Model Membrane Formations: Interaction of Sialoglycoprotein from Human Red Cell Membranes with Lipid monolayers *

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Summary

By penetration of enriched sialoglycoprotein preparations from human red cell membranes into a monolayer of red cell membrane lipids it is possible to form planar lipoprotein complexes. By deposition of two such monolayers on mica a bilayer is formed which, after freeze-fracturing and etching, closely resembles fractured red cell membranes with respect to the size and distribution of intramembrane particles. A clear correlation is seen between the particle number per unit area and the increase of surface pressure by the penetration of the protein into the lipid film. The amount of penetrated protein strongly depends on the initial film pressure of the lipid. Above 30 dynes/cm progressively less protein can penetrate the film. The results strongly support the hypothesis that the sialoglycoprotein fractions represent the particles seen in the native membrane.

The understanding of the detailed architecture of biological membranes depends largely on the progress of studies of protein-lipid interactions. Those proteins, which spand the lipid bilayer (e.g. the sialoglycoproteins and band III of the red cell membrane) are of interest because of their prospective role in membrane functions such as ion-transport or transmittance of signals from the extracellular compartment to the cell interior.

Penetration of isolated lipid free membrane proteins from the aqueous subphase into lipid monolayers at water/air surface has recently been shown to be a valuable technique in the study of protein lipid interaction [1,2,3]. The visualization of protein-lipid bilayer complexes by freeze-etching electron microscopy has revealed the appearence of particles in the fracture face similar to those observed in native membrane [4]. This indicates that monolayer penetration by membrane apoproteins may be considered as one of the most useful techniques for membrane reconstitution. Indeed Rothfield [5] obtained a functional reconstitution of the galactosyl transferase complex from E. coli after penetration of the respective inactive membrane components into a lipid monolayer.

In our previous work red cell membrane proteins have been added to the subphase as a solution of the apoproteins in 2-chloroethanol. This technique was previously shown in bulk recombination systems to ensure retention of the lipid-binding capacity of the proteins [6,7]. It was also shown that recombination of proteins and lipids was best achieved at *p*H values below 3, where an increased amount of glycoproteins was incorporated into the complex and where the number of particles in the fractured hydrophobic faces was highest [8]. We have now further investigated some membrane protein fractions of human red cells by a modified monolayer penetration technique and in the following will report experiments with the partly purified sialoglycoprotein fraction (PAS-bands I, II and III according to Steck [9]).

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Materials and Methods

All chemicals if not otherwise indicated have been obtained as analytical grade from Merck. Water was deionized and destilled before use.

Purification and characterization of the sialoglycoprotein fraction and isolation of human red cell membrane lipid

Human red cell membranes were isolated according to Doge et al. [10] and the sialoglycoprotein fraction was prepared according to Hamaguchi et al. [11]. The final aqueous solution of the sialoglycoprotein was freed of aggregates by a prolonged centrifugation at 100.000 g (4 h) before transfer to a 10 mM citrate buffer at *p*H 2 by dialysis (0.05% NaN_3 was included in the buffer as a bacterizide). This solution was stored at 4 °C under nitrogen at a protein concentration of 2.5 mg/ml (protein assay according to Folin [12]). SDS-acrylamide gel electrophoresis was prepared according to Neville et al. [13]. The gel concentration was 5.81%. The gels of 3 mm diameter and 6 cm in length were stained with either Coomassie Brilliant Blue or periodic acid Schiff reagent (PAS) according to Fairbanks et al. [14]. The phosphorus-assay was performed by the method of Lowry et al. [15]. Total lipid extracts of the red cell membranes were obtained by the method of Folch [16]. The solution in chloroform was stored at − 20 °C at a concentration of 2 mg total lipid/ml under nitrogen.

Surface pressure measurements (see Fig. 1)

A two compartment Teflon trough was used, each measuring 100 × 100 × 10 mm. The surface pressure of the film was measured by a platinium plate of 39.6 mm perimeter coated with platinium gray according to Wilhelmy [17]. The plate was suspended from a Statham Universal Transducing Cell, Model UC-2. The transducer was fitted with a micro-scale accessory. The amplified signal was recorded by a Phillips recorder.

Preparation of the lipid monolayer and penetration of protein

The monolayer trough (see Fig. 1) was placed in a thermostated cabinet at 20 ± 0.1 °C and the surface of the buffer (10 mM Na-citrate, 0.05% NaN_3 *p*H 2.0) was cleaned. The lipid solution in chloroform was added in μl portions by a Hamilton-micropipett on to the surface of the buffer until the desired surface pressure was reached (π_i). The protein solution was added through a hole in the movable barrier to the subphase to a final concentration of 10 μg protein/ml. During the whole process the subphase was stirred by a magnetic stirrer (12 mm long and 5 mm large) at 100 rpm. After equilibration of the surface pressure the film was transferred to the second trough containing 2 M NaCl in order to eliminate adsorbed but not penetrated protein from the film. After 30 min the film was then washed 4 times with water. Surface pressure during the whole washing phase was held constant.

Preparation of the lipoprotein bilayer and freeze-etching

The washed film (see Fig. 1) was then deposited on a freshly split and wetted mica, which was dipped into the subphase with a micromanipulator. No change in surface pressure was observed during this procedure. The mica was then slowly drawn up through the monolayer. This deposited the first layer with the lipid polar groups oriented toward the mica. The plate was air-dried for several minutes and then dipped back through the monolayer, whereby the second layer was deposited resulting in a bilayer model membrane.

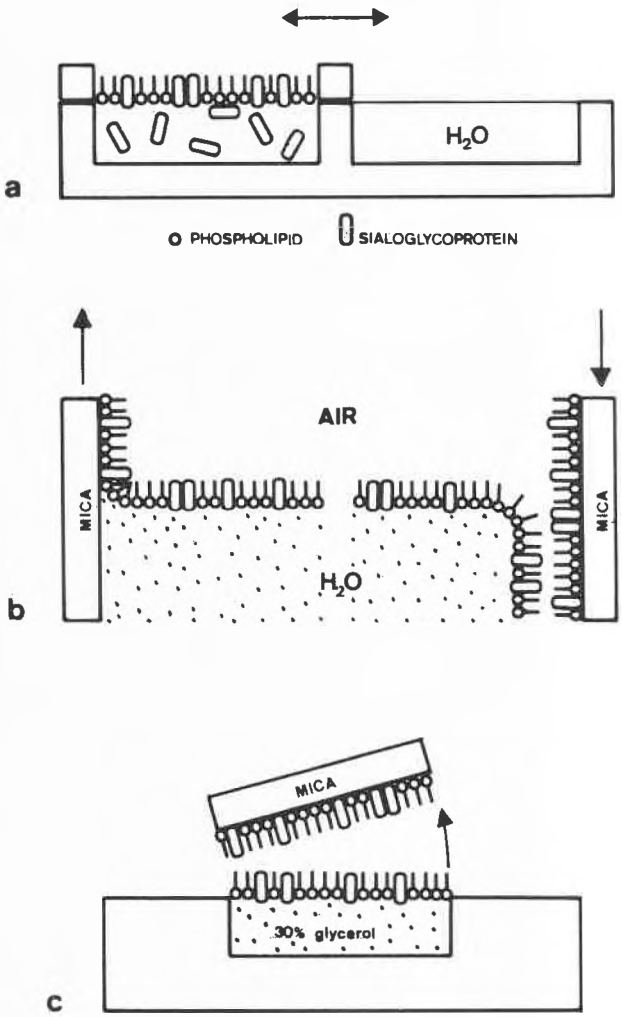

Fig. 1: a) Langmuir-through (Teflon) composed of two compartments 100 × 100 × 10 mm separated by a removable barrier: The monolayer can be transferred from one compartment to the other by the disposable barrier
b) Dipping technique with the hydrophilic mica using a micromanipulator
c) Fracturing of the bilayer by tearing off the mica from the U-shaped brass holder. The second monolayer remains on the frozen 30% glycerol

The mica plates were then transferred in water to a large container where they were attached to a U-shaped brass holder. The holder with the mica was rinsed with 30% glycerol, rapidly frozen in Freon 22 and then

placed on the pre-cooled (− 150 °C) stage of a Balzers BA 360 freeze-etch apparatus. Under vacuum (10^{-6} torr), the mica plate was broken away from the ice with the knife assembly (see Fig. 1). The exposed surface was etched briefly (60 sec at − 100 °C), and shadowed with platinium and carbon.

Electron microscopy

The carbon replica corresponding to the hydrophobic fracture face of the second monolayer deposited on the mica was placed on a 200 mesh copper net and used for electron microscopy in a Phillips EM 200 at 60 kV. All micrographs were obtained at a scale of 26,900× with subsequent photographic enlargement of 2.4× giving a final magnification of 64.560×.

Results

1. Characterization of the sialoglycoprotein fraction

Gel electrophoresis of the purified sialoglycoprotein-fraction revealed the typical bands (PAS-I, II, III and IV) by carbohydrate staining (see Fig. 2). However by protein staining a considerable amount of spectrin (band I and II) was observed in fresh extracts. During storage of the preparation in the cold most of it disappeared from the gels indicating progressive breakdown (see Fig. 2).

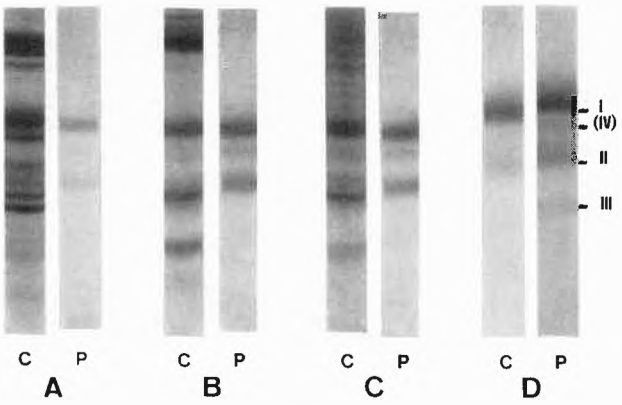

Fig. 2: SDS-polyacrylamide gel electrophoresis of:
A human stroma (C = Coomassie-blue, P = PAS-stain)
B fresh sialoglycoprotein extract
C sialoglycoprotein extract after 6 days storage in the cold
D delipidized sialoglycoprotein extract after 6 days storage in the cold

The purified sialoglycoprotein fractions showed a content of 0.4 to 1 % of phosphorus indicating various amounts of bound phospholipids. In order to remove most of the lipid before penetration experiments the fractions were chromatographed in 90 % 2-chloroethanol on Sephadex LH-20 (ref. 6). Three preparations have been used for the experiments with the lipid monolayer; they contained 0.12 %, 0.48 % and 0.8 % phosphorus corresponding to lipid contents of 3 %, 12 % and 20 % respectively (see Fig. 3).

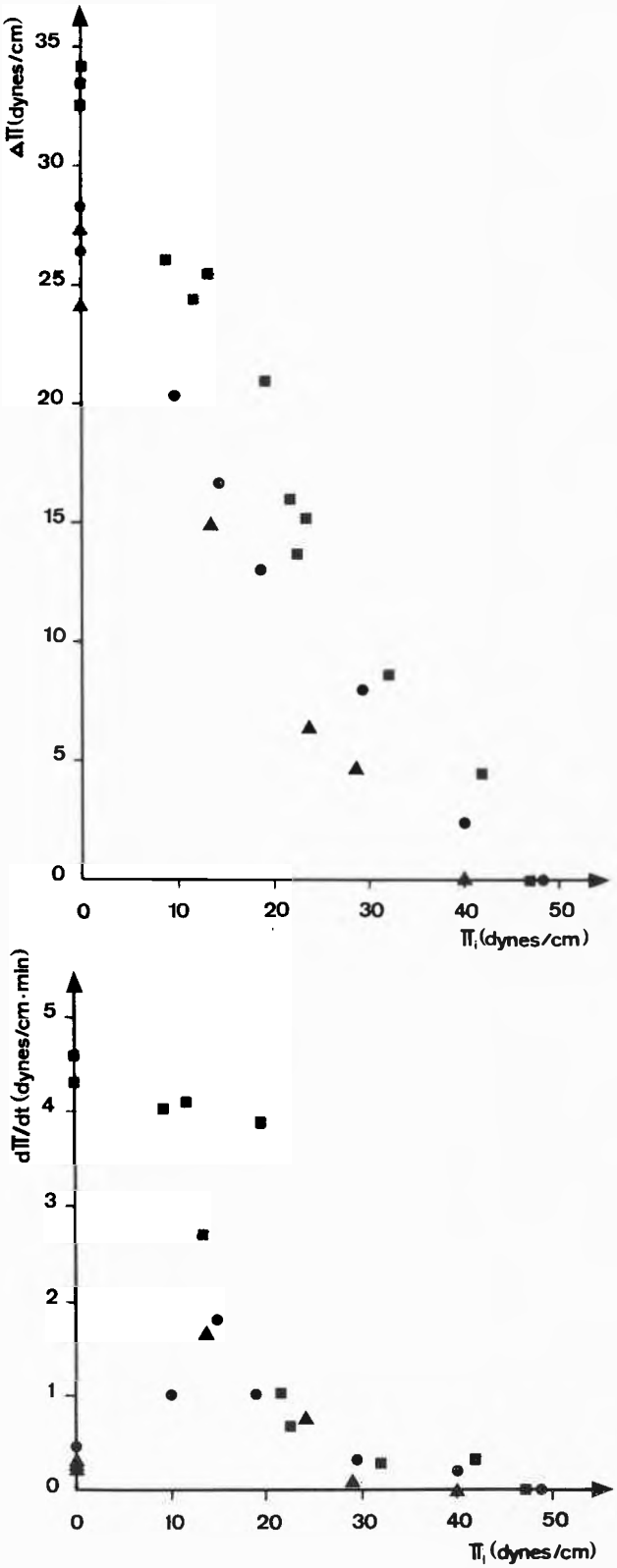

Fig. 3: Data on surface pressure increase ($\Delta\pi$) by penetration of the sialoglycoprotein fraction into a lipid monolayer of a given initial surface pressure (π_i) (see also methods)
a) dependence of $\Delta\pi$ on π_i
b) dependence of the rate of penetration ($d\pi/dt$) on π_i
■ *protein preparation containing 20 % lipid*
● *protein preparation containing 12 % lipid*
▲ *protein preparation containing 3 % lipid*

2. Penetration experiments

The penetration of the three preparations with varying lipid-content was studied as a function of the initial lipid surface pressure (π_i). From Fig. 3 it can be seen that the change in surface pressure ($\Delta\pi$) due to penetration of protein decreases with the increase of π_i. Near the collapse pressure of the lipid film (at 45 to 50 dynes/cm) no penetration of protein is observed. At low pressure ($\pi_i < 10$ dynes/cm where the film is no longer homogeneous the values measured for $\Delta\pi$ become inconsistent. In the absence of lipid ($\pi_i = 0$) one measures the surface activity of the protein alone which varies between 25 and 35 dynes/cm depending on the lipid content of the preparation. High lipid content leads to the highest values for $\Delta\pi$ over the full range of π_i. The velocity of penetration $d\pi/dt$ also depends on the lipid content. With 2% and 12% lipid the protein penetrates only slowly into the lipid free surface ($\pi_i = 0$). In the presence of a lipid monolayer the rate of penetration into the surface is considerably increased with a maximum around $\pi_i = 15$ dynes/cm decreasing again at higher film pressures.

Sialoglycoprotein preparations with a high lipid content show high penetration rates also when no lipid monolayer is present.

3. Electronmicroscopy

In Fig. 4 (a to d) a number of control experiments are shown. Native red cell membranes reveal the well known pattern of particles on the outer fracture face (OFF) and of the dense, scattered particles in the inner fracture face (IFF) (Fig. 4a). 30% glycerol is recognized as a smooth surface, building blocks during fracturing (Fig. 4b). Water shows the irregular surface with water crystals (Fig. 4e) and in Fig. 4c the protein free lipid layer has a highly smooth and even surface consistent with other observations [4, 18].

Particle size of the native red cell membrane in the fracture face (Fig. 4a) is comparable with the particles seen in Fig. 5 a to c from penetrated films. The diameter in both is 80 to 100 Å. The density of the particles in the recombined model membranes is dependent on the initial film pressure π_i. The more protein penetrating the film (high $\Delta\pi$) the more particles can be seen in a given area. However in comparison to the native membrane the particles in the model membrane are more aggregated. Very few particles can be seen at a initial film pressure of 30 dynes/cm and no particles are visible when penetration is tried near the collapse pressure of the film.

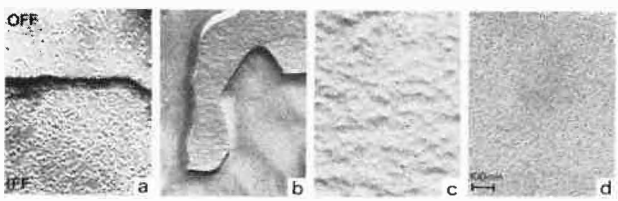

Fig. 4: Electron micrographs of four control experiments

a) freeze-etch view of isolated human red cell membrane
upper part: outer fracture face (OFF)
lower part: inner fracture face (IFF)
b) 30% glycerol after freeze-etching
c) water after freeze-etching
d) lipid of human red cell membranes spread as a monolayer and prepared as a bilayer according to methods after freeze-fracturing and -etching ($\pi_i = 30$ dynes/cm)

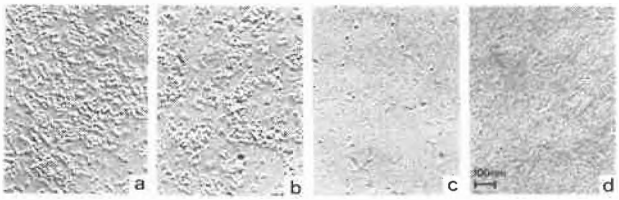

Fig. 5: Electron micrographs of the fracture face of synthetic model membranes prepared by penetration of sialoglycoprotein fractions into a lipid monolayer of varying initial surface pressure. Preparation of the bilayer and freeze-etching according to methods

a) $\pi_i = 13$ dynes/cm b) $\pi_i = 20$ dynes/cm
c) $\pi_i = 30$ dynes/cm d) $\pi_i = 48$ dynes/cm

Discussion

We have calculated the lipid content of the three sialoglycoprotein fractions on the basis of their phosphorus content. One has, however, to consider that part of the phosphorus may be non-lipid-phosphorus (e.g. phosphorylated protein). From unpublished data we estimate this amount to be roughly 0.08%, which would reduce our calculated value for the lipid content by about 2%. Due to the presence of spectrin which is known to contain ATP ase activity [19] the degree of phosphorylation may be even larger.

It is not surprising that the penetration rate is highest with the preparation showing the high lipid content (Fig. 3b). Even with no initital lipid layer being present ($\pi_i = 0$) the lipid-rich preparation shows a high surface activity. It is interesting to note that for the two other preparations the rate of penetration into the surface is increased by the presence of a lipid monolayer if compared to the surface activity of the protein alone ($\pi_i = 0$). This may be interpreted as a specific affinity of these preparations for lipid surfaces.

It is difficult from our results to assess the effect of the contaminating spectrins on the penetration characteristics. From other studies we know that isolated spectrins show high penetration rates into lipid films.

Although our sialoglycoprotein preparation with the low lipid content (3%) was practically devoid of spectrin we cannot exclude that the lowering of $\Delta\pi$ and $d\pi/dt$ is not also influenced by the above effect.

On the other hand we may interpret the clustering of the particles (as seen in Fig. 5) as an indication that spectrins interact only poorly with the penetrated sialoglycoproteins [21, 22]. In any case, the amount of contaminating spectrin is very low. and it could be shown that breakdown (as also described by Toekes and Chambres [20]) further decreases this amount.

The fact that penetration stops at about 35 dynes/cm further supports the hypothesis that native membranes show surface pressures of about 33 dynes/cm [23]. At lower initial film pressures we find a good correlation between $\Delta\pi$ (indicating the amount of penetration) and particle number. The great resemblence of the particles with those of the original membrane support the idea that the sialoglycoprotein fractions may represent (at least to a large extent) the particles seen in the fractured native red cell membrane.

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