Mechanisms of Protein-Lipid Interaction

ASSOCIATION OF APOLIPOPROTEINS A-I AND A-II WITH BINARY PHOSPHOLIPID MIXTURES*

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Studies of the recombination of apo A-I and apo A-II, the major protein components of human high density lipoprotein, with binary mixtures of dimyristoyl phosphatidylcholine (DMPC) and distearoyl phosphatidylcholine (DSPC) were performed. Recombination was observed to occur only near the lower bound temperature of the phase transition for each mixture. Similar experiments using binary mixtures of DMPC and 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) demonstrated that recombination occurs in a temperature range which is believed to approximate the lower bound of the phase transition for this mixture as well. The reactivity of both types of mixtures toward recombination with apolipoproteins was found to decrease with decreasing proportions of DMPC, even though the effect of DMPC was to decrease the transition temperature in DMPC/POPC mixtures and to increase the transition temperature in DMPC/DSPC mixtures. A mechanism for insertion of apolipoproteins into lipid bilayers is proposed in which the protein gains entry to the interior of the bilayer through a defect resulting from equilibrium fluctuations of state at the onset temperature of acyl chain melting.

The insertion of proteins into lipid bilayers is a critical event for the assembly of the plasma lipoproteins and the hydrolysis of phospholipids by pancreatic phospholipase A2 (1), as well as the addition of protein components to membranes (2). An important, but unfulfilled, goal is to understand the mechanism of protein insertion and how the properties of component lipids in the membranous bilayer modulate this process.

As a model system for studying this problem, we have investigated the interaction of the major apolipoproteins of human high density lipoprotein, apo A-I and apo A-II, both with pure phospholipids and with binary mixtures of dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine (3, 4). These studies have demonstrated the profound dependence of protein insertion upon the physical state of the phospholipid, an observation reported previously by Pownall et al. (5, 6). Additionally, our data have suggested that protein insertion is dependent on the cooperativity of the gel-liquid crystalline phase transition, such that apo A-I can associate more readily with phospholipids which show highly cooperative melting than with those which do not.1

This report concerns the interaction of apo A-I and apo A-II with binary mixtures of DMPC2 and distearoyl phosphatidylcholine (DSPC), as well as DMPC and 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC). Interest in these mixtures stems from several factors. First, we have found that both DSPC and POPC are quite refractory by themselves to interaction with apo A-I or apo A-II, as evidenced by clearing of turbidity (6), and it was of interest to see whether the presence of DMPC, a lipid which is highly reactive with these apolipoproteins, would facilitate protein-lipid interaction. Second, binary mixtures, such as DMPC/DSPC, undergo lateral phase separation over a wide temperature interval (7-9) similar to that observed with biomembranes (2); consequently, such mixtures appear to be better models for biomembranes than do pure phospholipids. Third, reconstitution of apolipoproteins with a synthetic phospholipid like POPC, which bears an unsaturated acyl chain, has not previously been reported, and should produce a lipid-protein complex with properties more similar to those found under physiological conditions than those formed with phospholipids in which both acyl chains are saturated.

MATERIALS AND METHODS

High density lipoprotein was isolated from the d = 1.063 to 1.21 fraction of fresh human plasma by ultracentrifugation. This fraction was delipidated by solvent extraction (10) and the apolipoproteins were purified by gel filtration column chromatography (3). Prior to use, apo A-I and apo A-II were denatured in 6 M guanidine hydrochloride and refolded by slow removal of the denaturing agent (3).

Dimyristoyl phosphatidylcholine and distearoyl phosphatidylcholine were purchased from Calbiochem (La Jolla, CA) and 1-palmitoyl-2-oleoyl phosphatidylcholine was obtained from Supelco (Bellafonte, PA). Cholesterol was purchased from Applied Science (State College, PA). These lipids were found to give single spots with I2 vapor when chromatographed on thin layers of silica gel using CHCl3:CH2OH:2.5 n NH2OH (70:30:5).

Lipid mixtures were prepared by mixing the appropriate lipids in benzene/methanol (4:1) and evaporating the solvent under N2 at room temperature. Any remaining solvent was removed in a vacuum oven at 40°C over a period of 2 to 4 h. Aqueous buffer was added (0.01 M Tris-HCl, pH 7.4, 8.5% KBr, 0.01% azide, 0.01% EDTA) to bring the concentration of phospholipid to 0.25 mg/ml and the sample was warmed to above its transition temperature. The lipids were then dispersed with a Vortex mixer for 1 min, placed in a low power sonication bath (ultrasonic cleaner, Branson Scientific) for 10 min, and dispersed again with a Vortex mixer for 30 s to obtain turbid dispersions of multilamellar vesicles.

Kinetic studies of recombination employing temperature programming were performed (6). Aliquots from the lipid dispersion (1 ml) were placed in three thermostated spectrophotometer cells and, after equilibration at the desired temperature, 0.1 ml of apo A-I (1.0 mg/

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1 The abbreviations used are: DMPC, dimyristoyl phosphatidylcholine; DSPC, distearoyl phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl phosphatidylcholine; Tm, temperature at which a maximum rate of recombination is achieved during a programmed temperature rise; XPOPC, the mole fraction of POPC in a binary mixture with DMPC.
RESULTS

Interaction of Apo A-I and Apo A-II with DMPC/DSPC Mixtures—Fig. 1 shows measurements of turbidity for aqueous dispersions of DMPC/DSPC mixtures as a function of temperature during a programmed heating or cooling experiment (12°C/h) following the addition of apo A-I or apo A-II. Cooling curves indicate a significant difference in the onset of the phase transition, and the cooling curves tend to be sharper than those observed with apo A-I. Maximum absorbance at 325 nm was measured as a function of time during the time interval of the experiment. The cooling curves (Fig. 1) indicate a significant difference between apo A-I and apo A-II with respect to recombination with these phospholipid mixtures. Rapid rates of recombination with apo A-I are observed within a few degrees of the transition temperature, as demonstrated by the cooling curves, whereas rates of recombination with apo A-II are sluggish, even at its transition temperature. When low proportions of cholesterol were incorporated into the lipid mixture, recombination was greatly facilitated and reached a maximum between 0 and 11% cholesterol. As the cholesterol proportion was increased above 11%, a marked reduction in recombination rate occurred; at a proportion of cholesterol above 19%, the dispersions failed to clear during the heating program. Similar results have been reported for apo A-I recombination with DMPC/cholesterol mixtures (16).

Interaction of Apo A-I and Apo A-II with DMPC/POPC Mixtures—Phospholipid components of the plasma lipoproteins generally possess a saturated acyl chain in position 1 and an unsaturated chain in position 2. Accordingly, Roseman et al. (17) have proposed that 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) would be a good model lipid, since these acyl groups are especially common in phospholipids of membranes and the plasma lipoproteins. We have determined the transition temperature of POPC by differential scanning calorimetry and found it to occur at $-2.5^\circ$C. Temperature-programmed studies of pure POPC with either apo A-I or apo A-II gave no evidence for clearing with heating or cooling programs between 2°C and 25°C.

Consequently, studies similar to those described above were performed with binary mixtures of DMPC and POPC (Fig. 5). As before, rapid clearing of turbidity was observed only over a narrow temperature interval with both heating and cooling programs. As the percentage of POPC in the mixture is increased, the temperature at the inflection point decreases. Of particular significance is the observation that, as the mole fraction of POPC in the mixture is increased, the rate of recombination, estimated as the slope at the inflection point or the total change of absorbance over the course of the experiment, decreases. This effect was particularly evident with apo A-II, but at approximately 0.69, neither apo A-I nor apo A-II appears to react with the lipid mixture during the time interval of the experiment. Contrary to our studies of DMPC/DSPC mixtures and studies of other phospholipids, the rates of recombination with DMPC/POPC mixtures decrease with decreasing inflection point temperature. We believe that this observation is not attributable merely to the decreased kinetic energy of the system, since dilauroyl phosphatidylcholine was found to be very reactive with A-1 at 3°C and ditridecanoyl phosphatidylcholine was very reactive at 13.5°C (where its gel-liquid crystalline transition occurs).

Data for the inflection point temperature obtained from heating curves with apo A-I as a function of X_{POPC} are plotted in Fig. 6. These data reveal a smooth curve reminiscent of a solidus curve for a phase diagram. If these data do, indeed, reflect the solidus curve, the phase diagram for this mixture would more closely resemble that of a DMPC/DPPC mixture (8, 9) than that of a DMPC/DSPC mixture (Fig. 4), suggesting that the DMPC/POPC combination is more miscible than is the DMPC/DSPC pair.

When recombination experiments were performed with a X_{POPC} = 0.31 mixture containing various proportions of cholesterol, results similar to those obtained with DMPC/DSPC mixtures were obtained. Specifically, the ability of apo A-I to...
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**DISCUSSION**

The use of binary phospholipid mixtures in studies of recombination with apolipoproteins was selected in order to attempt incorporation of a wider range of phospholipids than have heretofore been reported, and to obtain well defined recombinant particles whose lipid components resemble as closely as possible those occurring naturally, such as POPC and DSPC. Although we were unable to form complexes between apo A-I or apo A-II with these lipids in their pure state, binary mixtures of these phospholipids with DMPC appeared to recombine with apolipoproteins, as judged by clearing of turbidity. Previous studies have consistently demonstrated a correlation between clearing of phospholipid dispersions by apolipoproteins and the formation of small recombinant particles (4-6, 18-20). Furthermore, through the use of radioactively labeled DSPC and DMPC, we have demonstrated equal incorporation of these lipids into small complexes with apo A-I (21).

Temperature-programming studies demonstrate that for
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apo A-I and, to a lesser extent, for apo A-II, recombination with phospholipid mixtures occurs only over a narrow temperature interval (Figs. 1 and 5). Upon heating, the temperature of most rapid reaction is found to agree well with the known onset temperature for the phase transition of DMPC/ DSPC mixtures (8, 9). Based on these data and on previous results, we would speculate that Fig. 6, which shows the inflection point temperatures as a function of lipid composition for DMPC/POPC mixtures, should provide a good approximation of the solidus curve for this phospholipid pair.

Studies with pure phospholipids have previously shown preferential reaction toward recombination with apo A-I near the gel-liquid crystalline transition (5, 6), and this has been interpreted to mean that protein insertion occurs in a lattice defect at a gel-liquid crystal interface (6, 21). Theoretical treatments of the phase transition process have demonstrated that the fraction of boundary lipid between fluid and solid domains is maximal at the midpoint of the phase transition, and it has been postulated that defects arising at this boundary due to incompatible energy states are responsible for the maximal permeability properties observed at these temperatures (22-24).

It has been established that phospholipids undergo the phase transition in groups or clusters and that the size of these clusters is proportional to the cooperativity of the transition (25). A convenient expression for cooperativity is the cooperative unit size, which represents the apparent number of molecules which undergo the phase transition in concert. The size of the lattice defect where insertion is assumed to occur has been related to the size of these clusters (23). Based on recombination studies with a variety of phospholipids, we believe that the rate of recombination is proportional to the cooperativity of the particular phospholipid under study. We propose that the greater rates of recombination observed with apo A-II, relative to apo A-I, reflect mainly the smaller size of this protein and a less stringent requirement for a large lattice defect. It is also possible that the greater hydrophobicity of apo A-II also promotes faster kinetics of recombination.
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Fig. 5. Turbidity as a function of temperature at a programmed heating rate of 1°C/h for DMPC/POPC mixtures alone (A), or in the presence of apo A-I (B) or apo A-II (C). Apo A-I or apo A-II (0.1 mg) was added to phospholipid (0.24 mg) which had been equilibrated at the initial temperature of the heating program. The mole fraction of POPC in the mixture was: A, 0.18; B, 0.31; C, 0.47; and D, 0.69. Arrows indicate the estimated temperature at the inflection point.

In the present investigations, the rate of recombination of apo A-I or apo A-II with DMPC/DSPC mixtures was found to decrease as the proportion of DMPC decreased. Since DMPC has been shown to possess a much larger cooperative unit size than does DSPC (25), we believe that the results of Fig. 1 indicate that the cooperativity of the transition decreases as the proportion of DMPC in the mixture decreases. Likewise, we expect that POPC has a smaller cooperative unit size than does DMPC, and that the cooperativity of the POPC/DMPC mixture decreases as the proportion of POPC increases. It is noteworthy that with all other phospholipids and phospholipid mixtures studied, the rate of recombination was found to increase as the transition temperature decreased; this led us to consider the possibility that the rate of recombination was proportional to the lifetime of the lattice defect. By contrast, the observed decrease in reaction rate for POPC/DMPC mixtures of decreasing transition temperature suggests that the cooperativity of the phospholipid is a stronger determinant of the recombination rate than the lifetime of the lattice defect.

We deem it a salient finding that recombination is maximal at the onset temperature of the phase transition. This is to be distinguished from studies where analysis of the proportion of boundary lipid as a function of temperature (23) leads to a prediction that recombination should be maximal at the midpoint of the phase transition. We therefore believe that defects large enough to accommodate these proteins require the presence of a preponderance of solid phase lipid.

Based upon mathematical treatment of calorimetric data, Freire and Biltonen (24) have shown that throughout the phase transition interval, phospholipid molecules are found in clusters which undergo rapid fluctuations between solid and fluid phases, resulting in localized volume fluctuations. The number of such local defects would be maximal at the midpoint of the phase transition; therefore, to explain our data, we have extended the model of Freire and Biltonen in the fashion depicted in Fig. 7.

Upon heating, a cluster of phospholipid molecules melts to form a fluid domain surrounded by solid phase lipid (Fig. 7, State II). Since the fluid phase is less densely packed, some rearrangement of the solid phase is required, possibly resulting in packing defects (26), but these would likely be too small to
permit insertion of larger proteins like apo A-I. Due to the phase fluctuations (24), the fluid clusters have a high probability of reverting back to the solid phase; upon solidification, a large defect would be created from the remaining "excess" volume (State III). This excess volume can be calculated from the per cent volume change accompanying the phase transition and the size of the cluster, if known. The defect created by the excess volume could be expected to have a long lifetime only near the onset temperature of the phase transition, where it would be surrounded by immobilized solid phase lipid, and this would provide an opportunity for a protein to become inserted in the bilayer. Thus, our data appear to provide experimental verification of the role of phase fluctuations, as predicted by Freire and Biltonen, and indicate the importance of the onset temperature of chain melting for protein insertion.

In addition, we believe that the property of lateral phase separation (2) could also contribute to the observed maximal rate of recombination at the onset temperature of acyl chain melting. Since the composition of the fluid phase is indicated by the intersection of the horizontal tie line with the fluidus curve of the phase diagram (27), raising the temperature of a given mixture above the onset temperature of melting would result in a progressively decreasing proportion of DMPC in the fluid phase. Since it is reasonable to assume that the cooperativity of the fluid phase lipid would be proportional to the DMPC content (in the case of a DMPC/DSPC mixture), the maximum cooperativity would be found at the onset temperature of melting (solidus curve), thus promoting a maximum rate of recombination.

The presence of cholesterol in a bilayer containing DMPC and DSPC (1:1, w/w) was found to alter profoundly the rate of recombination with apo A-I (Fig. 4). The observed rate enhancement at low proportions of cholesterol with subsequent rate diminution at higher levels is difficult to explain. In the context of our model, we would propose that the fundamental effect of cholesterol is to lower the rate of reaction by decreasing the cluster size and hence the size of the volume fluctuations occurring over the phase transition interval. Mabrey et al. (28) have reported decreasing cooperativity for DPPC/cholesterol mixtures as the proportion of cholesterol is increased, as have others (29). It has been proposed that a 4:1 lecithin:cholesterol phase is formed, so that 20 mol % cholesterol, the highly cooperative lecithin phase is abolished and is supplanted by a relatively noncooperative lecithin:cholesterol phase. This could, then, explain the marked reduction in recombination at higher proportions of cholesterol. A possible explanation for the rate enhancement observed at low cholesterol levels might be that a small amount of cholesterol or lecithin:cholesterol component could promote the conversion between States II and III (Fig. 7), or possibly it might ensure that the excess volume resulting from the conversion from State II to State III is not fragmented into multiple small lattice defects. As the proportion of cholesterol in the bilayer is increased, this effect would be swamped out by reduction in excess volume resulting from a decreased net cooperativity of the lipid mixture.

In summary, a model is proposed in which the insertion of apolipoproteins into phospholipid bilayers is interpreted as occurring at pockets of excess volume which accompany equilibrium fluctuation of phase states; it is proposed that these pockets of excess volume exist long enough for protein insertion only near the onset temperature of acyl chain melting. The size of these lattice defects is proportional to the cooperativity of the phospholipid, so that lipids of higher cooperativity are more reactive toward recombination. The effect of temperature on the composition of the fluid phase also promotes maximum cooperativity, and hence maximum reactivity, at the onset temperature of melting for lipid mixtures. From a practical standpoint, these studies have demonstrated that appropriate mixtures of phospholipids can be prepared which have a sufficiently large net cooperativity to allow lipophilic proteins to associate with inherently unreactive phospholipids of low cooperativity, thus facilitating the production of a variety of lipid-protein complexes for further study. Characterization of a few examples of such complexes is reported in the accompanying study (21).
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REFERENCES