# Mechanism of Lipid–Protein Interaction in the Plasma Lipoproteins: Lipid-Binding Properties of Synthetic Fragments of Apolipoprotein A-II†

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ABSTRACT: To delineate the basic structural unit for the binding of phospholipids by the plasma apolipoproteins, we have synthesized peptides of human high-density apolipoprotein A-II (apoA-II) and tested them for their ability to interact with single bilayer vesicles of dimyristoylphosphatidylcholine (DMPC). The fragments corresponding to residues 65–77, 56–77, 47–77, and 40–77 in the apoprotein were prepared by solid-phase peptide synthesis. The binding of phospholipid by these synthetic fragments and by the native tryptic peptide 56–77 was studied by changes in conformation, as determined by circular dichroism and by fractionation of peptide–DMPC mixtures in density gradients of KBr. Fragments 65–77 and 56–77 (synthetic and native) had disordered structures in the absence and presence of DMPC and no significant amount of peptide–phospholipid complexes was isolated. When fragments 47–77 and 40–77 were incubated with DMPC, there were marked conformational changes; the $\alpha$-helical content of fragment 47–77 increased from 25 to 48% and fragment 40–77 from 23 to 48%. After ultracentrifugation in KBr, phospholipid complexes with fragments 47–77 and 40–77 were isolated between $d$ 1.07 and 1.10 g/mL. The molar ratios of DMPC to fragments 47–77 and 40–77 were 25 and 44, respectively. The results of these studies and examination of space-filling models of apoA-II suggest that an amphipathic $\alpha$ helix which contains a nonpolar face and a polar face with at least one juxtaposed acidic and basic group is required for phospholipid binding by apoA-II.

Human plasma high-density lipoproteins (HDL)[1] contain two major protein constituents designated apolipoprotein A-I (apoA-I) and apolipoprotein A-II (apoA-II). The physicochemical and lipid-binding properties of these proteins have been reviewed by Jackson et al. (1976) and Morrisett et al. (1977). ApoA-II, the protein used in the present study, consists of two identical polypeptide chains of 77 amino acids each that are linked by a single disulfide bond at residue 6 (Figure 1). Previous reports from this laboratory (Jackson et al., 1973a,b; Lux et al., 1972a) have shown that either the dimeric form of apoA-II or reduced-carboxymethylated apoA-II interact with phosphatidylcholines to form lipid–protein complexes. Based, in part, on the lipid-binding properties of the two cyanogen bromide fragments of apoA-II (Figure 1), it was concluded in these earlier studies (Jackson et al., 1973a) that apoA-II contained phospholipid-binding regions in the COOH-terminal half of the molecule. From these results, we have suggested that apoA-II, as well as the other plasma apolipoproteins of known sequence, has a structural feature which may account for its ability to bind phosphatidylcholine (Segrest et al., 1974; Jackson et al., 1974a, 1975; Baker et al., 1975). The fundamental feature of this structure is that of an amphipathic $\alpha$ helix which contains two clearly defined faces, a polar and a nonpolar one. A space-filling model of the amphipathic helices of apoA-II is shown in Figure 1. A characteristic of the polar face of the helix is the distribution of acidic and basic amino acid residues; the acidics occur along the center of each polar face, while the basics are located along the lateral edges separating the polar and nonpolar faces. With this distribution of residues, zwitterionic phospholipids could orient perpendicular to the long axis of the helix such that the polar-head groups interact with the juxtaposed acidic and basic amino acid residues and the fatty acyl chains with the hydrophobic face of the helix. In apoA-II (Figure 1), there are two proposed regions which contain an amphipathic helix and at least two juxtaposed ion pairs per helix. In the earlier report (Segrest et al., 1974), two ion pairs per helix were required before the helix could be considered as a phospholipid-binding structure. Since we have previously shown (Jackson et al., 1973a,b; Lux et al., 1972a) that the COOH-terminal cyanogen bromide fragment (residues 27–77) bound phosphatidylcholine, it was of considerable interest to design experiments to further delineate the binding sites in this region of the molecule. In the present report, we have prepared fragments corresponding to residues 65–77, 56–77, 47–77, and 40–77 and tested them for their ability to interact with dimyristoylphosphatidylcholine.

## Materials and Methods

*Preparation of ApoA-II and Native Fragments.* ApoA-II was isolated from HDL as described previously (Mao et al., 1975). The two cyanogen bromide fragments corresponding to residues 1–26 and 27–77 were obtained from reduced-carboxymethylated apoA-II (Jackson et al., 1973b). The peptides extending from residues 55 to 77 and 56 to 77 were obtained from a tryptic digestion of the COOH-terminal cyanogen bromide fragment (residues 27–77); the tryptic peptides were fractionated by chromatography of the digest on Sephadex G-50 in 0.10 M Tris-HCl, pH 8.0, containing 5.4 M urea and

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[1] Abbreviations used are: HDL, high-density lipoproteins; apoA-I and apoA-II, apoprotein constituents of HDL; DMPC, dimyristoylphosphatidylcholine; CD, circular dichroism; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; DEAE, diethylaminoethyl; DMF, dimethylformamide; EDTA, (ethylenedinitrilo)tetraacetic acid.

on DEAE-cellulose as described previously (Jackson and Gotto, 1972).

*Preparation and Purification of Synthetic Fragments of ApoA-II.* Four synthetic fragments were prepared in the present study and correspond to residues 65–77, 56–77, 47–77, and 40–77 in the native apoprotein (Figure 1). The rationale for choosing to synthesize the fragment extending from 56 to 77 was based on the fact that the same native fragment could be isolated. The fragments corresponding to 40–77 and 47–77 were chosen because of their potential involvement in an amphipathic helix.

The peptides were synthesized with a Schwarz Bio-Research Peptide Synthesizer on a modified polystyrene resin as described by Sparrow (1976). The program shown in Table I was used to incorporate the *tert*-butoxycarbonyl (*t*-BOC) protected amino acid stepwise as the symmetrical anhydride generated in situ in 50% DMF/methylene chloride with dicyclohexylcarbodiimide. Five grams of *t*-BOC-glutamine resin was loaded into the shaker vessel of the synthesizer and the resin was acetylated with acetic anhydride/pyridine. The *tert*-butoxycarbonyl group was removed with 40% trifluoroacetic acid in methylene chloride and the next *t*-BOC-amino acid was coupled to the resin after neutralization with 5% diisopropylethylamine in methylene chloride. Any unreacted amino groups were blocked with acetic anhydride/pyridine before the synthesis was continued. The following protecting groups were used for the polyfunctional amino acids: benzyl ethers for threonine and serine, benzyl esters for glutamic acid, 2,6-dichlorobenzyl for the phenolic hydroxyl of tyrosine, and 2-chloro-*Z* for the $\epsilon$-amino group of lysine. The progress of the synthesis was monitored by total acid hydrolysis (12 M HCl/propionic acid, 1:1 (v/v), 2 h, 135 °C) of sample peptide resins with subsequent amino acid analysis. A portion of solvated resin (8 g) was removed after the coupling of residues 65, 56, and 47. The resins were washed with methylene chloride and dried in vacuo over $P_2O_5$.

To cleave the protecting groups and to remove the peptides from the resin, 1 g of dried resin was transferred to a Kel-F vessel and treated with 20 mL of anhydrous hydrogen fluoride (HF) containing 2 mL of anisole for 30 min at 0 °C. After evaporating the HF, the peptides were extracted with anhydrous trifluoroacetic acid (3 × 50 mL) and the trifluoroacetic acid was evaporated in vacuo at 20 °C; the peptides were precipitated with diethyl ether. After centrifugation, the ether was removed by decantation and the peptides were dissolved in 10 mL of 1 M Tris, pH 11, containing 6.0 M urea. After the peptide dissolved, the pH of the sample was 8.2.

Fragments were initially fractionated to remove small truncated peptides by chromatography on Bio-Gel P-10 equilibrated with 0.1 M Tris-HCl, 6.0 M urea, pH 8.2. The appropriate fractions were pooled and desalted on a column of Bio-Gel P-2 in 0.1 M ammonium bicarbonate. Peptides were further purified by ion-exchange chromatography on DEAE-cellulose or sulfopropyl-Sephadex as described in the legends to the figures. The purity of the peptide fragments was determined by polyacrylamide gel electrophoresis at pH 8.2 and by amino acid analysis.

*Phospholipid-Binding Studies.* The phospholipid-binding properties of fragments were studied using single bilayer vesicles of dimyristoylphosphatidylcholine (DMPC). The lipid was prepared by sonication for 1 h at 24 °C under ultrapure nitrogen of a suspension of DMPC (Sigma) (40 mg/mL) in a standard buffer containing 0.01 M Tris-HCl, 0.10 M NaCl, 0.001 M $NaN_3$, and 0.001 M EDTA, pH 7.4. 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. Chromatography of the sonicated dispersion on Sepharose 4B indicated that 90% of the DMPC was in small single bilayer vesicles. The fragments (1 mg) and DMPC (3 mg) in 1.5 mL of standard buffer were incubated for 12 h at 23.5 °C. The incubation mixtures were then subjected to gradient ultracentrifugation in KBr (Morriset et al., 1973). The gradient solutions consisted of the appropriate quantity of KBr in the standard buffer and were prepared in 5-mL polyallomer tubes with a Buchler peristaltic pump, gradient maker, and Densiflow. The samples were immediately centrifuged in a Beckman SW 50.1 rotor at 45 000 rpm and 25 °C for 96 h. The contents of each tube were fractionated with a Buchler fractionator; 0.30-mL fractions were collected. Each fraction was analyzed by absorbance at 280 nm for phospholipid (Bartlett, 1959) and for density as determined by refractive index measured on a Bausch and Lomb refractometer.

*Other Methods.* Circular dichroism (CD) was measured at 25 °C on a Cary 61 spectropolarimeter using cells of 0.5-mm path length as described previously (Morrisett et al., 1973). The percent $\alpha$-helical content was estimated from the $[\theta_{222}]$ by the relative % $\alpha$ helix = $(\theta_{222} + 3000)/(36\,000 + 3000)$. Peptide concentrations were determined by amino acid analysis.

TABLE I: Synthetic Program: Symmetrical Anhydrides.

| Operation[a] | Reagent[b] | Applications | Time (min) |
|---|---|---|---|
| 1 | $CH_2Cl_2$ | 2 | 1 |
| 2 | $F_3AcOH$–$CH_2Cl_2$ (2:3)[c] | 1 | 1 |
| 3 | $F_3AcOH$–$CH_2Cl_2$ (2:3) | 1 | 10 |
| 4 | $CH_2Cl_2$ | 3 | 1 |
| 5 | $CH_3CH_2OH$ | 3 | 1 |
| 6 | $CH_2Cl_2$ | 5 | 1 |
| 7 | $F_3AcOH$–$CH_2Cl_2$ (2:3) | 1 | 1 |
| 8 | $F_3AcOH$–$CH_2Cl_2$ (2:3) | 1 | 10 |
| 9 | $CH_2Cl_2$ | 5 | 1 |
| 10 | DIEA–$CH_2Cl_2$ (5:95) | 1 | 1 |
| 11 | DIEA–$CH_2Cl_2$ (5:95) | 1 | 5 |
| 12 | $CH_2Cl_2$ | 5 | 1 |
| 13 | BOC-amino acid–$CH_2Cl_2$[d] | 1 | 1 |
| 14 | DCC–DMF[e] | 1 | 60 (120)[f] |
| 15 | $CH_3CH_2OH$–$CH_2Cl_2$ (1:9) | 3 | 1 |
| 16 | $CH_2Cl_2$ | 3 | 1 |
| 17 | $CH_3CH_2OH$ | 3 | 1 |
| (18–27) | Repeat operations 9–17 | | |
| 28 | $CH_2Cl_2$ | 5 | 1 |
| 29 | $(CH_3CO)_2O$–pyridine (1.0 M) | 1 | 1 |
| 30 | $(CH_3CO)_2O$–pyridine (1.0 M) | 1 | 10 |
| 31 | $CH_2Cl_2$ | 5 | 1 |

[a] All operations were carried out at 25 °C. [b] Purity of reagents: $F_3AcOH$ (trifluoroacetic acid), redistilled from calcium sulfate; DIEA (diisopropylethylamine), distilled from napthyl isocyanate; $(CH_3CO)_2O$ (acetic anhydride), distilled (bp 140 °C); pyridine, distilled (bp 115 °C); $CH_2C_2$ and $CH_3CH_2OH$, reagent grade; BOC-amino acids (*tert*-butoxycarbonyl), obtained from Bachem and Peninsula Labs and used as received; DOC (dicyclohexylcarbodiimide), obtained from Schwarz/Mann and used as received; DMF, distilled from $CaH_2$ and stored at 4 °C over molecular sieves. [c] Volume ratio of components. [d] An 8:4 molar ratio of BOC-amino acid and DCC to amino component was used, except for Ala-75, Gly-71, Leu-70, Tyr-66, Gly-57, Ala-56, Pro-51, Leu-49, Glu-47, and residues 46 to 40 where a 4:2 ratio was employed. The BOC-Gln and BOC-Asn were coupled once as the *p*-nitrophenyl active ester for 18 h in DMF. [e] DMF was used as a solvent for DCC in the cases where an eightfold excess of BOC-amino acid was employed (see *d* above). [f] Coupling time (120 min) was used for 8:4 ratio couplings.

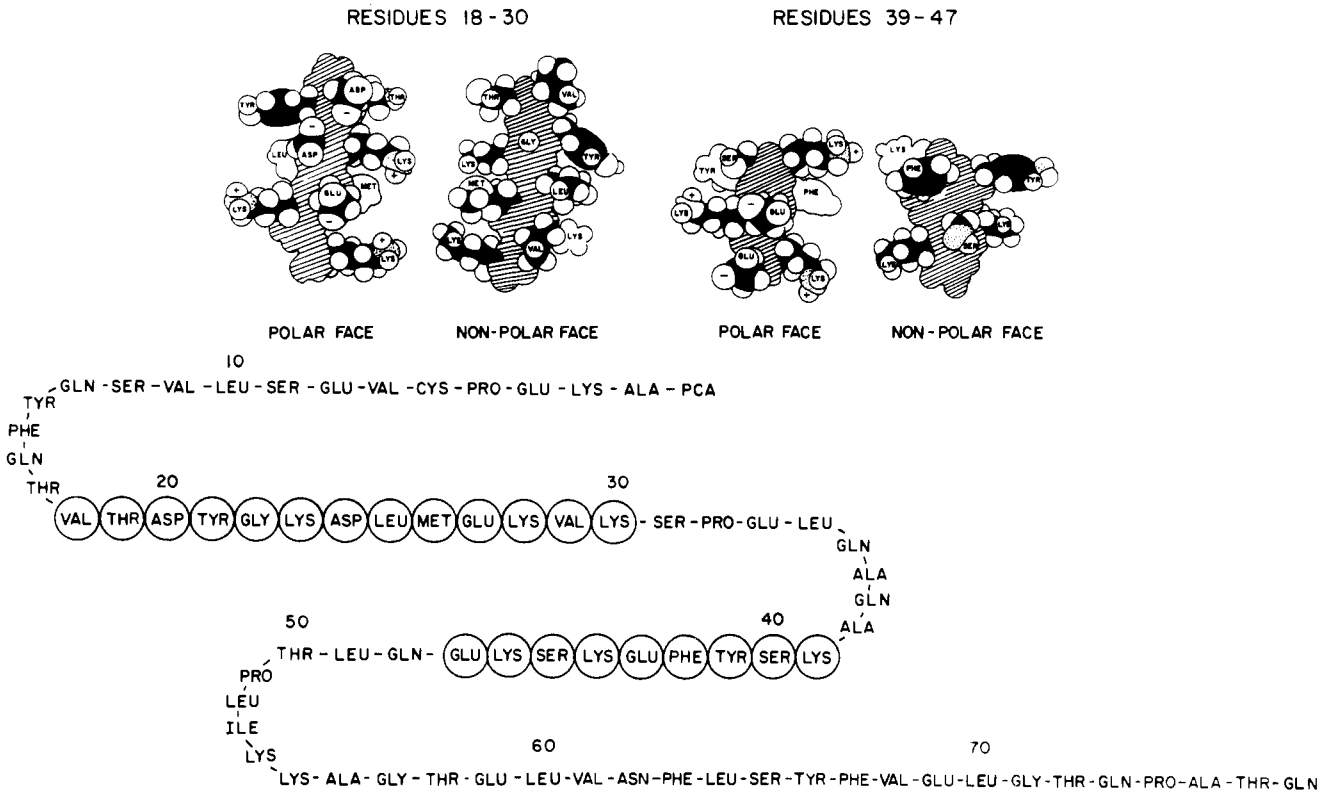


FIGURE 1: Amino acid sequence of human high-density apolipoprotein A-II (apoA-II) as described by Brewer et al. (1972) and Lux et al. (1972b). The presumed helical regions 18–30 and 39–47 previously described by Segrest et al. (1974) are indicated by circles. Each helix was built with Ealing CPK space-filling models. A right-handed $\alpha$-helical backbone was constructed with 3.6 residues per turn. For each amphipathic region, amino acid residues were added onto the $\alpha$ carbon in their proper order. Each helix is shown with its axis oriented parallel to the plane of the page and its $NH_2$ terminus toward the top of the page. The polar and nonpolar faces of each helix are shown.

TABLE II: Amino Acid Compositions of Synthetic and Native Fragments of ApoA-II.[a]

| Amino acid | Synthetic fragment 65–77 | Synthetic fragment 56–77 | Native fragment 56–77 | Synthetic fragment 47–77 | Synthetic fragment 40–77 |
|---|---|---|---|---|---|
| Asp | | 1.0 (1) | 1.1 (1) | 1.0 (( | 1.1 (1) |
| Thr | 2.1 (2) | 2.8 (3) | 2.9 (3) | 3.8 (4) | 3.8 (4) |
| Ser | 1.0 (1) | 1.0 (1) | 0.9 (1) | 1.0 (1) | 2.5 (3) |
| Glu | 3.0 (3) | 3.9 (4) | 4.3 (4) | 5.8 (6) | 6.8 (7) |
| Pro | 1.0 (1) | 1.0 (1) | 1.1 (1) | 1.8 (2) | 2.2 (2) |
| Gly | 1.0 (1) | 2.0 (2) | 2.1 (2) | 2.0 (2) | 2.2 (2) |
| Ala | 1.0 (1) | 2.0 (2) | 2.0 (2) | 2.0 (2) | 2.3 (2) |
| Val | 1.0 (1) | 1.6 (2) | 1.6 (2) | 2.1 (2) | 1.8 (2) |
| Ile | | | | 0.9 (1) | 0.9 (1) |
| Leu | 0.9 (1) | 3.0 (3) | 2.9 (3) | 5.2 (5) | 5.3 (5) |
| Tyr | 0.9 (1) | 0.9 (1) | 1.1 (1) | 1.1 (1) | 1.8 (2) |
| Phe | 0.9 (1) | 1.9 (2) | 2.0 (2) | 2.0 (2) | 3.0 (3) |
| Lys | | | | 1.7 (2) | 3.8 (4) |
| Total | 13 | 22 | 22 | 31 | 38 |

[a] Amino acid analyses were obtained on peptide samples subjected to hydrolysis (24 h, 110 °C) in sealed evacuated tubes employing 6 N HCl. Analysis was performed on a Beckman Model 117 or 119 analyzer equipped with an Autolab integrator. The values represent the average of two determinations.

## Results

*Synthesis and Purification of Fragments.* The strategy used for the synthesis of the four fragments of apoA-II can be summarized as follows: (1) the use of an improved resin (Sparrow, 1976) which incorporates a *p*-bromomethylphenylacetamido-(11-hendecanamido)$_2$ spacer group; (2) all couplings performed with symmetrical anhydrides of the BOC-amino acids generated in situ with dicyclohexylcarbodiimide, except BOC-Gln and BOC-Asn which were coupled as active esters; (3) a strategy of maximum protection for amino acid side chain functions; (4) a program (Table I) which included swelling and shrinking of the resin between the double couplings; and (5) acetylation to block unreacted amino groups. At the appropriate residue, a portion of the peptide resin was removed and treated with HF. After an initial purification on Bio-Gel P-10 to remove truncated peptides, the fragments corresponding to residues 65–77, 56–77, and 47–77 (Figure 1) were subjected to chromatography on DEAE-cellulose (Figure 2); the fragment representing residues 40–77 was purified on sulfopropyl-Sephadex. The amino acid composition

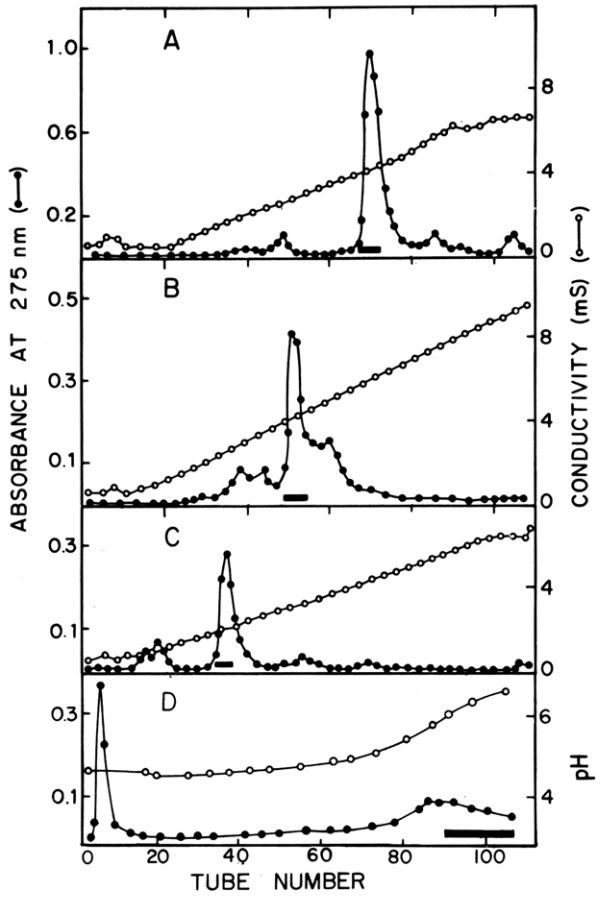


FIGURE 2: Purification of synthetic peptides of apoA-II by DEAE-cellulose (panels A–C) or SP-Sephadex (panel D). (A) Fragment 65–77; (B) fragment 56–77; (C) fragment 47–77; (D) fragment 40–77. The samples (50 mg) were applied to a column (1.6 × 30 cm) of DEAE-cellulose (DE-52 from Whatman) at a concentration of 5 mg/mL. After the sample entered the resin, it was eluted with the following gradient: (A) 400 mL of 0.01 M Tris-HCl, pH 8.2, and 400 mL of the same buffer containing 0.1 M NaCl; (B) 400 mL of 0.01 M Tris-HCl, pH 8.2, which contained 6 M urea and 400 mL of the same buffer containing 0.2 M NaCl; (C) same as B except the NaCl was 0.1 M; (D) the SP-Sephadex column (1.6 × 30 cm) was equilibrated with 0.02 M sodium acetate, pH 4.5. The 40–77 fragment was applied to the column in 20 mL of equilibrating buffer. The peptide was eluted with a pH gradient consisting of 300 mL of 0.2 N sodium acetate, pH 4.5, and 300 mL of 0.2 N sodium phosphate, pH 7.0. In all columns, 5-mL fractions were collected and the flow rates were 25 mL/h. The fractions under the bars were pooled, desalted, and characterized by amino acid analyses and gel electrophoresis.

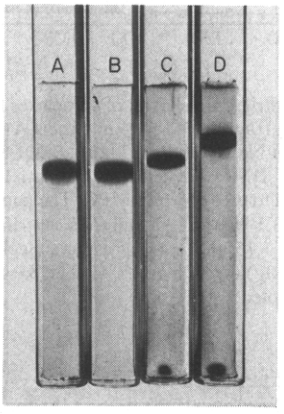


FIGURE 3: Polyacrylamide gel electrophoresis of synthetic fragments. The gels were run on 7.5% acrylamide gels in a 0.03 M Tris–glycine buffer containing 8 M urea, pH 8.2, for 3 h at 3 mA/gel. Each gel was loaded with 40 μg of fragment; the gels were stained with Coomassie blue. (A) Native fragment 56–77; (B) synthetic fragment 56–77; (C) synthetic fragment 47–77; (D) synthetic fragment 40–77.

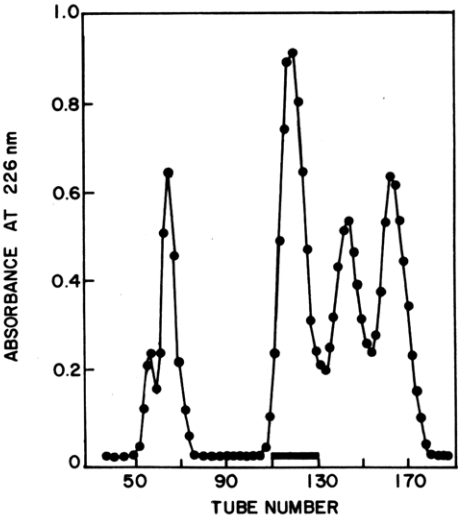


FIGURE 4: Chromatography on Sephadex G-50 of tryptic peptides of the cyanogen bromide peptide (residues 27–77, Figure 1) of apoA-II. The CNBr fragment (50 mg) was digested with 1 mg of trypsin (TPCK, Worthington) for 30 min at 23 °C. The digest was applied directly to a column (2.6 × 200 cm) equilibrated with 0.1 M Tris-HCl, pH 8.0, containing 5.4 M urea. The flow rate was 35 mL/h and 5.6-mL fractions were collected. The fractions indicated by the bar were pooled, desalted on Bio-Gel P-2 in 0.1 M ammonium bicarbonate, and lyophilized.

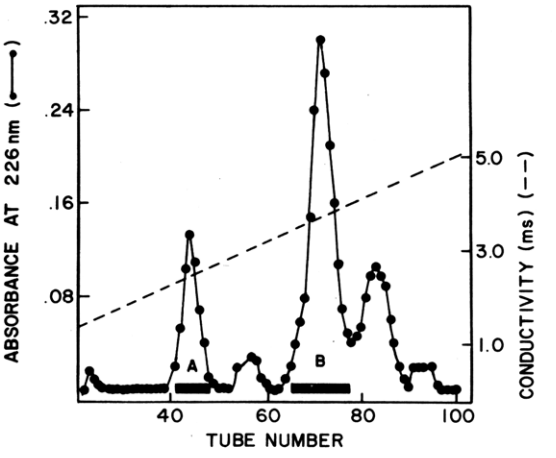


FIGURE 5: Chromatography of Sephadex G-50 fraction (Figure 4) on DEAE-cellulose. The sample was applied to a column (1.6 × 30 cm) of DEAE-cellulose (DE-52, Whatman) and was eluted with the same gradient as described in Figure 2 for synthetic fragment 47–77. The fractions represented in peak A correspond to residues 55–77 in apoA-II and peak B to residues 56–77.

of each fragment is given in Table II. Each of the four peptides gave compositions which were in close agreement with the theoretical values. By polyacrylamide gel electrophoresis at pH 8.2 in 8 M urea, fragments 40–77, 47–77, and 56–77 gave single bands (Figure 3). The synthetic fragment 56–77 migrated to the same position as the native one.

As a further comparison of the properties of the 56–77 fragment, the corresponding peptide was obtained by a tryptic digestion of the COOH-terminal cyanogen bromide fragment of apoA-II. Chromatography of the tryptic digest on Sephadex G-50 (Figure 4) yielded a fraction which had a composition consistent with the sequence of residues 56–77 but, in addition, it contained 0.4 residue of lysine, suggesting that trypsin had also cleaved between residues 54 and 55. Separation of these two fragments (55–77, 56–77) was achieved by chromatography on DEAE-cellulose (Figure 5); the fragment representing residues 56–77 eluted at the same conductivity as the

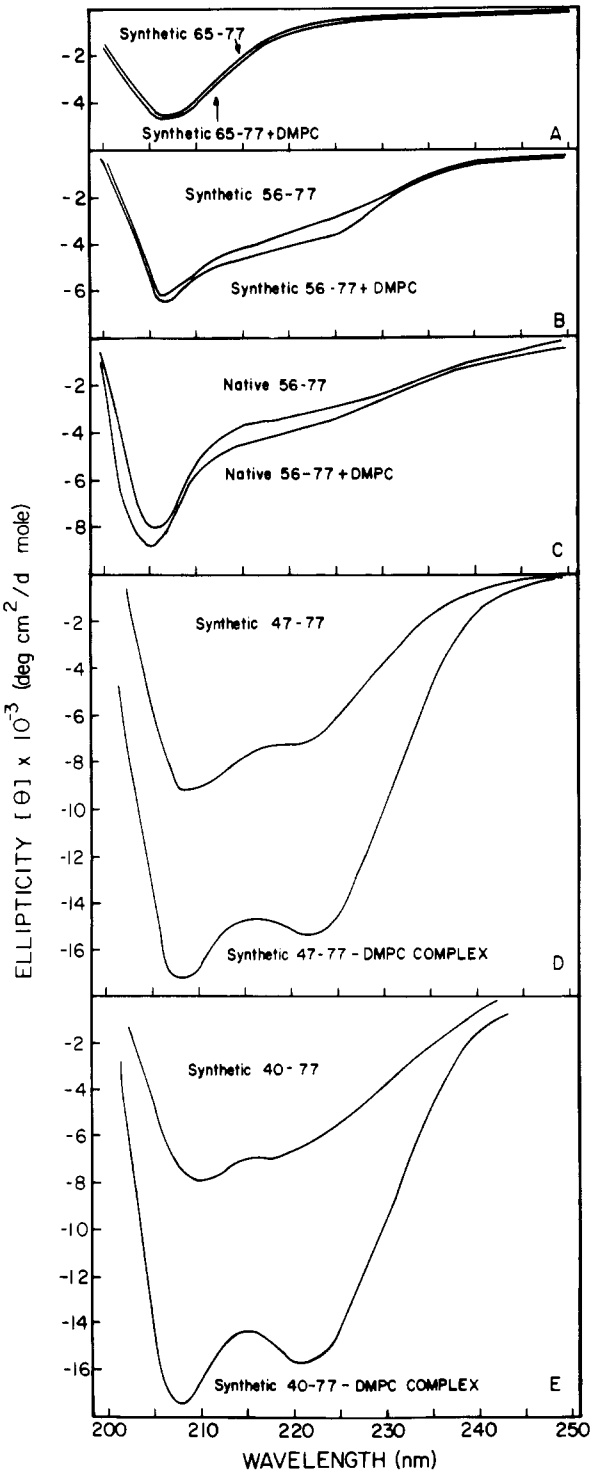


FIGURE 6: Circular dichroic spectra of fragment and fragment-DMPC complexes. The spectra shown in panels A, B, and C are those of fragments alone or fragments to which DMPC was added in a ratio of 3:1 (w/w) fragment:DMPC. The mixtures (1.5 mL) were incubated at 23.5 °C for 12 h before the spectra were recorded. The spectra shown in D and E for the DMPC-fragment complexes are those of the isolated complexes shown in Figure 7.

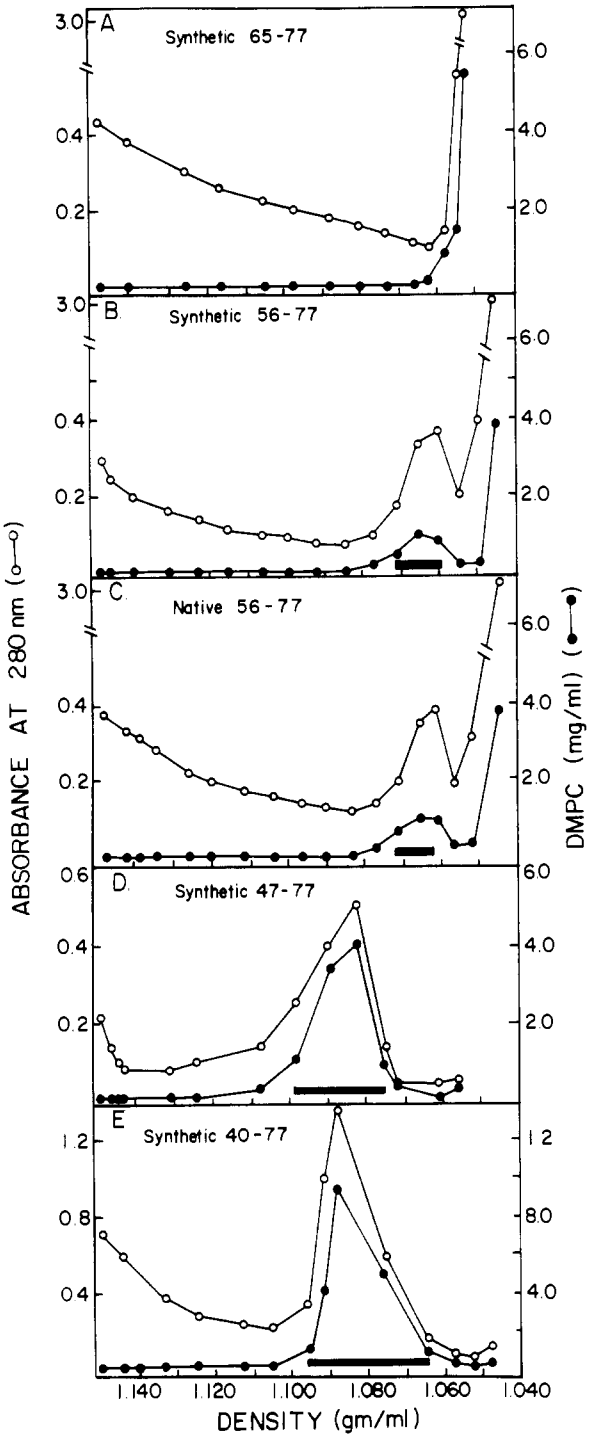


FIGURE 7: Ultracentrifugal behavior of complexes formed by DMPC and fragments. To each fragment (1 mg) in panels A-D in 1.42 mL of 0.01 M Tris-HCl, 0.10 M NaCl, 0.001 M $NaN_3$, 0.001 M EDTA, pH 7.4, was added 0.075 mL of DMPC vesicles (40 mg/mL). In panel E, 2 mg of fragment was added to 0.15 mL of DMPC. The peptide-DMPC mixtures were incubated at 23.5 °C for 12 h and then centrifuged in a KBr density gradient between *d* 1.15 and 1.04 by using a Beckman SW 50.1 rotor, 45 000 rpm (248 000*g*) for 96 h at 25 °C. The bars represent those fractions which were pooled.

corresponding synthetic peptide. The amino acid composition of the native 56-77 is given in Table II.

*Phospholipid-Binding Properties of Fragments.* In the absence of lipid, all of the synthetic and native fragments had primarily disordered structures as evidenced by the trough between 200 and 210 nm in the CD spectra (Figure 6). After incubation of each fragment with DMPC vesicles, peptides 47-77 and 40-77 had CD spectra which are characteristic of helical structure; peptides 65-77 and 56-77, both native and synthetic, showed only a slight or no CD spectral changes with the addition of lipid. The CD spectra (not shown) of native fragment 55-77 were identical to 56-77 either in the absence or presence of DMPC. Since previous studies (Jackson et al., 1975) have shown that phospholipid binding occurs concomitantly with an increase in $\alpha$-helical structure, it was of interest

TABLE III: Conformation and Composition of Fragments and Fragment-DMPC Complexes.

| Synthetic fragment | % helix | | DMPC:fragment[a] (molar ratio) |
|---|---|---|---|
| | Fragment | Fragment-DMPC complex | |
| 47-77 | 25 | 48 | 25 |
| 40-77 | 23 | 48 | 44 |

[a] The % helix and molar ratios were determined on the isolated complexes shown in Figure 7.

to isolate the potential lipid-peptide complexes. Each of the DMPC-peptide mixtures from the CD experiments was subjected to density-gradient ultracentrifugation in KBr (Figure 7). The majority of fragments 65-77 and 56-77 (synthetic and native) were found unassociated with lipid and extending from the bottom of the tube ($d$ 1.15) to $d$ 1.08. Inspection of the ultracentrifugation profiles for the 56-77 fragments indicated that a complex was present between $d$ 1.08 and 1.06 (Figure 7); however, the measured absorbance at 280 nm was due to light scattering and the fraction contained less than 5% of the peptide which was added to the centrifuge tube. Fragments 47-77 and 40-77 both formed complexes with DMPC (Figure 7) which were isolated between densities 1.07 and 1.10 g/mL; the isolated complexes contained greater than 70% of the peptide added to the tubes. Calculations of the molar ratios of phospholipid to peptide gave values of 25 and 44 for fragments 47-77 and 40-77, respectively, on the pooled complexes (Table III). The helical content of the peptides in the isolated complexes was 48% for both the 47-77 and 40-77 fragments compared to 25 and 23%, respectively, in the absence of lipid (Table III).

## Discussion

In previous reports from this laboratory (Segrest et al., 1974; Jackson et al., 1975), it was suggested that the plasma apolipoproteins contain amphipathic $\alpha$-helical segments and that these helices represent the basic lipid-binding units for this class of lipid-associating proteins. To obtain direct proof for this hypothesis, we have synthesized various peptide fragments of apoA-II (Figure 1) and have examined them for their capacity to interact with DMPC. Neither the synthetic nor the native fragment 56-77 interacted with phospholipid; there were no changes in the CD spectra with the addition of lipid and no significant amount of complex was isolated. Fragments 47-77 and 40-77, however, both interacted with DMPC as evidenced by the dramatic increases in $\alpha$-helical content and by the isolation of peptide-DMPC complexes.

The finding that the 47-77 fragment interacted with phospholipid was somewhat surprising, since in our original description of the amphipathic helical model (Segrest et al., 1974) we predicted that apoA-II contained only two such regions (Figure 1); the 47-77 segment was not considered as being an amphipathic helix. However, in the initial report, we required the amphipathic helix to contain at least two ion pairs per helix. For example, the helical segment between residues 18 and 30 has three ion pairs, i.e., Lys-23-Asp-20, Lys-28-Asp-24, and Lys-30-Glu-27, and the segment between 39 and 47 has two, Lys-44-Glu-43 and Lys-46-Glu-47. The residues between Pro-51 and Leu-70 can be placed into an amphipathic helix with one side of the helix being hydrophobic and the other side hydrophilic (Figure 8). However, in this segment, there is only one ion pair. Although fragment 56-77 contains part but not all of the amphipathic helix shown in Figure 8, it did not interact with phospholipid (Figure 7). The native tryptic fragment 55-77 has a lysine at residue 55 and based on space-filling models could form an ion pair with Glu-59 (Figure 8). However, it did not interact with DMPC. When the residues Glu-Gln-Leu-Thr-Pro-Leu-Ile-Lys were added to the 55-77 fragment, the peptide bound phospholipid. Assuming that the phospholipid-binding site is helical and that Pro-51 is the first residue in the helix, it seems likely that the addition of residues 51-54 (Pro-Leu-Ile-Lys) gives the polypeptide chain the necessary information to make it a lipid-binding peptide. The addition of the residues Pro-Leu-Ile-Lys to the 55-77 fragment would also add two hydrophobic amino acids to the nonpolar face of the helix. Whether it is the addition of the lysine residue, the hydrophobic residue, or both which conveys lipid-associating properties to the 55-77 fragment is not known. The importance of basic amino acid residues for lipid-binding has been shown by Jackson et al. (1974). These investigators found that maleylation of apoA-II completely abolished the lipid-associating properties of the apoprotein. These chemical-modification studies and the finding in this study that the absence of the two lysine residues gives a fragment which does not interact with DMPC add support to the amphipathic helical model of lipid binding. Studies are currently in progress to synthesize fragments which contain other residues between positions 51 and 56 and to test these for their lipid-binding properties. Finally, it should be mentioned that caution must be taken in extrapolating the results with the synthetic fragments to the intact apoprotein. It should also be mentioned that the concept that apolipoproteins contain amphipathic helical structures still remains a hypothesis and final proof must await three-dimensional analysis.

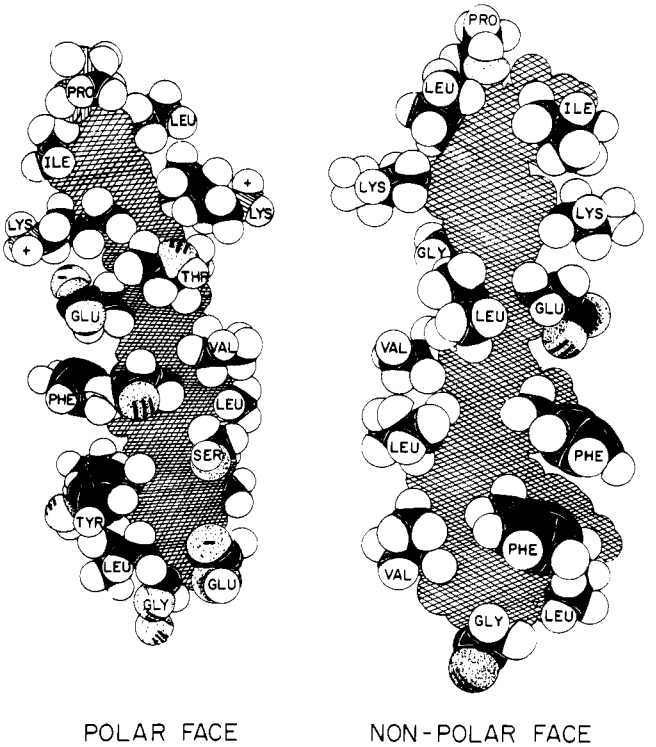


FIGURE 8: Drawing of a CPK space-filling model of residues Pro-51-Leu-70 in apoA-II. The residues were placed into an $\alpha$ helix as described in the legend to Figure 1.

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# Proton Nuclear Magnetic Resonance Study of the Decay of Transbilayer Compositional Asymmetry Generated by a Phosphatidylcholine Exchange Protein†

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ABSTRACT: Transbilayer compositional asymmetry was generated in single-lamellar vesicles formed from $-N(CD_3)_3$ egg phosphatidylcholine by incubation with erythrocyte ghost membranes in the presence of a purified phosphatidylcholine exchange protein prepared from beef liver. In a series of experiments, between 50 and 85% of the $-N(CD_3)_3$ phosphatidylcholine in the external face of the bilayer vesicles was replaced by $-N(CH_3)_3$ phosphatidylcholine from ghost membranes after a 24-h incubation at 37 °C. Proton NMR studies utilizing $Pr^{+3}$ as a shift reagent showed that 82 to 89% of the exchanged $-N(CH_3)_3$ phosphatidylcholine was found on the external face of the vesicle wall. The decay of the transbilayer compositional asymmetry by exchange migration of $-N(CH_3)_3$ and $-N(CD_3)_3$ phosphatidylcholines, as followed by NMR spectroscopy over a 5-day period at 23 °C, was shown to be a slow process with a half-time of 26 days. The half-times consistent with ±1 standard deviation were 16 and 69 days. Appropriate controls established the integrity of the vesicles throughout the 5-day period. The cholesterol content of the erythrocyte ghost membranes used to prepare the asymmetric vesicles was reduced 40 to 60% by prior incubation with phosphatidylcholine vesicles. During preparation of the asymmetric vesicles, spontaneous cholesterol movement from the cholesterol-depleted ghosts resulted in final cholesterol concentrations in the asymmetric vesicles of between 5 and 16 mol %.

Three approaches have been utilized for measuring transbilayer migration of phosphatidylcholine in small, single-lamellar vesicles. Spin-labeled phosphatidylcholine has been incorporated into phosphatidylcholine vesicles and the outer bilayer spin-label paramagnetism abolished with ascorbate (Kornberg and McConnell, 1971). The rate of accessibility to external ascorbate of the remaining spin label initially on the inner bilayer surface suggested a half-time for transbilayer equilibration of spin label as short as 6.5 h at 30 °C. Second, a chemical-labeling technique has been utilized in which the outer monolayer phosphatidylethanolamine present in phosphatidylcholine vesicles is converted to its amidine derivative by reaction with isethionyl acetimidate. Transbilayer movement of the unreacted inner monolayer phosphatidylethanolamine, measured with 2,4,6-trinitrobenzenesulfonic acid, showed the half-time to be greater than 80 days at 22 °C (Roseman et al., 1975). A third approach introduced by Johnson and co-workers (1975) and by Rothman and Dawidowicz (1975) employed a phosphatidylcholine exchange protein to generate a compositional asymmetry of isotopically labeled phospholipid in a vesicle bilayer. The subsequent "exchange-out" kinetics of the labeled phospholipid was used to establish the half-time for the transbilayer equilibration of this lipid. Johnson and co-workers (1975) reported a half-time

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