins, probably due to steric hindrance. Electrostatic repulsion may support this effect; however, it is not a necessary condition, as was shown by a neuraminidase digestion, which only cleaves the negatively charged sialic acid residues and did not change the shape of the EPR spectra. Only nonaggregated glycophorin is able to separate a large amount of cholesterol from a mixed membrane. Proteins without N-termini probably aggregate, and prior bound cholesterol will then be dispersed in the lipid bilayer membrane.

The following concept for phospholipid–cholesterol–glycophorin interactions combines our results with those of others. In a homogeneous mixture of DMPC and cholesterol ($X_{Ch} < 0.08$), a low glycophorin content already induces a reorganization of the lipid matrix into cholesterol-rich and cholesterol-depleted domains. The cholesterol-rich domains were identified as the liquid-ordered phase by DSC measurements. This phase behaves like a liquid phase with respect to the lipid diffusional rate; however, the phospholipid hydrocarbon chains are in a well-ordered "all trans" conformation. Cholestane spin-label, when substituted for cholesterol, segregates into these ordered domains where it becomes clustered. This local concentration increase enhances the spin-exchange interaction, which in turn causes the observed tremendous line broadening.

The molecular interaction mechanism leading to the glycophorin-induced formation of the cholesterol-enriched l_o phase is not yet clear, and several aspects have to be discussed. First, the strong hydrophobic protein segment may serve as a nucleus for the formation of the l_o phase. A motionally restricted lipid component interacting directly with the intramembraneous protein surface may force other membrane components with a competence for the l_o phase to join this ordered phase.

A second aspect is the steric adaption between the lipid phase and the hydrophobic protein segment, which may be estimated to span 3.3 nm (Tampé et al., 1989a). Assuming 2.3 nm for the membrane thickness of the l_o phase and 3.0 nm for the ordered phase (Lewis & Engelmann, 1983), it is convincing to assume that lipids may adapt their length by the formation of a gel-like ordered structure (Tampé et al. 1989a). Such an effect has also been observed for another membrane-bound protein, the photoreaction center from *Rhodopseudomonas sphaeroides* into pure phospholipid membranes (Rieger & Möhwald, 1986). Such an adaption may be also valid for the cholesterol-enriched l_o phase.

A third point that has to be considered is the increased hydration of the lipids in the l_o phase. This is correlated with a larger free volume and a higher mobility of the phospholipid head groups (Kusumi et al., 1986). The penetration of water into the hydrophobic membrane regions is enhanced. Glycophorin forms a glycocalyx-like coat of the membrane surface and may increase the hydration of the lipid head-group region. Such an effect has been observed for sialic acid containing glycolipids, which stabilize the hydrated phase in phosphatidylethanolamine membranes (Ollmannn & Galla, 1988; Ollmann et al., 1988). The increased hydration near the bulky N terminus of glycophorin that carries the negatively charged oligosaccaride chains may, in addition, favor the formation of the l_o phase in the immediate vicinity of the protein.

This may also serve to interpret some experimental results obtained with biological membranes. Our results and those of others (Mühlebach & Cherry, 1982; Simmonds et al., 1982) indicate that cholesterol interacts with hydrophobic interfaces of erythrocyte proteins. Gordon et al. (1983) discussed a model for a lipid phase separation in plasma membranes into cholesterol-rich and cholesterol-depleted domains. Such a lipid phase separation was observed not only in human erythrocyte membranes but also in human platelet and rat liver plasma membranes. EPR studies showed that the temperature-induced lipid phase separation occurs below 38 °C. Gordon et al. (1984) reported that the protective effect of low temperature during a cold-induced hypertonic lysis of intact erythrocytes may be due to the formation of cholesterol-enriched domains that stabilize the membranes.

Electron microscopy using gold-labeled con A showed a domain movement in reconstituted erythrocyte membranes (He & Hui, 1985). These results indicate that components of the erythrocyte membrane tend to form moving domains and that this motion is related to a lipid phase separation within the bilayer. Pasynkov and Alekseev (1986) presented a model for a lipid phase separation at the gel-to-liquid-crystalline phase transition that allows a discussion of the dynamics of the lipid-domain formation in bilayer membranes. It was proposed that membrane-bound protein molecules exchange energy and information about their conformational rearrangements via lipid-domain interactions. This domain-controlled protein–protein interaction may be responsible for the connectivity between membrane proteins like glycophorin, band 3, band 4.1, and cytoskeletal proteins. The latter are mainly anchored to the lipid membrane via protein–protein interactions. Lipids may also play an important role for this anchoring process, which then causes the stability of the erythrocyte membrane, for example, against osmotic pressure.

The band 3 protein, the anion transporter of the erythrocyte membrane, is known to interact with cholesterol. Klugermann et al. (1984) suggested that the conformation of the band 3 protein is influenced by the level of cholesterol in the bilayer. Other authors postulated that the band 3 protein possesses a high-affinity steroid-binding site that modulates anion transport (Schubert & Boss, 1982). The postulated cholesterol domain may be the controlling factor and the trigger for a protein–protein interaction not only in erythrocyte membranes but also in other biological membranes as well.

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Order and Dynamics in Mixtures of Membrane Glucolipids from *Acholeplasma laidlawii* Studied by ^{2}H NMR†

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ABSTRACT: The two dominant glucolipids in *Acholeplasma laidlawii*, viz., 1,2-diacyl-3-*O*-(α-D-glucopyranosyl)-*sn*-glycerol (MGlcDG) and 1,2-diacyl-3-*O*-[α-D-glucopyranosyl-(1→2)-*O*-α-D-glucopyranosyl]-*sn*-glycerol (DGlcDG), have markedly different phase behavior. MGlcDG has an ability to form nonlamellar phases, whereas DGlcDG only forms lamellar phases. For maintenance of a stable lipid bilayer, the polar headgroup composition in *A. laidlawii* is metabolically regulated in vivo, in response to changes in the growth conditions [Wieslander et al. (1980) *Biochemistry 19*, 3650; Lindblom et al. (1986) *Biochemistry 25*, 7502]. To investigate the mechanism behind the lipid regulation, we have here studied bilayers of mixtures of unsaturated MGlcDG and DGlcDG, containing a small fraction of biosynthetically incorporated perdeuterated palmitic acid, with ^{2}H NMR. The order-parameter profile of the acyl chains and an apparent transverse spin relaxation rate (R_2) were determined from dePaked quadrupole-echo spectra. The order of the acyl chains in DGlcDG-d_{31} increases upon addition of protonated MGlcDG, whereas the order of MGlcDG-d_{31} decreases when DGlcDG is added. The variation of order with lipid composition is rationalized from simple packing constraints. R_2 increases linearly with the square of the order parameter (S^2) up to $S \approx 0.14$; then, R_2 goes through a maximum and decreases. The increase in R_2 with S^2, as well as the magnitude of R_2, is largest for pure MGlcDG-d_{31}, smallest for DGlcDG-d_{31}, and similar for mixtures with the same molar ratio of MGlcDG/DGlcDG but with the deuterium label on different lipids. The relaxation data indicate the presence of slow reorientational motions, such as collective bilayer fluctuations and/or lipid lateral diffusion over a curved bilayer surface. The variation of acyl-chain order and bilayer curvature and/or fluctuations with sample composition are discussed in relation to the tendency of MGlcDG to form nonlamellar phases in vitro and in relation to the lipid regulation in vivo.

The presence of a lipid bilayer in several biological membranes of both eukaryotic and prokaryotic origin has been demonstrated by X-ray diffraction studies [reviewed by Shipley (1973)]. The hydrocarbon chains of the lipids in a biological membrane, or at least a major fraction of them, are usually in a disordered liquid-like state (Melchior, 1982). Investigations of the cell-wall-less prokaryote *Acholeplasma laidlawii* have shown that these structural conditions of the membrane lipids are actively maintained by metabolic regulation of the polar headgroup and, to a certain extent, the hydrocarbon chain composition. A minimum fraction of the hydrocarbon

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