Lipid Binding by Fragments of Apolipoprotein C-III-1 Obtained by Thrombin Cleavage†

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ABSTRACT: We have used thrombin to cleave apolipoprotein C-III-1 into two fragments constituting residues 1-40 (apoLP-C-III-A) and 41-79 (apoLP-C-III-B). The lipid binding properties of these fragments with dimyristoyl- and 1-palmitoyl-2-oleoylphosphatidylcholines have been determined using circular dichroic and intrinsic tryptophan fluorescence spectroscopy. The peptide-phospholipid mixtures were fractionated by density gradients of cesium chloride. ApoLP-C-III-A showed disordered structure in the absence and presence of DMPC and no significant amount of peptide-phospholipid complex was isolated. ApoLP-C-III-B showed conformational changes in the circular dichroic spectrum and a shift in the intrinsic tryptophan fluorescence spectrum. Ultracentrifugation in cesium chloride gradients yielded peptide-phospholipid complexes isolated between density 1.10 and 1.18. The molar ratio of lipid to protein was 12:1. The results of these studies and the examination of space filling models of apoLP-C-III provide evidence that an amphipathic α helix which contains a nonpolar face and a polar face is the basic structural unit for binding of phospholipid by the plasma apolipoproteins. These results also provide direct evidence that the hydrophobicity of the nonpolar face is important in lipid binding since the nonpolar face of residues 1-40 is considerably less hydrophobic than the nonpolar face of residues 41-79.

The amino acid sequence (Figure 1) of apolipoprotein C-III (apoLP-C-III),[1] a major protein constituent of human plasma very low density lipoproteins, has been determined (Shulman et al., 1974; Brewer et al., 1974). The sequence contains 79 amino acid residues and has a carbohydrate chain attached to Thr-74. At least two forms of the protein, called apoLP-C-III-1 and apoLP-C-III-2, have been isolated; they differ only in their content of sialic acid. We have previously studied the interactions of phosphatidylcholine with apoLP-C-III-1 and have shown that the binding of phosphatidylcholine is associated with changes in the intrinsic tryptophan fluorescence maximum and circular dichroic spectrum of the apoprotein (Morrisett et al., 1973). These changes are consistent with the formation of an α helix and with a shift of the tryptophan residues to a nonpolar environment. A phospholipid-protein complex can be isolated by density gradient ultracentrifugation or by chromatography on Sepharose 4B; the complex contained approximately 40 mol of phosphatidylcholine per mol of apoLP-C-III-1 (Morrisett et al., 1974). Using solid phase techniques, we have synthesized the carboxyl-terminal half of apoLP-C-III (residues 41-79) and have found that it binds phosphatidylcholine similar to apoLP-C-III-1 (Sparrow et al., 1973a,b). We, therefore, concluded that the major phospholipid binding region resides in this half of the protein. In agreement with our results, Shulman et al. (1973) have reported that limited tryptic digestion of a phospholipid-apoLP-C-III complex released only those tryptic fragments that could be obtained from the amino-terminal half (residues 1-40); the carboxyl-terminal half was protected by the phospholipid from tryptic cleavage and could be isolated by delipidation of the complex. We now report the isolation in good yield of the fragments of apoLP-C-III-1 produced by thrombin cleavage at Arg-40-Gly-41 (Figure 1); their interaction with dimyristoyl- and 1-palmitoyl-2-oleoylphosphatidylcholine is compared with intact apoLP-C-III-1.

Materials and Methods

Dimyristoyl-L-α-phosphatidylcholine (DMPC) was obtained from Sigma. 1-Myristoyl-2-[^{3}H]myristoyl- and 1-palmitoyl-2-[^{3}H]oleoyl-L-α-phosphatidylcholine (POPC) were synthesized in our laboratory from lysomyristoyl- and lysopalmitoylphosphatidylcholine, respectively, by the procedure of Robles and van den Berg (1969). All lipids were judged pure by thin-layer chromatography. Amino acid analyses of 6 N HCl hydrolysates (110 °C, 24 h) were performed on a Beckman 119 analyzer equipped with an Autolab integrator. Urea and guanidine hydrochloride were Schwarz/Mann Ultra Pure. Solutions of 6 M urea were passed through a mixed-bed resin shortly before use. Apolipoprotein C-III-1 was purified from the very low density lipoproteins of patients with primary type IV or V hyperlipoproteinemia, as previously described (Brown et al., 1969).

Thrombin Cleavage of ApoLP-C-III-1. Fifty milligrams of apoLP-C-III-1 was dissolved in 6 mL of 0.1 M Tris-HCl, 6 M guanidine hydrochloride, pH 8.5, and immediately desalted on a Bio-Gel P-2 column (4 × 35 cm) equilibrated in 0.1 M NH_4HCO_3. Fractions containing the protein were pooled (~50 mL) in a 125-mL Erlenmeyer flask. A solution of bovine thrombin (6.75 mg, 840 NIH units, ICN Pharmaceutical Inc., highly purified) and soybean trypsin inhibitor (10.1 mg,

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[1] Abbreviations used are: apoLP-C-III, an apolipoprotein constituent of human plasma very low density lipoproteins; apoLP-C-III-A, residues 1-40 of apolipoprotein C-III; apoLP-C-III-B, residues 41-79 of apolipoprotein C-III; DMPC, dimyristoylphosphatidylcholine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; UV, ultraviolet; CD, circular dichroism; EDTA, ethylenediaminetetraacetic acid; Bistris, 1,3-bis-[tris(hydroxymethyl)amino]propane; Tris, tris(hydroxymethyl)aminomethane.

APOLIPOPROTEIN-C-III-1

H_2N-Ser-Glu-Ala-Glu-Asp-Ala-Ser-Leu-Leu-Ser-Phe-Met-Gln-Gly-Tyr-Met-Lys-His-Ala-Thr-Lys-Thr-Ala-Lys-Asp-Ala-Leu
1 5 10 15 20 25

Ser-Ser-Val-Gln-Ser-Gln-Gln-Val-Ala-Ala-Gln-Gln-Arg-Gly-Trp-Val-Thr-Asp-Gly-Phe-Ser-Ser-Leu-Lys-Asp-Tyr-Trp-
30 35 40 45 50

Ser-Thr-Val-Lys-Asp-Lys-Phe-Ser-Glu-Phe-Trp-Asp-Leu-Asp-Pro-Glu-Val-Arg-Pro-Thr-Ser-Ala-Val-Ala-Ala-COOH
55 60 65 70 75 79

FIGURE 1: Amino acid sequence of human very low density apolipoprotein C-III-1 (apoLP-C-III-1) as described by Shulman et al. (1974) and Brewer et al. (1974). The carbohydrate chain is attached to residue 74. Thrombin cleaved the protein at Arg-40–Gly-41 to give two fragments which we have termed apoLP-C-III-A (residues 1–40) and apoLP-C-III-B (residues 41–79).

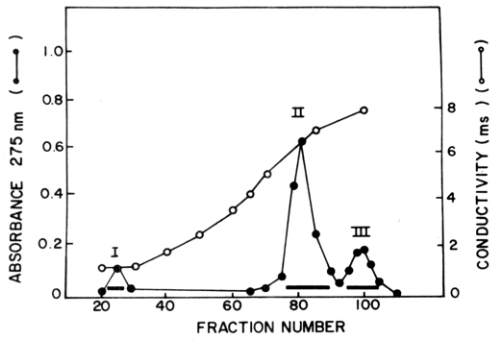

FIGURE 2: Elution profile from DEAE-cellulose (Whatman DE52) of the thrombin digest of apoLP-C-III-1. After applying the digest in 0.01 M Tris-HCl, 6 M urea, pH 8.2, the column was developed with a linear gradient consisting of 400 mL of 0.01 M Tris-HCl, 6 M urea and 400 mL of 0.125 M NaCl, 0.01 M Tris-HCl, 6 M urea, pH 8.2. (Peak I) Fractions 21–29; (peak II) fractions 78–88; and (peak III) fractions 96–104 were pooled separately and desalted on a Bio-Gel P-2 column (4 × 35 cm) in 0.1 M NH_4HCO_3.

Worthington Biochemical Corp.) in 4 mL of 0.15 M NH_4HCO_3 was added. After incubating at 37 °C for 6 h, the solution was frozen and lyophilized.

Purification of ApoLP-C-III-A (Residues 1–40) and ApoLP-C-III-B (Residues 41–79). The thrombin-treated apoLP-C-III-1 was chromatographed on a DEAE-cellulose column (1.6 × 35 cm) which was equilibrated in 0.01 M Tris-HCl, 6 M urea, pH 8.2. The protein fragments were eluted with a linear gradient of 400 mL of the equilibrating buffer and 400 mL of this buffer containing 0.125 M NaCl (Figure 2). Fractions which contained protein were located by measuring the adsorbance at 275 nm. The fractions indicated by the solid bars in Figure 2 were pooled; they were then desalted and lyophilized. Each peak was further purified on a Bio-Gel P-10 column (2.6 × 90 cm) equilibrated with 0.1 M Tris-HCl, 6 M urea, 0.001 M EDTA, pH 8.2. The protein containing fractions were desalted and lyophilized. The residue was dissolved in 2 mL of distilled H_2O and an aliquot taken for amino acid analysis. The analysis (Table I) indicated that peak I contained 12.4 mg of apoLP-C-III-A (residues 1–40) and peak II contained 12.1 mg of apoLP-C-III-B (residues 41–79). The peaks were pure judged by their migration as a single band on polyacrylamide gel electrophoresis in urea at pH 8.2 (Figure 3) and by their UV spectra (Figure 4). Peak III (analysis not given) was determined to be carbamoylated apoLP-C-III-B as evidenced by the presence of homocitrulline and the absence of one residue of lysine in the amino acid analysis.

Circular Dichroic Spectroscopy. Before recording the circular dichroic spectrum, each protein fragment was dissolved in 6 M guanidine hydrochloride and desalted on a Bio-Gel P-2 column equilibrated in 0.01 M Bistris-HCl buffer, 0.1 M NaCl, pH 7.4. The concentration of apoprotein in the pooled effluent was approximately 0.7 mg/mL; 1.5 mL of this solution containing approximately 1 mg of protein was used in 0.5-mm circular cells to record the CD spectrum from 250 nm to 205 nm on a Cary 61 spectropolarimeter. In four separate experi-

TABLE I: Amino Acid Composition of Fragments of ApoLP-C-III-1.[a]

Amino acid	Peak I ApoLP-C-III-A	Peak II ApoLP-C-III-B
Asp	2.43 (2)	4.81 (5)
Thr	2.09 (2)	2.84 (3)
Ser	5.98 (6)	5.08 (5)
Glu	7.60 (8)	2.27 (2)
Pro	(0)	2.17 (2)
Gly	1.20 (1)	1.84 (2)
Ala	7.00 (7)	2.60 (3)
½-Cystine	(0)	(0)
Val	2.31 (2)	3.66 (4)
Met	1.78 (2)	(0)
Ile	(0)	(0)
Leu	3.06 (3)	2.00 (2)
Tyr	1.01 (1)	0.96 (1)
Phe	1.02 (1)	2.83 (3)
Lys	2.91 (3)	2.86 (3)
His	0.96 (1)	(0)
Arg	0.93 (1)	0.89 (1)
Trp	*b* (0)	*c* (3)
Total	40	39

[a] Amino acid analyses were obtained on peptide samples subjected to 24 h, 6 N HCl hydrolysis at 110 °C in sealed evacuated tubes. Analyses were performed on a Beckman 119 analyzer equipped with an Autolab integrator. The columns of numbers represent the values obtained from two determinations and the expected values (in parentheses). [b] Not present in elution profile. [c] Present in elution profile but not quantitated.

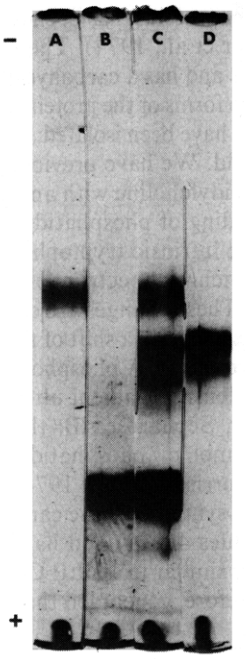

FIGURE 3: Polyacrylamide gel electrophoresis of thrombin generated fragments of apoLP-C-III-1. The gels were run in 8 M urea at pH 8.2 for 3 h and contained approximately 20 μg of protein; the gels were stained with Coomassie blue. (A) Peak I from DEAE (apoLP-C-III-A); (B) peak II from DEAE (apoLP-C-III-B); (C) apoLP-C-III-1 plus the thrombin digest before DEAE-cellulose chromatography; (D) apoLP-C-III-1.

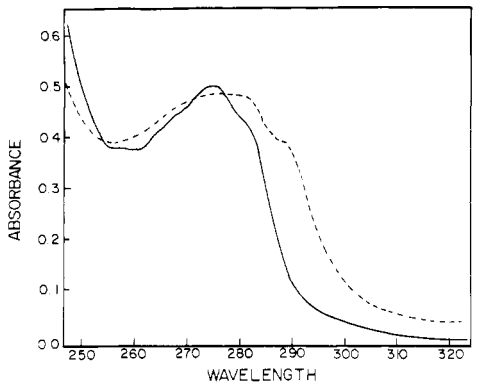

FIGURE 4: Ultraviolet absorption spectra of apoLP-C-III-A (—) and apoLP-C-III-B (- - -). The spectra were recorded on a Beckman Acta V spectrophotometer and the concentration of apoLP-C-III-A was approximately 1.6 mg/mL and of apoLP-C-III-B was 0.13 mg/mL.

ments, aliquots (50 μL) of sonicated dispersions (10 mg/mL) of dimyristoylphosphatidylcholine or 1-palmitoyl-2-oleoylphosphatidylcholine were added and the spectrum was recorded after each addition (Figures 5 and 6) until no further changes were observed in the CD spectra. The final concentration of lipid to protein was 3 to 1 on a weight basis.

Fluorescence Spectroscopy. The intrinsic tryptophan fluorescence spectrum of apoLP-C-III-B was recorded on an Aminco-Bowman spectrofluorimeter. A fluorescence maximum of 352 nm was observed; this maximum shifted to approximately 340 nm upon the addition of either phospholipid.

Density Gradient Ultracentrifugation. The volume of each protein–lipid mixture from the CD titration was adjusted to 2.5 mL with 0.5 M CsCl. This solution containing 1 mg of protein and 3 mg of phospholipid was placed in the lower density side of a Buchler gradient former. The high density side contained 2.5 mL of a 1.73 M CsCl solution with 0.01 M Bistris-HCl, 0.1 M NaCl, 0.002 M NaN_3. After each gradient was formed with the aid of a Buchler peristaltic pump and densiflow, the tubes were centrifuged in a Beckman SW-50.1 Rotor at 45 000 rpm for 4 days at 23 °C. After ultracentrifugation, the gradients were fractionated from the bottom of the polyallomer tube using a Buchler gradient fractionator. Fractions of approximately 250 μL each were collected. Protein was estimated by measuring the absorbance at 275 and 280 nm. The density was estimated by refractometry on a Bausch and Lomb refractometer. Phospholipid radioactivity was measured in a Beckman LS-265 liquid scintillation counter.

Microcalorimetry. The enthalpies of interaction of apoLP-C-III-A and apoLP-C-III-B with DMPC and POPC were measured on an LKB 10070 batch microcalorimeter equipped with gold cells as previously described (Pownall et al., 1977). Experimental values were corrected for the heat of dilution of lipid and of peptide, and for the frictional heat of mixing. In a typical experiment, 0.15–0.20 μg of the peptide in the 2-mL compartment of the sample cell was mixed with 2.0–2.7 mg of phospholipid in the 4-mL compartment. The 2- and 4-mL compartments, respectively, of the reference cell were filled with buffer and with an amount of lipid equal to that in the sample cell. This last procedure internally compensated for the heat of dilution of the lipid. The enthalpy of interaction was calculated by comparison of the integrated recorder signal after correction for the heat of dilution of the peptide and the frictional heat of mixing with that of an electrical calibration. The accuracy of the electrical calibration was periodically checked by measuring the known enthalpy of dilution of sucrose (Zimmer and Biltonen, 1972). Between experiments the cells were rinsed with dilute hydrochloric acid, dilute sodium hydroxide, and repeatedly with distilled water until the pH of the rinse had returned to neutrality; the cells were then dried with a gentle stream of nitrogen. When not in use the cells were kept filled with distilled water.

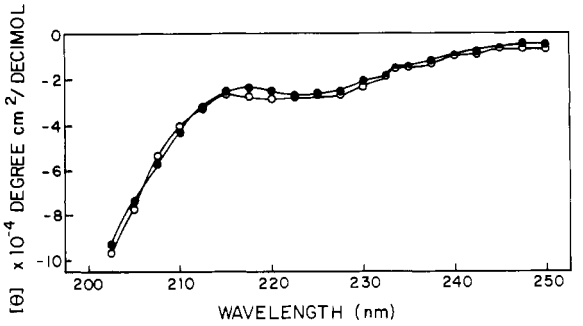

FIGURE 5: Circular dichroic spectra of apoLP-C-III-A (○—○) and apoLP-C-III-A plus 3 mg of DMPC or POPC (●—●). The spectra were recorded on a Cary 61 spectropolarimeter in 0.01 M Bistris-HCl, 0.1 M NaCl, pH 7.4.

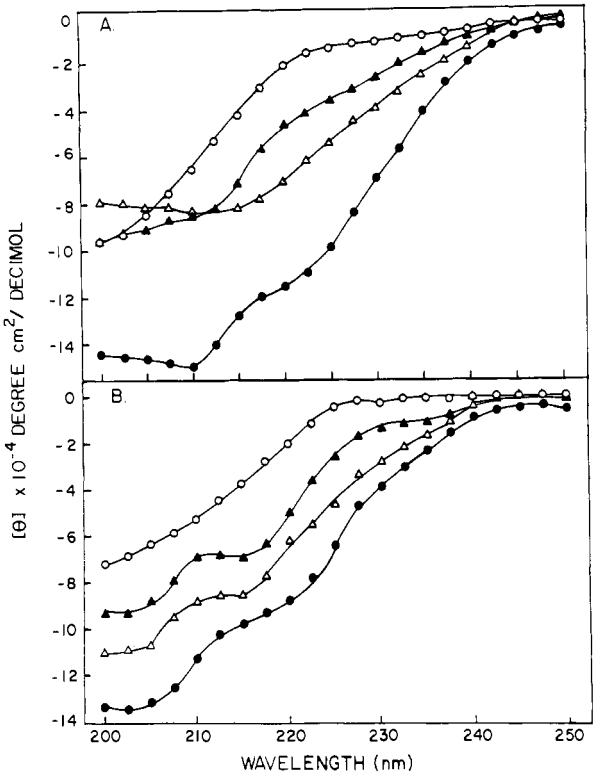

FIGURE 6: Circular dichroic spectra of apoLP-C-III-B. (A) ApoLP-C-III-B plus DMPC. Aliquots of a solution containing 10 mg/mL of DMPC dispersed by sonication in 0.01 M Bistris-HCl, 0.1 M NaCl, pH 7.4, were added, and the spectrum was recorded after each addition: 100 μL (▲—▲); 200 μL (△—△); 300 μL (●—●). The final lipid to protein ratio was 3 to 1 (w/w). Apoprotein alone (○—○). (B) ApoLP-C-III-B plus POPC. Aliquots of a sonicated dispersion of POPC (10 mg/mL) in 0.01 M Bistris-HCl, 0.1 M NaCl, pH 7.4, were added and the spectrum recorded after each addition: 100 μL (▲—▲); 200 μL (△—△); 300 μL (●—●). The final lipid to protein ratio was 3 to 1 (w/w). Apoprotein alone (○—○).

Results

The cleavage of apoLP-C-III by thrombin proceeded in good yield to give the two halves of the protein molecule which were separated by chromatography on DEAE-cellulose (Figure 2). Amino acid analysis and UV spectroscopy confirmed the

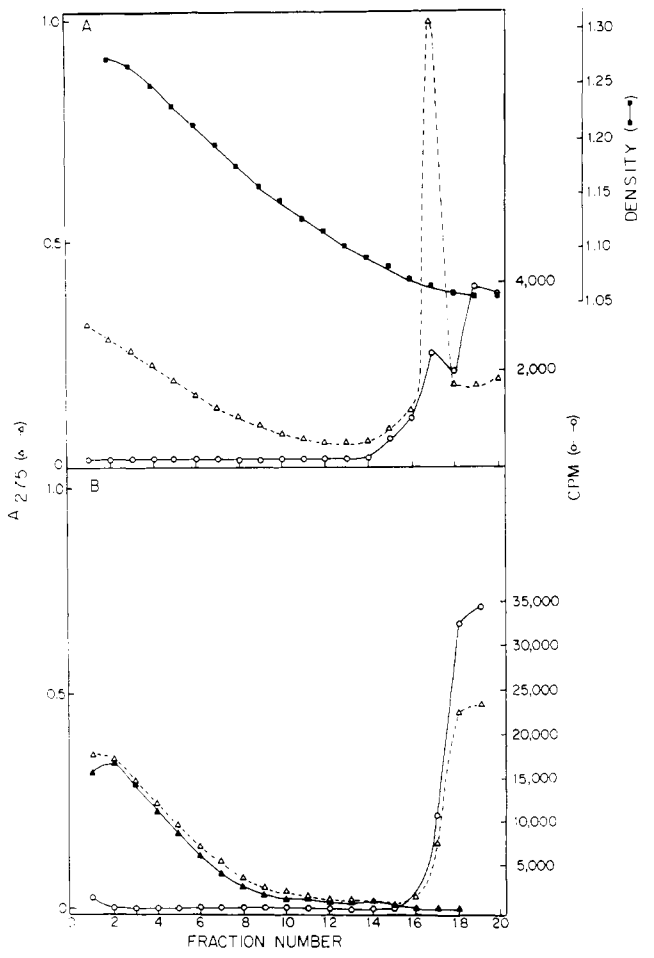

FIGURE 7: Density gradient ultracentrifugation of apoLP-C-III-A and phospholipid. The gradients were fractionated and protein, lipid, and density determined on each fraction (see Experimental Section). (A) ApoLP-C-III-A plus DMPC (Δ- - -Δ). (B) ApoLP-C-III-A plus POPC (Δ—Δ); apoLP-C-III-A alone (▲—▲).

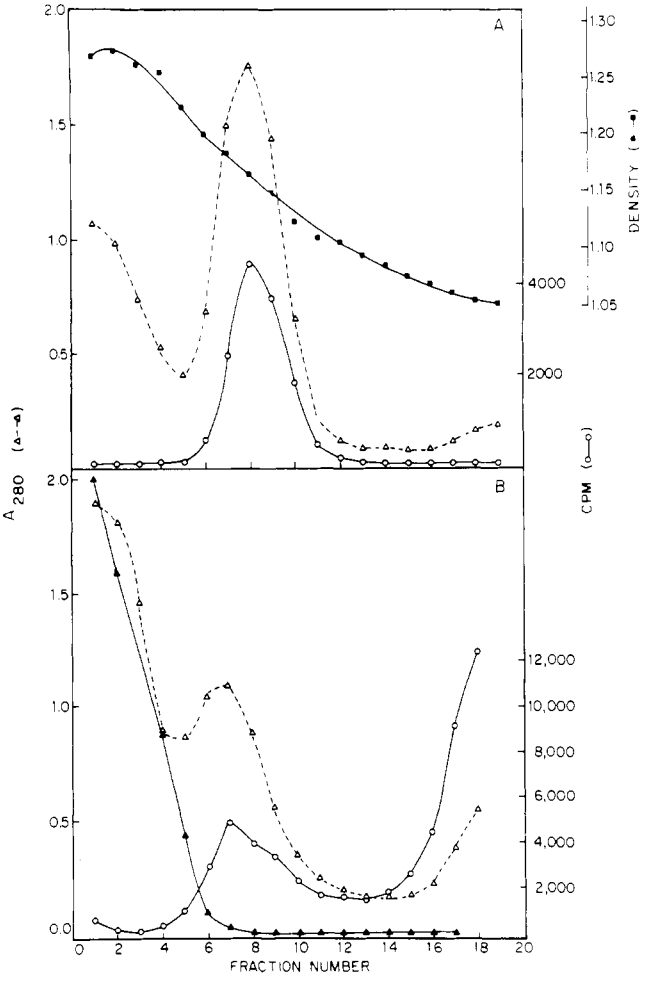

FIGURE 8: Density gradient ultracentrifugation of apoLP-C-III-B and phospholipid. The gradients were fractionated and protein, lipid, and density determined on each fraction (see Experimental Section). (A) ApoLP-C-III-B plus DMPC (Δ- - -Δ). (B) ApoLP-C-III-B plus POPC (Δ—Δ); apoLP-C-III-B alone (▲—▲).

composition of peak I as residues 1–40 (apoLP-C-III-A) and peak II as residues 41–79 (apoLP-C-III-B). ApoLP-C-III-A showed a typical Tyr absorption spectrum with no evidence of Trp absorption (Figure 4). Its amino acid analysis showed an absence of Pro and Trp and a high content of Ala and Glu (Table I). ApoLP-C-III-B had a characteristic Trp absorption (Figure 4); the amino acid analysis indicated the presence of Trp and the absence of Met and His (Table I). The peptides were homogeneous on disc gel electrophoresis in urea at pH 8.2 (Figure 3).

ApoLP-C-III-A does not bind to either a saturated or an unsaturated phosphatidylcholine based on the absence of change in the circular dichroic spectrum (Figure 5) and the failure to form a complex that could be isolated by density gradient ultracentrifugation (Figure 7). Acid hydrolysis and subsequent amino acid analysis of the phospholipid containing fractions at the top of the tube confirmed that there was no protein associated with the lipid.

In contrast, apoLP-C-III-B exhibited the changes expected to occur as a consequence of binding phospholipid. Circular dichroic spectroscopy showed changes consistent with an increase in α-helical content from 13% to 38% (Figure 6). The intrinsic tryptophan fluorescence maximum shifted from 352 nm to 340 nm, indicating a transfer of these residues to a more hydrophobic environment. This fragment formed a complex with phosphatidylcholine that was isolated by density gradient ultracentrifugation (Figure 8). All the saturated lipid was found associated with the peptide as a complex having a lipid to protein ratio of 12:1 at d = 1.16 g/mL (Figure 8A). Two complexes were formed with the unsaturated phospholipid (Figure 8B); one at d = 1.18 g/mL had a ratio of approximately 6:1 mol of phospholipid/mol of protein and the other at d = 1.06 g/mL contained approximately 29 mol/mol of protein.

The enthalpy of interaction of apoLP-C-III-A and apoLP-C-III-B with DMPC and POPC was measured using a >100-fold molar ratio of the lipid (Table II). The enthalpy was relatively small (−35 kcal/mol) for the binding of DMPC to apoLP-C-III-A. A large exothermic heat of interaction for the binding of apoLP-C-III-B with DMPC (−170 kcal/mol) was observed. The enthalpy of interaction of apoLP-C-III-A and apoLP-C-III-B with POPC was very small in both cases, although it was significantly larger in the latter case.

Discussion

The specificity of thrombin for arginine-glycine bonds (Dorman et al., 1972; Gladner, 1968; Andreatta et al., 1971) led us to investigate the cleavage of apoLP-C-III-1 at Arg-40–Gly-41 (Figure 1). Disc gel electrophoresis showed that thrombin cleaved apoLP-C-III-1 into two fragments which subsequently could be separated by chromatography on DEAE-cellulose. Amino acid analysis and UV spectroscopy of the separated peptides indicated that the two fragments

TABLE II: Enthalpy of Interaction of Fragments of Apolipoprotein C-III with Various Lipids.

	ΔH_I/mol of peptide	ΔH/residue
ApoLP-C-III-A + DMPC	−35	−0.9
ApoLP-C-III-A + POPC	−4	−0.1
ApoLP-C-III-B + DMPC	−170	−4.3
ApoLP-C-III-B + POPC	−25	−0.6
ApoLP-C-III + DMPC	−240	−3.0
ApoLP-C-III + POPC		

comprised the amino-terminal 40 residues, apoLP-C-III-A, and the carboxyl-terminal 39 residues, apoLP-C-III-B. The purified peptides were used to investigate the phospholipid binding properties of apoLP-C-III. Based on model building and theoretical grounds, Segrest et al. (1974) suggested that only the carboxyl-terminal half of apoLP-C-III should bind phospholipids. The present study represents the first direct test of this prediction.

Circular dichroic and fluorescence spectral changes of apoLP-C-III-B are indicative of the formation of an α helix with the tryptophans placed in a hydrophobic environment. The α helicity of apoLP-C-III-B changes from 13% to 38% upon interacting with phospholipid and the intrinsic tryptophan fluorescence maximum shifts 12 nm from 352 nm to 340 nm. These changes are comparable to those reported previously for synthetic apoLP-C-III-B (Sparrow et al., 1973a,b). Apolipoprotein C-III-1 shows increases in its α helicity from approximately 26% to 70% upon binding phospholipid and the intrinsic tryptophan fluorescence maximum shifts from 350 nm to 338 nm (Morrisett et al., 1973). The formation of a phospholipid–protein complex was confirmed by isolation of the complex by density gradient ultracentrifugation. The phospholipid complex with apoLP-C-III-B was somewhat more dense than that isolated from apoLP-C-III-1, floating at d = 1.16 g/mL and having a lipid:protein ratio of 12:1 with dimyristoylphosphatidylcholine. A comparable complex with apoLP-C-III-1 occurs at d = 1.09 g/mL and contains 40 phospholipids per apoLP-C-III-1 (Morrisett et al., 1974).

ApoLP-C-III-A does not bind either to a saturated or a mixed chain phospholipid based on the lack of changes in the circular dichroic spectrum and the inability to isolate a defined complex with either phospholipid. The low enthalpy (−35 kcal/mol) of interaction of apoLP-C-III-A with DMPC further collaborates the evidence presented for its inability to interact with phospholipids since Pownall et al. (1977) have shown that apoLP-C-III-1 has a high enthalpy change upon interaction with DMPC. The high enthalpy (−170 kcal/mol) of interaction of apoLP-C-III-B is consistent with this finding; the sum of enthalpies of interaction of apoLP-C-III-B and A is nearly equal to that of apoLP-C-III-1 (Table II).

From Ealing CPK models, it is apparent that there is a potential amphipathic helical region between residues 7 and 30 in apoLP-C-III-A. Likewise, there is an amphipathic region between residues 41 and 67 in apoLP-C-III-B. The calculated average hydrophobicities per residue using the hydrophobicity assignments for the amino acids of Bull and Breeze (1974) are −609 cal/residue for apoLP-C-III-A and −893 cal/residue for apoLP-C-III-B. If one assumes that only residues 41–67 are binding to the phosphatidylcholine (Segrest et al., 1974), the calculated hydrophobicity of this fragment is −1030 cal/residue. Therefore, from these projected hydrophobicities, it is predicted that apoLP-C-III-B would bind to lipids whereas apoLP-C-III-A would not. Of particular interest in apoLP-C-III-A is the low hydrophobicity near the Lys-24–Asp-25 ion pair which is predicted by Segrest et al. (1974) to be one of the determinants for lipid binding. The hydrophobicity of this region is below that sufficient for binding of the phospholipids by model peptides in the studies of Sparrow et al. (1975). The hydrophobicity between residues 7–20 is much higher, but the only ion pair present is Lys-17–His-18 which has not been implicated in phospholipid binding to date.

We feel our results support the conclusion that the hydrophobicity of the apolipoprotein is an important determinant in its ability to bind phosphatidylcholine. Our results are also consistent with the amphipathic helical model of apolipoprotein interaction with phospholipid. According to this model, the α helix is amphipathic in that one side contains the polar residues and the other side contains the nonpolar residues. The present findings support the concept that the hydrophobicity of the nonpolar side of this amphipathic helix is important in the binding of the phospholipid, and, in our opinion, is likely the major interaction between the apolipoprotein and the phospholipid in the human plasma lipoproteins.

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