Kinetics and Mechanism of Association of Human Plasma Apolipoproteins with Dimyristoylphosphatidylcholine: Effect of Protein Structure and Lipid Clusters on Reaction Rates†

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ABSTRACT: We have used a series of lipid-associating proteins with similar p*I*'s and with molecular weights between 2280 and 28 000 to study the mechanism of lipid–protein association. All of these polypeptides spontaneously associate with dimyristoylphosphatidylcholine (DMPC) to give quasi-discrete products. The reaction of the apoproteins with unsaturated lecithins is slow, if it occurs at all. Our data support the Kanehisa–Tsong cluster model [Kanehisa & Tsong (1978) *J. Am. Chem. Soc. 100*, 424] of lipid permeability in many of its qualitative aspects. These are (a) that the rate of lipid–protein association increases with decreasing polypeptide molecular weight, (b) that there is a small temperature dependence for the rate of association of small peptides with DMPC but with large polypeptides the temperature at which association with lipid is rapid is confined to the solid → fluid transition temperature (T_c) of DMPC, and (c) that the rate is asymmetric about T_c, with the change in the rate with respect to temperature below T_c being greater than at $T > T_c$. In addition, we have shown that unfolded monomeric proteins with a large number of exposed hydrophobic residues associate with DMPC faster than self-associated and/or folded proteins. Our data suggest that the association of some of the apoproteins with phospholipids is subject to kinetic control.

The human plasma lipoproteins are the primary vehicles for the transport of cholesteryl esters, triglycerides, cholesterol, and phospholipids and are operationally defined according to their densities as the high, low, and very low density lipoproteins (HDL, LDL, and VLDL, respectively).[1] In addition to lipid, the lipoproteins are composed of specialized proteins (apolipoproteins) which solubilize lipids into small macromolecules with molecular weights between 10^5 and 10^7 (Smith et al., 1978). It is now recognized that the sparingly soluble apoproteins and lipids can freely exchange among lipoproteins and phospholipid vesicles by a mechanism that probably involves transport of monomers through the aqueous phase (Smith et al., 1978; Doody et al., 1980; Charlton et al., 1976; Leto et al., 1980; Roseman & Thompson, 1980).

In addition to transporting lipid, some of the apoproteins are activators of lipid-catabolizing enzymes while others have been implicated in a receptor-mediated uptake of cholesterol-rich lipoproteins by cultured cells; thus the transport of apolipoproteins among lipoproteins is an important component of a rational understanding of several aspects of lipid metabolism. More specifically, the association of apoproteins with lipids would appear to play a major role in lipoprotein dynamics. Numerous investigators have shown that apoA-I spontaneously associates with dimyristoylphosphatidylcholine (DMPC) single and multilamellar vesicles to give a small lipid–protein complex ($M_r \sim 10^5$) (Jonas & Krajnovich, 1977; Tall et al., 1976). The rate of formation of the product is stimulated by small amounts of cholesterol and is fastest at the transition temperature ($T_c = 23.9°$) of the lipid (Pownall et al., 1978a,b, 1979). The association of apoA-I with more physiologic lecithins is much slower, if it occurs at all, and there has been considerable controversy surrounding this question (Jonas & Krajnovich, 1977; Reynolds et al., 1977; Pownall et al., 1978a,b; Assmann & Brewer, 1974; Tall et al., 1976). We reasoned that a more thorough investigation of the kinetics and mechanism of lipid–apoprotein association might resolve some aspects of this problem; i.e., is the association of physiologic lecithins with apoproteins kinetically controlled?

In this paper, we compare the rates of association of DMPC with four common apolipoproteins (Table I) and with a model lipid-associating protein of 20 residues, LAP-20, which is (Pownall et al., 1980)

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1                   5                  10
Val-Ser-Ser-Leu-Leu-Ser-Ser-Leu-Lys-Glu
                   15                  20
Tyr-Trp-Ser-Ser-Leu-Lys-Glu-Ser-Phe-Ser
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Materials and Methods

DMPC (99+) was obtained from Calbiochem, La Jolla, CA. 1-Palmitoyl-2-palmitoleoyl-PC (PPOPC) and 1-stearoyl-2-oleoyl-PC were prepared by the acylation of their respective lysolecithins. 1,2-Dielaidoyl-PC and 1,2-dimyristoleoyl-PC were prepared by the method of Patel et al. (1979). All lecithins were purified by preparative liquid chromatography on a Waters Prep/LC-500 chromatograph (Patel & Sparrow, 1978).

The ^{3}[H]cholic acid (New England Nuclear, Boston, MA) from the vendor contained considerable amounts of radioactive impurities which could not be removed by dialysis. Both the unlabeled and the radioactive cholic acids were purified by chromatography on Brinkmann silica gel 60 by using a mixture of isooctane, 2-propanol, acetic acid, and water (72:25:1:2).

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[1] Abbreviations used: HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low density lipoprotein; PC, phosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; T_c, gel → liquid-crystalline transition temperature; apoA-I, apoprotein A-I, the most abundant protein of human HDL; apoA-II, apoprotein A-II, the second most abundant protein human HDL; RCM-A-II, the monomer of apoA-II obtained by reduction and carboxymethylation; apoC-III, the major soluble apoprotein of VLDL; Ans, 2-anilinonaphthalene-6-sulfonate; Gdn·HCl, guanidine hydrochloride; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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Sodium cholate was prepared by titration of the cholic acid with sodium hydroxide.

ApoA-I and apoA-II were obtained according to published procedure (Pownall et al., 1978a,b). ApoC-III$_1$ was isolated according to Brown et al. (1969). LAP-20 was synthesized by solid phase methods (Pownall et al., 1980; Sparrow et al., 1977; Sparrow, 1976). Reduced and carboxymethylated apoA-II (RCM-A-II) was prepared by the method of Jackson et al. (1973).

Complexes of PPOPC and apoA-I were prepared as follows: PPOPC (13.7 μmol) was dispersed in 1.0 mL of buffer by vortexing briefly and combined with 137 nmol of apoA-I in 0.5 mL of buffer. The highly turbid sample was divided into two equal portions and the temperature raised to 37 °C. Sodium cholate was added to one sample until complete clarification of liposomal turbidity was observed. Typically, 40 μL of a 20% cholate solution is sufficient, although a small excess did not effect the final product. The sample was passed over a column of Bio-Rad P-4 and the complex collected in the void volume. The cholate elutes much later as a micelle. The amount of cholate remaining with the complex is less than 0.25 mol/mol of apoA-I (<0.1%). All isolated complexes and the control sample without added cholate were chromatographed on a 1.6 × 30 cm column of Sepharose CL-4B at 24 °C.

DMPC–Apoprotein Assembly. Kinetic turbidimetric measurements were identical with those of Pownall et al. (1978a, 1979). DMPC (20 mg) was vortexed briefly above its T_c in 1 mL of buffer composed of 8.5% KBr, 0.01% sodium azide, 0.01% EDTA, and 0.01 M Tris, pH 7.4. This buffer and liposome preparation were used throughout unless otherwise indicated. The KBr gives a solution of sufficient density to prevent sedimentation of the liposomes.

For slow kinetic experiments, DMPC and the apoprotein were preincubated in quartz spectrophotometer cells (1-cm path length) in a thermostated cell compartment of a Cary 15 spectrophotometer for 10 min. The protein and lipid were then mixed and lipid–protein association followed by monitoring the rate of clearing of lipid turbidity measured at 325 nm with the spectrometer operated in the absorbance mode. For experiments that were too fast to be measured by this method, a Durrum stopped-flow unit was used. The Durrum stopped-flow unit, also in the absorbance mode, was interfaced with a Biomation 805 transient recorder whose output was fed to an X-Y recorder. DMPC and the apoprotein were loaded into separate syringes of the instrument. After the bath surrounding the syringes and all compartments reached thermal equilibrium, numerous mixings of 250 μL each of lipid and protein were performed until a reproducible trace was obtained. Details of concentrations are in the figure legends.

Column Chromatography. Chromatography of DMPC–apoprotein complexes was conducted with Sepharose CL-4B over a 1.6 × 30 cm water-jacketed column; the column temperature was maintained at 24 °C. The column effluent was monitored by absorbance at 280 nm and phosphorus analysis. The recovery of apoproteins and DMPC was >95%.

Results

Column Chromatography of DMPC–Apoprotein Complexes and the Correlation of Turbidity with Complex Formation. All of the apolipoproteins in Table I spontaneously associate with highly turbid DMPC liposomes to give small lipid–protein complexes which have a low optical absorbance. The relationship between absorbance at 325 nm and the fraction of lipid and protein associated is not linear, but as the example in Figure 1 shows, there is good correlation. Addition

Table I: Composition of Chromatographically Isolated DMPC–Apolipoprotein Complexes[a]

	M_r	lipid–protein ratio[b] w/w	lipid–protein ratio[b] m/m	PC incorporated (%)
apoA-I	28400	2.2	92	100
apoA-II	17400	2.3	60	100
apoC-III	9300	2.5	32	100
RCM-A-II[d]	8700	2.0	26	100
apoA-I + PPOPC[c]		2.0	85	>95
LAP-20	2280	10.1	20	100

[a] Taken from data of Figure 2. Initial lipid–protein ratios were 2:1 (w/w). [b] w/w = weight/weight; m/m = molar ratio. [c] Prepared by cholate method. [d] Reduced and carboxymethylated according to Jackson et al. (1976).

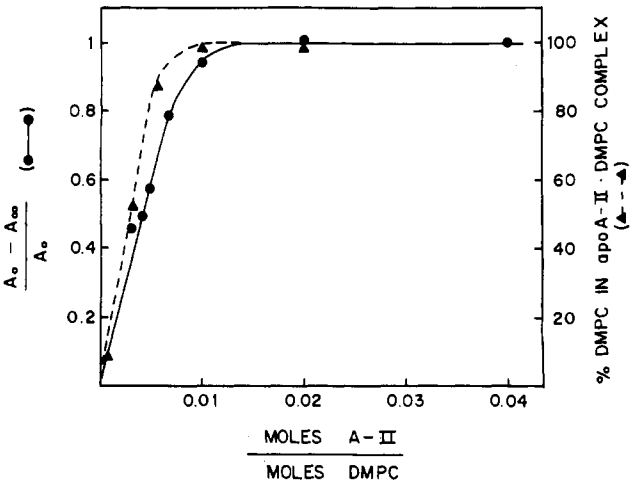

FIGURE 1: Amount of lipid incorporated into a DMPC–apoA-II complex as a function of the initial protein–lipid ratio. Various molar ratios of apoA-II were added to a fixed concentration of DMPC (0.5 mg/mL in 3 mL) at 24 °C. Two parameters were measured. One is the turbidity of the mixtures defined as $(A_0 - A_\infty)/A_0$ where A_0 and A_∞ are the initial and final values of the absorbance at 325 nm. The second parameter is the % DMPC in the complex, which is the amount of DMPC in a DMPC–apoA-II complex isolated in a water-jacketed column (1.6 × 40 cm) of Sepharose CL-4B divided by the amount of DMPC in the incubation mixtures.

of apoA-II to liposomal suspensions of DMPC resulted in partial or complete clearing of turbidity, indicating the dissolution of large phospholipid liposomes into smaller DMPC–apoA-II complexes.[2] Figure 1 shows the percent of DMPC found in DMPC–apoA-II complexes and the relative turbidity of the lipid and protein mixtures obtained when different molar ratios of apoA-II are added to a fixed concentration of DMPC at 24 °C. The percent of DMPC incorporated into the DMPC–apoA-II complex is calculated from the ratio of DMPC in a lipid–protein complex isolated by Sepharose CL-4B chromatography to the total amount of lipid contained in the initial incubation mixture. Unassociated DMPC is found at low apoA-II to DMPC molar ratios. However, when the starting molar ratio of DMPC to apoA-II was 100 or less, all liposomal turbidity was cleared, and 95–100% of the

[2] Turbidity data must be considered with some caution; the liposome mixtures used in these studies were polydisperse, and the total light-scattering intensity from these mixtures represents different contributions from each size of liposome weighted according to their relative abundance. As seen in Figure 1, the light-scattering intensity is only a qualitative representation of the amount of complex formed. The relationship between liposome size, amount of complex formed, and light-scattering cross section is nonlinear so that the changes in light-scattering intensity with time would not be expected to be exponential even for a first-order reaction.

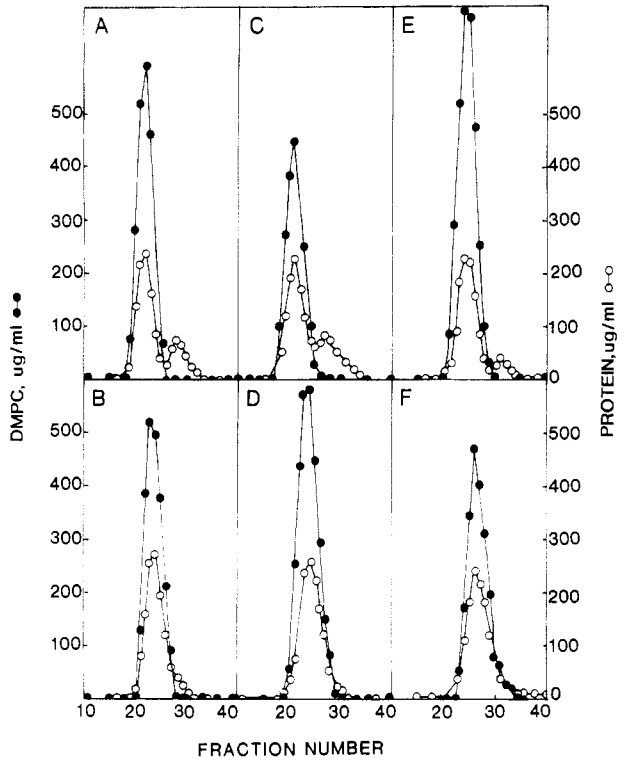

FIGURE 2: Chromatography of PC polypeptide/apoprotein complexes on Sepharose CL-6B. DMPC multishelled liposomes (5 mg) were mixed with the peptide or protein (2.5 mg) and incubated at T_c for 1 h or until clarification occurred. The sample in 1 mL was applied to a thermostated column (24 °C) and eluted with buffer. Fractions (1.5 mL) were collected and analyzed for protein and lipid. (A) ApoC-III; (B) apoA-II; (C) RCM-A-II; (D) apoA-I; (E) LAP-20; (F) apoA-I/PPOPC complex prepared by cholate method as described under Materials and Methods. More than 95% of the lipid and protein applied to the column was recovered. The void and total volumes appeared at fractions 14 and 42, respectively.

DMPC coeluted with apoA-II when chromatographed on Sepharose CL-4B. We have defined the relative turbidity as $\{(A_0 - A_\infty)/A_0\}$, where A_0 and A_∞ represent the initial and equilibrium values, respectively, for the optical absorbance at 325 nm. Comparison of the variations of $(A_0 - A_\infty/A_0$ with the percent DMPC isolated in an DMPC–apoA-II complex vs. moles of apoA-II/moles of DMPC curves indicates that the parameter, $(A_0 - A_\infty/A_0$, gives a qualitatively reliable representation of the amount of a lipid–protein complex formed. Therefore, changes in turbidity can be used to follow the rate of formation of a DMPC–apoprotein complex, particularly when systems are compared where the differences are between 1 and 3 orders of magnitude different (see below). At a lipid to protein ratio (w/w) of 2, all of the apoproteins reduce the absorbance at 325 nm of a 1 mg/mL dispersion of DMPC from 1.0 to less than 0.05 if the reaction goes to completion. Also, if allowed to go to completion, >95% of the lipid in these mixtures was found in a complex, as verified by chromatography of the lipid and protein on Sepharose CL-4B (Figure 2). The elution volumes of the complexes were much larger than those of multishelled liposomes in the starting mixture; this finding suggests that the complexes are much smaller than liposomes or vesicles and is consistent with the much lower light scattering of these complexes as assessed from the reduced absorbance at 325 nm.

A similar series of experiments was performed with apoA-I and several unsaturated lecithins. These were 1,2-dielaidoyl-PC, 1-stearoyl-2-oleoyl-PC, 1,2-dimyristoleoyl-PC, and PPOPC. When the same spectral and chromatographic criteria given above was used, little or no association of these

Table II: Comparative Rates of Association of Apolipoproteins with DMPC

apoprotein	rate constant, $K_{1/2}$ (min^{-1}) $T < T_c$ (22.5 °C)	$T = T_c$ (24 °C)	$T > T_c$ (27 °C)
apoA-I	0.002	0.08	0.003
apoA-II	0.02	3.0	2.0
apoC-III	1.0	12	7.0
RCM-A-II	5.0	25 (5)	3
LAP-20	60	100	116

lipids with apoA-I could be demonstrated at room temperature. At 24 °C, there was no clearance of optical turbidity, and during chromatography on Sepharose CL-4B, the lipid appeared in the void volume and the protein as a separate peak coincident with that of free apoA-I. However, if one mixes PPOPC with apoA-I in the presence of cholate and removes the cholate by chromatography on Bio-Rad P-4 (not shown), >95% of the lipid and protein coelute as a complex that exhibits very little light scattering; the details of this procedure and a characterization of the complex will appear elsewhere (H. J. Pownall et al., unpublished results). Although we could not obtain a good yield of lipid–protein complexes from mixtures of apoA-I and PPOPC, Sepharose CL-4B chromatography of the PPOPC/apoA-I complex after the cholate (Figure 2F) was removed demonstrated that the resulting complex had an elution volume similar to those formed by DMPC and each of the native apolipoproteins. The chromatographic data are summarized in Table I.

Kinetics of Lipid–Protein Association. We have monitored the rate of association of each of these polypeptides with DMPC by measuring the rate of disappearance of liposomal turbidity. There is a strong temperature dependence in the reaction rate in the vicinity of the T_c of DMPC. Some representative curves are shown in Figure 3. These curves did not obey any simple kinetic expression, although their shapes were similar, suggesting, though not proving, that similar mechanisms are operative. For the sake of comparison, we have defined a rate constant, $k_{1/2} = 1/t_{1/2}$, where $t_{1/2}$ is the time required for the disappearance of half of the relative turbidity, $\{(A_0 - A_\infty)/A_0\}$. At all temperatures, there was complete clearance of turbidity, $\{(A_0 - A_\infty)/A_0\} = 1$. This result and the data of Figure 1 suggested that the reaction of DMPC had gone to completion but that the time required for each was different. Comparing the rates below, at, and above T_c (Table II), we found that for a given apoprotein the rate was always fastest at T_c. The strong temperature dependence is dramatically illustrated in the Arrhenius plots of Figure 4. First, these data demonstrated that the rate was fastest at T_c for all five polypeptides. Second, the temperature dependence was stronger below T_c than above T_c. Third, there were marked differences among the individual apoproteins; in general, the smaller polypeptides associated with DMPC faster than did the large ones. Finally, the temperature range for the maximal rate of association, which was narrowest for apoA-I, the largest of the apolipoproteins studied, decreased as the molecular weight of the apoproteins decreased.

The rate of lipid–protein association was also dramatically influenced by the addition of Gdn·HCl. In Figure 5, the rate constant for the clearance of DMPC turbidity by each of the apolipoproteins is plotted as a function of the guanidine hydrochloride concentration in the reaction medium. Between 0 and 2.0 M denaturant, the rate increased by a factor of 2 and 5, respectively, for apoC-III and RCM-A-II. For both there was a sharp increase at about 0.3 M Gdn·HCl. By contrast, both apoA-I and apoA-II exhibited a pair of inflection

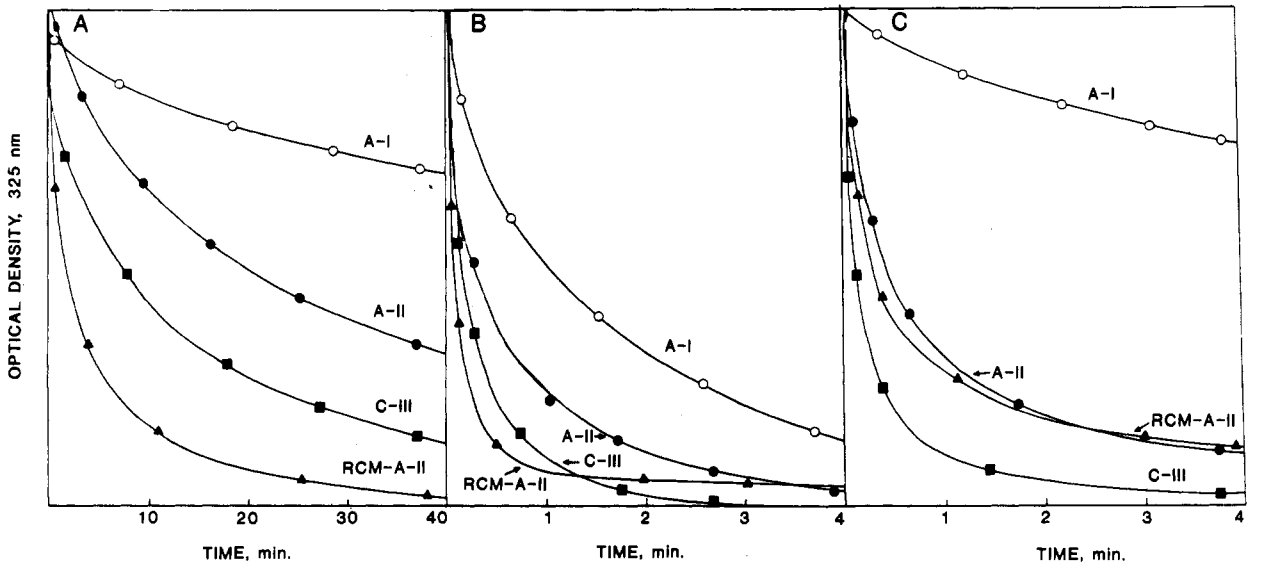

FIGURE 3: Representative traces of clarification of DMPC turbidity by apoproteins. DMPC (0.5 mg/mL) and the apoprotein (0.25 mg/mL) were preincubated for 10 min at a given temperature and equal volumes mixed at the same temperature in a 1-cm path length spectrophotometer cell. The decrease in absorbance was recorded as a continuous function of time. (A) 23 °C; (B) 24 °C; (C) 30 °C.

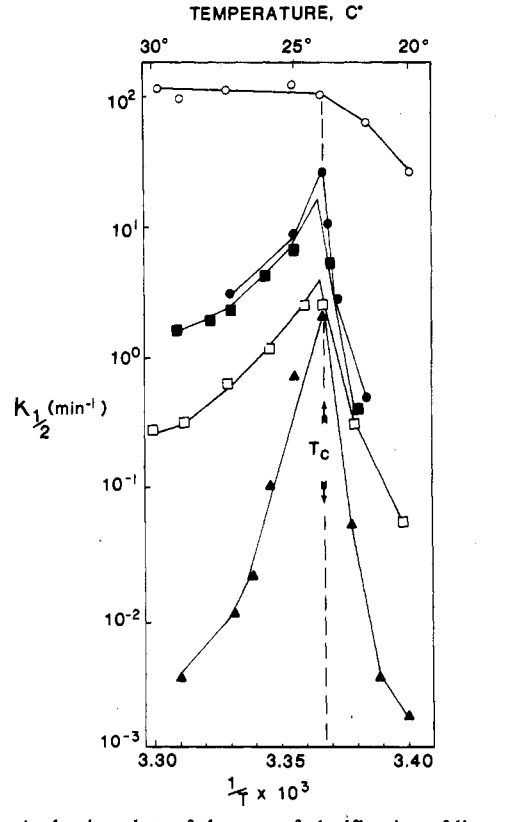

FIGURE 4: Arrhenius plots of the rate of clarification of liposomal turbidity by apoproteins and LAP-20. Data were taken from Figure 3, and a larger body of data that were collected in the same way at other temperatures. (○) LAP-20; (●) RCM-A-II; (■) apoC-III; (□) apoA-II; (▲) apoA-I.

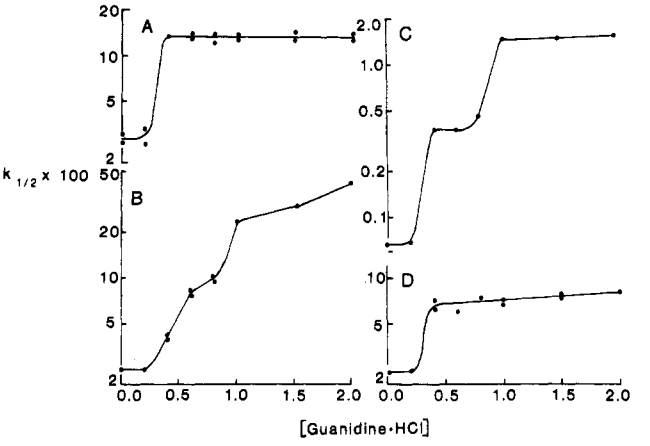

FIGURE 5: Effect of Gdn·HCl on the rate of clearance of liposomal turbidity by the apolipoproteins. DMPC and protein concentrations were the same as for Figure 3. (A) RCM-A-II; (B) apoA-II; (C) apoA-I; (D) apoC-III. Temperature of the experiment was 24 °C.

points in the plot of rate constant vs. Gdn·HCl concentration. These appeared at 0.3 and 1.1 M for apoA-I and 0.4 and 0.9 M for apoA-II. Over the same concentration range, the change in the rate of association of LAP-20 and DMPC at 24 °C was nil. Furthermore, similar experiments with apoA-I in the presence of 2.0 M sodium chloride instead of Gdn·HCl slowed the reaction by 50%.

Discussion

The gel filtration studies on Sepharose CL-4B demonstrated that all of the polypeptides investigated spontaneously associated with DMPC to give a stable isolatable complex. In each instance, the elution position of the complex was similar, and each isolated complex contained >95% of the phospholipid. In the chromatography of phospholipid complexes of apoA-I and apoA-II, nearly all of the protein coeluted with the complex. However, with LAP-20, RCM-A-II, and apoC-III, free protein peaks elute after the complex, suggesting that the stoichiometric lipid–protein ratio is greater for these proteins (see also Table I). Although apoA-I and PPOPC did not spontaneously associate, a complex prepared by an indirect method had an elution volume similar to that of DMPC and apoA-I. All of the complexes were sufficiently small to be included in Sepharose CL-4B and had estimated molecular weights of 150 000–400 000. As a consequence, they scattered little or no light when compared to the initial preparation of multishelled liposomes. Therefore, the disappearance of liposomal turbidity correlated well with the formation of a lipid–protein complex.

A great many phenomena have been reported to be accelerated at the transition temperature of a lipid and several theories have been proposed to accommodate these observations. These phenomena include solubilization of DMPC by lysolecithin (Harlos et al., 1977), the passive permeation of ions (Blok et al., 1975; Papahadjopoulas et al., 1973), sucrose (Papahadjopoulas et al., 1973), valinomycin (Wu &

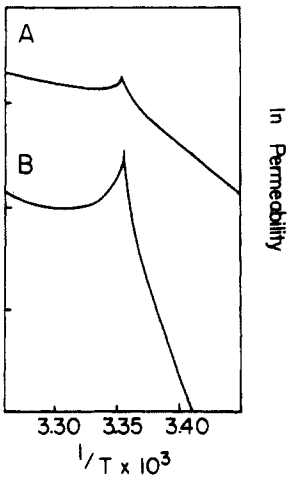

FIGURE 6: Theoretical curves for the temperature dependence of the permeability of lipid bilayers based upon the cluster model, assuming a positive correlation between permeability and the rate of penetration of apoproteins into DMPC. These curves are representative of the permeation rates of small molecules, ions, and nonhydrophobic molecules. The plot and parameters used are identical with those used by Kanehisa & Tsong (1978), except that the data are plotted against the reciprocal of the absolute temperature for comparison with Figure 4. In this model, the gel and liquid-crystalline phases are impermeable; penetration of the lipid matrix occurs only at the boundary between two phases. (A) Penetration rate for small amphiphiles with $l_{min} = 1$ and $E_b = 8$ kcal/mol; (B) large amphiphiles with $l_{min} = 6$ and $E_b = 25$ kcal/mol. The other parameters used were $\epsilon = 3$ kcal/mol, $s_0\gamma = 3$ kcal/mol, $s_0\omega = 9.6$ eu, $l_{max} = 300$, and $T_m' = 298$ K. Ordinate is on an arbitrary scale. In (B), the minimum cluster size, l_{min}, must be larger than in (A) in order to form pores that are large enough to accomodate large molecules; a larger activation energy, E_b, is also required for this process.

McConnell, 1973), tempocholine (Marsh et al., 1976) and the fluorescent dye, Ans (Tsong, 1975), into membranes, and the preferential hydrolysis of DMPC by porcine pancreatic phospholipase A_2 (op den Kamp et al., 1975). Pownall et al. (1978a, 1979) and Swaney (1980) have shown that apoA-I preferentially associates with phospholipid bilayers at their respective T_c's.

Our kinetic data can be best understood in terms of the Kanehisa–Tsong cluster model of lipid phase transitions which has been applied to the passive permeation of molecules through phospholipid bilayers (Kanehisa & Tsong, 1978). In that model, there are three hypothetical phases; two of these are the solid (S) or gel phase and the fluid (F) or liquid-crystalline phase, both of which are ordered. The disordered phase represents the boundary region between S and F phases. At the phase transition where both S and F phases exist, a large fraction of the molecules form a boundary region between both S and F phases. At $T \neq T_c$, the model also allows for lipid clusters, which are defined as a microdomain of the nondominant phase. Below T_c, a cluster is a group of F states within a sea of S states while the converse is true above T_c. These clusters give rise to additional boundary regions in what would be considered a macroscopically pure phase.

Several studies have suggested that the permeability of the lipid matrix is the rate-limiting determinant in the association of apoA-I with phospholipids (Pownall et al., 1978a, 1979; Swaney, 1980). Therefore, a good model for lipid permeability should also predict the rate of lipid–apoprotein association. The cluster model predicts different rates of permeation which are related to the size of the permeant and the temperature of the system with respect to the transition temperature of the lipid. In Figure 6, we provide theoretical plots based upon the cluster theory and adapted from the work of Kanehisa & Tsong (1978). The two cases shown correspond to the temperature dependence of permeability of a lipid bilayer to small and large molecules. The effects of permeant size on the theoretical rate vs. temperature profiles correspond very well with our data in Figure 4. First, the cluster model predicts that small molecules will penetrate the lipid matrix faster than large ones. The rates of association of the apoproteins decrease in the order LAP-20 (M_r 2280) > RCM-A-II (M_r 8700) > apoC-III (M_r 9300) > apoA-II (M_r 17 400) and apoA-I (M_r 28 400). Second, the model predicts that the rate of penetration of a permeant into the lipid matrix will be fastest at the transition temperature of the lipid. With the exception of LAP-20, all of the apoproteins preferentially associate with DMPC at T_c. This exception is also consistent with predictions based upon the Kanehisa–Tsong cluster model. According to that model, very small permeants exhibit little or no effects in rate even at T_c. This effect is probably due to the requirement of a larger interstice for permeation of large proteins; small clusters, therefore, do not produce interstices that are sufficiently large for the penetration of the larger molecules. With small molecules the rate maximum at T_c is predicted, though it is expected to be much smaller than that of large molecules, so there is still a good correlation in this case. Finally, the rate vs. temperature profile is predicted to be asymmetric about the transition temperature and to be very sharp for large molecules and broad for smaller ones. This again is supported by our data; the breadth of the rate vs. temperature plot increases in going from apoA-I to the smaller polypeptides, and the increase in rate in going from $T_c - 2$ to T_c is always greater than that observed going from $T_c + 2$ to T_c.

The fact that apoA-I is associated with physiologic lecithins in HDL suggests that the association is thermodynamically favorable; our isolation of a PPOPC/apoA-I complex prepared by an alternative method supports this point. We propose that the association of apoA-I with lecithins is kinetically controlled, and the Kanehisa–Tsong cluster model aids our understanding of this process. Our assembly experiments with PPOPC were conducted >25 °C above the T_c of that lipid. The lack of spontaneous association of apoA-I with PPOPC may be due to the large difference in the transition temperature of PPOPC relative to our experimental temperatures, although it may not be valid to use the behavior of lipids that have saturated acyl chains to predict the behavior of those containing an unsaturation or greater acyl chain length.

Effect of Exposed Hydrophobic Residues on the Rate of Lipid–Protein Association. The rates of association of DMPC with all four of the native apoproteins of this study change as a function of the concentration of Gdn·HCl. The denaturant concentrations where the rapid changes in the rates occur appear to correspond to the same concentrations where the apoproteins undergo major structural changes (Osborne & Brewer, 1977). ApoA-I and apoA-II (Reynolds, 1976) undergo an oligomer to monomer reaction at about 0.3 M Gdn·HCl succeeded by a helix → coil transition between 1.0 and 1.5 M Gdn·HCl.[3] In RCM-A-II (Osborne et al., 1975) and apoC-III (W. W. Mantulin and H. J. Pownall, unpublished results), these two processes occur simultaneously and appear around 0.3 M Gdn·HCl. Since both of these structural changes involve the exposure of additional hydrophobic regions, we speculate that the rate of association may be controlled by

[3] Although the point was not addressed in the manuscript, the data in Figures 4 and 5 in the Reynolds paper (1976) revealed changes not only for unfolding of apoA-I but also for dissociation; at low concentrations of Gdn·HCl, there is an increase in the negative ellipticity at 222 nm.

the hydrophobicity of the amphiphile and that the number of exposed hydrophobic residues is an important determinant in the mechanism of lipid–protein association. This hypothesis, however, requires a more rigorous test.

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References

Assmann, G., & Brewer, H. B., Jr. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 989–993.
Blok, M. C., van der Neut-kok, E. C. M., van Deenen, L. L. M., & de Gier, J. (1975) *Biochim. Biophys. Acta 406*, 187.
Brown, W. V., Levy, R. I., & Fredrickson, D. S. (1969) *J. Biol. Chem. 244*, 5687–5694.
Charlton, S. C., Olson, J. S., Hong, K.-Y., Pownall, H. J., Louis, D. D., & Smith, L. C. (1976), *J. Biol. Chem. 251*, 7952–7955.
Doody, M. C., Pownall, H. J., Kao, Y. J., & Smith, L. C. (1980) *Biochemistry 19*, 108.
Harlos, K., Vaz, W. L. C., & Kovatchev, S. (1977) *FEBS Lett. 77*, 7.
Jackson, R. L., Morrisett, J. D., Pownall, H. J., & Gotto, A. M., Jr. (1973) *J. Biol. Chem. 248*, 5218–5224.
Jackson, R. L., Morrisett, J. D., & Gotto, A. M. (1976) *Physiol. Rev. 56*, 259.
Jonas, A., & Krajnovich, D. J. (1977) *J. Biol. Chem. 252*, 2194.
Kanehisa, M. I., & Tsong, T. Y. (1978) *J. Am. Chem. Soc. 100*, 424.
Leto, T. L., Roseman, M. A., & Holloway, P. W. (1980) *Biochemistry 19*, 1911.
Marsh, D., Watts, A., & Knowles, P. F. (1976) *Biochemistry 15*, 3570.
op den Kamp, J. A. F., Kauerz, M. T., & van Deenen, L. L. M. (1975) *Biochim. Biophys. Acta 406*, 169.
Osborne, J. C., & Brewer, H. B. (1977) *Adv. Protein Chem. 31*, 253.
Osborne, J. C., Palumbo, G., Brewer, H. B., & Edelhoch, H. (1975) *Biochemistry 14*, 3741.
Papahadjopoulas, D., Jacobson, K., Nir, S., & Isac, T. (1973) *Biochim. Biophys. Acta 311*, 330.
Patel, K., & Sparrow, J. T. (1978) *J. Chromatogr. 150*, 542.
Patel, K., Morrisett, J. D., & Sparrow, J. T. (1979) *J. Lipid Res. 20*, 674.
Pownall, H. J., Massey, J. B., Kusserow, S. K., & Gotto, A. M., Jr. (1978a) *Biochemistry 17*, 1183.
Pownall, H. J., Pao, Q., Rohde, M., & Gotto, A. M., Jr. (1978b) *Biochem. Biophys. Res. Commun. 85*, 408.
Pownall, H. J., Massey, J. B., Kusserow, S. K., & Gotto, A. M., Jr. (1979) *Biochemistry 18*, 574.
Pownall, H. J., Hu, A., Albers, J. J., Gotto, A. M., & Sparrow, J. T. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3154.
Reynolds, J. A. (1976) *J. Biol. Chem. 251*, 6013.
Reynolds, J. A., Tanford, C., & Stone, W. L. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3796–3799.
Roseman, M. A., & Thompson, T. E. (1980) *Biochemistry 19*, 439–444.
Smith, L. C., Pownall, H. J., & Gotto, A. M. (1978) *Annu. Rev. Biochem. 47*, 751.
Sparrow, J. T. (1976) *J. Org. Chem. 41*, 1350–1353.
Sparrow, J. T., Pownall, H. J., Sigler, G. F., Smith, L. C., Soutar, A. K., & Gotto, A. M., Jr. (1977) *Pept., Proc. Am. Pept. Symp., 5th*, 149.
Swaney, J. B. (1980) *J. Biol. Chem. 255*, 8791.
Tall, A., Shipley, G. G., & Small, D. M. (1976) *J. Biol. Chem. 251*, 3749.
Tsong, T. Y. (1975) *Biochemistry 14*, 5409.
Wu, S. H., & McConnell, H. M. (1973) *Biochem. Biophys. Res. Commun. 55*, 484.

Phase Equilibria in Binary Mixtures of Dimyristoylphosphatidylcholine and Cardiolipin†

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ABSTRACT: Paramagnetic resonance spectra of the spin-label 2,2,6,6-tetramethylpiperidinyl-1-oxy have been used to study phase separations in binary mixtures of dimyristoylphosphatidylcholine and cardiolipin. Two different samples of cardiolipin were used: (i) One sample contained calcium ions at a mole ratio of calcium:cardiolipin = 1:2; the experimental data support the view that cardiolipin is present in the bilayer membrane as calcium ion linked dimers, $(CL)_2Ca^{2+}$. (ii) A calcium-free sodium cardiolipin sample yielded remarkable spin-label partition data that were quite different from those obtained in the presence of Ca^{2+}. In both cases the spin-label data provide evidence for compound formation and for fluid–fluid immiscibility in the bilayer membrane.

There have been numerous studies of the physical properties of binary mixtures of lipids. In a number of cases phase diagrams describing lateral phase separations in planar bilayer membranes have been described [see, e.g., Shimshick & McConnell (1973), Grant et al. (1974), Luna & McConnell (1977), Gent & Ho (1978), Lentz et al. (1978), Mabrey et al. (1978), Oldfield & Chapman (1972), and Ladbrooke & Chapman (1969)]. The most common type of two-phase equilibria that has been encountered is that in which the temperatures and compositions are such that a "solid" phase is in equilibrium with a "fluid" phase. For the purposes of the present paper, we consider a fluid lipid bilayer to be one

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