Studies of Synthetic Peptide Analogs of the Amphipathic Helix

EFFECT OF CHARGED AMINO ACID RESIDUE TOPOGRAPHY ON LIPID AFFINITY

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The amphipathic helix hypothesis for plasma lipoproteins was investigated using synthetic peptides. The lipid-associating properties of two potentially amphipathic model peptides and two analogs were studied by incubating synthetic peptides with small unilamellar vesicles and protein-lipid association examined by equilibrium density centrifugation, leakage of liposome-entrapped fluorescence compounds, intrinsic tryptophan fluorescence, and circular dichroism spectroscopy. The analog peptides were designed to determine the significance of the number and specific location of the charged residues in amphipathic domains of plasma lipoproteins to protein-lipid association. Based on the four procedures used to examine protein-lipid interactions, the two model peptides ("Aa, As) were found to associate strongly with liposomes; the two analog peptides ("As, As), differing only with respect to the number and/or position of their charged residues, failed to demonstrate similar lipid binding properties.

These findings support the earlier suggestions of the importance of the charged residues, but do not define the precise mechanisms involved. Such amino acids may help initiate the lipid-protein association by electrostatic interactions, contribute to the hydrophobicity of the nonpolar face of the helix by the acyl portion of lysine and arginine, and/or complement the charge distribution in the polar head regions of the phospholipid molecules.

The amphipathic helix hypothesis in its original form (1) was proposed as a mechanism to explain certain features of protein-lipid interactions in the plasma lipoproteins. The model defines a general structural accommodation of amino acid residues resulting in helical domains, termed amphipathic helices, containing opposing polar and nonpolar faces. A specific distribution of the charged residues was suggested, with the positive occurring along the interface between the polar and nonpolar faces, and the negative along the center of the polar face. Such an arrangement of the charged residues allows for the contribution of the lysine or arginine acyl side chains to the hydrophobicity of the nonpolar face. The charged residues also appear to form topographically close complementary ion pairs, the number of which may prove to be significant. In addition, the model allows for ionic interactions between positively charged side chains and the phosphate group of the phospholipid, as well as negatively charged residues and positive substituents on the phospholipid, i.e. choline in the case of phosphatidylcholine (1). These ionic interactions may play a significant role in initiating or contributing to the stability of the peptide-lipid complex. The general features of this model have been supported by a reasonable body of experimental evidence (1–13) since the theory first appeared. However, this evidence is not unequivocal. The most uncertain aspect of the theory concerns the function of the distinctive topographical distribution of charged residues along the polar face (1). It is our contention that this distribution is important to lipid association by the amphipathic helical domain (5). Not all authors have agreed with this contention (14, 15). Because the theory of the amphipathic helix is general enough to allow the design and synthesis of analog peptides, we have undertaken such studies with the ultimate goal of defining more precisely the functional role of the various features of the model. These peptides provide a means of studying, by simple amino acid sequence modification, the protein structural features capable of controlling lipid affinity and lipid micelle size and shape.

MATERIALS AND METHODS

Synthesis of Peptides—Four peptides were prepared ("Aa, As, "As, and "As), two of which ("Aa, "As) corresponded to the amphipathic helix model (Table I). In this nomenclature, the superscript 18 represents the residue length of the amphipathic peptide chain (16). The COOH-terminal amino acid was esterified to the amino group was used for NH₂-terminal protection. The abbreviations used are: Boc, tert-butyloxycarbonyl; DCC, dicyclohexylcarbodiimide; SUV, small unilamellar vesicles; PC, phospholipid; HSA, human serum albumin; Cl, choline; CHL, cholesteryl linoleate; CTAB, cetyltrimethylammonium bromide; and CHS, cholesterol sulfate. The complete synthesis details are given in Table I. The model defined a general structural accommodation of amino acid residues resulting in helical domains, termed amphipathic helices, containing opposing polar and nonpolar faces. A specific distribution of the charged residues was suggested, with the positive occurring along the interface between the polar and nonpolar faces, and the negative along the center of the polar face. Such an arrangement of the charged residues allows for the contribution of the lysine or arginine acyl side chains to the hydrophobicity of the nonpolar face. The charged residues also appear to form topographically close complementary ion pairs, the number of which may prove to be significant. In addition, the model allows for ionic interactions between positively charged side chains and the phosphate group of the phospholipid, as well as negatively charged residues and positive substituents on the phospholipid, i.e. choline in the case of phosphatidylcholine (1). These ionic interactions may play a significant role in initiating or contributing to the stability of the peptide-lipid complex. The general features of this model have been supported by a reasonable body of experimental evidence (1–13) since the theory first appeared. However, this evidence is not unequivocal. The most uncertain aspect of the theory concerns the function of the distinctive topographical distribution of charged residues along the polar face (1). It is our contention that this distribution is important to lipid association by the amphipathic helical domain (5). Not all authors have agreed with this contention (14, 15). Because the theory of the amphipathic helix is general enough to allow the design and synthesis of analog peptides, we have undertaken such studies with the ultimate goal of defining more precisely the functional role of the various features of the model. These peptides provide a means of studying, by simple amino acid sequence modification, the protein structural features capable of controlling lipid affinity and lipid micelle size and shape.

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and its removal was effected with 50% trifluoroacetic acid. Side chain protection was as follows: 2,6-dichlorobenzyl for tyrosine, benzyl for serine, \( \beta \)- and \( \gamma \)-benzyl esters for aspartic and glutamic acids, and benzoyloxycarbonyl for lysine. Tryptophan was introduced without resin. Unreacted amino groups were blocked with acetic anhydride for 2 h and were repeated when positive Kaiser tests were obtained.

The peptides were removed from the resin with hydrogen fluoride (15 ml/g of resin), containing anisole (2.5 ml) and 1,2-ethanediol (2 ml), at 0°C for 75 min. The crude peptides were dissolved in 20% acetic acid and lyophilized following extractions with ether for removal of anisole and 1,2-ethanediol. The synthetic peptides were desalted initially on a Bio-Gel P-2 (Bio-Rad) column (2.5 \( \times \) 23 cm) equilibrated with 0.1 M \( \text{NH}_4\text{HCO}_3 \)/6 M urea, pH 8.2, and eluted with the same solvent. (All purification procedures were carried out in the presence of urea to avoid aggregation of the peptides. Urea was removed at the completion of the purification procedures by passing the peptides through a Bio-Gel P-2 column.) After desalting, the highest molecular weight material was fractionated on a Bio-Gel P-4 column (6 \( \times \) 70 cm) using 0.1 M \( \text{NH}_4\text{HCO}_3 \)/6 M urea, pH 8.2 (Fig. 1). The fractions corresponding to the first eluted peak (47 to 52) were pooled, lyophilized, and further purified by ion-exchange chromatography on a Cellex D (0.75 meq/g; Bio-Rad) column (3 \( \times \) 16 cm) using linear gradients.

Peptides "As' and "As', were dissolved in 0.01 M \( \text{NH}_4\text{HCO}_3 \)/6 M urea, pH 8.2, and chromatographed on a column pre-equilibrated with the same buffer using a linear gradient from 0.01 M to 0.2 M \( \text{NH}_4\text{HCO}_3 \)/6 M urea, pH 8.2 (Fig. 3). Parts I, II, and III corresponding to sequence of elution of the peptide(s) from the column, were lyophilized separately and rechromatographed. A three-step linear gradient was used from 0.01 M to 0.05 M, followed by 0.05 M to 0.08 M and finally from 0.08 M to 1.0 M \( \text{NH}_4\text{HCO}_3 \)/6 M urea, pH 8.2. In each chromatogram, two well separated peaks of different size and peptide content were obtained. Both peptides "As' and "As', behaved almost identically under the above chromatographic procedures and were each time found to correspond to the main peak of part II.

When peptide "As' was submitted to ion exchange under the same conditions, a higher concentration gradient of 0.01 M to 1.0 M \( \text{NH}_4\text{HCO}_3 \)/6 M urea, pH 8.2, was necessary for elution (Fig. 2). Amino acid analyses of the separate fractions indicated the presence of the desired peptide in the slowest eluted position. Fractions 98 to 191 were pooled and lyophilized. All three pure peptide preparations were then passed through a Bio-Gel P-2 column (2.5 \( \times \) 23 cm) to remove the urea. A single peak was obtained in each case monitoring the eluent by its UV absorbance at 280 nm and tryptophan fluorescence (excitation, 280 nm; emission, 470 nm). The process of ion exchange and gel chromatography were repeated to demonstrate homogeneity of final products. The products contained a single component as indicated by thin layer chromatography in BAW (1-butanol/acetic acid/water 4:1:1) with \( R_f \) values 0.75 for "As', 0.64 for "As", and 0.59 for "As", as well as BPAW (1-butanol/pyridine/acetic acid/water 15:10:3:12) where the \( R_f \) value (0.63) was common for all three peptides. The amino acid analyses of the synthetic peptide hydrolysatess (6 N HCl, 110°C, for 16 h) appear in Table II.

Association of Synthetic Peptides with Phospholipid Bilayers—

The phospholipid associating properties of the synthetic peptides were studied using small unilamellar vesicles of egg phosphatidylcholine, DMPC, dimyristoyl phosphatidylcholine; TES, N-[\( \text{tris} \) (hydroxymethyl)methyl]2-aminoethanesulfonic acid, CF, carboxyfluorescein; \( \gamma \), transition temperature.

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**TABLE I**

Primary structure of synthetic peptides

| Peptide | H-(Ala)2 | Ala | Asp | Trp | Leu | Lys | Ala | Phe | Tyr | Asp | Lys | Val | Ala | Glu | Lys | Leu | Lys | Glu | Ala | Phe | (Ala)2-OH | (Ser)2-OH |
|--------|---------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| "As"  | H-(Ser)2 | Ser | Asp | ---- | ---- | ---- |---- |---- |---- |---- |---- |---- |---- |---- |---- |---- |---- |---- |---- |---- |---- |---- |
| "AsA" | ----     |     |-----|----- |     |----- |---- |---- |---- |---- |---- |---- |---- |---- |---- |---- |---- |---- |---- |---- |---- |---- |
| "AsS" |        |     |     |     |     |-----|---- |---- |---- |---- |---- |---- |---- |---- |---- |---- |---- |---- |---- |---- |---- |---- |
Synthetic Amphipathic Helical Peptides

Table II
Amino acid analyses of synthetic peptides

| Amino acid | \(^{14}Aa\) | \(^{14}As\) | \(^{14}Aa\) | \(^{14}As\) |
|------------|------------|------------|------------|------------|
| Lysine     | 4          | 4          | 4          | 3          |
| Aspartic acid | 2          | 1          | 2          | 1          |
| Serine     | 5          | 4          | 6          | 5          |
| Glutamic acid | 2          | 2          | 2          | 2          |
| Alanine    | 8          | 8          | 4          | 5          |
| Valine     | 1          | 1          | 1          | 1          |
| Leucine    | 1          | 1          | 2          | 2          |
| Tyrosine   | 3          | 4          | 1          | 1          |
| Phenylnaline | 2          | 1          | 2          | 2          |

RESULTS

Ultracentrifugation—Equilibrium density gradient centrifugation studies were used as one criterion for peptide-lipid association. The synthetic peptides (Table I) were incubated with small unilamellar PC vesicles and the mixture was subjected to ultracentrifugation as already described. Peptides \(^{14}Aa\) and \(^{14}As\) formed peptide-lipid complexes as indicated in Figs. 4 and 5c, respectively. For \(^{14}Aa\) (Fig. 4), all the PC but only a portion of the peptide is present in the complex; the remainder of the peptide is in the bottom of the gradient. The presence of unassociated radiolabeled peptide was found in all protein/lipid molar ratios of \(^{14}Aa\) examined (1:50, 1:30, 1:20, 1:10). For \(^{14}As\) (Fig. 5c), three separate protein-lipid complexes are seen with protein/lipid molar ratios of 1:16, 1:28, and 1:82. The presence of free protein (Fig. 4) and multiple complexes (Fig. 5c) is consistent with kinetic dissociation of peptide-lipid complexes formed by \(^{14}Aa\) and \(^{14}As\), respectively, with PC during the course of ultracentrifugation. This possibility will be considered in more detail under “Discussion.” Peptides \(^{14}Aa\) and \(^{14}As\) failed to exhibit lipid binding properties (Fig. 5, a and b).

Liposome Leakage—The integrity of the PC liposomes was followed as a function of peptide-lipid interaction using the CF technique (23, 24). CF is a fluorescent dye which under ordinary circumstances has only a slight tendency to leak from PC liposomes (approximately 1% leakage in 24 h at room temperature). The leakage of CF can be measured as an increase in fluorescence, due to dequenching, as the dye is diluted into the extraliposomal medium. Disruption of the CF-containing liposomes with Triton X-100 results in total dequenching of the CF and provides the fluorescent equivalent of 100% leakage.

As shown in Fig. 6, peptide \(^{14}Aa\) produces large perturba-

until the solution was clear) or (b) a Branson W200P probe sonicator, at 0°C for 1 h at 50% output. When radiolabeled lipid was output, 0.01 pCi of uniformly labeled \(^{14}C\)IPC from Applied Science (State College, Pa.) was added to the PC before rotary evaporation. Radiolabeled \(^{125}I\)-peptide (\(^{125}I\)Aa) was prepared using the iodine-125-monochloride method (20). Iodine-125-monochloride was purchased from New England Nuclear. Apolipoproteins A-I and C-111 were isolated from human serum using standard chromatographic procedures (21, 22).

The peptide (or apolipoprotein) was dissolved in a small volume of Tes-histidine buffer (2 mM Tes, 2 mM histidine, 0.15 M NaCl, pH 7.4) to give concentrations ranging from 0.01 to 3 M. Peptide-lipid association was examined by incubating the peptide and lipid together at 23°C for a period of 24 h prior to equilibrium density gradient centrifugation.

Equilibrium Density Gradient Centrifugation of Peptide-Lipid Complexes—The incubation mixtures were subjected to density gradient centrifugation in self-forming gradients of 4% or 5% CsCl. Two different centrifugation procedures were used following overnight incubation of the peptide with the lipid (1:20 molar ratio). (a) The samples (300 ml) were layered on top of the CsCl (4.2 M) followed by a 0.6-mI overlay of water and the tubes were immediately centrifuged in a Beckman AH 650 swinging bucket rotor for 20 h at 50,000 × g. (b) The samples (200 ml) were layered on top of the CsCl (4.8 ml) and were centrifuged in a Beckman 550 swing bucket rotor for 20 h at 350,000 × g. Centrifugations were performed on an OTD-2 ultracentrifuge from DuPont-Sorvall. The contents of each tube were fractionated by downward flow into 0.3-ml fractions. The density of each fraction was estimated by measuring the refractive index of fractions from a gradient containing no complex on an Abbe refractometer.

Liposomal Leakage Measurements by Fluorescence Dequenching—PC was hydrated in 2 mm Tes, 2 mm histidine, 0.15 M NaCl, pH 7.4, containing 200 mM carboxyfluorescein (Eastman Kodak, recrystallized from ethanol) and sonicated as described. Small unilamellar PC vesicles containing trapped CF were separated from larger vesicles and from free CF by gel filtration on a column (2 × 41 cm) of Sepharose 4B. Unilamellar vesicles of 250 to 300 Å in diameter (measured by Stokes radius calculations) were consistently obtained. Fluorescence spectra on an Aminco SPF 500 corrected spectra spectrofluorometer were recorded as a function of time at 23°C using a 492-nm excitation and a 525-nm emission. Baseline leakage was established for CF-containing PC vesicles alone. Peptide-induced leakage was measured as an increase over baseline at 525 nm fluorescence after addition of synthetic peptides or controls. Leakage (100%) was set equal to fluorescence intensity at 525 nm following addition of 100 μl of 10% Triton X-100.

Intrinsic Tryptophan Fluorescence—Fluorescence spectra were recorded at 23°C with excitation at 287 nm. Spectra were recorded for peptide alone and following addition of small unilamellar vesicles. Tryptophan fluorescence emission maxima for the peptides alone in solution were recorded to within 1.0 nm of the quoted value for free tryptophan in solution.

Circular Dichroism—CD was measured at 23°C on a Cary 60 spectropolarimeter in cells 0.5-mm pathlength using 0.02 M sodium phosphate buffer, pH 7.4. The sensitivity setting was 0.04 and the spectra were run with a time constant of 3.0 s. Following determination of the CD spectra, light scattering of the complex was measured by recording the absorption spectrum in a Cary 14 spectrophotometer between 185 and 300 nm for the peptide-lipid complexes. Spectroscopic and absorption spectra were also recorded for the peptides and PC alone at concentrations equal to those in the peptide-lipid complexes.

FIG. 3. Ion exchange chromatography of peptide \(^{14}Aa\) (\(^{14}As\) similar), on Cellex D. Column (3 × 16 cm), equilibrated in 0.01 M NaHCO₃. Peptide was collected over 3 min. A second gradient of 100 ml of 0.1 M to 100 ml × 0.1 M NH₄HCO₃/6 M urea started at fraction 100 did not elute additional peptide material.
Synthetic Amphipathic Helical Peptides

**FIG. 4. Ultracentrifugal behavior of \(^{14} \text{As}: \text{PC} \) complexes in CsCl density gradients.** The peptide-PC mixtures were incubated overnight at 23°C and then centrifuged in a 4% CsCl gradient using a Sorvall TV 865 vertical rotor for 5 h at 370,000 × g. ○—○, lipid; ▲—▲, peptide. (The peptide alone sedimented to the bottom of the gradient.)

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**FIG. 5. Ultracentrifugal behavior of \(^{14} \text{As} (A), \(^{14} \text{As} (B), \) and \(^{14} \text{AS} (C) \) complexes with PC.** The peptide-PC mixtures were incubated overnight at 23°C and then centrifuged in a 5% CsCl gradient using a Beckman AH 850 swinging bucket rotor for 20 h at 350,000 × g. ▲—▲, lipid; ○—○, peptide. Panel A includes peptide (○—○) and lipid (●—●) centrifuged separately as reference.

As shown in Fig. 8, the addition of peptide \(^{14} \text{As} \) results in a biphasic leakage process; there is an initial, rapid leakage component, followed by a much slower one. Addition of a second pulse of \(^{14} \text{As} \), equal to the first, produces a second rapid leakage again followed by a slower component. The second rapid phase is identical to the first if the slower leakage is taken into account. The slope of the second slow leakage component is twice that of the first slow component. A reasonable interpretation of these data is that the rapid phase represents leakage due to the active process of insertion of peptide \(^{14} \text{As} \) (and \(^{14} \text{As} \), data not shown) into the PC liposome outer monolayer, a process essentially complete in approximately 2 min; the slower phase represents leakage due to disorder induced by the steady state presence of the peptide in the PC liposome outer monolayer. Negative stain electron microscopy (Fig. 9) shows that liposomal integrity is maintained after \(^{14} \text{As}: \text{PC} \) complex formation.
**Synthetic Amphipathic Helical Peptides**

**Fig. 6.** Leakage of PC vesicles, as a function of time, upon interacting with synthetic peptide and apolipoprotein AI (molar ratio of peptide to lipid 1:20). Leakage was quantitated relatively to 6-CF fluorescence (100%) upon addition of 1% Triton X-100. - - As peptide; - - A-I; - - As, peptide; - - A-I. 

**Fig. 7.** Rate of leakage of PC vesicles as a function of As (○ - - ○), apo A-I ( ● - - A-I), and apo C-III (△ - - △) concentration. Spectrum was obtained at 23°C with excitation at 492 nm and emission at 525 nm.

**Fig. 8.** Sequential leakage produced by peptide As on addition to PC vesicles. Spectrum obtained at 23°C with excitation at 492 nm and emission at 525 nm.

**Intrinsic Tryptophan Fluorescence**—Studies of the intrinsic fluorescence of the single tryptophan residue contained in peptide As (Fig. 10) show a large blue shift (toward the UV) in the fluorescence peak (352 nm to 333 nm) and an increase in quantum yield with the addition of PC liposomes, consistent with movement of the tryptophan residue on the nonpolar face of the “amphipathic” peptide (see Fig. 15 and “Discussion”) from the aqueous medium into the hydrocarbon region of the PC liposome bilayer. Peptide As gives similar results; no shift is seen for analog peptides AsI and AsII. 

**Fig. 9.** Negative stain electron microscopy of As:PC (1:20 molar ratio) at approximately 0.1 mg/ml phospholipid. Bar is equal to 1000 Å.

**Fig. 10.** Intrinsic Trp fluorescence as a function of PC association of As and As. Lower magnitude spectrum corresponds to the peptide in aqueous solution and higher magnitude to the peptide-PC complex. Spectra were measured at 23°C with excitation at 287 nm.

This shows that the degree of UV shift in fluorescence is a function of the protein-lipid concentration, being maximal at lower concentrations of protein; these data are consistent with a partial saturation phenomenon at higher protein concentrations.
FIG. 11. Changes in fluorescence maximum of Trp as a function of \(^{18}\)Aa:PC molar ratio. Spectra were measured at 23°C with excitation at 287 nm.

FIG. 12. CD spectroscopy of \(^{18}\)Aa versus \(^{18}\)Aa:PC (1:1 molar ratio) complex. Spectra were obtained at 23°C on a Cary 60 Spectropolarimeter in 0.5-mm pathlength cells in 0.02 M sodium phosphate at pH 7.4.

Circular dichroism—Circular dichroism spectroscopy (Fig. 12) suggests that peptide \(^{18}\)Aa in solution has approximately 9% \(\alpha\)-helical content; this increases to 46% upon addition of sonicated PC liposomes (calculation from the molar ellipticity at 222 nm; 100% helix = 34 \(\times\) 10\(^{-3}\) degrees cm\(^2\)/dmol) (36). Amphipathic peptide \(^{18}\)As shows similar changes. These changes are qualitatively similar to those seen upon the association of apolipoprotein, such as apolipoprotein A-I and apolipoprotein C-III, to sonicated PC liposomes. The maximum helicity of 43% represents 10 to 11 residues of the 18-residue “amphipathic” segment. Peptides \(^{18}\)As and \(^{18}\)As, did not undergo any measurable structural changes in the presence of sonicated PC liposomes, as indicated by a CD spectrum unchanged following addition of liposomes to the peptide in solution.

Interactions with DMPC—DMPC was used in place of egg PC in one set of experiments. Small unilamellar vesicles were

FIG. 13. Phase transition of sonicated DMPC vesicles (a) alone, and (b) in association with \(^{18}\)Aa peptide (20:1 molar ratio). Vertical axis is in relation to \(A_{350}\). Peptide was added at 20°C. Phase transition was monitored by measuring light scattering changes at 350 nm.

FIG. 14. Negative stain electron microscopy of (upper) sonicated DMPC liposomes alone, and (lower) upon addition of peptide \(^{18}\)Aa.
formed from DMPC under the conditions used for egg PC. Peptides were added to a solution of sonicated DMPC liposomes and the DMPC order-disorder transition (Tc) monitored by measuring light scattering changes (ΔA sno) as a function of temperature using a Hitachi 100-20 spectrophotometer with a water-jacketed cuvette. Sonicated DMPC vesicles alone showed a well defined, reversible transition at approximately 24°C (Fig. 13b). The addition of peptide 18AA to the same DMPC liposomes at 20°C (Fig. 13b) resulted in an initial small drop in turbidity; as the temperature was increased, a major decrease occurred near the Tc of DMPC (22–24°C). When the temperature was brought back to 20°C from 37°C, a small drop in turbidity; as the temperature was increased, a marked, irreversible decrease occurred near the Tc, for DMPC; associated with this complex formation is a concomitant decrease in the light scattering properties of the peptide complexes with the DMPC vesicles, most avidly at the Tc for DMPC; associated with this complex formation is a concomitant decrease in the light scattering properties of the peptide complexes (as measured by light scattering changes). These studies suggest that peptide 18AA (and peptide 18AS, data not shown) forms complexes with DMPC vesicles, most avidly at the Tc for DMPC; associated with this complex formation is a concomitant marked, irreversible decrease in the light scattering properties of the sonicated DMPC liposomes.

Because of the marked, irreversible decrease in light scattering induced in DMPC by 18AA, the structure of the 18AA-DMPC complex was examined by negative stain electron microscopy. Fig. 14 (upper) shows that sonicated DMPC liposomes alone are unilamellar vesicles of 250 to 400 Å in diameter. The addition of peptide 18AA (Fig. 14, lower) results in a significant alteration in the negative stain appearance of the DMPC from that of vesicles to that of stacked discs and sheets. We conclude that peptide 18AA destabilizes DMPC liposomes to the point of forming "bicycle tire micelles" (12, 20) similar to those produced by the addition of human apolipoprotein A-I to DMPC liposomes (26).

**DISCUSSION**

Fig. 15 shows a space-filling model of the amphipathic portion of peptide 18AA (or 18AS) constructed with an α-helical conformation. The design of this segment of the amphipathic peptides was based on a segmental statistical distribution of amino acid residues in putative amphipathic helical domains from four plasma apolipoproteins for which amino acid sequences are known (5). The amphipathic helical structure of these peptides was further refined visually by construction of CPK space-filling models prior to synthesis.

Note in Fig. 15 the location of the tryptophan residue on the nonpolar face and the positions of the charged residues on the polar face; the positively charged residues (lysyls) are along the lateral interfaces between faces and the negatively charged residues (aspartyls and glutamyls) are centrally located on the polar face. In analog peptide 18AS (Table I), the second aspartyl and the third lysyl residue distant to the NH2 terminus have been replaced with neutral residues (seryl and alanyl, respectively); the result is the loss of the two close ion pairs in the middle of the putative amphipathic helix. In analog peptide 18AS, 4 lysyl residues have been positioned centrally in place of the 4 negatively charged residues and aspartyl and glutamyl residues have been positioned peripherally in place of the 4 positively charged residues; we have previously referred to this as a reversed amphipathic helix (5).

We earlier suggested, based on our computer analysis of the general occurrence of amphipathic helix patterns in proteins with known amino acid sequences, that a reversed amphipathic helix would have a lower affinity for phospholipid association compared to a "standard" amphipathic helix (5). The results reported here, that 18AS associates with PC liposomes and the reversed amphipathic analog 18AS does not, clearly support the notion that the topomolecular pattern of the charged residues on the polar face contributes significantly to the association of amphipathic helix domains with phospholipid.

It is useful at this juncture to compare the lipid-associating properties of peptides 18AA and 18AS with the apolipoproteins A-I and C-III. Of the methods employed in the present study, we consider the CF leakage experiments to be the most sensitive measure of protein-lipid interactions of a hydrophobic nature (27). On the basis of liposomal leakage/mmol of peptide, peptide 18AS causes greater perturbations in liposomal bilayer structure than either apolipoprotein A-I or apolipoprotein C-III (Fig. 7).

On the other hand, the equilibrium density gradient centrifugation studies (Figs. 3 and 5c) suggest that peptides 18AA and 18AS dissociate from the liposomes during the time course of the ultracentrifugation run. This indicates that the free energy of association of the amphipathic peptides with liposomes is less than that of the apolipoproteins A-I and C-III, which fail to dissociate under similar conditions (28, 29).

Table III lists some of the properties of amphipathic helix domains of the synthetic amphipathic peptides and plasma apolipoproteins. In the first two columns are numbers indi-

![Fig. 15. CPK space-filling model of Phe-Asp 21 of 18AA and 18AS peptides. The residues appear in the form of an amphipathic helix with the polar and nonpolar faces shown (top). The complete sequence of 18AA is shown with the amphipathic portion denaturated with circles.](image-url)
cating the relative hydrophobicity of the nonpolar face of each amphipathic helical domain, expressed as mean hydrophobicity per residue (or per unit helix surface area). On the same scale, the α-helical, membrane spanning domains of glycoporphin and the B-coat protein of filamentous bacteriophages have mean hydrophobicities per residue (or per unit helix surface area) of between 2.5 and 3.0. Note that the nonpolar face of the synthetic amphipathic peptides Aa and As has a higher mean hydrophobicity than the equivalent portions of plasma apolipoprotein-derived putative amphipathic helix domains.

The residue length of the amphipathic domains from plasma apolipoproteins varies from 11 to 28 with a mean of 19.6. The amphipathic helical domains from those apoproteins containing a single domain have a mean length of 26 residues; further all apolipoproteins contain at least one domain longer than 20 amino acid residues.

It would appear, therefore, that differences in the lipid affinity of the synthetic peptides Aa and As, compared to native apolipoproteins, are not due to deficiencies in peptide hydrophobicity or the number or position of ion pairs. Differences could, however, be due to a less than optimal amphipathic peptide length (real or effective) for peptides Aa and As.

How can the leakage differences be explained? We have suggested, based on the results of Fig. 6, that amphipathic helix insertion into a PC bilayer is a disruptive and, therefore, a more leaky process than the steady state presence of the amphipathic helix in the bilayer. If amphipathic peptides Aa and As have a significantly lower lipid affinity than apolipoproteins A-I and C-III, then there would be a greater tendency for the peptides to undergo dynamic exchange between the aqueous phase and the lipid bilayer; this would result in an increased leakage for the peptides compared to apolipoproteins A-I and C-III, the latter having an equilibrium tending more towards the lipid-associated state. Also, the steady state binding of a stable amphipathic helix (apolipoproteins A-I and C-111) might have less impact on bilayer permeability than the steady state binding of a less stable amphipathic helix (peptides Aa and As), due to a disruptive containing helix to coil transition in the latter.

Certain nonapoprotein peptides, such as glugagon (30), have been shown to have regions of amino acid sequence compatible with amphipathic helical domains and, under certain conditions, have been shown to associate with lipid. However, the domains reported so far (30-32) do not have the characteristic topomolecular distribution of charged residues found in apolipoproteins. Further, glucagon, for example, interacts with DMPC only below the phase transition (33), whereas apoproteins, such as apo A-I, interact best at or above the phase transition (34).

A number of possible mechanisms have been suggested previously to explain the significance of the specific topomolecular distribution of charged residues on the polar face of the amphipathic helix: (a) the distribution is complementary to the charge distribution in the polar head regions of phospholipid molecules (1); (b) the distribution is important in initiating association of the apolipoprotein with the surface of aqueous phospholipid structures via electrostatic interactions; hydrophobic association follows this initiating event (2, 27). (c) the lysyl and arginyl residues are localized to the lateral interface between opposing faces because of the hydrophobicity of the bulk acyl portion of these amino acids; aspartyl and glutamyl are considerably less hydrophobic (5); (d) the distribution of charged residues on the polar face gives the amphipathic helix the potential to associate with adjacent helices via lateral electrostatic interactions (26).

Of these four possibilities for the functional significance of the topography of the polar face, possibility (d) seems unlikely to influence protein-lipid interactions (35) to the degree suggested by the studies reported here. Possibility (c) clearly would seem to be a factor, since a comparison of column 1 with column 2 in Table III shows that lysyl and arginyl residues in the lateral position contribute significantly to the overall hydrophobicity of the nonpolar face.

New evidence has been presented here in support of the amphipathic helix hypothesis. Known factors contributing to protein-lipid associations, such as hydrophobic or protein intrachain interactions, have been taken into consideration in constructing the amphipathic helix mode. In this respect, the amphipathic helix hypothesis is not inconsistent with the traditionally accepted factors which contribute to protein-lipid associations, but has incorporated them into the structure of the amphipatic model itself. This is not to deny the possible importance in native apolipoproteins of other factors, such as long range cooperative interactions, that may contribute to the association of apolipoproteins with lipid.

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Synthetic Amphipathic Helical Peptides

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