# Mechanism of Dissociation of Human Apolipoproteins A-I, A-II, and C from Complexes with Dimyristoylphosphatidylcholine As Studied by Thermal Denaturation†

D.-J. Reijngoud‡ and M. C. Phillips*

ABSTRACT: The effect of temperature on the structure of human apolipoprotein A-I (apo A-I), apo A-II, and the combined apo C fraction in the absence and presence of dimyristoylphosphatidylcholine (DMPC) has been investigated. The thermal denaturation of the apolipoproteins was monitored by circular dichroism spectroscopy. In the absence of lipid, the apolipoproteins A-I and A-II denature over a wide temperature range, giving van't Hoff enthalpies of 33 ± 4 kcal/mol of apo A-I and 17.8 ± 0.2 kcal/mol of apo A-II. These enthalpies are independent of the protein concentration, although a decrease in molar ellipticity was observed on increasing the protein concentration from 0.01 to 1 mg/mL. No effect of temperature could be observed on the combined apo C fraction because at 0.01 and 1 mg/mL the apo C's were essentially random coiled. In the presence of DMPC, thermal denaturation could be measured for apo A-I above 70–75 °C and for apo A-II and apo C above about 45 °C. In general, the denaturations were biphasic reactions for all apolipoproteins tested, with only a third, minor intermediate phase for apo A-I/DMPC denaturation. The two major kinetic phases are identified as an unfolding reaction of the apolipoprotein bound to the complex followed by a desorption step. The relaxation times ($\tau$) associated with the latter step are dependent on the molecular weight of the apoprotein: when the temperature is increased from 70 to 90 °C, $\tau$ decreases from 400 to 1 min for apo A-I, while for apo A-II and apo C as the temperature is increased from 50 to 70 °C, $\tau$ decreases from 15 to 1 min. The activation energies for the desorption of apoprotein decrease with decreasing molecular weight: the values are 71 ± 2 kcal/mol of apo A-I, 28 ± 3 kcal/mol of apo A-II, and 22 ± 3 kcal/mol of apo C. The thermal denaturation of apo A-I/DMPC is a thermodynamically irreversible process whereas the denaturations of apo A-II/DMPC and apo C/DMPC complexes are reversible with midpoints of 71 and 54 °C, respectively. The van't Hoff enthalpies are 16.8 ± 0.6 kcal/mol of apo A-II ($T < 70$ °C), 86 ± 2 kcal/mol of apo A-II ($T > 70$ °C), and 22.3 ± 0.8 kcal/mol of apo C. On the basis of the above findings, a model to describe the association and dissociation of apolipoproteins with DMPC has been derived. It is assumed that, on a molecular level, the association reaction is determined by two parameters: (1) the intrinsic rate constant describing the insertion of an apolipoprotein into a "vacancy" in the phospholipid matrix ("on rate") and (2) the probability of the colliding apoprotein molecule encountering a vacancy in the phospholipid bilayer. Alterations in either of these two parameters change the macroscopic rate constant of association. Desorption ("off rate") involves the protein leaving from a constant phospholipid environment because the perturbed adjacent lipid molecules render this process insensitive to the physical state of the remainder of the bilayer. The similarities in the van't Hoff enthalpies associated with the reversible desorption of apo A-II and apo C to literature values for the calorimetric enthalpies of association of these proteins with DMPC suggest that the desorption is a two-state process.

In human serum lipoproteins, the surface monolayer which surrounds the core of apolar lipids comprises mainly phospholipids, cholesterol, and apolipoproteins (Edelstein et al., 1979). The surface location of apolipoproteins is facilitated by the presence of amphipathic $\alpha$-helices in the secondary structure of the apolipoproteins, as has been inferred from the amino acid sequence (Segrest et al., 1974). The structural predictions on the content of $\alpha$-helix in apolipoproteins agree quite well with the values obtained from circular dichroism spectra [cf. Osborne & Brewer (1977) and Sparrow & Gotto (1982)]. This type of $\alpha$-helix arises when apolar and polar residues in the amino acid sequence of the apolipoprotein are located on opposite sides of the helix. The amphipathic nature of the helix allows the apolipoprotein to concentrate at interfaces of phospholipids and cholesterol by intercalation between the surface constituents [for a review, see Osborne & Brewer (1977)]. In contrast to this quite detailed understanding of the structural aspects of the apolipoprotein/lipid interaction, the thermodynamics of the interactions are far less well understood. The interaction of free apolipoproteins with phospholipids has been studied the most intensively. Thus, it has been observed by microcalorimetry (Massey et al., 1981; Pownall et al., 1981b) and column chromatography (Jonas & Mason, 1981) that the association with phospholipids of apolipoprotein A-I (apo A-I),[1] apo A-II, reduced and carboxymethylated apo A-II, and apo C-III occurs most avidly near the gel to liquid-crystal transition temperatures of the lipids for the homologous series of dilauroyl- to dipalmitoylphosphatidylcholines. Furthermore, it was found that large enthalpies are associated with this recombination. At odds with this, it has been observed that the partition of reduced and carboxymethylated apo A-II between the aqueous and dimyristoylphosphatidylcholine (DMPC) phases is constant

† From the Department of Physiology and Biochemistry, The Medical College of Pennsylvania, Philadelphia, Pennsylvania 19129. *Received June 27, 1983.* This work was supported by a NATO fellowship from the Netherlands Organization for the Advancement of Pure Research (ZWO) to D.-J.R. and by National Institutes of Health Grant PPG HL22633.

‡ Present address: Physiological Chemistry Laboratory, Rijksuniversiteit, Bloemsingel 10, Groningen, The Netherlands.

[1] Abbreviations: apo A-I, apolipoprotein A-I; apo A-II, apolipoprotein A-II; apo C, apolipoprotein C; DMPC, dimyristoylphosphatidylcholine; EDTA, ethylenediaminetetraacetic acid; Gdn·HCl, guanidine hydrochloride; $NaDodSO_4$, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PC, phosphatidylcholine; Tris, tris(hydroxymethyl)-aminomethane; HDL, high-density lipoprotein.

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over the temperature range 5–37 °C (Pownall et al., 1981). This occurs although the gel to liquid-crystal transition temperature of DMPC [24 °C; cf. Phillips (1972)] is included and large enthalpies of association are observed at this point; application of the Gibbs–Helmholtz equation predicts that a change in the free energy of binding and partitioning of apoprotein should occur at 24 °C.

It is necessary to consider the denaturation of preformed complexes of apolipoproteins and phospholipids with increasing temperature in order to understand the molecular processes involved and to resolve the above anomaly. By this approach, it is possible to relate the observed thermodynamic parameters to particular steps in the processes of association and dissociation of apolipoproteins and phospholipids. Here, the influence of temperature on the denaturation of complexes of DMPC with human apo A-I ($M_r$ 28 000) (Scanu et al., 1975), apo A-II ($M_r$ 17 500), and the combined apo C fraction ($M_r$ 7000–10 000) has been investigated. In this way, insights are gained into the effects of apolipoprotein molecular weight on the interaction with DMPC. The denaturation was followed by circular dichroism spectroscopy. This study shows that apo A-I/DMPC complexes denature in an irreversible fashion. In contrast, complexes of apo A-II and apo C with DMPC exhibit a reversible denaturation. The van't Hoff enthalpies of these equilibrium denaturations compare well with the published heats of association for apo A-II and apo C-III with DMPC measured above the phase transition of the lipids in the complexes. The kinetics of denaturation of apolipoprotein/DMPC complexes are essentially biphasic: there is a rapid initial reaction followed by a second far-slower reaction. By comparison with previous data on the Gdn·HCl-induced denaturation of apo A-I/DMPC complexes, where similar biphasic kinetics were observed (Reijngoud & Phillips, 1982), this second phase is attributed to desorption of apolipoprotein from the surface of the lipid/protein complex.

## Experimental Procedures

### *Materials*

Total HDL ($1.063 < d < 1.21$ g/mL) was isolated from outdated human plasma from the blood bank by using sequential ultracentrifugation (Havel et al., 1955). Total HDL was delipidated with 3:2 (v/v) ethanol:diethyl ether at 0 °C (Scanu & Edelstein, 1971) and apo A-I, apo A-II, and the combined apo C fraction were isolated by using a modification of the original method of Scanu et al. (1969); the protein was chromatographed on Sephacryl S300 columns (90 × 5 cm) using 6 M urea, 1 M NaCl, 10 mM Tris-HCl, pH 8.6, 1 mM EDTA, and 0.02% $NaN_3$ as the elution buffer. The fractions corresponding to the positions of the different apolipoproteins were pooled, dialyzed against distilled and deionized water, and lyophilized. Purification of the different apolipoproteins was achieved by rechromatography on a Sephacryl S200 column (180 × 1 cm) eluted with the above buffer. The peak fractions containing different apolipoproteins were pooled, dialyzed against distilled and deionized water, and lyophilized. The protein was stored at −20 °C. The resultant apo A-I and apo A-II gave single bands on NaDodSO$_4$–PAGE.

Apolipoproteins were dissolved in buffer (0.15 M NaCl, 25 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 0.02% $NaN_3$) supplemented with 4 M Gdn·HCl. The solutions were desalted by passing them through a Bio-Rad P2 column (5 × 1 cm) and were spun at $95000g_{max}$ for 2 h. After centrifugation, the protein concentration was determined by using the NaDodSO$_4$–Lowry method of Markwell et al. (1978). The protein solutions were then diluted to the requisite concentrations. The remaining materials have been described previously (Reijngoud & Phillips, 1982).

### *Methods*

*Formation of Lipid/Protein Complexes.* Complexes of the different apolipoproteins and DMPC were prepared by incubation of the protein with a hand-shaken DMPC dispersion at an initial molar ratio of 1:100 at 24 °C overnight in buffer supplemented with 0.3 M Gdn·HCl. Gdn·HCl was added to prevent self-association of apolipoproteins from interfering in the recombination of lipid and protein; 0.3 M Gdn·HCl does not affect the resulting lipid/protein complexes (Edelstein & Scanu, 1980; Reijngoud & Phillips, 1982; unpublished results). After incubation, the samples were centrifuged for 2 h at $95000g_{max}$ to remove excess lipids. NaBr was added to the supernatant to increase the density to 1.21 g/mL, and the samples were centrifuged for 18 h at $170000g_{max}$ to remove excess protein. After centrifugation, the top 2 mL was collected and dialyzed against buffer. After dialysis, the solution was analyzed for protein as before, and phospholipid (as phosphorus) was determined according to Sokoloff & Rothblat (1974).

*Circular Dichroism.* Thermal denaturation was recorded with a JASCO 41A spectropolarimeter by the change in molar ellipticity at 220 nm. The denaturation was started by adding the sample, kept at 24 °C, to the cuvette which was maintained at the temperature in the range 25–90 °C by a Haake E12 circulating pump (Haake, Saddle Brook, NJ). The time required for the thermal equilibration of the sample was ≤1 min. The temperature was monitored inside the cuvette with a chromel–constantan thermocouple connected to a digital display (Omega, Stanford, CT). The molar ellipticity at 220 nm was calculated according to

$$[\theta]_{220} = \frac{(\mathrm{MRW})\theta_{220}}{10lc}$$

where $\theta_{220}$ is the observed ellipticity at 220 nm in degrees, MRW the mean residue molecular weight, $l$ the path length in centimeters (0.1 cm), and $c$ the protein concentration in grams per milliliter. The MRW values for apo A-I and apo A-II were 114.2 and 113.1, respectively (Scanu et al., 1975). For the combined apo C fraction, a value for the MRW of 112 was used.

*Analysis of Kinetic Data.* The decay of $[\theta 1_{220}$ was digitized, and the fractional decrease in the signal was plotted semilogarithmically against time. If necessary, analysis of these plots was carried out by "peeling back" the various kinetic phases in order of decreasing time constant (Hagerman & Baldwin, 1976). In order to account for the amplitude of the reaction completed within the dead time of the method, the sum of the amplitudes was normalized to unity. Calculation of the equilibrium constant of denaturation from the curves of $[\theta]_{220}$ vs. temperature was done in the usual way, by assuming that the denaturation was a two-state reversible process. Since kinetically detectable intermediate states were observed, the assumption implies that at equilibrium these states are occupied only to a minor degree (Lapanje, 1978; Tanford, 1970). The denaturation constant is

$$K_D = \frac{\theta_N - \theta}{\theta - \theta_D} \quad (1)$$

where $\theta$ is the observed $[\theta]_{220}$ at the indicated temperature and $\theta_N$ and $\theta_D$ are the values for the $[\theta]_{220}$ for the native and unfolded forms of the apolipoprotein, respectively, obtained by extrapolation. The extrapolations of $\theta_N$ and $\theta_D$ are shown as dashed lines in the figures. Standard free energies ($\Delta G°$)

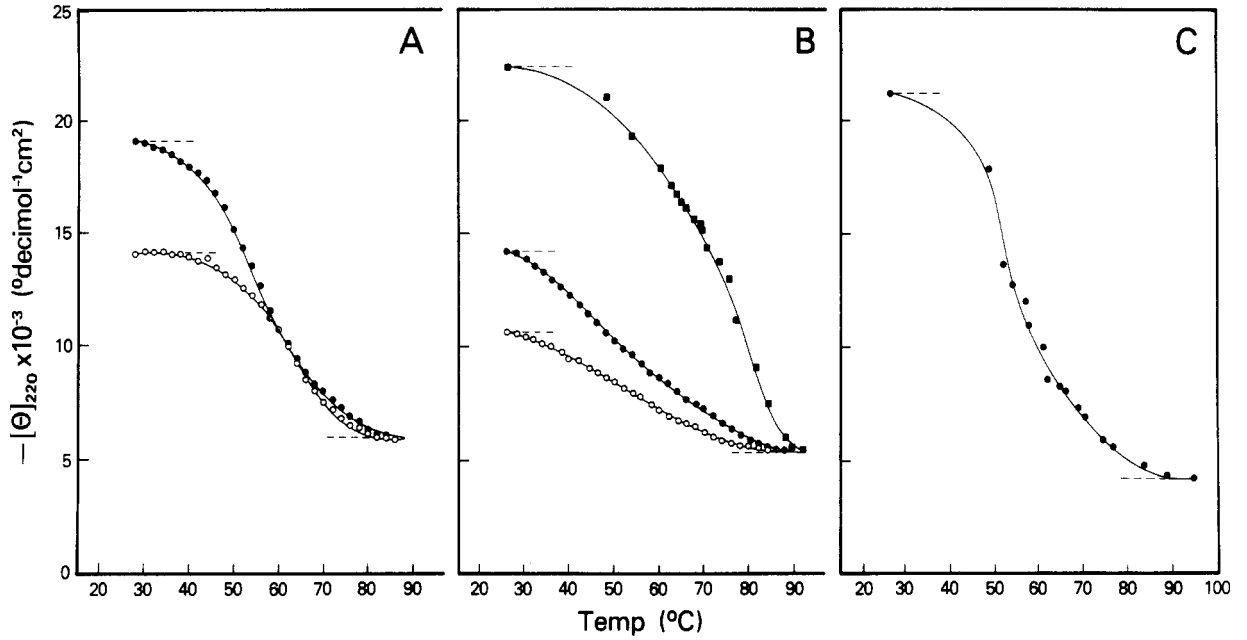



FIGURE 1: Influence of temperature on the molar ellipticity, $[\theta]_{220}$, of human apolipoproteins dissolved in buffer solution (0.15 M NaCl, 25 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 0.02% $NaN_3$) either as free protein or as complexes with DMPC. (A) Free apo A-I at 1.0 (●) and 0.01 (○) mg/mL. (B) Free apo A-II at 1.0 (●) and 0.01 (○) mg/mL; 1:85 (mol/mol) apo A-II/DMPC complex (■). (C) 1:50 apo C/DMPC complex.

of denaturation for the free apolipoprotein were calculated according to

$$\Delta G° = -RT \ln K_D \quad (2)$$

Standard free energies of denaturation for DMPC/apolipoprotein complexes were calculated according to a procedure which gives this potential in unitary terms [see Pownall et al. (1981) and Tanford (1973)]. For the denaturation

$$\Delta G° = -RT \ln \frac{X_{H_2O}}{X_{PC}} \quad (3)$$

where $X_{H_2O}$ and $X_{PC}$ are the mole fractions of denatured protein in the aqueous phase and native protein in the phospholipid phase, respectively. It should be noted that this calculation does not include any contribution to the total free energy change from discoidal sections of bilayer sealing and rearranging to the vesicular structure:

$$X_{H_2O} = \frac{n_{prot}^{H_2O}}{n_{H_2O}} \quad (4)$$

$$X_{PC} = \frac{n_{prot}^{PC}}{n_{PC}} \quad (5)$$

where $n_{prot}^{H_2O}$ and $n_{prot}^{PC}$ are the number of protein molecules in the water and phospholipid phases, respectively, and $n_{H_2O}$ and $n_{PC}$ are the number of water and phospholipid molecules, respectively, in 1 mL of solution. Substitution of eq 1 and 5 in eq 3 and rearranging lead to

$$\Delta G° = -RT \ln \left( \frac{n_{prot}^{H_2O}}{n_{prot}^{PC}} \frac{n_{PC}}{n_{H_2O}} \right) \quad (6)$$

When the denaturation of apolipoprotein/DMPC complexes is accompanied by a release of denatured protein (see Discussion), the partition coefficient $n_{prot}^{H_2O}/n_{prot}^{PC}$ is equal to the denaturation constant $K_D$ so that

$$\Delta G° = -RT \ln \left( K_D \frac{n_{PC}}{n_{H_2O}} \right) \quad (7)$$

At the applied protein concentrations and the molar ratios of apolipoproteins to DMPC, the correction term $n_{PC}/n_{H_2O}$ is equal to $(4.9 \pm 0.2) \times 10^{-5}$ for apo A-II and $(6.5 \pm 0.3) \times 10^{-5}$ for the combined apo C fractions.

The calculation at 25 °C of the standard enthalpy ($\Delta H°$), standard entropy ($\Delta S°$), and standard free energy ($\Delta G°$) of association of apolipoprotein with DMPC was carried out by subtracting the value for complex denaturation from the value for free apolipoprotein denaturation.

## Results

*Free Apolipoproteins.* The thermal denaturation of free apo A-I is shown in Figure 1A as the increase in $[\theta]_{220}$ with increasing temperature. This denaturation was measured at two protein concentrations, 0.01 and 1 mg/mL; at the former concentration at 25 °C, apo A-I is monomeric (>90%) while at the latter concentration it is predominantly (~70%) oligomeric (Swaney & O'Brien, 1978). The tendency to self-associate at 28 °C is reflected in the decrease in $[\theta]_{220}$ from $-14.1 \times 10^3$ deg cm$^2$ dmol$^{-1}$ at 0.01 mg/mL to $-19.0 \times 10^3$ deg cm$^2$ dmol$^{-1}$ at 1 mg/mL, indicating the increase in $\alpha$-helix content upon self-association [cf. Massey et al. (1981) and Osborne & Brewer (1977)]. It is obvious from Figure 1A that at both concentrations the melting range is about 40–80 °C, with a midpoint at 61 °C for 0.01 mg/mL and at 56 °C for 1 mg/mL (Gwynne et al., 1975). The calculated van't Hoff enthalpies are 35 ± 1 kcal/mol of apo A-I and 29.9 ± 0.2 kcal/mol of apo A-I at 0.01 and 1 mg/mL, respectively (Table I). Although the enthalpies are slightly lower than those observed by Tall et al. (1975, 1977), the melting points are the same. Furthermore, the data in Figure 1A show that self-association of apo A-I does not enhance its thermal stability. In this respect, it is of interest to note that Swaney & O'Brien (1978) observed that the degree of self-association depends on the incubation temperature in a way very similar to that depicted for $[\theta]_{220}$ in Figure 1. The $\Delta G°$ values of denaturation of apo A-I at 25 °C at 0.01 and 1 mg/mL are 3.3 ± 0.2 and 2.9 ± 0.1 kcal/mol of apo A-I, respectively (Table I). Similar values have been observed for Gdn·HCl-

Table I: Thermodynamic Parameters[a] for the Denaturation of Apolipoproteins and Their Complexes with DMPC at 25 °C

| apolipoprotein | $\Delta H°$ (kcal mol$^{-1}$) | $\Delta S°$ (cal mol$^{-1}$ deg$^{-1}$) | $\Delta G°$ (kcal mol$^{-1}$) |
|---|---|---|---|
| A-I | | | |
| free[b] | 33 ± 4 | 100 ± 10 | 3.1 ± 0.3 |
| complex | *c* | *c* | *c* |
| A-II | | | |
| free | 17.8 ± 0.2 | 55 ± 1 | 1.5 ± 0.1 |
| complex | | | |
| $T < 70$ °C | 16.8 ± 0.6 | 29 ± 5 | 8.2 ± 0.4 |
| $T > 70$ °C | 86 ± 2 | 225 ± 10 | 19 ± 1 |
| C | | | |
| free | *d* | *d* | *d* |
| complex | 18.1 ± 0.4 | 36 ± 2 | 7.2 ± 0.2 |

[a] The thermodynamic parameters are expressed as (moles of apolipoprotein)$^{-1}$. [b] Average of values obtained at 0.01 and 1 mg of apoprotein/mL. [c] Irreversible denaturation. [d] Completely unfolded.

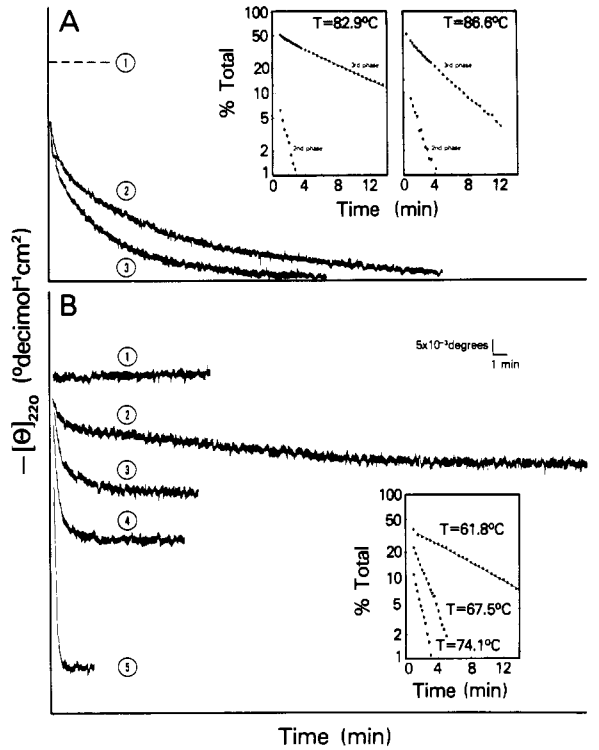



FIGURE 2: Kinetics of the increase of the molar ellipticity ($[\theta]_{220}$) of 1:90 (mol/mol) apo A-I/DMPC and 1:85 apo A-II/DMPC complexes during thermal denaturation after rapid heating to the indicated temperatures. (A) Apo A-I/DMPC: Time courses of the changes of $[\theta]_{220}$ at 25.3, 82.9, and 86.6 °C are represented by curves 1, 2, and 3, respectively. The two insets show the first-order decays at two temperatures of the two slower kinetic phases obtained by curve peeling. (B) Apo A-II/DMPC: Curves 1, 2, 3, 4, and 5 represent the time courses at 26.1, 61.8, 67.5, 74.1, and 92.3 °C, respectively. The inset shows the first-order kinetics of the slow second phases observed at 61.8, 67.5, and 74.1 °C. The remaining conditions are as given in the legend to Figure 1.

induced denaturation (Edelstein & Scanu, 1980; Reijngoud & Phillips, 1982).

The thermal denaturation studies of apo A-II are summarized in Figure 1B. Two concentrations of free apo A-II were used in order to evaluate the effects of self-association on its thermal stability; at 0.01 mg/mL, about 30% of the apo A-II is dimeric, and this increases to about 70% at 1 mg/mL (Swaney & O'Brien, 1978). As a consequence of this self-association, $[\theta]_{220}$ at 25 °C decreases from $-10.6 \times 10^3$ to $-14.2 \times 10^3$ deg cm$^2$ dmol$^{-1}$ [cf. Gwynne et al. (1975)]. Similar to

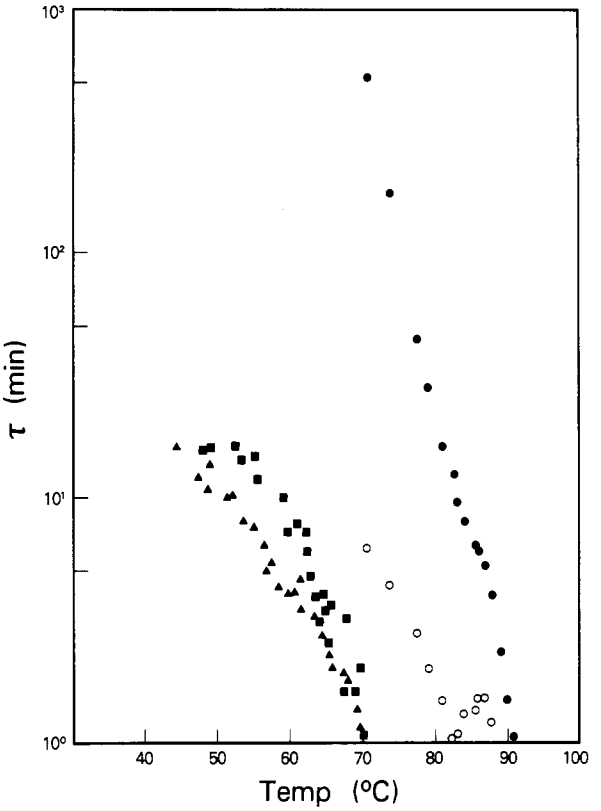



FIGURE 3: Relaxation times ($\tau$) of thermal denaturation of apolipoprotein/DMPC complexes. The $\tau$ values were derived for the various kinetic phases identified in kinetic data such as those shown in Figure 2. (○) $\tau_2$(apo A-I); (●) $\tau_3$(apo A-I); (■) $\tau_2$(apo A-II); (▲) $\tau_2$(apo C).

the denaturation of apo A-I, the transition of apo A-II occurs over an ~60 °C temperature range with a midpoint of 53 °C for both concentrations. Consequently, a low van't Hoff enthalpy of 17.8 ± 0.2 kcal/mol of apo A-II is observed in both cases (Table I). As with apo A-I, the increased self-association of apo A-II has no influence on its thermal stability. The observations of Swaney & O'Brien (1978) on the temperature sensitivity of the self-association of apo A-II are very similar to the present data for $[\theta]_{220}$. At 25 °C, $\Delta G°$ for the denaturation of apo A-II is 1.5 ± 0.1 kcal/mol of apo A-II (Table I). Reynolds (1976) reported a value of 1.0 kcal/mol of apo A-II on the basis of the Gdn·HCl-induced denaturation of apo A-II. It seems that the folded structure of apo A-II is only marginally more stable than the completely unfolded protein.

When similar experiments were performed with the combined apo C fraction, $[\theta]_{220}$ at 25 °C showed only a slight decrease when the protein concentration was raised 100-fold; at 0.01 mg/mL, $[\theta]_{220} = -4.1 \times 10^3$ deg cm$^2$ dmol$^{-1}$, and at 1 mg/mL, $[\theta]_{220} = -7.1 \times 10^3$ deg cm$^2$ dmol$^{-1}$. This indicates that at both concentrations the protein is mainly random coil. Accordingly, no thermally induced denaturation could be observed.

*Complexes of Apo A-I and DMPC at a Molar Ratio of 1:90.* The thermal denaturation of complexes of apo A-I and DMPC is an irreversible process. At temperatures at which it was possible to measure the $[\theta]_{220}$ after thermal denaturation was complete ($T > 78$ °C), it was observed that this final value of $[\theta]_{220}$ was independent of the temperature when corrected for the $[\theta]_{220}$ of free apo A-I. This indicates that partly unfolded apo A-I is unable to reassociate with DMPC at these temperatures. The time courses for this irreversible denaturation, the time constants ($\tau$), and related amplitudes ($\alpha$) are shown as a function of temperature in Figures 2A, 3, and 4A,

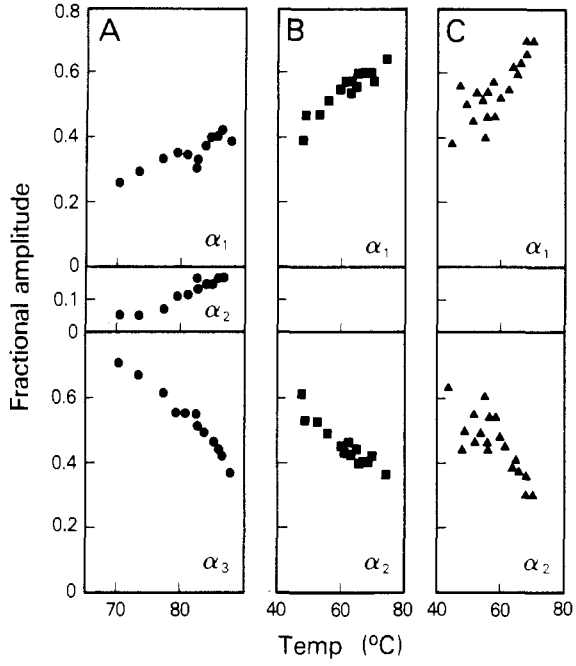



FIGURE 4: Amplitudes ($\alpha$) of the various kinetic phases obtained by curve-peeling data such as those in Figure 2 for the thermal denaturation of apolipoprotein/DMPC complexes. (A) Apo A-I; (B) apo A-II; (C) apo C.

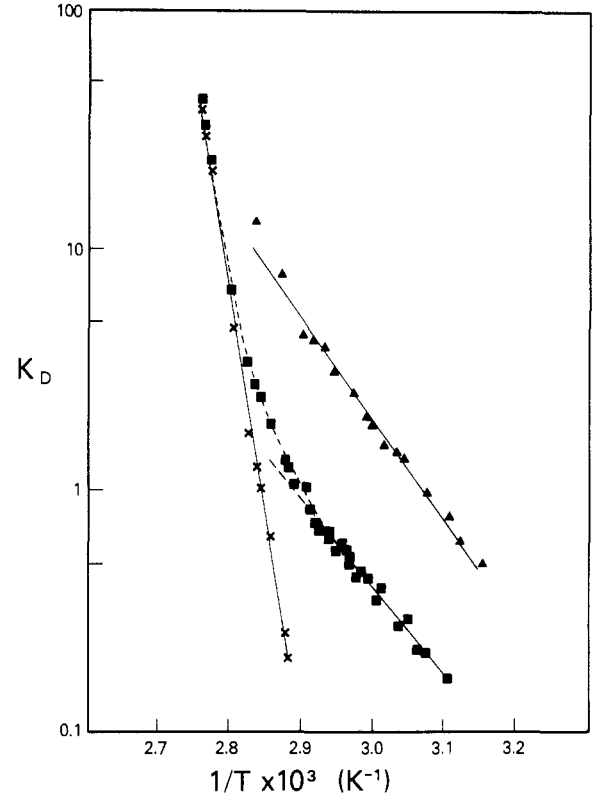



FIGURE 5: van't Hoff plot of the denaturation constants $K_D$ (see eq 1) for the reversible thermal denaturation of apo A-II/DMPC and apo C/DMPC complexes. (■) Experimental points for apo A-II/DMPC; (×) points corrected for the contribution from the low-temperature region ($2.9 \times 10^{-3} < 1/T < 3.1 \times 10^{-3}$ K$^{-1}$); (▲) experimental points for apo C/DMPC. The lines are fitted by linear regression. The derived van't Hoff enthalpies are listed in Table I.

respectively. At least three phases can be discerned in the denaturation of apo A-I/DMPC complexes. The first phase is essentially completed within the dead time of thermal equilibration and is followed by a minor second phase ($0.04 \leq \alpha \leq 0.15$). The subsequent third phase, together with the first phase, accounts for the major part of the observed decay. It is apparent from Figure 3 that measurable rates of denaturation can be observed only above 70 °C. Activation energies ($E_a$) were derived from Arrhenius plots of the data in Figure 3; $E_a = 24 \pm 4$ and $71 \pm 2$ kcal/mol of apo A-I for the second and third phases, respectively.

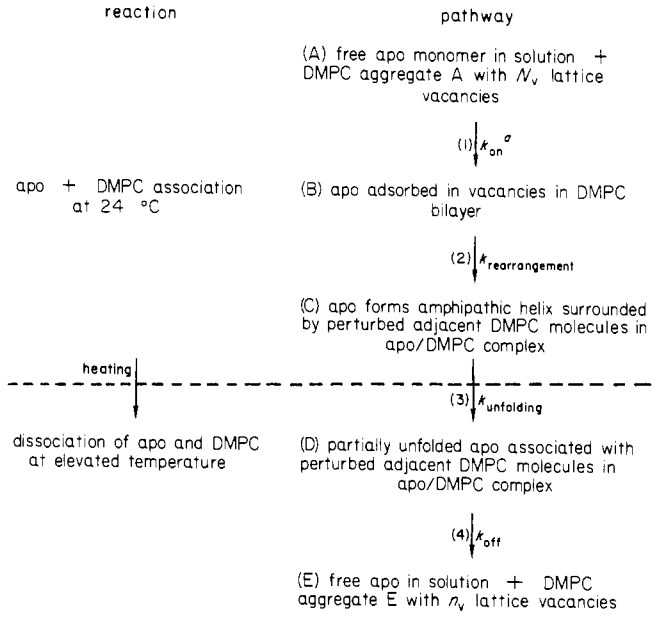
*Complexes of Apo A-II and DMPC at a Molar Ratio of 1:85.* The thermal denaturation of apo A-II complexed with DMPC as monitored by $[\theta]_{220}$ is shown in Figures 1B and 2B. Since the final value of $[\theta]_{220}$ is lower in the presence than in the absence of lipids at all temperatures below which complete denaturation occurs ($T < 90$ °C), it follows that partly denatured apo A-II and DMPC are able to reassociate at temperatures far above the gel to liquid-crystal transition temperature of DMPC. Furthermore, experiments in which heat-denatured apo A-II/DMPC complexes were cooled down to allow reassociation to occur gave the same final value for $[\theta]_{220}$. The complex denatures over a wide temperature range with a midpoint of 71 °C. A van't Hoff analysis of the thermal denaturation of apo A-II/DMPC complexes is shown in Figure 5, and a change in the slope of the van't Hoff plot is apparent. This change can be interpreted most reasonably by assuming that several equilibria with different van't Hoff enthalpies occur in parallel. The thermal denaturation below 70 °C is determined by a single van't Hoff enthalpy ($\Delta H° = 16.8 \pm 0.6$ kcal/mol of apo A-II, Table I). Correction of the thermal denaturation at temperatures above 70 °C for the contribution of this equilibrium shows that this part of the plot can also be accounted for by a single van't Hoff enthalpy (Figure 5; $\Delta H° = 86 \pm 2$ kcal/mol of apo A-II, Table I). The latter enthalpy is associated with the last 35% of the transition. At 25 °C, $\Delta G°$ for the denaturation of apo A-II/DMPC complexes is $8.2 \pm 0.4$ kcal/mol of apo A-II for the extrapolation of the van't Hoff plot below 70 °C and $19 \pm 1$ kcal/mol of apo A-II for the extrapolation of the corrected plot above 70 °C (Table I).

The kinetics of thermal denaturation are biphasic (Figure 2B); the first phase is completed within the time of thermal equilibration of the sample. The time constant $\tau_2$ of the second phase and the amplitudes of both phases are shown in Figures 3 and 4B, respectively. The linear portion of the Arrhenius plot of these data gives an activation energy of $28 \pm 3$ kcal/mol of apo A-II.

*Complexes of the Combined Apo C Fraction with DMPC at a Molar Ratio of 1:50.* For complexes of apo C and DMPC, the thermal denaturation exhibits essentially the same characteristics as the denaturation of apo A-II except that the midpoint is somewhat lower at 54 °C (Figure 1C). As with apo A-II/DMPC complexes, the thermal denaturation of apo C/DMPC complexes is a reversible process because apo C can recombine with DMPC at temperatures above the gel to liquid-crystal transition of DMPC. Control experiments in which heat-denatured complexes were cooled down gave values for $[\theta]_{220}$ similar to those observed in the heat denaturation reaction. The van't Hoff plot describing the thermal denaturation of apo C/DMPC complexes is a straight line giving an enthalpy of $18.1 \pm 0.4$ kcal/mol of apo C (Figure 5). At 25 °C, $\Delta G°$ for the denaturation of the complex is $7.2 \pm 0.2$ kcal/mol of apo C (Table I).

As is the case for apo A-II/DMPC complexes, the kinetics of thermal denaturation of apo C/DMPC complexes are biphasic; the time constant of the second phase is given in Figure 3 while the amplitudes for both phases are given in Figure 4. The kinetic parameters for the denaturation of apo C/DMPC complexes are similar to those for apo A-II/DMPC; the second phase has an activation energy of $22 \pm 3$ kcal/mol of apo C

Scheme I: General Reaction Scheme for Formation of Apolipoprotein/DMPC Complexes and Their Thermal Denaturation




[a] $k$ represents the rate constant for the reaction described.

compared to 28 ± 3 kcal/mol of apo A-II for apo A-II/DMPC complexes. It is noteworthy that these values are similar to the activation energy of the minor second phase in the denaturation of apo A-I/DMPC complexes.

## Discussion

*Reaction Mechanisms.* The thermal denaturation of apoprotein/DMPC complexes involves two major kinetic phases, and the molecular events underlying these are summarized, together with a hypothetical mechanism of the association reaction of monomeric apolipoprotein with DMPC, in Scheme I. The details of the various reactions 1–4 in this scheme are discussed below.

*Step 1.* Studies in several laboratories have established that the physical state of the PC plays a critical role in both the rate and degree of association of apoprotein with PC dispersions. In particular, variables such as temperature (Pownall et al., 1978, 1981a–c), lateral phase separation (Swaney, 1980), or vesicle curvature (Wetterau & Jonas, 1982) which affect the PC molecular packing and the number of lattice defects or vacancies (Kanehisa & Tsong, 1978; Freire & Biltonen, 1978) can have dramatic effects on the rate of recombination. In other words, this step depends upon the lateral properties of PC bilayers because the association reaction involves the insertion of apolipoproteins into vacancies ($N_v$ is the number of lattice vacancies in the DMPC bilayer before association with protein) in the bilayer. For given concentrations, the rate of adsorption of monomeric apoprotein to PC aggregates will be proportional to the product $k_{on}N_v$. Because $N_v$ is maximum at the gel to liquid crystal-transition temperature ($T_c$ = 24 °C) of DMPC (Kanehisa & Tsong, 1978), the rate of association of apoprotein with DMPC is maximal at the same temperature (Pownall et al., 1981b). The rate constant $k_{on}$ describes the insertion of an apoprotein molecule into any bilayer vacancy it encounters: this process is assumed to be probably limited by the diffusion coefficient of the protein molecule and will not be sensitive to any phase transitions in the PC bilayer.

*Step 2.* Once the apoprotein molecule has adsorbed, it will rearrange and penetrate the DMPC bilayer. The penetration of apoprotein is accompanied by an increase in the α-helix content to the maximum permitted by the amino acid sequence. The amphipathic helices embedded in the PC bilayer are surrounded by adjacent lipid molecules whose packing is perturbed and which undergo hindered rotational motion compared to DMPC molecules farther away in the bilayer (Mantulin et al., 1981). The presence of apoprotein molecules in the bilayer leads to disruption of the bilayer and formation of small discoidal sections. This process causes a decrease in turbidity when the initial DMPC aggregate is a multilamellar liposome (Pownall et al., 1978). Random coil to helix transitions in proteins occur very rapidly (Schwarz, 1965) so that the rate-limiting reaction in step 2 will be the disruption of the PC bilayer.

A similar situation occurs when a turbid oil/water/surfactant emulsion is cleared by addition of a cosurfactant and a stable microemulsion is formed. DeGennes & Taupin (1982) have argued that for such systems the flexibility of the interface is a crucial factor in determining the particle size under conditions of a vanishing surface tension. Assuming that the surface tension of the phospholipid bilayer is close to zero throughout [cf. Tanford (1979)], the action of apolipoproteins on DMPC bilayers can be envisioned as causing an increase in the elasticity perpendicular to the plane of the bilayer so that a transition to a "discoidal" phase is induced. The compositional data show that with decreasing apoprotein molecular weight, a higher surface concentration of protein is required to accomplish this transition. An extremely low surface tension at the lipid/water interface is maintained by continued adsorption of apoprotein on to the freshly exposed apolar surface. The curvature–elastic modulus of an egg PC bilayer is a value characteristic of a "stiff" interface (Servuss et al., 1976; DeGennes & Taupin, 1982) and is consistent with pure PC forming large planar bilayers. DeGennes and Taupin have calculated that if addition of a suitable cosurfactant (e.g., an apolipoprotein) decreases the curvature–elastic modulus by a factor of 5, the enhanced surface flexibility leads to the formation of particles of ~100-Å diameter.

*Step 3.* Once the apolipoprotein/DMPC complex is heated to high temperatures, thermal denaturation of the apoprotein molecules occurs as indicated by the data in Figures 2–5. Similar effects have been observed by Tall et al. (1977) using calorimetry and by Epand (1982) using fluorescence polarization. The initial fast phase observed in the present kinetic data and in earlier Gdn·HCl denaturation studies from this laboratory (Reijngoud & Phillips, 1982) can be attributed to a rapid helix to random-coil transition in apoprotein molecules still associated with DMPC.

*Step 4.* As in Gdn·HCl denaturation of apo A-I/DMPC complexes (Reijngoud & Phillips, 1982), the second kinetic phase of thermal denaturation (e.g., Figures 3 and 4) can be attributed to desorption of apoprotein molecules from the lipid/water interface [cf. Tall et al. (1977) and Epand (1982)]. The amphipathic helical regions will desorb from an adjacent layer of perturbed DMPC molecules which causes the apoprotein molecules to be isolated from the bulk of the DMPC bilayer. Consequently, the protein molecules always desorb ($k_{off}$) from similar surroundings, largely independent of the physical state of the remainder of the DMPC molecules (i.e., unlike the rate of adsorption, the rate of desorption is not a function of $N_v$). The desorption of denatured apolipoprotein from disks exposes apolar surfaces to water. The increase in surface tension is compensated by fusion of disks which reduces the exposure of apolar hydrocarbon chains to water. According to a recent molecular model of vesicle formation during detergent removal (Lasic, 1982), when there is insufficient

apoprotein to shield the exposed apolar surfaces, the hydrophobic free energy starts to exceed the elastic curvature energy, causing the bilayer to curve and eventually vesiculate ($n_v$ is the number of lattice vacancies in the DMPC bilayer after denaturation and dissociation of protein).

*Effects of Thermal History.* The DMPC aggregates A and E in Scheme I are unlikely to be the same because they are formed under different conditions and probably $N_v \neq n_v$. Consequently, cooling from stage E to stage A is likely to result in hysteresis because the kinetics of association of apoprotein and DMPC are sensitive to $N_v$ and $n_v$ (see Step 1). As $n_v$ approaches zero, the rate of reassociation of apoprotein with DMPC upon cooling will become progressively slower due to an increased energy barrier opposing penetration of protein residues into the bilayer. If the apoprotein cannot fold into an $\alpha$-helix because the temperature is too high, then reassociation with DMPC will be inhibited additionally. It is clear that the kinetics of the steps in Scheme I are very sensitive to the physical states of both the DMPC and apoprotein components [cf. Pownall et al. (1981), Epand (1982), and Wetterau & Jonas (1982)].

*Thermodynamic Reversibility.* If steps 1–4 in Scheme I can be repeated in reverse order with the same values for the thermodynamic potentials characterizing them when the system is cooled from high temperature, then the thermal denaturation is thermodynamically reversible (Prigogine & Defay, 1962). However, the reaction will be thermodynamically irreversible if, for instance, stage D with bound and partially unfolded apoprotein molecules is unattainable upon cooling so that stage E passes directly to stage C.

When the binding of monomeric apoprotein and DMPC is thermodynamically reversible, the Gibbs–Helmholtz equation describes the relation between the enthalpy and free energy of binding as a function of temperature. In its most simple form as given by Scheme I, it is apparent that the binding constant is given by the ratio $k_{on}/k_{off}$. In view of the diffusional nature of the reactions involved, this ratio is unlikely to be strongly temperature dependent so that the free energy of binding will be largely invariant with temperature, and from the Gibbs–Helmholtz equation, the enthalpy of binding will be small. The large calorimetric enthalpy of interaction observed around 24 °C arises from the formation of lattice vacancies in the bilayer (Massey et al., 1981).

*Kinetic Parameters.* A previous report (Epand, 1982) has shown that the relaxation time for movement of Trp residues from an apolar to polar environment when apo A-I/DMPC complexes are denatured at 55 °C in 0.15 M Gdn·HCl is about 3 h. This is in reasonable agreement with the $\tau_3$ values of 400 min decreasing to 1 min when apo A-I/DMPC complexes are denatured in the increasing temperature range 70–90 °C (Figure 3). In contrast, apo A-II or apo C desorbs from the DMPC complex as the temperature is increased from 50 to 70 °C, and the $\tau_2$ values are shorter and decrease from 15 to 1 min (Figure 3). This indicates that the smaller apo A-II and apo C molecules desorb more rapidly, presumably because of the greater probability of the appropriate conformational changes occurring in a short time and the higher diffusion coefficients of the lower molecular weight proteins. It is interesting that the rate of lipid/apoprotein association also increases with decreasing polypeptide molecular weight (Pownall et al., 1981b).

The activation energies for desorption decrease with decreasing molecular weight. The activation energy derived from the temperature dependence of $\tau_3$ for the major desorption phase of apo A-I is about 70 kcal/mol, which is much larger than the equivalent figures of approximately 30 and 20 kcal/mol for apo A-II and apo C, respectively. The activation energy is probably associated with the process of overcoming the hydrophobic effect and desorbing apolar amino acid residues from the lipid/water interface and will be a function of the number of such residues which must be removed before the apoprotein molecule is free to diffuse away.

Table II: Thermodynamic Potentials[a] of Association of Apolipoproteins with DMPC at 25 °C

| apolipoprotein | $\Delta H^\circ$ (kcal mol$^{-1}$) | $\Delta S^\circ$ (cal mol$^{-1}$ deg$^{-1}$) | $\Delta G^\circ$ (kcal mol$^{-1}$) |
|---|---|---|---|
| A-II | | | |
| $T < 70$ °C | 1.0 ± 0.8 | 26 ± 6 | −6.7 ± 0.5 |
| $T < 70$ °C | −68.2 ± 2.2 | −170 ± 11 | −17.5 ± 1.1 |
| C | −18.1 ± 0.4 | −36 ± 2 | −7.2 ± 0.2 |

[a] The term mol$^{-1}$ refers to (moles of apolipoprotein)$^{-1}$.

*Thermodynamic Parameters.* While the midpoint temperatures ($T_m$) for the heat denaturation of free apo A-I and apo A-II in solution lie in the range 50–60 °C, $T_m$ is increased when the apoprotein is complexed with DMPC. This enhanced stability presumably arises because more energy is required to unfold the $\alpha$-helices when they are constrained to the lipid/water interface. The additional free energy of binding of apoprotein to DMPC (Tables I and II) has to be overcome in order for helical segments to unfold and desorb. Since apo A-I has 13 sections of amphipathic helix, compared to 6 or less for apo A-II and apo C (Sparrow & Gotto, 1982), anchoring the molecule in the lipid/water interface, it is relatively difficult to denature the apo A-I/DMPC complex, and this process only commences above 70 °C.

Since the denaturation of apo A-II/DMPC and of apo C/DMPC complexes is reversible, van't Hoff enthalpies calculated from the denaturation data can be compared with the heats of association measured by calorimetry. No differential scanning calorimetric transitions associated with the denaturation of apo A-II/DMPC complexes have been observed. The reasons for this are that (1) the enthalpies involved are relatively small and (2) the temperature range over which the denaturation occurs is extremely wide. When apoprotein and DMPC are combined in a batch microcalorimeter, the observed heats of association are strongly dependent on the incubation temperature around the transition temperature of DMPC. Only when the temperature is above 30 °C are the heats of association of apo A-II with DMPC (Massey et al., 1981) and apo C-III with DMPC (Pownall et al., 1981c) exothermic and constant with temperature.

The thermodynamic potentials in Table II describe the association reaction of apo A-II and apo C with DMPC; these values are calculated from the potentials given in Table I. For the combined apo C fraction, the van't Hoff heat of association is −18.1 kcal/mol of apo C compared to a calorimetric heat of association of about −12 kcal/mol of apo C-III [see Figure 3 in Pownall et al. (1981c)]. The similarity in these values suggests that the denaturation of apo C is a two-state process [cf. Lapanje (1978) and Tanford (1970)]. The situation is more complicated for the case of apo A-II/DMPC complexes since two equilibria determine the association/dissociation reaction, as measured by circular dichroism. Massey et al. (1981) observed a calorimetric heat of association at temperatures above the melting point of DMPC in the complexes ($T > 30$ °C) of −62 kcal/mol of apo A-II. This value corresponds reasonably well with the van't Hoff enthalpy of association derived for the equilibrium which determines the thermal denaturation above 70 °C (see Table I). This again

suggests a two-state denaturation and that this equilibrium is determined by apo A-II acting as a cooperative unit. A major source of the enthalpy associated with the interaction of apo A-II and DMPC is the transition from helix to coil of apo A-II, and when this process is involved in the dissociation reaction, it contributes about 2.9 kcal/(1% $\alpha$-helix unfolded) to the enthalpy (Massey et al., 1981).

Similar values are obtained for the $\Delta G°$ of association of apo A-II calculated from the thermal denaturation above 70 °C ($\Delta G° = -17.5 \pm 1.1$ kcal/mol: see Table II) and calculated from the distribution data of Pownall et al. (1981c) for the reduced and carboxymethylated apo A-II ($\Delta G°$ for apo A-II $\approx 2 \times \Delta G°$ for reduced apo A-II $= 14.8 \pm 0.8$ kcal/mol of apo A-II). It is interesting that this $\Delta G°$ value for reduced and carboxymethylated apo A-II is similar to the $\Delta G°$ observed for the second denaturation reaction of apo A-II/DMPC disks at $T < 70$ °C and for denaturation of apo C/DMPC complexes (Table I). This implies that a single polypeptide chain of apo A-II is involved in the second denaturation of apo A-II/DMPC disks. Furthermore, the observed activation energies for the denaturation of apo A-II and apo C complexed with DMPC relate to that part of the denaturation reaction in which a single polypeptide chain is assumed to act as a cooperative unit.

Similar calculations are not possible for the apo A-I/DMPC system because, unlike complexes of DMPC with apo A-II and apo C which give reversible two-state desorption from DMPC, the equivalent apo A-I denaturation is formally irreversible so that the free energy of binding cannot be calculated without approximation [cf. Privalov (1982)] by the approaches used here. This irreversibility arises because apo A-I cannot penetrate into the liquid-crystalline bilayer at the high temperatures achieved at the end of the thermal denaturation (see Scheme I) whereas apo A-II and apo C can. Studies by Pownall and co-workers (1981b) of the association of apoproteins with DMPC at 30 °C indicate that apo A-II and apo C bind with $t_{1/2} = 1$–3 min whereas apo A-I has a $t_{1/2} \simeq 10^3$ min. The reasons for this are not entirely clear, but since the complete denaturation of apo A-I/DMPC complexes requires heating to 90 °C, it may be that nucleation of $\alpha$-helix formation in the free apo A-I molecule is inhibited so that as cooling commences the protein molecules cannot reassociate with the DMPC aggregate.

*Physiological Significance.* In vivo, apolipoproteins redistribute between different classes of lipoprotein particle, and we have suggested previously (Reijngoud & Phillips, 1982) that desorption of apolipoproteins from lipoprotein surfaces can occur when the surface pressure increases to values above the collapse pressure of the monolayer surrounding the apolar core. Lipolysis of core lipids would facilitate this process. The present thermal denaturation data imply that the time constant for apo A-I dissociation would lie in the range 1–400 min, depending upon conditions, while the equivalent values for the lower molecular weight apo A-II and apo C molecules would be in the range 1–15 min. This investigation of the association and dissociation indicates that desorption of pure apolipoproteins is thermodynamically unfavorable, especially for apoproteins of higher molecular weight. Thus, the free energies for the reversible dissociation of apo A-II and apo C are about 7 kcal/mol. The flexibility of the surface monolayer of a lipoprotein particle might become an important parameter for the movement of apoprotein molecules. At an appropriate protein/phospholipid molar ratio, the flexibility of the monolayer might be such that discoidal complexes would become the most stable configuration, thereby making the surface monolayer configuration inherently unstable. Spontaneous release of disks would not occur because it would expose apolar core triglyceride and cholesterol ester molecules to water. Lipolysis of core lipids could remove these apolar molecules so that release of phospholipid/apoprotein disks becomes possible.

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References

DeGennes, P. G., & Taupin, C. (1982) *J. Phys. Chem. 86*, 2294–2304.
Edelstein, C., & Scanu, A. M. (1980) *J. Biol. Chem. 255*, 5747–5754.
Edelstein, C., Kezdy, F. J., Scanu, A. M., & Shen, B. W. (1979) *J. Lipid Res. 20*, 143–153.
Epand, R. M. (1982) *Biochim. Biophys. Acta 712*, 146–151.
Freire, E., & Biltonen, R. (1978) *Biochim. Biophys. Acta 514*, 54–68.
Gwynne, J., Brewer, H. B., Jr., & Edelhoch, H. (1970) *J. Biol. Chem. 250*, 2269–2274.
Gwynne, L., Palumbo, G., Osborne, J. C., Jr., Brewer, H. B., Jr., & Edelhoch, H. (1975) *Arch. Biochem. Biophys. 170*, 204–212.
Hagerman, P. J., & Baldwin, R. H. (1976) *Biochemistry 15*, 1462–1473.
Havel, R. J., Eder, H. A., & Bragdon, J. H. (1955) *J. Clin. Invest. 34*, 1345–1353.
Jonas, A., & Mason, W. R. (1981) *Biochemistry 20*, 3801–3905.
Kanehisa, M. J., & Tsong, T. Y. (1978) *J. Am. Chem. Soc. 100*, 424–432.
Lapanje, S. (1978) *Physicochemical Aspects of Protein Denaturation*, p 187, Wiley-Interscience, New York.
Lasic, D. D. (1982) *Biochim. Biophys. Acta 692*, 501–502.
Mantulin, W. W., Massey, J. B., Gotto, A. M., Jr., & Pownall, H. J. (1981) *J. Biol. Chem. 256*, 10815–10819.
Markwell, M. A. K., Haas, S. M., Bieber, L. H., & Tolbert, N. E. (1970) *Anal. Biochem. 87*, 206–210.
Massey, J. B., Gotto, A. M., Jr., & Pownall, H. J. (1981) *Biochemistry 20*, 1575–1584.
Osborne, J. C., Jr., & Brewer, H. B., Jr. (1977) *Adv. Protein Chem. 31*, 253–337.
Phillips, M. C. (1972) *Prog. Surf. Membr. Sci. 5*, 139–221.
Pownall, H. J., Massey, L. B., Kusserow, S. K., & Gotto, A. M., Jr. (1978) *Biochemistry 17*, 1183–1188.
Pownall, H. J., Massey, J. B., Hsu, F.-J., & Gotto, A. M., Jr. (1981a) *Can. J. Biochem. 59*, 700–708.
Pownall, H. J., Pao, Q., Hickson, D., Sparrow, L. T., Kusserow, S. K., & Massey, J. B. (1981b) *Biochemistry 20*, 6630–6635.
Pownall, H. J., Hickson, D., & Gotto, A. M., Jr. (1981c) *J. Biol. Chem. 256*, 9849–9854.
Prigogine, I., & Defay, R. (1962) *Chemical Thermodynamics*, p 31, Longmans, Green and Co., London.
Privalov, P. L. (1982) *Adv. Protein Chem. 35*, 1–104.
Reijngoud, D.-J., & Phillips, M. C. (1982) *Biochemistry 21*, 2969–2976.
Reynolds, J. A. (1976) *J. Biol. Chem. 251*, 6013–6015.
Scanu, A. M., & Edelstein, C. (1971) *Anal. Biochem. 44*, 576–588.
Scanu, A. M., Toth, L., Edelstein, C., Koga, S., & Stiller, E. (1969) *Biochemistry 8*, 3309–3316.

Scanu, A. M., Edelstein, C., & Keim, P. (1975) in *Plasma Proteins* (Putnam, F. W., Ed.) 2nd ed., pp 312-481, Academic Press, New York.
Schwarz, G. (1965) *J. Mol. Biol. 11*, 64-77.
Segrest, L. P., Jackson, R. L., Morrisett, J. D., & Gotto, A. M. (1974) *FEBS Lett. 38*, 247-273.
Servuss, R. M., Harbich, W., & Helfrich, W. (1976) *Biochim. Biophys. Acta 436*, 900-903.
Sokoloff, L., & Rothblat, G. H. (1979) *Proc. Soc. Exp. Med. Biol. 146*, 1166-1168.
Sparrow, J. T., & Gotto, A. M., Jr. (1982) *CRC Crit. Rev. Biochem. 13*, 87-107.
Swaney, J. B. (1980) *J. Biol. Chem. 255*, 8791-8797.
Swaney, J. B., & O'Brien, K. (1978) *J. Biol. Chem. 253*, 7069-7077.
Tall, A. R., Shipley, G. G., & Small, D. M. (1975) *J. Biol. Chem. 251*, 3749-3755.
Tall, A. R., Small, D. M., Deckelbaum, R. J., & Shipley, G. G. (1977) *J. Biol. Chem. 252*, 4701-4711.
Tanford, C. (1970) *Adv. Protein Chem. 24*, 1-95.
Tanford, C. (1973) *The Hydrophobic Effect*, p 4, Wiley, New York.
Tanford, C. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3318-3319.
Wetterau, J. R., & Jonas, A. (1982) *J. Biol. Chem. 257*, 10961-10966.

# Purification and Characterization of β-Leptinotarsin-h, an Activator of Presynaptic Calcium Channels†

Richard D. Crosland,‡ Ting H. Hsiao, and William O. McClure*

ABSTRACT: A new neuroactive protein, β-leptinotarsin-h, has been purified to near-homogeneity from the hemolymph of the beetle *Leptinotarsa haldemani* by column chromatography. β-Leptinotarsin-h has a molecular weight of 57 000. Rat brain synaptosomes incubated with appropriate radioactive precursors release acetylcholine (ACh), norepinephrine, and 4-aminobutyrate when exposed to β-leptinotarin-h, but do not release lactate dehydrogenase. Release of ACh has been examined in some detail. Release of ACh varies with the concentration of β-leptinotarsin-h in a rectangular hyperbolic fashion. Half-maximal release is stimulated by a concentration of 50 ng/mL. Altering the ionic composition of the bathing solution affects the release in a manner which suggests that neither $Na^+$ channels nor $K^+$ channels are affected by β-leptinotarsin-h but that the β-leptinotarsin-h acts to increase permeability to $Ca^{2+}$. Varying the concentration of $Ba^{2+}$, $Sr^{2+}$, $Co^{2+}$, and $Cd^{2+}$ indicates that β-leptinotarsin-h acts to open the voltage-sensitive presynaptic $Ca^{2+}$ channel. β-Leptinotarsin-h may be a useful tool for studying the $Ca^{2+}$ channel associated with the release of neurotransmitters.

In 1969 Hsiao and Fraenkel reported that the hemolymph of various species of the beetle *Leptinotarsa* was lethal to houseflies and mice. Subsequently, Hsiao (1978) found that partially purified fractions from the hemolymph of seven species of *Leptinotarsa* were also lethal to houseflies and mice. From these seven species, a partially purified fraction from the hemolymph of *Leptinotarsa haldemani* proved to be the most toxic. McClure et al. (1980) examined the effect of the partially purified toxin from *L. haldemani*, designated leptinotarsin-h, on the neuromuscular junction of the rat. Leptinotarsin-h caused a massive, biphasic increase in the frequency of miniature end-plate potentials. Further examination of the effect of leptinotarsin-h revealed that the first phase of release of acetylcholine (ACh)[1] accounted for about 10% of the total release and was abolished by removal of $Ca^{2+}$ from the bathing medium. The second phase of release, however, was little affected by the absence of $Ca^{2+}$. The data suggest that leptinotarsin-h can induce two modes of quantized release at the neuromuscular junction.

The neurochemical characteristics of both leptinotarsin-h and a partially purified preparation of leptinotarsin from the hemolymph of *L. decemlineata*, designated leptinotarsin-d, were investigated by using synaptosomes from rat brain (McClure et al., 1980; Yoshino et al., 1980). Both preparations caused the preferential release of ACh over that of choline, were inactivated by heat, and were dependent on the presence of $Ca^{2+}$ in the incubation medium. In this paper we report further purification and characterization of the neuroactive component from the hemolymph of *L. haldemani*. These studies were undertaken to clarify the mechanism of action of this toxin in causing the release of neurotransmitters.

## Materials and Methods

Hemolymph was extracted from fourth instar larvae of *L. haldemani* and lyophilized. $^{45}CaCl_2$ (18.2 mCi/mg), [*methyl*-$^3$H]choline chloride (80 Ci/mmol), 4-[$^3$H]aminobutyric acid ([$^3$H]GABA) (34.5 Ci/mmol), and [$^3$H]norepinephrine

† From the Section of Neurobiology, University of Southern California, Los Angeles, California 90089 (R.D.C. and W.O.M.), and the Department of Biology, Utah State University, Logan, Utah 84322 (T.H.H.). *Received July 13, 1983.* This research was supported by funds from the National Science Foundation, the National Institutes of Health, the Max and Victoria Dreyfus Foundation, and Nelson Research.

‡ Present address: Department of Pharmacology, School of Medicine, University of California, Los Angeles, CA 90024.

[1] Abbreviations: ACh, acetylcholine; GABA, 4-aminobutyric acid; DEAE, diethylaminoethyl; di-O-$C_5$-(3), 3,3′-dipentyl-2,2′-oxacarbocyanine; LDH, lactate dehydrogenase; NE, norepinephrine; PS, physiological saline solution; Tris, tris(hydroxymethyl)aminomethane; Tris-PS, physiological saline solution with Tris-HCl instead of sodium phosphate; TTX, tetrodotoxin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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