Thermodynamics of Lipid–Protein Interactions: Interaction of Apolipoprotein A-II from Human Plasma High-Density Lipoproteins with Dimyristoylphosphatidylcholine†

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ABSTRACT: Apolipoprotein A-II (apoA-II), the second most abundant protein of the human high-density lipoproteins, spontaneously associates with dimyristoylphosphatidylcholine (DMPC) to give multiple products whose composition, structure, and properties are a sensitive function of the temperature and of the initial lipid to protein ratio at which they are formed. We have studied the thermodynamics of this association by calorimetric and spectroscopic methods. Complexes having a DMPC/apoA-II molar ratio of 75:1 are formed at 20 and 24 °C; a 240:1 complex is also formed at 24 °C, and a 45:1 complex is formed at 30 °C. Additionally, in the presence of a large excess of lipid at 24 °C, the 75:1 complex can be converted to a 240:1 complex. These temperature regions are respectively below, at, and above the transition temperature, T_c, of DMPC (23.9 °C). According to our analysis of the differential scanning calorimetric data, the 75:1 and 240:1 complexes contain a "boundary layer" of 45 lipid molecules/molecule of ApoA-II whereas the 45:1 complex has a boundary of about 37 DMPC molecules/molecule of apoA-II. Batch calorimetry of the association of DMPC with apoA-II at 23.45, 24.5, and 30.0 °C gives values of +90, −260, and −62 kcal/mol of apoA-II. The enthalpy of association of *lyso*myristoylphosphatidylcholine (LMPC) with apoA-II at 30.0 °C was identical with that of DMPC. Correlation of circular dichroic and calorimetric data shows that the enthalpy of α-helix formation of apoA-II which accompanies its association with DMPC is exothermic with a value of −2.9 kcal/1% α-helical development (−2.0 kcal/α-helical residue). This is the major source of enthalpy in the association of apoA-II with DMPC at 30 °C and with LMPC at all temperatures. Between the T_c of DMPC and that of its complexes with apoA-II the enthalpy of association is highly exothermic (−260 kcal/mol of apoA-II); the enthalpy in this temperature range is assigned to the sum of protein-induced, isothermal acyl chain crystallization and α-helix formation. Below T_c, the association is endothermic (+90 kcal/mol of apoA-II) but occurs spontaneously. Therefore, the entropic contribution ($+T\Delta S$) to the free energy of association is greater than −90 kcal/mol of apoA-II. This value compares favorably with the calculated free energy of transfer (−98 kcal/mol of apoA-II) of hydrophobic amino acid side chains of the nonpolar faces of amphipathic α-helical regions of apoA-II from an aqueous to a hydrophobic environment. These results demonstrate that the energetics of apolipoprotein association with phospholipids are a function of accompanying structural changes in both the lipid and the protein.

Because the apoproteins of the human plasma lipoproteins are in a state of dynamic equilibrium (Schaefer et al., 1978; Smith et al., 1978), studies on the energetics of lipid–protein interactions are an important consideration in predicting the distribution of apoproteins among different lipoprotein classes and the extravascular compartment. This distribution of the apolipoproteins may be subject to kinetic or thermodynamic control. Many physical techniques have been used to study lipid–protein interactions (Morrisett et al., 1977), with the use of differential scanning calorimetry (DSC)[1] and batch microcalorimetry being particularly useful because they provide quantitative data on the energetics of lipid–protein interactions.

DSC has been used in studying the lipid–protein recombinants of DMPC–apoA-I (Tall et al., 1975, 1977a), DMPC–cholesterol–apoA-I (Tall & Lange, 1978), and DMPC–porcine apoHDL (Andrews et al., 1976). ApoA-I–lipid recombinants show two thermal transitions; one of these has been assigned to a phospholipid gel → liquid crystalline transition of DMPC within the recombinant, and the other has been attributed to a thermal denaturation of the recombinant. Several thermodynamic parameters have been derived from the studies of thermal denaturation of lipid-free apoA-I (Tall et al., 1976) and apoA-I in HDL (Tall et al., 1977b).

The enthalpy of association of apoproteins and lipids has been measured by microcalorimetry. Using microcalorimetry, Rosseneu et al. (1976a,b) estimated the affinity of apoproteins for phospholipids and established a relationship between self-association and phospholipid binding of human and baboon apoA-I (Rosseneu et al., 1977). In an indirect way, microcalorimetry has been useful for studying the mechanism of lipid–protein complex formation (Roth et al., 1977; Pownall et al., 1977; Rosseneu et al., 1978). However, none of these studies provided experimental evidence permitting a quantitative distinction between the enthalpy of lipid–protein association and that of other processes which are concurrent with lipid binding; moreover, none of these studies produced a

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[1] Abbreviations used: HDL, high-density lipoproteins; 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 of human HDL; apoC-III, apoprotein C-III, a minor protein of human HDL; LMPC, lysomyristoylphosphatidylcholine; DSC, differential scanning calorimetry; CMC, critical micelle concentration; Gdn–HCl, guanidine hydrochloride; VHDL, very high density lipoprotein; HDL_2, high-density lipoprotein subfraction 2; HDL_3, high-density lipoprotein subfraction 3.

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correlation between the enthalpy of these processes and the nature of the complex formed.

We have shown that apoA-II interacts with DMPC to form three different, isolatable lipid–protein complexes (Massey et al., 1980, 1981). The effects of temperature with respect to the gel → liquid crystalline transition of DMPC and the initial lipid to protein molar ratio are the two major determinants of what type of lipid–protein complex is formed. In this paper we have measured the enthalpy of association of apoA-II and DMPC by microcalorimetry and correlated that with the thermotropic properties of the isolated complexes as measured by DSC. We have been able to assign the enthalpy of association of apoA-II and DMPC to the sum of the enthalpies of increased α helicity developed by the protein upon complex formation and that due to either the "melting" or "freezing" of DMPC molecules which accompanies the formation of a lipid–protein complex. From our data we now report the first reasonable estimate for the entropic contribution to the free energy of lipid–apolipoprotein association. We have also developed a model based on the concept of the amphipathic helix (Segrest et al., 1974) which attributes the entropic part of the free energy of association to the transfer of certain amino acid side chains from an aqueous to an hydrophobic environment. This interpretation provides a model for the energetics of lipid–protein interaction which can be used in assessing the physicochemical bases for the distribution of apoprotein components between plasma lipoproteins.

Experimental Procedures

Materials

All materials and their methods of preparation have been described in previous papers in this series (Massey et al., 1980, 1981).

Methods

Circular Dichroism. Circular dichroic (CD) spectra were obtained with a Cary Model 61 spectropolarimeter. Mean residue ellipticities were calculated by using eq 1, where $[\theta]_\lambda$

$$[\theta]_\lambda = 113[\theta]_{obsd}/(10LC) \quad (1)$$

is the mean residue ellipticity at wavelength λ; $[\theta]_{obsd}$, the observed ellipticity; 113, the mean residue molecular weight of apoA-II; L, the path length of the cell in centimeters; and C, the concentration of protein in g/mL. In cases where the buffer did not permit scanning wavelengths below 220 nm, the % α helicity was measured by eq 2 (Pownall et al., 1977).

$$\% \ \alpha \text{ helix} = ([\theta]_{222} + 3000/39\,000) \times 100 \quad (2)$$

Batch Microcalorimetry. The enthalpy of interaction (ΔH) of apoA-II and DMPC liposomes was measured on an LKB 10070 batch microcalorimeter equipped with gold cells as previously described (Morrisett et al., 1977; Rosseneu et al., 1975); enthalpy values have been corrected for the heat of dilution of lipid and protein and for the friction of mixing. The heat of mixing was minimized by dialyzing the lipid and protein against a common buffer at room temperature prior to the calorimetric experiment. In a typical experiment, the total heat release was between 1 and 6 mcal. Extreme care was taken to clean the calorimeter cells between each experiment. Each cell was rinsed with dilute acid and base several times and then with distilled water several times after which they were dried with a stream of nitrogen. When not in use, the cells contained distilled water. The enthalpy of reaction was obtained by comparison with an electrical calibration. Periodic checks of the electrical calibrations were made by measuring the enthalpy of dilution of sucrose (Zimmer & Biltonen, 1972). The microcalorimeter contains two compartments in which apoA-II was placed in the 4-mL compartment and the other reactant in the 2-mL compartment.

Differential Scanning Calorimetry (DSC). Differential scanning calorimetry was performed on a Perkin-Elmer DSC-2 equipped with a subambient cooling unit. The calorimeter was calibrated with an indium standard. The accuracy of these measurements was ±0.3 °C. The T_c's were obtained at a scan rate of 2.5 °C/min and were corrected for thermal lag by extrapolation of the measured T_c obtained at 0.62, 1.25, 2.5, and 5.0 °C/min heating rates to zero. Enthalpy measurements were determined from the area under the endotherms by weighing the trace enclosed by the peak and the base line; this area was compared with that obtained from indium. Samples were sealed in 75-μL stainless-steel pans; the reference pan contained an equal amount of buffer to minimize the effects of heat capacity changes. Samples were scanned at least four times. Samples were isolated and concentrated by either pressure ultrafiltration in an Amicon ultrafiltration cell with UM-10 filters or by vacuum filtration using 25 000 molecular weight cutoff collodion bags from Schleicher and Schuell, Inc. (Keene, NY).

Results

Circular Dichroism. DMPC–apoA-II recombinants were isolated as previously described (Massey et al., 1980) and dialyzed against buffer (0.1 M NaCl and 10 mM Tris, pH 7.4) to remove KBr which interferes with the measurement of the circular dichroic spectra below 220 nm. Figure 1 shows CD spectra of apoA-II and DMPC–apoA-II complexes recorded at 25 °C. ApoA-II and DMPC–apoA-II complexes having stoichiometries[2] of 45:1, 75:1, and 240:1 mol of lipid/mol of protein have $[\theta]_{222}$ of −10.0 ± 0.5, −15.3 ± 0.5, −25.0 ± 0.5, and −24.0 ± 0.5 × 10^3 deg cm^2/dmol, respectively. These values correspond to calculated α helicities of 33%, 47%, 72%, and 69%, respectively. The inserts in Figure 1 illustrate the effect of temperature on the CD spectra of apoA-II and DMPC–apoA-II complexes. The spectra have been normalized to that observed at 25 °C. As seen in Figure 1A, the values for apoA-II go through a maximum value of $[\theta]_{222}$ between 20 and 25 °C, an effect that has been previously documented (Gwynne et al., 1975) and postulated to be due to changes in the monomer–dimer association of apoA-II with temperature. All three DMPC–apoA-II complexes exhibit a linear decrease in the $[\theta]_{222}$ with temperature. Over the temperature range 5–40 °C, there is no abrupt break in these plots which might be construed as changes induced by the phase transition of lipid in the complex or as thermal denaturation of the complex; moreover, although the ellipticity changes with temperature, there is no change in shape of the CD spectrum, indicating no significant changes in the relative amounts of protein secondary structure. The effect of temperature is probably due to thermal "breathing" of the α-helical structure, an effect which has been seen previously (Pownall et al., 1974).

We studied the effect of Gdn–HCl on the CD spectra of the protein in the three complexes. From the CD data of Figure 2 the relative stability of apoA-II in water and in the complexes can be assessed. ApoA-II is readily denatured by Gdn–HCl with a 50% change in α helicity observed at approximately 0.7 M. This observation agrees with those of other workers (Reynolds, 1976; Gwynne et al., 1975). The protein

[2] Throughout this paper, the proportions of dimyristoylphosphatidylcholine to apoA-II are given as a molar ratio of lipid to protein.

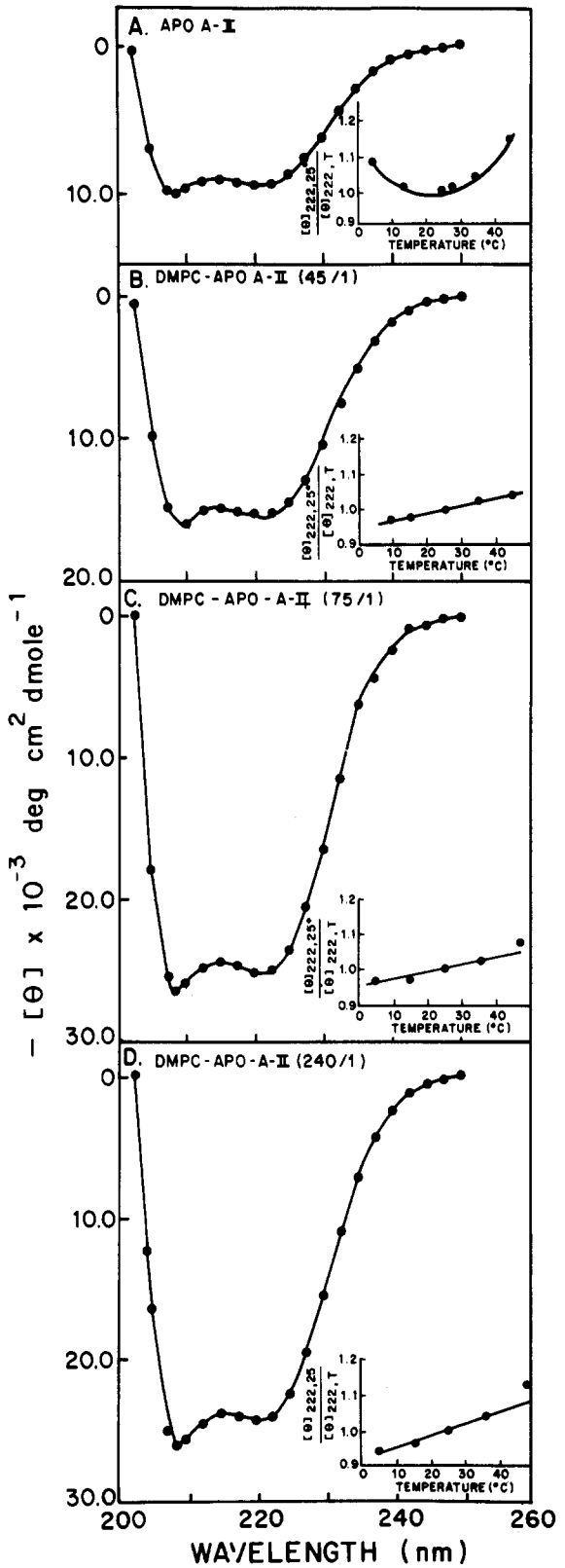

FIGURE 1: Circular dichroic spectra of apoA-II, DMPC–apoA-II (45:1), DMPC–apoA-II (75:1), and DMPC–apoA-II (240:1). The inserts are plots of $[\theta]_{222,25}/[\theta]_{222,T}$ vs. temperature where $[\theta]_{222,25}$ is the ellipticity at 25 °C and $[\theta]_{222,T}$ is the ellipticity at 222 nm at temperature T.

in a DMPC–apoA-II complex is more stable to Gdn–HCl denaturation. The 45:1 complex has a single broad transition curve which begins at 1 M and ends at 7 M with a midpoint at around 4 M. Relatively speaking, the 75:1 and 240:1 complexes both have sharper transitions which begin around 2 M and end at 6 M with the midpoints at 4 M Gdn–HCl.

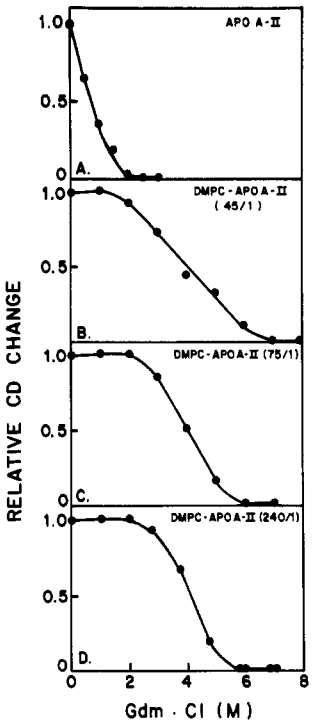

FIGURE 2: Circular dichroism ($[\theta]_{222}$) of apoA-II, DMPC–apoA-II (45:1), DMPC–apoA-II (75:1), and DMPC–apoA-II (240:1) as a function of Gdn–HCl concentration. Concentrations of protein are 0.15 mg/mL for apoA-II alone, 0.15 mg/mL for apoA-II in the 45:1 complex, 0.15 mg/mL for apoA-II in the 75:1 complex, and 0.15 mg/mL for the 240:1 complex. Samples were individually prepared by the addition of an aliquot of apoA-II or a DMPC–apoA-II complex to a certain Gdn–HCl concentration at room temperature (22 °C) and incubation of the samples for 4 h before measuring the circular dichroic spectrum. The buffer was 0.1 M NaCl and 10 mM Tris, pH 7.4.

Their similar helical contents and Gdn–HCl denaturation curves indicate that the secondary structures of apoA-II in the 75:1 and 240:1 complexes are very similar. The secondary structure of apoA-II in the 45:1 complex must be different from that of the 75:1 and 240:1 complexes since its helical content and Gdn–HCl induced transition curve differs from those of the other two complexes.

Differential Scanning Calorimetry. Samples of DMPC–apoA-II complexes were prepared by two different procedures. In one method, DMPC in chloroform was placed in the DSC pan and the chloroform evaporated. Concentrated apoA-II was added, and the pans were sealed and incubated for 24 h at 24 °C. In the second method, complexes were isolated by column chromatography and concentrated. Both methods gave similar results so that only measurements obtained on isolated complexes will be presented here. Figure 3B–D shows typical DSC traces of isolated complexes which may be compared with those of DMPC liposomes in curve A. The thermal transitions are due to the gel → liquid crystalline transition of DMPC in the complexes, because no abrupt changes in the secondary structure of the protein in the complexes due to denaturation are seen over the same temperature range. Additionally, fluorescence polarization studies using the lipophilic fluorescence probes diphenylhexatriene and α- and β-parinaric acids show cooperative lipid transitions in the same temperature range as the thermal transitions (Mantulin et al., 1980). The thermal properties of the DMPC–apoA-II complexes differ from those of the DMPC liposomes in that the former have lower enthalpies of melting, a broader melting range, and a higher transition temperature. The thermal transitions are totally reversible, and no other thermal transitions were found

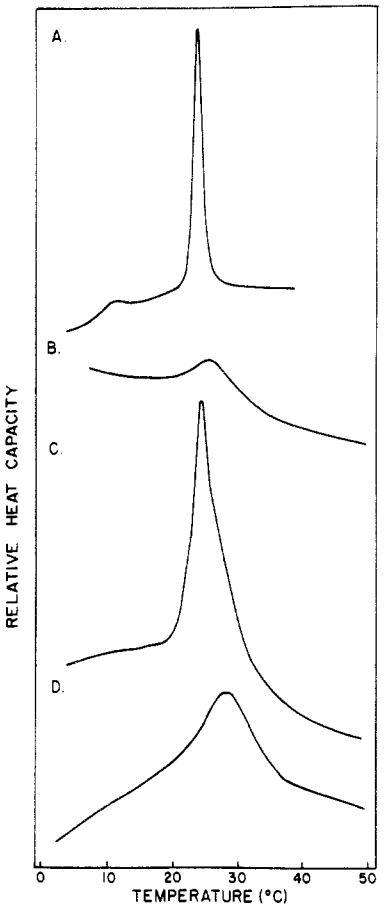

FIGURE 3: DSC heating curves of DMPC–apoA-II complexes. The samples are (A) DMPC (2.5 mg of DMPC), (B) a 75:1 DMPC–apoA-II complex formed and isolated at 20 °C (2.7 mg of DMPC), (C) a 240:1 DMPC–apoA-II complex formed and isolated at 24 °C (6.6 mg of DMPC), and (D) a 45:1 DMPC–apoA-II complex formed and isolated at 33 °C (2.2 mg of DMPC). All samples were scanned at 2.5 °C/min at a sensitivity of 0.5 mcal/s for DMPC, the 75:1 DMPC–apoA-II complex, and the 240:1 DMPC–apoA-II complex and a sensitivity of 0.2 mcal/s for the 45:1 DMPC–apoA-II complex. The scans are not directly comparable as far as the relative heat capacity is concerned since they were measured on different sensitivities and with different sample concentrations.

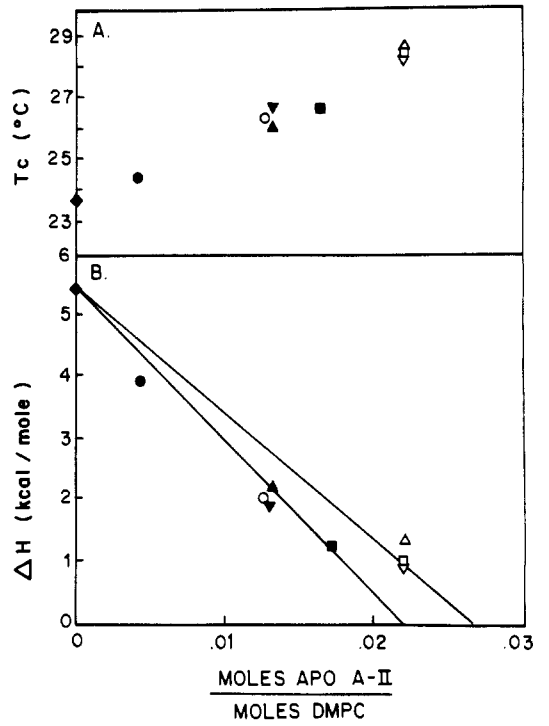

FIGURE 4: Thermal properties of isolated complexes of DMPC–apoA-II. Panel A shows the peak temperature (T_c) and panel B shows the enthalpy (ΔH) of the gel → liquid crystalline transition of DMPC in the complexes as a function of moles of apoA-II/mole of DMPC. The samples are DMPC (♦), a 240:1 complex isolated by column chromatography at 24 °C (●), a 76:1 complex isolated by pelleting the excess lipid by low-speed centrifugation at 20 °C (▲), a 77:1 complex isolated by column chromatography at 20 °C (▼), a 77:1 complex isolated by chromatography at 24 °C (○), a 45:1 complex isolated by column chromatography at 30 °C (□), 33 °C (△), and 35 °C (▽), and a 58:1 complex which did not contain excess lipid in the starting mixture but had an initial lipid to protein ratio of 60:1 which was formed and isolated by column chromatography at 24 °C (■). The lines were drawn by using assumptions which are described under Discussion. The line through the 75:1 complex intersects zero enthalpy at 45 mol of DMPC/mol of apoA-II and the line through the 45:1 complexes intersects zero enthalpy at 37 mol of DMPC/mol of apoA-II. The samples were concentrated as discussed under Methods. The collodion bags were used more often because the samples could be concentrated to smaller volumes than with an Amicon ultrafiltration cells.

even when the complexes were heated to 100 °C. A plot of the enthalpy of the transition and the transition temperature, T_c, vs. protein to lipid molar ratio is given in Figure 4. A linear extrapolation of ΔH vs. moles of apoA-II/mole of DMPC for the complexes that have a similar protein conformation as assessed from their CD spectra (75:1 and 240:1 complexes) gives a value of ~45 mol of lipid/mol of protein at zero enthalpy of the transition. This finding indicates that 45 mol of lipid/mol of protein in these two complexes do not undergo a gel → liquid crystalline transition. An extrapolation for the 45:1 complex gives a value for zero enthalpy at 37 mol of DMPC/mol of apoA-II.

Effect of Lipid/Protein Ratio on Binding Enthalpy. We have measured the enthalpy of interaction of apoA-II and DMPC when a constant quantity of apoA-II is mixed with increasing amounts of DMPC at 22.0, 24.0, and 30.0 °C. In all of the microcalorimetric experiments, apoA-II at 0.15 mg/mL (4 mL) is mixed with lipid (2 mL) such that the final concentration of apoA-II is 0.1 mg/mL. Studies on the self-association of apoA-I (Teng et al., 1978) have shown that apoA-II is at least 90% monomeric at this concentration. Control experiments where the lipid solution was replaced with buffer indicated that the heat of dilution of apoA-II was negligible when compared to experiments conducted with lipid present. Moreover, the enthalpy of self-association of apoA-II must be quite small since sedimentation equilibrium studies (Teng et al., 1978) have shown no effect of temperature on the self-association of apoA-II within the temperature range used in this study. At this concentration of protein (Massey et al., 1981) we have found complexes identical with those characterized in the earlier paper (Massey et al., 1980). In Figure 5, typical microcalorimeter traces for runs at 23.45, 24.5, and 30.0 °C and an electrical calibration is shown. The electrical calibration lasts only 10 s; the time constant of the instrument is 67 s. At all temperatures the traces return to base line within 15–20 min. Therefore, the heat released from the mixing of apoA-II and DMPC must occur on a similar time scale as the calibration pulse because the processes are both over at approximately the same time. Therefore, the enthalpic events accompanying the association of apoA-II and DMPC must be on the order of a few minutes or less. The rate of association of LMPC and apoA-II is also on the same order of magnitude as that of DMPC and apoA-II. From the traces, it can also be seen that at 23.45 °C the interaction of apoA-II and DMPC is endothermic whereas at 24.5 and 30.0 °C it is exothermic. The interaction of apoA-II and LMPC at 20.2 °C is exothermic whereas with DMPC it is endo-

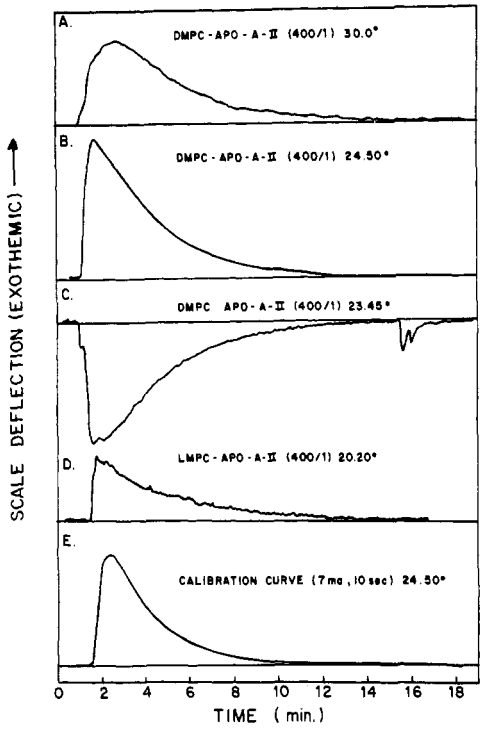

FIGURE 5: Experimental tracing from batch calorimetry. (A) DMPC and apoA-II (400:1) at 30 °C on the 10-μV scale; (B) DMPC and apoA-II (400:1) at 24.5 °C on the 30-μV scale; (C) DMPC and apoA-II (400:1) at 23.45 °C on the 10-μV scale (heat of mixing is shown after the experimental trace); (D) LMPC and apoA-II (400:1) at 20.2 °C on the 10-μV scale; (E) electrical calibration produced by a 7-mA current over a 10-s interval at 24.5 °C on the 30-μV scale.

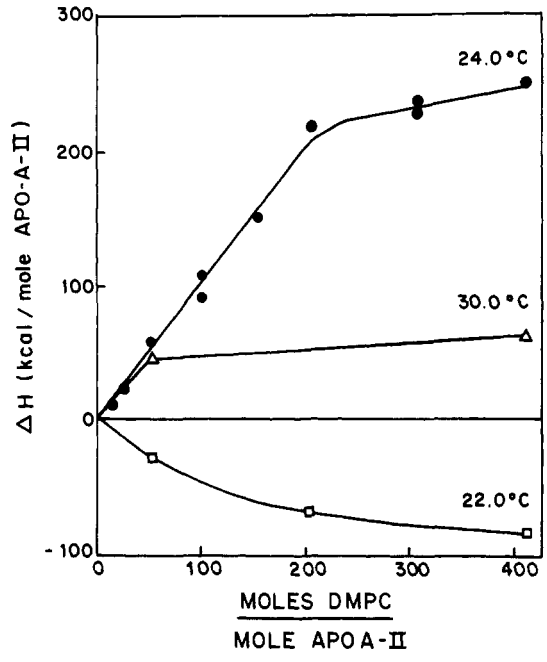

FIGURE 6: Enthalpy of apoA-II and DMPC interaction as measured by batch microcalorimetry as a function of the initial lipid to protein molar ratio. The experiments were performed at 24.0 °C (●), 30.0 °C (△), and 22.0 °C (□). The final concentration of apoA-II is 0.100 mg/mL.

thermic. In Figure 6, the enthalpy of association is plotted as a function of the moles of DMPC in the reaction mixture per mole of apoA-II. At 24 °C, the enthalpy appears to increase linearly with the DMPC/apoA-II ratio up to 200:1, where the enthalpy levels off. However, at 30.0 °C, it appears to level off after a ratio of 50:1 is reached; at 22.0 °C it does not appear to have completed leveling off even at 400:1 ratio.

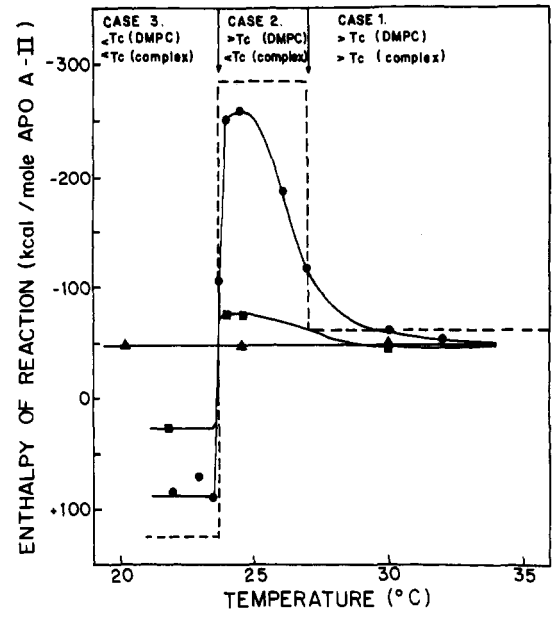

FIGURE 7: Enthalpy of apoA-II and DMPC association as a function of temperature. DMPC was at either a 400:1 (●) or 50:1 (■) molar ratio of lipid to protein and LMPC was at a 400:1 (▲) molar ratio. A model to predict the enthalpy of association is indicated by the dashed line. In this model the temperature range was divided into three regions where vastly different structures were formed (Massey et al., 1980) with different enthalpies. The final concentration of apoA-II is 0.128 mg/mL.

We assign the nonstoichiometric relationship between the enthalpy and the isolated lipid–protein complex to the fact that the protein can only bind at completely equivalent sites on the surface of the liposomes at very high ratios of lipid to protein. At high lipid to protein ratios we observed a constant enthalpy of association per mole of apoA-II even with additional DMPC.

Effect of Temperature on Binding Enthalpy. The enthalpy of association of apoA-II and DMPC was measured as a function of temperature. ApoA-II was mixed with DMPC at 50:1 and 400:1 molar ratios of lipid to protein and with LMPC at a 400:1 molar ratio. Figure 7 shows that the enthalpy of association of LMPC and apoA-II is constant, within experimental error, over the temperature range from 20 to 30 °C. However, the enthalpy found when DMPC is added to apoA-II changes dramatically over the temperature range studied. Below T_c (T_c = 23.9 °C; Mabrey & Sturtevant, 1976), the reaction is endothermic, being approximately +90 kcal/mol of apoA-II for the 400:1 molar ratio. Slightly above T_c, the reaction enthalpy is highly exothermic, being around −260 kcal/mol of apoA-II; at temperatures a few degrees above T_c, the enthalpy diminishes to a constant level around −50 kcal/mol of apoA-II. The reaction at a 50:1 molar ratio shows the same general effect; however, this effect is not as pronounced as that at a 400:1 molar ratio. Enthalpy measurements could not be performed below 22 °C because the rate of association is too slow (Massey et al., 1981) to be followed in the microcalorimeter.

Effect of α-Helicity Changes on Binding Enthalpy. To determine the amount of heat released due to conversion of random coil to α-helical regions, we measured the enthalpy of binding as a function of the α-helical change between apoA-II in solution and in a complex. The α-helical content of apoA-II in solution was controlled by the addition of various concentrations of Gdn–HCl, up to 1 M, while KCl was added to maintain an ionic strength of μ = 1.0 M. As seen in Table I, the isolated DMPC–apoA-II complexes in different con-

Table I: Correlation of Change in α-Helical Content and Enthalpy of Interaction in Lipid–ApoA-II Complex Formation

sample	buffer	temp (°C)	stoichiometry[a] initial	stoichiometry[a] complex	$[\theta]_{222} \times 10^{-3}$ (deg cm²/dmol) protein	$[\theta]_{222} \times 10^{-3}$ (deg cm²/dmol) complex	α helix[b] (%) protein	α helix[b] (%) complex	α helix (%)	enthalpy, $-\Delta H$ (kcal/mol)
LMPC[c]	8.6% KBr	30.0	400:1		8.4	16.0	29.2	48.7	19.5	49
DMPC	8.6% KBr	30.0	400:1	45:1	8.4	15.9	29.2	48.4	19.2	61
DMPC	0.25 M Gdn-HCl	30.0	400:1	45:1	11.1	16.4	36	49.7	13.6	40
DMPC	0.50 M Gdn-HCl	30.0	400:1	45:1	10.54	17.2	34.7	51.7	17.0	42
DMPC	0.75 M Gdn-HCl	30.0	400:1	45:1	8.94	16.9	30.6	51.0	20.4	55
DMPC	1.0 M Gdn-HCl	30.0	400:1	45:1	6.22	16.7	24.0	50.4	23.6	64

[a] Stoichiometry is in moles of lipid per mole of protein. [b] % α helix calculated by eq 1. [c] We did not attempt to isolate a complex of LMPC and apoA-II.

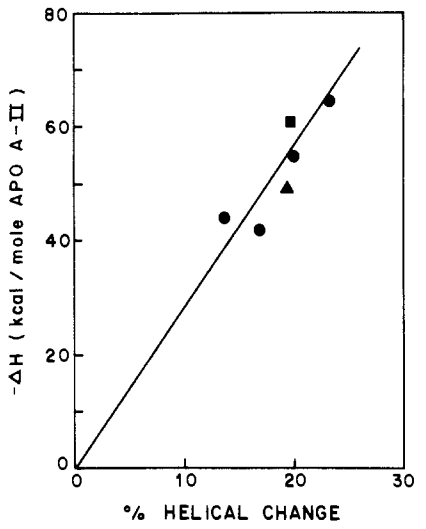

FIGURE 8: Enthalpy of association as a function of % helical change. The change in % α-helical structure between apoA-II in solution and apoA-II complexed to lipid at 30 °C was plotted as a function of the enthalpy of association. The reactants were apoA-II and DMPC in 8.6% KBr (■), apoA-II and LMPC in 8.6% KBr (▲), and apoA-II and DMPC in various concentrations of Gdn-HCl (●). The data are summarized in Table I. The complexes in Gdn-HCl buffers were isolated by low-speed centrifucation where the liposomes are pelleted and the complexes are in the supernatant (Tall et al., 1977a; Pownall et al., 1979).

centrations of Gdn-HCl have the same stoichiometry and α-helical content. However, the α helicity of the lipid-free protein decreases with increasing Gdn-HCl. In Figure 8, the enthalpy of reaction at 30 °C is plotted as a function of the α-helicity change. From the linear correlation between the change in α-helical structure and the enthalpy of interaction of apoA-II and DMPC we may write

$$\Delta H_t = \%\ \alpha\ \text{helix} \times \Delta H_\alpha + \Delta H_b$$

where ΔH_t is the observed enthalpy per mole of apoA-II, ΔH_α is the average enthalpy due to the conversion of 1% of the apoA-II from a random to an α-helical conformation, and ΔH_b is the enthalpy of association exclusive of changes in the protein conformation. Calculated from the slope of the line in Figure 8 there is a −2.9 kcal/mol of apoA-II change per 1% helical change which corresponds to −2.0 kcal/helical residue; from the intercept (zero) we conclude that there are no other processes other than α-helicity changes that substantially contribute to the enthalpy of association ($\Delta H_b \simeq 0$) ($T > T_c$ complex).

Discussion

Origin of the Enthalpy of DMPC–ApoA-II Association. ApoA-II and DMPC interact under different conditions to form three different isolatable complexes (Massey et al., 1980, 1981). Although the energetics of the reaction of apoA-II and DMPC appear to be complex, they can be assigned to enthalpic contributions of the gel → liquid crystalline transition of DMPC and that of α-helix formation by apoA-II.

We make one tentative assumption, the validity of which will be supported by the internal consistency of the data. That assumption is that DMPC molecules intimately surrounding or within one or two layers of the protein ("boundary" lipid) do not readily undergo a gel → liquid crystalline transition and that there is another domain of lipid in the complex that can undergo a gel → liquid crystalline transition with the same enthalpy [ΔH = 5.4 kcal/mol of DMPC; Mabrey & Sturtevant (1976)] as that seen in DMPC multilayer liposomes. A similar type of analysis has been performed on DMPC–apoA-I recombinants (Tall et al., 1977a) to predict the enthalpy of the transition as a function of protein to lipid ratio. This assumption is vital in interpreting the DSC data in Figure 4, because it enables one to predict that the enthalpy of melting seen in the complexes is a linear combination of these two types of lipid in the complexes. The different stoichiometry and protein structure observed in the 45:1 complex suggests that this lipid–protein complex is distinctly different from the 240:1 and 75:1 complexes. For the 240:1 and 75:1 complexes where apoA-II has a similar conformation, the enthalpy of melting vs. moles of apoA-II/mole of DMPC extrapolates to a value of ~45 mol of DMPC/mol of apoA-II (Figure 4); we assign this ensemble of lipid as nonmelting or "boundary" lipid whereas the rest of the lipid in the two complexes is bulk or "meltable" lipid. The linear plots of ellipticity vs. temperature for the three complexes (Figure 1) substantiate the concept of "boundary" lipid: there are DMPC molecules in the complexes that undergo a gel → liquid crystalline transition but there is no effect on protein conformation in the temperature region of the thermal transition. Thus, the lipid adjacent to the protein must not undergo a thermal transition. From the similar conformation of apoA-II in these complexes and the 45:1 value obtained for the number of "boundary" DMPC molecules in both complexes, we conclude that the 240:1 and 75:1 complexes contain a structurally similar 45:1 lipid–protein "structural unit". With the 45:1 complex, this type of analysis yields a value of ~37 mol of DMPC/mol of apoA-II as "boundary" lipid.

We studied the thermodynamics of α-helix formation as related to the enthalpy of association of DMPC and apoA-II and obtained a value of −2.9 kcal/mol of apoA-II per 1% helical change (−2.0 kcal/α-helical residue). Under slightly different conditions of ionic strength and temperature, we have previously obtained a value of −1.3 kcal/mol of amino acid residues converted from a random coil to α-helix structure for the association of apoA-II and apoC-III with several lysolecithins and short-chain lecithins (Massey et al., 1979). These values are of the same order of magnitude as that value found

in the thermal unwinding of apoA-I as measured by DSC [−1.2 kcal/α-helical residue (Tall et al., 1975)], the value of −1.2 kcal/α-helical residue for the KCl shock of poly-L-lysine measured by microcalorimetry (Chou & Sheraga, 1971), and the −2.0 kcal/α-helical residue obtained from pH jump measurements of apoA-I (Pownall et al., 1977). Even though there is a great deal of scatter in our data in Figure 8, it agrees reasonably well with other published values.[3] Therefore, we use the value of −2.9 kcal/mol of apoA-II 1% α-helical change with some degree of confidence. It is relevant to note that the enthalpy of LMPC and apoA-II association is rather constant over the temperature range 20–30 °C. Since LMPC does not have a thermal transition in this temperature range, the enthalpy must be solely due to formation of α-helical structure. However, with DMPC, the thermal transition causes the large changes in the measured enthalpy of association. This view is supported by our previously published data on the association of apoA-II and apoC-III with other lipids above their thermal transition (Massey et al., 1979) and the fact that a similar value was observed for the enthalpy of association of apoA-II with DMPC at 400:1 and 50:1 molar ratios and LMPC at 30 °C, which is 6 °C above the T_c of DMPC. Moreover, extrapolation of the data of Figure 8 to zero helical change gives a value close to zero for the enthalpy of association, which indicates other enthalpic events accompanying lipid protein association are small compared to α-helix formation (Massey et al., 1979).

Using the amount of "boundary" lipid obtained from our analysis of the DSC data and knowing the amount of α-helical change which accompanies binding of DMPC to apoA-II, we can predict the enthalpy of reaction of apoA-II with DMPC as a function of temperature; i.e., the enthalpy of reaction is equal to the sum of the enthalpy of melting of the "nonboundary" or "meltable" lipids or the formation of "boundary" lipid in a complex and that of the accompanying α-helix formation of apoA-II. For simplicity, we divided the temperature into three ranges, as shown in Figure 7.

In case 1, the temperature is above the transition temperature of both the lipid reactants (DMPC liposomes; T_c = 23.9 °C and that of the lipid in the lipid–protein complex (T_c = 28 °C). At 30 °C, the lipids in both the reactant liposomes and the complexes are in the liquid crystalline state so that there is no enthalpy to be gained or lost by "freezing" or "melting" of lipids during the association of DMPC and apoA-II; the observed enthalpy is due solely to α-helix formation. The α-helical change between the lipid-free protein and protein in the complex is ~20%, so the calculated enthalpy is (20% × −2.9 kcal/1% helix)/mol of apoA-II = −58 kcal/mol of apoA-II (observed enthalpy = −62 kcal/mol of ApoA-II).

In case 2, the temperature range is above the transition temperature of DMPC liposomes but below that of the lipid in the complex (75:1 complex). At 24 °C the lipid in the liposome (reactant) is in the fluid state but the "meltable" lipid in the complex (product) is in the gel state. Therefore, there is a protein-induced, isothermal conversion of liquid crystalline lipid to gel phase lipid with an exothermic enthalpy of −5.4 kcal/mol of "meltable" DMPC. There are 30 "meltable" lipid molecules per mole of apoA-II (75 mol of total lipid/mol of apoA-II − 45 mol of "boundary" lipid/mol of apoA-II = 30 mol of "meltable" lipid/mol of apoA-II). Therefore, (30 mol of lipid/mol of apoA-II) × −5.4 kcal/mol of lipid = −167 kcal/mol of apoA-II, and from a 42% α-helicity change (Figure 1), 42% α-helix × (−2.9 kcal/1% α-helix)/mol of apoA-II = −122 kcal/mol of apoA-II or a total of −289 kcal/mol of apoA-II (observed enthalpy = −260 kcal/mol of apoA-II).

In case 3, the temperature is below the transition temperature of both DMPC liposomes and DMPC–apoA-II complexes. The "meltable" lipid is in the gel state in both the liposomes and the complex, and so there would be no expected enthalpy change from these lipid molecules. However, the protein must isothermally convert 45 "frozen" lipid molecules to "boundary" molecules with an endothermic enthalpy change of 5.4 kcal/mol of "boundary" DMPC. Therefore, (45 mol of DMPC/mol of apoA-II) × +5.4 kcal/mol of DMPC = +243 kcal/mol of apoA-II plus the 42% α-helical change; 42% α-helix × (−2.9 kcal/1% α-helix)/mol of apoA-II = −122 kcal/mol of apoA-II or a total of +121 kcal/mol of apoA-II (observed enthalpy = +90 kcal/mol of apoA-II).

The predictions based on the schematic model illustrated in Figure 7 are in excellent agreement with the experimental results. The separation between the temperature range of case 3 and that of case 2 is very sharp because the thermal transition of DMPC liposomes is sharp ($T_{1/2max}$ = 0.2 °C; Mabrey & Sturtevant, 1976); the temperature dependence of the enthalpy of association separating case 2 from case 1 is more gradual due to the breadth of the thermal transition of the 75:1 lipid protein complex (Figure 3).

Below T_c of the complex (cases 2 and 3) the "nonboundary" lipid is in the gel state, and in both of these temperature ranges the initial complex, isolated by chromatography, is a 75:1 complex (Massey et al., 1980). Thus, the 75:1 complex is formed only at those temperatures where the "nonboundary" lipid is in the lower energy gel state in the complex. However, when lipid and protein are combined above the T_c of the 75:1 complex, only a 45:1 complex can be isolated. We attribute this difference to the temperature-dependent apoA-II induced freezing of "nonboundary" DMPC molecules upon complex formation below the T_c of the complex. These processes are schematically illustrated in Figure 9.

Contribution of Enthalpy to the Formation of the 240:1 Complex from the 75:1 Complex in Excess DMPC. We have shown that the 75:1 complex converts to a 240:1 complex in the presence of excess DMPC at T_c (Massey et al., 1980) although the reaction is too slow to measure in the calorimeter. We speculate that this conversion is also determined by the difference in the enthalpy of the free lipid and that of DMPC in the 240:1 complex. The enthalpy of melting of the 240:1 complex is 4 kcal/mol of DMPC whereas that of the 75:1 complex is 2.1 kcal/mol. This represents an exothermic enthalpy difference of 1.9 kcal/mol of DMPC when more DMPC and the 75:1 complex are isothermally converted to the 240:1 complex. From hydrodynamic data, there are ~9 mol of apoA-II/mol of 240:1 complex and 5 mol of apoA-II/mol of 75:1 complex, so that two 75:1 complexes plus additional molecules of DMPC must form the larger complex. A portion of the predicted enthalpy of formation would be 2 × (75 mol of DMPC/mol of apoA-II) × −1.9 kcal/mol of DMPC = −285 kcal/mol of apoA-II. There are 90 mol of

[3] In a previous paper from this laboratory (Pownall et al., 1977), a value of −8.0 kcal/1% helical change was reported for the association of DMPC and apoA-I by microcalorimetry. That interpretation did not take into proper account an endothermic contribution, thought to be due to dissociation of apoA-I, of 40 kcal/mol of apoA-I observed at low ratios of lipid to protein (Rosseneu et al., 1976b). In Figure 4 of the paper by Pownall et al. (1977), it is clear that the ΔH vs. moles of DMPC/mole of apoA-I curves at pH 7.4 and 3.1 are different. If the endothermic value of 40 kcal/mol of apoA-I is added to the difference in enthalpy (−80 kcal/mol) at a 100:1 molar ratio at pH 7.4 and 3.1, the ΔH difference for a 1% helical change is −4.0 kcal/mol or −1.6 kcal/α-helical residue. This value is closer to the value we have measured in this paper.

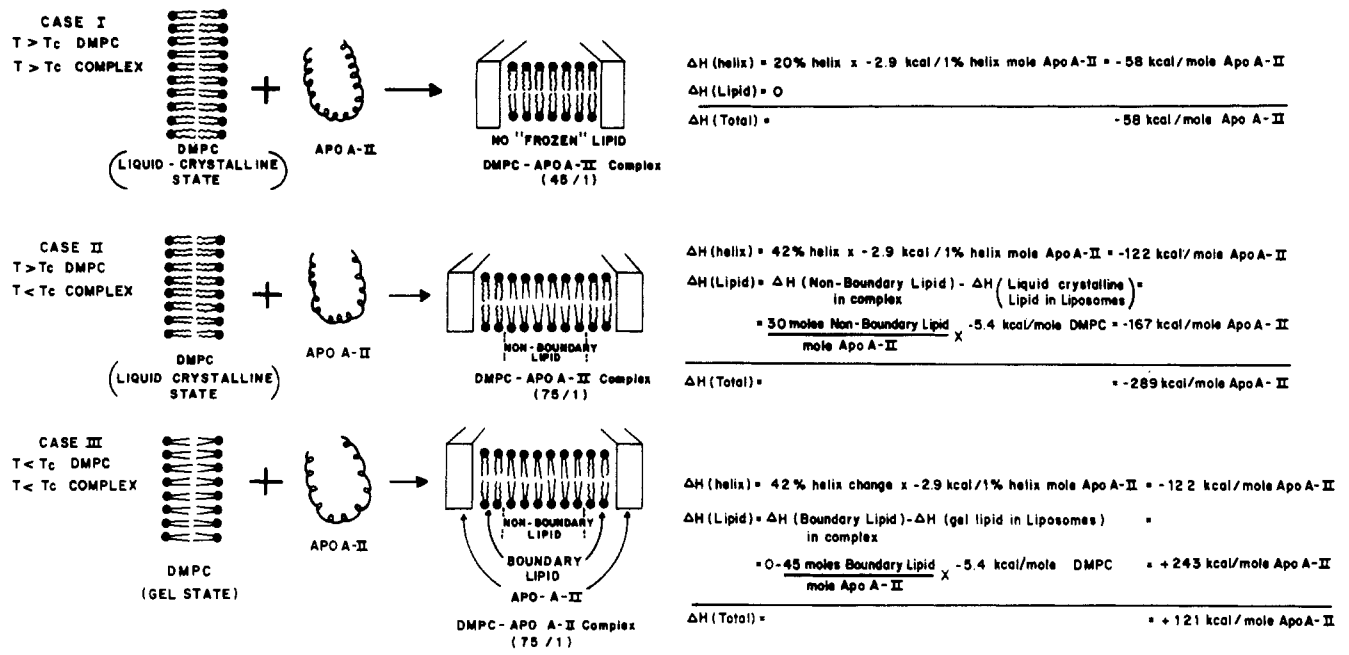

FIGURE 9: A schematic model that relates the enthalpic contributions of structural changes in DMPC (freezing) and apoA-II (helix formation) to the total enthalpy of complex formation. The three cases are discussed under Discussion. The complexes are represented as disks with protein on the edge. There is no evidence to support this structural model; however, it is the simplest model that can be used to discuss the data.

liposomal DMPC [(240 mol of DMPC/mol of apoA-II) − (2 × 75 mol of DMPC/mol of apoA-II) = 90 mol of DMPC/mol of apoA-II] added to form a complex. If the lipid is above T_c of DMPC but below T_c of the 240:1 complex then liquid-crystalline lipid is isothermally converted to gel-phase lipid. Therefore, with an exothermic enthalpy change of 5.4 kcal/mol of DMPC, we can calculate (90 mol of DMPC/mol of apoA-II) × −5.4 kcal/mol of DMPC = −486 kcal/mol of apoA-II. At $T < T_c$ of DMPC we have the conversion of gel phase lipid ($\Delta H_{melting}$ = 5.4 kcal/mol) to lipid in the 240:1 complex ($\Delta H_{melting}$ = 4.0 kcal/mol) which would give an endothermic enthalpy change of 1.4 kcal/mol. For this change we calculate (90 mol of DMPC/mol of apoA-II) × (+5.4 kcal/mol of DMPC in liposome − 4 kcal/mol of DMPC in 240:1 complex) = +126 kcal/mol of apoA-II. Between the T_c of DMPC and that of the 240:1 complex the total calculated enthalpy for the addition of lipid to a pair 75:1 complexes to form a 240:1 complex is −285 kcal/mol of apoA-II − 486 kcal/mol apoA-II = −771 kcal/mol of apoA-II. At $T < T_c$ (DMPC liposomes) the corresponding value is −285 kcal/mol of apoA-II + 126 kcal/mol of apoA-II = −159 kcal/mol of apoA-II. In both temperature ranges, the formation of a 240:1 complex is enthalpically favored. However, in the temperature range $T > T_c$ (240:1 complex) > T_c (DMPC liposomes), the enthalpy of this conversion would be negligible due to the absence of the isothermal "freezing" of DMPC molecules.

In other studies (Massey et al., 1980) we have shown that a 75:1 complex converts to a 240:1 complex; however, a 45:1 complex does not. The reason for this may be due to the structure of the protein in each complex. The secondary structures of apoA-II in the 75:1 and 240:1 complexes are similar but different from that of the 45:1 complex. The inability of the 45:1 complex to accept more DMPC to form a 240:1 complex could be due to the high activation barrier required to rearrange the protein; this might represent a formidible kinetic barrier for the conversion of a 45:1 complex to a 240:1 complex even though the latter is energetically favored.

Estimation of the Entropy of DMPC–ApoA-II Association. The formation of a complex must be entropically driven presumably via the hydrophobic effect. This conclusion is based on the fact that complex formation is spontaneous below the T_c of DMPC where this process is highly endothermic (+90 kcal/mol of apoA-II). The relationship between the free energy of lipid–protein complex formation (ΔG) and the entropy (ΔS) and enthalpy (ΔH) of complex formation is represented by the standard equation, $\Delta G = \Delta H - T\Delta S$. For a spontaneous reaction, ΔG is less than zero. For the association of apoA-II and DMPC to occur spontaneously below T_c, $T\Delta S$ must be greater than +90 kcal/mol of apoA-II, and so we can conclude that protein–lipid complex formation is strongly entropically driven.

Energetics of the Amphipathic Helical Model of Lipid Associating Proteins. Two helical lipid-binding regions of apoA-II have been proposed by both model building and lipid-binding studies of synthetic and natural peptides of apoA-II (Chen et al., 1979; Mao et al., 1977). The concept of the amphipathic helix is that there is a nonpolar face that penetrates the lipid phase and a polar face that interacts with the aqueous phase (Segrest et al., 1974). It was also originally proposed that the charged amino acids, i.e., lysine and glutamic acid, can form ionic interactions with charged groups of the polar head group of the lipid. On theoretical grounds this explanation is untenable. Since water can penetrate to the glycerol backbone of the lipid (Griffith et al., 1974), the charged groups probably would be hydrated when the protein is free in solution or bound to lipid. Ion–ion interactions are very weak in media of high polarity and dielectric constant and would be unlikely to displace ion–water interactions. Therefore, ionic interactions between protein and lipid would be predicted to be enthalpically small, in agreement with our interpretation that the enthalpy of association is due mainly to changes in the secondary structure of the protein and in the physical state of the lipid.

Figure 10 contains schematic representation of the amphipathic helical segments of apoA-II as predicted and experimentally determined (Chen et al., 1979; Mao et al., 1977). We propose that a major driving force for lipid–protein com-

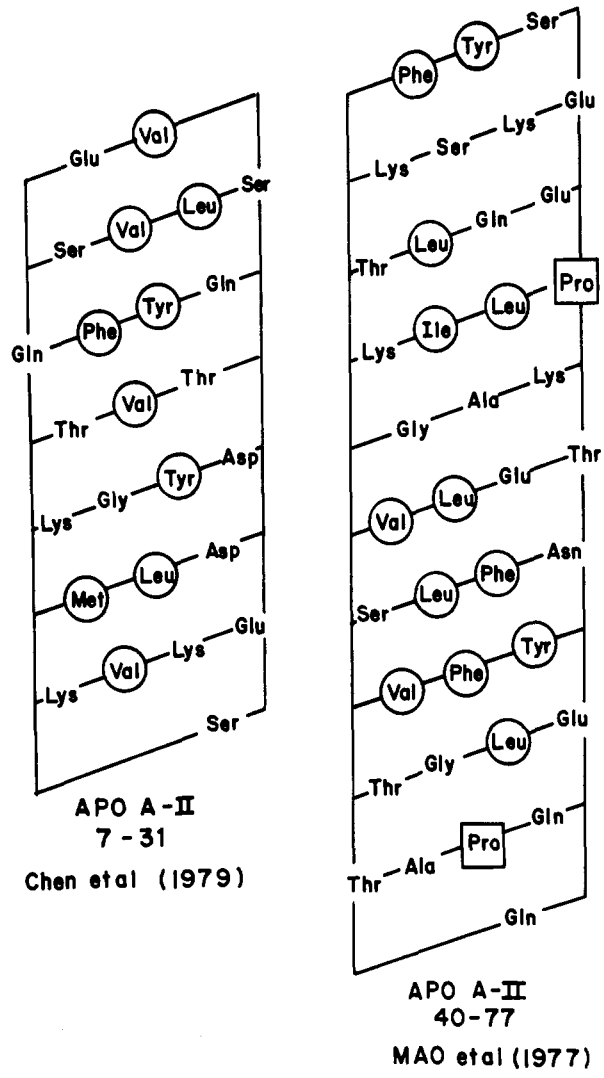

FIGURE 10: A schematic representation of the predicted and experimentally determined hydrophobic faces of the lipid-binding region of apoA-II. (A, left) The 7–31 sequence of apoA-II demonstrated to be a lipid-binding region by Chen et al. (1979). (B, right) The 40–77 sequence of apoA-II demonstrated to be a lipid-binding region by Mao et al. (1977). The representation is of an α-helical region in two dimensions where the hydrophobic residues are represented by the circled amino acids. The assumption that most of the protein is α helical is based on the CD data where the protein in the 75:1 complex is $\simeq$75% α helical. The region between residues 40 and 77 is represented as one long helix; however, proline residues at positions 51 and 75 would prevent this section from being totally helical.

plex formation is due to the transfer of hydrophobic clusters of amino acid side chains on the nonpolar face from water into the nonpolar region of the lipid. From the Gdn–HCl denaturation of apoA-II, Reynolds (1976) has concluded that most of the hydrophobic residues of the apolipoprotein are exposed to the aqueous phase. We have calculated the hydrophobic free energy of transfer of apoA-II as the sum of the free energy of transfer of the individual amino acids from an aqueous to a nonpolar environment using the free-energy values of Bull & Breese (1974). We obtained a value of −98 kcal/mol of apoA-II when we sum up the values for each amino acid on the hydrophobic face. The hydrophobic free energy contribution must be of this order of magnitude or greater for lipid and protein to spontaneously associate at $T < T_c$ (DMPC liposomes) where the enthalpy of reaction is +90 kcal/mol of apoA-II. The correlation between this model and our calorimetric data suggests that the association is, in part, entropically driven by the transfer of the hydrophobic side chains of the protein from water to the hydrocarbon region of the lipid matrix.

In conclusion, the enthalpy of apolipoprotein association with phospholipids is a function of accompanying structural changes in both the lipid and the protein and can be either exothermic or endothermic. The entropic part of the free energy of association can be contributed in part to the transfer of certain amino acid side chains from an aqueous to a hydrophobic environment. The direct measurement of the free energy will give exact values for the entropy, to further quantitate the thermodynamics of the amphipathic helical model of lipid–apolipoprotein association.

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Semisynthesis of Phospholipase A_2. Preparation and Properties of Arginine-6 Bovine Pancreatic Phospholipase A_2†

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ABSTRACT: The major differences between porcine and bovine pancreatic phospholipases A_2 are the low affinity of the bovine enzyme for lipid–water interfaces and its low capacity to penetrate more densely packed monolayers of lecithins. In the proposed binding site for lipid–water interfaces the porcine enzyme has an Arg residue at position 6 which is Asn in the bovine enzyme. In order to study whether this difference affects the above-mentioned properties, a hybrid bovine phospholipase A_2 that has Arg at position 6 was prepared. Bovine pancreatic prophospholipase A_2 was converted into the fully ϵ-amidinated zymogen (AMPREC) which produced enzymatically active ϵ-amidinated phospholipase A_2 (AMPA) upon limited proteolysis. CNBr cleavage of AMPREC at the unique Met residue at position 8 gave des(Ala^1–Met^8)AMPA, a protein completely devoid of all enzymatic activity. Met^8 was reintroduced by coupling of the latter protein with Boc-Met-*N*-hydroxysuccinimide ester followed by treatment with trifluoroacetic acid, yielding des(Ala^1–Gly^7)AMPA. Subsequently Boc-Ala-Leu-Trp(For)-Gln-Phe-Arg-Gly, synthesized by the solid-phase technique, was coupled by using the mixed-anhydride method. Removal of the protecting groups and purification gave semisynthetic bovine [Arg^6]AMPA in 30% yield. The feasibility of this procedure was proven unambiguously by the retroconversion of des(Ala^1–Met^8)AMPA into the original bovine AMPA, being identical in all respects including enzymatic activity with the starting AMPA. Both the affinity of bovine [Arg^6]AMPA for lipid–water interfaces and its ability to penetrate more densely packed monolayers of lecithin are considerably increased as compared to the bovine AMPA. In these respects bovine [Arg^6]AMPA was found to be almost identical with the porcine AMPA. Moreover, bovine [Arg^6]AMPA possesses enhanced enzymatic activity as compared to bovine and porcine AMPA. It can be concluded that substitution of Asn^6 by Arg in bovine phospholipase A_2 improves the binding for lipid–water interfaces. The concomitant increase in enzymatic activity strongly suggests an effect of the lipid binding site on the active site.

Phospholipase A_2 (EC 3.1.1.4) specifically catalyzes the hydrolysis of the 2-acyl ester linkage in 3-*sn*-phosphoglycerides (van Deenen & de Haas, 1964). The pancreatic enzyme occurs as a zymogen, prophospholipase A_2, which is converted into the active enzyme by splitting the N-terminal heptapeptide (Figure 1) upon limited proteolysis (de Haas et al., 1968). Both proteins have low, though comparable, activities toward monomeric substrate, indicating the presence of a functionally active site in the zymogen as well (Pieterson et al., 1974; Volwerk et al., 1979). When substrate is present as a certain organized lipid–water interface, like, e.g., micelles, there is a large increase in enzymatic activity of phospholipase A_2 but not for its zymogen (Pieterson et al., 1974). Various direct binding studies with micellar substrate analogues demonstrated that phospholipase A_2, in contrast to the zymogen, binds to these lipid–water interfaces (Pieterson et al., 1974; van Dam-Mieras et al., 1975; Soares de Araujo et al., 1979). Therefore, the pancreatic phospholipases A_2 were proposed to possess a specific binding site for lipid–water interfaces called the interface recognition site (IRS).[1] A conformational change

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[1] Abbreviations following the IUPAC–IUB Commission on Biochemical Nomenclature recommendations (*Biochemistry 6*, 362, 3287 (1967); *11*, 1726 (1972)) were used throughout. Other abbreviations used: AMPA, ϵ-amidinated phospholipase A_2; AMPREC, ϵ-amidinated prophospholipase A_2; tosyl, *p*-toluenesulfonyl; Boc, *tert*-butyloxycarbonyl; DCC, dicyclohexylcarbodiimide; For, formyl; LC, liquid chromatography; iBuOCOCl, isobutyl chloroformate; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; IRS, interface recognition site; TFA, trifluoroacetic acid; CPK model, Corey, Pauling, and Koltun space-filling molecular model; CIDNP, chemically induced dynamic nuclear polarization; TSP, [2,2,3,3-$^2H_4$]trimethylsilylpropionate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; cmc, critical micellar concentration; EtOH, ethanol; DIEA, *N*,*N*′-diisopropylethylamine; DMF, dimethylformamide; NMR, nuclear magnetic resonance; pH*, uncorrected pH meter readings in ^2H_2O; ppm, parts per million.

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