Formation of Domains of Cationic or Anionic Lipids in Binary Lipid Mixtures Increases the Electrostatic Coupling Strength of Water-Soluble Proteins to Supported Bilayers†

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ABSTRACT: The electrostatic binding strength of water-soluble proteins having either an excess positive (cytochrome *c*) or negative (*β*-lactoglobulin) electric charge to oppositely charged supported planar bilayers (SPBs) was studied as a function of the bilayer phase state (fluid or gel phase) by IR-ATR spectroscopy. The bilayer consisted of mixtures of zwitterionic DEPC with either cationic DMTAP or anionic DMPG. We observed drastic differences in the binding strength of both proteins for the two bilayer phase states, with the gel phase exhibiting a higher binding strength than the fluid phase, under conditions where the two lipid components had different hydrophobic chain lengths resulting in a nonideal mixing behavior. In addition, for *β*-lactoglobulin we observed a strong binding to a gel phase SPB comprising DEPC/DMTAP, while raising the temperature of the SPB above the chain melting transition temperature of the mixture resulted in a complete unbinding of the protein. In contrast, for DMPC/DMTAP having the same cationic charge content but no hydrophobic chain mismatch, no phase-dependent coupling strength of the protein to the SPB was observed. Our results suggest that the formation of charge-enriched domains by partial demixing of the bilayer lipids at the transition to the gel state is crucial for modulation of the protein binding strength to the SPB, while the intrinsic charge of the solid support surface is of minor importance.

The phase transition in phospholipid model membranes between the crystal-like L_β phase or gel phase and the fluidlike L_α phase is a well-known phenomenon and has been studied in great detail over the past two decades (*1, 2*). Lateral diffusion of lipids along the membrane plane, the best-established dynamical phenomenon in lipid bilayers, has been shown to be remarkably fast in the L_α phase ($D_l = 2 \times 10^{-12}-1 \times 10^{-11}$ m^2/s) while virtually frozen in the L_β phase (*3*−*5*). Phospholipid bilayers on a solid support exhibit a quite similar behavior with respect to lateral diffusion (*6*) and show a phase transition similar to that of the classical model membrane systems (*7*). The phase transition temperature is about 2 °C smaller for supported bilayers than for MLV[11] but is still very cooperative. This is ascribed to the presence of an ultrathin water film of 15−30 Å thickness, which separates the bilayer from the solid surface and thus retains the lateral diffusivity of the lipids (*8*). In the case of supported bilayers containing cationic as well as zwitterionic lipids, we showed that the incubation time of the solid support with the SUV coating solution has to be kept short in order to keep the transition behavior comparable to that of the single-component SPB (*9*). Bilayers consisting of more than one lipid species can form domains in the gel state of the lipids, resulting in a heterogeneous distribution of the lipid species over the bilayer surface (*10*). It has been demonstrated very recently that the size of these domains is below 10 nm (*11*), and so each domain consists of only a few hundred lipid molecules. If the bilayer temperature is raised above the melting temperature (T_m) of the lipid mixture, the domains will disappear due to the onset of lateral diffusion, giving rise to a rather homogeneous lipid distribution in the fluidlike L_α phase.

If the headgroups of one of the lipid species are zwitterionic while the other species are either cationic or anionic, domain formation is equivalent to a change of the local surface charge density of the bilayer. This is due to the fact that the driving force for domain formation, the demixing of the two lipids at the transition to the gel state, causes an enrichment of one species and the depletion of the other species within the domain. This process is theoretically well described by regular solution theory (*12, 13*), which is analogous to the description of mixing in metal alloys (*14*). Since the domain diameter is still large as compared to the size of water-soluble proteins of less than 50 kDa molecular mass, this change in charge density at the phase transition can cause the coupling and uncoupling of such proteins to/from the domain surface via Coulomb interaction. In a previous paper, we have shown that, for the case of porous silica beads coated with domain-forming bilayers in a standard chromatography column, this effect can be used for

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[1] Abbreviations: ATR, attenuated total reflection; DEPC, 1,2-dielaidoyl-*sn*-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol; DMTAP, 1,2-dimyristoyl-3-trimethylammoniumpropane; DSTAP, 1,2-distearoyl-3-trimethylammoniumpropane; IR, infrared; MLV, multilamellar vesicle; SPB, supported planar bilayer; SUV, small unilamellar vesicle; T_m, main phase transition temperature.

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a new type of charge-selective bioseparation where the elution process is controlled by column temperature rather than by ionic strength or pH value of the buffer medium (*15, 16*). A very recent work by Heimburg et al. suggests that cytochrome *c* binding to charged membranes in the fluid phase is modulated by the mixing behavior of the lipid components (*17*).

Nevertheless, important questions about the influence of the negative charge of the solid support on this effect and about the mixing behavior of the two lipid species in terms of their mutual hydrophobic chain mismatch with respect to the domain formation are still open. To tackle these problems, we have used supported planar bilayers of different lipid composition and of different chain mismatch where the planar support consisted of the germanium plate of an attenuated total reflection infrared spectroscopy setup. Using a special ATR flow cell, this enabled a very sensitive quantitative analysis of protein coupling to the SPB as a function of bilayer phase state, lipid composition, and protein excess charge by analyzing the amide band signals of the proteins. A major aim of these experiments was the provision of evidence that the attractive Coulomb interaction potential between a water-soluble protein and an oppositely charged bilayer is considerably larger for the gel phase (i.e., domain patterned) bilayer than for the homogeneous fluid phase bilayer, independent of the charge of the solid support.

MATERIALS AND METHODS

Materials and Sample Preparation. DMPC, DEPC, DMPG, DMTAP, and DSTAP were obtained from Avanti Polar Lipids (Alabaster, AL). β-Lactoglobulin (bovine milk) and cytochrome *c* (bovine heart) were purchased from Fluka (Buchs, Switzerland). Deuterium oxide was from Deuchem GmbH (Leipzig, Germany).

All lipid mixtures were prepared by dissolving appropriate amounts of dry phospholipids in chloroform and evaporating the solvent by overnight vacuum desiccation. MLVs were obtained by swelling the lipid mixtures in buffered D_2O (buffer A: 20 mM HEPES, 0.5 mM EDTA, pH 7.0) and incubating them under gentle vortexing for 30 min at a temperature well above their main phase transition temperature, T_m. SUVs were obtained by treatment of the MLV suspension with a titanium rod sonifier in a thermostated water bath at temperatures 10 °C above the T_m of the mixture. Sonication was done at continuous power mode until the SUV solution appeared optically transparent in white light. HPLC analysis of the lipids right after the sonication and after 5 days of storage at 4 °C showed no detectable degradation of the lipids.

SPBs were prepared by SUV fusion at the support surface. The two large faces of a germanium ATR plate (52 × 30 × 2 mm, 45° aperture) mounted inside a home-built flow cell of about 1 mL liquid volume were used as the solid support. Prior to the SUV incubation, the plate was hydrophilized by immersion in 2% SDS solution for 2 days followed by washing in purified water (Milli-Q System, Millipore, Bedford, MA). For SPB formation, the SUV solution (2 mL volume, lipid concentration 2 mg/mL) was flushed into the cell while the temperature was maintained above the T_m of the SUV solution. After 15 min incubation time, the whole ATR unit was cooled below the lipid main phase transition temperature (T_m) and afterward flushed with buffer A to remove all bulk vesicles. The rather short incubation time is important to avoid any substantial enrichment or depletion of charged lipids in the supported bilayer formed on top of the ATR plate (*9*).

Protein adsorption was achieved by flushing 2 mL of the protein dissolved in buffer A into the cell (protein concentration 0.3 mg/mL) and allowing incubation with the gel phase SPB (temperature below T_m) for 15 min. After this, the cell was flushed with a minimum of 15 mL of buffer A to remove all unbound protein. Hence, the measured IR signals of the amide I′ and amide II′ bands arose solely from proteins bound to the SPB.

To unbind the proteins by reducing the electrostatic attraction between protein and SPB, the cell was flushed with buffer A of stepwise increased NaCl concentration (15 mL of buffer A for each NaCl concentration). After finishing each increment in salt concentration, before commencing the IR measurement the cell was additionally flushed with 12 mL of buffer with no NaCl. During these unbinding series, the cell and buffer A temperature was kept at either 1 °C (gel phase SPB) or 40 °C (fluid phase SPB) throughout the series. This allowed a direct comparison of the amount of protein adsorbed to the SPB at a given salt concentration for the two phase states.

Methods. Our home-built vertical ATR cell assembly consisted of two identical flow cells arranged on top of each other and sharing the same large face of the ATR plate. This arrangement was the same on both sides of the ATR plate in order to double the area accessible for the evanescent IR field for each cell. The height of each cell was 13 mm, and therefore two of them fitted well on the ATR plate with a height of 30 mm. A sample shuttle controlled by the IR data acquisition system directed the IR beam either through the upper cell containing the reference buffer or through the lower cell with the sample SPB plus proteins. The temperature of the whole ATR setup (both flow cells) was controlled on-line by a water bath thermostat interfaced to the acquisition computer and was constant within 0.2 °C. The shuttle system allowed us to measure sample (SPB with or without adsorbed proteins in buffer A) and background (pure buffer A) almost simultaneously to minimize background drift and contributions from the rotational bands of water vapor interfering with the amide signals. This principle is similar to the horizontal shuttle system described by Flach et al. (*18*) for external reflection IR measurements of monolayers.

Infrared spectra were obtained with a Perkin-Elmer System 2000 infrared spectrometer equipped with a DTGS detector. Typically, 16 scans were collected with a resolution of 2 cm^{-1}. ATR spectra of the sample and of the buffer background were recorded sequentially with a time separation of about 4 min. The latter were used to normalize the sample spectra. By subtracting the normalized spectra of the supported bilayer from that of the bilayer with the adsorbed proteins, we obtained the amide spectra. The spectra were linearly baseline corrected and analyzed with Grams 3.01B software (supplied with the spectrometer).

The electrostatic binding of cytochrome *c* to anionic supported bilayers was further evaluated in terms of its log *P* value using a commercial kit (Transil-A) purchased from Nimbus Biotech, Leipzig, Germany. This kit consisted of porous, bilayer-coated silica beads (diameter 30 μm, pore

size 400 nm) with a lipid composition identical to that used in the infrared experiments (2% DMPG and 98% DEPC). The beads were dissolved in buffer A and titrated into a solution of cytochrome *c* in buffer A. After each titration step, the mixture was allowed to equilibrate for 5 min under gentle vortexing, followed by a short centrifugation step (30 s in a desk top centrifuge). The sediment contained all Transil-A beads with the bound protein fraction. The concentration of the unbound cytochrome *c* after each titration step was determined by UV/VIS spectroscopy (absorption at 410 nm) of the supernatant. A plot of the unbound protein concentration vs the titrated lipid concentration gave a sigmoid-shaped binding curve from which the log *P* value was determined according to (*19*)

$$\log P = \log \frac{c(\text{prot})_{\text{lipid}}}{c(\text{prot})_{\text{buffer}}} = \log \frac{n(\text{prot})_{\text{lipid}} V_{\text{buffer}}}{n(\text{prot})_{\text{buffer}} V_{\text{lipid}}} \quad (1)$$

where

$$c(\text{prot})_{\text{lipid}} = \frac{n(\text{prot})_{\text{lipid}}}{V_{\text{lipid}}}$$

and

$$c(\text{prot})_{\text{buffer}} = \frac{n(\text{prot})_{\text{buffer}}}{V_{\text{buffer}}}$$

P is the partition coefficient between the concentrations of protein bound to the lipid membrane and dissolved in buffer. Here $n(\text{prot})_{\text{lipid}}$ and V_{lipid} are the amount of protein bound to lipid and the lipid volume, respectively. Accordingly $n(\text{prot})_{\text{buffer}}$ and V_{buffer} are the amount of protein dissolved in buffer and the buffer volume. At 50% protein binding to the lipid membrane, we have $n(\text{prot})_{\text{lipid}} = n(\text{prot})_{\text{buffer}}$, i.e., equal amounts of protein are bound and unbound. For this case, the partition coefficient is solely the ratio of the buffer volume (V_{buffer}) to the lipid volume (V_{lipid}). The titration series were performed at two temperatures, giving the log *P* value at 1 °C (gel phase bilayer) and at 40 °C (fluid bilayer).

RESULTS

Cytochrome c Binding to Anionic SPB. In a first set of experiments, we investigated the temperature-dependent coupling of positively charged cytochrome *c* (pI = 9.6) to an anionic SPB composed of 2% DMPG (C_{14} chains) and 98% DEPC (C_{18} chains). The SPB was incubated with the same amount of protein under fluid and gel phase conditions. Figure 1 shows the ATR spectra (amide band region) of the protein after the adsorption to the SPB for the fluid (40 °C) and for the gel state (1 °C). At both temperatures, the ATR cell was flushed after the cytochrome *c* adsorption to the bilayer with buffer A of increasing ionic strength (0, 50, 100, and 150 mM NaCl). After each flushing, ATR spectra were acquired. The unbinding of protein with increasing ionic strength is reflected by the decrease of amide band intensity as shown in Figure 1. A plot of the amide I′ band intensity at 1651 cm^{-1} from Figure 1 versus the salt concentration in the buffer is shown in Figure 2 for both temperatures. It demonstrates that for the case of salt-free buffer A (0 mM NaCl) about 35% more cytochrome *c* adsorbed to the gel phase bilayer than to the fluid one. With increasing salt concentration, the total amount of protein adsorbed decreases for both phase states, and the difference in amide I′ intensity between the two temperatures becomes negligible at 150 mM NaCl.

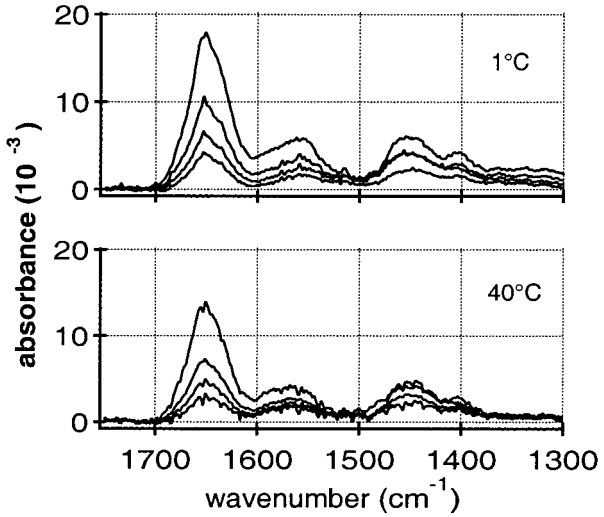

FIGURE 1: Unbinding of cytochrome *c* from a negatively charged SPB (2% DMPG/98% DEPC) under fluid (T = 40 °C) and gel phase (T = 1 °C) conditions observed by infrared ATR spectroscopy. The amide band region of the protein is shown for a series of stepwise increases of the NaCl concentration in the buffer medium at the corresponding temperature. From the top trace to bottom trace: 0.00, 0.05, 0.10, and 0.15 M NaCl.

By contrast, the lipid bilayer did not show any changes by the flushing procedure. An unchanged carbonyl absorption band at 1740 cm^{-1} arising from the bilayer lipids demonstrated that the lipid content of the SPB was a constant.

Nevertheless, the SPB was indeed in the fluid state at 40 °C and in the gel state at 1 °C. This was indicated by the frequency difference of the asymmetric CH_2-stretching band of the lipids by 4.5 cm^{-1} between the two temperatures (2918.0 cm^{-1} at 1 °C and 2922.5 cm^{-1} at 40 °C).

As a test of whether the phase state of a zwitterionic lipid alone can account for the different adsorption of cytochrome *c*, the above experiment was repeated by using a SPB of pure DEPC at the same two temperatures. For salt-free buffer, we obtained amide I′ signals of the same intensity (Figure 3) for both phase states, and their decrease with increasing NaCl was the same for both temperatures (data not shown). In this case, the attraction of cytochrome *c* was dominated by the electrostatic potential arising from the negative surface charge of the germanium ATR crystal serving as solid support. The surface charge may induce the P−N dipoles of the PC headgroups to adopt an orientation that is slightly attractive for cytochrome *c*. The results indicate that the presence of anionic lipids in the SPB is a prerequisite for a phase-state-dependent cytochrome *c* coupling.

Further support for the temperature-dependent binding of cytochrome *c* to supported anionic bilayers was obtained by a direct determination of the partition coefficient of the protein when using a bilayer of 2% DMPG and 98% DEPC. This was achieved by doing a titration series using a commercial supported bilayer material (Transil-A) of this lipid composition at the two temperatures, 1 and 40 °C. The result is shown in Figure 4. The log *P* values determined from these titration series were 3.3 (1 °C) and 2.7 (40 °C),

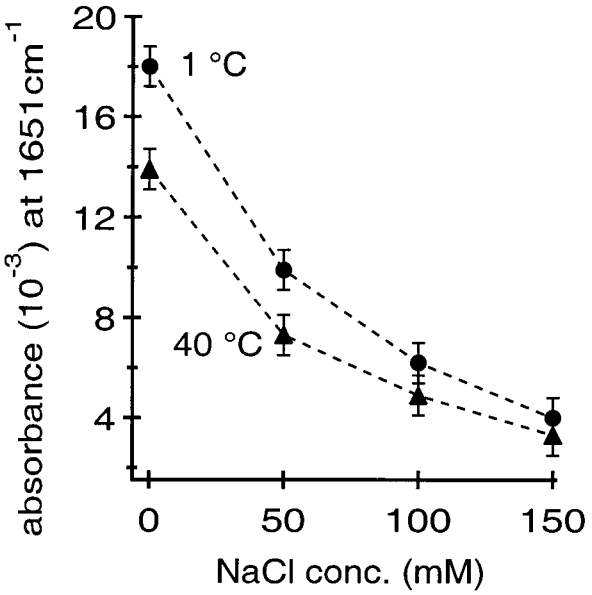

FIGURE 2: Intensity of the amide I′ band of cytochrome *c* at 1651 cm^{-1} taken from the spectra shown in Figure 1 as a function of NaCl concentration in the buffer for the gel phase (1 °C, circles) and for the fluid phase (40 °C, triangles). Dashed lines were drawn to guide the eye.

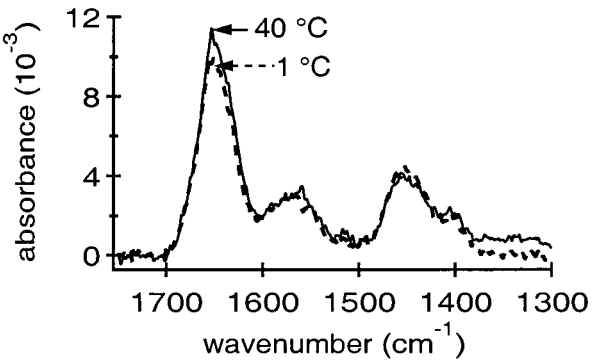

FIGURE 3: Amide I′ band region of cytochrome *c* bound to a pure zwitterionic DEPC SPB (no NaCl in the buffer) for the fluid phase (40 °C, full line) and for the gel phase (1 °C, dashed line).

indicating significantly higher binding of cytochrome *c* to the anionic bilayer under gel phase conditions.

β-Lactoglobulin Binding to Cationic SPB. In a second set of experiments, we studied the binding of negatively charged β-lactoglobuline (pI = 5.2) to a cationic SPB [8% DMTAP (C_{14} chains)/92% DEPC (C_{18} chains)] under identical conditions as reported above for cytochrome *c*. However, it should be noted that now the negative charge of the solid germanium ATR surface caused a Coulombic repulsion of the protein, while the cationic lipids in the bilayer gave rise to electrostatic attraction of β-lactoglobulin. Consequently, a control measurement with a SPB of pure DEPC gave no measurable protein adsorption, independent of the salt concentration, due to the repulsive action of the negative Ge surface charge (data not shown).

A plot of the amide I′ intensity at 1636 cm^{-1} versus the salt concentration is shown in Figure 5 for both the fluid (40 °C) and the gel phase (1 °C) of the cationic SPB. The change of the bilayer phase state between the two temperatures was again verified by observing the shift in the asymmetric CH_2-stretching band of the lipids from 2918.0 (1 °C) to 2922.5 cm^{-1} (40 °C). It is interesting to note that

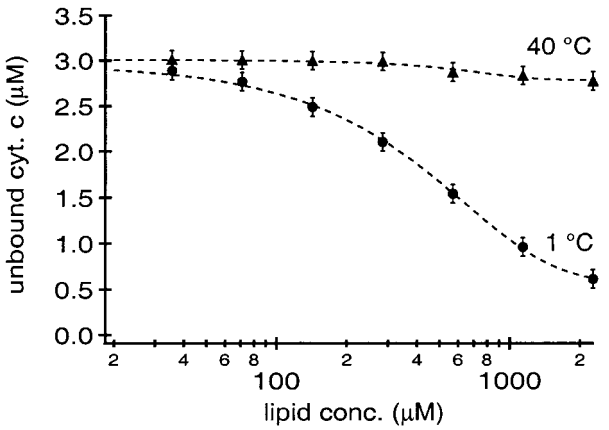

FIGURE 4: Binding curve of cytochrome *c* to an anionic supported bilayer (2% DMPG/98% DEPC) where the support consisted of porous silica beads (Transil-A), measured by titration under fluid (T = 40 °C, triangles) and gel phase (T = 1 °C, circles) conditions. Dashed lines were drawn to guide the eye.

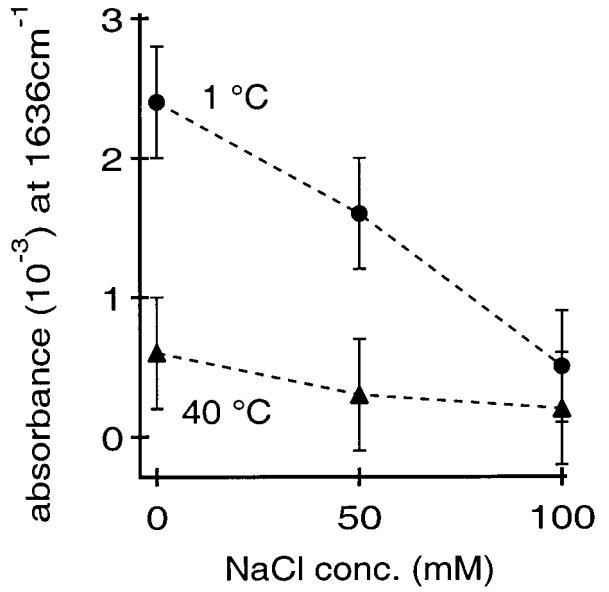

FIGURE 5: Intensity of the amide I′ band of β-lactoglobulin bound to a positively charged SPB (8% DMTAP/92% DEPC) as a function of NaCl concentration in the buffer for the gel phase (1 °C, circles) and for the fluid phase (40 °C, triangles). Dashed lines were drawn to guide the eye.

β-lactoglobulin was found to bind exclusively to the gel phase SPB, while in the fluid phase no binding was observed at the lowest salt concentrations studied.

One should note that in the above experiment, the lipids having the longer chains (DEPC) were zwitterionic while the shorter chain lipids (DMTAP) carried a positive charge. In a control experiment, we reversed this arrangement by using a DSTAP (C_{18} chains, 9 mol %) and DMPC (C_{14} chains, 91 mol %) SPB and did the analogous experiment. The results with respect to the β-lactoglobulin binding at 40 and 1 °C were qualitatively similar to those obtained with the DMTAP/DEPC mixture, although we observed a still detectable amount of the protein at 40 °C (Figure 6). The likely reason for this is that the DSTAP/DMPC mixture is not completely fluid at this temperature, as is indicated by the IR frequency of 2922.0 cm^{-1} at 40 °C, 0.5 cm^{-1} less than for the DMTAB/DEPC mixture.

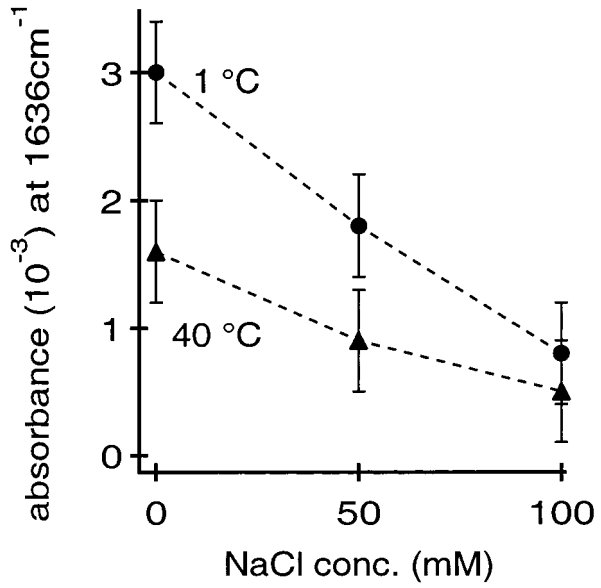

FIGURE 6: Intensity of the amide I′ band of β-lactoglobulin bound to a positively charged bilayer (9% DSTAP/91% DMPC) where the hydrophobic chain mismatch is similar to the mixture from Figure 4 but with the cationic lipid featuring the longer chains. The unbinding is again shown as a function of NaCl concentration in the buffer for the gel phase (1 °C, circles) and for the fluid phase (40 °C, triangles). Dashed lines were drawn to guide the eye.

In a further experiment, we investigated whether the mixing properties of the SPB lipids were crucial for the binding of β-lactoglobulin in the gel phase. This was achieved by using a cationic SPB where both lipid components had identical chain lengths. We used a bilayer of 10% DMTAP and 90% DMPC (i.e., both lipids had C_{14} chains, thus no chain mismatch) that showed only a slight nonideality in its mixing properties. Although the cationic charge content of this bilayer was comparable to the above experiment, we observed no difference in β-lactoglobulin binding in salt-free buffer A between gel and fluid phase conditions (data not shown).

DISCUSSION

The above results provide evidence that the lipid phase state of a binary SPB is crucial for the coupling of water-soluble proteins by Coulomb interactions under conditions of nonideal mixing of the two lipid components. This effect was observed both for anionic and cationic SPBs.

One could be tempted to argue that the increase of lipid packing density at the transition from the fluid to the gel phase may provide a sufficiently high increase of the surface charge density to cause a Coulomb attraction between the oppositely charged proteins and the SPB. However, the negative result of our experiment using cationic and zwitterionic lipids of identical (C_{14}) chain length (DMTAP/DMPC) does not support this surmise. Furthermore, a significant contribution of closer lipid packing to the protein binding is not expected from electrostatic theory, as is shown by an estimate of the electrostatic potential increase connected with the change of lipid packing density at the phase transition. Assuming a reduction of the molecular area by 25% at the transition to the gel phase [from 63 (fluid) to 48 Å^2 (gel)] equation (*20*), we can expect for a binary lipid mixture having one charged component an increase of the surface charge density by approximately the same amount. For the case of a bilayer containing 10 mol % cationic lipids, this is tantamount to an increase from about 0.16 to 0.20 charges/nm^2. According to the Graham equation (*21*)

Table 1: Theoretical Estimate of the Change in Electrostatic Potential $\Psi_z(T)$ at a Distance z from the Bilayer Surface Due to the Area Reduction at the Transition to the Gel Phase for a DEPC Bilayer Containing 10 mol % Cationic DMTAP[a]

phase	A_l (Å^2)	σ (10^{-1} C/m^2)	T (°C)	κ^{-1} (nm)	Ψ_0 (mV)	$\Psi_{z=5Å}$ (mV)	$\Psi_{z=10Å}$ (mV)
fluid	63	2.6	40	2.2	67	47	38
gel	48	3.2	1	2.1	69	46	37

[a] Equations 2 and 3 are used assuming ideal mixing of the lipids. A_l is the area per lipid, σ is the surface charge density, T is the bilayer temperature, κ^{-1} is the Debye screening length, and Ψ_0 is the surface potential.

$$\Psi_0 = \frac{2kT}{e}\operatorname{arcsin} h\left(\frac{\sigma}{\sqrt{8\epsilon\epsilon_0 kT}\sqrt{[\mathrm{NaCl}]}}\right) \qquad (2)$$

this causes a slight potential increase at the bilayer surface from $\Psi_0^{(T=40°C)} = 67$ meV (fluid) up to $\Psi_0^{(T=1°C)} = 69$ meV (gel) for a buffer containing 20 mM NaCl. By using the Gouy−Chapman equation

$$\Psi_z \approx \frac{4kT}{e}\gamma e^{-\kappa z}$$

where

$$\gamma = \tanh(e\Psi_0/4kT) \qquad (3)$$

we obtain the potential $\Psi_z(T)$ as a function of distance z from the bilayer surface at a given temperature T, where κ^{-1} is the Debye screening length. For reasonable values of z between 0.5 and 1.0 nm (note that the protein cannot be considered as a point charge at close distances to the SPB), this corresponds to a potential energy difference of (cf. Table 1)

$$e\Delta\Psi_{z=0.5nm} \approx e\Delta\Psi_{z=1.0\mathrm{nm}} \approx 1 \text{ meV} \qquad (4)$$

This energy difference is clearly negligible in comparison to the thermal energy (1 kT = 27 meV), and the assumed 25% shrinkage in area is already the upper expectation of the packing density increase. It follows that the homogeneous (i.e., ideal mixing) condensation of a bilayer at the transition to the gel phase is highly unlikely to account for the effects observed.

Therefore, formation of two-dimensional bilayer heterogeneities (so-called domains) at the transition to the gel phase by partial demixing of the lipid components is the most likely process to dominate the protein coupling at low temperature. This process can give rise to a substantial enrichment of one lipid component within the domain at the expense of the other component and thus may cause a drastic increase of local charge density over the domain. Our results using DMTAP/DEPC or DMPG/DEPC mixtures suggest that a chain mismatch resulting in a sufficiently strong nonideality of the lipid mixing under gel phase conditions is crucial for protein coupling. Domains in gel phase bilayers are tiny, quasi two-dimensional objects, the size of which was recently studied by our group. A combination of neutron techniques

and atomic force microscopy suggested gel domain sizes of less than 10 nm (*11*). Since the size of the proteins used in our work is considerably less than 10 nm [3 nm for cytochrome *c* (*22*) and only slightly more for β-lactoglobulin as estimated from its molecular weight], we can safely assume that each protein can couple to only one domain. Hence, the charge enrichment within the domain by partial demixing must be sufficiently high to provide an attractive electrostatic potential energy at or above the thermal energy. The energy for this significant charge enrichment within the domain must be provided by the demixing process, i.e., the energy of van der Waals attraction between the chains of like lipids must be sufficiently high to overcome the Coulomb repulsion between charged headgroups. Since the latter scale with an inverse square distance law while van der Waals forces scale with r^{-6}, a chain mismatch can indeed be assumed to be the driving force of this process.

Furthermore, our results obtained for β-lactoglobulin binding to a SPB where the chain length mismatch between the two lipids was reversed indicate that the total mismatch dominates the domain formation while the exact assignment—which species which chain—is probably of rather minor importance. This finding is in agreement with the established knowledge on binary phase diagrams of nonideally mixing lipids (*12*).

To summarize, domain formation and dispersion induced by the phase transition of a binary lipid mixture in a SPB can modulate the electrostatic coupling between water-soluble proteins and the bilayer surface. Although gel phase bilayers are not a feature of biological membranes, domains may also at least temporarily exist in fluid, multicomponent systems. One has to consider that the cell membrane is made up of a wide variety of neutral and charged lipid species having different unsaturated chain lengths. In this case, charged networks such as the cytoskeleton of eukaryotic cells, which couple closely to the membranes, may provide a driving force for temporal demixing.

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