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Interaction of Plasma "Arginine-Rich" Apolipoprotein with Dimyristoylphosphatidylcholine†

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ABSTRACT: Very low density lipoproteins isolated from the plasma of cholesterol-fed rabbits contain abnormally high amounts of cholesterol, phospholipid, and an apoprotein referred to as the "arginine-rich" protein (ARP). It is generally assumed that the major interaction between apolipoproteins and lipids is between the protein and the phospholipids. Therefore, we have studied in the present report the lipid-binding properties of ARP to dimyristoylphosphatidylcholine (DMPC) vesicles in order to determine the importance of this interaction for ARP. The interaction was studied by ultracentrifugal flotation, circular dichroism, and microcalorimetry. The binding studies were performed using low protein-to-lipid ratios so as to minimize protein–protein interaction and vesicle disintegration. The ARP–DMPC complexes were isolated by salt density ultracentrifugation in KBr and had an average DMPC to protein molar ratio of 625 to 1. The complexes were stable for several days. The addition of DMPC to ARP induced an increase in the α helicity of the protein; the maximal change (from 45% to 65%) in α-helical content required 90 min with a $t_{1/2}$ of approximately 15 min. The enthalpy of association of ARP with DMPC was highly exothermic with a value $\Delta H = -614$ kcal/mol of protein. The rate of heat release in this measurement was time dependent, requiring in excess of 20 min; however, the enthalpic changes were totally finished when the helical increase was only about one-half complete. Based on the kinetics of interaction, we suggest that the high enthalpy of binding may be associated with the increase in helicity of the protein; these two processes, though, are not sufficiently concomitant to account unequivocally for the heat release in terms of either protein–lipid interaction or protein structural changes.

Very low density lipoproteins (VLDL)[1] of human plasma contain several apolipoproteins in variable amounts. (For a review, see Scanu 1972a,b; Scanu et al., 1975; Jackson et al., 1976.) These apoproteins have been designated apoB, apoC-I, apoC-II, apoC-III (Alaupovic, 1971) and a protein rich in arginine termed the "arginine-rich" protein (ARP) (Shore and Shore, 1972, 1973) or apoE (Utermann, 1975). Normal subjects, as well as those with hyperlipidemia, show individual quantitative differences in these VLDL proteins which may reflect variances in genetic, hormonal, and dietary factors. The "arginine-rich" protein of normal human VLDL, which was originally described by Shore and Shore (1970) and characterized by Shelburne and Quarfordt (1974), comprises about 5–15% of the total VLDL proteins. Shore et al. (1974) found that the proportion of ARP is preferentially increased in VLDL

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[1] Abbreviations used are: VLDL, very low density lipoproteins; ARP, "arginine-rich" protein; apoB, apoC-I, apoC-II, apoC-III, and ARP, apoprotein constituents of VLDL; DMPC, dimyristoylphosphatidylcholine; CD, circular dichroism; DSC, differential scanning calorimetry; T_c, gel → liquid crystalline transition temperature; R_s, Stokes radius; LP-X_1 and LP-X_2, two lipoproteins isolated from plasma of obstructive jaundice patients; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl.

from cholesterol-fed rabbits; the amount of ARP increased from 10 to 15% of the total protein in normal VLDL to 50% in cholesterolemic rabbit VLDL. The induction of ARP by dietary cholesterol and its localization in cholesterol-rich particles raises several interesting questions concerning the role of the apoprotein in the transport of this lipid. In addition, ARP may also have a role in the regulation of cholesterol synthesis. The purpose of the present report is to describe the interaction of ARP with dimyristoylphosphatidylcholine (DMPC), and to determine the effects of binding upon the protein and lipid structures. The rationale for using phospholipid is based on previous findings (Scanu, 1972a,b) which suggested that the major interaction between the plasma apolipoproteins and lipids was one between the proteins and phospholipids. This concept is also consistent with recent studies on the intact lipoproteins as described in a recent review (Morrisett et al., 1977). Although VLDL does not contain significant amounts of DMPC, this phospholipid was chosen because its physical form and properties are well defined (Newman and Huang, 1975; Lentz et al., 1976), and its thermotropic transition at about 23 °C makes it particularly suitable for study.

Materials and Methods

Isolation and Purification of Rabbit "Arginine-Rich" Protein (ARP). Blood was collected in 1% EDTA by cardiac puncture from 12-h-fasted New Zealand white rabbits that had been maintained on a diet of commercial rabbit chow plus 2% cholesterol (Sigma) for 2 months. The plasma cholesterol concentrations were typically greater than 1500 mg/100 mL. Very low density lipoproteins (VLDL) were isolated from the hypercholesterolemic plasma by ultracentrifugation at plasma density for 18 h at 55 000 rpm and 5 °C in a Beckman L2-65 using a 60 Ti rotor. Following separation of the top and bottom fractions by tube slicing, the VLDL top fraction was further purified by centrifugation using a SW 50.1 rotor at 45 000 rpm at 5 °C for 40 h. The VLDL obtained from this ultracentrifugation was analyzed by Geon-Pevikon block electrophoresis (Mahley et al., 1975) and shown to consist primarily of β-migrating material. The VLDL were delipidated by diethyl ether–ethanol (3:1) as previously described for human VLDL (Brown et al., 1969) and the lipid-free proteins dissolved in a buffer containing 8 M guanidine hydrochloride, 10 mM Tris-HCl, 1 mM EDTA, 1 mM NaN_3, pH 7.6. The soluble proteins were subjected to chromatography at room temperature on a column of Sephadex G-200 (2.6 × 260 cm) equilibrated with the same buffer. The fraction corresponding to ARP, as determined by polyacrylamide gel electrophoresis, was dialyzed overnight against 8 M urea, 10 mM Tris-HCl, 1 mM EDTA, 1 mM NaN_3, pH 8.2, and was then applied to a column)1.6 × 60 cm) of DEAE-cellulose equilibrated with the same dialysis buffer. ARP was eluted from the column with a continuous gradient from 0 to 0.25 M NaCl in the urea buffer. ARP was then dialyzed against 0.15 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, 1 mM NaN_3, pH 7.6 (referred to hereafter as the standard buffer), concentrated to 0.23 mg/mL by ultrafiltration using an Amicon cell equipped with a UM-10 membrane, and stored at 4 °C. Although great care was taken in these studies to avoid conditions which might give rise to aggregated states of ARP, we were unable to show that all of the apoprotein was in a monomeric form. The purity of the isolated ARP was demonstrated by polyacrylamide electrophoresis in sodium dodecyl sulfate.

Synthesis of [^{14}C]DMPC and Preparation of Vesicles. [^{14}C]DMPC was synthesized from glycerol phosphorylcholine (Sigma) and [^{14}C]myristic anhydride (Amersham/Searle) as described by Robles and van den Berg (1969). Bilamellar vesicles were prepared by dissolving 10 mg of DMPC in 1 mL of benzene and then drying the phospholipid by lyophilization; 1 mL of standard buffer was then added and the lipid was subjected to sonication for 30 min at 30 °C. Titanium particles were removed by slow speed centrifugation. Sonically irradiated preparations of DMPC contained about 90% small bilamellar vesicles and about 10% multilamellar structures as judged by chromatography on Sepharose 4B. Rechromatography of the small bilayer vesicles yielded a preparation which contained 5–10% large vesicles; this finding suggested that an equilibrium between small quantities of multilamellar structures and the bilamellar vesicles cannot be avoided. DMPC was not degraded by sonication as demonstrated by thin-layer chromatography on silica gel in a solvent system of $CHCl_3$/MeOH/H_2O, 65:25:4. All experiments were performed at least twice using well-sonicated but nonchromatographed vesicles as well as chromatographically isolated vesicles with no significantly different results.

Preparation of ARP–DMPC Complexes. To 1.0 mL of ARP (~0.20 mg/mL in standard buffer) was added 0.075 mL of DMPC (~40 mg/mL in standard buffer) at 23 °C. After vortexing for 10 s, the mixture was placed directly into a Cary 61 spectropolarimeter cuvette which was maintained at 26 °C and the CD spectrum recorded as described below. After 5 h, the mixture was removed and the ARP–DMPC complex was separated from residual free lipid and free protein by gradient ultracentrifugation in KBr. The gradient solutions consisted of the appropriate quantity of KBr in the standard buffer and were prepared in 5 mL nitrocellulose tubes with a Buchler peristaltic pump and gradient maker. The samples were immediately centrifuged in an SW 50.1 rotor at 45 000 rpm and 25 °C for 60 h. The content of each tube was fractionated with an ISCO fractionator. Each fraction was analyzed for absorbance at 280 nm, for phospholipid ^{14}C radioactivity, and for KBr density (Bausch and Lomb refractometer).

Circular Dichroism. Circular dichroism was measured at 26 °C on a Cary 61 spectropolarimeter using cells of 1-mm path length as described previously (Morrisett et al., 1973). The α-helical content was estimated from the $[\theta]_{222}$ by the relation % α helix = $(\theta_{222} + 3000)/(36\,000 + 3000)$ (Morrisett et al., 1973). For experiments where changes in $[\theta]_{222}$ were observed over time, the spectropolarimeter monitored the ellipticity at 222 nm for ~30 s at each time interval. Protein concentrations were determined by amino acid analysis.

Gel Permeation Chromatography of ARP–DMPC Complexes. Chromatography of complexes were performed on columns (1.6 × 30 cm) of Sepharose 4B (Pharmacia) equilibrated with standard buffer at 23 °C and eluted at an approximate flow rate of 12 mL/h under a hydrostatic pressure of <15 cm. The Stokes radius was measured as described by Patsch et al. (1977) on a column of Sepharose 4B (1.6 × 100 cm) at 4 °C in standard buffer eluted at 2 mL/h; fractions were analyzed for phospholipid by scintillation counting.

Analytical Ultracentrifugation. Sedimentation velocity experiments were performed on a Beckman Model E analytical ultracentrifuge at 27 °C; 0.42 mL of complex (2.5 mg/mL) was run in standard buffer at 52 640 rpm. The refractive index gradient was observed using schlieren optics, and the results were recorded on metallographic plates. The photographic plates were read on an LP-6 profile projector equipped with a Nikon X-Y stage, goniometer, and micrometers. The sedimentation coefficient was calculated on a Hewlett-Packard 9810 programmable calculator.

Intrinsic Tryptophan Fluorescence. Fluorescence measurements were determined on 1-mL samples in a quartz cuvette using an Aminco-Bowman spectrofluorimeter with a

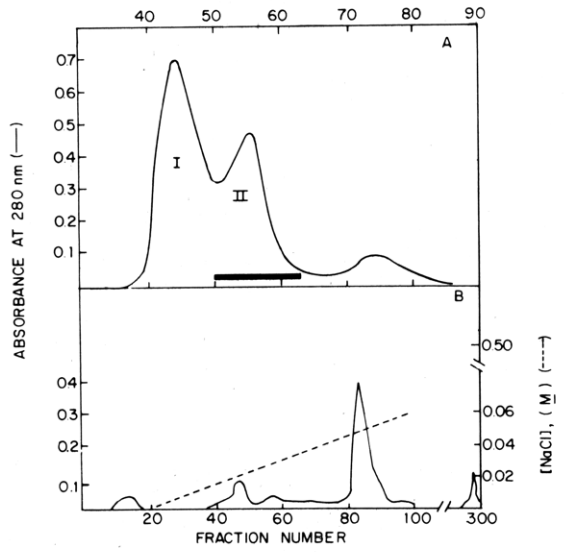

FIGURE 1: Profiles of ARP isolated from apoVLDL. A is the profile of apoVLDL chromatographed on Sephadex G-200 (as described in Materials and Methods). Peak II contains the apoARP and the fractions indicated by the bar were pooled. B is the profile of peak II from the G-200 chromatographed on DEAE-cellulose (as described in Materials and Methods). Fractions 80–90 contain pure apoARP.

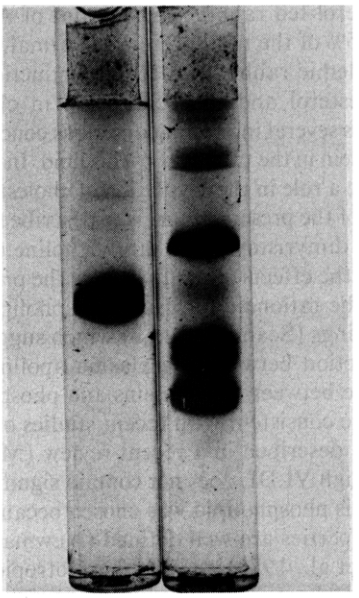

FIGURE 2: Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of apoARP (left) and a reference gel (right) of bovine serum albumin, apo-A-I and apoA-II (the three major bands, from top to bottom). Gels were 7.5% acrylamide, 0.1% sodium dodecyl sulfate, pH 7.2.

thermostated cell compartment at 23 °C. The excitation slit widths were 0.5 mm and the emission slit widths 1.0 mm for most measurements on samples containing ARP in concentrations of between 0.03 and 0.30 mg/mL. Samples were excited with 280-nm light, and emission spectra recorded from 300 to 400 nm.

Pyrene fluorescence measurements were carried out with the Aminco-Bowman fluorimeter as described above. Pyrene was first dissolved in absolute ethanol (10 mg/mL) and then 5 μL of an appropriate dilution was added to preparations of DMPC or to isolated complexes in standard buffer; the final concentration of pyrene was 0.5% (g of pyrene/g of phospholipid). Samples were equilibrated with pyrene for 15 min at 24 °C before spectra were recorded; samples were excited at 335 nm. The intensity of monomer fluorescence was measured at 392 nm and excimer fluorescence at 470. Excitation and emission slit widths were 1.0 mm. DMPC vesicles were used at concentrations between 5 and 10 mg/mL; concentrations of the complexes were about 0.25 mg/mL (lipid concentration + protein concentration). Spectra were recorded at 2–3 °C intervals between 4 and 36 °C with increasing and then decreasing temperature; the samples were incubated in the thermostated cell compartment for 15 min at each temperature to permit thermal equilibration before recording the spectra.

Differential Scanning Calorimetry. Differential scanning calorimetry (DSC) was performed on a Perkin-Elmer DSC-2 calibrated with cyclohexane and indium. The isolated complex was concentrated using an Amicon Centriflo CF-50 cone centrifuged at 2000 rpm. Thermograms were obtained from 10 μL of ARP–DMPC complex (12.4 mg/mL) or DMPC vesicles (16.0 mg/mL), both in standard buffer; 10 μL of buffer was in the reference pan. Heating and cooling traces were recorded on a scale of 0.1 mcal/s. The heating and cooling rates were 2.5 °C/min and the heat flow was monitored between 7 and 47 °C. T_c values were determined from the intersection of a line extrapolated through the endotherm and the baseline. Enthalpy (ΔH) values were determined from the peak areas.

Microcalorimetry. Microcalorimetry was performed on an LKB 2107 microcalorimeter. All enthalpies were recorded at 27.6 °C with a Keithley 150B current amplifier on the 10-μV scale using a recorder scale of 0.1 V and a chart speed of 0.5 in./min. Electrical calibration of the calorimeter was achieved with a 6-mA pulse of 14-s duration. The large and small sample compartments of the calorimeter were filled with 2 mL of ARP (0.23 mg in 2 mL of standard buffer) and 4 mL of DMPC (5 mg in 4 mL of buffer), respectively. The reference compartments were filled with 2 mL of buffer and 4 mL of DMPC solution, thereby compensating for the enthalpic contribution of the heat of dilution of vesicles. The heat of dilution of ARP was quantitated separately by filling the sample compartments with 2 mL of ARP solution (0.23 mg) and 4 mL of buffer, and both reference compartments with buffer. The heat of friction due to mixing was measured following each experiment by rotating the drum a second time after the trace had returned to the initial baseline. The tracing for the ARP–DMPC complex formation was continued for 30 min with a baseline drift of only 0.1 μV. The enthalpy of interaction was calculated by integrating the experimental trace after subtracting the contributions from the heats of dilution and mixing.

Results

Isolation of ARP. "Arginine-rich" protein was isolated from VLDL of cholesterol-fed rabbits by chromatography of apoVLDL on Sephadex G-200 and DEAE-cellulose (Figure 1); 150 μg of isolated ARP gave a single band by polyacrylamide gel electrophoresis in sodium dodecyl sulfate with a molecular weight of 33 000 (Figure 2). It should be mentioned, however, that, although a single band was found for ARP, isoelectric focusing of the same preparation gave a mixture of polymorphic forms. This finding is consistent with the results of Utermann (1975). The amino acid analysis of isolated ARP (Table I) is in excellent agreement with the published work of Shore and Shore (1973); the ARP contained approximately 10% arginine.

Spectral Characterization of ARP and ARP–DMPC Mixtures. A typical CD spectrum of ARP in standard buffer is shown in Figure 3. The pronounced troughs at 208 and 220 nm, indicating a significant content of α-helical conformation,

TABLE I: Amino Acid Composition (Moles/10^3 Moles of Amino Acids) of Rabbit "Arginine-Rich" Protein.[a]

	ARP (this study)	VLDL Subfraction R2[b]	VLDL Subfraction R3[b]
Asp	45	50	48
Thr	33	31	30
Ser	75	58	57
Glu	221	227	229
Pro	34	32	33
Gly	75	59	59
Ala	125	118	120
1/2-Cys	0	0	0
Val	58	62	62
Met	24	30	30
Ile	10	10	9
Leu	114	109	112
Tyr	17	15	15
Phe	18	15	15
His	11	10	9
Lys	44	44	43
Trp	ND[c]	26	ND[c]
Arg	96	101	102

[a] The values represent duplicate analysis of 24-h acid hydrolysates. [b] Shore et al. (1974). [c] ND, not determined.

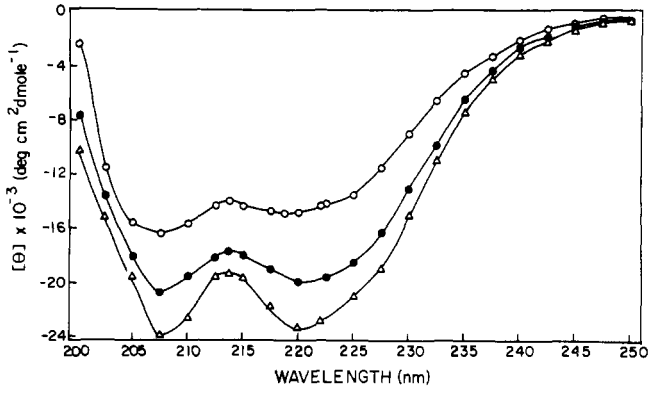

FIGURE 3: Circular dichroic spectra of ARP (top trace), ARP-DMPC final mixture (middle trace), and the lipid-protein complex isolated by ultracentrifugation (as described in Materials and Methods) (bottom trace). Ellipticity traces have been replotted as mean residue ellipticities.

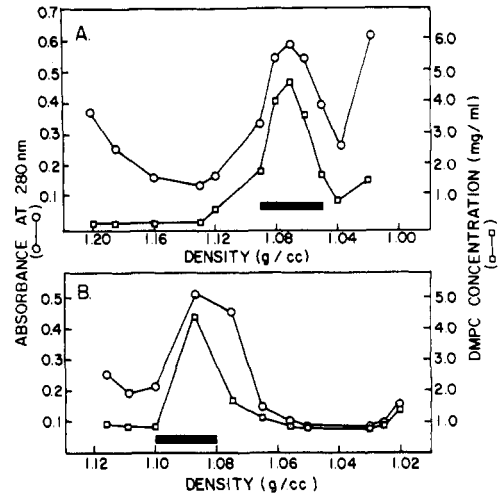

FIGURE 4: Profiles of an ARP-DMPC mixture after gradient ultracentrifugation. A is the profile of a final mixture recovered after circular dichroic analysis which was centrifuged in a linear KBr gradient from d = 1.006 g/cm^3 to d = 1.21 g/cm^2. Five 0.3-mL fractions (indicated by the bar) were pooled and recentrifuged in a shallow KBr gradient from 1.02 to 1.12 g/cm^3. B is the profile of the isolated complex from A after the second centrifugation. The fractions indicated by the bar were pooled.

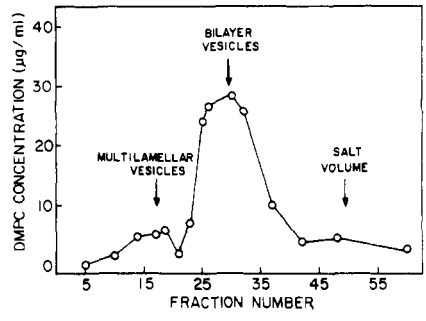

FIGURE 5: Analytical gel filtration elution profile of an ultracentrifugally isolated ARP-DMPC complex. Chromatography was performed on a 1.6 × 30 cm column of Sepharose 4B. Fractions (1.3 mL) were collected at a rate of about 12 mL/h. Multilamellar and bilamellar vesicles of DMPC and apoARP were chromatographed prior to the complex for calibration and the peak tubes eluted as indicated by the arrows.

correspond to 45% α helix as calculated from the $[\theta]_{222}$ value of $-14\,500$ deg cm^2 dmol^{-1}. The addition of DMPC vesicles to ARP was associated with an increase in the negative mean residue ellipticity at 222 nm to a final value of $\sim -20\,000$ deg cm^2 dmol^{-1}. The CD spectrum of the protein 5 h after the addition of phospholipid is given in Figure 3. As discussed below, the final mean residue ellipticity reflects contributions from both lipid-bound ARP and lipid-free ARP.

Isolation of ARP-DMPC Complex. The ARP-DMPC complexes formed as described above were then isolated by ultracentrifugation in KBr between densities 1.006 to 1.210 g/cm^3 (Figure 4A). The isolated complex had a hydrated density of about 1.08 g/cm^3 and was more dense than the free DMPC vesicles (1.04 g/cm^3). Recentrifugation of the complex between d = 1.02 and 1.12 g/cm^3 reproducibly yielded a complex with a density of 1.08–1.09 g/cm^3 (Figure 4B). Since no free protein or lipid was removed during the second spin, it suggested that the complex was stable to the high salt conditions of centrifugation. Analytical ultracentrifugation of the complex gave a symmetrical schlieren pattern (a single 8 S peak) with no evidence of slower sedimenting protein-free vesicles (6 S). Four isolated complexes, whose compositions varied between 4% and 10% protein according to the ratios of ARP and DMPC used in each mixture, showed an average lipid-to-protein ratio of 11.4 g/g or 625 mol/mol. Assuming that each bilayer vesicle contains an average of 2350 DMPC molecules per vesicle, there are 3–4 molecules of ARP bound per vesicle. The CD spectrum of the purified complex exhibited a negative mean residue ellipticity at 222 nm of about $-23\,000$ deg cm^2 dmol^{-1}, corresponding to $\sim$67% α-helical conformation (Figure 3). Based on four separate binding experiments, the average helical content of the protein increased from 45% in the absence of DMPC to 65% in the isolated complex.

Characterization of ARP-DMPC Complexes by Gel Permeation Chromatography. The ultracentrifugally isolated complexes (Figure 4) were chromatographed on a column of Sepharose 4B. As shown in Figure 5, the isolated complex eluted from the column in the region of the bilamellar DMPC vesicles, well separated from the elution volumes of larger aggregates or smaller micelle-like products. Under the conditions described in Materials and Methods section, free apoprotein eluted near the salt volume. The composition of the complex was identical with that of the complex prior to chro-

TABLE II: Characterization of ARP, DMPC Vesicles, and Isolated Complexes.

	% helix	Trp fluorescence max (nm)	Stokes radius (Å)	$s_{20,w} \times 10^{-13}$ (S)	T_c (°C)[a] (DSC)	T_c (°C)[b] (pyrene I_E/I_M)	ΔH of binding (kcal/mol)
ARP	44.8 ± 0.9	344.0					
DMPC vesicles			125 ± 4	5.50 ± 0.15	24.5	23.5–25.0	
Isolated APR-DMPC vesicles	65.4 ± 3.7	343.5	131 ± 17	8.53 ± 0.12	23.0	23.0–25.0	614 ± 76

[a] As determined by differential scanning calorimetry. [b] As determined by pyrene fluorescence.

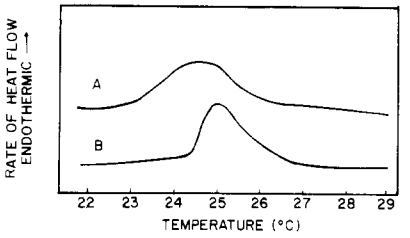

FIGURE 6: DSC heating traces of (A) ARP-DMPC complex; (B) DMPC vesicles (see text for details).

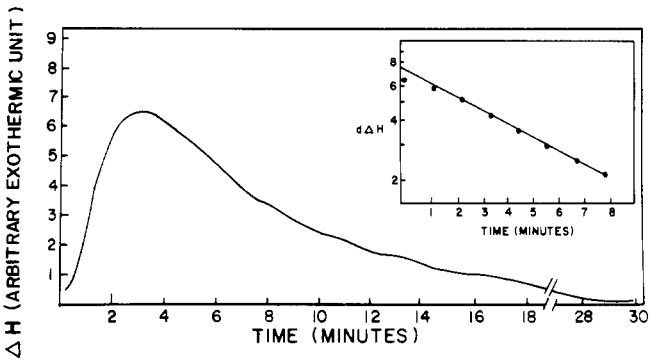

FIGURE 7: Microcalorimetric spectrum of the heat released after mixing DMPC vesicles and ARP. Quantitation of ΔH was performed by comparison with an electronic calibration standard as described in Materials and Methods. The inset is a plot of the decay curve of the increase in ΔH. Zero time for the inset is at the beginning of the decay curve in the actual calorimetric trace (3.33 min after mixing). The inset plot is linear, indicating a first-order process. The rate constant was calculated from this plot according to first-order kinetics.

matography; 95% of the protein and phospholipid was recovered.

Further Characterization of the Isolated Complexes. Stokes Radius. A Stokes radius (R_s) for the isolated complex was calculated from its elution volume using a calibrated Sepharose 4B column. This determination was made four days after the complex was formed. The column was calibrated as described previously by Patsch et al. (1977) with egg yolk phosphatidylcholine (R_s = 103 Å), turnip yellow mosaic virus (R_s = 132 Å), LP − X_1 (R_s = 339 Å), and LP − X_2 (R_s = 343 Å). The standard curve was a plot of Stokes radii of the calibration particles (as obtained independently by laser light scattering) vs. $\text{erfc}^{-1}\sigma$ (where $\sigma = (V_e - V_0)/V_i$; V_e = particle elution volume; V_0 = void volume; V_i = included volume). Using the slope b_0 and the y intercept a_0 of the calibration curve, the Stokes radius of the complex was calculated from its elution volume V_e by the relation $R_s = a_0 + b_0\text{erfc}^{-1}\sigma$. A Stokes radius of 131 ± 17 Å was obtained for the complex (Table II). Bilamellar DMPC vesicles alone gave a Stokes radius of 125 ± 4 Å indicating that the binding of 3–4 molecules of ARP did not significantly alter the size of the vesicle.

Analytical Ultracentrifugation. To further characterize the isolated ARP-DMPC vesicle, one preparation of the complex was analyzed by analytical ultracentrifugation. A $s_{20,w}$ of 8.53 ± 0.12 S was determined for a complex containing 25 g of DMPC per g of ARP (Table II); by this method, the complex also appeared homogenous. In these experiments, the $s_{20,w}$ value for DMPC alone was 5.80 ± 0.20 S. The complex was analyzed 6 days after it was formed.

Intrinsic Tryptophan Fluorescence. Since ARP contains 7–8 residues of tryptophan, it was of interest to monitor the changes in tryptophan fluorescence which accompanied binding. The fluorescence maximum for ARP alone was 344 nm (Table II). The average maximum for four ARP-DMPC complexes was virtually identical (343.5 nm) with that of ARP.

Transition Temperature as Measured by Pyrene Fluorescence and Differential Scanning Calorimetry. To determine the effect of ARP on the thermal properties of DMPC, we used differential scanning calorimetry and pyrene fluorescence to identify the transition temperature, T_c, at which the DMPC reorganizes from the gel state to the liquid-crystalline state. Increase in the pyrene excited dimer (excimer) emission at the expense of the monomer emission is diffusion controlled and, thus, varies inversely with the viscosity of the probe's environment. The transition temperature may be observed as a change in the slope of the plot of the excimer:monomer intensity ratio (I_B/I_M) vs. temperature (Pownall and Smith, 1973). The T_C obtained by this method was in the range of 23–25 °C for both pure DMPC vesicles and isolated ARP-DMPC complexes (Table II), indicating that the apoprotein had little effect on the lipid structure. The sum of the relative fluorescent intensities decreased according to a smooth, nonlinear curve with increasing temperature to a final value at 37.5 °C of about 35% of the intensities at 6 °C. Similar results were also obtained by DSC (Table II). DMPC vesicles alone exhibited a T_c at 24.5 ± 0.5 °C; the T_c of the isolated complex was at 23.0 °C (Figure 6). These values are not considered to be significantly different. In these experiments, the heating endotherm for the complex was broadened by only about 1 °C relative to that of DMPC vesicles alone. Enthalpies for both vesicles alone and the complex were 8.5 cal/g (35.6 J/g), somewhat lower than that described for DMPC by Hinz and Sturtevant (1972).

Thermodynamics of Binding. The enthalpy of binding was determined by microcalorimetry. A typical recorder trace of the heat flow is shown in Figure 7. The total heat of binding of 0.23 mg of ARP to 5 mg of DMPC in this experiment was 2.886 mcal. The amount of ARP which actually interacted with DMPC was determined by Sepharose 4B chromatography of the mixture from the calorimetry sample cell. Based on the amount of ARP-DMPC complex which is isolated, the calculated enthalpy for the reaction was ca. −614 kcal/mol of protein (Table II).

Whereas the calibration curve had returned to baseline in less than 5 min, the experimental binding curve was significantly broadened (Figure 7) beyond the 67-s time constant for

the calorimeter response, indicating that the exothermic process was taking place relatively slowly. Kinetic analysis of this curve according to a first-order process (Figure 7, inset) indicated a linear relationship with a first-order rate constant of $2.7 \times 10^{-3}\ s^{-1}$.

Since the rate at which heat was evolved was slow, it was of interest to obtain the rate constant for helix formation and to compare it with the rate constant for heat released. ARP (0.2 mg) was mixed with 3.0 mg of DMPC in standard buffer and the ellipticity at 222 nm was monitored at 30-min intervals for several hours (Figure 8). Initially, the ellipticity change was rapid but not instantaneous, as half of the increase was completed after about 10 min. The rate of increase then became slower, and 90–120 min was required before the increase was maximal. Kinetic analysis (Figure 8, inset) indicated that the helical increase was first order, with a rate constant of about $7.6 \times 10^{-4}\ s^{-1}$.

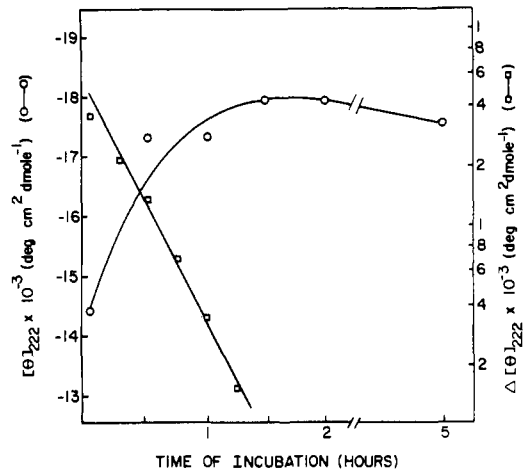

FIGURE 8: Change in the negative mean residue ellipticity of ARP at 222 nm as a function of time of incubation with DMPC. Closed circles represent the calculated mean residue ellipticity values. Open squares represent values for the differences ($[\theta]_{222}$ at time t minus $[\theta]_{222}$ at the final time) plotted on a log scale vs. the time of incubation. This plot is linear, indicating a first-order process; the rate constant was calculated according to first-order kinetics.

Discussion

Rabbits fed a cholesterol-diet develop hypercholesterolemia and severely atherosclerotic vessels. They are an excellent source of ARP; within 3–4 weeks after beginning a 2% cholesterol diet, serum cholesterol levels were typically in excess of 1500 mg/100 mL and the VLDL, greatly elevated to carry the additional cholesterol, were preferentially enriched in the ARP. ARP is also associated with several cholesterol-induced lipoproteins in the hypercholesterolemic dog, pig, rat, and Patas monkey (Mahley et al., 1974, 1975, 1976). In man, ARP is elevated in the serum of patients with types III and V hyperlipoproteinemia (Havel and Kane, 1973; Curry et al., 1976) and in patients with familial LCAT deficiency (Utermann et al., 1974). The VLDL obtained in the present study from cholesterol-fed rabbits were enriched in ARP and abnormal in electrophoretic migration. Although it was not examined in the present report, Shore et al. (1974) found that the abnormal VLDL in the cholesterol-fed rabbit contain significantly more cholesteryl esters and less triglycerides than normal VLDL. Whether the increased amount of ARP is somehow related to the elevated cholesteryl esters is unknown at present. Studies are currently in progress to determine the binding properties of ARP to phospholipid–cholesterol and cholesteryl ester mixtures.

Although the synthesis and structure of VLDL and the mechanism by which ARP becomes part of the VLDL particle are not completely understood, a reasonable model includes the binding of ARP to the surface of a particle containing a core of neutral lipid and a surface monolayer of phospholipid–cholesterol. The present studies were designed to test the affinity of ARP for phospholipid and to assess the changes in the protein and the lipid as a consequence of their association. Similar lipid-binding studies involving DMPC interactions with apolipoproteins A-I (Tall et al., 1975; Rosseneu et al., 1976; Morrisett et al., 1977), A-II (Tall et al., 1975; Rosseneu et al., 1976; Morrisett et al., 1977), C-I (Rosseneu et al., 1976; Morrisett et al., 1977), C-II (Morrisett et al., 1977), and C-III (Morrisett et al., 1973; Rosseneu et al., 1976) have been reported previously, but have not been carried out with the "arginine-rich" protein. We specifically designed these experiments using low protein-to-lipid ratios so as to characterize the interaction without the complicating factors of protein–protein interactions within the complex, exchange processes between free and bound ARP or vesicle destruction. Low concentrations of the vesicles (5–10 mg/mL phospholipid) avoided vesicle aggregation. Although these vesicles mimic the bulk protein and lipid ratios in VLDL, they do not reflect the specific lipids and proteins found in the native particle, nor does the phospholipid bilayer represent the most probable basic structure for a VLDL particle. It is very probable, however, that the interactions studied in this model system are similar to those between proteins and polar lipids on the surface of a native lipoprotein particle.

An increase in α-helical content of the protein upon binding phospholipid is characteristic of all the apoproteins studied to date. Segrest et al. (1974) and Jackson et al. (1975) have interpreted this characteristic in terms of maximizing the content of amphipathic structure which stabilizes lipid vesicles in solution. The binding of ARP to DMPC vesicles was also shown in the present study to induce a significant increase in the helical content of an apoprotein which is already quite helical. Isolated complexes from gradient ultracentrifugation always had greater negative mean residue ellipticity values than the crude mixture, whose CD spectrum contained contributions of both free and bound ARP.

Blue shifts in intrinsic tryptophan fluorescence have also been associated with the binding processes in previous studies (Morrisett et al., 1977). These blue shifts have been interpreted to reflect relocation of the fluorescent tryptophan residues from the aqueous medium to a more hydrophobic environment with lipid binding. For ARP and a typical ARP–DMPC complex, the fluorescence maxima listed in Table II were virtually identical. This suggests that the protein may not be integrated into the bilayer to the same extent as are the smaller apolipoproteins.

The complexes formed in these studies contained 5–10% protein by weight and were shown by gel filtration and sedimentation velocity methods to have retained the dimensions of the original vesicle. DMPC vesicles without ARP were not stable for long periods of time; within 2 to 3 days there was significant aggregation even for dilute solutions. ARP–DMPC complexes retained the vesicle size for several days which suggests that the protein may stabilize the particles such that the bilamellar structure is retained. By analytical ultracentrifugation, it is shown that the complex has not aggregated or dissociated into free lipid and protein at 6 days after formation. In contrast, Aune et al. (1977) found that binding of 0.08 g of apoC-III to 1.0 g of DMPC resulted in disintegration of the vesicles into much smaller particles of uncertain structure. Binding of ARP did not cause the vesicle to lose its orig-

inal thermotropic behavior. The complexes retained their cooperative melting properties and were as fluid above the transition temperature as vesicles alone (indicated by the equally observable pyrene excimer state). It was also significant that the heating endotherm for the complex was broadened by only about 1 °C. Similar experiments using dipalmitoylphosphatidylcholine vesicles indicated that binding of the major myelin proteolipid N-2 or Gramicidan A restructures the lipid bilayer so as to decrease cooperative melting; this was observed by DSC as a broadening of the transition range (Papahadjopoulos et al., 1975). Broadening has similarly been observed by pyrene fluorescence and electron paramagnetic resonance for the binding of apoC-III to DMPC vesicles (Novosad et al., 1976) when the particles contained between 25 and 35% protein by weight.

The large enthalpy associated with the binding process and the time course of heat release raises several interesting points. The heat could be derived from the actual protein–lipid interaction, from a structural change in the protein, from a structural change in the lipid, or from a restructuring of the water of hydration. If the enthalpy change is a result of electrostatic and/or hydrophobic interactions between the protein and adjacent lipid molecules during binding, then the binding process must itself by very gradual. However, it is also possible that the binding is extremely rapid and that the heat derives from some of the other processes subsequent to binding. The kinetics of the helical change raise questions concerning the relationship between binding and helix formation. The rate constants indicate that the rate of helix formation is four times slower than the rate of heat release, and that the helical increase is only a little more than half complete when the enthalpic reaction is totally completed. It is therefore difficult to assess the role of helix formation in the actual binding process as opposed to a role in stabilizing the particle. Since the two processes are not concomitant, it is possible that binding may be the driving force for the protein's structural reorganization. On the other hand, if binding is indeed immediate, it may be that the subsequent changes in lipid structure or hydration properties suggested above are responsible for the protein's conformation change. Experiments to test these possibilities are currently under study.

Acknowledgments

The authors are most grateful for the assistance of Dr. Kirk C. Aune in obtaining the sedimentation coefficient measurements, for the help of Ms. Debbie Mason in preparing the manuscript, and for the help of Ms. Kaye Shewmaker in preparing the figures.

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