Binding Steps of Apolipoprotein A-I with Phospholipid Monolayers: Adsorption and Penetration†

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ABSTRACT: In whole HDL particles, the arrangement of apoA-I relative to phospholipids is of crucial interest with respect to the physiological formations of HDL. We report here new data concerning the nature of the interaction of apoA-I with condensed phospholipid (PL) monolayers (phosphatidylcholine and phosphatidylserine). The use of alternative current polarography allowed the detection for the first time of different binding steps which are dependent on apoA-I concentration. At low concentration (below 10 μg/mL), apoA-I adsorbs on PL polar headgroups, through electrostatic interactions. Above this threshold concentration, apoA-I penetrates within the monolayer (i.e., part of apoA-I crosses the PL polar headgroup/hydrocarbon chain interface). The process of penetration described here brings experimental evidence supporting Segrest's "snorkel" model. Penetrated helices are lying at the interface, their apolar face in contact with PL hydrocarbon chains and their polar face in contact with PL polar headgroups. In the absence of cholesterol, a second level of penetration was detected at higher apoA-I concentrations. It was facilitated in the presence of phosphatidylserine in comparison to phosphatidylcholine and disappeared in the presence of cholesterol. It is proposed that the C-terminal domain is involved in the first binding steps and that hinged domains may also be implicated. Furthermore, we propose that the apoA-I binding states stabilize the protein/phospholipid layer complex. These different binding states are discussed with respect to their roles in HDL metabolism.

Lipoproteins allow the transport of water insoluble lipids in the blood plasma. High-density lipoproteins (HDL[1]), which have various biological origins and functions, all contain apolipoprotein A-I (apoA-I) as their major protein constituent (*1*). From a structural point of view, like several other related exchangeable apolipoproteins, apoA-I (whose sequence is known) consists of repetitive amphipathic α-helices (*2*), some of them belonging to class A (*3*), which has a distinct pattern of biological properties from other classes (*4*).

The conformation of apoA-I in HDL can critically affect interactions with plasma proteins such as lecithin-cholesterol acyltransferase (LCAT) (*5*, *6*, *7*), cholesteryl ester transfer protein (CETP) (*8*), and hepatic lipase (*9*), as well as the ability of HDL to remove cholesterol from cells (*10*) or to bind to cell membranes (*10*, *11*). Moreover, the different arrangements of apoA-I relative to different phospholipid environments may play a role in the apoA-I movements which occur during metabolism, such as the transfer of apoA-I from triglyceride-rich particles to HDL during lipolysis (*1*).

Since native HDL are very heterogeneous, the interaction was mainly studied on reconstituted HDL (*6*, *7*, *12*). Several structural models of discoidal and spherical HDL have been proposed (*13*, *14*). ApoA-I undergoes conformational changes during its interaction with phospholipids (increase in α-helical content) and during HDL remodeling which is associated with modifications in the lipid composition (phospholipid, cholesterol) and apoA-I content. Nevertheless, the arrangement of apoA-I relative to phospholipids (PL) in the particles is not yet defined, even though it is of crucial interest with respect to the physiological functions of HDL. The present literature conveys the hypothesis whereby the helices of apoA-I are located along the edge of the disks (*6*, *15*, *16*). Two possible orientations have been discussed (*17*). The axes of the helices can be oriented either parallel to the acyl chains of the phospholipids, with two pieces of experimental evidence (*15*, *18*), or perpendicular, as proposed previously in the case of the "bicycle tire" model (*19*) or more recently (*20*). In fact, the structural assembly of HDL is due mainly to the lipid−apolipoprotein interactions in the outer mono-

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[1] Abbreviations: apoA-I, apolipoprotein A-I; HDL, high-density lipoproteins; chol, cholesterol; PL, phospholipid; PC, phosphatidylcholine; PS, phosphatidylserine; ac, alternative current; dc, the differential capacity; HMDE, hanging mercury drop electrode; Hg, mercury.

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layer of these particles.

In vitro, apoA-I has been shown to interact with PL leading to the formation of lipid−protein complexes (*12*). Generally, the understanding of the interaction of exchangeable apolipoproteins with PL is based on the amphipathic helix theory (*3*, *17*). Indeed the nonpolar face of the α-helices is buried in the hydrocarbon milieu, and the polar face (exposed to the aqueous surroundings) lies laterally between PL polar headgroups, interacting with their charged residues. This implies the penetration of apoA-I in the PL layer, which involves electrostatic and hydrophobic interactions, as proposed in the "snorkel" model of Segrest et al. (*3*). Nevertheless there is no direct experimental evidence of the penetration of apoA-I at the phospholipid−solution interface, and no study was undertaken on the different steps which lead to this binding state in the formation of the apolipoprotein−phospholipid complexes.

We used alternative current (ac) polarography in order to investigate interactions between apoA-I and PL. It has the particular advantage of allowing discrimination between the modes of binding of a hydrosoluble protein at the PL polar headgroups−hydrocarbon chains interface, in terms of penetration or adsorption, as was shown in previous papers (*21*, *22*). We use this method under restricted conditions which allow the measurement of the differential capacity (dc) of the electrode in contact with a PL condensed monolayer. Thus, the conditions of application of the method are consistent with the condensed state of PL in biological membranes. Such a system has potentialities of a biosensor. It has a high selectivity to the interaction between various PL and proteins with their different domains (*21*). This method has recently been used to show that the blood coagulation factor Va is engaged in both electrostatic and hydrophobic interactions with phospholipid monolayers, depending on bulk factor Va concentration (*21*).

In the present paper, we report the occurrence of the various binding steps of apoA-I with condensed PL monolayer (when varying apoA-I concentration) and the influence of cholesterol on the binding. While all the studies made on disks or spheres gave information on the final phase of the association, the electrochemical method used in this work allows the investigation of the initial mechanism of the apoA-I−phospholipid association.

EXPERIMENTAL PROCEDURES

Materials. All reagents were of the highest grade commercially available. The different inorganic salts and acids were of analytical grades. Mercury was purified and doubly distilled under vacuum. Ultrapure water was obtained from a Millipore Super Q system.

Isolation of Apolipoprotein A-1. The human apolipoprotein A-I was isolated from apo HDL, by Sephacryl S-200 (Pharmacia) gel permeation chromatography, using 0.03 M Tris-HCl buffer, pH 8.0, containing 6 M urea as the elution buffer (*23*). The eluates were collected and dialyzed against 5 mM ammonium bicarbonate solution at 4 °C. The dialyzates were lyophilized and analyzed by SDS−PAGE. The apoA-I preparations yielded a single band, as shown by coomassie blue staining. Protein concentration was determined according to Lowry et al. (*24*) using bovine serum albumin as a standard.

Monolayer Preparation. Chromatographically pure egg lecithin (PC) and ox brain phosphatidylserine (PS), grade I, were purchased from Lipid Products (Nutfield, United Kingdom) and supplied in chloroform−methanol solution. Phospholipid samples in the right studied ratio were evaporated in a stream of nitrogen; the lipid content was determined by weight and then samples were dissolved in hexane, for monolayer spreading. A 2-fold excess of lipid was spread over an aqueous solution, containing 0.15 M NaCl and 25 mM Tris at pH 7.4. The excess was relative to the condensed state obtained according to surface pressure experiments (*25*). This excess lipid formed collapsed structures in equilibrium with the spread monolayer and had a negligible contribution to the electrochemical measurements. Experiments were performed at room temperature, where the lipids are in liquid-crystalline phase (*26*).

Electrochemical Measurements. Alternative current (ac) polarography was carried out as described previously (*21*), with the polarographic instrument already mentioned and in a Metrohm polarographic cell. A hanging mercury drop electrode (HMDE) was positioned in contact with the phospholipid monolayer; an Ag/AgCl saturated KCl electrode was the reference, and a platinum gauge was the auxiliary electrode. The potential was scanned at a rate of 50 mV/s, and the frequency of ac modulation (10 mV peak to peak) was 80 Hz. The starting potential was chosen so as to be in the stable region of the monolayer, namely, at −300 mV relative to the Ag/AgCl saturated KCl electrode (Figures 1−4). Once the differential capacity was invariant, the equilibrium was reached.

RESULTS

Variation of the Differential Capacity Versus Potential, for the Mercury Electrode in Contact with Phospholipids, Proteins, or Electrolyte. As previously reported (ref *21*, and references cited therein), the ac polarography method allows the study of the perturbations which can occur in a membrane by measuring the differential capacity variations of a mercury (Hg) electrode in direct contact with a condensed PL monolayer (spread at the surface of the solution) while macromolecules, like proteins, are introduced into the solution beneath. In fact, at a potential imposed between working (Hg) and reference electrodes, dc depends on the polarizability of the molecules in contact with the electrode. As shown in Figure 1, the highest differential capacity corresponds to the pure electrolyte, so that, when a macromolecule adsorbs at the electrode, dc decreases. This is the case for condensed monolayers of proteins or of phospholipids adsorbed on a mercury electrode located above. With PL, dc is low around the zero charge potential (−0.45 V), where the lipids are adsorbed at the electrode by their hydrophobic part. With proteins, dc is higher than with PL. Thus, if the introduction of a protein in the solution underneath a PL monolayer leads to an increase in dc, this is indicative of a perturbation of the dense PL layer by the interacting proteins. This can be interpreted in terms of a penetration model, since condensers are in parallel (*21*), according to the following equation:

$$C = C_L + \theta(C_P - C_L) \quad (1)$$

where C_L and C_P are the differential capacity of the intact

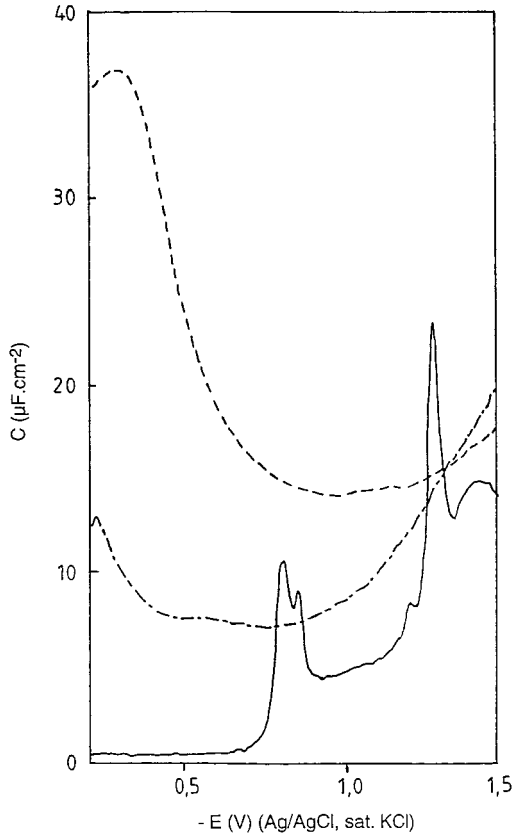

FIGURE 1: Variation of the differential capacity with various types of molecules in contact with the electrode. Differential capacity versus electrical potential curves of the HMDE in contact with electrolyte (− − −), or a condensed monolayer of apoA-I (−•−•), or of phosphatidylcholine (—)

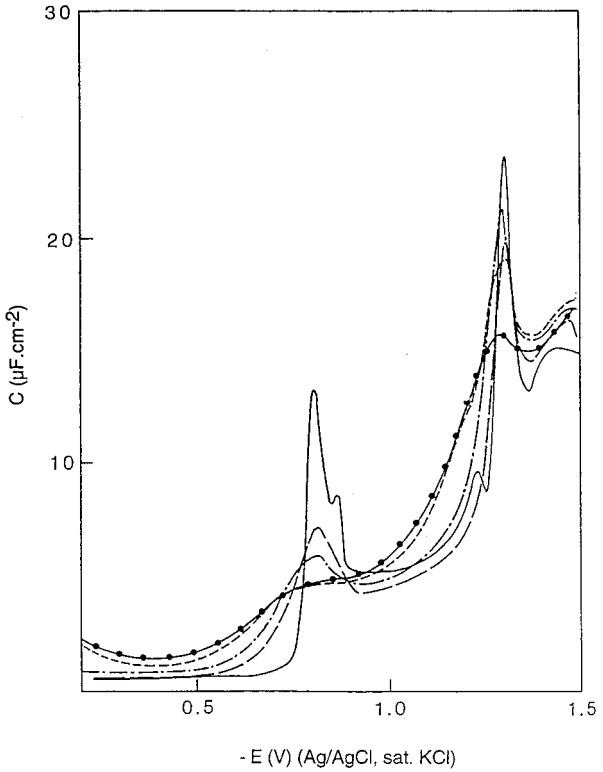

FIGURE 2: Effect of apoA-I concentration on its interaction with PC monolayer. Differential capacity versus electrical potential curves of the HMDE in contact with a monolayer containing 100% PC, at initial bulk apoA-I concentrations (—) 0, (− − −) 4, (−•−•) 10, (---) 16, (−●−) 28 μg/mL, injected underneath. The full line represents the condensed lipid monolayer in the absence of protein added.

condensed lipid and protein monolayers, respectively (Figure 1), and θ is the fraction of lipid monolayer which has been penetrated by the protein.

As expected from the structure of apoA-I, no disulfide bond (S−S) reduction peak is observed at around −0.6V.

Interaction of apoA-I with Phospholipid Monolayers Containing PC. The PL monolayer is the most stable around the zero charge potential (−0.45 V). In the case of monolayers containing 100% PC (Figure 2), at low concentrations of apoA-I added (up to 4 μg/mL), there is no change of dc, while a significant increase of dc can be detected above 10 μg/mL. This observation leads to the conclusion that apoA-I molecules are penetrating the monolayer only above a threshold apoA-I concentration in solution. Below this concentration, the constant differential capacity measured is not due to a lack of sensibility of the method but rather to another mode of interaction of the protein with PL or to the absence of interaction. Around −0.45 V, the potential has no influence on the forces involved in the interaction, while at more negative value, it starts to interfere with these forces since dc increases already around −0.6 V. It is possible that the potential facilitates penetration or/and the reorganization of the layer.

Between −0.7 and −0.9 V, two peaks appear which were proposed to concern phase transitions of the layer (*27*). The height of these peaks decreases clearly when apoA-I concentration increases (Figure 2). This observation means that apoA-I interacts with the PL monolayer, even at low apoA-I concentrations (below 4 μg/mL). Thus, as at low concentrations, apoA-I interacts with the monolayer (as shown at the −0.85 V peak) and, as it does not penetrate (as shown at −0.45 V), this indicates that apoA-I adsorbs at the PL−solution interface.

In the potential range more negative than −1.2 V, a desorption peak appears. Indeed, in this range, adsorption of cations from the electrolyte replaces the adsorption of phospholipids at the electrode, in the absence of protein.

In Figure 3, parts A and B, the height of the peaks between −0.7 and −0.9 V decreases distinctly in the presence of cholesterol (1 chol/10 PC, Figure 3A), as well as in the presence of low apoA-I concentrations (Figure 3B). This suggests that apoA-I and cholesterol have a similar effect on the PL layer adsorbed at the electrode. This decrease indicates from an electrochemical kinetic point of view a slower process at the electrode. Since cholesterol is known to rigidify the fluid layer, we propose that low apoA-I concentrations in a similar way would stabilize the new layer formed in this range of potential (*27*).

Cholesterol Effect on Penetration of apoA-I into Phospholipid Monolayer Containing PC. Since cholesterol is present at the monolayer surface of HDL, it was of interest to investigate the effect of cholesterol on the interaction of apoA-I with the PL monolayer. Two extreme cholesterol−phospholipid ratios (in weight) involved in HDL were used (*28*).

For a monolayer containing 1 chol/10 PC (Figure 4), the differential capacity of the monolayer around the zero charge potential does not significantly increase with low apoA-I

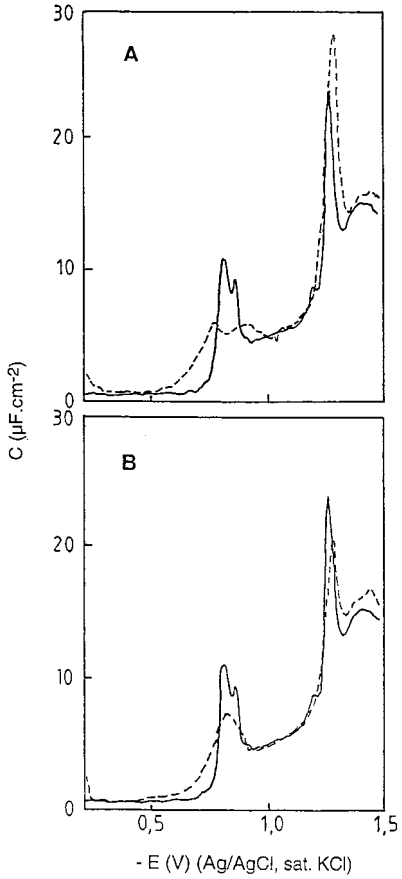

FIGURE 3: Comparison effect of apoA-I and cholesterol on PC monolayer. Differential capacity versus electrical potential curves of the HMDE in contact with a phosphatidylcholine-containing monolayer (full line), in the presence of cholesterol (1 chol/10 PC) (Figure 3A) or of 4 μg/mL of apoA-I (Figure 3B) (dotted lines).

concentrations (below 7.5 μg/mL), thus indicating a lack of significant penetration. At more negative potentials than −0.7 V, the ac signal changes with increasing protein concentrations, thus indicating a binding of apoA-I. Consequently, apoA-I adsorbs, like in Figure 2. At higher apoA-I concentration (above 7.5 μg/mL), the differential capacity around the zero charge potential increases, thus indicating penetration of apoA-I in the monolayer.

In Figure 5 plots of the differential capacity variation (at −0.45 V and at equilibrium) are represented as a function of apoA-I bulk concentration for phospholipid monolayers containing PC alone or two cholesterol content relative to PC. Above 10 μg/mL a cooperative interaction appears to be involved in the penetration process of apoA-I into PC alone or 1 chol/10 PC monolayers, as indicated by the sigmoidal nature of the plots. Above around 40 μg/mL of apoA-I, saturation of the process occurs as there is almost no further change in dc with increasing protein concentration; a plateau is reached in the presence of cholesterol. Then, in the absence of cholesterol, after a short plateau, a second increase in dc is observed at around 80 μg/mL, until a second plateau is reached. In the presence of 1 chol/10 PC, this second increase is suppressed. With 1 chol/4 PC the suppression extends to the first increase (see inset of Figure 5). Consequently, the penetration of apoA-I into a phosphatidylcholine monolayer is inhibited with high cholesterol content.

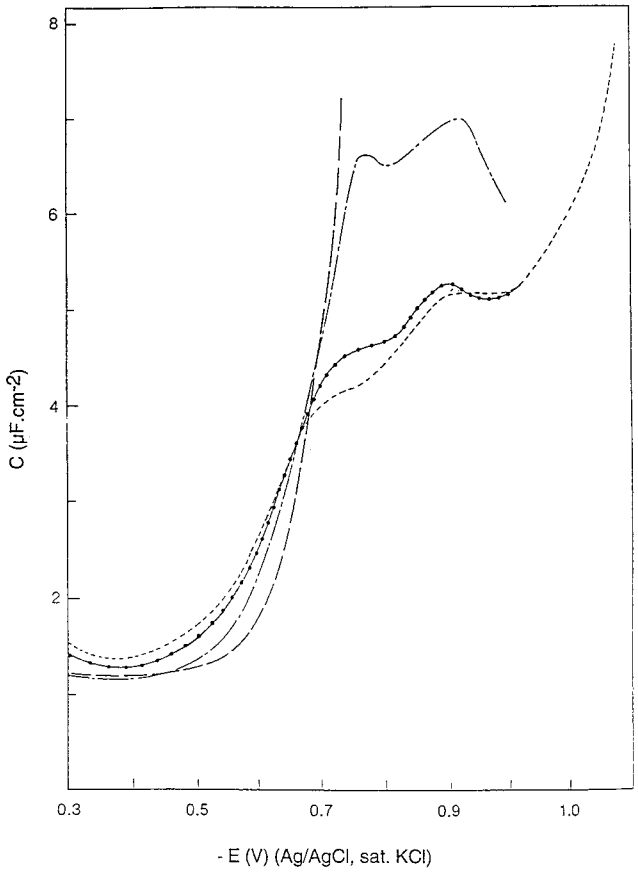

FIGURE 4: Effect of low apoA-I concentrations on their interaction with monolayer containing 1 chol/10 PC. Differential capacity versus electrical potential curves of the HMDE in contact with a monolayer containing 1 chol/10 PC, at initial bulk apoA-I concentrations (− − −) 0, (−•−•) 5, (−●−) 7.5, (---) 10 μg/mL, injected underneath.

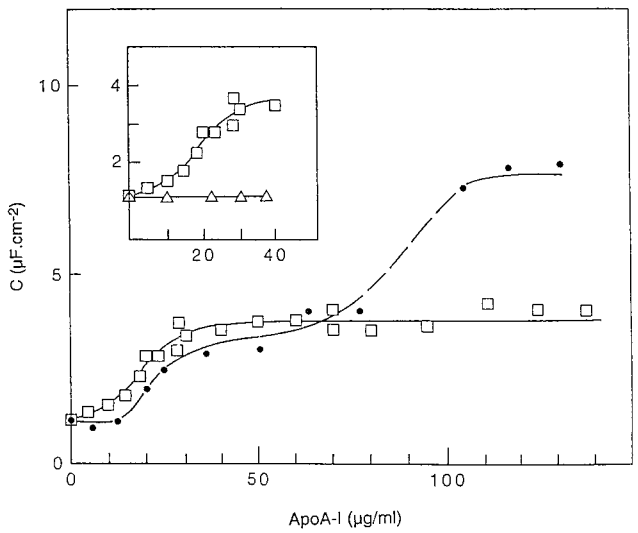

FIGURE 5: Differential capacity of condensed PC monolayers with different contents of cholesterol, as a function of the apoA-I concentration injected underneath the monolayer, at −0.45 V relative to saturated Ag/AgCl electrode: (□) 1 chol/10 PC, (△) 1 chol/4 PC, and (●) 100% PC.

Effect of Polar Headgroup on the Penetration of apoA-I into Phospholipid Monolayers. To investigate the influence of the charge of the polar headgroup on the interaction of apoA-I with the PL monolayer, we used comparative monolayers of pure PS and PC. With increasing apoA-I bulk

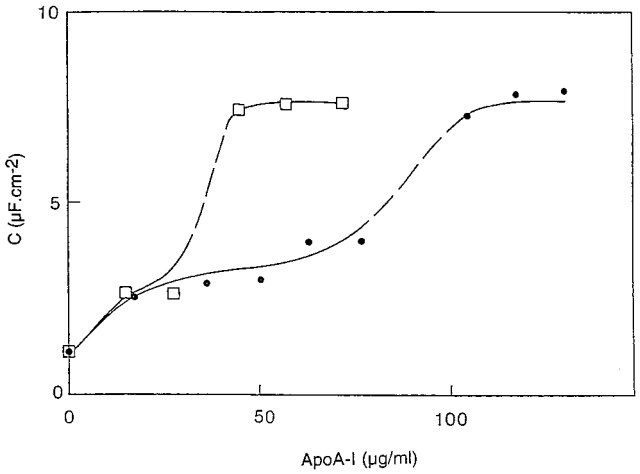

FIGURE 6: Polar headgroup effect on penetration of apoA-I into phospholipid monolayer. Differential capacity of (□) PS or (●) PC containing condensed monolayers, at −0.45 V relative to saturated Ag/AgCl electrode, as a function of the initial bulk apoA-I concentration injected underneath.

concentrations, the differential capacity increased, thus indicating penetration of apoA-I into the PS and PC monolayer (Figure 6). Dc increases until a first plateau is reached at around 30 μg/mL for both PL and a higher second plateau is observed at 40 and 100 μg/mL of apoA-I concentration for PS and PC monolayers, respectively. It may be noted that the level of the second plateau is similar with both lipids, despite the shift of their threshold. Measurements were made on both sides of the threshold concentrations, where they are more stable.

DISCUSSION

We report here the first application of ac polarography to the study of interactions between an exchangeable apolipoprotein (apoA-I) and PL monolayers. Increasing apoA-I concentration induces differential capacity variations of the PL monolayer, which are indicative of structural changes in the PL monolayer (due to apoA-I binding). To our knowledge, this gives the first experimental evidence of distinct binding steps, that is, adsorption of apo A-I on PL polar headgroups and its penetration across the polar headgroups−hydrocarbon chains interface of the condensed PL monolayer (*21*). This can be investigated by the present ac polarography method which, in this case, supersedes the method measuring the surface pressure. It is recalled that the differential capacity is the second derivative of the interfacial tension respective to the potential (*29*).

The principle of the method and the relationship between the electrical parameters and the molecular events have been previously discussed (*21*). In the present paper, we used an extended potential range toward more negative values. This gives new possibilities to the method, allowing checking for the integrity of the condensed PL monolayer and detection of the adsorption of the protein at the PL monolayer (which may precede the penetration step).

Several data give evidence on the integrity of the condensed PL monolayer adsorbed at the electrode:

(1) In the absence of any protein, the differential capacity at zero charge potential of the pure PL monolayer corresponds to that of a condensed monolayer (*21*), since the right quantity of PL was spread at the surface of the solution as assessed by surface pressure (*22*). It is much lower than that for the electrolyte (Figure 1).

(2) Below a threshold apoA-I concentration (where there is no penetration), the apoA-I molecules adsorbed at the interface do not alter the permeability of the monolayer, since a significant change of dc at zero charge potential was not observed (cf. comments of Figure 2 in results).

(3) Above the apoA-I concentration threshold, the increase of dc at zero charge potential suggests that apoA-I does not penetrate in pre-existing defects (previously occupied by electrolyte) of the PL monolayer, since, if the electrolyte is replaced by apoA-I, the dc should decrease (because the dc of apoA-I is lower than that of the electrolyte, as shown in Figure 1).

(4) Furthermore, increasing apoA-I concentrations do not significantly alter permeability properties of the lipid monolayers (deposited on the electrode), since dc remains constant on a range of apoA-I concentrations (first plateau in Figure 5). Moreover, this range is broader in the presence of cholesterol, when the second plateau disappears.

Penetration of a protein into the PL monolayer (i.e., a part of the protein must cross the polar headgroups−hydrocarbon chains interface) induces an increase of dc, at the zero charge potential (since condensers, corresponding for one part to the PL monolayer and for the other to the protein, are in parallel) (*21*). In the case of **adsorption** (i.e., the protein does not cross the interface), dc should decrease at the zero charge potential (since the two condensers, formed by the full PL monolayer and the adsorbed proteins, are in series) (*21*). As in fact, we did not detect dc decreasing, with the resolution of our experiments, we utilized the variations of the ac signal at the −0.8 V peak.

In the adsorption state (i.e., low apoA-I concentration), apoA-I does not cross the polar headgroups−hydrocarbon chains interface. Consequently, apoA-I interacts only with the polar headgroups of phospholipids, by electrostatic interactions through hydrophilic amino acids (which are present on the surface of lipid-free apoA-I) of the nonhelical C-terminal part (between residues 190 and 243) or/and of the polar faces of the amphipathic α-helices (*30*). This second hypothesis requires that the nonpolar faces of the amphipathic α-helices be hidden. This can be the case if two or more helices bind to each other, which is consistent with the existence of the three paired helices already proposed (*15*). Thus, they would lie on the surface with their axis perpendicular to the hydrocarbon chains.

Two penetration steps have been detected at threshold concentrations (10 and 70 μg/mL, respectively). Such threshold has been previously reported in the case of the interaction of prothrombin with PL (*22*).

The first penetration step is probably associated with a partial conformational change of apoA-I. It has been proposed that apolipoproteins may easily interact electrostatically with the PL polar headgroups and induce in turn an increase of helicity and a rearrangement of helices leading to additional hydrophobic interactions (*13*). In our model, such a conformational change could be involved in the shift from the adsorbed to the penetrated states. This conformational change may result from the helix formation of the C-terminal part (*31*), already adsorbed, and from uncoupling of the adsorbed helices when they penetrate into the monolayer. This is consistent with thermodynamic consid-

erations since the hydrophobic interaction of one helix with another helix is weaker than with PL, which bring a supplementary electrostatic interaction, as shown in the "snorkel" model (*3*, *14*, *17*).

In our model, the penetrating helices are lying on the surface because they cannot penetrate with their axis parallel to the hydrocarbon chain, since their length is twice that of a monolayer. As the penetration state corresponds to condensers in parallel, we can determine from eq 1 that 25% of the surface is covered by such helices. Consequently, on the basis of the areas covered by one helix lying on the surface and one cross-section of phospholipid (*32*), one helix is surrounded by 30 phospholipids. If one or two paired helices per apoA-I molecule penetrate, the ratio PL−apoA-I is consistent with the one in disks (*12*, *33*), while it corresponds to vesicles (*12*, *33*) in the adsorbed state.

Therefore, our *adsorption−penetration* model may be interpreted in light of recent data of the literature, obtained on disks. The helices of the C-terminal moiety are widely thought to be involved in the first binding step of apoA-I with PL (*34*−*39*), whereas the N-terminal part is not necessary for this binding (*40*). These data are consistent with our hypothesis of the role of the extreme C-terminal part, in the first binding step (adsorption), which induces important structural modifications leading to subsequent penetration of other helices (*31*).

The presence of a second plateau when apoA-I concentration increases may be explained by the following hypothesis: (i) a free apoA-I molecule (from the solution) interacts with an already bound apoA-I molecule; and (ii) this interaction induces an additional penetration either of a part (not yet penetrated) of a first bound apoA-I molecule or of the second apoA-I molecule. From eq 1, and assuming the additional binding of helices, we can calculate that they covered around 50% of the monolayer surface, so that one helix is surrounded by about 10 phospholipids. This is the maximal possible arrangement which can occur. If the eight helices (*6*) are embedded in the membrane the ratio PL−apoA-I would be 80/1, which is consistent with that found in disks (*12*, *33*).

The disappearance of this second plateau in the presence of cholesterol can be interpreted as a decrease of the number of penetrated helices. This may be related to the literature's data concerning the effect of cholesterol which increases the immunoreactivity of the central domain between residue 99 and 143 (*41*), designated as the hinged domain (*3*, *16*). It is a mobile domain in HDL as strongly suggested using monoclonal antibodies (*16*, *42*−*44*). The potential role of the hinged domain is also consistent with the shift of the second plateau to the left with PS (Figure 6), which may be explained by a facilitated interaction between negatively charged PS and the positive charges of the class Y helices of the hinged domains (*14*).

In the presence of low content of cholesterol, we could also detect different binding steps: adsorption followed by penetration. When we increased the chol−PL ratio, we did not observe penetration of apoA-I. This may be explained by the presence of molecules of cholesterol which tends to rigidify the PL monolayer, thereby leading to the incapacity of PL to associate with some penetrating domains of apoA-I.

Moreover it was proposed that low apoA-I concentrations already stabilize the new protein−phospholipid complex formed, without cholesterol (Figure 3) and with low content of cholesterol (Figure 4). This stabilization of the phospholipid layer by apoA-I is in agreement with what was proposed by Tytler et al. (*4*), who demonstrated that class A helices of apoA-I inhibited the lytic effect of amphipathic peptides of class L.

How to correlate this information to the understanding of some steps of the HDL metabolism? According to our results, when apoA-I interacts with phospholipids (*1*), it first adsorbs then penetrates. Lipid-bound apoA-I would stabilize the nascent HDL, in a penetrated or adsorbed state. Thus, cholesterol molecules would enter in a stable particle (disk). When the reaction with LCAT occurs, the cholesterol content in the phospholipid monolayer decreases because it is converted to esterified cholesterol which enters the core of the particle. Thus, it is plausible that the presence of apoA-I transiently stabilizes the particle, while LCAT can catalyze fatty acid transfer to cholesterol, and CETP the esterified cholesterol transfer to triglyceride-rich lipoproteins. Penetration of part of apoA-I in the lipid layer may be an important event in order to facilitate the above-mentioned reactions, combining a well-anchored protein with a mobile part involved in the reactivity of the enzyme.

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