Apolipoprotein E: Phospholipid Binding Studies with Synthetic Peptides from the Carboxyl Terminus†

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ABSTRACT: We have previously shown that the synthetic peptide apoE(129–169) forms lipid–peptide complexes with dimyristoylphosphatidylcholine (DMPC) with an L:P molar ratio of 125:1; the peptide in the isolated complex contains ~56% α-helicity. These results verify the presence of an amphipathic α-helix in this region of apoE as predicted by Chou–Fasman analysis and hydrophobicity calculations. To further define the lipid binding regions of apoE, we have synthesized four peptides, apoE(211–243), -(202–243), -(267–286), and -(263–286), from the carboxyl terminus of apoE and studied their lipid binding properties; apoE(202–243) contains two potential amphipathic helices. Although all four peptides formed α-helices in the helix-forming solvent 30% hexafluoropropanol, we found that only apoE(263–286) formed a stable complex with DMPC. The peptide contained ~80% α-helicity, and its Trp fluorescence spectrum was blue-shifted by 20 nm in the complex which had an L:P ratio of 163:1. We conclude that this sequence is a newly identified lipid binding region of apoE and that the amphipathic helices 203–221 and 226–243 are too hydrophilic to bind phospholipid.

Human plasma chylomicrons, very low density lipoproteins, and high-density lipoproteins contain apolipoprotein E (apoE)[1] which is believed to be necessary for their clearance (Mahley, 1978). ApoE isoforms, which are defective in their ability to interact with the apoB cellular receptors [for a review, see Mahley and Innerarity (1983)], have been reported (Rall et al., 1983; Innerarity et al., 1983) and occur in the lipoprotein disorder type III hyperlipoproteinemia (Mahley & Angelin, 1984). The apoprotein must be bound to phospholipid for receptor binding to occur (Pitas et al., 1980). In the presence of phospholipid, a high α-helical content (65%) is observed for the protein by CD spectroscopy (Innerarity et al., 1984). Amphiphathic helical regions are predicted to occur at positions 60–78, 130–150, 203–221, 226–243, and 245–266 (Rall et al., 1982) and are believed to be responsible for the interaction of apoproteins with phospholipid (Segrest et al., 1974).

Recently, the X-ray structure of the amino-terminal 191 residues of apoE has been reported (Wilson et al., 1991) and showed this segment of apoE to contain 5 helices, comprising more than 80% of the residues, arranged in a 4-helix bundle; residues 1–20 and 165–191 are absent from the crystal structure and are believed to be disordered. The apoE helix bundle contains helices of 19, 28, 36, and 35 amino acids, each lying antiparallel to the adjacent helices, a topology found in most 4-helix bundles. The first two helices (24–42 and 54–84) are connected by a short helix (44–53). Residues 82–86 form a poorly defined β-turn between helix 2 and helix 3 (87–122) which is kinked at Gly_{105}. The LDL receptor binding region is contained in the well-defined helix 4 (130–164); the high positive charge density in this region is a prominant feature in the X-ray structure. There is no clear indication from the X-ray structure of a lipid binding domain per se. Only the interaction of the hydrophobic faces of the helices implies the existence of regions which could interact with phospholipid.

It is known that apoE binds heparin in the presence of calcium, and we have shown that apoE(129–169) and -(202–243) can also bind to highly purified heparin (Cardin et al., 1986; Weisgraber et al., 1986) and show changes in their secondary structure upon binding (Cardin et al., 1989); the binding is also calcium-dependent. In the intact protein, the heparin binding by the 202–243 region is blocked in the presence of phospholipid while the binding by the 129–169 region is not.

We have reported on the phospholipid binding of several synthetic fragments of apoE which contain the putative receptor binding domain (140–150) and the amphipathic helix (130–150) (Sparrow et al., 1985). We found that only the longest peptide, apoE(129–169), can form a stable complex with dimyristoylphosphatidylcholine (DMPC). There was an increase in the α-helical content of the peptide (~56% helical), and a complex of 125 mol of lipid/mol of peptide was isolated by density gradient ultracentrifugation.

To further define the lipid binding regions of apoE, we have studied the properties of synthetic peptides from the carboxyl terminus of apoE including the two amphipathic helical regions 203–221 and 226–243. We report here our results on the synthesis, purification, and interaction of these synthetic apoE peptides with DMPC.

Materials and Methods

The solvents used in the peptide synthesis and for the HPLC purification and analysis were purchased from Burdick and Jackson (Muskegan, MI). The *t*-Boc amino acids were purchased from Bachem (Torrance, CA). Trifluoroacetic acid

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[1] Abbreviations: apoE, apolipoprotein E isolated from human plasma very low density lipoproteins; apoE(202–243), synthetic peptide comprising residues 202–243 of apolipoprotein E; CD, circular dichroism; DMPC, dimyristoylphosphatidylcholine; HFP, hexafluoropropanol; HPLC, high-performance liquid chromatography; *t*-Boc, *tert*-butyloxycarbonyl; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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was from Halocarbon Corp. (Hackensack, NJ) and was distilled. Diisopropylethylamine and acetic anhydride were purchased from Aldrich (Milwaukee, WI) and were distilled before use. The polystyrene resin was from Lab Systems (San Mateo, CA) and was modified according to Sparrow (1976). Cesium chloride was purchased from Alfa (Danvers, MA). Buffer salts were from Fisher, TES was from Sigma (St. Louis, MO), and ultrapure Tris base and guanidine hydrochloride were purchased from Schwarz/Mann (Spring Valley, NJ). The 0.01 M ammonium phosphate buffer was prepared from HPLC-grade water (Burdick and Jackson) and phosphoric acid (Fisher, Pittsburgh, PA) by titrating it with ammonium hydroxide to pH 3.0 and then stabilizing against bacterial contamination with 100 μL of 1 M sodium azide per 4 L of buffer.

Peptide Synthesis. The peptides were synthesized by solid-phase methods (Barany & Merrifield, 1980) using a Schwarz/Mann Bioresearch synthesizer modified for computer control (Edelstein et al., 1981) or an Applied Biosystems, Inc., Model 430A. The *t*-Boc amino acid was attached to aminoundecanoylaminomethylpolystyrene through the oxymethylphenylacetyl linkage (Sparrow, 1976). The peptides were synthesized using the sequence of reactions and washes previously reported (Sparrow et al., 1985) or those supplied by Applied Biosystems for the 430A. Resin was removed after the attachment of residues 211 and 202 in the synthesis starting with Leu_{243} and residues 267 and 263 in the one starting with Ala_{286}, deprotected with TFA, and cleaved from the resin (1 g) by treatment for 3 h at −20 °C with 20 mL of anhydrous HF containing 2 mL of anisole and 0.2 mL of ethanedithiol. The HF was removed under vacuum, and the peptide and resin were washed with ether; the peptide was dissolved in TFA and the resin removed by filtration. The TFA was evaporated in vacuo and the peptide precipitated with ether. After centrifugation, the peptide was dissolved in 1 M Tris–6 M guanidine hydrochloride. In those peptides containing Trp, the formyl protecting group was removed from Trp by cooling the above solution to 4 °C and adding ethanolamine to 1 M; after 5 min, the pH was adjusted to 8 with concentrated HCl. In all cases, the peptide was desalted on a 5 × 50 cm column of BioGel P-2 equilibrated with 0.1 M ammonium bicarbonate; the peptide-containing fractions were lyophilized.

Peptide Purification. The crude peptide was purified by semipreparative reversed-phase HPLC on a 1 × 25 cm column of Vydac 214TP-C_4 equilibrated in 0.01 M $NH_4H_2PO_4$, pH 3.0, at a flow rate of 2.5 mL/min. The crude peptide (~50 mg) was injected in 10 mL of 0.1 M $NH_4H_2PO_4$ and 6 M guanidine hydrochloride, pH 3.0, on a Spectra Physics 8000B liquid chromatograph. The column was developed by stepping to 15% 2-propanol and then running a 120-min linear gradient to 40% 2-propanol. The peptides were eluted between 60 and 90 min and were detected by monitoring the effluent at 280 nm. The major peptide peak was collected, solid guanidine hydrochloride added to a concentration of 4 M, the pH adjusted to 8, and the peptide desalted on a BioGel P-2 column equilibrated with 0.1 M ammonium bicarbonate in HPLC-grade water. The peptide fractions were pooled and lyophilized. Analytical HPLC was performed on a Spectra Physics 8100 chromatograph equipped with an autosampler and fitted with a 0.46 × 25 cm Vydac 214TP-C_4 column; the effluent was monitored at 220 nm. A 1-h linear gradient from 0.01 M $NH_4H_2PO_4$, pH 3.0, to 50% 2-propanol at a flow rate of 1.5 mL/min was used to elute the peptide.

Phospholipid Binding. Approximately 0.5 mg of peptide was dissolved in 1 mL of 0.1 M KF and 0.005 M TES, pH 7.2, and the concentration was determined from the optical density at 280 nm and the theoretical extinction coefficient of each peptide and by amino acid analysis of an aliquot. A dispersion of dimyristoylphosphatidylcholine was prepared by sonicating 140 mg of DMPC in 3.0 mL of 0.005 M TES–0.1 M KF buffer, using a probe sonicator. The dispersion was maintained at 24 °C under nitrogen during the 45 min required to clarify the solution. The dispersion was centrifuged for 30 min at 18 000 rpm in a Beckman J-21 centrifuge to remove any titanium. A modified microphosphorus analysis (Hess & Derr, 1975) was used to determine the concentration.

DMPC and peptide were mixed in appropriate quantities to obtain a molar ratio of 100:1. The screw-cap tubes were placed in a 24 °C bath for 24 h. The CD spectrum was recorded before and after the addition of DMPC as well as after the incubation. The volume of the peptide–lipid mixture was adjusted to 1.5 mL with 0.005 M TES–0.10 M KF, pH 7.2. A solution (2.5 mL) containing 0.005 M TES and 0.928 M CsCl, pH 7.2, was placed in the bottom of a 5-mL polyallomer tube and the peptide–lipid solution 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 added to the top of the centrifuge tube. The tubes were placed in the buckets of a Beckman SW 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-μL aliquots using a peristaltic pump, Densiflow, and a drop counting fraction collector. Each tube was analyzed for peptide by a modified fluorescamine assay (Bohlen et al., 1973) using the peptide for a standard curve and for phospholipid by a modified microphosphorus assay (Hess & Derr, 1975). The density of each fraction was determined from the refractive index using a refractive index calibration curve. The results were plotted in Figures 3 and 4 as milligrams per milliliter of peptide and lipid.

Circular Dichroism. The circular dichroic spectrum was recorded with a Cary 61 spectrometer with an E.G. and G. Princeton Applied Research lock-in amplifier or a Jasco 500 spectrometer interfaced with an IBM computer. The instruments were calibrated with D(−)-pantoyl lactone. The peptide concentration was determined by UV spectroscopy, amino acid analysis of an aliquot, and fluorescamine assay. The cell path length was 0.05 cm, and the spectrum was recorded between 260 and 190 nm. The helicity was estimated by the equation: % α-helix = $[([\theta]_{222} + 3000)/39\,000] \times 100$ (Morrisett et al., 1973).

Results

Peptide Purification and Characterization. After desalting and lyophilization, the peptides eluted from the semipreparative Vydac C_4 column by a linear gradient of 0.01 M $NH_4H_2PO_4$, pH 3.0, and 2-propanol were shown to be 99% pure by analytical high-performance liquid chromatography (Figure 1). Amino acid analyses indicated the peptides had the expected composition (Table I). The yield of purified material varied from 5 to 10% based on the loading of *t*-Boc amino acid on the resin.

Phospholipid Binding. In the absence of phospholipid, the CD spectrum of each peptide showed very little ellipticity at 208 and 222 nm (Table II). In the presence of 30% hexafluoropropanol, the peptides had approximately 87–95% α-helicity. The addition of DMPC to apoE(263–286) induced an increase in the α-helical content to 85% (Figure 2); an enhancement of the intensity and a 20-nm blue shift were observed in the intrinsic Trp fluorescence spectrum (Figure 2, inset). The fluorescence spectrum and the helicity of the

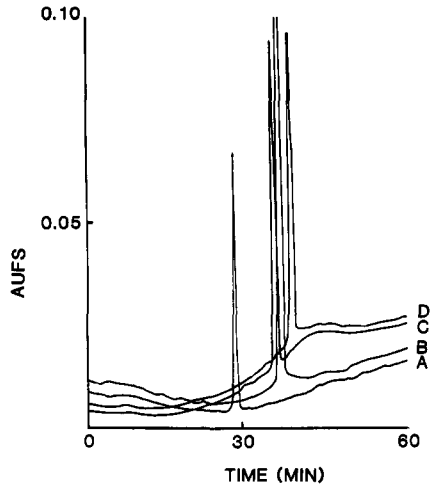

FIGURE 1: Analytical reversed-phase HPLC analysis of the purified peptides. Approximately 100 μg of peptide in 6 M guanidine hydrochloride was injected on a 0.46 × 25 cm Vydac C_4 column. A linear gradient over 1 h of 0.01 M $NH_4H_2PO_4$, pH 3.0, to 50% 2-propanol was used to elute the peptides. The flow rate was 1.5 mL/min, and the effluent was monitored at 220 nm. (A) ApoE-(211–243); (B) apoE(202–243); (C) apoE(267–286); (D) apoE-(263–286).

Table I: Amino Acid Composition of Synthetic ApoE Peptides

amino acid[a]	211–243	202–243	267–286	263–286
aspartic acid	1.84 (2)[c]	1.99 (2)	0.98 (1)	1.00 (1)
threonine[b]	0.83 (1)	0.91 (1)	– (0)	– (0)
serine[b]	0.91 (1)	0.90 (1)	– (0)	0.92 (1)
glutamic acid	7.72 (7)	10.85 (10)	5.50 (5)	6.60 (6)
proline	– (0)	0.87 (1)	0.96 (1)	1.08 (1)
glycine	1.94 (2)	1.99 (2)	0.94 (1)	1.18 (1)
alanine	2.83 (3)	4.85 (5)	2.89 (3)	3.18 (3)
valine	3.00 (3)	3.00 (3)	3.29 (3)	3.24 (3)
methionine	1.72 (2)	1.68 (2)	0.90 (1)	0.86 (1)
leucine	3.00 (3)	4.00 (4)	1.95 (2)	2.15 (2)
phenylalanine	– (0)	– (0)	– (0)	1.12 (1)
lysine	2.04 (2)	2.14 (2)	0.94 (1)	1.01 (1)
arginine	7.21 (7)	8.34 (8)	0.98 (1)	1.01 (1)
tryptophan	– (0)	nd[d] (1)	nd (1)	nd (2)

[a] Amino acid analyses were performed on an LKB analyzer after hydrolysis for 24 h in 6 N HCl at 110 °C. [b] Uncorrected for destruction. [c] The numbers in parentheses are the theoretical values. [d] nd, not determined, but present by UV spectroscopy.

Table II: Ellipticity of Synthetic Fragments of Apolipoprotein E

	mean residue ellipticity,[a] $[\theta]_{222}$				
	peptide				
	aqueous	with DMPC	with HFP	isolated complex	lipid:peptide ratio
apoE(211–243)	–4090	–2975	–36941	NC[b]	NC
apoE(202–243)	–5232	–3190	–33292	NC	NC
apoE(267–286)	–3538	–3669	–31236	NC	NC
apoE(263–286)	–3507	–30040	–34430	–28063	163:1

[a] The mean residue ellipticity at 222 nm, $[\theta]_{222}$, is the average from three experiments and is calculated from $[\theta]_{222} = MRW\theta_{222}/10\,Pc$ where c is the peptide concentration, P is the cuvette path length, MRW is the mean residue weight of the peptide, and θ_{222} is the measured ellipticity angle at 222 nm; MRW's for apoE(211–243), -(202–243), -(267–243), and -(263–243) are 118.6, 118.9, 113.4, and 117.4, respectively. [b] No complex.

remaining peptides did not change significantly. Density gradient ultracentrifugation demonstrated the formation of stable lipid–peptide complexes with apoE(263–286) (Figure 4B); the other peptides did not form complexes (Figures 3A,B and 4A). Analysis for lipid and peptide of the DMPC–apoE(263–286) complex indicated a molar ratio of 163:1 (Table II). The CD spectrum of the isolated complex indicated

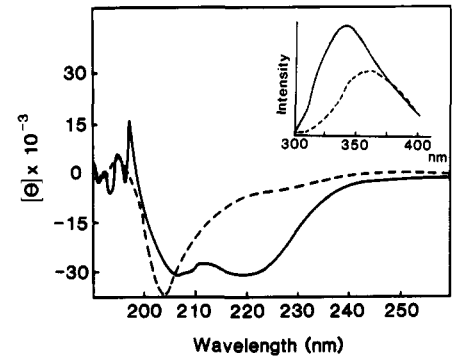

FIGURE 2: Circular dichroic and fluorescence spectroscopy of apoE-(263–286). Circular dichroic spectrum between 190 and 260 nm of apoE(263–286). ApoE(263–286) in buffer (dashed line); after the addition of DMPC (solid line). Inset: The corresponding fluorescence spectra between 300 and 400 nm; the excitation wavelength was 290 nm.

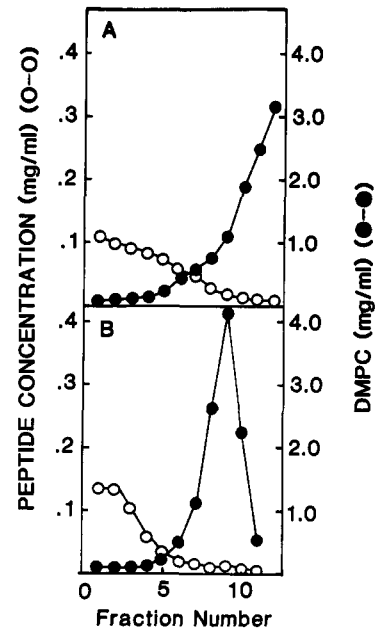

FIGURE 3: Density gradients of apoE peptide–DMPC mixtures. (A) ApoE(211–243); (B) apoE(202–243). Peptide (O); phosphorus (●).

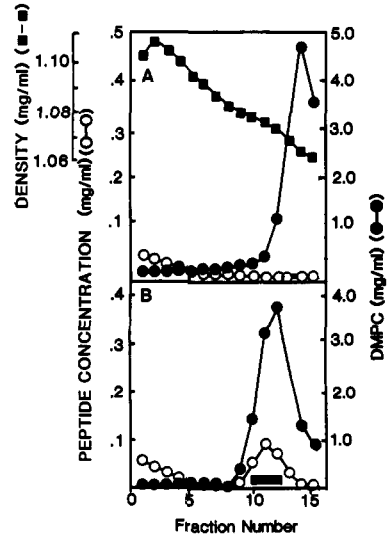

FIGURE 4: Density gradients of apoE peptide–DMPC mixtures. (A) ApoE(267–286); (B) apoE(263–286). Density (■); peptide (O); phosphorus (●). The fractions indicated by the solid bar were pooled for CD spectroscopy and determination of the lipid to peptide ratio.

80% α-helicity for the peptide (Table II), and the Trp fluorescence spectrum was also blue-shifted by 20 nm.

Discussion

Apolipoprotein E is known to have a requirement for bound phospholipid for interaction with the apoB/E receptor of fibroblasts (Innerarity & Mahley, 1978). We have previously shown that a synthetic peptide containing the putative receptor binding region apoE(129–169) will form a phospholipid–peptide complex with a molar ratio of 125:1 after isolation by density gradient ultracentrifugation (Sparrow et al., 1985); the α-helicity of the peptide in the complex is ~56%. However, in preliminary experiments to study the interaction of this peptide with the apoB/E receptor of fibroblasts or the apoE receptor of hepatocytes, we observed an extremely high nonspecific binding which we attribute to transfer of the peptide from the complex to other lipid surfaces. In order to better understand the lipid binding properties of apoE, we have synthesized four peptides from the carboxyl terminus, two of which contain proposed amphipathic lipid binding regions. We have studied their interaction with DMPC by CD and fluorescence spectroscopy and density gradient ultracentrifugation.

The CD spectrum of these peptides indicates little α-helix formation in the absence of phospholipid (Table II). In the presence of the helix-forming solvent 30% hexafluoropropanol, these peptides assume approximately 87–95% α-helicity. In the presence of DMPC, apoE(263–286) shows increases in ellipticity that are indicative of α-helix formation upon interaction with phospholipid (Table II and Figure 2); the enhanced intensity and the 20-nm blue shift of the fluorescence spectrum (Figure 2, inset) are attributed to the two tryptophans being placed in a more hydrophobic environment when the amphipathic helix interacts with the phospholipid bilayer. Density gradient ultracentrifugation (Figures 3A,B and 4A,B) resulted in the isolation of stable complexes only from apoE-(263–286). These complexes had a molar ratio of lipid to peptide of 163:1. Circular dichroic spectroscopy of the complex indicated a helicity of 80% and the Trp fluorescence spectrum was blue-shifted by 20 nm to 340 nm. These results demonstrate that only apoE(263–286) formed a stable lipid–peptide complex. The lack of complex formation by apoE-(202–243) indicates that lipid binding by the putative amphipathic helices in this region does not occur and can most probably be attributed to the low hydrophobicity of this segment of the protein. However, the more hydrophobic apoE-(263–286) does form a stable complex containing a peptide with high helical content, identifying this segment of apoE as an avid lipid binding segment.

Acknowledgments

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References

Barany, G., & Merrifield, R. B. (1980) in *The Peptides: Analysis, Synthesis, Biology* (Gross, E., & Meienhofer, J., Eds.) pp 3–284, Academic Press, New York.

Bohlen, P., Stein, S., Dairman, W., & Undenfriend, S. (1973) *Arch. Biochem. Biophys. 155*, 213.

Cardin, A. D., Hirose, N., Blankenship, D. T., Jackson, R. L., Harmony, J. A. K., Sparrow, D. A., & Sparrow, J. T. (1986) *Biochem. Biophys. Res. Commun. 134*, 783–789.

Cardin, A. D., Jackson, R. L., Sparrow, D. A., & Sparrow, J. T. (1989) *Ann. N.Y. Acad. Sci. 556*, 186–193.

Edelstein, M. S., McNair, D. S., & Sparrow, J. T. (1981) in *Peptides: Synthesis–Structure–Function* (Rich, D. H., & Gross, E., Eds.) pp 217–220, Pierce Chemical Co., Rockford, IL.

Hess, H. H., & Derr, J. E. (1975) *Anal. Biochem. 63*, 607.

Innerarity, T. L., & Mahley, R. W. (1978) *Biochemistry 17*, 1440–1447.

Innerarity, T. L., Freidlander, E. J., Rall, S. C., Jr., Weisgraber, K. H., & Mahley, R. W. (1983) *J. Biol. Chem. 258*, 12341–12347.

Innerarity, T. L., Weisgraber, K. H., Arnold, K. S., Rall, S. C., Jr., & Mahley, R. W. (1984) *J. Biol. Chem. 259*, 7261–7267.

Mahley, R. W. (1978) in *Disturbances in Lipid and Lipoprotein Metabolism* (Dietschy, J. M., Gotto, A. M., Jr., & Ontko, J. A., Eds.) pp 181–197, American Physiological Society, Bethesda, MD.

Mahley, R. W., & Innerarity, T. L. (1983) *Biochim. Biophys. Acta 737*, 197–222.

Mahley, R. W., & Angelin, B. (1984) *Adv. Intern. Med. 29*, 385–411.

Morrisett, J. D., David, J. S. K., Pownall, H. J., & Gotto, A. M., Jr. (1973) *Biochemistry 12*, 1290–1299.

Pitas, R. E., Innerarity, T. L., & Mahley, R. W. (1980) *J. Biol. Chem. 255*, 5454–5460.

Rall, S. C., Jr., Weisgraber, K. H., & Mahley, R. W. (1982) *J. Biol. Chem. 257*, 4171–4178.

Rall, S. C., Jr., Weisgraber, K. H., Innerarity, T. L., Bersot, T. P., Mahley, R. W., & Blum, C. B. (1983) *J. Clin. Invest. 72*, 1285–1297.

Segrest, J. P., Jackson, R. L., Morrisett, J. D., & Gotto, A. M., Jr. (1974) *FEBS Lett. 38*, 247.

Sparrow, J. T. (1976) *J. Org. Chem. 41*, 1350–1353.

Sparrow, J. T., Sparrow, D. A., Culwell, A. R., & Gotto, A. M., Jr. (1985) *Biochemistry 24*, 6984–6988.

Weisgraber, K. H., Rall, S. C., Jr., Mahley, R. W., Milne, R. W., Marcel, Y. L., & Sparrow, J. T. (1986) *J. Biol. Chem. 261*, 2068–2076.

Wilson, C. W., Wardell, M. R., Weisgraber, K. H., Mahley, R. W., & Agard, D. A. (1991) *Science 252*, 1817–1822.