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INTERACTION OF INFLUENZA VIRUS PROTEINS WITH PLANAR BILAYER LIPID MEMBRANES

I. CHARACTERIZATION OF THEIR ADSORPTION AND INCORPORATION INTO LIPID BILAYERS

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Alterations in the surface potential difference (ΔU) of asolectin planar bilayer lipid membranes were measured following the adsorption of isolated matrix protein (M-protein) or neuraminidase of influenza virus. The method used was based upon measurement of the bilayer lipid membrane capacitance current second harmonic. The ΔU dependence on the M-protein and neuraminidase concentration indicates different mechanisms of adsorption of these viral proteins by the lipid bilayer. The conductance (G_0) dependence of the bilayer lipid membrane with different compositions on the concentration of isolated surface glycoproteins, hemagglutinin and neuraminidase, M-protein or neuraminidase was investigated. The change in G_0 for M-protein was observed only after adsorption saturation had been achieved. Neuraminidase alone does not affect the membrane conductivity. The surface charge and lipid composition of the lipid bilayer influences the adsorption and incorporation of influenza virus M-protein and surface glycoproteins. The reversibility of protein incorporation into the bilayers was investigated by a perfusion technique. The results show reversibility of surface glycoprotein incorporation while M-protein binding appears to be irreversible.

Introduction

In order to understand the mechanism of self assembly of influenza virions in infected cells it is necessary to understand the interaction of individual viral proteins with the lipid bilayer. The shell of the influenza virus particle consists of a spherical lipid bilayer (membrane) to which three major proteins are bound: the surface glycoproteins, hemagglutinin and neuraminidase forming spikes on the outer surface of the particle, and matrix protein (M-protein) covering the inner side of the viral membrane [1]. The hemagglutinin apparently interacts and spans the lipid bilayer of the viral particle through a hydrophobic region near the C-terminus of the small hemagglutinin polypeptide (HA_2) with several amino acids at the C-terminus situated on the inner surface of the membrane [2]. Neuraminidase appears to be bound to the membrane through its hydrophobic N-terminal region [3]. Experimental data suggest that hemagglutinin and neuraminidase interact with and penetrate the lipid bilayer in different ways [4].

Influenza virus glycoproteins are synthesized in

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an infected cell, incorporated into the endoplasmic reticulum membranes and then migrate towards the plasma membrane where they appear to form clusters [5]. Matrix protein molecules are adsorbed by the membrane at these sites where the clusters have been formed. Following binding of the viral ribonucleoprotein complexes to these sites of the membrane, modified by M-protein clusters, maturation of the viral particles is completed by budding of the virions from the cell surface [5].

In our previous paper we reported results on the interaction of isolated influenza viral proteins with planar bilayer lipid membranes (BLM) made of oxidized cholesterol [6]. The 'BLM-viral protein' system was found to be a suitable model for simulation of some features of virus self-assembly on the membrane of the host cell. We studied effects of viral proteins on the ionic conductivity of bilayer lipid membranes and showed the cooperative nature of the membrane modification by M-protein and glycoproteins. It was also shown that the binding of M-protein on one side of the membrane facilitates the binding of the glycoproteins on the opposite side of the membrane.

In this paper we focus on study of the mechanism(s) of interaction of surface glycoproteins and M-protein with bilayer lipid membranes. Purified influenza viral proteins including M-protein, total glycoproteins and purified neuraminidase were interacted with bilayer lipid membranes. An increase in the membrane permeability was observed on adsorption of the proteins on the membrane and subsequent incorporation into the bilayer. The influence of lipid membrane composition on these processes was also studied.

Materials and Methods

Virus. The MRC-11 (H3N2) strain of influenza virus (a recombinant of A_2/Port Chalmers/1/73 and A/PR/8/34) was grown in 9-day-old embryonated eggs and purified as previously described [7]. Purity was monitored by SDS-polyacrylamide gel electrophoresis [8] and electron microscopy.

Surface glycoproteins. Surface glycoproteins of influenza virus were purified as follows: a virus suspension (2.5 mg/ml) in distilled water was incubated 10 min at 37°C with 4% octylglucoside (Sigma). This treatment solubilizes both hemagglutinin and neuraminidase [4]. Viral cores were separated from surface glycoproteins by centrifugation in the SW-65 rotor (centrifuge L5-65, 20 min, 40 000 rpm, $t = 4$°C). Detergent was removed by exhaustive dialysis against distilled water. Neuraminidase was separated as described [9].

Matrix protein. The matrix protein of influenza virus was isolated by preparative electrophoresis of total viral proteins (after disruption of virus in 1% SDS) in cylindrical polyacrylamide gels (1.5×10 cm) in 0.1% SDS without reducing agents [10]. Detergent was removed by continuous ultrafiltration as described earlier [11].

Purity of viral protein preparations was checked by SDS-polyacrylamide gel electrophoresis. Protein concentration was determined by the Lowry assay using bovine serum albumin (Koch Light) as a standard [12].

Planar lipid bilayers. Planar lipid bilayers were formed on a 0.8 mm diameter aperture in a teflon partition separating two stirring thermostated compartments [13] containing 0.2 M KCl + 5 mM Tris-HCl buffer (pH 7.4). Membrane formation was observed under reflected light with a low-power microscope. Electrical measurements were made with Ag/AgCl electrodes connected with both compartments via 3 M KCl-agar bridges in series with Pasteur pipettes containing agar in 0.1 M NaCl. A high impedance operational amplifier circuit was used to clamp the membrane voltage (15 mV) and monitor the transmembrane current. Current versus time plots were made on an *X-Y* recorder. The experiments were carried out at 25°C.

The membranes were formed from asolectin (Associated Concentrates, Woodside, NY), a lipid fraction of soybeans, containing approximately equal amounts of phosphatidylethanolamine, phosphatidylcholine and phosphatidylinositol [14], or ox brain phosphatidylethanolamine (Koch-Light) with cholesterol (Sigma), or α-monoolein (glyceryl *cis*-9-octadecenoate) (Sigma). Heptane was used as a solvent with a lipid concentration of 20–30 mg/ml). The membrane made from the mixture of phosphatidylethanolamine and cholesterol contained the lipids at the weight ratio of 2 : 1.

Protein adsorption on bilayer lipid membranes

was investigated using a method based on the phenomenon of electrostriction. Adsorption of charged particles on one side of a membrane brings about a surface potentials difference, jumping ΔU. If alternative current is applied to the membrane, the nonlinearity of the system (caused by electrostriction in this case) results in the appearance of multiple frequency current components and, in particular, the component with doubled frequency, the second harmonic [15]. The magnitude of the second harmonic is a linear function of ΔU and the membrane elasticity module which can be calculated from the magnitude of the third harmonic of the capacitance current [16].

The scheme of the instrument used for measurements of higher harmonics is shown in Fig. 1. A signal with a frequency of 160 Hz from a generator (1) with low harmonic distortions is applied to the membrane through an electrode (6). The other electrode (6) is connected with an alternative current amplifier (7). The current's amplitude after the amplifer is proportional to the amplitude of the first harmonic which characterizes the bilayer lipid membrane's capacitance. The second and third harmonics are selected from the signal with the use of resonance filters (9). Rectified signals are registered by a multichannel recorder (11). The ΔU value is measured using the compensation technique. The direct current, from an independent power supply, applied to the membrane is amplified by an electrometric amplifier (8) and registered by the same recorder (11).

To check the reversibility of the protein binding we used a perfusion technique. The solution bathing the membrane and containing the protein was gradually replaced by the solution of the same composition but without the protein. Two peristaltic pumps were used for the perfusion.

The experimental methods used in this work do not permit calculation of the quantities of protein molecules adsorbed on or incorporated into the bilayer lipid membrane directly, without any additional measurements. Therefore, we express the protein adsorption and incorporation in terms of surface potential difference and membrane conductance which are proportional to the degree of adsorption or incorporation.

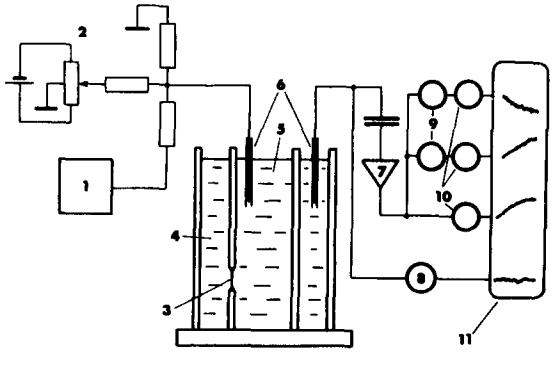

Fig. 1. Diagram of instrument for measurement of first, second and third harmonics capacitance current and constant current through membrane. (1) generator, (2) power supply, (3) aperture, (4) cell with solution bathing membrane (outer compartment), (5) inner compartment, (6) silver chloride (AgCl) electrodes with agar bridge, (7) amplifier of alternating current (8) electrometric amplifier of alternating current, (8) electrometric amplifier, (9) filters for selection of second and third harmonics, (10) detector.

Results

In this paper we report results of the study of particular characteristics of interactions of influenza virus 'shell' proteins with bilayer lipid

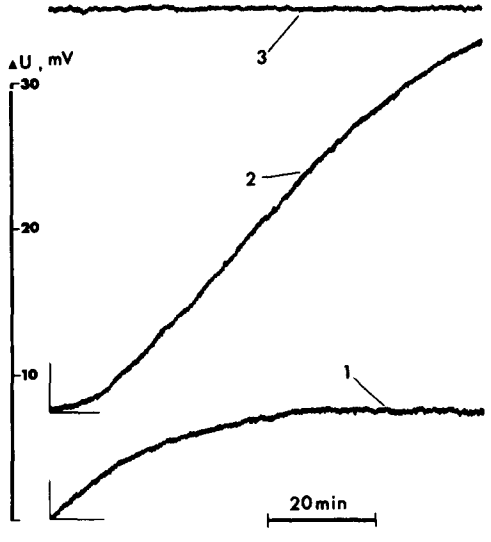

Fig. 2. The kinetics of surface potential difference change. All curves for an asolectin bilayer lipid membrane after addition to one of its sides of the following: (1) neuraminidase (3 μg/ml) and (2) M-protein (0.6 μg/ml). The bilayer lipid membrane is formed in a solution containing KCl (0.2 M), Tris buffer (5 mM, pH 7.4 at 20°C). (3) The current through the membrane.

membranes of different lipid compositions. Fig. 2 shows the surface potentials change after additions of neuraminidase (curve 1) or M-protein (curve-2) to one of the sides of a membrane made of asolectin. The curves differ significantly from each other and reflect the kinetics of adsorption of the proteins, inducing asymmetry, on the surface charges of the membrane. It is important to note that the addition of the proteins at low concentrations results in binding of the proteins by the membrane but the conductivity of the membrane (i.e., the current through it) does not increase (curve 3).

The dependence of M-protein (curve 2) and neuraminidase (curve 1) adsorption on their concentrations are shown in Fig. 3. Curve 2 is obviously different from Langmuir's isotherm which is characteristic of systems with a finite amount of independent binding sites where the bound particles can interact with the binding sites only but not with each other. Curve 2 in Fig. 3 is peculiar to heterogeneous surfaces or to systems with lateral interactions between binding centers of adsorbed particles.

Fig. 4 shows the dependence of conductivity of a bilayer lipid membrane made from (a) asolectin or (b) monoolein on the concentration of (1) M-protein and (2) the total fraction of surface glycoproteins. Neuraminidase alone does not affect the membrane conductivity even at high concentrations (up to 10 μg/ml). It may be suggested therefore that the augmentation of bilayer lipid membrane conductivity is caused by hemagglutinin incorporation or a concerted action of both glycoproteins upon the bilayer lipid membrane. Furthermore, analysis of the curves demonstrates that the cooperativity of the incorporation process in the case of total glycoproteins is higher than that for M-protein ($\Delta \lg G_0/\Delta \lg[C] = 13.6$ and 6.3, re-

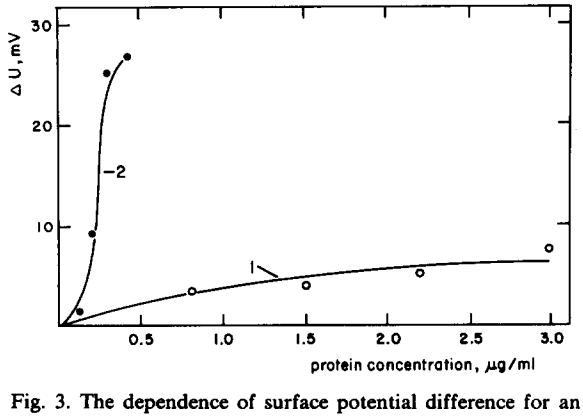

Fig. 3. The dependence of surface potential difference for an asolectin bilayer lipid membrane on the (1) neuraminidase and (2) M-protein concentrations which are added to one side of the membrane. For conditions, see the legend to Fig. 2.

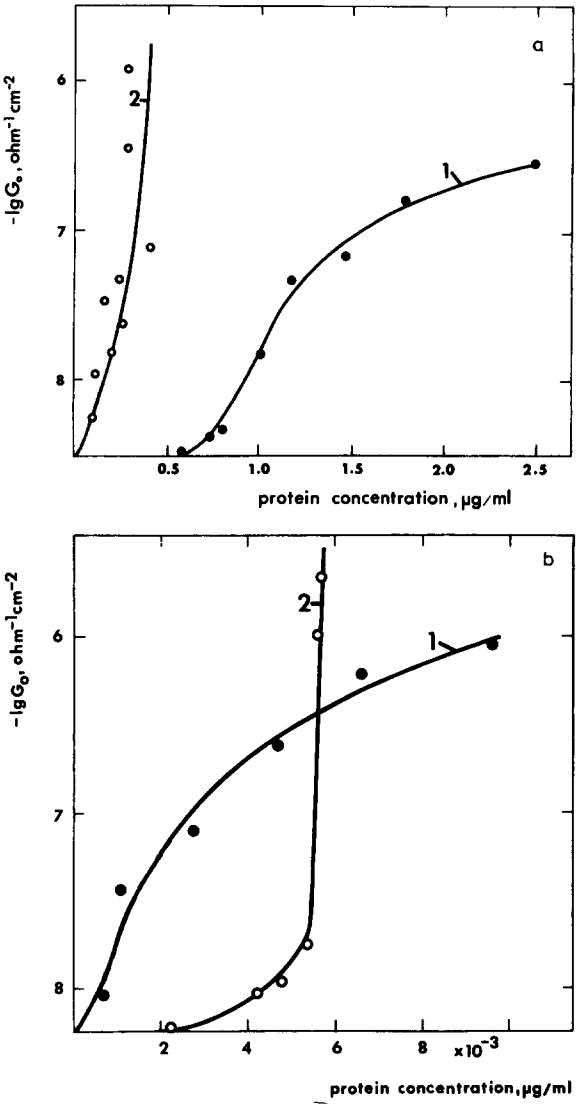

Fig. 4. (a) The dependence of the asolectin bilayer lipid membrane conductance on the concentration of (1) M-protein and (2) total surface glycoproteins, For conditions, see the legend to Fig. 2. (b) Dependence of the monoolein bilayer lipid membrane conductance on (1) concentration of M-protein and (2) total fraction of surface glycoproteins. For conditions, see the legend to Fig. 2.

spectively). Comparing Figs. 4a and 4b, one can conclude that the shape of the curves for M-protein and total glycoproteins does not depend on the membrane composition and is completely determined by the nature of the protein. However, the concentration ranges at which the proteins induce membrane conductivity depend on its lipid composition.

The conductivity of asolectin membranes in the presence of M-protein increases after saturation of the isothermic adsorption of M-protein has been achieved, i.e. at M-protein concentrations = 0.6 μg/ml (curve 1 in Fig. 4a and curve 2 in fig. 3).

In Table I, the minimal concentrations are listed at which M-protein and total glycoproteins alter the membrane conductivity. The data suggest that the proteins penetrate into homogeneous bilayer lipid membranes easier than into non-homogeneous membranes and that electrostatic interactions are important in binding proteins by membranes because a decrease in the ionic strength of the solution resulted in an increase of the modifying protein concentration.

The reversibility of the protein binding by bilayer lipid membranes was investigated by means of a perfusion technique. The solution bathing the membrane was gradually substituted with a solution which did not contain protein. When applied to asolectin bilayer lipid membranes modified by glycoproteins, the technique resulted in a gradual restoration of low membrane conductivity (Fig. 5a). The time-course of the conductivity change may correspond to the kinetics of desorption of surface glycoproteins. In the case of M-protein modified membranes, perfusion resulted in an increase in conductivity only if it was begun immediately after the initial 'jump' in conductivity caused by membrane modification. If even a short period of time (4–5 min) passed after the 'jump', perfusion did not restore the initial conductivity of the membrane. These results imply reversibility of

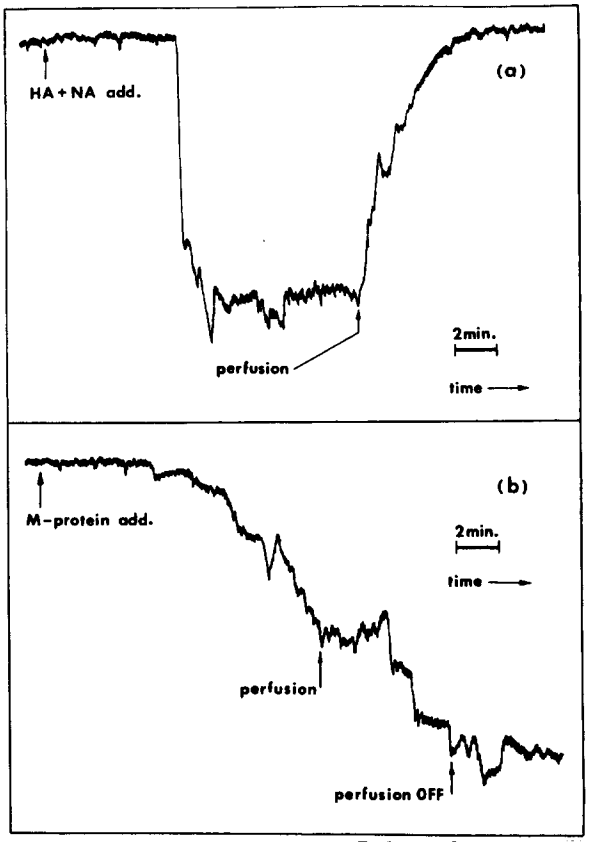

Fig. 5. Effect of perfusion on asolectin bilayer lipid membrane conductance for (a) total glycoproteins and (b) M-protein. For conditions, see the legend to Fig. 2. HA, hemagglutinin; NA, neuraminidase.

TABLE I

CONCENTRATIONS (μg/ml) OF SURFACE GLYCOPROTEINS AND M-PROTEIN NECESSARY FOR MODIFYING CONDUCTIVITY OF BILAYER LIPID MEMBRANES WITH DIFFERENT COMPOSITIONS (AT $G_0 = 10^{-8}$ ohm^{-1}·cm^{-2})

Protein	Membrane composition				
	Oxidized cholesterol	Mono-olein	Asolectin		Cephalin + cholesterol
			200 mM KCl	20 mM KCl	
HA+NA	$4.8 \cdot 10^{-3}$	$4.3 \cdot 10^{-3}$	0.17	0.4	0.7
M-protein	$0.8 \cdot 10^{-3}$	$1.7 \cdot 10^{-3}$	0.9	20	1.2

surface glycoprotein binding while M-protein binding appears to be irreversible.

Discussion

The interaction of viral proteins as well as many other proteins with natural and artifical membranes is a multistage process with the separate stages-approach, adsorption and incorporation-under the control of different physicochemical processes. Our results suggest that adsorption of proteins on the membrane surface precedes their incorporation into the bilayer. The adsorption is controlled primarily by electrostatic interactions. If the bilayer lipid membrane and the protein molecules have electrical charges of the same sign (both positive or both negative), an increase of bilayer lipid membrane surface charge density results in a reduction of the number of molecules adsorbed. This observation could explain the increase in modifying protein concentration for asolectin membranes (surface charge density = $-10\ \mu C/cm^2$ [17]), in comparison with oxidized cholesterol membranes (surface charge density = $-1\ \mu C/cm^2$, [18]).

It is known that a decrease of the ionic strength of a solution causes an increase in the electrostatic component contribution to interaction in dispersed systems (in particular, it causes an increase of the energy barrier for adsorption). As can be seen in Table I, lowering the KCl concentration from 200 mM to 20 mM causes a significant increase in the modifying protein concentration, especially in the case of M-protein.

M-protein adsorption results in a bigger rise in the surface potentials difference than neuraminidase adsorption. These results suggest that electrostatic interactions play an important role in membrane binding of M-protein as compared with neuraminidase although presumably M-protein is more hydrophobic.

Electrostatic interaction is not the only force controlling protein incorporation into membranes. Practically neutral membranes made from a mixture of phosphatidylethanolamine and cholesterol can be modified by the same concentrations of proteins as electrically charged asolectin membranes. Both the specific interactions of proteins with polar lipid groups and the fatty acid chain packing in hydrophobic regions of bilayer lipid membrane may play a role in this phenomenon. It seems that the tighter the packing, the higher the energy of the bilayer distortion which accompanies protein penetration into the bilayer lipid membrane. This is in agreement with the observation that lipids in asolectin bilayer lipid membranes are packed more tightly than in monoolein bilayer lipid membranes.

An increase in ionic conductance of modified membranes may be caused by formation of hydrophilic pores by protein molecules having penetrated deeply enough into the hydrophobic region of the lipid bilayer. However, not all polypeptides and proteins which can adsorb to membranes are able to produce an increase in their ionic conductance. We have recently shown that the yeast glycoprotein, invertase, does not change the conductance on adsorption to an asolectin bilayer lipid membrane, up to concentrations as high as 400 $\mu g/ml$. A similar observation was made with the interaction of neuraminidase with the bilayer lipid membrane. On the other hand, the total fraction of surface glycoproteins increases the ionic conductance of the bilayer lipid membrane, a confirmation of the hypothesis of the membrane-spanning character of hemagglutinin [2].

Our data also show cooperative interaction between the surface glycoproteins and M-protein with lipid membranes during both the adsorption and incorporation stages. The sigmoidal shape of the adsorption isotherm for M-protein suggests the positive cooperativity of this process, which may be a consequence of interactions between protein molecules resulting in formation of clusters of molecules on the membrane. It may be concluded that assembly of influenza virus particles on the infected cell surface should also display positive cooperativity.

The observation of the effect of facilitation of surface glycoprotein incorporation into the bilayer lipid membrane when M-protein is added to the opposite side of the bilayer lipid membrane [6] may be a result of transmembraneous interactions between these proteins which may occur during virus assembly at the membrane of the infected cell.

It is natural to assume that the membrane having adsorbed surface glycoproteins and M-protein

on its opposite side would by asymmetric with respect to its physiochemical properties [19]. An increase of local interphase tension as a consequence of the asymmetric protein binding may lead to an increase in local membrane curvature and facilitate formation of a viral particle. Clustering of viral proteins on the cell membrane and the subsequent alteration of the lipid molecule packing could expel cell membrane proteins from the region of virus assembly. The reversibility of the binding of surface glycoproteins suggests that the binding of the glycoproteins and M-protein on opposite sides of the membrane may be responsible for stability of the viral envelope. Another proposal published recently [20,21] on the ionic regulation of endocytosis of blood lipoproteins may be applicable to the question of virus maturation at the cell surface and subsequent release of the virus particle. The local increase of membrane ionic permeability in the regions of cluster formation may result in local ion concentration changes, in particular an increase in calcium ion concentration which can trigger the contractile system of the cytoskeleton and facilitate formation of a separate virus particle.

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