APOLIPOPROTEIN/LIPID INTERACTIONS: STUDIES WITH SYNTHETIC POLYPEPTIDES

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I. INTRODUCTION

The lipoproteins are the major carriers of triglycerides, cholesterol, cholesteryl esters, and phospholipids in human plasma.[1-5] Traditionally, they have been separated by ultracentrifugation into four density classes which are termed chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL). Each lipoprotein density class contains different ratios of lipid and protein (Table 1) and can be subfractionated by ultracentrifugation or column chromatography into discrete particles with more or less constant composition. The incidence of coronary heart disease has been positively correlated with high levels of LDL cholesterol and negatively correlated with high levels of HDL cholesterol. The proteins and phospholipids are thought to form a monolayer on the surface of the particle surrounding a core of neutral lipids. The apolipoproteins (the delipidated lipoproteins) can be separated by molecular exclusion and/or ion exchange chromatography; the individual apolipoproteins interact with lipid to form lipid-protein complexes.[1-5] Several of the apolipoproteins are known to influence lipid degradation and synthesis. Apolipoprotein C-II activates lipoprotein lipase (LPL) to hydrolyze the triglycerides in chylomicrons and VLDL.[6,7] Apolipoproteins A-I and C-I are thought to activate lecithin:cholesterol acyltransferase (LCAT) which catalyzes the synthesis of cholesteryl esters.[8,9] LDL have been extensively investigated by Brown and Goldstein,[10] who have shown that LDL interact with cell-surface receptors for the control of cholesterol synthesis. Lipolysis experiments in vitro indicate that the C-apolipoproteins transfer with some phospholipid and cholesterol to the HDL density class suggesting a highly dynamic system of protein and lipid exchange.[11]

Many laboratories have studied the changes that occur in the apolipoprotein and the lipid upon complex formation using a wide variety of techniques.[1-5] In the presence of phospholipid, the apolipoproteins increase their α-helical structure as measured by increases in ellipticity at 208 and 222 nm in the circular dichroic spectrum. Upon binding to phospholipid, a shift occurs in the intrinsic tryptophan fluorescence of the apolipoprotein from ~350 to ~335 nm, indicating a transfer to a more hydrophobic environment. A decrease in lipid turbidity also occurs as complexes are formed. Density-gradient ultracentrifugation or column chromatography have been used to isolate the lipid-protein complexes. These experimental results have been interpreted to mean that

Table 1
COMPOSITION OF HUMAN PLASMA LIPOPROTEIN

	Chylomicrons	VLDL	LDL	HDL
Density range	<.95	0.95—1.006	1.006—1.063	1.063—1.210
Major lipids	Exogenous triglycerides	Endogenous triglycerides	Cholesterol Cholesteryl esters	Phospholipids Cholesteryl esters
Protein (%)	2	10	25	50
Major apoproteins	ApoA-I[a] ApoB ApoC	ApoB ApoC-I[a] ApoC-II[b] ApoC-III ApoE	ApoB	ApoA-I[a] ApoA-II
Minor proteins	ApoA-II ApoE[c] PRP[d]	ApoA-I[a] ApoA-II ApoD[e]	ApoC	ApoC-I[a] ApoC-II[b] ApoC-III ApoD[e] ApoE[c]

[a] Activator of lecithin:cholesterol acyltransferase.
[b] Cofactor for lipoprotein lipase.
[c] Also termed arginine-rich protein (ARP).
[d] Proline-rich protein.
[e] Also termed thin-line protein and apoA-III.

the apolipoproteins form α-helical regions with their tryptophan residues inserted into hydrophobic regions of the lipid and that the lipid dispersions have been reorganized into discrete particles.

In an attempt to explain the association of the apolipoproteins with phospholipids, Segrest et al.[12] proposed that the apolipoproteins form a unique structure which allows them to interact with phospholipids. Using CPK models of predicted helical regions of the apolipoproteins, these authors concluded that parts of the apolipoprotein sequence contain an α-helix with two distinct faces, which they call amphipathic. The polar face (Figure 1) of this helix contains the acidic residues near the center which are frequently paired with basic residues at the edge. The nonpolar face (Figure 1) contains the hydrophobic residues with frequent pairings of aromatic amino acids. The authors postulate that there may be an ionic interaction between the pairings of acidic and basic residues and the zwitterionic polar head group of phospholipids. They also propose that the hydrophobic surface of the protein can interact strongly with the fatty acyl chains of phospholipids. With this unique arrangement, the apolipoprotein can present a polar surface to the aqueous environment of the polar head groups of phospholipids and the plasma, and a nonpolar surface to the hydrophobic fatty acyl chains of phospholipids. Evidence from several laboratories supports the role of hydrophobic interactions in complex formation.[13-16] However, to date, there is no evidence for ionic interactions between the paired acidic and basic residues and the zwitterionic polar head group of phospholipids.

Several portions of these proteins have been synthesized to gain insight into the protein structural requirements for the apolipoprotein-lipid and apolipoprotein-enzyme interactions that occur in this family of important plasma proteins. These studies constitute the subject of this review.

II. APOLIPOPROTEIN C-III

Apolipoprotein C-III (apoC-III) is isolated from delipidated chylomicrons and VLDL by gel permeation and ion exchange chromatography.[17] This apolipoprotein occurs in

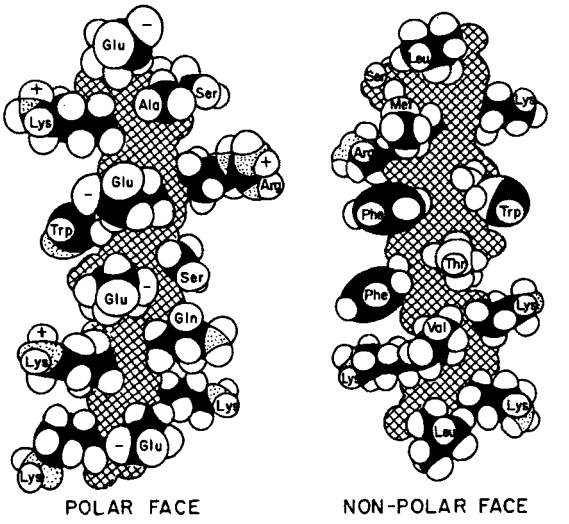

FIGURE 1. Artist illustration of a CPK model of the amphipathic helical segment of apolipoprotein C-I between residues 32 and 53. The acidic residues are at the center of the polar face paired with the basic residues at the edge. The hydrophobic residues form the nonpolar face.

at least three forms which differ only in the content of sialic acid. The amino acid sequence has been reported by Brewer et al.;[18] apoC-III contains 79 residues with a carbohydrate chain attached to Thr_{74} (Figure 2). Conformational analysis by the technique of Chou and Fasman[19] indicates α-helix between residues 1 to 39 and 54 to 69; there are probable β-turns at 39 to 42 and 72 to 75. Segrest et al.[12] have proposed that residues 40 to 67 form an amphipathic helix and therefore contain the phospholipid-binding region of apoC-III. The interaction of apoC-III with phospholipid has been studied extensively by Morrisett et al.[20-22] who have reported that the α-helicity of the protein increases from 26 to 70% and that the intrinsic tryptophan fluorescence is shifted from 350 to 337 nm upon binding to phosphatidylcholine vesicles. Complexes with a molar ratio of phospholipid to protein of 40 to 1 have been isolated by density-gradient ultracentrifugation.

Sparrow et al.[23] have reported that thrombin cleavage of apoC-III at $Arg_{40}Gly_{41}$ produces two peptides which are separable by DEAE-cellulose chromatography. Upon mixing with phospholipid, apoC-III(41 to 79*) increases it α-helical content from 13 to 38%; apoC-III(1 to 40) shows no changes in the circular dichroic (CD) spectrum upon the addition of phospholipid. The tryptophan fluorescence maximum of apoC-III(41 to 79) shifts from 352 to 340 nm. With dimyristoylphosphatidylcholine (DMPC), a complex of 12 to 1 has been isolated by density-gradient ultracentrifugation; no lipid-protein complex is isolated with apoC-III(1 to 40). The value for the enthalpy of association of complex formation, as measured with batch microcalorimetry, is relatively low (−35 kcal/mol) for apoC-III(1 to 40) and DMPC. This finding supports the lack of binding of apoC-III(1 to 40). The high enthalpy (−170 kcal/mol) of association of apoC-III(41 to 79) with DMPC is consistent with the binding of this peptide; apoC-III

* This indicates the beginning and end of the amino acid sequence. The word "to" may easily be replaced by a dash or hyphen.

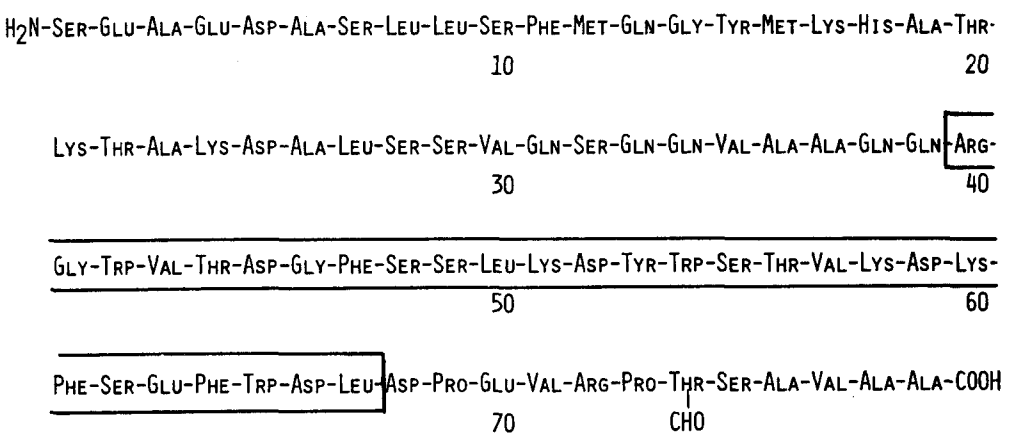

FIGURE 2. The amino acid sequence of apolipoprotein C-III (apoC-III) reported by Brewer et al.[18] There is a carbohydrate chain attached to Thr_{74}. The amphipathic helix proposed by Segrest et al.[12] is within the box.

has an enthalpy of association of −240 kcal/mol. The authors conclude that residues 41 to 79 contain the phospholipid-binding site of apoC-III.

Since both peptides 1 to 40 and 41 to 79 contain potential amphipathic helices, Sparrow et al.[23] has calculated the mean residue hydrophobicity for these fragments of apoC-III in an attempt to explain the differences in binding. Using the hydrophobicity scale of Bull and Breese,[24] the calculated hydrophobicity index for apoC-III(1 to 40) is −609 cal per residue and for apoC-III(41 to 79) is −893 cal per residue. The calculated hydrophobicity index of the proposed binding region, i.e., apoC-III(41 to 67), is −1030 cal per residue. These differences suggest that the hydrophobicity of the amphipathic segment plays an important role in the association of the peptide with the phospholipid matrix.

To localize the phospholipid-binding region, Sparrow et al.[25] have synthesized four peptide fragments from the carboxyl half of apoC-III using Merrifield solid-phase methodology.[26] These synthetic peptides include residues 61 to 79, 55 to 79, 48 to 79, and 41 to 79. Hydrogen fluoride is used to cleave the peptide from the solid support and ethanedithiol has been used to prevent the oxidation of tryptophan during acidolysis and HF cleavage.[27] Purification of the peptides on DEAE-cellulose in the presence of urea under basic conditions at room temperature probably lowered the yields of peptide since urea is known to carbamylate the apolipoproteins under these conditions.[28] The peptides migrate as a single band on polyacrylamide gels at pH 8.2 and the amino acid analyses are consistent with the theoretical values.

Phospholipid binding by the synthetic peptides has been assessed in the following ways:

1. By monitoring fluorescence[29] and CD spectral changes in the presence and absence of phosphatidylcholine (PC).
2. By attempting to isolate a complex by ultracentrifugal flotation at d = 1.064 g/mℓ.
3. By the inhibition of reactivation of β-hydroxybutyrate apodehydrogenase.

In the presence of phospholipid, the tryptophan fluorescence spectrum of apoC-III(48 to 79) and (41 to 79) shifts from 350 to 344 and 338 nm, respectively; no changes are observed in the spectrum of the two shorter peptides. The CD spectrum of apoC-III(61 to 79) and (55 to 79) does not change in the presence of phosphatidylcholine; the peptides appear to be disordered. In the presence of PC, apoC-III(48 to 79) has a CD spectrum

with no distinct trough at 222 nm, whereas apoC-III(41 to 79) has a CD spectrum with a trough at 208 and 222 nm which has been attributed to α-helical structure in proteins;[30] the α-helicity calculated from the CD spectrum of the lipid-peptide mixture is 23%. The CD spectrum of apoC-III(41 to 79) in the isolated peptide-lipid complex has an α-helicity of 35% which compares favorably with that of 38% determined in the lipid-peptide complex formed by the equivalent native peptide isolated after thrombin cleavage of apoC-III.[23]

Ultracentrifugal flotation from the bottom of the tube results in the isolation of lipid-peptide complexes at the top of the tube with apoC-III(48 to 79) and (41 to 79); about 85% of the 41 to 79 peptide is found associated with the phospholipid. However, this technique does not permit the separation of the lipid-peptide complexes from phospholipid vesicles. Inhibition of reactivation of β-hydroxybutyrate apodehydrogenase indicates that apoC-III(48 to 79) binds phospholipid to a lesser extent than apoC-III(41 to 79); apoC-III(55 to 79) and (61 to 79) do not bind. The specificity of this indirect enzyme assay is unknown. The authors suggest that apoC-III(48 to 79) contains the minimal sequence of apoC-III for phospholipid binding.[25]

If residues 40 to 67 of apoC-III form an amphipathic helix as proposed by Segrest et al.,[12] an increase in the helicity of apoC-III(48 to 79) and (41 to 79) to approximately 62% and 70%, respectively, would be required; the observed helicity of the lipid-apoC-III(41 to 79) complex is ~38%. This discrepancy, between the observed helicity of the native and synthetic peptides and the calculated value from the number of residues in the proposed binding region, has not been addressed, but probably indicates that fewer residues are involved in the amphipathic helix than originally thought by Segrest et al.

In addition to the phospholipid binding, the immunoreactivity of the synthetic peptides with anti-apoC-III antibody has been investigated using Ouchterlony diffusion plates.[25] A precipitin line of identity is formed between apoC-III(41 to 79) and apoC-III, indicating that a major antigenic determinant resides in this region of apoC-III. Mao et al.[31] have since reported on the antigenicity of the native fragments isolated from thrombin cleavage at $Arg_{40}Gly_{41}$, and have corroborated by radioimmunoassay that apoC-III(41 to 79) contains a major antigenic reactive site of apoC-III.

III. APOLIPOPROTEIN C-II

Apolipoprotein C-II (apoC-II) is an important apolipoprotein also isolated from VLDL and has been shown to bind phospholipids and to activate LPL for the hydrolysis of chylomicron and VLDL triglycerides.[6,7] The helicity of apoC-II increases in the presence of phospholipids from 35% to 59%.[32,33] ApoC-II forms an equimolar complex with LPL with reported dissociation constants of $10^{-10}M$[34] and $10^{-8}M$;[35] the association is reportedly disrupted by high concentrations of chloride.[36] The sequence of apoC-II contains 78 residues (Figure 3).[37] Chou-Fasman analysis[19] of the amino acid sequence predicts a high probability of helices forming between residues 13 to 22, 28 to 39, and 42 to 51.[32] CPK models indicate that the helices are amphipathic and therefore are potential phospholipid-binding regions. Chou-Fasman analysis also indicates a high probability of β-turns at residues 9 to 12, 23 to 26, and 52 to 55, while a β-sheet region is predicted from 60 to 74. The cyanogen bromide peptides, apoC-II(1 to 9) and (10 to 59), do not activate lipoprotein lipase;[38] however, a third peptide, apoC-II(60 to 78), gives a 3.9-fold stimulation at 4.8 μM. Tryptic cleavage at Lys_{75} reduces the activity to near zero, as does acetylation.[39]

To characterize the LPL activation region, fragments of apoC-II have been synthesized by solid-phase techniques[38,40] on an improved polystyrene support[41] containing a spacer chain and a more stable resin linkage. Each peptide has been purified

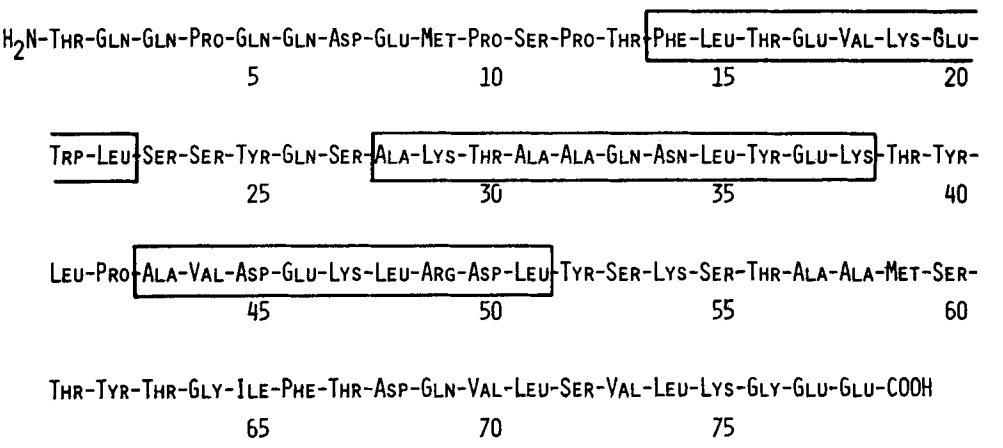

FIGURE 3. The sequence of apolipoprotein C-II (apoC-II) according to Jackson et al.[37] Amphipathic helices are proposed between 13 to 22, 28 to 39 and 42 to 51.

by ion exchange chromatography and characterized by a single band on polyacrylamide[38] and isoelectric focusing gels.[40] The amino acid composition is in good agreement with the expected values.

ApoC-II(55 to 78) activates LPL to 90% of the level of apoC-II in a gum arabic stabilized triolein emulsion.[38] In addition, apoC-II(60 to 78) gives a 2.5-fold activation of LPL compared to 3.9-fold by the corresponding cyanogen bromide peptide (60 to 78) from apoC-II; apoC-II(60 to 75) and (66 to 78) do not activate. Kinnunen et al. conclude that residues 55 to 78 contain the site of interaction of apoC-II with LPL. Since the addition of five residues to apoC-II(60 to 78) greatly increases the activity, it has been postulated that residues 55 to 65 are involved in the activation of the enzyme. Furthermore, the fact that tryptic digestion of apoC-II(60 to 78) greatly reduces the activity raises the possibility that there may be an ionic interaction between the COOH-terminal glutamic acid residues and the enzyme. From the amphipathic nature of helical segments between 10 and 49, Kinnunen et al. suggest that this region is involved in binding to the phospholipid in the chylomicron and VLDL particles. In support of this suggestion, Smith et al.[42] have reported that the activation of LPL by synthetic peptides in a phospholipid-stabilized triolein emulsion is dependent on phospholipid binding. Activation by apoC-II(55 to 78) is 20% of that of apoC-II and apoC-II(50 to 78) has 50% of the activity, while apoC-II(43 to 78) has 100% of the activity of apoC-II. These results suggest that the phospholipid-binding region plays an essential role in activation.

Catapano et al.[40] have observed that synthetic peptides of apoC-II enhance lipolysis of VLDL from a patient deficient in apoC-II. Normal rates of ester hydrolysis are achieved in apoC-II deficient VLDL regardless of which of the three activating peptides, apoC-II(55 to 78), (50 to 78), or (43 to 78) is added; at 0.5 μM concentration, sevenfold activation of LPL is achieved. The authors conclude that high affinity binding to the VLDL is not required for triglyceride hydrolysis since the synthetic peptides differ greatly in binding, but have similar rates of fatty acid release. Using ^{125}I-labeled peptides, these authors report that only apoC-II and apoC-II(43 to 78) are able to bind to apoC-II deficient VLDL.

To investigate the phospholipid binding, Sparrow et al.[43-44] have studied several synthetic fragments of apoC-II by CD spectroscopy and density-gradient ultracentrifugation. ApoC-II(50 to 78) and shorter peptides do not bind to DMPC by density-gradient ultracentrifugation. However, the addition of seven residues, i.e., apoC-II(43

to 78), produces an amphipathic peptide which does bind lipid; a lipid-peptide complex with a molar ratio of 36:1 can be isolated at d 1.08 g/mℓ. A helical content of approximately 30% is estimated from the CD spectrum of the isolated complex. This value correlates well with residues 43 to 51 forming an amphipathic helix. All of the synthetic peptides of apoC-II are hydrophobic (hydrophobicity index >850 cal per residues), but those lacking an amphipathic segment do not bind lipid, showing the importance of this unique structural feature to apolipoprotein-phospholipid complex formation. These results are the first report of separate functional domains in an apolipoprotein, i.e., LPL activation is located in residues 55 to 78 of apoC-II and phospholipid binding occurs in residues 43 to 51.

Smith et al.[45] recently postulate that the potential β-sheet between 63 and 74 and a weak β-turn at 60 to 63 are important to LPL activation. In order to investigate the importance of the β-turn and Tyr_{62} to activation, three peptides have been synthesized with Trp, Phe, and Gly substituted for Tyr_{62} in apoC-II(55 to 78). The substitution of Phe and Trp decreases the calculated probability of a β-turn between 60 to 63 and can therefore drastically modify the protein secondary structure. However, the peptide with Gly at residue 62 has an enhanced β-turn probability and therefore has been synthesized to serve as a control. The three peptides have only 33% of the activation of the native sequence. These results suggest that the presence of an aromatic residue is not sufficient for activation but that the hydroxyl group of Tyr_{62} is important.

To investigate the importance of the β-sheet between 63 and 74, several peptides have been synthesized which delete residues 69 to 78 of apoC-II. ApoC-II(50 to 68), (43 to 68), and (35 to 68) activate LPL 50, 100, and 100%, respectively, of apoC-II; apoC-II(60 to 68) and (55 to 68) were inactive.[45] To explain these results, Smith et al. suggest that once the peptide is bound to substrate it can still activate by concentrating the apolipoprotein at the interface with LPL. Additional investigation is needed to substantiate this hypothesis. However, Bengtsson and Olivecrona[46] have recently demonstrated that LPL does bind to lipid substrates and suggest the activator orientates the enzyme and/or lipid for effective hydrolysis.

IV. APOLIPOPROTEIN C-I

Apolipoprotein C-I (apoC-I) is a small, structurally interesting peptide which is isolated from the apoC peptides of VLDL by DEAE-Sephadex® chromatography[17] and is obtained in purest form by rechromatography on SP-Sephadex® at pH 3.7 in 6 *M* urea.[47] ApoC-I contains 57 residues[48,49] (Figure 4) and in addition to binding phospholipid,[50] it activates LCAT which catalyzes the esterification of cholesterol.[9] Jackson et al.[50] have shown that the α-helical content of intact apoC-I increases from 56 to 73% upon the addition of egg yolk phosphatidylcholine; a concomitant blue-shift of 6 nm occurs in the tryptophan fluorescence spectrum. Chou-Fasman analysis predicts the protein to be highly helical, and Segrest et al.[12] propose that residues 7 to 14, 18 to 20, and 33 to 53 (Figure 1) contain the lipid-binding regions of apoC-I. ApoC-I(1 to 38), a cyanogen bromide fragment, has an increase in helicity from 35 to 65% in the presence of PC; apoC-I(39 to 57) changes from 15 to 25% and the fluorescence spectrum shifts 3 nm. Complexes may be isolated by density-gradient ultracentrifugation. These results confirm that apoC-I(1 to 38) contains a phospholipid-binding site while apoC-I(39 to 57) appears to bind weakly;[50] neither peptide activates LCAT.

Recently, Chen et al.[16] have described the nitroxyl and ^{13}C labeling of apoC-I at Met_{38} and reported the effect of DMPC binding. Upon binding DMPC, the rotational correlation time (T_c) in the EPR increases from .22 to .35 nsec; the spin-lattice relaxation time (T_1) in the ^{13}C-NMR decreases to 380 msec and the line width (υ ½) increases to

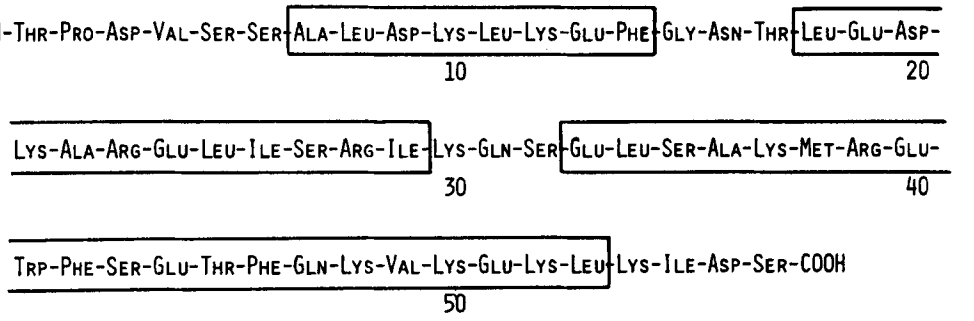

FIGURE 4. The amino acid sequence of apolipoprotein C-I (apoC-I) as reported by Shulman et al.[49] and confirmed by Jackson et al.[48] Segrest et al.[12] proposed residues 7 to 14, 18 to 20, and 32 to 53 form amphipathic helices.

4.7 Hz. These results suggest that Met_{38} is inserted into the fatty acyl chain region of DMPC with a reduction in its rotational freedom.

Harding et al.[51] and Sigler et al.[52] have independently published the complete solid-phase synthesis of apoC-I. The purified synthetic protein of both groups has the predicted amino acid composition. Harding et al.[51] find that the tryptic map of their material is in good agreement with that of the natural protein. Sigler et al.[52] have shown that their material is a single band on polyacrylamide gels at acidic and basic pH. Both groups find that the synthetic apoC-I activates LCAT. Sigler et al. have also investigated the phospholipid binding by their synthetic material. The α-helical content increases from 30 to 40% in the presence of phospholipid and a shift in the tryptophan fluorescence maximum occurs from 344 to 339 nm. A complex with a molar ratio of phospholipid to protein of 23 to 1 was isolated by density-gradient ultracentrifugation; a complex of 19:1 is isolated with the native protein.

Sparrow et al.[53] and Soutar et al.[54] have investigated the activation of LCAT and the phospholipid binding of shorter fragments of apoC-I. After purification by chromatography on SP-Sephadex®, each peptide migrates as a single band on polyacrylamide gels at acidic and basic pH; the amino acid composition is in good agreement with the expected values. ApoC-I(17 to 57) is as active as apoC-I in LCAT activation while apoC-I(24 to 57) and (32 to 57) have approximately 50% of the activity of apoC-I; apoC-I(39 to 57) is inactive. Therefore, the authors believe that apoC-I(17 to 57) contains all the necessary protein structural requirements for LCAT activation. ApoC-I(32 to 57), (24 to 57) and (17 to 57) display a 12 nm shift in the tryptophan fluorescence spectrum in the presence of DMPC; apoC-I(39 to 57) has no shift. The helicity of the three longer peptides increases from 18 to 35% for (32 to 57) and (24 to 57) and from 27 to 45% for apoC-I(17 to 57). The binding of apoC-I(32 to 57) and longer peptides to phospholipid has been confirmed by density-gradient ultracentrifugation; apoC-I(39 to 57) does not form a complex. The isolated complexes contain 30 mol of lipid per mole of peptide. These results verify that apoC-I(32 to 57) contains one of the lipid binding regions predicted by Segrest et al.[12] However, the α-helical content does not agree between that predicted by the model and that observed in the synthetic peptides.

V. APOLIPOPROTEIN A-II

Apolipoprotein A-II is isolated from delipidated HDL and is a dimer of 77 residues per chain (Figure 5).[55] Chou-Fasman analysis[19] predicts residues 24 to 30, 42 to 47, and 51 to 58 (weak) to be α-helical; 10 to 20 and 60 to 70 to be β-sheet; and 4 to 7, 20 to 23, and 31 to

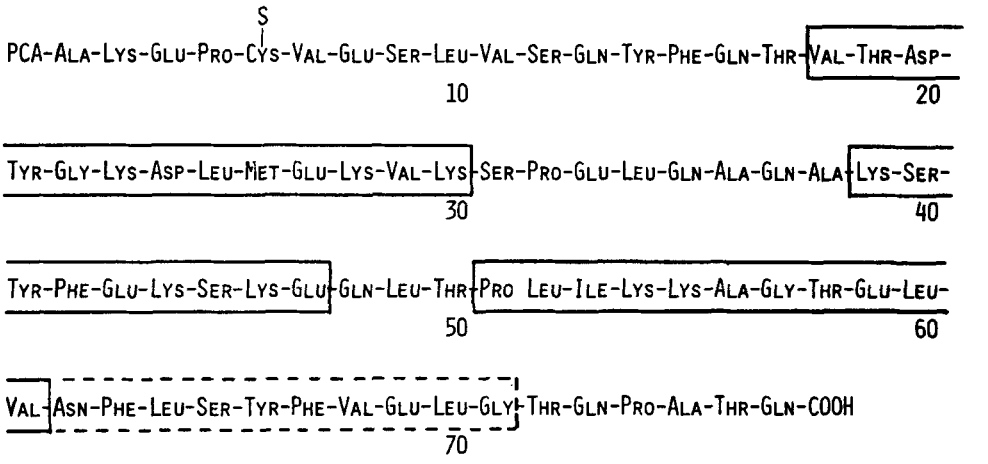

FIGURE 5. Lux et al.[55] reported the amino acid sequence of apolipoprotein A-II (apoA-II). Residues 17 to 30 and 39 to 47 have been proposed as amphipathic helices.[12] Residues 51 to 70 can be placed in an amphipathic helix,[59] but C.D. measurements[60] of lipid-peptide complexes and Chow-Fasman analysis suggest only residues 51 to 60 are helical.

34 to be β-turns. Segrest et al.[12] have predicted that residues 17 to 30 and 39 to 47 bind phospholipid. Jackson et al.[56] have reported that upon association with phospholipid apoA-II increases its α-helical content from 49 to 64% and that a complex isolated by density-gradient ultracentrifugation has a molar ratio of 24 to 1. Residues 1 to 26 of apoA-II do not interact with phospholipid, while residues 27 to 77 form lipid-peptide complexes with a ratio of 15 to 1. In the presence of phospholipid, the helicity of apoA-II(27 to 77) increases from 18 to 38% while that of apoA-II(1 to 26) does not change.

In a search for the lipid-binding region between residues 17 and 31, Chen et al.[57] have synthesized by solid-phase methodology, three peptides from the amino terminal of apoA-II. After purification by SP-Sephadex® chromatography, the peptides, apoA-II(17 to 31), (12 to 31), and (7 to 31), have the expected amino acid composition and are single bands on polyacrylamide gels at basic pH. ApoA-II(7 to 31) is obtained in 17.5% yield based on the first residue incorporated onto the resin. Kroon and Kaiser[58] have reported the synthesis of apoA-II(22 to 31). Their purified material is a single spot in three TLC solvent systems. The peptide has the expected amino acid composition, amino terminal, and correct sequence by Edman degradation with no preview. The CD spectrum in chloroethanol is indicative of a predominantly random structure.

Chen et al,[57] have reported that synthetic apoA-II(17 to 31) does not bind phospholipid; likewise, Kroon et al.[58] find no binding by apoA-II(22 to 31). However, Chen et al.[57] have shown that the addition of five residues to apoA-II(17 to 31) produces a peptide, apoA-II(12 to 31), that binds phospholipid. This peptide has a helicity of 20% in a complex with a phospholipid to protein ratio of 14 to 1. The phospholipid binding by this peptide confirms the location of a binding region in the amino terminal of apoA-II as proposed by Segrest et al.[12] They have also isolated a peptide-lipid complex with apoA-II(7 to 31). ApoA-II(7 to 31) contains 29% α-helix in the complex which consists of 27 mol of phospholipid per mole of peptide. The observed α-helical content of apoA-II(12 to 31) and (7 to 31) is much less than that calculated for residues 17 to 30 being an amphipathic helix as predicted by Segrest et al.[12] However, the value is consistent with residues 24 to 30 being in an α-helix as proposed by Chou-Fasman analysis.

Mao et al.[59] have investigated the lipid binding of the carboxyl terminal region of apoA-II by synthesizing apoA-II(65 to 77), (56 to 77), (47 to 77), and (40 to 77). These

peptides, prepared by solid phase and purified by DEAE-cellulose chromatography, have the expected amino acid composition and migrate as a single band on polyacrylamide gels at pH 8.2. Upon the addition of DMPC, apoA-II(47 to 77) and (40 to 70) exhibit increases in α-helicity from 25 to 48% and 23 to 48%, respectively; apoA-II(65 to 77 and 56 to 77) remain disordered. The 47 to 77 and 40 to 77 peptides form complexes with molar ratios of lipid to protein of 25 and 47:1, respectively; the 65 to 77 and 56 to 77 peptides do not bind phospholipid. These authors conclude that residues 47 to 77 contain a third lipid-binding region of apoA-II, one not predicted by Segrest et al.[12] They speculate that an amphipathic helix can be formed between residues 50 and 70 of apoA-II.

Subsequently, Mao et al.[60] have described the solid-phase synthesis of apoA-II fragments between 56 to 77 and 47 to 77, i.e., apoA-II(54 to 77), (52 to 77), and (50 to 77). They report that apoA-II(50 to 77) displays an increased ellipticity at 222 nm in the presence of phospholipid, calculated as 41% α-helicity; the spectra of the other peptides are unchanged. A lipid-peptide complex with apoA-II(50 to 77) has been isolated by density-gradient ultracentrifugation with a molar ratio of 41:1. It appears, therefore, that apoA-II(50 to 77) is the minimal sequence of apoA-II which will bind phospholipid since apoA-II(52 to 77) and (54 to 77) do not. An amphipathic helical structure is predicted between residues 51 to 60 of apoA-II. The α-helicity estimated from Chou-Fasman analysis[19] agrees well with that observed in the lipid-peptide complex.

These authors[60] have also investigated the importance of hydrophobic and charged residues for phospholipid binding by synthesizing two apoA-II(50 to 77) peptides containing amino acid substitutions; one has AlaAla substituted for $Leu_{52}Ile_{53}$ and another with SerSer substituted for $Lys_{54}Lys_{55}$. These peptides are purified by DEAE-Sephadex® and exhibit single bands on polyacrylamide gels. The SerSer peptide has a helicity of approximately 38% in the complex; the AlaAla peptide is disordered in the presence of DMPC. The SerSer peptide forms complexes with phospholipid of 38:1 which may be isolated by density-gradient ultracentrifugation; the AlaAla peptide does not form an isolable complex. This evidence suggests that the hydrophobic center provided by $Leu_{52}Ile_{53}$ is an important determinant for binding while charged residues are less important. However, due to the complexity of the CD spectrum, the possibility of another structure contributing to the binding of the SerSer derivative can not be excluded.

Mao et al.[61] have used synthetic fragments of apoA-II to investigate the relationship of lipid-binding sites to antigenic sites. They find that tryptic digestion of apoA-II yielded three peptides, 4 to 23, 31 to 39 and 56 to 77 which contain 28, 10 and 25%, respectively, of the antigenicity of apoA-II; a synthetic fragment containing residues 40 to 46 has 10% of the immunological reactivity. Furthermore, a synthetic peptide of residues 60 to 77 gives a precipitin line of complete identity with apoA-II(56 to 77). From previously reported lipid-binding studies[59,60] these authors conclude that the immunological reactions of the carboxyl terminal of apoA-II are independent from the lipid-binding region which they believe is between residues 51 and 60.

VI. APOLIPOPROTEIN A-I

Apolipoprotein A-I is the major apolipoprotein of HDL and is reported to be the major activator of LCAT for the synthesis of cholesteryl esters.[9] The protein which binds phospholipid and phospholipid-cholesterol has been studied extensively.[1-5] Two sequences have been reported for apoA-I which differ primarily in the amino terminal cyanogen bromide peptide and with smaller discrepancies at other points in the sequence[62,63] (Figures 6 and 7). The two larger cyanogen bromide fragments have been

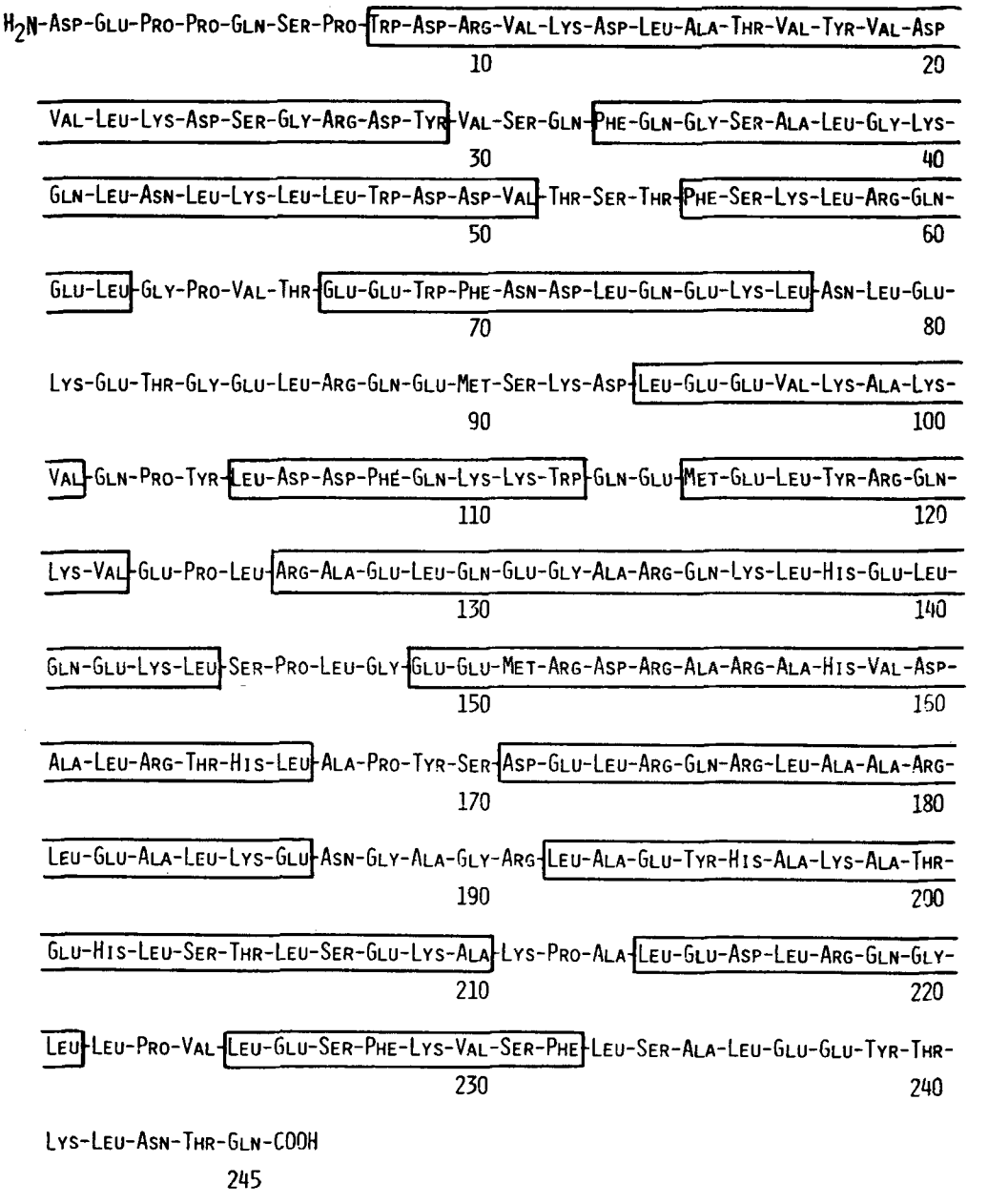

FIGURE 6. Amino acid sequence of apolipoprotein A-I (apoA-I) as reported by Baker et al.[62] The amphipathic helices are within the boxes.

shown to activate LCAT, the smaller two do not.[9] Analysis of apoA-I for homologous sequences has shown that there are six twenty-two residues repeating units which are amphipathic and are thought to contain the lipid-binding regions of the protein.[64–66]

Sparrow et al. have reported[44,67] the solid-phase synthesis of peptide fragments from the carboxyl-terminal cyanogen bromide peptide. After purification by ion exchange chromatography, the peptides are reported to be a single band on polyacrylamide gels. ApoA-I(227 to 245), with a hydrophobicity index of −1016 cal per residue, does not bind phospholipid as evidenced by a lack of CD spectral changes and the inability to isolate a

NH_2-Asp-Glu-Pro-Pro-Gln-Ser-Pro-Trp-Asp-Arg-Val-Lys-Asp-Leu-Ala-Thr-Val-Tyr-Val-Asp-
10 20
Val-Leu-Lys-Asp-Ser-Gly-Arg-Asp-Tyr-Val-Ser-Gln-Phe-Gln-Gly-Ser-Ala-Leu-Gly-Lys-
30 40
Gln-Leu-Asn-Leu-Lys-Leu-Leu-Asp-Asn-Trp-Asp-Ser-Val-Thr-Ser-Thr-Phe-Ser-Lys-Leu-
50 60
Arg-Glu-Gln-Leu-Gly-Pro-Val-Thr-Gln-Glu-Phe-Trp-Asp-Asn-Leu-Glu-Lys-Glu-Thr-Glu
70 80
Gly-Leu-Arg-Gln-Glu-Met-Ser-Lys-Asp-Leu-Glu-Glu-Val-Lys-Ala-Lys-Val-Gln-Pro-Tyr-
90 100
Leu-Asp-Asp-Phe-Gln-Lys-Lys-Trp-Gln-Glu-Glu-Met-Glu-Leu-Tyr-Arg-Gln-Lys-Val-Glu-
110 120
Pro-Leu-Arg-Ala-Glu-Leu-Gln-Glu-Gly-Ala-Arg-Gln-Lys-Leu-His-Glu-Leu-Gln-Glu-Lys-
130 140
Leu-Ser-Pro-Leu-Gly-Gln-Gln-Met-Arg-Asp-Arg-Ala-Arg-Ala-His-Val-Asp-Ala-Leu-Arg
150 160
Thr-His-Leu-Ala-Pro-Tyr-Ser-Asp-Glu-Leu-Arg-Gln-Arg-Leu-Ala-Ala-Arg-Leu-Glu-Ala
170 180
Leu-Lys-Glu-Asn-Gly-Gly-Ala-Arg-Leu-Ala-Glu-Tyr-His-Ala-Lys-Ala-Thr-Glu-His-Leu
190 200
Ser-Thr-Leu-Ser-Glu-Lys-Ala-Lys-Pro-Ala-Leu-Glu-Asp-Leu-Arg-Gln-Gly-Leu-Leu-Pro-
210 220
Val-Leu-Glu-Ser-Phe-Lys-Val-Ser-Phe-Leu-Ser-Ala-Leu-Glu-Glu-Tyr-Thr-Lys-Lys-Leu-
230 240
Asn-Thr-Gln-COOH

FIGURE 7. The amino acid sequence of apoA-I recently published by Brewer et al.[63]

complex with DMPC. However, apoA-I(220 to 245) displays an increase in α-helicity from 25 to 36% in the presence of DMPC; the longer peptides, 213 to 245, 204 to 245, and 197 to 245 show similar increases in α-helicity in the presence of DMPC. Lipid-peptide complexes, isolated by density-gradient ultracentrifugation, have ratios of lipid to peptide varying from 40:1 to 100:1. Complexes are also formed with DMPC-cholesterol dispersions, but these are inactive in the LCAT assay. Therefore, these authors conclude that apoA-I(220 to 245) contains one of the lipid-binding regions of apoA-I, but does not contain the necessary sequence for the activation of LCAT.

Sparrow, et al.[44] have also reported the solid-phase synthesis of five peptides between residues 142 and 185; the peptides are apoA-I(164 to 185), (157 to 185), (152 to 185), (148 to 185), and (145 to 185). After purification by ion exchange chromatography, these peptides bind to dispersions of phospholipid and phospholipid:cholesterol and complexes are isolated by density-gradient ultracentrifugation; CD spectral data are not reported. The peptides apoA-I(157 to 185), (152 to 185), (148 to 185), and (145 to 185) activate LCAT 8, 6, 19, and 24%, respectively, of that of apoA-I; apoA-I(164 to 185) is inactive. These findings suggest that apoA-I(148 to 185) is involved in the activation of

LCAT and apoA-I(164 to 185) contains another of the lipid-binding regions of apoA-I.

Kroon and Kaiser[58] also have synthesized by solid phase several peptides from apoA-I. These include apoA-I(114 to 133), (158 to 168), (158 to 168)Arg_{160}, and (144 to 165). The peptides are purified by gel filtration and ion exchange chromatography and display a single spot on thin layer chromatography in several solvent systems. ApoA-I(158 to 168) has a disordered CD spectrum even in the presence of chloroethanol. ApoA-I(147 to 168) and (114 to 133) have a helical content of 10 to 15% in aqueous buffers which increases to 40 and 33%, respectively, in 50% trifluoroethanol.

Kroon et al.[68] have studied the phospholipid binding by these peptides. Binding is determined by column chromatography or by an ultrafiltration assay. Both methods indicate complex formation with a ratio of phospholipid to peptide of 34:1 by apoA-I(147 to 168), but no complex formation is observed for apoA-I(158 to 168). The experimental details on the ultrafiltration assay are sketchy, and therefore it is difficult to judge the results of the technique, particularly in cases where binding may be weak and/or dependent on ionic strength. ApoA-I(114 to 133) binds below pH 5 and apoA-I(147 to 168) is lost from the complex above pH 7. The authors point out the importance of the ionization state of the peptide to binding affinity. The affinity of these peptides appears to be greatest near their isoelectric point where the peptide is neutral and less soluble in aqueous media. It should be noted the pH dependence of lipid binding by the apolipoproteins appears to be small.

Fukushima et al.[69] have reported the synthesis of apoA-I(124 to 167) and compared its lipid binding to apoA-I(147 to 168). The peptide, synthesized by solid phase and purified by gel filtration and ion exchange chromatography, has the expected amino acid composition and is a single spot on high voltage electrophoresis and thin layer chromatography. The correct sequence is found by automated Edman degradation. The yield of purified peptide is 2% based on the initial level of amino acid on the resin. ApoA-I(124 to 167) has an apparent molecular weight of 6600 by gel permeation chromatography, which is close to the expected monomer molecular weight of 5069. The helicity is calculated as approximately 17% at 222 nm; in the presence of 50% trifluoroethanol an helicity of 60% is observed. The helicity has not been reported in the presence of phospholipid, since significant values for the ellipticity could not be obtained. Other investigators have been able to obtain circular dichroic data on lipid-peptide and lipid-protein complexes and in several cases find a reasonable correlation with the Chou-Fasman predicted helicity.[1-5,44]

The surface properties of apoA-I(148 to 168) and (124 to 167) have been investigated by monolayer techniques.[69] These authors find both peptides to form monolayers with characteristic collapse pressures of 12 and 8 dyn/cm, respectively, compared to 22 dyn/cm for apoA-I. Both peptides appeared to be monomeric and have molecular areas of 20.8 $Å^2$ per amino acid and 18.8 $Å^2$ per amino acid, respectively. Apo-I(147 to 168) displays a marked pH dependence in its binding to mixed cholesterol-phospholipid vesicles as reported earlier for phospholipid vesicles.[68] However, apoA-I(124 to 167) shows no pH effects for either vesicle. In complexes with phospholipid, the shorter peptide has a molar lipid-peptide ratio of 106:1, and the longer a ratio 42:1. The ratios are 178:1 and 94:1, respectively, with phospholipid:cholesterol. ApoA-I(124 to 167) has approximately 30% of the activity of apoA-I for esterification of cholesterol by LCAT and has 12% of the phospholipase activation. Since apoA-I(147 to 168) does not bind near neutral pH to PC-cholesterol vesicles, it was not possible to determine the LCAT activation; however, phospholipase A_2 activation is observed to be 33% of that of apoA-I(124 to 167), i.e., 4% of that of apoA-I. From these results, the authors suggest that the depth of pentration of the peptide into the organized monolayers is the determining factor in enzyme activation. They speculate that the optimal depth should occur when $\frac{1}{3}$

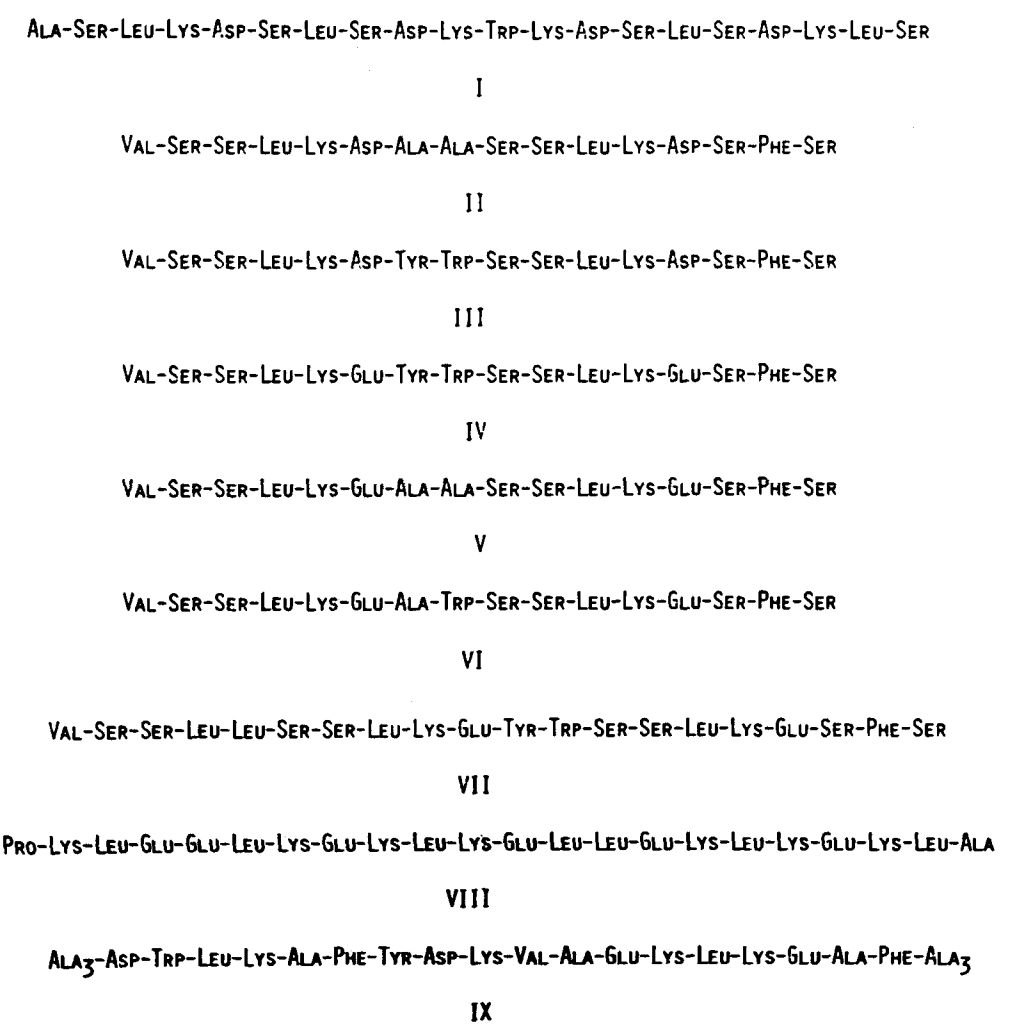

FIGURE 8. Peptides designed to model the amphipathic helix. Peptides I to VII, Sparrow et al.[53,70] Peptides VIII, Fukushima et al.[72] Peptide IX, Kanellis et al.[75]

of the helical surface is hydrophobic and that more hydrophobic proteins such as apoA-II do not activate LCAT due to their greater penetration into the phospholipid monolayer.

VII. PEPTIDE MODELS

Sparrow et al.[70] have reported the first attempt to model the amphipathic helix with small peptides containing a minimum number of different amino acids (peptides I-III, Figure 8). The purified peptides are studied for phospholipid binding by CD and fluorescence spectroscopy and density-gradient ultracentrifugation. Peptide I does not show any change in the CD or fluorescence spectra in the presence of phospholipid; the fluorescence maximum is 353 nm and the calculated helicity is approximately 10%. Similarly, peptide II has approximately 12% helicity in the absence or presence of phospholipid. However, peptide III in the presence of phospholipid displays a 6 nm blue shift in the fluorescence spectrum and an increase from 15 to 28% helicity based on

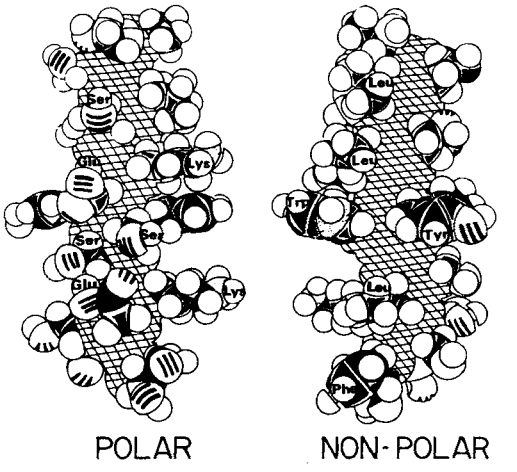

FIGURE 9. Artist illustration of the amphipathic nature of peptide VII.

ellipticity changes in the CD spectrum. A lipid-peptide complex could be isolated by density-gradient ultracentrifugation with peptide III, but none is observed with peptides I or II.

These three peptides contain aspartic acid which decreases the calculated helix potential and could result in poorer binding of lipid. However, Sparrow et al.[53] have synthesized and studied the same peptides (IV to VI) with glutamic acid, a better helix-former. In the presence of phospholipid, peptide IV exhibits a fluorescence shift of 10 nm and a calculated α-helicity of 27%. The insertion of four residues, two Ser and two Leu, at the amino terminal to form peptide VII, results in a dramatic increase in binding. Peptide VII (Figures 8 and 9) has a 19 nm shift in the fluorescence spectrum and becomes 70% helical in the phospholipid-peptide mixture. Density-gradient ultracentrifugation indicates that the majority of the peptide has been incorporated into a complex with a 12:1 molar ratio of lipid to protein. The authors conclude from these two investigations that hydrophobicity and peptide chain length are important to the stabilization of the lipid-peptide complex.

Pownall et al.[71] have published a more detailed study of peptide VII and its interaction with DMPC. As the final purification step, the peptide is subjected to granulated gel isoelectric focusing and is a single band on analytical isoelectric focusing gels with a pI of 6.2. Peptide VII has a 22 nm shift in the intrinsic tryptophan fluorescence. There is a strong temperature dependence of the fluorescence intensity at 330 nm and of the light scattering at 280 nm. At 40° the sample becomes very turbid, but upon cooling to the transition temperature of DMPC reclarifies. The calculated helical content from the CD spectrum of the complex is 90%. Column chromatography at 12 and 45° shows that a lipid-peptide complex is formed at and below the transition temperature of DMPC, but the complex is dissociated at 45°. Peptide VII-cholesterol-phospholipid complexes have been tested as substrates for lecithin:cholesterol acyltransferase. On the basis of an equal amino acid content, peptide VII is 65% as active as the native activator, apoA-I. When peptide VII is mixed with apoA-I-cholesterol-DMPC the resulting complex is 30% more active than those complexes containing only apoA-I. The temperature dependence of the LCAT activity is different with apoA-I since a distinct break occurs in the Arrhenius

plot at the transition temperature of DMPC that is not observed in the peptide VII plot. These authors conclude that a peptide must have a high hydrophobicity, a chain length of ~20 residues, a high helical probability, and a sequence such that an amphipathic helix is formed as prerequisites for binding phospholipid. They suggest that the activation of LCAT is exerted through an effect of the peptide on the boundary lipid and not by association of the activator peptide with the enzyme.

Fukushima et al.[72,73] have described the synthesis of peptide VIII which was designed to have surface properties similar to apoA-I. The peptide is synthesized by solid phase and purified by ion exchange chromatography. Its purity has been determined by Edman degradation, high voltage electrophoresis, and thin layer chromatography. The helical content is 61% in trifluoroethanol and 50% in phosphate buffer. The peptide binds to egg yolk phophatidylcholine vesicles at a ratio of 48 peptides per vesicle with a dissociation constant of $1.9 \times 10^{-6} M$. The peptide forms monolayers at the air-water interface with a collapse pressure of 22 dyn/cm, indistinguishable from that of apoA-I. The peptide is able to penetrate phospholipid monolayers and increases the surface pressure from 14 to 24 dyn/cm. Fukushima et al.[73] find a larger dissociation constant for the binding of peptide VIII to phosphatidylcholine:cholesterol vesicles than for binding to phosphatidylcholine. The resulting complex is a substrate for LCAT. They suggest that repulsion between the cholesterol hydroxyl group and the hydrophobic surface of peptide VIII could account for the decreased affinity.

In a more detailed study of peptide VIII, Yokoyama et al.[74] have compared the binding and LCAT activation of peptide VIII with that of apoA-I. With phosphatidylcholine vesicles, they find a binding capacity $N = 1.51 \times 10^{-2}$ peptides/phosphatidylcholine for peptide VIII and $N = 1.74 \times 10^{-3}$ protein/phosphatidylcholine for apoA-I. With cholesterol:phosphatidylcholine vesicles (1:4 *M/M)* the binding capacity increases to 2.27×10^{-2} peptides/PC and 3.7×10^{-3} proteins/PC. The cholesterol/lipid/peptide complexes are 18% as active as the cholesterol/PC/apoA-I complex for cholesterol esterification and 50% as active for phospholipase A2 cleavage. Both Yokoyama et al.[74] and Pownall et al.[71] suggest that the peptide activator affects the phospholipid matrix so as to allow LCAT more ready access to the phospholipids and is not interacting with the enzyme.

Kanellis et al.[75] recently have synthesized another series of peptides to investigate the role of the amphipathic helix and charged residues on phospholipid binding. The peptides are based on modifications to the sequence of peptide IX in Figure 8. The purified peptides have good amino acid analyses and are a single component by thin layer chromatography in two solvent systems. Peptide IX and another with serine in place of the terminal alanines associate with phospholipids by density-gradient ultracentrifugation. Some free peptide IX is found at all ratios of peptide and lipid examined. However, the serine containing peptide is completely incorporated in phospholipid and forms three complexes with molar ratios of 16:1, 28:1, and 82:1. The peptides with reversal of charged residues or with a serine substituted for Asp 11 and an alanine for Lys 16 do not bind lipid as assessed by density-gradient ultracentrifugation. The leakage of entrapped carboxyfluorescein from phospholipid vesicles is induced by peptide IX and the serine analogue, but not by the other two peptides. The intrinsic tryptophan fluorescence of the former peptides show a large blue shift from 352 to 333 nm; no shift is seen with the analogues. The α-helicity of peptide IX and peptide X $(Ser)_8$ increases from 9 to 46% in the presence of PC; there is no ellipticity change observed for the other two peptides. Light scattering experiments with small DMPC vesicles indicate that peptide IX binds at the transition temperature of DMPC. These authors suggest that the topography of the charged residues are important to the binding properties of the amphipathic helix.

VIII. SUMMARY

The understanding of complex interactions which occur in the serum lipoproteins has been greatly aided by using peptide synthesis to obtain fragments of the apolipoproteins which are unobtainable by other means. The results from lipid-binding studies with these synthetic materials have generally supported the amphipathic helical hypothesis of Segrest et al.[12] for the interaction of phospholipid with the apolipoprotein. However, CD results from these same experiments suggest that the amphipathic helices may not be as large as originally proposed. The contribution of other protein structural features, e.g. β-sheets and β-turns, to lipid binding has not been systematically investigated. The importance of hydrophobicity to lipid-protein interaction is strongly supported by the experimental data. Indeed, there is preliminary evidence[44,60] that the hydrophobic residues positioned beneath the paired acidic and basic residues on the amphipathic helix are extremely critical to the interaction with phospholipid. The role of charged residues in binding is less clear and needs further investigation. The importance of the structural features previously mentioned can be elucidated through the synthesis of appropriately substituted peptides. However, the final proof of the protein structural features involved in protein-lipid interaction must await x-ray diffraction analysis and detailed NMR measurements.

As more peptides are synthesized and studied, the authors feel that the complexities of lipid transport and metabolism will be better understood. The surface properties of peptide fragments of the apoproteins are presently being investigated and could lead to important findings on the exchange of apoproteins between lipoprotein classes. The interactions of synthetic peptides with the enzymes which control lipid synthesis and degradation have increased the understanding of protein-protein and protein-lipid interactions which control these important processes. The ability of a synthetic peptide to accelerate lipolysis in an apoC-II deficient lipoprotein offers the potential for treating these patients with synthetic material to reduce their hypertriglyceridemia. The ability to model the amphipathic helix opens new vistas for the study of the role of hydrophobicity, peptide length, helix potential, and charged residues in lipid binding. The observation of Pownall et al.[71] and Yokayama et al.[74] that phospholipid-cholesterol complexes of these model peptides can serve as substrates for LCAT suggests several exciting avenues for further study of cholesterol metabolism and transport. As these studies increase knowledge of lipid transport, the potential exists to intervene therapeutically with potent synthetic lipid-binding peptides to reduce serum cholesterol or to remove cholesterol from arterial lesions.

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