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Thermal Dependence of Apolipoprotein A-I–Phospholipid Recombination†

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ABSTRACT: Studies of the recombination of apolipoprotein A-I (apo A-I), the major protein constituent of human high-density lipoprotein, and various synthetic phospholipids, both alone and in mixtures, have been performed. Pure diacyl phospholipids containing homologous fatty acids of the C_{12}, C_{13}, C_{14}, and C_{15} series, as well as the two positional isomers containing C_{14} and C_{16} fatty acids in positions 1 and 2, undergo reaction with the apo A-I protein only near their gel–liquid-crystalline transition temperatures; the degree of reactivity of these phospholipids toward recombination was observed to decrease as the transition temperature increased. The presence of lysolecithin in the incubation mixtures at proportions of 5 mol/mol of protein or lower was not found to have a significant effect on the rate of recombination. Binary mixtures of dimyristoylphosphatidylcholine and dipalmitoylphosphatidylcholine at various proportions react maximally with apo A-I at the onset of the phase transition, as judged by comparison with published phase diagrams; in this case also, the rate of recombination was observed to decline for mixtures with higher phase transition temperatures. These results are interpreted in terms of protein insertion at lattice defects arising from the presence of phospholipid clusters undergoing the phase transition; these clusters are derived from the cooperative and simultaneous melting of a number of molecules, the cooperativity being dependent upon the nature of the phospholipid. It is postulated that phospholipids which melt in a more highly cooperative fashion are more capable of interacting with the apolipoproteins since these phospholipids will possess larger lattice defects during the phase transition.

A great deal of interest has been focused in recent years on the recombination[1] of apolipoproteins, especially apolipoprotein A-I (apo A-I) from the high-density lipoprotein, with synthetic phospholipids. The apo A-I is of particular interest because it is the major protein component of human high-density lipoprotein (Shore & Shore, 1969); the high-density lipoprotein of several other mammalian species (Edelstein et al., 1976; Jonas, 1972; Cox & Tanford, 1968) also contain as virtually their sole protein complement an equivalent apolipoprotein. Apo A-I has also been found to be the major protein component of a disk-shaped high-density particle secreted by rat intestine (Green et al., 1978).

While numerous studies of recombination of apo A-I with well-defined phospholipids have been performed, extensive binding of lipid by apo A-I has only been found for dimyristoylphosphatidylcholine [DMPC, di(14:O)PC][2] (Hauser et al., 1974; Andrews et al., 1976; Middelhoff et al., 1976; Tall et al., 1977; Jonas & Krajnovich, 1977; Pownall et al., 1978; Swaney, 1980a) and for didecanoylphosphatidylcholine [di-(10:O)PC] (Reynolds et al., 1977). Especially relevant to the

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[1] Recombination is used here in the general sense of reformation of a lipid–protein complex, even though the synthetic lipids used here are not found in significant amounts in naturally occurring lipoproteins.

[2] Abbreviations used: DMPC or di(14:O)PC, dimyristoylphosphatidylcholine; DLPC or di(12:O)PC, dilauroylphosphatidylcholine; DPPC or di(16:O)PC, dipalmitoylphosphatidylcholine; di(13:O)PC, di-(tridecanoyl)phosphatidylcholine; di(15:O)PC, di(pentadecanoyl)phosphatidylcholine; PMPC, 1-palmitoyl-2-myristoylphosphatidylcholine; MPPC, 1-myristoyl-2-palmitoylphosphatidylcholine; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

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mechanism of protein–lipid interaction was the finding by Pownall et al. (1978) that the rate of association of A-I with DMPC was appreciable only near the gel–liquid-crystalline transition temperature of this phospholipid (23.9 °C); similar findings were also reported for the C-III apolipoprotein by this group (Pownall et al., 1977). This result is similar to the finding of maximal rates of phospholipid hydrolysis by pancreatic phospholipase A_2 near the transition temperature (Op den Kamp et al., 1975) and suggests that protein insertion is facilitated by volume changes which occur at the melting temperature (Linden et al., 1973).

Attempts to recombine apo A-I with dipalmitoylphosphatidylcholine [DPPC, di(16:O)PC] at, below, or above its transition temperature have been largely unsuccessful (Pownall et al., 1978; Middelhoff et al., 1976). Thermal denaturation studies by Tall et al. (1975) indicate that thermal denaturation of apo A-I does not begin to occur until 43 °C, indicating that protein unfolding is an unlikely cause of the poor reactivity with DPPC.

On the basis of studies of single-tailed amphiphile interaction with apo A-I, Reynolds et al. (1977) have suggested that this protein requires preliminary binding of 4 mol of a fatty acid or lysolecithin to permit cooperative binding of phospholipids. Furthermore, it is suggested that the variable extents of recombination with different phospholipids may be due to variable levels of lysolecithin contamination in the lipid preparations used in different laboratories.

The present studies have been undertaken to establish whether the major factor limiting apo A-I–phospholipid recombination is the state of the phospholipid or the state of the protein. We have also attempted to determine whether phospholipid mixtures may facilitate formation of recombinant particles containing DPPC.

Materials and Methods

Apolipoprotein A-I. To obtain purified apo A-I, we first isolated the high-density lipoprotein (HDL) (*d* 1.063–1.21) from fresh human plasma by ultracentrifugation. Following delipidation with ethanol–ether (3:1), the apo HDL was applied to a 2.5 × 190 cm column of Sephadex G-150 in 0.2 M Tris-HCl, pH 8.2, 6 M urea (ultrapure), and 0.1% sodium decyl sulfate. Usually, this was sufficient to yield pure apo A-I; however, for some preparations it was necessary to use ion-exchange chromatography on DEAE-cellulose to obtain a pure preparation, as judged by $NaDodSO_4$ gel electrophoresis (Swaney & Kuehl, 1976) and amino acid analysis. Before use, the apo A-I was renatured from guanidine hydrochloride, as previously described (Swaney & O'Brien, 1978).

Phospholipids. For these studies L-α-dimyristoyl-1-[^{14}C]-phosphatidylcholine was obtained from Applied Science (State College, PA) and purified by preparative thin-layer chromatography on silica gel using $CHCl_3$–CH_3OH–2.5 N NH_4OH (70:30:5) as the developing solvent; autoradiography was used to locate the di(14:O)PC, and the lipid was scraped off the plate and eluted with methanol. L-α-Dipalmitoyl[9,10-^{3}H]-phosphatidylcholine was also obtained from Applied Science, and its purity was established by autoradiography. Unlabeled di(16:O)PC and di(12:O)PC were obtained from Sigma Chemical Co., and unlabeled DMPC was obtained from Calbiochem (LaJolla, CA). Di(tridecanoyl)phosphatidylcholine [di(13:O)PC] and di(pentadecanoyl)phosphatidylcholine [di(15:O)PC] were obtained from Supelco (Bellafonte, PA). 1-Stearoyl-2-oleoylphosphatidylcholine was purchased from Applied Science. 1-Palmitoyllysophosphatidylcholine was obtained from Serdary Laboratories (London, Ontario). All phospholipids were subjected to thin-layer chromatography in the aforementioned solvent system; only those preparations which yielded a single spot after visualization with Zinzadze reagent (Phospray, Supelco) were used. 1-Palmitoyl-2-myristoylphosphatidylcholine (PMPC) and 1-myristoyl-2-palmitoylphosphatidylcholine (MPPC) were provided by Drs. K. M. W. Keough and P. J. Davis.

Methods. Solutions of phospholipids in benzene–methanol (4:1 v/v) were placed in a test tube, and the solvent was evaporated with N_2; any remaining solvent was removed by placing the tube in a vacuum oven at 40 °C for 3–6 h. Buffer (8.5% KBr, 0.01% EDTA, and 0.01 M Tris-HCl, pH 7.5) was added to bring the concentration to 0.25 mg/mL, and the lipids were dispersed with a vortex mixer for 1.0 min, then sonicated in a low-power sonication bath (ultrasonic cleaner, Model 220, Branson Scientific) for 10 min, and again dispersed with a vortex mixer for 0.5 min to produce multilamellar liposomes. The salt concentration used (8.5% KBr) was selected to maintain the phospholipid in suspension during the course of the kinetic experiments.

For nonisothermal kinetic experiments employing temperature programming, 1 mL of the phospholipid dispersion was placed in each of four thermostated spectrophotometer cells; these cells were heated or cooled by a Neslab circulating water bath which was equipped with an electronic temperature programmer (Neslab Instruments, Portsmouth, NH). At the beginning of the experiment 100 μL of apo A-I (1 mg/mL) was added with mixing to the desired cuvettes.

Since the interaction of apolipoprotiens with multilamellar phospholipid dispersions results in a clearing of turbidity due to formation of small protein–lipid complexes (Träuble et al., 1974), we have utilized absorbance as an index of recombination in these studies. The absorbance at 325 nm was measured in a Gilford 240 spectrophotometer, while the temperature was raised linearly with time by the temperature programmer using the approach of Pownall et al. (1978, 1979); a rate of 30 °C/h was used in Figure 1, and a rate of 12 °C/h was used for all other experiments. The temperature in the cuvette was determined with a thermocouple microprobe (Bailey Instruments, Saddle Brook, NJ).

For isothermal kinetic studies, the phospholipid dispersion was equilibrated at the desired temperature for 20–30 min, apo A-I was added at a 1:2.5 weight ratio to phospholipid, and the absorbance at 325 nm was measured as a function of time.

For the study of lipid–protein complexes a 1:1 (w/w) mixture of [^{3}H]di(16:O)PC and [^{14}C]di(14:O)PC was incubated with apo A-I (phospholipid/protein = 2.5:1 w/w) for 24 h at 29.8 °C. This sample was applied to a 1.6 × 90 cm column of agarose A-5m (Bio-Rad Laboratories), and the column was eluted with 0.02 M Tris-HCl, pH 7.2, 0.01% EDTA, 0.01% azide, and 0.15 M NaCl. The column effluent was monitored for absorbance at 280 nm, and the radioactivity in collected fractions was determined by dual isotope scintillation counting.

For studies of the effects of 1-palmitoyllysophosphatidylcholine on protein–lipid complex formation, aliquots of this lipid in benzene–methanol (4:1 v/v) were added to a solution of di(15:O)PC in the same solvent to reach the desired proportion, and an aqueous dispersion of phospholipids was prepared as described above. For these studies, as with temperature-programmed studies of pure phospholipids, four samples were run simultaneously in thermostated spectrophotometer cells by means of an automatic sample programmer, thus facilitating side by side comparison of samples.

In order to verify the existence of protein–lipid interactions in the recombinant particles, fluorescence spectroscopy was

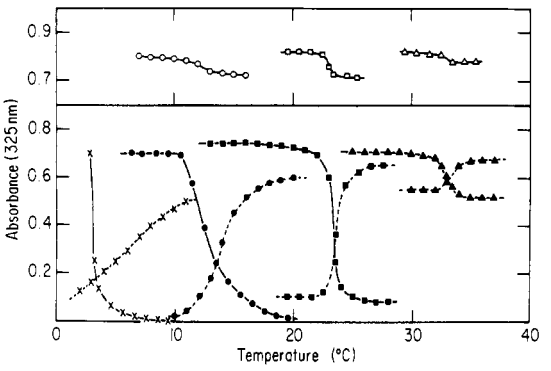

FIGURE 1: Turbidity as a function of temperature at programmed heating (solid lines) and cooling (dashed lines) rates (30 °C/h) for di(12:O)PC (×), di(13:O)PC (●, ○), di(14:O)PC (■, □), and di-(15:O)PC (▲, △) both with (closed symbols) and without (open symbols) added apo A-I. One milliliter of each phospholipid dispersion was placed in a thermostated cuvette at 2.8 °C with or without added apo A-I, and all four samples were monitored simultaneously by means of an automatic sample programmer as the programmed temperature rise was performed. No absorbance change upon heating was noted for the sample of di(12:O)PC in the absence of apo A-I so these data are not shown in the figure. Cooling runs for all samples began at 39 °C.

performed with selected samples in the presence of varying amounts of guanidine. Samples of apo A-I or apo A-I–phospholipid complexes were placed in a Perkin-Elmer MPF-3 fluorescence spectrophotometer which was thermostated to 24 °C, and the wavelength of maximum intrinsic tryptophan fluorescence was measured with excitation at 278 nm. Aliquots of 8 M guanidine hydrochloride or weighed portions of solid guanidine hydrochloride were added to adjust the solution to various concentrations of the denaturant, as described previously (Swaney, 1980a) and the resultant spectra were recorded.

Results

Studies of Apo A-I Recombination with Pure, Homologous Lecithins. Figure 1 shows the effect of monitoring the turbidity of di(12:O)PC, di(13:O)PC, di(14:O)PC, and di(15:O)PC following the addition of apo A-I, both with heating and with cooling at a rate of 30 °C/h; all four phospholipid samples were run simultaneously and carried through the entire heating or cooling program, although data are shown only where changes in absorbance took place.

Upon heating, clearing of turbidity occurs only near the transition temperature of the individual phospholipid. The known transition temperatures of the phospholipids are 13.5 °C and 33–34 °C for di(13:O)PC and di(15:O)PC, respectively (Silvius et al., 1979), 23.9 °C for di(14:O)PC, and −1.8 °C for di(12:O)PC (Mabrey & Sturtevant, 1976). Di-(12:O)PC cleared quickly at the lowest accessible temperature (2.8 °C), which is several degrees above its transition temperature.

Heating curves of the lipids alone showed small decreases in turbidity in the same temperature intervals as the samples containing apo A-I. This change in absorbance is attributed to a change in the net polarizability which accompanies the change of state; this is the result of both changes in volume and alterations of intrinsic polarizability of the phospholipid molecules upon melting (Behof et al., 1978). These results confirm the presence of a phase change at the indicated temperature and provide a correction factor for estimating the extent of recombination from the absorbance change, in samples containing apo A-I.

Data for programmed cooling of A-I–phospholipid samples are also shown in Figure 1. Temperatures for the maximum rate of recombination agree well for heating and cooling of di(15:O)PC and di(14:O)PC but somewhat less well for di-(13:O)PC; di(12:O)PC behaved rather anomalously by this criterion. This probably reflects the known instability of the structures formed by this phospholipid (Hauser & Barratt, 1973) since the low energy of interaction which maintains the bilayer structure may allow rupture by thermal motions. Thus significant rates of recombination are observed at temperatures above the transition temperature of DLPC, as contrasted with phospholipids which form more stable structures.

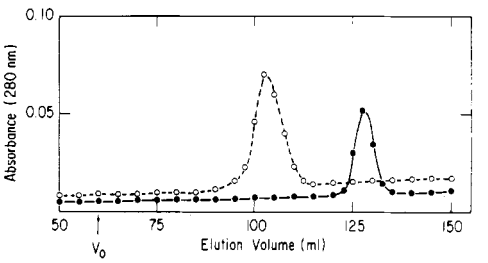

FIGURE 2: Gel filtration chromatography of apo A-I (●) and a 2.5:1 (w/w) incubation mixture of DLPC–A-I (O). A 1.6 × 88 cm column of agarose A-5m was employed, and the effluent was monitored at 280 nm. The arrow indicates the void volume of the column (V_0).

Of particular significance is the result that the extent of recombination during this interval of study, as judged from the change in absorbance upon heating, decreases progressively as the length of the acyl chains is increased, reflecting a decreasing rate of recombination. It should be noted that recombination in the context of these experiments is defined as that degree of lipid–protein association which results in the clearing of turbidity through formation of smaller particles. Any lipid–protein interactions which do not lead to the breakdown of the light-scattering particles would not be detected by our technique. However, previous studies with A-I and DMPC at elevated lipid/protein ratios have demonstrated that little or no protein is associated with the large phospholipid structures (Swaney, 1980a); equivalent results with DMPC and DPPC have also been reported by Pownall et al. (1978).

We have considered the possibility that the observed differences in behavior between different phospholipids arise from the use of a constant weight ratio of phospholipid to protein rather than a constant molar ratio. However, the observed differences are conserved when a constant molar ratio is employed.[3]

Further studies were undertaken with the shorter chain phospholipids to verify that the clearing of turbidity observed in Figure 1 reflects the formation of lipid–protein complexes, as has previously been shown with A-I and DMPC; two experimental approaches were employed for this purpose.

In the first, gel permeation chromatography was performed on samples of apo A-I or a mixture of apo A-I and DLPC incubated at 0 °C for 18 h (Figure 2) or a mixture of apo A-I and di(13:O)PC incubated at 13.5 °C for 18 h (not shown). Absorbance measurements of the protein demonstrated that apo A-I eluted in a volume equal to 2.07 times the void volume of the column, while the incubation mixture of A-I and DLPC yielded a complex which eluted in a volume 1.67 times the void volume. These values are quite close to those reported previously for apo A-I and the A-I–DMPC complex prepared in the same way (Swaney, 1980a). Thus the A-I appears to reside in a complex with DLPC which is larger than apo A-I

[3] This point is further established by Figure 8, in which isomeric phospholipids are studied and differences in kinetic behavior are observed despite the use of equivalent molar ratios of phospholipid to protein.

Table I: Fluorescence Emission Wavelength Maxima (in Nanometers) for Apo A-I Alone or following Incubation of Apo A-I with Phospholipids[a]

sample	guanidine concn (M)			
	0	2	4	6
apo A-I	334	349	350	350
A-I-DMPC[b]	332	333	336	347
A-I-di(13:0)PC[b]	333	336	344	348
A-I-DLPC[b]	333	335	343	348

[a] Spectra were obtained on a Perkin-Elmer MPF-3 fluorescence spectrophotometer at an excitation wavelength of 278 nm; all samples at 0 molar concentration of guanidine contained 0.042 mg/mL of A-I protein. Dilution due to addition of guanidine hydrochloride yielded protein concentrations of 0.032, 0.021, and 0.0.019 mg/mL at guanidine concentrations of 2 M, 4 M, and 6 M, respectively. [b] Recombinant particles were prepared by incubating the indicated phospholipids overnight at a 3:1 (w/w) ratio with apo A-I at 24 °C for DMPC, 13.5 °C for di (13:O)PC, and 0 °C for DLPC.

alone, but smaller than a phospholipid vesicle, which elutes in a volume 1.05 times the void volume (Swaney, 1980a). Equivalent results were obtained with di(13:O)PC. When samples of these mixtures were incubated for 18 h at 24 °C, instead of at the phospholipid transition temperature, clearing of turbidity failed to occur, thus visually confirming the failure to form small recombinant particles at temperatures removed from the phase transition region, even after extended time periods.

In a second approach, the wavelength of maximum tryptophan fluorescence emission was measured in the presence of various concentrations of guanidine hydrochloride (Table I). As has been reported previously, apo A-I is conformationally unstable (Reynolds, 1976) and completely unfolds in 2 M guanidine to yield a limiting value of 350 nm for this parameter (Verdery & Nichols, 1974). We have previously demonstrated that the complexes of A-I and DMPC require concentrations of guanidine above 6 M to achieve the same degree of unfolding (Swaney, 1980a). The data in Table I indicate that incubation of A-I with DLPC or with di(13:O)PC also produces complexes which require high concentrations of guanidine to achieve unfolding, although the data suggest that these complexes may not be quite as stable as those involving DMPC.

Effect of Lysolecithin on Apo A-I–Di(15:O)PC Recombination. Since Reynolds et al. (1977) have suggested that low levels of single-tailed amphiphiles such as lysolecithin might profoundly enhance the reactivity of apo A-I with phospholipid, we studied recombination of apo A-I with preparations of di(15:O)PC to which lysolecithin had been added in various proportions (Figure 3). Lysolecithin was added to the phospholipid mixture in benzene–methanol (4:1 v/v) prior to dispersion in aqueous buffer to ensure its integration into the phospholipid multilamellar vesicles. Low proportions of lysolecithin to lecithin did not significantly alter the kinetic profile obtained by the temperature-programming method. Higher proportions of lysolecithin led to a dramatically enhanced reactivity and a shift in the temperature of maximum reactivity to lower values. Thus, when present in appreciable amounts, lysolecithin is capable of significantly increasing the ability of di(15:O)PC to bind A-I. A similar effect of lysolecithin on the binding of egg yolk lecithin by apo A-I has been observed by Nichols et al. (1974). It should be noted that in the absence of added protein a small reversible change in absorbance is seen in the region of the transition temperature for samples containing from 1% to 20% lysolecithin; the magnitude of this change is comparable to that observed for

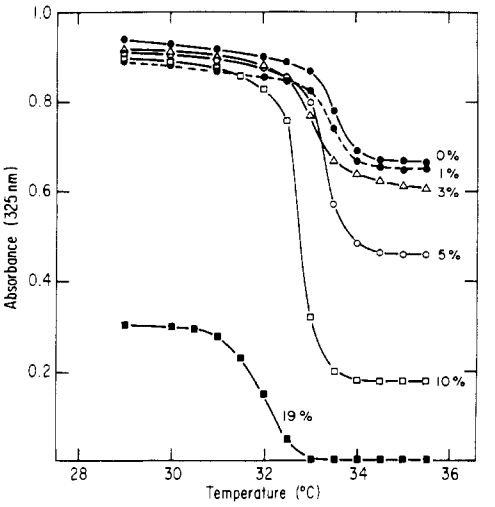

FIGURE 3: Effect of 1-palmitoyllysophosphatidylcholine on the recombination of apo A-I with di(15:O)PC. Absorbance of phospholipid to which apo A-I was added [di(15:O)PC/A-I = 2.5:1 (w/w)] was monitored during a programmed temperature rise (12 °C/h). The mole percentages of lysolecithin incorporated into the phospholipid dispersion were 0% (●–●), 1% (●- - -●), 3% (△–△), 5% (○–○), 10% (□–□), and 19% (■–■). The highest proportions of lysolecithin produced less turbid dispersions, indicating smaller vesicle sizes.

di(15:O)PC alone (Figure 1, upper panel).

Studies were also performed in which lysolecithin was incubated in various proportions with A-I for 1 h prior to mixing with di(15:O)PC. When temperature-programming experiments were performed on these samples, no difference was observed between the profiles in the absence or in the presence of lysolecithin up to a level of 23 mol of lysolecithin/mol of A-I [19% (mol/mol) of phospholipid present in the incubation]. These data suggest that lysolecithin exerts its effect through modification of the physical properties of the phospholipid and that integration of lysolecithin into the lecithin bilayer does not occur to an appreciable extent during the time course of our experiments if these lipids are not mixed prior to dispersal in the aqueous phase. These results are similar to those of Pownall et al. (1977) who showed that DMPC and DPPC possessed different properties when mixed prior to the formation of aqueous vesicles than when mixed after the formation of vesicles. This is visually apparent in our system by the fact that the presence of 19% lysolecithin (mol/mol) in a lecithin preparation yields a much less turbid solution (Figure 3) than a lecithin solution of comparable concentration to which aqueous lysolecithin is added to proportion of 19% (mol/mol); integration of lysolecithin into the lecithin bilayer evidently causes a reduction in the average size of the vesicles, thus attenuating the scattering of light by these preparations.

Studies of Apo A-I Recombination with DMPC–DPPC Mixtures. Since reactivity of phospholipids decreases with increasing chain length and since it has been shown that DPPC is virtually unreactive with apo A-I (Pownall et al., 1978), we have attempted to form recombinant particles with this phospholipid when it is mixed with DMPC (Figure 4). In the absence of added protein, a small change in turbidity occurs in the region of the transition temperature for each phospholipid mixture, but no changes are observed at the transition temperature for pure DMPC or DPPC (23.9 and 41.4 °C, respectively), suggesting complete miscibility of these components.

Addition of apo A-I to the phospholipid mixtures results in a much greater change in absorbance. The inflection point of these curves, which we estimate visually, corresponds to the

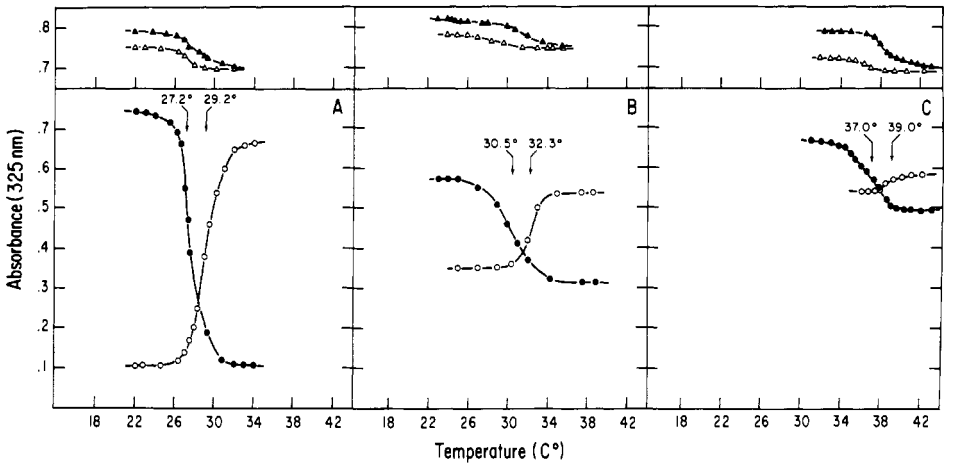

FIGURE 4: Turbidity as a function of temperature at programmed heating and cooling rates (12 °C/h) for phospholipids both in the presence (○, ●) and in the absence (△, ▲) of apo A-I. Apo A-I (0.1 mg) was added to DPPC–DMPC mixtures (0.24 mg) equilibrated at the initial temperature of the heating (solid symbols) or cooling (open symbols) programs. The mole fractions of DPPC in the mixtures were (A) 0.27, (B) 0.56, and (C) 0.87. It should be noted that the absorbance values at the start of the heating and cooling programs differ in the A-I-containing samples because of differences in the absorbance of the phospholipid alone at these two temperatures; likewise, differences occur at the ends of the programmed runs in those samples containing appreciable amounts of unreacted phospholipid. Arrows indicate the estimated temperature for the maximum rate of absorbance change for the heating (T_{max}^{H}) and cooling (T_{max}^{C}) curves.

temperature at which the maximum rate is observed (T_{max}). This inflection point is found to occur at slightly different temperatures for heating and cooling curves (T_{max}^{H} and T_{max}^{C}, respectively) for samples with the same lipid composition (Figure 4). For several values of the mole fraction of DPPC (X_{DPPC}) experiments were also performed at scan rates of 4 °C/h and 18 °C/h; values of T_{max}^{H} and T_{max}^{C} similar to those obtained at a rate of 12 °C/h were obtained in all cases.

As the mole fraction of DPPC in the lipid mixture is raised and the transition temperature is correspondingly increased, two features of significance become evident. First, the temperature at which the maximum rate of recombination occurs is increased. Second, the rate of reconstitution, as evidenced by the slope at the inflection point or the total change in absorbance during the temperature program (expressed as a percentage of the initial absorbance), is decreased. It should be noted that the lack of symmetry between heating and cooling curves is due to the change in absorbance of the uncomplexed phospholipid itself upon heating and cooling.

Isothermal Kinetic Studies. In order to verify that the temperature of maximum reaction as determined by temperature-program studies agrees with that determined by isothermal kinetic studies, we studied a number of phospholipid mixtures by conventional isothermal kinetic analysis at a variety of temperatures; a representative mixture (X_{DPPC} = 0.36) is depicted in Figure 5. In these studies, it was found that the fastest rate of recombination was achieved only at the T_{max}^{H} and that slower rates were obtained at all other temperatures, including the T_{max}^{C}. We take these results to mean that the temperature-programming technique is a reliable method for determining the temperature at which a maximum rate of recombination occurs. This method is especially valuable for comparing the properties of different phospholipids, since anomalous Arrhenius plots are obtained by isothermal kinetic studies (Pownall et al., 1978) making it extremely difficult to pick appropriate temperatures for comparing the recombination kinetic properties of these lipids.

Correlation with Phase Diagram Data. Since it has been suggested from recombination studies of A-I with DMPC that rapid rates of reconstitution are obtained only at the liquid-crystalline–gel transition temperature (Pownall et al., 1978), we have plotted the T_{max}^{H} data obtained in experiments like those shown in Figure 4 on a phase diagram (Figure 6)[4] de-

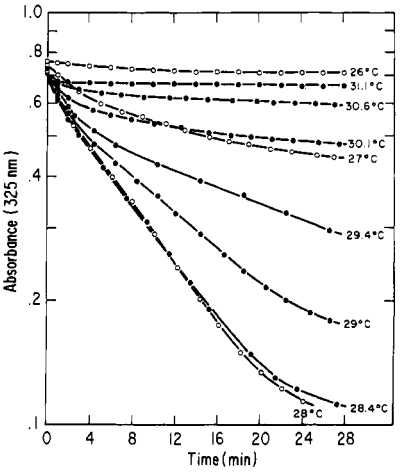

FIGURE 5: Isothermal studies of the reconstitution of apo A-I with an aqueous solution of DPPC–DMPC (X_{DPPC} = 0.36). After the lipid mixture was equilibrated at the indicated temperatures for 30 min, apo A-I was added and the decrease in turbidity was followed with time.

rived from the calorimetric data of Mabrey & Sturtevant (1976) and the electron spin resonance data of Shimshick & McConnell (1973). It can be seen that for this binary mixture, the T_{max}^{H} data closely follow the solidus curve of the phase diagram.

Isolation of A-I–DMPC–DPPC Complexes. Since it had been previously reported that DPPC combines poorly with apo A-I (Pownall et al., 1978), we undertook to establish whether DPPC in a binary mixture with DMPC is actually incorporated into a lipid–protein complex or whether the observed reduction in turbidity resulted merely from reaction of apo A-I with DMPC alone. After incubation of A-I with a 1:1 (w/w) mixture of DMPC and DPPC (X_{DPPC} = 0.48), the incubation mixture was chromatographed on agarose A-5m (Figure 7). A single protein–lipid complex was observed which eluted earlier than uncomplexed apo A-I. The molar

[4] In addition to the mixtures shown in Figure 4, experiments were also performed at X_{DPPC} = 0.12, 0.44, and 0.74; the results obtained consistently followed the trend which is evident in Figure 4, and inflection point temperature data are included in Figure 6.

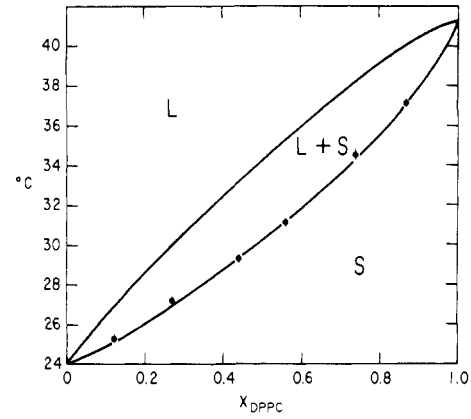

FIGURE 6: Data for T_{max}^{H} (●) plotted on a phase diagram for DPPC–DMPC mixtures obtained from data reported by Mabrey & Sturtevant (1976) and by Shimshick & McConnell (1973) (solid curves). The experimental points presented are the average of two experiments; error bars indicate the range of values obtained.

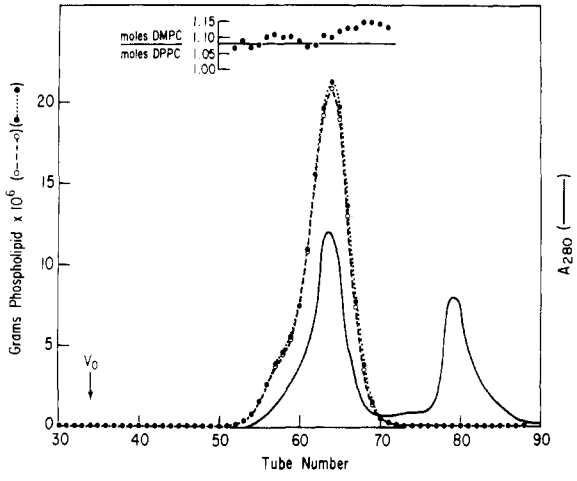

FIGURE 7: Gel filtration chromatography of the reconstitution products from an incubation mixture of apo A-I (0.78 mg) and a 1:1 (w/w) mixture of [^{3}H]DPPC–[^{14}C]DMPC (1.83 mg). In this experiment, a 1.6 × 85 cm column of agarose A-5m was employed. Absorbance at 280 nm (solid curve) reflects the protein distribution; scintillation counting was used to quantitate [^{3}H]DPPC (●) and [^{14}C]DMPC (○). The insert shows the calculated DMPC/DPPC molar ratio in the area of the protein–lipid complex. The horizontal line, corresponding to a DMPC/DPPC ratio of 1.08, reflects the phospholipid ratio in the incubation mixture.

ratio of DMPC/DPPC across this peak was within 8% of the DMPC/DPPC ratio in the initial incubation mixture. Virtually no phospholipid was found in the void volume where uncombined phospholipid elutes. Approximately 82% of the applied radioactivity for each phospholipid was found in the peak corresponding to the protein–lipid complex.

Studies of Apo A-I Recombination with MPPC and PMPC. An interesting structural property of phospholipids which has only recently been demonstrated is the steric nonequivalence of acyl chains in positions 1 and 2 (Hitchcock et al., 1974; Seelig & Seelig, 1975). To investigate the significance of this property with respect to recombination, we have carried out kinetic studies with mixed-chain phospholipids bearing palmitoyl and myristoyl acyl chains [1-myristoyl-2-palmitoylphosphatidylcholine (MPPC) and 1-palmitoyl-2-myristoylphosphatidylcholine (PMPC)], which have shown to possess transition temperatures intermediate between those of DMPC and DPPC (Keough & Davis, 1979). Temperature-programmed kinetics (Figure 8) reveal reactivities which differ from each other. The temperatures at the inflection points

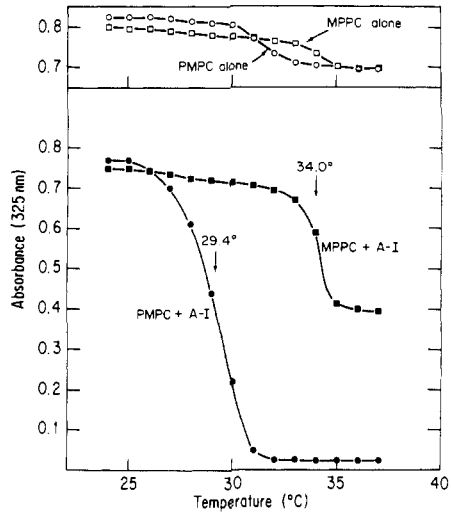

FIGURE 8: Turbidity as a function of temperature at a heating rate of 12 °C/h for PMPC (●, ○) or MPPC (■, □), either with (filled symbols) or without (empty symbols) added apo A-1. The estimated inflection point temperatures are indicated by the vertical arrows.

(30 °C for PMPC and 34 °C for MPPC) are in good agreement with the midpoint temperatures for the enthalpic peaks observed by differential scanning calorimetry of these particular preparations.[5]

Discussion

The data presented here clearly establish the importance of phospholipid phase properties in the initiation of interaction with apo A-I, since recombination occurs only near the transition temperature for di(12:O)PC, di(13:O)PC, di(14:O)PC, and di(15:O)PC (Figure 1); this is in agreement with the findings of Pownall et al. (1978) for their study of di(14:O)PC. At the same time it is difficult to rule out the possibility that conformational changes in apo A-I may also be important for phospholipid binding, since undetectably low levels of lysolecithin contamination of our lipid preparations could be sufficient to induce such a conformational change (Reynolds et al., 1977). We have, however, examined the effect of including lysolecithin in a preparation of di(15:O)PC to ascertain its effect on recombination with apo A-I (Figure 3). Levels of lysolecithin up to 5 mol % (which in our protocol corresponds to 5 mol of lysolecithin/mol of A-I) were found to have a very modest effect on the recombination reaction. However, higher levels of lysolecithin markedly increase the extent of reaction and lower the temperature of the maximum reaction rate. The latter effect is probably due to a lowering of the phase transition temperature of the phospholipid, since additions of lysolecithin at comparable levels directly to the A-I protein, prior to mixing with lecithin, had little or no effect on the kinetic pattern obtained by temperature programming. Consequently, we believe that the major determinant of protein interaction with and breakdown of lecithin vesicles is the phase state of the lipid rather than induced conformational changes in the A-I protein.

It is also apparent from Figure 1 that there is a progressive decrease in reactivity with apo A-I, even at the transition temperature, as the length of the acyl chain increases. Since longer chain phospholipids, like DPPC, are virtually unreactive with apo A-I, we attempted to incorporate DPPC into a

[5] Transition temperatures reported by Keough & Davis (1979) for PMPC (27.2 °C) and MPPC (35.5 °C) were obtained by extrapolation. Actual preparations used in this study had 10–22% acyl migration, resulting in somewhat altered transition temperatures.

complex with apo A-I through the maneuver of forming binary mixtures of this lipid with a highly reactive phospholipid (DMPC). Clearing of the liposome turbidity of the mixtures occurred in the region of the transition temperature (Figure 4) with both heating and cooling, although the inflection point temperatures were always 1–2 °C higher for the cooling curves than for the heating curves. Isothermal kinetic studies (Figure 5) show that the maximum rate is found only at the temperature where a maximum rate is observed by heating in the temperature-programming experiments. Differences in heating and cooling inflection point temperatures may be due to hysteresis effects, such as those observed by Pownall et al. (1978) and by Behof et al. (1978). We note that these differences in inflection point temperatures are much smaller for most pure phospholipids than for the binary mixtures, suggesting that these differences may reflect properties of the phospholipids, rather than characteristics of the experimental technique. Cooling curves establish the fact that recombination does not proceed at an appreciable rate at temperatures significantly above the transition temperature.

A plot of the heating curve inflection point temperature vs. the mole fraction of DPPC (Figure 6) duplicates very well the solidus curve of the phase diagram for this binary mixture as obtained by physcial techniques (Mabrey & Sturtevant, 1976; Shimshick & McConnell, 1973; Ladbrooke & Chapman, 1969). Although the temperature interval over which the phase transition occurs is fairly narrow, the data lie distinctly at the temperature of onset of the gel–liquid-crystalline transition. This has been shown more clearly with mixtures of DMPC and DSPC (Swaney, 1980b) where the phase transition occurs over a temperature interval as wide as 20 °C.

It is especially noteworthy that as the transition temperature for DMPC–DPPC mixtures (Figure 4) is increased, the rate of reaction decreases. We have considered the possibility that reaction occurs exclusively with DMPC so that the rate would be influenced by the proportion of this component; however, the demonstration that both DMPC and DPPC are incorporated equally into a protein–lipid complex (Figure 7) excludes this possibility.

We have also investigated mixed-chain isomers (PMPC and MPPC) since the "internal" mixing of acyl groups on these isomers is complementary to the "external" mixing of DMPC–DPPC pairs (Figure 8). This study was of particular interest because of the differences in transition temperature between the positional isomers. Again, maximum recombination occurs at the transition temperature, and, in agreement with Keough & Davis (1979), we find a difference of several degrees between the transition temperatures of these isomers. It is known that in phosphatidylcholines the acyl chain in position 2 is offset from the acyl chain in position 1 by ~3 Å (Büldt et al., 1978; Seelig & Seelig, 1975; Gaber et al., 1978). In order to explain the differences in transition temperature between PMPC and MPPC, Keough & Davis (1979) have postulated that the acyl chain in position 1 of PMPC is folded back upon itself to compensate for the partial void in position 2, thus reducing the interactions between the acyl chains thorugh a net shortening of the bilayer thickness. Our data with mixed-chain isomers and with homologous-chain phospholipids reveal a consistent inverse correlation between reactivity toward recombination and the transition temperature of the phospholipid. Thus the positional distribution of acyl groups can be seen to significantly affect the kinetics of recombination with apolipoproteins.

We have considered possible explanations for the observed variability in rates of recombination with phospholipids containing various acyl chains. Because recombination occurs only near the phase transition temperature, we assume that some, or all, of the protein must be inserted into a hole, or defect, in the lattice structure (Op den Kamp et al., 1974; Pownall et al., 1978). It therefore would follow that the rate of recombination would be dependent upon properties of the defect, such as lifetime, size, number, or possibly shape. Because of the apparent relationship between reactivity and transition temperature, the dependence on defect lifetime is an attractive possibility, since thermal motion at higher temperatures could decrease the lifetime of a lattice defect. However, we have found that apo A-I is unreactive with 1-stearoyl-2-oleoylphosphatidylcholine (unpublished results) even though this phospholipid undergoes the phase transition at 2–4 °C (as estimated from the dependence of turbidity on temperature). Also, binary mixtures of DMPC and 1-palmitoyl-2-oleoylphosphatidylcholine show decreasing reactivity as the transition temperature is lowered (Swaney, 1980b). It thus appears that factors other than thermal effects per se predominate in influencing the rate of reaction.

Several theoretical and semiempirical treatments have been reported which describe the characteristics of the phospholipid gel–liquid-crystalline phase transition (Marsh et al., 1976; Kanehisa & Tsong, 1978; Freire & Biltonen, 1978). These authors suggest that insertion of molecules into or through the bilayer is most likely to occur at defects which arise at the boundary between fluid and solid phase regions. For a pure phospholipid, the fraction of molecules located at the boundary between gel and liquid-crystalline phases is maximal at the phase transition temperature; the number of lattice defects is proportional to this fraction of boundary lipid (Marsh et al., 1976), and therefore recombination is also expected to be maximal at the transition temperature. Furthermore, many of the phospholipids we have studied are known to melt cooperatively in clusters, rather than independently (Hinz & Sturtevant, 1972); a convenient expression for the cooperativity is the cooperative unit size, which represents the apparent number of molecules which undergo the phase transition in concert. It has been suggested that the size of the lattice defect where insertion is thought to occur is proportional to the size of these clusters and hence is a function of the degree of cooperativity (Kanehisa & Tsong, 1978; Freire & Biltonen, 1978). However, since the fraction of boundary lipid is inversely proportional to cluster size, it is apparent that the number of defects is inversely proportional to their size.

Measurements of cooperativity of the phospholipid phase transition for DLPC, DMPC, DPPC, and distearoylphosphatidylcholine have shown a progressive decrease as the length of the acyl chains is increased (Mabrey & Sturtevant, 1976). On the basis of the foregoing discussion, one would therefore predict that, if the number of defects is the limiting property, reactivity toward recombination would increase in going from di(12:O)PC to di(16:O)PC, whereas reactivity would decrease if defect size is the critical parameter. The data in Figure 1 are most compatible with the latter hypothesis, and, on the basis of this result, we would expect the cooperativity of the phase transition to be a useful predictor of reactivity toward recombination with apolipoproteins. A reverse pattern was reported for the small amphiphilic dye, anilinonaphthalenesulfonic acid, by Tsong (1975), who found that bilayers of DPPC were more permeable to this dye than DMPC bilayers; presumably, permeability of this molecule is less dependent on the size of the lattice defect than it is on the number of sites available for penetration. Apparently, in the case of apoliprotein insertion a substantial portion of the

molecule must be inserted into the bilayer in order to initiate complex formation, thus making defect size the critical parameter.

Thus, a major implication of these studies is that the cooperativity of the phospholipid phase transition is of crucial importance in determining the receptivity of lipid bilayers to protein insertion. Modeling of the phase transition suggests that maximum recombination should occur at a temperature where the fraction of boundary lipid is maximal. However, results with binary mixtures of DMPC and DPPC indicate that a maximal rate is obtained at the onset temperature of melting, where a small amount of fluid phase is in equilibrium with solid phase, rather than at the midpoint of the transition where the fraction of boundary lipid is highest. A model to explain these observations has recently been proposed (Swaney, 1980b).

Acknowledgments

We thank Drs. K. M. W. Keough and P. L. Davis for the generous gift of PMPC and MPPC used in this study. We also thank Dr. Howard Eder for helpful discussions.

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