Mechanism of Lipid–Protein Interaction in the Plasma Lipoproteins: Identification of a Lipid-Binding Site in Apolipoprotein A-II†

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ABSTRACT: Apolipoprotein A-II (apoA-II) is a dimeric 77-residue apoprotein of human high-density lipoproteins. Previous studies indicate that residues 56–77 in the apoprotein do not bind phospholipid whereas residues 47–77 form a complex with dimyristoylphosphatidylcholine (DMPC). To further delineate the lipid-binding region between residues 47 and 77, we have prepared synthetic fragments of apoA-II corresponding to residues 54–77, 52–77, and 50–77 and have tested each fragment for its ability to interact with vesicles of DMPC. The interaction of the fragments was determined by changes in secondary structure as measured by circular dichroism and by isolation of peptide–DMPC complexes by ultracentrifugation in density gradients of KBr. By these criteria, only fragment 50–77 binds DMPC; there is an increase in α helicity from 17% to 41% when the fragment associates with lipid. Since the 56–77 fragment does not associate with phospholipid, we propose that the addition of residues Thr-Pro-Leu-Ile-Lys-Lys (corresponding to residues 50–55) to the 56–77 fragment gives the peptide the necessary sequence information for lipid binding. To further identify the important amino acid residues in the region of 50–55, we have substituted Leu-Ile with Ala-Ala. This substitution totally abolishes the lipid-binding capacity of the 50–77 fragment. On the other hand, substitution of Lys-Lys with Ser-Ser does not alter the lipid-binding capacity of the peptide. We conclude that residues 50–55 are important in lipid binding and that the hydrophobic center formed by Leu-Ile plays an important role.

In previous reports from this laboratory (Jackson et al., 1974, 1975; Segrest et al., 1974; Sparrow & Gotto, 1980), we suggested that the plasma apolipoproteins contain a unique structural feature which accounts for their ability to bind and transport lipid. The fundamental feature of this structure is that of an amphipathic α helix which contains two clearly defined faces, a polar and a nonpolar one. To obtain direct proof of this hypothesis, we have utilized apoA-II from human high-density lipoproteins (HDL)[1] and have attempted to delineate the phospholipid-binding regions within the protein (Mao et al., 1977; Chen et al., 1979). In the earlier study (Mao et al., 1977), we showed that a tryptic fragment corresponding to residues 56–77 in the protein (Figure 1) did not interact with phospholipid. However, a synthetic fragment consisting of residues 47–77 did interact, suggesting that the residues between 47 and 56 were important to the lipid-binding properties of the peptide; residues between Pro-51 and Val-61 can be placed into an amphipathic helix with one side of the helix being hydrophobic and the other side hydrophilic. To further delineate the lipid-binding site between residues 47 and 56, we have now prepared synthetic fragments corresponding to residues 54–77, 52–77, and 50–77 and have tested each of these fragments for their ability to interact with dimyristoylphosphatidylcholine (DMPC). The results of these studies show that the fragment 50–77 is the minimal sequence capable of forming an apoprotein–lipid complex. We also show that Leu and Ile at sequence positions 52 and 53 play an important role in the lipid-binding properties.

Materials and Methods

Preparation and Purification of Synthetic Fragments of ApoA-II. Five synthetic fragments were prepared in the present study (Figure 1) and correspond to residues 50–77, 52–77, and 54–77, and 50–77 with substitution of Ala-Ala for Leu-Ile (positions 52–53) and Ser-Ser for Lys-Lys (positions 54–55). The peptides were synthesized with a Schwarz Bio-Research peptide synthesizer on a modified polystyrene resin as described by Sparrow (1976). The program used for the synthesis of these peptides and the procedures used to remove them from the resin have been described (Mao et al., 1977, 1979). Peptides were initially fractionated on Bio-Gel P-10 equilibrated with 0.1 M Tris-HCl and 6.0 M urea, pH 8.2, desalted on Bio-Gel P-2 in 0.1 M ammonium bicarbonate, and then chromatographed on DEAE-cellulose as described in the legend to Figure 2. The purity of each fragment was determined by polyacrylamide gel electrophoresis at pH 8.2 and by amino acid analysis.

Phospholipid-Binding Studies. The lipid-binding properties of the fragments were studied by using single bilayer vesicles of dimyristoylphosphatidylcholine (DMPC). The lipid was prepared by sonication for 30 min at 24 °C under ultrapure nitrogen of a suspension of DMPC (40 mg/mL) in a standard buffer containing 0.1 M Tris-HCl, 0.1 M NaCl, 0.001 M NaN_3, and 0.001 M EDTA, pH 7.4. The fragments (0.5 mg) and DMPC (2.5 mg) in 1.5 mL of standard buffer were incubated for 12 h at 24.5 °C. The incubation mixtures were then subjected to density gradient ultracentrifugation in KBr as described previously (Morrisett et al., 1973; Mao et al., 1977, 1979).

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[1] Abbreviations used: HDL, high-density lipoproteins; apoA-I and apoA-II, apoprotein constituents of HDL; DMPC, dimyristoylphosphatidylcholine; CD, circular dichroism.

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Lys - Lys .....Gln

Leu - Ile - Lys - Lys ..... Gln

Thr - Pro - Leu - Ile - Lys - Lys ..... Gln

Thr - Pro [Ala - Ala] Lys - Lys ..... Gln

Thr - Pro - Leu - Ile [Ser - Ser] ..... Gln

50 51 52 53 54 55 77

FIGURE 1: Amino acid sequence of synthetic peptides of human apoA-II prepared in the present study. The complete sequence of apoA-II has been reported by Brewer et al. (1972) and Lux et al. (1972).

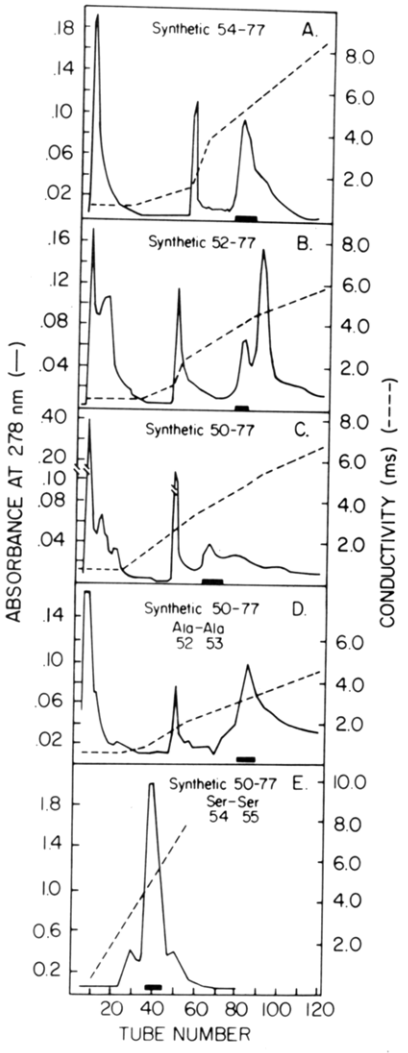

FIGURE 2: Purification of synthetic peptides of apoA-II on DEAE-cellulose. (A) Fragment 54–77; (B) fragment 52–77; (C) fragment 50–77; (D) fragment 50–77 substituted with alanine and alanine at residue 52 and 53; (E) fragment 50–77 substituted with serine and serine at residue 54 and 55. The peptides were eluted with nonlinear gradients of NaCl in a buffer of 0.02 M Tris-HCl and 6 M urea, pH 8.2. In panels A–D, the peak at tubes 5–10 was nonpeptide in nature, and the peak at tube 50 was a truncated peptide.

Other Methods. Circular dichroism (CD) was measured at 25 °C on a Cary 61 spectropolarimeter using cells of 0.5-mm path length. The percent α-helical content was estimated from the $[\theta_{222}]$ by the relation % α helix = $(\theta_{222} + 3000/36000 + 3000)$. Peptide concentrations were determined by amino acid analysis. Phosphorus was determined by the method of Bartlett (1959). Prediction of secondary structure of synthetic fragment was analyzed according to Chou & Fasman (1974, 1977, 1978).

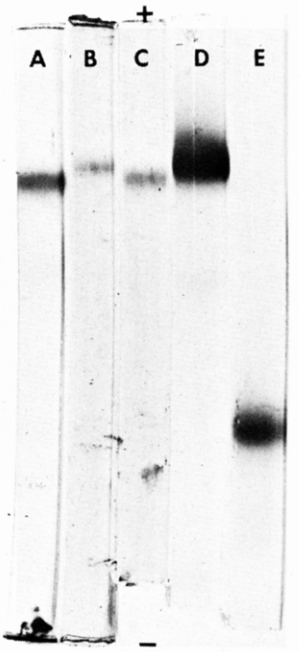

FIGURE 3: Polyacrylamide gel electrophoresis of synthetic fragments. The gels were run on 7.5% acrylamide in the presence of 8 M urea, pH 8.2. Each gel was loaded with 20 μg of peptide. (A) Fragment 54–77; (B) fragment 52–77; (C) fragment 50–77; (D) fragment 50–77 substituted with alanine and alanine at residues 52 and 53; (E) fragment 50–77 substituted with serine and serine at residues 54 and 55.

Table I: Amino Acid Compositions of Synthetic Fragments of ApoA-II[a]

amino acid	synthetic fragment 54–77	52–77	50–77	Ala-Ala substitution 50–77	Ser-Ser substitution 50–77
Asp	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)
Thr	2.9 (3)	2.5 (3)	3.4 (4)	3.7 (4)	3.5 (4)
Ser	0.9 (1)	0.9 (1)	0.9 (1)	1.0 (1)	2.7 (3)
Glu	4.3 (4)	4.7 (4)	4.8 (4)	4.1 (4)	4.2 (4)
Pro	1.1 (1)	1.0 (1)	1.9 (2)	2.0 (2)	2.1 (2)
Gly	2.1 (2)	2.1 (2)	2.0 (2)	2.0 (2)	2.0 (2)
Ala	2.0 (2)	2.1 (2)	2.0 (2)	4.2 (4)	2.0 (2)
Val	2.0 (2)	2.2 (2)	2.0 (2)	2.0 (2)	2.2 (2)
Ile		0.9 (1)	0.9 (1)		0.9 (1)
Leu	3.0 (3)	4.0 (4)	4.0 (4)	3.0 (3)	3.96 (4)
Tyr	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	0.9 (1)
Phe	2.0 (2)	2.2 (2)	2.0 (2)	2.0 (2)	2.1 (2)
Lys	1.9 (2)	2.3 (2)	2.0 (2)	2.0 (2)	
total	24	26	28	28	28

[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; the values in parentheses are the theoretical values.

Results

Preparation and Isolation of Synthetic Fragments of ApoA-II. Synthetic fragments corresponding to residues 54–77, 52–77, 50–77, and 50–77 with Ala-Ala and Ser-Ser substitutions (Figure 1) were prepared by solid-phase peptide synthesis methodologies. After peptides were removed from the resin, they were chromatographed on Bio-Gel P-10 to remove truncated peptides, and then the appropriate fractions were desalted on Bio-Gel P-2. The peptides were subjected to chromatography on DEAE-cellulose (Figure 2). After the appropriate fractions were pooled, the peptides were desalted on Bio-Gel P-2, and their purity was determined by poly-

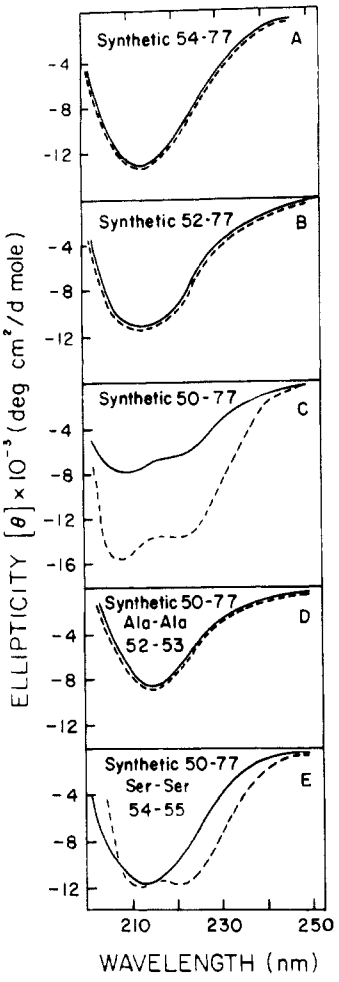

FIGURE 4: Circular dichroic spectra of fragment (—) and fragment/DMPC complexes (---). The spectra shown in panels A, B, and D are those of fragments alone or fragments to which DMPC was added in a ratio of 5:1 (w/w) of DMPC/fragment. The spectra shown in C and E (---) are those of the isolated complexes shown in Figures 5 and 6.

acrylamide gel electrophoresis (Figure 3) and by amino acid analysis (Table I). Each synthetic peptide gave a single major band by electrophoresis. In addition, the amino acid compositions of the fragments were in close agreement with the theoretical values.

Conformation and Phospholipid-Binding Properties of Synthetic Fragments. The secondary structure of the synthetic fragments was determined by circular dichroism; the spectra are shown in Figure 4. On the basis of the trough at 215 nm, the fragments corresponding to residues 52–77 and 54–77 and the Ala-Ala and Ser-Ser substitutions contained predominantly β structure. In contrast, the 50–77 fragment was disordered with a trough at 208 nm. After each of the synthetic fragments was incubated with DMPC, there was little or no change in the CD spectra for the 52–77 or 54–77 fragment or the Ala-Ala substituted fragment. However, the 50–77 fragment and Ser-Ser substituted fragment showed an increase in the α-helical structure with the addition of DMPC and in the isolated complex (Figure 4).

For isolation of the lipid–peptide complexes, the mixtures were subjected to density gradient ultracentrifugation in KBr. As shown in Figures 5 and 6, peptide–DMPC complexes were isolated between d 1.095 and 1.070 g/mL for the 50–77 fragment and the fragment containing the Ser-Ser substitution for Lys-Lys (Figure 1); the 54–77 and 52–77 fragments and Ala-Ala substituted fragment were not associated with lipid. DMPC alone was isolated at $d < 1.07$ g/mL. The lipid-

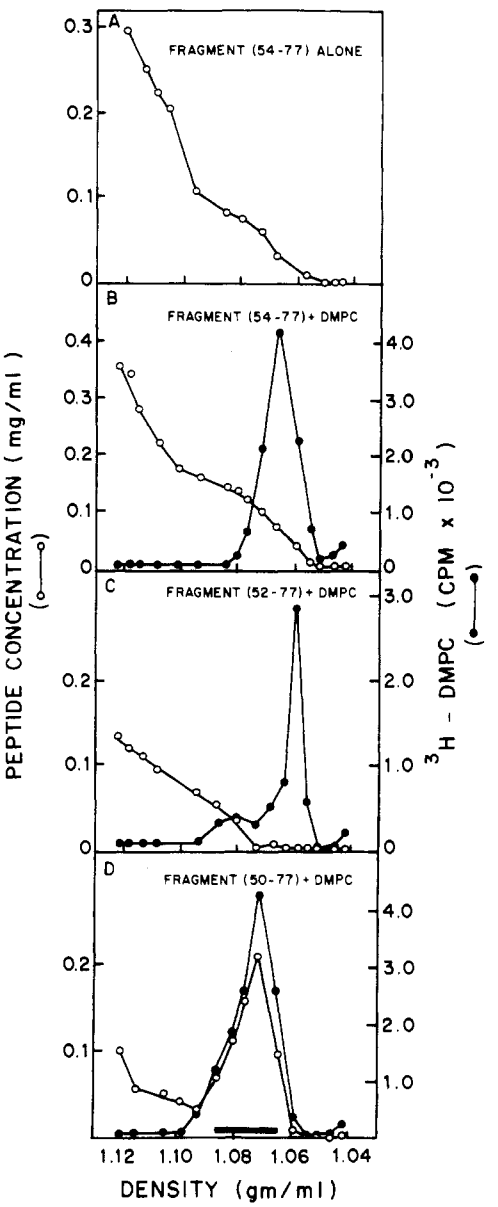

FIGURE 5: Ultracentrifugal behavior of complexes formed by synthetic fragments and DMPC. Panel A represents the 54–77 fragment (0.5 mg) alone. In panels B and D, 0.5 mg of the fragments in 1.42 mL of 0.01 M Tris-HCl, 0.1 M NaCl, 0.001 M NaN_3, and 0.001 M EDTA, pH 7.4, was added to 2.5 mg of DMPC vesicles (50 mg/mL). In panel C, 0.3 mg of the 52–77 fragment was added to 1.5 mg of DMPC. The mixture was incubated at 24.5 °C for 12 h and then centrifuged in a KBr density gradient between d 1.12 and 1.04 in a total volume of 4.8 mL. The bar represents those fractions which were pooled.

peptide complexes formed with the 50–77 fragment and the Ser-Ser substituted fragment were analyzed for conformation and composition. The molar ratio of DMPC to peptide was 41 and 37 for the 50–77 fragment and Ser-Ser substitution, respectively. In addition, the isolated complexes showed an increase in ellipicity at 222 nm (Figure 4). The calculated α helicity of the 50–77 fragment increased from 17% in the absence of lipid to 41% in the isolated lipid–peptide complex. The Ser-Ser substituted fragment contained 38% α-helical structure in the isolated complex.

Discussion

In a previous report (Mao et al., 1977), it was shown that a synthetic peptide corresponding to residues 47–77 in apoA-II formed a lipid–peptide complex with phospholipid vesicles while the 56–77 fragment did not interact with lipid. To

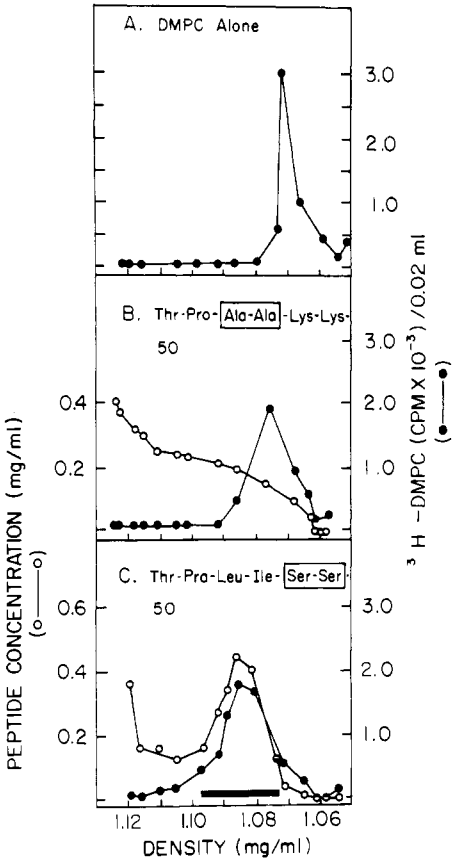

FIGURE 6: Ultracentrifugal behavior of complexes formed by the substituted 50–77 fragments and DMPC. (A) DMPC alone; (B) fragment 50–77 substituted with alanine and alanine at residue 52 and 53; (C) fragment 50–77 substituted with serine and serine at residue 54 and 55. One milligram of each peptide was added to 5 mg of DMPC (50 mg/mL). Experimental conditions were the same as described in Figure 5.

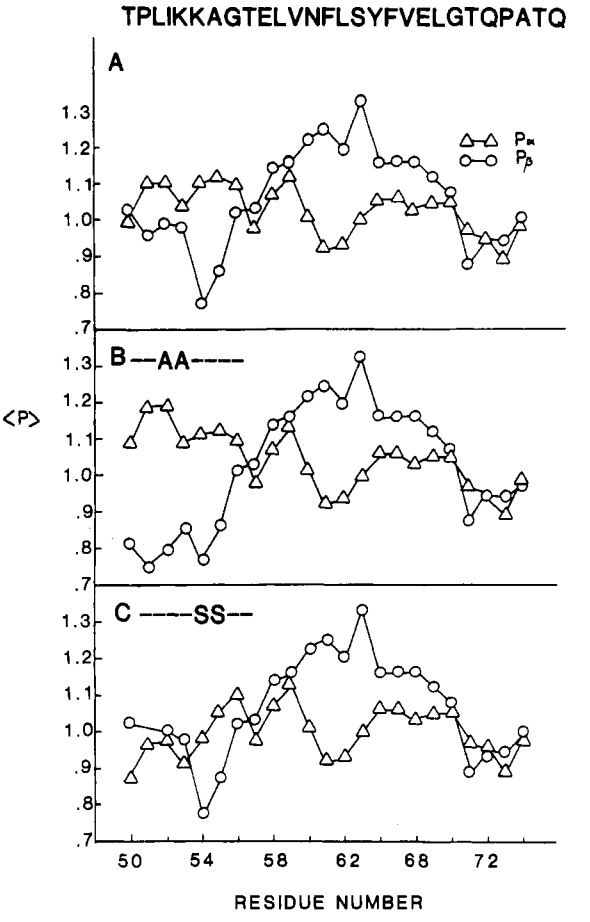

FIGURE 7: Computer plot of predicted secondary structure of apoA-II fragments. The program used the parameters of Chou & Fasman (1978). (Panel A) Fragment 50–77. The prediction indicates that residues 51–60 are potentially α helix but contain no nucleation site, 61–69 are β sheet, and 71–73 is a potential β turn. (Panel B) Fragment 50–77 substituted with Ala-Ala. There is an increase in helical probability between 51 and 60 and a decrease in β-sheet probability. (Panel C) Fragment 50–77 substituted with Ser-Ser. A decrease in helical probability occurs with an increase in β-sheet potential between 50 and 53.

further delineate the minimal sequence between residues 47 and 56 that is required for lipid binding, we have extended the 56–77 fragment, prepared peptides corresponding to residues 54–77, 52–77, and 50–77 (Figure 1), and tested each of these fragments for their ability to bind lipid. By the criteria used in this study, only the 50–77 fragment interacts with phospholipid. The 50–77 fragment is the only one of the three fragments which exhibits disordered structure in the absence of lipid. Fragments 54–77 and 52–77 contain primarily β structure; β structure is observed even in the presence of 2-chloroethanol (data not shown). In the presence of phospholipid, only the 50–77 fragment showed a marked increase in the α-helicity from 17% to 41% (Figure 4).

The addition of the Thr-Pro-Leu-Ile-Lys-Lys sequence to the 56–77 fragment adds two additional hydrophobic amino acids (Leu-Ile) and provides the necessary amino acid sequence for binding. The mean residues hydrophobicity increases from −951 to −995 cal/residue. In this regard, it is of interest that Sparrow et al. (1977) have shown with model amphipathic helical peptides that a peptide of ~20 residues with a mean residue hydrophobicity of >−900 cal/residue is required in order for the peptide to interact with phospholipid.

On the basis of calculations as described by Chou & Fasman (1974, 1977, 1978), it is predicted that the 50–77 region of apoA-II is primarily β structure from 61 to 69 (Figure 7); there is low helical probability from 51 to 60 but no clear nucleation site. From the CD spectrum of the peptide in the presence of phospholipid, we estimate that the phospholipid binding site contains 11 residues in an α helix, and we assume Pro-51 is the first residue in the helix. From the illustration in Figure

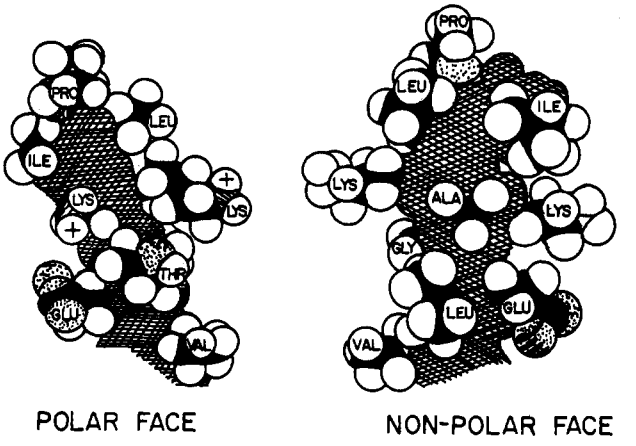

FIGURE 8: Drawing of a CPK space-filling model of residues Pro_{51}–Val_{61} in apoA-II. A right-handed α-helical backbone was constructed with 3.6 residues/turn. The amino acid residues were added onto the α carbon in their proper order. The polar and nonpolar faces of the segment are shown.

8 of a CPK space-filling model, we see that residues 51–61 form a typical amphipathic helix (Segrest et al. 1974), with one side of the helix being hydrophobic and the other side hydrophilic. The presence of the two Lys residues on the polar face and exposed to the aqueous environment is consistent with

the recent cross-linking studies of Stoffel & Preissner (1979).

To determine the importance of the hydrophobicity of Leu-Ile to the lipid-binding properties of this region of apoA-II, we substituted Ala-Ala for Leu_{52}–Ile_{53}. As a result of this substitution, the mean residue hydrophobicity is lowered to −840 cal/residue, and there is an increase in the calculated Chou–Fasman helical probability (Figure 7B). In the CD spectrum of the Ala-Ala substituted fragment, a reduced amount of β structure is observed, but the structure evident in the CD spectrum of the 50–77 fragment is absent. No spectral changes are observed in the presence of lipid (Figure 4), and no complex is isolated (Figure 6). The inability of the Ala-Ala substituted peptide to bind phospholipid strongly suggests that the Leu-Ile hydrophobic center on the nonpolar face has an important role in lipid–protein interaction. The observed spectral differences between peptides in the absence of phospholipid may occur as a result of the aggregation of the hydrophobic 50–77 fragment with a resultant increase in ellipticity, as has been reported for the apolipoproteins (Osborne et al. 1975; Osborne & Brewer, 1977).

To assess the importance of ionic interaction in phospholipid binding, we substituted Ser-Ser for Lys_{54}–Lys_{55}. This substitution slightly increases the predicted β structure (Figure 7C) which is observed in the CD spectrum of the peptide alone. However, in the presence of phospholipid, an increase in α-helical content to ~38% occurs, and a complex is isolated. The fact that the Ser-Ser substitution for Lys-Lys does not affect the lipid-binding properties of the peptide suggests that ionic interactions between the lysine residues in the peptide and the polar head group of the phosphatidylcholine do not play an important role in lipid binding. However, due to the complexity of the CD spectrum, we cannot exclude the possibility that another mechanism of binding other than the formation of an amphipathic helix is occurring.

Acknowledgments

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