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18 May 1990; accepted 17 July 1990

Surface-Induced X-Ray Reflection Visualization of Membrane Orientation and Fusion into Multibilayers

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The fusion of lipid membranes at the air-water interface has been detected with the use of x-ray reflection as a high-resolution, surface-sensitive technique. The rate of this fusion for dimyristoylphosphatidylcholine (DMPC) bilayers is the highest at 29°C, which coincides with the chain-melting phase-transition temperature for the top membrane layers. After 6 hours of incubation a stack of at least ten surface-ordered membrane bilayers in equilibrium with the bulk vesicle suspension is formed. Such fusion is thus surface-catalyzed but not restricted to the first surface layer. The process involves partial membrane dehydration near the solution surface which decreases toward the bulk.

Molecular function and reorganization in the biological systems often involve interfaces. Membrane fusion provides good examples for this. To clarify the detailed physical principles that control the structural reorganization near the biological surfaces—and the role of interfaces in general—we have monitored the ordering and fusion of lipid vesicles into multibilayers near the air-water interface. We have used x-ray reflection as a high-resolution, surface-sensitive technique for this purpose (*1*). Our results indicate that both membrane ordering and membrane fusion depend on surface phenomena but are not always restricted to the top layer in close contact to the air. The rate of the observed structural reoganization is temperature-dependent. It changes abruptly a few degrees above the membrane chain-melting phase-transition temperature. The precise value of such a characteristic temperature is surface-sensitive because of the partial surface-induced membrane dehydration and concomitant isothermal phase transitions and fusion. In our case, this temperature coincides with the value that has previously been reported to give rise to the spontaneous formation of single bilayers under different conditions (*2*). We believe that this is a manifestation of a general principle of the surface catalysis. The relatively short range of the observed surface effects, which is probably less than 80 nm, provides a means for the preparation and structural investigation of the surface-supported, well-oriented membrane specimen.

Dimyristoylphosphatidylcholine (DMPC; Boehringer Mannheim) and water (>18 MΩ cm) of the highest available purity (>99.9%) were used for all experiments. In independent measurements, the order-disorder, chain-melting phase transition of the lipid suspensions was confirmed to be 23.5° ± 0.2°C for a 1-day-old bulk sample; the corresponding value for a sample aged for 4 weeks in humid air above pure water in a closed compartment at ambient temperature was measured by differential scanning calorimetry to be 29° ± 1°C, suggesting a partial sample dehydration. The transition enthalpies in both cases were not significantly different (26 ± 1.5 kJ mol^{-1}).

X-rays ($\lambda = 0.138$ nm) were directed nearly parallel to the surface of the lipid suspension at a series of incident angles ($1 \leq \theta \leq 60$ mrad). The resulting initial reflectivity of the vesicle suspension surface was found to be similar to that characteristic of the lipid monolayers deposited at the air-water interface. For a specimen kept below 26°C such a picture persists for at least one-half day (and probably longer). Conversely, the reflectivity measured at 29°C after less than 1 hour is already different, especially in the low-angle region. After a few hours a series of sharp, asymmetric but equidistant peaks evolves from the initial state (Fig. 1). To clarify the nature of these peaks we have also calculated the reflectivity of a series of layers (consisting of an electron-dense headgroup region and two adjacent, electronically less dense hydrocarbon and water layer zones) under the assumptions that such layers normally form bilayers and that the overall contrast decreases exponentially toward the bulk. From the detailed comparison of the measured and calculated data for various combinations of the layer structure parameters we deduce that the observed peaks reflect the formation of multilamellar, stacked membranes. The calculated number of coplanar layers pertaining to Fig. 1 is 11 to 12, the repeat distance of these membranes being 6.2 nm at 29°C; this value is slightly less than the characteristic intermembrane distance in the bulk in excess water, 6.25 nm. Once created, the peaks keep growing for hours, even at temperatures below 29°C.

The shape of the maxima in the reflectivity curves is temperature-independent within the investigated range (20° to 32°C). The

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rate of the peak growth changes with temperature quite dramatically, however (Fig. 1, inset). This rate is for fresh samples the greatest at 29°C, the precise time dependence varying somewhat from preparation to preparation. [The characteristic temperature at which the initial rate-maximum is observed, in general, seems to be little affected by the lipid concentration, as concluded from the ellipsometric measurements with relatively dilute vesicle suspensions (<1 mg of lipid per milliliter).]

The positions of the peaks in the reflectivity curve exhibit a different characteristic temperature variation. The peak shifts toward smaller wave vectors between 26° and 25°C upon very slow cooling (<0.25 degrees per hour); specifically for a 14-hour-old sample the shift is by 5%. These effects are all indicative of the chain-ordering phase transition in lipid layers near the sample surface. The observed characteristic transition temperature (for our approximately 11-layer system) is thus intermediate between the chain-melting phase-transition temperature of the fully hydrated, bulk DMPC suspension (23.5°C) and the critical temperature of the sample in equilibrium with wet air (29°C). This result suggests that our surface-associated specimens are typically slightly dehydrated.

Vesicle morphology is of secondary importance for such surface-induced reorganization. The use of unilamellar of oligolamellar suspensions or of freshly prepared (and thus defect-rich) or aged vesicles (with partly annealed defects) all yielded qualitatively similar results. Surface-associated bilayers (or monolayers) do not form, however, if the lipid (1 mg/ml) is inside a dialysis bag, which prevents the diffusion of lipid vesicles throughout the sample but should not hamper appreciably the diffusion of lipid monomers.

The following picture emerges based on these experimental observations. Individual membrane vesicles are attracted from the suspension to the air-water interface (irrespective of whether or not the latter is covered by a lipid monolayer). Once close to the water surface they tend to deform and align partly along the surface plane. When the layer of adsorbed vesicles is sufficiently compact it hampers the exchange of the solvent molecules between the interfacial layers and the bulk and thus transiently reduces the local water activity between the membranes. The resulting gradient causes the uppermost lipid layers to dehydrate partly in a temperature-dependent manner (*3*). This leads to a position-dependent chain-melting phase-transition shift and causes the critical temperature of the surface-associated layers to decrease gradually from 29°C (near water-saturated air) to 23.5°C, characteristic of the bulk (*3, 4*). The precise spatial dependence can also depend on the relative humidity of the air.

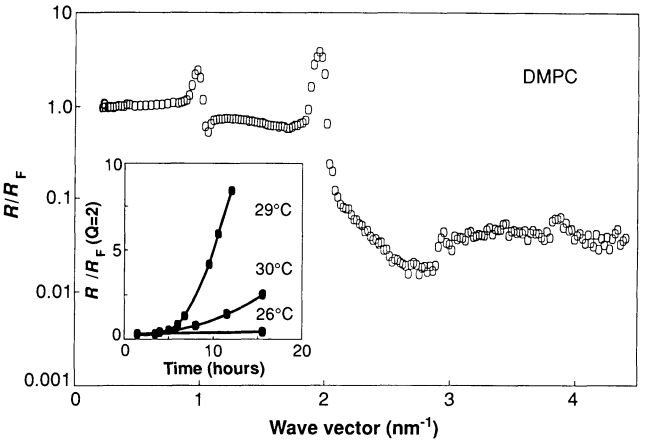

Fig. 1. X-ray reflectivity of the "air-water interface" above a suspension of DMPC vesicles in water as a function of the wave vector transfer $Q = 4\pi \sin\theta/\lambda$. The measured reflectivities were divided by the calculated Fresnel reflectivity of the bulk phase. Inset: Temporal evolution of the intensity of the second ($Q = 2.0123$ nm^{-1}) "Bragg peak" in the reflectivity curve, which is indicative of the multimembrane stack formation near the sample surface at different temperatures. Vesicle suspensions to be studied were obtained by mixing 100 mg of lipid with 5 ml of water and ultrasonication. They were filled directly into a round (8 mm by 5 cm) polyvinylchloride trough situated in the horizontal x-ray reflectivity instrument D4 in HASYLAB at Deutsches Elektronen Synchrotron (DESY) in Hamburg.

Now consider the membrane structure when the ambient temperature is close to the upper limit of ~29°C. The surface-bound lipids then are within the chain-melting phase-transition region, whereas the more deeply located membranes are not. Critical fluctuations caused by the phase transition (*5*) therefore arise predominantly in the surface-adsorbed bilayer. In combination with the surface alignment they promote fusion of the vesicles near the surface and, ultimately, lead to the formation of uniform, extended, surface-associated membranes. For further from the surface layers, closer to the bulk a similar situation is encountered at slightly lower temperatures.

Our experimental data thus are diagnostic of the surface-catalyzed, temperature-dependent, slow metamorphosis from individual membrane vesicles into a multilayer stack of surface-oriented, coplanar lipid bilayers.

Several years ago Gershfeld (*2*) has proposed, on the basis of radioactive tracer and surface tension measurements, that at some lipid-dependent critical temperature (*6*)—which for DMPC coincides with the value of 29°C, as detected in our study—single bilayers should form spontaneously at the air-water interface. Gershfeld later showed that related critical phenomena exist for the lipid extracts from the biological membranes (*7, 8*). But to date no molecular explanation for the whole process has been found and the results as such have attracted too little attention, perhaps because of the circumstantial experimental evidence (*9*).

We have succeeded in persuing the surface-catalyzed formation and orientation of the lipid bilayers directly by means of x-ray reflection. This method has helped us identify one possible source of the temperature anomaly. Furthermore, it has allowed us to show that under appropriate conditions the process of the surface-induced bilayer formation may involve vesicle fusion and can result in multilamellar, not only unilamellar, interface-bound membranes.

X-ray reflection is a well-established technique for studying the surface structures of liquids and solids (*10*). It has been used, among other methods, to investigate the lipid monolayers at the air-water interface with appreciable detail (*1, 11*). But in all such surface-reflectivity studies the sample composition and configuration were imposed by the experimenter; lipids were spread from organic solvents and, on water, as a rule, formed monolayers; the experimental conditions and the setup fixed or constrained the degrees of membrane freedom. In contrast, we track the lipid vesicles on their "natural way" into a thermodynamic, surface-dependent equilibrium, including both the surface-bound and the bulk membrane subportions.

Spontaneous adsorption of the detergent molecules from highly concentrated micellar suspensions into surface-bound multilayers has been reported recently (*10, 12*). We infer that this observation, as well as ours, is a manifestation of a surface-induced molecular reorganization.

The basic principle of such surface-induced catalysis and of the corresponding structural membrane reorganization, studied here for the air-water interface, should be also applicable to other artificial or biological interfaces. We suggest that the corresponding supramolecular layering, such as near the surface of membranes or protein arrays, can increase the propensity for structural reorganizations under suitable conditions. It is, moreover, quite plausible to assume that in the "coplanar" regions, where

the mutual membrane-surface and, of course, membrane-membrane attraction is the strongest, certain subclasses of the membrane components with special properties can be enriched preferentially. (One example of this involves molecules with similar hydration properties.) The resulting edge-inhomogeneities could also ultimately facilitate macromolecular insertion, membrane reorganization, and fusion.

In practical terms our findings suggest that one can generate nonsupported aligned samples consisting of lipids and other biological materials. Depending on the precise experimental conditions the resulting membrane systems should contain one, a few, or many bilayers—all essentially parallel to the surface of the probe-suspension. Surface-catalyzed orientation of the membrane samples at the air-water interface—preferably in combination with the x-ray reflectivity technique—can prove useful for the structural investigations of the model, reconstituted, and native membranes and contribute to the clarification of the physical and physicochemical principles underlaying supramolecular reorganization and fusion at interfaces.

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13. We thank E. Burkel and F. Grey for their support as well as J. Als-Nielsen from Riso and HASYLAB at Deutsches Elektronen Synchrotron (DESY) in Hamburg for offering us their technical facilities. This study has been financed in part by the Deutsche Forschungsgemeinschaft through grants C8/SFB 266 and Ce 19/1-1 to G.C. and C9/SFB 266 to W.F.

24 April 1990; accepted 5 July 1990

Heart Rate Regulation by G Proteins Acting on the Cardiac Pacemaker Channel

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Heart rate is determined by pacemaker currents, of which the most important is the hyperpolarization-activated current I_f. Heart rate and I_f are increased by β-adrenergic agonists and decreased by muscarinic agonists released from cardiac sympathetic and vagal nerves, respectively. The hypothesis that the receptors for each agonist are directly coupled to I_f channels by G proteins was tested. Under substrate-free conditions, preactivated G protein G_s stimulated and preactivated G protein G_o inhibited I_f channels of sinoatrial node pacemaker cells. These effects were mimicked by the corresponding preactivated α subunits of the G proteins. Unexpectedly, the two G proteins acted simultaneously, with G_o being the more potent. This result may explain in molecular terms the classical observation in cardiac physiology, that vagal inhibition of heart rate is much greater on a background of sympathetic stimulation.

THE MAMMALIAN HEARTBEAT ARISES from spontaneous, pacemaker currents in sinoatrial (S-A) node cells and is neurally regulated by the stimulatory, β-adrenergic effects of the transmitter norepinephrine and the inhibitory, muscarinic cholinergic effects of the transmitter acetylcholine released from the cardiac sympathetic and vagus nerves, respectively. The key pacemaker current is the hyperpolarization-activated current I_f (*1, 2*). Muscarinic agonists decrease and β-adrenergic agonists increase I_f, and it is proposed that G proteins act upon adenylyl cyclase (AC) to change the phosphorylation of I_f channels (*3, 4*). Since the heart rate can change from second to second (*5*), if phosphorylation and dephosphorylation of the channel cause this change, these reactions must occur at subsecond rates. This is unlikely (*6*), and an alternative hypothesis has been proposed in which G proteins couple these receptors to pacemaker channels by faster, more direct, membrane-delimited pathways (*7*). A necessary condition for the direct coupling hypothesis would be that G proteins directly regulate I_f channels in S-A node cells. We report here that the G protein G_s preactivated with guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) (G_s^*) or its preactivated α subunit (α_s^*) (*8*) stimulated I_f channels in inside-out (IO) membrane patches excised from S-A node cells under substrate-free conditions. The preactivated G proteins G_o^* and G_i^*(*8, 9*) or their respective preactivated α subunits α_o^* and α_i^* inhibited I_f channels with G_o^* being more potent. G_o^* and G_s^* or their α subunits seem to act simultaneously on an apparently shared set of I_f channels. The simultaneous action may provide a molecular explanation for why vagal inhibition of heart rate is greater in the presence of cardiac sympathetic nerve stimulation (synergistic effect) and why cardiac sympathetic stimulation of heart rate is smaller in the presence of cardiac vagal nerve stimulation (antagonistic effect) (*10*).

The current I_f can be recorded from cell-free, IO membrane macropatches (*11*). If we assume that single-channel conductance is 1 pS, patch I_f originated from about 200 channels (*12*). While still cell-attached (CA), patch I_f had the same waveform and voltage-dependence as whole-cell (WC) I_f (*11*) (Fig. 1A, trace a) and ran down slowly with time. Upon excision to the IO configuration, patch I_f fell immediately and then resumed its slow decline (Fig. 1, A, trace b, and B). To test whether autonomic receptors were coupled to I_f by G proteins in these cell-free patches, we added the β-adrenergic agonist isoproterenol (iso) to the pipette solution and changed guanosine triphosphate (GTP) in the bath. Concentration jumps of GTP increased I_f (parts c and d of Fig. 1, A and B). The effects were reversible and Mg^{2+}-dependent ($n = 4$). The time course could be determined by the difference in current between the traces before and immediately after the concentration jump of GTP. This time course indicated a delay of 50 ms and a time constant of 800 ms. Both rate and magnitude of the increase in I_f varied directly with the concentration of GTP; the increase could be as great as 50% (37.3 ± 9%, mean ± SD; $n = 3$). With carbachol (carb) in the pipette, the same series of tests produced almost identical results except for a change in sign (Fig. 2A); I_f was reduced by

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