Use of Synthetic Peptide Analogues to Localize Lecithin: Cholesterol Acyltransferase Activating Domain in Apolipoprotein A-I

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The major protein of high density lipoprotein (HDL), apolipoprotein (apo) A-I, is the major activator of the plasma enzyme: lecithin: cholesterol acyltransferase (LCAT). A consensus amino acid sequence has been defined for the eight, 22-residue long, tandem amphipathic helical repeats located in the carboxy-terminal region of apo A-I. A series of 22 and 44mer synthetic peptide analogues of the consensus domain, differing only in their 13th amino acid residue, were prepared and tested for LCAT activation. One of the peptides was found to equal apo A-I in LCAT activation. This is the first time a peptide activator for LCAT that rivals the activity of apo A-I in the vesicular and discoidal egg phosphatidylcholine assay systems has been synthesized. Based on these results, we propose that the major LCAT-activating domain of apo A-I resides in the 22mer tandem repeats, each containing Glu at the 13th residue and located between residues 66 and 121 in the native apolipoprotein.

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Coronary artery disease is inversely correlated with levels of plasma high density lipoprotein (HDL) and the major HDL protein component, apolipoprotein (apo) A-I. It has been proposed that HDL helps prevent coronary artery disease by removal of cholesterol from the arterial wall via a process called reverse cholesterol transport. The plasma enzyme lecithin: cholesterol acyltransferase (LCAT) is thought to mediate reverse cholesterol transport by trapping cholesterol in the form of cholesteryl ester in the HDL particle for removal by the liver. Apo A-I, a 243 amino acid residue protein, is the major cofactor for activation of LCAT (Table 1). Many apolipoproteins can also serve as co-factors for the enzyme LCAT. However, the extent of activation for these proteins is similar to that of the nonspecific synthetic peptide activators (Table 1). Human apo A-I has, therefore, been studied extensively to localize the LCAT activating domain(s) in its sequence. The strategy has been to compare native fragments or synthetic peptide analogues with apo A-I in in vitro assay systems in an attempt to mimic the activity of the intact apolipoprotein. None of these studies have achieved LCAT activation greater than that achieved with nonspecific, nonhomologous peptide analogues of the amphipathic helix—i.e., no greater than approximately 30% of the activity of intact apo A-I (Table 1). These studies thus provide no information on the localization of the LCAT activating domain(s) in apo A-I.

The entire 198 residue carboxy-terminal domain of apo A-I, representing the fourth exon in the apo A-I gene, seems to have evolved by eight tandem duplications of a primordial sequence of 22 amino acid residues. The primordial 22mer is itself the result of the duplication of an even more primordial sequence of 11 amino acid residues. In every case but one, the eight tandem 22mer repeats are punctuated either by single proline residues at the aminoterminus of each 22mer or by two unpaired 11mers inserted between the 22mers (Figure 1A).

Each tandem 22mer repeat in apo A-I is an amphipathic helix, a secondary structural motif believed to be important for lipid association. Because each tandem 22mer in apo A-I appears to represent a duplication of a primordial gene sequence, as part of our studies of the molecular properties of amphipathic helixes, a consensus sequence approximation of the primordial 22mer, called A-Icons, was derived from the amino acid sequence of human apo A-I: Pro Val Leu Asp Glu Phe Arg Glu Lys Leu Asn Glu X Leu Glu Asp Leu Lys Gin Lys Leu Lys. This sequence was constructed by selection of the most prevalent residue at each position. Compared to the other 21 residues in the consensus sequence, residue 13, denoted by X, has a marked and unusual amino acid polymorphism. When
Table 1. Percent LCAT Activation by Different Activators in Different Assay Systems Relative to Apolipoprotein A-I

<table>
<thead>
<tr>
<th>Activator</th>
<th>Vesicular Egg PC</th>
<th>Discoidal Egg PC</th>
<th>DMPC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apo A-I</td>
<td>100 (Chen et al., 1985)</td>
<td>100 (Steinmetz, 1985)</td>
<td>100* (Steinmetz, 1985)</td>
</tr>
<tr>
<td>Apo A-IV</td>
<td>28 (Steinmetz, 1985)</td>
<td>143 (Steinmetz, 1985)</td>
<td>18 (Jonas, 1986)</td>
</tr>
<tr>
<td>Apo A-II</td>
<td>2 (Steinmetz, 1985)</td>
<td>18 (Jonas, 1986)</td>
<td>34 (Chung, 1985)</td>
</tr>
<tr>
<td>Apo C-I</td>
<td>12 (Jonas, 1986)</td>
<td>18 (Jonas, 1986)</td>
<td>30 (Chung, 1985)</td>
</tr>
<tr>
<td>Apo C-II</td>
<td>3 (Jonas, 1986)</td>
<td>85 (Kumari, 1987)</td>
<td>460 (Epand, 1988)</td>
</tr>
<tr>
<td>Apo C-III-1</td>
<td>5.4 (Jonas, 1986)</td>
<td>4 (Jonas, 1986)</td>
<td></td>
</tr>
<tr>
<td>Apo C-III-2</td>
<td>4 (Jonas, 1986)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAP-20</td>
<td>18 (Yokoyama, 1980)</td>
<td>50 (Pownall, 1980)</td>
<td></td>
</tr>
<tr>
<td>AP</td>
<td>18 (Jonas, 1986)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GALA</td>
<td>18 (Chung, 1985)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[Glu^{18}Leu^{117}]18A</td>
<td>34 (Epand, 1987)</td>
<td>460 (Epand, 1988)</td>
<td></td>
</tr>
</tbody>
</table>

*35% as active as apo A-I in egg PC vesicular assay system.

LCAT = lecithin:cholesterol acyltransferase, Apo = apolipoprotein, PC = phosphatidylcholine, DMPC = dimyristoylphosphatidylcholine.

the consensus sequence is organized as a helical wheel (Figure 1B). All amino acid residues are radially distributed as a typical apolipoprotein amphipathic helix, except for residue 13. Although residue 13 is positioned on the nonpolar face of the consensus sequence, six out of the eight tandem repeats have charged residues at this position, and a seventh is polar. None of the other apolipoproteins, including apo E and A-IV, which also have tandem 22mer repeats, have polar residues at defined positions on the nonpolar faces of their consensus amphipathic helical domains. Although both apo E and apo A-IV activate LCAT, when vesicular egg phosphatidylcholine (PC) is used as the substrate, neither is more effective than the most effective synthetic nonspecific amphipathic helixes, i.e., 30% of apo A-I (Table 1).

The anomalous nature of residue 13 in the consensus sequence suggested to us the possibility that residue 13 in several or all of the tandem 22mer repeats might play a role in the LCAT-activating properties of apo A-I. To investigate this possibility, four 22mer peptide analogues of the consensus sequence differing only in the 13th residue, and four 44mer peptides representing three homo- and one heterodimeric combinations of the four 22mer peptides, were synthesized. Stereo pair α-helical models for two of the monomers, [Glu^{18}A-I_{18}]_{18} and [Arg^{17}A-I_{17}]_{17}, are shown in Figure 2. The rationale for synthesis of dimeric peptide analogues was our previous observation that dimeric analogues of model amphipathic helices more effectively mimic many of the properties of apo A-I than do the monomeric analogues. We describe here the properties of these peptides with respect to: 1) their relative affinity for phospholipid and 2) their relative ability to activate LCAT by using two different egg lecithin assay systems.

Methods

Apo A-I was purified as described earlier. The enzyme LCAT was purified according to the method described elsewhere.

Peptide Synthesis

Peptides were synthesized by the solid phase synthesis procedure described elsewhere. The crude peptides were dissolved in 6 M of guanidine hydrochloride, dialyzed against water (Spectrapor dialysis membrane, 1000 Mr cutoff), lyophilized, and purified by using high-performance liquid chromatography (HPLC).

Peptide Characterization

The sequence and purity of peptides were confirmed by amino acid analysis, peptide sequencing, and analytical HPLC. Table 2 gives amino acid analyses of the eight synthetic peptides used in these studies. A representative HPLC purification profile of a 22mer and a 44mer analogue and the analytical HPLC of the purified peptides are given in Figure 3.

Peptides were dissolved in guanidine hydrochloride (6 M) to obtain a concentration of about 5 mg/ml. These solutions were dialyzed against phosphate buffer (pH 7.4) by using 1000 Mr cutoff dialysis bags to ensure the proper pH of solutions of peptides. The concentrations of the solutions were determined by quantitative amino acid analysis.

Electron Microscopy of Peptide-Dimyristoyl Phosphatidylcholine Complexes

Peptides complexed with multilamellar vesicles of dimyristoyl phosphatidylcholine (DMPC, Avanti Polar Lipids, Birmingham, AL) at a 1:2.5 (peptide/DMPC) weight ratio were stained with 2% potassium phosphotungstate, pH 5.9 and were examined with a Philips EM400 microscope on carbon-coated Formvar grids.

Binding of Peptide/Apolipoprotein A-I to Egg Phosphatidylcholine/Cholesterol Small Unilamellar Vesicles

Peptides and apo A-I were dissolved in guanidine HCl buffer dialyzed against Tris buffer (pH 7.5), and the
concentrations of solutions were determined as described above. Egg lecithin and unesterified cholesterol (9:2 molar ratio) containing a trace of $^3$H-labeled cholesterol were sonicated in a bath sonicator for 1 hour at 23°C. Binding of the peptide dimers and apo A-I to the lipid was measured by the procedure of Yokoyama et al. The results of these studies are summarized in Table 3.

**LCAT Activation**

**Egg Phosphatidylcholine Vesicular Assay System**

Egg PC vesicles containing the unesterified cholesterol (preparation described above) were used to determine the percent conversion of free cholesterol to cholesteryl ester. The assay was conducted by incubating a mixture of 45 nM of egg lecithin and 10 nM of unesterified cholesterol, 1 mM of ethylenediaminetetraacetic acid (EDTA), 4 mM 2-mercaptoethanol, 2.5 mg of bovine serum albumin, (pH 7.4), and varying amounts of activator (peptide or apo A-I) in a total volume of 150 μl for 1 hour at 37°C. The reaction was initiated by adding 50 μl of suitably diluted purified enzyme and incubating for 1 hour at 37°C. Free cholesterol and cholesteryl ester were separated by thin-layer chromatography on silica.
Figure 2. Photographs of consensus sequences modeled as α helices on the Evans and Sutherland PS 300 using the TRIPOS SYBYL software package. A. [Glu13]A-1opp. B. [Arg13]A-Uopp. Stereo pairs viewed from the carboxyl-terminal end. The nonpolar face is oriented toward the top of the photograph. (Lower) Stereo pairs viewed from the polar face. The amino terminus is oriented toward the top of the photograph.
gel. The percent conversion of cholesterol to ester was calculated for increasing amounts of activator.

**Preparation of Peptide or Protein/Egg Phosphatidylcholine Discoidal Particles**

Egg PC containing \(^{3}H\)-cholesterol (9:2 molar ratio) was vortexed and mixed with apo A-I or peptides at a peptide/egg PC ratio of 1:2 (wt/wt). The mixture was solubilized in an excess of 1% sodium cholate (approximately 300 \(\mu\)t) and was dialyzed against Tris buffer overnight by using 50 000 Mr cutoff dialysis bags. Analysis of the concentration of proteins (by amino acid analysis), cholesterol (by radiolabel remaining), and phospholipid\(^{29}\) indicated no loss of any of them.

**Egg Phosphatidylcholine Discoidal Assay System**

The peptide or protein/egg PC complexes were incubated with purified LCAT at 37°C for varying periods of time. Cholesterolester formation was assayed as described for the sonicated vesicles.

**Results**

As one means of determining the relative affinity for phospholipid, the different consensus peptides were mixed with multilamellar vesicles of DMPC, and their ability to convert the multilamellar vesicles to minimal size discoidal particles was determined. Apo A-I and various peptide analogues of the amphipathic helix have been shown to act as polypeptide detergents to convert turbid suspensions of multilamellar DMPC vesicles into clear solutions containing small discoidal protein-lipid complexes.\(^{14,15,21}\) The conversion to discoidal particles was followed by light-scattering analysis, negative stain electron microscopy, and nondenaturing gradient gel electrophoresis.\(^{14,15,21}\) The results of the electron microscopic studies of the peptide-DMPC complexes for the eight different consensus sequence peptides are shown in Figure 4; results consistent with these were found by light-scattering and gradient gel analyses (data not shown). As determined in earlier studies from this laboratory,\(^{14}\) the diameters of the discoidal peptide-DMPC particles vary inversely with the lipid affinity of the peptide analogues. Although the consensus sequence analogues differ by only one amino acid residue in the 13th position, they have widely varying lipid affinities as judged by the size of the discoidal complexes. The 44mer dimer analogues had a higher affinity than did the 22mer monomer analogues. The approximate rank order lipid affinities for the dimers were ([(His\(^{13}\)A-I\(^{\text{lon}}\)]\(^{2}\)) (HH) > ([Arg\(^{13}\)A-I\(^{\text{lon}}\)]\(^{2}\)) (RR) > ([Arg\(^{13}\)A-I\(^{\text{lon}}\)]([Glu\(^{13}\]A-I\(^{\text{lon}}\)]) (EE); the rank order among monomers was ([Ala\(^{13}\]A-I\(^{\text{lon}}\)] (A) > [His\(^{13}\]A-I\(^{\text{lon}}\)] (H) > [Arg\(^{13}\]A-I\(^{\text{lon}}\)] (R) > [Glu\(^{13}\]A-I\(^{\text{lon}}\)] (E).

The peptide analogues EE, RE, RR, and HH and intact apo A-I were examined for their ability to bind to egg PC: cholesterol small unilamellar vesicles (SUV) used as the substrate for the LCAT activation studies. At the three concentrations of apo A-I and peptides studied, all four activators had essentially indistinguishable SUV binding characteristics (Table 3).

The consensus peptides were then compared with apo A-I for the ability to activate the enzyme LCAT. Criteria used to judge apo A-I mimicry were extent of esterification at equilibrium at low activator concentrations and rate of esterification. In the first set of experiments, the SUV assay system was used; sonicated egg lecithin containing unesterified cholesterol and a trace of radiolabeled cholesterol served as the substrate for LCAT. The reaction was carried to equilibrium. Apo A-I is known to be the best activator of LCAT in this assay system. Apo A-I is also a better activator in this system than in any other system (Table 1).

The results of these studies are shown in Figure 5. In general the 22mer monomers were found to be poorer activators of LCAT than the 44mer dimers. The R monomer and the RR dimer produce approximately the same degree of esterification. Both are considerably more effective than the E monomer; however, the EE dimer is the most effective of the peptides. At lower activator concentrations, EE equaled apo A-I activation at equilibrium.

### Table 2. Amino Acid Analysis of Synthetic Peptides

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>E</th>
<th>R</th>
<th>H</th>
<th>A</th>
<th>EE</th>
<th>RE</th>
<th>RR</th>
<th>HH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>1.3 (1)</td>
<td>1.1 (1)</td>
<td>1.2 (1)</td>
<td>2.5 (2)</td>
<td>2.3 (2)</td>
<td>2.4 (2)</td>
<td>2.3 (2)</td>
<td>2.5 (2)</td>
</tr>
<tr>
<td>Arg</td>
<td>1.2 (1)</td>
<td>2.1 (2)</td>
<td>0.6 (1)</td>
<td>1.2 (2)</td>
<td>1.8 (2)</td>
<td>2.6 (3)</td>
<td>4.0 (4)</td>
<td>1.5 (2)</td>
</tr>
<tr>
<td>Asp</td>
<td>2.3 (2)</td>
<td>2.3 (2)</td>
<td>1.4 (2)</td>
<td>1.7 (2)</td>
<td>3.5 (4)</td>
<td>3.3 (4)</td>
<td>4.1 (4)</td>
<td>4.4 (4)</td>
</tr>
<tr>
<td>Glu</td>
<td>7.2 (6)</td>
<td>5.4 (5)</td>
<td>5.1 (5)</td>
<td>5.8 (5)</td>
<td>12.0 (12)</td>
<td>9.9 (11)</td>
<td>10.9 (10)</td>
<td>11.5 (10)</td>
</tr>
<tr>
<td>Leu</td>
<td>5.8 (5)</td>
<td>3.4 (3)</td>
<td>4.6 (5)</td>
<td>5.4 (5)</td>
<td>10.3 (10)</td>
<td>10.6 (10)</td>
<td>10.6 (10)</td>
<td>11.3 (10)</td>
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<tr>
<td>Lys</td>
<td>3.9 (4)</td>
<td>4.9 (5)</td>
<td>3.7 (4)</td>
<td>3.4 (4)</td>
<td>8.1 (8)</td>
<td>8.4 (8)</td>
<td>7.5 (8)</td>
<td>8.6 (8)</td>
</tr>
<tr>
<td>Phe</td>
<td>1.1 (1)</td>
<td>0.9 (1)</td>
<td>0.9 (1)</td>
<td>0.7 (1)</td>
<td>1.8 (2)</td>
<td>1.8 (2)</td>
<td>1.8 (2)</td>
<td>1.9 (2)</td>
</tr>
<tr>
<td>Pro</td>
<td>1.3 (1)</td>
<td>1.1 (1)</td>
<td>0.7 (1)</td>
<td>1.3 (1)</td>
<td>1.8 (2)</td>
<td>1.7 (2)</td>
<td>2.0 (2)</td>
<td>1.5 (2)</td>
</tr>
<tr>
<td>Val</td>
<td>1.2 (1)</td>
<td>0.9 (1)</td>
<td>0.9 (1)</td>
<td>0.8 (1)</td>
<td>1.7 (2)</td>
<td>1.6 (2)</td>
<td>1.7 (2)</td>
<td>1.4 (2)</td>
</tr>
<tr>
<td>His</td>
<td>0.8 (1)</td>
<td>0.8 (1)</td>
<td>0.8 (1)</td>
<td>0.8 (1)</td>
<td>1.6 (2)</td>
<td>1.6 (2)</td>
<td>1.6 (2)</td>
<td>1.6 (2)</td>
</tr>
</tbody>
</table>

The calculated values are given in parentheses.

E = [Glu\(^{13}\]A-I\(^{\text{lon}}\], R = [Arg\(^{13}\]A-I\(^{\text{lon}}\], H = [His\(^{13}\]A-I\(^{\text{lon}}\].

Dimer peptides: EE = [[Glu\(^{13}\]A-I\(^{\text{lon}}\)[Glu\(^{13}\]A-I\(^{\text{lon}}\], RE = [[Arg\(^{13}\]A-I\(^{\text{lon}}\)[Glu\(^{13}\]A-I\(^{\text{lon}}\], RR = [[Arg\(^{13}\]A-I\(^{\text{lon}}\)\(^{2}\], HH = [[His\(^{13}\]A-I\(^{\text{lon}}\)]\(^{2}\].

Peptides were hydrolyzed by using 6 N HCl at 110°C for 24 hours. The amino acids were analyzed by using the PicOTAG system (Waters system, Millipore division, Millford, MA).
Croda

Figure 3. High-performance liquid chromatography (HPLC) of (A) crude [Glu$^{13}$]$A_{-10n}$, (B) purified [Glu$^{13}$]$A_{-10n}$, (C) crude ([Glu$^{13}$]$A_{-10n}$)$_2$, and (D) purified ([Glu$^{13}$]$A_{-10n}$)$_2$. HPLC was run on a reversed-phase C-18 column by using water/acetonitrile (in presence of 0.1% trifluoroacetic acid [TFA]) gradient of 30% to 50% at a gradient of 1% per minute.

Figure 4, and RE equaled apo A-I at higher activator concentrations.

Figure 6 compares negative stain electron micrographs of control liposomes and egg PC/cholesterol liposomes complexed with the two most active LCAT activators, EE and RE, at the highest concentrations of peptide studied. Examination of the incubation mixtures of all of the consensus peptides with egg lecithin SUV showed that none of the eight analogues formed discoidal particles from egg lecithin at any of the peptide concentrations examined.

To test the 44mer peptides in the discoidal system, discoidal complexes of peptide/egg lecithin were pre-

Table 3. Protein or Peptide Bound to Egg PC:Cholesterol Small Unilamellar Vesicles

<table>
<thead>
<tr>
<th>Protein or peptide in starting solution*</th>
<th>Apo A-I</th>
<th>EE</th>
<th>RE</th>
<th>RR</th>
<th>HH</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>4.2</td>
<td>3.2</td>
<td>2.7</td>
<td>3.7</td>
<td>3.2</td>
</tr>
<tr>
<td>10</td>
<td>7.0</td>
<td>5.1</td>
<td>5.5</td>
<td>5.9</td>
<td>5.8</td>
</tr>
<tr>
<td>25</td>
<td>15.0</td>
<td>14.4</td>
<td>13.2</td>
<td>13.6</td>
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</tr>
</tbody>
</table>

All values are micrograms.

*Small unilamellar vesicles of egg PC:cholesterol were prepared as described in the Methods, and 20 μl was mixed with different concentrations of activator peptides or apo A-I. Proteoliposomes were prepared by using the same conditions as in the egg PC vesicular lecithin:cholesterol acyltransferase assay system. Binding of peptides or apo A-I was analyzed according to the procedure of Yokoyama et al.19
Figure 4. (A) Negative stain electron microscopy of peptide dimyristoyl phosphatidylcholine (DMPC) complexes at a 1:2.5 weight ratio. (B) Morphographic analysis of diameters of discoidal complexes of analogue peptides with DMPC. 1. [Glu\(^{13}\)]A-l-con. 2. [Ala\(^{13}\)]A-l-con. 3. [Arg\(^{13}\)]A-l-con. 4. [His\(^{13}\)]A-l-con. 5. ([Glu\(^{13}\)]A-l-con\(^2\)). 6. [Arg\(^{13}\)]A-l-con\(^2\). 7. [His\(^{13}\)]A-l-con\(^2\). 8. ([His\(^{13}\)]A-l-con\(^2\)).

Discussion

The theory of the lipid-associating amphipathic helix originated with the proposal by our laboratory in 1974 that members of the class of exchangeable apolipoproteins contain amphipathic \(\alpha\) helical domains involved in lipid association.\(^{10}\) The amphipathic helix is defined as an \(\alpha\) helix with opposing polar and nonpolar faces.

Based on our initial observation, amphipathic helical domains have been described for other putative lipid-
Figure 5. Activation of lecithin:cholesterol acyltransferase by synthetic peptide analogues as measured by the egg lecithin small unilamellar vesicle procedure. 
1. (O—O) = [Glu^{3}]A-l\text{con}.
2. (□—□) = [Ala^{3}]A-l\text{con}.
3. (Δ—Δ) = [Arg^{3}]A-l\text{con}.
4. (V—V) = [His^{3}]A-l\text{con}.
5. (●—●) = ([Glu^{3}]A-l\text{con})_{2}.
6. (●—●) = [Arg^{3}]A-l\text{con}[Glu^{3}]A-l\text{con}.
7. (Δ—Δ) = ([Arg^{3}]A-l\text{con})_{2}.
8. (V—V) = ([His^{3}]A-l\text{con})_{2}.
9. (●—●) = apo A-l.

associating proteins, such as certain polypeptide hormones. The amphipathic helix class associated with the apolipoproteins, as opposed to that associated with polypeptide hormones, is unique. Positively charged residues cluster at the polar-nonpolar interface, and negatively charged amino acid residues cluster at the center of the polar face. As can be seen in Figure 2, the bulk of the van der Waals’ surface areas of the positively charged residues at the interface of the amphipathic helix are hydrophobic. We propose that these amphipathic basic residues, when associated with phospholipid, extend toward, and approximately perpendicular to, the polar face of the helix to insert their charged moieties into the aqueous milieu for aqueous solvation. We suggest, therefore, that essentially the entirety of the uncharged van der Waals’ surface of the amphipathic helices of apolipoproteins can be buried within the hydrophobic interior of a phospholipid monolayer. The positively charged cluster is believed to provide additional, favorable free energy of lipid association via the contribution of the significantly hydrophobic portion of the lysyl and arginyl side chains to the overall hydrophobicity of the amphipathic helix.

The results of several attempts to localize the LCAT activating domain in apo A-l are summarized in Table 1. CNBr fragments of apo A-l have been subjected to LCAT activation studies. There are three Met residues in the human apo A-l sequence at positions 86, 112, and 148. CNBr degradation thus results in two larger and two smaller fragments. The two larger CNBr fragments of apo A-l have been shown to activate LCAT only to the extent of 20% to 25% of apo A-l. The smaller two CNBr fragments have been shown to have essentially no LCAT activating ability.

Fukushima et al. have synthesized the 121 to 164 residue segment of human apo A-l and found that it was 30% as active as intact apo A-l in the activation of LCAT. Sparrow and Goto have synthesized five peptides, representing sequences located between residues 142 and 185 of apo A-l and have studied their ability to activate LCAT. The full-length peptide was only 24% as active as apo A-l in the activation of LCAT, and the activity of the intermediate-length peptides decreased directly with their lengths. De novo-designed synthetic peptide analogues of the amphipathic helix have also been shown to activate LCAT. Yokoyama et al. using de novo-designed amphipathic helical peptides and egg lecithin as substrate, were able to achieve a
maximal LCAT activation of 18% of that produced by apo A-I.

It is well known that saturated phospholipids such as DMPC are considerably poorer substrates than are unsaturated phospholipids such as egg lecithin for LCAT activated by apo A-I.28 In fact, in the DMPC assay system several synthetic peptides from our laboratory are markedly more active than apo A-I.27 However, these same peptides are, at most, only 30% as active as apo A-I in the egg lecithin assay system.26 Apo A-I has a strong affinity for hydrated egg lecithin (Table 3) but will not disrupt the vesicular structure of this lipid.15 By contrast, peptides with a weaker affinity for hydrated DMPC than apo A-I can, nevertheless, disrupt the vesicular structure of DMPC.15 Therefore, as we proposed previously, it is probable that the size of the DMPC discoidal particles produced (disc diameter is an inverse function of the lipid affinity of the peptide) determines the extent of conversion of cholesterol to cholesteryl ester in this system;16 the smaller discs have the higher LCAT activation.

Despite the drawbacks associated with the DMPC system, there are several reports in which this system has been used to compare LCAT activation by apo A-I with activation by other cofactors, such as synthetic peptides. Using the DMPC system, a synthetic peptide designed by Pownall et al.29 was reported to be 50% as active as apo A-I. In a recent report, again using the DMPC system, acidic amphipathic peptide analogues were claimed to be 85% as active as apo A-I.30 It has also been reported that apo A-IV is more active than apo A-I; however, when using unsaturated lipids, apo A-I is at least four times as active as apo A-IV.26 We believe that investigations of mechanisms of LCAT activation with the DMPC assay system are an inappropriate way to compare peptide analogues of the amphipathic helix with apo A-I.

Because each tandem 22mer in apo A-I appears to represent a duplication of a primordial gene sequence, as part of our studies of the molecular properties of amphipathic helices, a consensus sequence approximation of the primordial 22mer, called A1con, was derived from the amino acid sequence of human apo A-I. A consensus nucleotide sequence has been proposed for apo A-I.31 The peptide derived from this consensus sequence does not have an amphipathic helical structure and, therefore, the consensus amphipathic helical sequence was derived from the 22mer repetitive amino acid sequence.

In earlier studies,16 we observed that nonspecific amphipathic peptide discoidal complexes were only approximately 30% to 40% as good LCAT substrates as apo A-I discoidal complexes, even though the same peptide-discoidal complexes were better LCAT substrates than apo A-I-SUV complexes.16 Since the 44mer analogue EE equals the equilibrium LCAT activating ability of apo A-I at the same concentrations at which apo A-I also reaches its maximum, we conclude that the EE dimer is a close mimic of the LCAT activating ability of apo A-I in the SUV system. As noted, RR, the only other peptide analogue that equals the LCAT activating ability of apo A-I in the SUV system, does so only at higher concentrations.

The enzyme LCAT cleaves the acyl chain at the sn-2-position of a glycerophospholipid molecule and then transfers the chain to the hydroxyl group of cholesterol. The products, free fatty acid and cholesteryl ester, therefore, depend on the environment of the acceptor hydroxyl group. It is possible that not all the free fatty acid product is converted to cholesteryl ester. For the sake of simplicity of studying apo A-I mimicry, we choose to follow the conversion of labeled free cholesterol to its cholesteryl ester rather than following the formation of free fatty acid.

Our present results indicate that, in two different assay systems, the EE dimer closely mimicked apo A-I as an activator of LCAT. When appropriate criteria were used for comparison, this peptide was the most active LCAT-activating peptide analogue, synthetic or native fragment, yet described. While the RE dimer can produce cholesteryl ester in comparable amounts to that of EE, this occurred only at higher concentrations or after longer incubation periods. The RR dimer had significantly less effect on LCAT than either EE or RE. We therefore speculate that the Glu half of the Arg-Glu dimer is the active portion of this peptide, producing an equal esterification of cholesterol only at higher concentrations or after longer incubation times.

The distribution of the 22mer tandem repeats in the carboxy-terminal 198 residues of apo A-I (exon 4) is diagrammed in Figure 1C. Each repeat is identified by the amino acid residue at the 13th position. Repeats two and three (residues 66 to 121) separated by an unpaired 11mer contain the only two Glu residues present in the 13th position of the eight repeats. As noted earlier, examination of CNBr fragments of apo A-I for LCAT
activation have failed to identify a fragment with activity comparable to that of intact A-I.\textsuperscript{9} It should be noted, therefore, that two of the three Met residues in apo A-I are located between residues 66 and 121 and that the second Met is immediately adjacent to the second Glu; the putative LCAT activation domain would thus be extensively disrupted by CNBr fragmentation.

We therefore propose that a major LCAT-activating domain of apo A-I is associated with the two Glu-containing, 22mer tandem repeats located between residues 66 and 121 in the native apolipoprotein. Consistent with this proposal is the conservation of these two Glu in apo A-I from other species.\textsuperscript{32,33} Figure 8 shows a comparison of the two 22mers containing Glu at the 13th position and A-I\textsubscript{loop}. Since ([Glu\textsubscript{13}]A-I\textsubscript{loop})\textsubscript{2} differs from the native sequence of 66 to 121 in having an intervening Pro rather than an unpaired 11mer, and since the analogue EE dimer is a consensus sequence rather than the native sequence, studies are presently underway to test the hypothesis further by direct synthesis of 66 to 121.

This hypothesis does not necessarily imply that the other six 22mer tandem repeats in apo A-I may not also be involved in LCAT activation. While the EE consensus dimer closely mimics apo A-I in artificial LCAT activation systems, apo A-I in the intact HDL particle very likely has interactions with other apolipoproteins, such as apo A-II.\textsuperscript{34} We have shown that half of apo A-I can be removed from HDL without noticeably affecting the ability of that HDL to act as an LCAT substrate.\textsuperscript{16} The latter observation suggests that different microenvironments within an individual HDL particle, for example interactions with other apolipoproteins, change the ability of apo A-I to act as an activator of LCAT. Such interactions may be mediated by an easily reversible lipid-associating region, the hinged domain, that we have previously hypothesized for apo A-I.\textsuperscript{35} The other 22mer tandem repeats could, therefore, play an important role in mediation of interactions with other apolipoproteins, perhaps acting as the hinged domain or serving to regulate the LCAT-activating status of a given apo A-I molecule. Recent studies by Jonas et al.\textsuperscript{36} provide additional support for this hypothesis.

What might be the mechanism whereby the two tandem, Glu-containing, 22mer repeats of apo A-I produce LCAT activation? Perhaps the two Glu residues bind to a specific region at or near the active site of the LCAT molecule within the lipid interior. As part of this mechanism, or separately, the Glu residues might form hydrogen bonds with one of the substrates, for example cholesterol, to produce an activated intermediate. An alternative, and in our opinion a less likely, possibility is that a charged amino acid residue buried in the hydrophobic interior of a phospholipid monolayer or bilayer might alter the lipid organization in such a way as to allow LCAT easier access to one or both of its substrates—cholesterol and one fatty acyl chain of the phospholipid—which are also buried in the hydrophobic interior.\textsuperscript{21}

In conclusion, we propose that a major LCAT-activating domain of apo A-I resides in the two, Glu-containing, 22mer tandem repeats located between residues 66 and 121 in the native apolipoprotein. This model provides a simple working hypothesis testable by site-directed mutagenesis.

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References


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Use of synthetic peptide analogues to localize lecithin:cholesterol acyltransferase activating domain in apolipoprotein A-I.

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