The insertion of human apolipoprotein H into phospholipid membranes: a monolayer study

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Apolipoprotein H (ApoH) is a plasma glycoprotein isolated from human serum. The interactions of ApoH with lipid membrane were reported to be essential for its physiological and pathogenic roles. In this paper we studied the ability of ApoH to insert into phospholipid membranes using the monolayer approach. The results show that ApoH is surface active and can insert into the lipid monolayers. The insertion ability of ApoH is stronger when a higher content of negatively charged lipids is present in the membrane. The acidic-pH and low-ionic-strength conditions will also enhance ApoH insertion, but these factors

may not have much influence on the final insertion ability of ApoH, suggesting that, in the mechanism of ApoH insertion, not only electrostatic forces, but also hydrophobic interactions, are evidently involved. Modification by heat inactivation and reduction/alkylation does not change the critical insertion pressure (π_c) of ApoH, suggesting a stable domain, maybe a linear sequence motif, but not the native three-dimensional structure of ApoH, is responsible for its insertion. The extent to which insertion of ApoH into phospholipid membranes may facilitate the 'immune cleaning' of plasma liposomes is discussed.

INTRODUCTION

Apolipoprotein H (ApoH), also referred to as β_2 -glycoprotein I, is a plasma glycoprotein partially associated with lipoproteins. It was described for the first time in 1961 by Schultze et al. [1]. Human ApoH is a single-chain molecule consisting of 326 amino acid residues and five carbohydrate chains, and has a molecular mass of approx. 54 kDa [2–5]. The amino acid sequences of ApoH in human, bovine, mouse and rat appear to be highly conserved [6–9]. The protein contains five internal repeat units of 60 amino acid residues, each with two internal disulphide bonds known as the Sushi domain [10]. Since the solution structures of the Sushi domains in three other proteins have been solved by NMR and been found to be very similar [11–13], it is feasible to create the structural model of ApoH by analogy.

Although quite a lot is known about the structure of ApoH, its biological function remains unclear. It is known that ApoH can bind to negatively charged substances such as DNA, heparin and anionic phospholipids etc. *in vivo* [14–16], but the meaning of such interactions is still unclear. Puurunen et al. compared ApoH with complement Factor H, which also consists of Sushi domains, and found that these two proteins exhibited distinct functions [17]. It is known now that ApoH may serve as a major factor in clearing the plasma liposomes [18] as well as an anticoagulant in blood [19]. ApoH may also modulate the function of kidney and placenta. In these organs the membrane-binding ApoH was reported to control the interaction of some other substrates with lipids directly [20,21]. The abnormality of plasma ApoH level was shown to be associated with many diseases such as thrombocytopenia, arterial and venous thrombosis and recurrent abortion [22,23]. In addition, it has been found that, in hyperlipidaemic, diabetic or atherosclerotic patients, the concentration of plasma ApoH increased and the distribution of the protein among different types of lipoprotein

was perturbed [24,25]. Recently, research on ApoH was given further impetus by the discovery that the lipid-associated ApoH can bind to some pathogenic antigens or proteins such as hepatitis B surface antigen [26,27], proteins p18, p26 and gp160 of HIV [28] and anti-phospholipid antibodies [29,30]. These findings highlighted a potentially critical role of ApoH in the mechanism of infection in hepatitis B, AIDS, systemic lupus erythematosus etc.

The interactions of ApoH with phospholipids have been considered as a basic mechanism related to its physiological and pathogenic functions. That ApoH prefers to bind to negatively charged phospholipids has been demonstrated in several laboratories [16,31–33], but to our knowledge no previous studies on the insertion of ApoH into membranes during lipid–protein interaction have been reported. In comparison with liposomes, a lipid monolayer at an air/water interface is easier to use to mimic biological membranes. The composition, temperature and ionic conditions of the monolayer can be easily controlled. Many studies have shown that the monolayer technique is a powerful one in membrane-insertion analysis [34–36]. In the present paper the ability of ApoH to insert into membranes was studied using this technique, and the factors influencing the insertion behaviour were carefully analysed. This work was conducted in order to obtain further information about the molecular mechanisms underlying ApoH–lipid interactions. The results obtained may also help to explain the role of ApoH as a 'cleaner' in purging the plasmic liposomes derived from senescent cells and foreign particles [37].

MATERIALS AND METHODS

Materials

1,2-Dioleoyl-*sn*-glycero-3-L-phosphocholine (DOPC), 1,2-dipalmitoyl-*sn*-glycero-3-L-phosphocholine (DPPC), 1,2-dioleoyl-

Abbreviations used: ApoH, apolipoprotein H; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-L-phosphocholine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-L-phosphocholine; DPPS, 1,2-dipalmitoyl-*sn*-glycero-3-L-phosphoserine; DOPS, 1,2-dioleoyl-*sn*-glycero-3-L-phosphoserine; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-L-phosphoglycerol; CL, bovine heart cardiolipin (diphosphatidylglycerol); SPR, surface plasmon resonance; $\Delta\pi$, surface pressure increase; π_i , initial surface pressure; π_c , critical insertion pressure.

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sn-glycero-3-L-phosphoserine (DOPS), 1,2-dipalmitoyl-*sn*-glycero-3-L-phosphoserine (DPPS), 1,2-dipalmitoyl-*sn*-glycero-3-L-phosphoglycerol (DPPG) and diphosphatidylglycerol (cardiolipin, CL, from bovine heart) were all purchased from Sigma Chemical Co. Heparin–Sepharose CL-6B was obtained from Pharmacia. Mouse monoclonal antibody to human ApoH was made in our laboratory. All the other chemicals used were of analytical grade and were manufactured in China.

Purification of ApoH

ApoH was purified from human serum by the method of Polz et al. [38]. Briefly, 2.5 ml of perchloric acid (70 %, v/v) was added to 100 ml of fresh human serum at 4 °C. After stirring for 15 min, the sample was centrifuged for 20 min at 20000 *g*. Then the supernatant was neutralized with satd. Na₂CO₃ and dialysed against Tris/HCl buffer [0.02 M Tris/HCl (pH 8.0)/0.03 M NaCl] for at least 36 h. The dialysis residue was applied to the Heparin–Sepharose CL-6B column which had been equilibrated with the same buffer. The adsorbed proteins were eluted stepwise with 0.02 M Tris/HCl buffer, pH 8.0, with increasing molarity of NaCl. The fraction that was eluted in a linear NaCl gradient from 0.15 to 0.60 M was dialysed and further purified by reverse-phase HPLC. The protein sample, checked by SDS/PAGE, showed one protein band at an apparent molecular mass of 54 kDa after silver staining. Further assay by ELISA using the monoclonal antibody of ApoH confirmed that the purified protein was ApoH. After dialysis against bidistilled water, ApoH was freeze-dried and stored at –70 °C. The protein was re-dissolved in 0.05 M Tris/HCl buffer, pH 7.4, containing 0.025 M NaCl, before use, and its concentration was determined by absorption spectroscopy ($\epsilon_{280} = 1.0 \text{ ml} \cdot \text{mg}^{-1} \cdot \text{cm}^{-1}$) [6].

Monolayer experimental apparatus

The monolayer surface pressure (π), defined as the change of the surface tension after spreading a monolayer on a water surface, was measured with a NIMA 9000 (England) microbalance. A filter paper of 1.0 cm width was employed as the Wilhelmy plate [39]. The surface-pressure measurements were made with the plate in a fixed-height position, and the data was automatically collected and recorded by a personal computer.

To measure protein insertion, a circular Teflon trough with a volume of 4 ml and a surface area of 10 cm² was used. The trough was specifically designed by Dr. X. H. Han of this laboratory [40]. One advantage of this trough is that it can convert the vortex flow driven by the magnetic stirring bar into a laminar flow at the subphase surface. Therefore the Wilhelmy plate will not be disturbed by the flow of the subphase solution. Another advantage is that it has a smaller subphase volume and a larger surface area compared with the usual one, so that the sample assumption for each measurement can be much reduced. For measuring the molecular area of surface-spread ApoH, another large rectangular trough with a moving barrier was used. This trough had the dimensions 20 cm × 8 cm × 0.8 cm.

Assay of ApoH insertion into phospholipid monolayers

In general, the experiments were conducted as follows. Firstly, the circular trough used for insertion assay was filled with 4 ml of Tris/HCl buffer. Then the phospholipid monolayers were prepared by carefully spreading the lipid solution (2–3 μl) on to the buffer surface. After the film pressure stabilized at a constant value, ApoH was injected into the subphase through a side sample hole. The pressure changes were monitored until the surface pressure increase ($\Delta\pi$) had reached a maximal value,

usually within 2 h. Unless stated otherwise, the measurements were performed under the following conditions. The lipid monolayers were spread from a chloroform/methanol (3:1, v/v) solution of phospholipid (1.0 mM) to the desired initial surface pressure (π_i). The water was deionized, and the subphase buffer contained 0.05 M Tris/HCl, pH 7.4, and 0.025 M NaCl. During the measurement, the subphase was continuously stirred with a magnetic bar. The temperature was controlled at 24.0 ± 1.0 °C, and the system was protected by N₂.

In order to characterize the self-penetration of ApoH into the air/water interface without the spread lipid monolayer, the protein solution was injected into the subphase to a final concentration of 20, 50 and 80 nM respectively. The surface pressures were then measured and plotted versus the reaction time.

To measure the ability of ApoH to insert into phospholipid monolayers at the air/water interface, samples of DPPS, DPPC, DPPG, CL, DOPS and DOPC were used. In the experiments a certain amount of lipid was carefully spread on to the subphase surface to a desired π_i . Then ApoH was injected into the subphase to a final concentration of 50 nM and the increase in $\Delta\pi$ was recorded. For each sample, the values of $\Delta\pi$ as a function of various π_i values were obtained. The plot of $\Delta\pi$ versus π_i yields a straight line with negative slope which intersects the abscissa at a limiting surface pressure. This limiting surface pressure is defined as the critical insertion pressure (π_c) of ApoH for the corresponding lipid monolayer.

Effects of various treatments of ApoH on membrane insertion

For heat inactivation, ApoH (50 μl ; 43 μg) was incubated at 95 °C for 25 min, then placed in ice. For the reduction/alkylation study, ApoH (100 μl ; 87 μg) was denatured in 4 M (final concn.) guanidinium chloride. Reduction was performed by adding dithiothreitol to a final concentration of 40 mM. After incubation for 2 h at 37 °C, the sample was alkylated with 0.1 M iodoacetamide and then dialysed against 0.05 M Tris/HCl (pH 7.4) and 0.025 M NaCl overnight.

Surface-plasmon-resonance (SPR) technique

Surface plasmon resonance is an optical-surface-sensitive technique. The homemade set-up was operated by varying ϕ [41]. Using a semiconductor laser (wavelength 670 nm), the plane of polarization of the incident beam was controlled with a polarizer. A monolayer-containing slide was stuck with index-matching oil on to the base of the triangular prism (refractive index 1.8) which had been fixed on the small plate of a goniometer (a device for measuring angles). The detector was fixed on the big plate of the goniometer which shared its rotating axis with the small one. The angular accuracy of the rotating axis is 0.001°. The rotation of the goniometer was controlled by a computer. The reflected light intensity (R) was converted into electric signal so that the relationship between R and ϕ could be directly analysed by a computer.

According to Stenberg's calibration [42] and our calculations, the surface mass density of the adsorbed protein (ng/mm²) has a linear relationship with the shift of the resonance angle ($\Delta\phi_{\text{SPR}}$). Thus, if the molecular mass (M , in g) is given, the surface number density of the adsorbed protein (n , mm⁻²) can be obtained directly by the equation:

$$n = 6.023 \times 10^{15} \times 0.55 \times \Delta\phi_{\text{SPR}} / M \quad (1)$$

RESULTS

Self-penetration of ApoH into an air/water interface

The self-penetration of ApoH into an air/water interface without phospholipid monolayer was measured as shown in Figure 1. The Figure shows the surface pressure variation as a function of time. Three measurements were performed in which the bulk concentrations of ApoH were 20, 50 and 80 nM respectively. The experimental results show that ApoH can significantly increase the surface pressure, indicating a strong surface activity. From the curves of Figure 1 we can see that the rate of $\Delta\pi$ is greater when more ApoH was injected into the subphase. Since the maximum $\Delta\pi$ induced by the self-penetration of ApoH was found to be 14.7 mN/m, the π_i values of the phospholipid

Figure 1 π - t curves for spontaneous penetration of ApoH into an air/water interface

Figure 2 Time courses for ApoH inserting into DPPS and DPPC monolayers

π - t curves for the monolayers of DPPS (curves A and D), DPPS /DPPC (1:1, in molar ratio) (curves B and E) and DPPC (curves C and F) on a solution of ApoH are shown. The values for π_i were 20.0 ± 0.2 mN/m (curves A, B and C) and 15.0 ± 0.2 mN/m (curves D, E and F). The arrow indicates the time at which ApoH was injected into the subphase to a final concentration of 50 nM.

Figure 3 $\Delta\pi$ - π_i curves of ApoH inserting into phospholipid monolayers

ApoH at a final concentration of 50 nM was injected into the subphase buffer and the $\Delta\pi$ induced by its inserting into phospholipid monolayers consisting of DPPC, DPPS, DPPG or CL was measured at various π_i values.

monolayers in the following protein insertion experiments were kept at or above 15.0 mN/m.

Interactions of ApoH with DPPC, DPPS and their mixed monolayers

The interaction of ApoH with monolayers of DPPS, DPPC and their mixture (1:1 molar ratio) was studied with a 50 nM ApoH solution. The time course for the change in surface pressure is shown in Figure 2. The proteins were injected into the subphase under two different initial monolayer pressures, e.g. 15 and 20 mN/m. From the curves of Figure 2 we can see that the injected ApoH induces a $\Delta\pi$ in all measured samples. No change in surface pressure was observed when only pure buffer control was injected (results not shown). A comparison among the time courses of the increase in surface pressure shows that, at the same π_i , the $\Delta\pi$ for the DPPS monolayer is notably higher than that for the DPPC one. In the case of the monolayer consisting of a mixture of them, an intermediate value of $\Delta\pi$ is obtained. These results indicate that, under the same conditions, the insertion of ApoH into the monolayer becomes stronger when a higher content of negatively charged lipids is present, suggesting that ApoH prefers to interact with negatively charged phospholipids such as DPPS.

From the curves of Figure 2 we also observed that, after protein injection, the surface pressure increases rapidly at first, then gradually levels off and remains stable during 2 h. Thus the duration of the following insertion measurements was chosen to be 2 h. Some other experimental conditions were tested, and it was found that although the penetration kinetics depends on a number of parameters such as the stirring rate, the final value of $\Delta\pi$ is reproducible for a given value of π_i and a fixed concentration of ApoH.

Measurement of π_c

The π_c values of ApoH measured in DPPC, DPPS, DPPG and CL monolayers are shown in Figure 3. The numerical value of π_c for DPPC is 27.7 mN/m, whereas those for negatively charged monolayers DPPS, DPPG and CL are 33.5, 32.0 and 32.0 mN/m

Figure 4 Effect of pH on the insertion of ApoH

(a) $\Delta\pi$ -pH curves of ApoH inserting into DPPS monolayers under various pH values (π_i was 17.7 ± 0.2 mN/m); (b) the $\Delta\pi$ - π_i curves of ApoH inserting into DPPS monolayers at pH 5.1 and 7.4.

respectively. These results indicate that the ability of ApoH to insert into the negatively charged lipid monolayers is much stronger than that into the neutral one. For the three types of negatively charged lipid monolayer, the values of π_c for all of them are almost the same, while their $\Delta\pi$ - π_i behaviours present some differences. Under low initial pressures, the $\Delta\pi$ caused by ApoH insertion is higher for the CL monolayer than those for the DPPS and DPPG ones.

Effect of subphase pH value on ApoH insertion

The insertion of ApoH into DPPS monolayers under various pH conditions adjusted by HCl was studied. Figure 4(a) exhibits the function of $\Delta\pi$ with pH when the π_i of the DPPS monolayer was kept at a constant value, 17.7 ± 0.2 mN/m. It is clearly seen from the curve of Figure 4(a) that $\Delta\pi$ increases significantly as the bulk pH drops from 10.7 to 5.0. A maximum increase in $\Delta\pi$ was found to be at about pH 5.0. However, when the bulk pH value is lower than 5.0, the $\Delta\pi$ decreases, suggesting the ability of ApoH to insert becomes lower under such conditions.

In further experiments we selected two representative pH values, e.g. 7.4 and 5.1, under which the $\Delta\pi$ - π_i behaviours upon

Figure 5 Effect of Na^+ on ApoH insertion

$\Delta\pi$ - $[\text{Na}^+]$ curves of ApoH inserting into DPPS and DPPC monolayers under various Na^+ concentrations (π_i was 20.0 ± 0.2 mN/m).

ApoH insertion were compared, as shown in Figure 4(b). From Figure 4(b) we can see that although the two $\Delta\pi$ - π_i curves have obviously different values of slope, they intersect the abscissa at an identical point, indicating that the ApoH molecule has the same ability to insert under these two pH conditions.

Effect of the univalent electrolyte NaCl on ApoH insertion

Figure 5 shows the features of ApoH insertion into DPPS and DPPC monolayers under different Na^+ concentrations. In the case of the DPPS monolayer (DPPS curve in Figure 5), the value of $\Delta\pi$ first increases as the bulk concentration of Na^+ goes from 0 to 0.025 M. Then it reaches its maximum value at an Na^+ concentration of about 0.025 M, which coincides with the result obtained by Wurm [16]. When the Na^+ concentration increases beyond 0.025 M, the $\Delta\pi$ decreases gradually. From the DPPS curve we can see further that, although the value of $\Delta\pi$ varies with a downward trend when the bulk concentration of Na^+ increases continually, it remains at least 60% of its maximum even at a very high concentration of Na^+ such as 0.5 M. This result indicates that only a small part of the adsorbed ApoH molecules can be detached by increasing the ionic strength. In contrast with the DPPS monolayer, the DPPC curve of Figure 5 shows that the $\Delta\pi$ value is not sensitive to the Na^+ concentration, reflecting the fact that the electrostatic interaction is less important in the case of the DPPC monolayer.

Effect of bivalent cation Ca^{2+} and Mg^{2+} on ApoH insertion

The properties of ApoH insertion into DPPS monolayers under various Ca^{2+} and Mg^{2+} concentrations were compared in another set of experiments (Figure 6). It was shown that $\Delta\pi$ induced by ApoH insertion reduces remarkably as the concentration of Ca^{2+} or Mg^{2+} increases to 0.01 M. For Mg^{2+} , such a detachment effect looks a little more pronounced. However, further increase of the subphase Ca^{2+} or Mg^{2+} concentration does not cause so much of a decrease in $\Delta\pi$. Again, at very high ionic concentrations such as 0.5 M Ca^{2+} or Mg^{2+} , the $\Delta\pi$ remains as high as 60% of the

Figure 6 Effects of Ca^{2+} and Mg^{2+} on ApoH insertion

Insertion of ApoH into DPPS monolayers under the indicated concentrations of Ca^{2+} and Mg^{2+} (π_i is 20.0 ± 0.2 mN/m).

Figure 7 Influence of the acyl-chain saturation of the phospholipids

$\pi_c - \pi_i$ curves for ApoH inserting into DPPC, DOPC, DPPS and DOPS monolayers are shown.

maximum in both cases. All the studies concerning the change of the subphase ionic strength clearly show that insertion of ApoH cannot be curtailed very much by weakening the electrostatic interaction between the protein and the lipid monolayers. Such an observation leads us to believe that, besides the electrostatic interaction, there is some other kind of interaction, more probably a hydrophobic force, strongly involved in the insertion process of ApoH.

Influence of the acyl-chain saturation of phospholipids

The influence of membrane fluidity on ApoH insertion was studied by varying the acyl-chain saturation of the phospholipids. The results are compared in Figure 7. For the neutral lipid monolayers composed of DPPC or DOPC, the values of $\Delta\pi$ upon ApoH inserting into DOPC monolayers are higher than those into DPPC ones for all measured π_i . As to the negatively charged lipids, the $\Delta\pi$ values are a little weaker for DOPS monolayers than for DPPS ones under conditions of low π_i .

Figure 8 Influence of heat treatment of ApoH on its insertion

$\Delta\pi - \pi_i$ curves for native (+) and heat-treated (●) ApoH inserting into DPPS monolayers are shown.

When the π_i is higher than 18.8 mN/m, the insertion of ApoH into DOPS monolayers becomes stronger than that into DPPS ones. The values of π_c obtained for DPPC and DOPC are 27.7 and 31.7 mN/m, and for DPPS and DOPS are 33.1 and 37.2 mN/m. It is clearly seen from these data that the ability of ApoH to insert is enhanced when the lipid monolayer contains unsaturated acyl chains. These results on the other hand suggest that the inserted segments of ApoH may, to a certain extent, touch the region of the hydrophobic tail of the lipid monolayer.

Influence of heat inactivation on ApoH insertion

π_c of the heat-treated ApoH was measured as shown in Figure 8. The results show that, with different values of π_i , the $\Delta\pi$ values induced by the insertion of the heat-treated ApoH are higher than those by that of untreated ApoH, whereas the π_c values in these two cases are identical. This interesting result suggests that the ability of ApoH to insert into the DPPS monolayer is not dependent on its three-dimensional structure.

Influence of reduction/alkylation on ApoH insertion

After reduction/alkylation treatment, π_c of the modified protein was measured. The results of Figure 9 show that, with varying values of π_i , the values of $\Delta\pi$ induced by the insertion of reduced/alkylated ApoH into DPPS monolayers are notably higher than those induced by the untreated protein. Again, we found that the π_c values in these two cases are almost the same.

Evaluation of molecular area of ApoH at the interface

Two different methods were used to measure the molecular area of ApoH at the interface. The first method is a self-penetration approach in which the technique of SPR was employed. In this case, using the circular trough, the protein monolayer was formed at the air/water interface by its self-penetration in the absence of lipids. After equilibrating the protein layer at its maximum surface pressure (14.7 mN/m) for 2 h, we horizontally transferred the protein layer on to the surface of the gold-film-covered slide. The shift in $\Delta\phi_{\text{SPR}}$ was then measured by SPR. The surface number density (n) of ApoH was calculated according to

Figure 9 Influence of reduction/alkylation of ApoH on its insertion

$\Delta\pi$ – π_i curves of native (+) and reduced (●) ApoH inserting into DPPS monolayers are shown.

eqn. (1) above, and a value of $(2.18 \pm 0.01) \times 10^{10} \cdot \text{mm}^{-2}$ was obtained under a surface pressure of 14.7 mN/m. Assuming that the self-penetrated protein at the air/water interface is in a monolayer, we can obtain the molecular area of ApoH by directly using the reciprocal of the value of n , namely $45.95 \pm 0.15 \text{ nm}^2$ ($4595 \pm 15 \text{ \AA}^2$).

The second method was a surface-spread approach. First, 10 μg of ApoH was denatured in a mixed solvent [water/chloroform/methanol (1:2:3, by vol.)] to a final volume of 70 μl overnight. Then the protein solution was carefully deposited on to the water surface with a microsyringe. After equilibration for 2 h, the pressure (π)–area (A) curve of the spread protein layer was recorded with the large rectangular trough (results not shown). Considering that the maximum surface pressure formed by the self-penetration of ApoH into the air/water interface is 14.7 mN/m (as shown in Figure 1), we directly measured the molecular area of ApoH, which had been denatured and spread at the air/water interface, from the π – A isotherm obtained under such a surface pressure. Thus we get the molecular area of ApoH in this case to be $109.7 \pm 0.1 \text{ nm}^2$ ($10970 \pm 10 \text{ \AA}^2$). Compared with the results of the self-penetration approach, we found that the area per surface-spread protein is much larger. The reason for such a difference between these two cases is discussed below.

DISCUSSION

The interactions of ApoH with lipid membranes have been considered in several laboratories [16,31–33] as a basic mechanism for the biological function of the protein. With lipid vesicles, Wurm [16] reported that negatively charged phospholipids interacted strongly with ApoH. With a supported planar bilayer, Willems et al. [33] examined the ApoH-mediated binding of anti-CL antibodies to lipid membranes. These results reported that the ApoH–lipid interaction seems to be critical for its physiological role, but the behaviour of ApoH at the membrane surface is still little known. The results of the present study show first that ApoH has a strong surface activity. Secondly its ability to insert into membranes was studied using the monolayer method. It is believed that the molecules which interact only with the headgroups of the lipid monolayer will not increase the surface pressure of the monolayer. Thus, when protein molecules are

injected into the subphase, the corresponding $\Delta\pi$ can be interpreted to be the result of the protein inserting into the monolayer. In such studies π_c is a very useful parameter. If the π_i of the monolayer is kept at or above π_c , the protein cannot insert into the membrane. Therefore, the value of π_c may quantitatively reflect the ability of the protein to insert into a certain phospholipid monolayer.

From Figure 3 we can see that the π_c values of ApoH inserting into the negatively charged lipid monolayers are significantly higher than that into the neutral ones. Figure 3 also shows some differences in the behaviour of ApoH when inserting into the different types of negatively charged membranes. Since a CL molecule contains two net charges, while a DPPS or DPPG molecule contains only one, the different values in $\Delta\pi$ under low π_i may reflect the different strengths of electrostatic interactions between ApoH and the lipids. Considering that the values of π_c for the different types of the negatively charged lipid membranes are almost the same, we propose that the electrostatic force is not the only factor that affects the $\Delta\pi$. It is possible that, under lower π_i , electrostatic interaction plays an important rôle in the insertion process, whereas when π_i increases, hydrophobic interactions become gradually more important.

A change in the subphase pH value and the ionic strength may affect the degree of the ionization of the protein and the lipids, thus affecting the interaction of ApoH with lipid membranes. From Figure 4(a) we can see a $\Delta\pi$ increase as the subphase pH value decreases from 10.7 to 5.0. Over this pH range the degree of ionization of the phospholipid headgroup will not change, since it was reported that the apparent pK_a of the carboxy group in the DPPS molecule is about 5.0 under our conditions [43]. Therefore the change in $\Delta\pi$ over such a pH range may mainly reflect an increase in the positive charges on the protein surface. When the subphase pH value decreased to less than 5.0, the $\Delta\pi$ upon ApoH insertion also diminished. One explanation is that the protein undergoes a conformational change which hinders its insertion. The other explanation may come from the deionization of the lipid itself. Our recent experiments revealed that, over the pH range 10.7–3.0, the conformation of ApoH changed little [44]. So such a decrease in $\Delta\pi$ over this pH range may mainly reflect the loss of negative charges in the lipid headgroups. Since π_c is a more influential factor in predicting the ability of a protein to insert into membranes, in further experiments, we compared the π_c values when ApoH inserted into DPPS monolayers at different pH values (7.4 and 5.1) (Figure 4b). The $\Delta\pi$ – π_i curves of Figure 4(b) show that, although the subphase pH value varies a lot, the π_c upon ApoH insertion is almost identical for the two cases. As the strength of the electrostatic interaction between the protein and the lipid is different under the two pH conditions, the same π_c obtained in Figure 4(b) strongly implies that, besides electrostatic interactions, hydrophobic interactions are also clearly involved.

The optimal Na^+ concentration upon ApoH inserting into DPPS monolayers is 0.025 M according to the results shown in Figure 5. As the subphase Na^+ concentration increased from 0 to 0.025 M, the apparent pK_a of DPPS decreased from about 6.0 to 5.0 [43]. So the degree of ionization of the lipid will increase in the neutral-pH conditions at 7.4, and the electrostatic attraction between the lipid and the protein will be enhanced. If the subphase Na^+ concentration increased further, the pK_a of DPPS would decrease further from 5.0 to approx. 3.7 at an Na^+ concentration of 0.5 M. In this range the degree of ionization of the lipid will no longer be affected. In contrast, a high Na^+ concentration may change the surface potential of the system. As a result, the electrostatic interaction between the protein and the lipid membrane will be weakened.

Concentrated bivalent ions such as Ca^{2+} and Mg^{2+} can affect the intermolecular electrostatic repulsion directly by binding to the lipid headgroups and bridging a pair of lipid molecules [43]. Therefore, these ions can influence the electrostatic interaction between protein and lipids more efficiently than the univalent ions. Combining the results in Figures 5 and 6, we can see that, although the $\Delta\pi$ upon ApoH insertion shows a downward trend when the bulk concentrations of ions increase continually, the values of $\Delta\pi$ remain at 60 % of their maximum values even under very high ionic-strength conditions. These findings, together with the influence of the acyl-chain saturation upon ApoH insertion shown in Figure 7, lead us to conclude that both electrostatic and hydrophobic interactions are essential for the insertion of ApoH into phospholipid membranes.

The pI of ApoH is 5–7 [45], so the protein is negatively charged under pH 7.4 conditions. It is unlikely that a negatively charged protein will preferentially insert into monolayers of negatively charged lipids. To reveal the structure base for ApoH insertion, we altered the conformation of ApoH by heat treatment and reduction/alkylation, and studied the influence of such treatments on ApoH insertion. The results shown in Figures 8 and 9 show that the $\Delta\pi$ upon ApoH insertion becomes higher after these treatments, whereas the π_c of ApoH insertion does not change, suggesting that the ability of ApoH to insert is independent of its three-dimensional or domain structure. It is evident that a heat-stable and disulphide-bond-independent motif, which may be a sequential region rich in positively charged residues, facilitates the interaction of ApoH with negatively charged lipids. Kertesz et al. [31] recently showed that a conserved sequence ($\text{C}^{281}\text{KNKEKKC}^{288}$) rich in basic residues is important in the ApoH–lipid interactions. Our previous study has shown that ApoH undergoes a large conformational change upon binding to negatively charged liposomes [44]. Combining these studies with the results obtained in the present work, we propose that the conformational change could expose hydrophobic regions of ApoH, allowing the interaction of ApoH with the monolayer to become more hydrophobic and, as a result, promoting the insertion of ApoH into the lipid monolayers.

The molecular area of surface-existing ApoH was measured by two methods. For the same surface pressure, the area occupied by one protein molecule in the self-penetration form (measured by SPR) is obviously smaller than that in the surface-spread form (measured from the π – A curve of the protein layer). The problem of the area of protein at the air/water interface has been studied in more detail by Cho and co-workers [46]. They carefully compared the area difference between proteins (such as BSA and pentylated BSA) in the self-penetration form and those in the surface-spread form. For each protein their results showed that the self-penetrated sample has smaller molecular area than the surface-spread one. With the aid of theoretical analysis and the results of various measurements, they indicated that the molecular area difference in these two forms is due to the unfolding of the protein to different extents. On the basis of Cho and co-workers' model we can reasonably conclude that the self-penetrated ApoH molecules unfolded less than the surface-spread ones. Consequently, the molecular area of the former ones is smaller.

It is reported that, *in vivo*, ApoH is one of the major proteins appearing in the very rapidly cleared large liposomes that contain phosphatidylcholine, cholesterol and negatively charged phospholipids (phosphatidylserine, phosphatidic acid or CL) [37]. Since these negatively charged phospholipids are normally found exclusively in the interior of cells, but become expressed when cells undergo apoptosis or programmed cell death, they are believed to be markers of cell senescence [47]. The immune

clearance rate of these liposomes from the circulation is associated with the amount of adsorbed ApoH and negatively charged lipids, but the reason is unknown. Our present results give direct proof that ApoH can specifically bind and penetrate into the negatively charged lipid monolayers. It is established that the bilayer–monolayer equivalence pressure is about 30.7–32.5 mN/m [48], so ApoH has the potential to insert into liposomes *in vivo*. ApoH may actually insert into these liposomes through strong interactions with the negatively charged phospholipids, then perform its biological functions as a 'cleaner' to purge the plasmic liposomes coming from senescent cells and foreign particles. Further studies are needed to determine the insertion domain of ApoH and its orientation on membranes.

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