Characterization of the permeability increase induced by the incorporation of glycophorin in phosphatidylcholine vesicles

Determination of the pore size

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Glycophorin incorporation into large unilamellar dioleoylphosphatidylcholine vesicles results in a greatly increased bilayer permeability for non-electrolytes of M_r-values up to 900. This suggests that glycophorin aggregates induce the formation of pores with a 15–18 Å diameter.

Glycophorin *Dioleoylphosphatidylcholine* *Vesicle* *Bilayer permeability* *Pore size*

1. INTRODUCTION

In general, incorporation of integral membrane proteins into model membranes such as lipid vesicles results in a non-specific increase in bilayer permeability [1–6]. Particularly in the case of transport proteins this non-specific leakage complicates interpretation of the specific permeation processes [2]. Glycophorin, the major sialoglycoprotein of the human red cell membrane, has been used to get an insight into the nature of this permeability increase. Incorporation of the protein, which has no known transport function, into dioleoylphosphatidylcholine vesicles, greatly increases the K^+ and glucose permeability of the bilayer [1]. This effect is lipid-dependent, as glycophorin-containing vesicles made of the total lipid extract of the red cell are much less permeable to these solutes [1]. Since incorporation of the tryptic hydrophobic fragment of glycophorin into lipid vesicles results in similar permeability changes, the membrane-spanning portion of the protein appears to be responsible for this effect [4]. Packing defects at the lipid–protein interface or channels present in protein aggregates, have been proposed as the most likely permeation pathways.

To acquire knowledge of the dimensions of this permeation pathway, we report in this study the effect of incorporation of glycophorin in dioleoylphosphatidylcholine vesicles on the permeability properties of the vesicles towards solutes of different sizes.

2. EXPERIMENTAL

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) was synthesized as in [7]. Glycophorin was purified from human erythrocytes as in [8]. Chemicals were purchased from the following commercial sources: [*carboxyl*-^{14}C]Dextran (M_r 70000, 0.99 mCi/g); [^{3}H(G)]inulin (175.5 mCi/g); [1,2-^{3}H]polyethyleneglycol 4000 (PEG 4000, 1.6 mCi/g); [1,2-^{3}H]polyethyleneglycol 900 (PEG 900, 4.5 mCi/g); L-[1-^{3}H(*N*)]glucose (10.7 Ci/mmol) and ^{3}H(G) raffinose (7.8 Ci/mmol) from New England Nuclear; [6,6′(*n*)-^{3}H]sucrose (9.8 Ci/mmol) from Amersham; Sephadex G-75 and G-150 from Pharmacia.

2.1. *Preparation of vesicles*

Glycophorin was incorporated into large unila-

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mellar lipid vesicles as in [9]. A mixture of glycophorin (0.65 mg) and DOPC (5 μmol) was dissolved in chloroform/methanol/water (150:75:1, by vol.) and dried by evaporation. After hydration of the mixed glycophorin–lipid in 0.5 ml buffer at 4°C, the unilamellar glycophorin-containing vesicles were separated from multilamellar, glycophorin-poor, vesicles by means of differential centrifugation [10]. These glycophorin–DOPC vesicles have a 400:1 lipid/glycophorin molar ratio and an average diameter of 2000–3500 Å [1].

2.2. *Efflux assay*

Efflux of ^{3}H-labeled solutes of different molecular mass was determined using the method in [1]. The vesicles were prepared, using the general procedure, in 100 mM NaCl, 0.2 mM EDTA, 0.2 mM NaN_3, 1 mM solute ($M_r < 900$) or 0.1% (g/v) solute ($M_r \geqslant 900$), 0.10% (g/v) dextran, 10 mM Tris–HCl (pH 7.4) buffer, containing 2 μCi ^{3}H-labeled solute/ml and 0.25 μCi [^{14}C]dextran/ml.

After the usual differential centrifugation steps carried out at 0°C, the resulting glycophorin-DOPC vesicles were washed 4-times by resuspending the vesicle pellet (~1 μmol lipid), obtained after centrifugation (0°C, 30 min, 35000 × *g*) in 1 ml ice-cold buffer without radioactive solutes. This washing procedure took 4 h and is essential to reduce the amount of non-trapped solute to acceptable levels. Finally the vesicle pellet was resuspended in 1 ml buffer from which samples for radioactivity [2] and phosphorus assay [11] were drawn.

In this way an estimate of the amount of solute still present in the vesicles after 4 h at 0°C, is obtained. This value is related to the trap of the large non-permeable solute [^{14}C]dextran [1]. The dextran trap of the vesicles was found to be 6.5–7.5 μl/μmol phospholipid.

A possible adsorption of solutes (especially PEG) to the vesicles was excluded by showing that upon mixing of vesicles no significant binding of solutes to the vesicles occurred.

2.3. *Influx assay*

The influx of radiolabeled solutes under equilibrium conditions was measured using a modified method in [11], described in detail in [2]. In this method, upon mixing of radiolabeled solutes with vesicles prepared in the usual buffer, the influx at 30°C is monitored, separating the vesicles from the extravesicular medium at appropriate time intervals by means of gel filtration at 0°C.

In general the assay medium contained 2.5–5 μmol phospholipid/ml and about 60 × 10^6 dpm ^{3}H-labeled solute/ml.

In the case of the raffinose influx assay, vesicles were made in 10 mM Na_2SO_4, 1 mM raffinose, 0.2 mM EDTA, 0.2 mM NaN_3, 10 mM Na/HEPES, pH 7.4. Gel filtration was performed with Sephadex G-75 columns (1.5 ml bed vol.). The vesicle suspension samples (50 μl) were eluted with 800 μl of ice-cold buffer. No significant elution of extravesicular [^{3}H(G)]raffinose occurred while the vesicle recovery was about 80%.

The influx assay of the larger molecule [1,2-^{3}H]PEG was performed in 10 mM Na_2SO_4, 0.1% PEG 4000, 0.2 mM EDTA, 0.2 mM NaN_3 and 10 mM Na/HEPES, pH 7.4. Since gel filtration over Sephadex G-75 resulted in a poor separation of vesicles and extravesicular medium, Sephadex G-150 columns (6 ml bed vol.) were used instead. The vesicle suspension sample (50 μl) was eluted with 1500 μl ice-cold buffer. The first 700 μl was rejected. In the following 800 μl, about 90% of the vesicles were eluted.

Glycophorin–DOPC vesicles prepared and assayed in 100 mM NaCl buffer, as used in the efflux assay, showed identical permeability properties as compared to vesicles prepared in 10 mM Na_2SO_4 buffer.

In both influx assays, radioactivity [2] and phosphorous content [11] were measured. Since the vesicle recovery of each column was variable, the phosphorous content in the eluent was used for normalizing the amount of radioactivity (dpm_t) to a constant vesicle recovery.

In order to get the proper kinetic parameters of the influx rates, dpm_t should be related to the trap of the vesicles as determined by [^{14}C]dextran (see section 2.2) or by prolonged incubation (18 h at 37°C) after the assay of the remainder of the vesicles with ^{3}H-labeled solute (trap at isotopic equilibrium = dpm_∞), followed by elution of the vesicles over ice-cold Sephadex.

In the case of the raffinose influx assay, the trap at isotopic equilibrium appeared to be about 28%

of the dextran trap, due to the loss of trapped raffinose during elution of the vesicles over the Sephadex columns. Therefore the observed dpm_t-values are corrected for this loss.

3. RESULTS AND DISCUSSION

In fig.1 the relative solute traps of the vesicles obtained after 4 h efflux at 0°C are shown. The relative trap of glucose appeared to be about 1%, equivalent to a loss of 99% trapped glucose, which is in agreement with results obtained in [1]. This means that the DOPC–glycophorin vesicles are extremely leaky towards a small solute like glucose. When the molecular radius of the solute is increased to 7.5 Å, the corresponding loss of trapped solute is only slightly decreased. However, when the radius of the solute exceeds 7.5 Å a dramatic decrease in permeability for these solutes (PEG 4000, inulin) occurs, since the loss of these trapped solutes is now only about 20%. From these results, the exclusion limit of the permeation pathway present in the DOPC–glycophorin vesicles is between M_r 900 and 4000, corresponding to a pore diameter of 15–18 Å.

The results obtained with the efflux assay which only gives relative traps after 4 h at 0°C, are extended by kinetic influx measurements. In fig.2 the influx of raffinose and PEG 4000 is shown. In

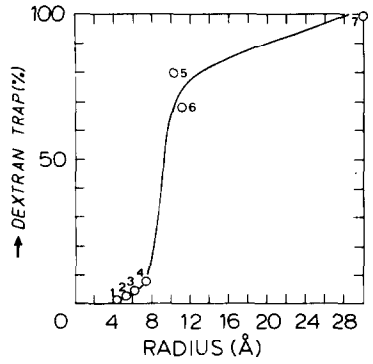

Fig.1. Relative solute/dextran traps as a function of solute radius. Solute (1) glucose; (2) sucrose; (3) raffinose; (4) polyethyleneglycol 900; (5) polyethyleneglycol 4000; (6) inulin; (7) dextran. The radii of solutes 1, 2, 3 and 6 are derived from [16,17], while the radii of solutes 4, 5 and 7 are calculated from the radius of raffinose using the cube root ratio of their M_r-values.

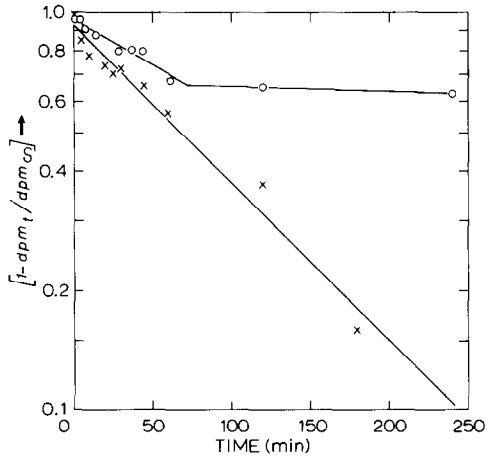

Fig.2. Influx assays of raffinose (×—×) and polyethyleneglycol 4000 (○—○) at 30°C. Vesicles were made and assay was performed as outlined in section 2.

agreement with the efflux measurements, raffinose enters all the vesicles at a relatively fast rate ($t_{½}$ = 76 ± 4 min), while PEG 4000 shows a slower influx rate ($t_{½}$ = 131 ± 16 min) and equilibrates only with about 20% of the vesicular volume (even when the vesicles are incubated for 38 h at 30°C with [^{3}H]PEG 4000). This latter value corresponds to the relative trap of about 80% in the efflux experiments.

When the influx assay was performed at 30°C, similar to the raffinose assay, but with $^{45}Ca^{2+}$ as the labeled solute (assay medium: 100 mM NaCl, 1 mM $CaCl_2$, 0.2 mM NaN_3, 10 mM Na/HEPES, pH 7.4), the resulting influx assay showed a very fast Ca^{2+} influx during 3 min, corresponding to about 20% of the vesicle trap, followed by a slow influx process with $t_{½}$ = 198 ± 31 min, corresponding to the remainder of the vesicle trap.

This result indicates that the glycophorin–DOPC vesicles, besides being leaky for the monovalent cation K^+ [1], are also leaky towards the divalent cation, Ca^{2+}.

Since pure DOPC vesicles do not show a significant permeability for both cations as well as the non-electrolytes described above, the incorporation of the protein in the DOPC bilayer has to be responsible for the increased permeability for these solutes. It has been suggested that this increased permeability is caused either by the formation of channels in protein aggregates, or by packing defects at the protein–lipid interface [1].

From the diameter of the permeation pathway

(pore) present in the glycophorin–DOPC bilayer, it can be calculated that the surface area ($\pm$ 210 Å^2) corresponds to the surface area of about 3 DOPC molecules (67 Å^2, [19]).

It might be expected that the magnitude of packing defects at the protein–lipid interface will resemble the magnitude of packing defects occurring between lipids at the liquid crystalline/gel state phase boundary. Since these packing defects result in a permeability increase for much smaller solutes [15] we favour the idea that the permeation of solutes through the glycophorin–DOPC bilayer occurs through channels, formed by glycophorin aggregates. Recently it was suggested that the sugar-containing externally located part of glycophorin may interact with the phospholipid bilayer [22]. Possibly this interaction may play a role in the formation of the permeation pathway, present in the glycophorin–DOPC vesicles. However, when glycophorin–DOPC vesicles are prepared in the presence of trypsin (10%, g/g relative to glycophorin) which cleaves under these conditions, the hydrophilic, sugar-containing part [23], the resulting DOPC vesicles, which contain the residual hydrophobic part of glycophorin, show similar permeability properties (cf. fig.1). Therefore, it is likely that aggregation of the hydrophobic part of glycophorin is responsible for the observed packing defect, in the glycophorin–DOPC vesicles. A possible participation of packing defects at the protein–lipid interface in the leak of the smaller solutes cannot be excluded. The presence of such defects is also suggested by the increased susceptibility of the phospholipids to phospholipases A_2 [21] and by the protein-induced transbilayer movement of lipids [1,20].

It is of interest to compare the present results with the permeability properties of pores occurring in biological membranes. For instance, pores existing in the outer membrane of *Salmonella typhimurium*, have diameters of about 12–15 Å [13], while a pore protein in the outer mitochondrial membrane of *Neurospora crassa* has a pore diameter of 20 Å [14]. When the B45 fragment of diphtheria toxin was cosonicated with liposomes consisting of 92:8 molar ratio egg phosphatidylcholine/bovine phosphatidic acid, the resulting vesicles were leaky and had pores with diameters of $\leqslant$18 Å [16].

Although the size of the pore induced by glycophorin in DOPC vesicles corresponds to the size of these naturally occurring pores, it should be realized that in the native erythrocyte membrane such pores do not exist. The formation of these pores must be the result of a different, possibly more aggregated, incorporation of the protein in the vesicles, probably in combination with the lack of an optimal lipid environment. These ideas are currently under active investigation.

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