Lipid-Peptide Association and Activation of Lecithin:Cholesterol Acyltransferase

EFFECT OF α-HELICITY*

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Gabriel Ponsins†, Leisha Hester, Antonio M. Gotto, Jr., Heany J. Pownall§, and James T. Sparrow

From the Department of Medicine, Baylor College of Medicine and The Methodist Hospital, Houston, Texas 77030

A series of apolipopeptides, in which single proline substitutions were made at various sites in the 20-residue sequence, have been synthesized and tested. These peptides have nearly the same hydrophobic content, but very different helical contents, in a structure-making solvent. The affinity of these peptides for phospholipids was evaluated on the basis of their intrinsic tryptophan fluorescence and equilibrium dialysis against model high density lipoproteins. Proline substitutions at one end of the peptide had little or no effect on the fluorescence, circular dichroism, affinity for model high density lipoproteins, or activation of human plasma lecithin:cholesterol acyltransferase. By contrast, there was a dramatic change in all of these variables as the site of substitution was moved progressively closer to the middle of the peptide. All of these data suggested that a helix breaker that is substituted at the midpoint of a helical surface-associating peptide will greatly reduce its affinity for phospholipid surfaces. These results demonstrate that helicity and hydrophobicity are independent determinants of the affinity of an apolipopeptide for a phospholipid surface.

EXPERIMENTAL PROCEDURES

Materials

The LAP-20,† which has the sequence, Val-Ser-Leu-Leu-Ser-Leu-Leu-Lys-Glu-Tyr-Trp-Ser-Ser-Leu-Lys-Glu-Ser-Phe-Ser, was synthesized as previously described (16). By the same methods, a family of peptides in which a single proline was substituted for leucine at residues 5, 8, and 15, for valine at residue 1, for phenylalanine at residue 19, and for tyrosine at residue 11 was also obtained. DMPC and POPC were obtained from Avanti Biochemicals, Birmingham, AL. [3H]Cholesterol and [3H]DMPC were purchased from New England Nuclear. Human apo-A-I was purified as previously described (17). Lecithin:cholesterol acyltransferase was purified to homogeneity by the method of Albers et al. (18). A buffer composed of 150 mM NaCl, 0.01% EDTA, 0.01% azide, and 10 mM Tris, pH 7.4, was used except where noted.

Methods

Fluorescence Measurements—Fluorescence spectra were recorded on an SLM photon counting spectrophotometer operated at ambient temperature using an excitation wavelength of 286 nm. Emission spectra were recorded between 300 and 450 nm using 2 nm slits throughout. Each peptide was incubated overnight at 23 °C with various concentrations of single bilayer vesicles of DMPC or POPC produced by sonication.

Density Gradient Ultracentrifugation—Approximately 0.5 mg of peptide was lyophilized in a 13-×-100-mm screw-cap culture tube. The peptide was solubilized in 1 ml of 0.1 M NaCl and 0.01 M Na2HPO4, pH 7.4, and the concentration was determined by recording the UV spectrum between 320 and 240 nm on a Cary 15 spectrophotometer. A dispersion of [3H]DMPC (22 μCi/mmol) was prepared by sonication of 30 mg in 2.8 ml of 0.1 M NaCl with a probe sonicator. The dispersion was maintained at 4 °C under nitrogen during the 15 min required to clarify the solution. The dispersion was centrifuged for 30 min at 14,000 rpm in a Beckman J-21 centrifuge to remove any titanium. The DMPC and peptide were mixed in appropriate quantities to obtain molar ratios of lipid to peptide of 50:1. The screw-cap tubes were placed in a 24 °C bath for 24 h. The volume of the peptide-lipid mixture was adjusted to 1.5 ml with 0.01 M Na2HPO4, and 0.10 M NaCl, pH 7.4. A solution (2.5 ml) containing 0.01 μM

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‡ Robert A. Welch Postdoctoral Fellow.

§ To whom correspondence should be addressed: Baylor College of Medicine, The Methodist Hospital, M.S. A-801, 6565 Fannin St., Houston, TX 77030.

*1The abbreviations used are: LAP, lipid-associating peptide; DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; POPC, 1-palmitoyl 2-oleoyl-sn-glycero-3-phosphocholine; apo, apoprotein; M-HDL, model high density lipoproteins.
Lipid-Peptide Interaction and Acyltransferase Activation

Na$_2$HPO$_4$, 0.10 M NaCl, and 1.31 M CsCl, pH 7.4, was placed in the bottom of a 5-ml polyallomer tube and the peptide-lipid solution was carefully added to the top. The tube containing the peptide-lipid mixture was rinsed with 1 ml of the CsCl solution and the rinse solution was added to the top of the centrifuge tube. The tubes were placed in the buckets of a Beckman 50.1 rotor and centrifuged at 45,000 rpm at 20°C for 72 h. The gradients were fractionated from the top of the tube into 250-ml aliquots with a peristaltic pump, Densiflow, and a drop-counting fraction collector. The density of each fraction was determined from the refractive index.

Equilibrium Dialysis—M-HDL were prepared by mixing POPC and apo-A-I (100:1 molar ratio) in the presence of cholate at room temperature; the detergent was removed as previously described (19). The peptides were radiiodinated by the chloramine-T method (20). One ml of M-HDL, at various concentrations, was placed inside the dialysis tubing. A trace of each labeled peptide was added to the outside compartment in 0.02 ml of 50% 2-propanol. At equilibrium, the concentration of POPC was determined (21), and the amount of peptide bound to M-HDL was calculated from the difference between the radioactivities of the inside and outside compartments. The concentration of POPC was determined (21), and the amount of peptide bound to M-HDL was calculated from the difference between the radioactivities of the inside and outside compartments. For each peptide, the equilibrium constant was calculated as $K = (B/\beta) ([W]/[P])$ where $B$ and $\beta$ represent the concentrations of peptide bound to M-HDL and in the aqueous phase, respectively, and [W] and [P] are the respective molar concentrations of water and POPC.

Lecithin-Cholesterol Acylation Transferase Assay—Enzyme activity was assayed by using a modification of the method of Glomset (22) in which minicolumns of silica gel (1 ml) constructed from Pasteur pipettes were substituted for thin layer plates (23). Further details are provided in the legend to Fig. 5.

Circular Dichroic Spectra—Circular dichroic spectra of the peptides in the helix-making solvent, hexafluoropropanol, were recorded on a Jasco J-500A spectropolarimeter operated under ambient conditions. The helical content of each peptide was calculated from the molar ellipticity at 222 nm (24).

RESULTS AND DISCUSSION

To investigate the importance of helical potential, we have synthesized a series of peptides with proline substituted at various positions of the previously reported LAP-20 peptide (12). Fig. 1 shows the Chou-Fasman predicted structures for these six peptides and their average hydrophobicities calculated using the hydrophobicity scale of Bull and Breese (25). Below, we present the results of our studies on the interaction of these peptides with phospholipids. These results demonstrate the importance of helical potential to protein-phospholipid interactions.

Lipid-Apolipoprotein Interaction—The spectroscopic changes that accompany the addition of POPC or DMPC to the model peptides were a function of the identity of the peptide. All peptides exhibited a blue shift that is characteristic of the transfer of the tryptophan to the hydrophobic environment of the lipid, although the magnitude of this effect varied markedly from one peptide to another (Fig. 2). A shift of 10 nm required the addition of 0.04, 1.2, 4.0, and 7.5 mM POPC to LAP-20, 5-Pro-, 15-Pro-, and 8-Pro-LAP-20, respectively. This indicated a decreasing affinity of the peptides for the lipid in the order given. Based upon the circular dichroic spectra, the helical contents of the peptides recorded in hexafluoropropanol varied from 32% for 11-Pro-LAP-20 to 59% for 1-Pro-LAP-20 (Table I). As the position of the proline was moved closer to the midpoint of the 20-residue peptide, the helical content decreased dramatically; this behavior was in fairly good agreement with the Chou-Fasman analysis in Fig. 1. The decreased helical content suggests that the helical potential is, as expected, a sensitive function of the location of the proline residues. Density gradient ultracentrifugation of the peptide:DMPC mixtures permitted isolation of complexes with these same peptides (Fig. 3). However, the 15-Pro-LAP-20 lipid-peptide complex was unstable to recentrifugation. In the complex with DMPC, the 1- and 5-Pro-LAP-20 formed the complexes with the highest helical contents, 63% and 54%, respectively; 19-Pro-LAP-20 had 47% helix. 15-Pro formed a quasi stable complex (24-38% $\alpha$-helix) that was unstable to recentrifugation. The 8- and 11-Pro-LAP-20 did not form a stable complex or show changes in their helical contents in the presence of DMPC. Therefore, the restriction of helix propagation in a hydrophobic peptide imposed by the introduction of a proline residue prevents the formation of a stable phospholipid-peptide complex when the helical segment is less than 15 residues, irrespective of the hydrophobicity of the peptide.

![FIG. 2. Effect of the concentration of POPC on the blue shift of tryptophan fluorescence of the apolipoproteins. LAP-20, ●; 5-Pro-, ○; 15-Pro-, □; 8-Pro-LAP-20, ▲. 2.5 μM was incubated overnight at 23°C in the presence of various concentrations of POPC liposomes. The wavelengths corresponding to the maximum of fluorescence (λmax) were determined after correction of the spectra for background due to POPC light scattering.](image)

**TABLE I**

| Peptide | Hexafluoropropanol | Buffer | +DMPC | Complex |
|---------|--------------------|--------|-------|---------|
| 1-Pro   | 200                | 67     | 170   | 210     |
| 5-Pro   | 150                | 24     | 130   | 180     |
| 8-Pro   | 98                 | 23     | 39    |         |
| 11-Pro  | 93                 | 18     | 20    |         |
| 15-Pro  | 110                | 10     | 120   | 63      |
| 19-Pro  | 120                | 86     | 130   | 150     |

**FIG. 1.** A computer representation of the Chou-Fasman predicted structure of Pro-LAP-20 peptides. The average hydrophobicity (H.I.) in calories/residue was calculated using the scale of Bull and Breese (25). In the fourth peptide, the labels of $\alpha$, $\beta$, and $\gamma$ represent the regions containing $\alpha$-helix, $\beta$-turn, and random structure, respectively. The labels are omitted in the other peptides.
faces of both parts could not be in simultaneous contact with the same surface. This observation could explain why the lipophilicity of a peptide containing a helix breaker in the middle of the primary structure is so much lower than one with a similar hydrophobicity but without a helix breaking residue.

**Lecithin:Cholesterol Acyltransferase Activation**—Apo-A-I and several synthetic peptides have been shown to activate lecithin:cholesterol acyltransferase in vitro (9, 12, 14, 15, 26, 27). Therefore, we compared the activation of each peptide with that of apo-A-I as follows. Initial velocities were measured in the presence of various concentrations of substrates, which consisted of either DMPC/[3H]cholesterol/apo-A-I (100:20:0.46, m/m) or DMPC/[3H]cholesterol/LAP-20 (100:2:5, m/m). These substrates give an equivalent mass for both apo-A-I and the peptides. The results of the substrate saturation experiments were expressed in terms of the Eadie-Hofstee representation, which is that $V_0 = \frac{(V_0/[S])}{(K)} + V_{max}$, where $V_0$ is the initial velocity, $K$ is the Michaelis-Menten constant, $[S]$ is the molar concentration of substrate, and $V_{max}$ is the maximum reaction velocity. With the exception of 8-Pro-LAP-20, all peptides activated lecithin:cholesterol acyltransferase. The values of the apparent $K$ and $V_{max}$ were calculated from the Eadie-Hofstee plots, which appeared reasonably linear (Fig. 5). The apparent $K$ values were approximately the same for all peptides and for apo-A-I; these were 0.21, 0.17, 0.23, and 0.08 mM for apo-A-I, LAP-20, 5-Pro-, 15-Pro-LAP-20, and 8-Pro-LAP-20, respectively. The values of $V_{max}$ were more variable with the highest value being observed with apo-A-I (2.4 nmol/h); the values observed with LAP-20, 5-Pro-, 15-Pro-, and 8-Pro-LAP-20 were 0.6, 0.07, 0.02, and 0.00 nmol/h, respectively. The activation correlated with that of the lipid-binding hypothesis of Segrest et al. (6), our results suggest that $\alpha$-helicity and calculated hydrophobicity are independent determinants of the affinities of model peptides for lipid surfaces. Studies of these peptides with space-filling models showed that portions of the peptide on either side of the proline could be placed in a helix, but that the hydrophobic

The binding of the synthetic peptides to M-HDL, which was further quantified by equilibrium dialysis, was independent of the M-HDL concentration (Fig. 4); the value of $K$ was obtained from the slopes of the indicated lines. These were 35,000 for LAP-20 and 1-Pro- (○), 5-Pro- (X), 15-Pro- (■), and 8-Pro-LAP-20 (▲) were done after 4 days of dialysis. For each peptide, $K$ was obtained from the slope of the regression line.

**Fig. 3.** Density gradients of mixtures of DMPC and proline-substituted LAPS. The samples were prepared as described under "Experimental Procedures." The identity of each peptide is indicated in each panel.

**Fig. 4.** Determination of the equilibrium constant, $K$, for the binding of apoepitopes to M-HDL, at 37 °C. Measurements of the ratio of M-HDL bound to free peptide ($B/F$) for LAP-20 (○), 1-Pro- (○), 5-Pro- (X), 15-Pro- (■), and 8-Pro-LAP-20 (▲) were done after 4 days of dialysis. For each peptide, $K$ was obtained from the slope of the regression line.

**Fig. 5.** Eadie-Hofstee plots of the lecithin:cholesterol acyltransferase activity. Lecithin:cholesterol acyltransferase activity was assayed at 37 °C by measuring the formation of [3H]cholesterol esters from substrates which consisted of DMPC/[3H]cholesterol (100:2, molar ratio) containing 0.46 mol% apo-A-I (○) or 5 mol% either LAP-20 (○), 5-Pro- (X), or 15-Pro-LAP-20 (■). The reaction was initiated by the addition of 10 μl of lecithin:cholesterol acyltransferase (80 μg/ml) and stopped after 15 min by adding 0.5 ml of methanol. For convenience, the concentrations of "substrate," $[S]$, were expressed in terms of DMPC molarity. The apparent $K$ and $V_{max}$ values are given by the slopes of the straight lines and their $y$ intercepts, respectively. To permit a clear visualization of the data, the inset shows 20 times magnification of both the $y$ and $x$ axes. The activation of lecithin:cholesterol acyltransferase by 8-Pro-LAP-20 was nil, and, therefore, the corresponding data do not appear on this figure.
of peptide binding to the M-HDL (Fig. 6). This confirmed that there was a direct relationship between binding and activation as previously noted by Fukushima et al. (9). This relationship was also noted with a series of peptides with similar helical potentials but different hydrophobicities (15), so that helical potential alone does not lead to activation. Rather, our results support the concept that the model apo peptides stimulate lecithin:cholesterol acyltransferase through the activation of a phospholipid surface by their bound helical segments.

The present results and the past results from this (15) and other laboratories (8-13) support the overall validity of the amphipathic helical theory of lipid-associating proteins; i.e. that both hydrophobicity and helical potential might function as independent determinants of this class of peptides with both being required for the interaction with phospholipid surfaces. Previously, we reported that the hydrophobicity of a peptide can be increased by the covalent attachment of a saturated acyl group to the amino terminus. The linear relationship between the length of the acyl group and the free energy of association with lipids suggested that the free energies of association of the peptide and acyl chains for lipid surfaces were additive (15). Subsequently, we found that the plasma turnover times of the synthetic peptides in the rat were a predictable function of their affinities for M-HDL (28); whereas those bound to HDL are taken up by tissues with HDL receptors. A similar finding was reported by Glass et al. (30) that helical potential alone does not lead to activation.

FIG. 6. Correlation between LCAT activation and lipid-peptide interaction. Values for Vₘₐₓ and Kₑq values were calculated from Figs. 2 and 3, respectively.

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