Apolipoprotein C-II$_{39-62}$ Activates Lipoprotein Lipase by Direct Lipid-Independent Binding†

Cait E. MacPhee,‡ Danny M. Hatters, William H. Sawyer, and Geoffrey J. Howlett*

Russell Grimwade School of Biochemistry and Molecular Biology, University of Melbourne, Parkville 3052, Victoria, Australia

Received November 1, 1999; Revised Manuscript Received December 30, 1999

ABSTRACT: Apolipoprotein C-II (apoC-II) is an exchangeable plasma apolipoprotein and an endogenous activator of lipoprotein lipase (LpL). Genetic deficiencies of apoC-II and overexpression of apoC-II in transgenic mice are both associated with severe hyperlipidemia, indicating a complex role for apoC-II in the regulation of blood lipid levels. ApoC-II exerts no effect on the activity of LpL for soluble substrates, suggesting that activation occurs via the formation of a lipid-bound complex. We have synthesized a peptide corresponding to amino acid residues 39−62 of mature human apoC-II. This peptide does not bind to model lipid surfaces but retains the ability to activate LpL. Conjugation of the fluorophore 7-nitrobenz-2-oxa-1,3-diazole (NBD) to the N-terminal α-amino group of apoC-II$_{39-62}$ facilitated determination of the affinity of the peptide for LpL using fluorescence anisotropy measurements. The dissociation constant describing this interaction was 0.23 μM, and was unchanged when LpL was lipid-bound. Competitive binding studies showed that apoC-II$_{39-62}$ and full-length apoC-II exhibited the same affinity for LpL in aqueous solution, whereas the affinity for full-length apoC-II was increased at least 1 order of magnitude in the presence of lipid. We suggest that while the binding of apoC-II to the lipid surface promotes the formation of a high-affinity complex of apoC-II and LpL, activation occurs via direct helix−helix interactions between apoC-II$_{39-62}$ and the loop covering the active site of LpL.

Human apolipoprotein C-II (apoC-II)[1] is an exchangeable 79-residue apolipoprotein found in blood plasma. ApoC-II activates lipoprotein lipase (LpL) and is an important regulator of blood lipid levels as indicated by the severe hypertriglyceridaemia associated with genetic deficiencies of apoC-II (*1*). Paradoxically, overexpression of apoC-II in transgenic mice also causes hypertriglyceridemia, an effect which can be attributed to the delayed clearance of VLDL triacylglycerol resulting from weaker binding of apoC-II rich, apoE poor lipoproteins to cell surface lipases (*2*). These studies imply a complex role for apoC-II in blood lipid homeostasis arising from the LpL activation and lipid binding capacities of apoC-II. In vitro studies show apoC-II does not modulate the activity of LpL against soluble substrates (*3*, *4*), suggesting that LpL activation requires the formation of an enzyme−cofactor complex at a lipid−water interface. In aqueous solution, dansylated apoC-II binds noncooperatively to each subunit of dimeric LpL with a dissociation constant (K_d) of 0.2 μM (*5*). In the presence of lipid, under conditions where both proteins are localized to the lipid surface, the affinity of apoC-II for the enzyme increases by approximately 2 orders of magnitude (*5*). Thus, the activation of LpL by apoC-II appears to be linked to the lipid binding properties of both LpL and the cofactor.

Our recent studies have shown that an N-terminal human apoC-II peptide (amino acid residues 19−39) interacts strongly with unilamellar phosphatidylcholine vesicles (K_d = 6 μM) and undergoes self-association on the lipid surface (*6*). This sequence has a high amphipathic helical propensity that may mediate the association of apoC-II with lipid surfaces. Structural algorithms predict that residues 46−52 of human apoC-II form an amphipathic α-helix that could determine the extent of lipid binding (*7*). Support for this idea comes from the observation that apoC-II$_{44-79}$ binds to apoC-II deficient VLDL particles (*7*) and adopts an α-helical conformation when bound to dimyristoylphosphatidylcholine vesicles (*8*). A peptide encompassing residues 55−79 does not bind to a lipid surface, suggesting that the putative helix between residues 46 and 52 is required for lipid-binding activity. However, under conditions where full-length apoC-II binds to apoC-II deficient VLDL, only 50% of apoC-II$_{44-79}$ binds, suggesting a weaker affinity for the lipid surface than for the full-length cofactor (*7*).

The C-terminal region of apoC-II also contains residues considered essential for binding to and activating lipoprotein lipase. Voyta and co-workers (*9*) studied the binding to LpL of dansylated peptides derived from the sequence of apoC-II. ApoC-II$_{65-79}$ has an affinity for LpL in aqueous

† Supported by a grant from the Australian Research Council. C.E.M. was the recipient of an Australian Postgraduate Research award.

* To whom correspondence should be addressed: Russell Grimwade School of Biochemistry and Molecular Biology, University of Melbourne, Parkville, Victoria 3052, Australia. Telephone: 61 3 9344 7632. Fax: 61 3 9347 7730.

‡ Current address: Oxford Centre for Molecular Sciences, New Chemistry Laboratory, University of Oxford, South Parks Road, Oxford OX1 3QT, United Kingdom.

[1] Abbreviations: apoC-II, apolipoprotein C-II; LpL, lipoprotein lipase; NBD, 7-nitrobenz-2-oxa-1,3-diazole; VLDL, very low-density lipoproteins; K_d, dissociation constant; LUVs, large unilamellar vesicles; SUVs, small unilamellar vesicles; EYPC, egg yolk phosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; TO, triolein; TGH, triacylglycerol hydrolase; GA, gum arabic; MBP, maltose binding protein; Et-OPPC, ether-linked 1-*O*-oleyl-2-*O*-palmityl-*rac*-glycero-3-phosphocholine; CD, circular dichroism; MRE, mean residue ellipticity.

10.1021/bi992523t CCC: $19.00
Published on Web 03/04/2000

solution of 25 μM, 1 order of magnitude lower than that of apoC-II$_{44-79}$, and 2 orders of magnitude lower than that of the full-length cofactor. ApoC-II$_{65-79}$ does not, however, activate LpL. Extending apoC-II$_{65-79}$ toward the N-terminus progressively restores the ability of the peptide to increase enzyme activity, with apoC-II$_{44-79}$ exhibiting full activation potential. Other studies with peptide derivatives of apoC-II indicate Tyr-63 is necessary for LpL activation since substitution with Trp, Phe, or Gly decreases cofactor activity by approximately 60−70% (*8*, *10*). In this paper, we explore the lipid and enzyme binding properties and enzyme activation of a synthetic peptide corresponding to amino acid residues 39−62 of mature human apoC-II. This peptide includes the putative amphipathic helix between residues 46 and 52 but lacks Tyr-63, which is considered important for LpL activation. Although unable to bind to model lipid surfaces, this peptide binds to and activates LpL.

MATERIALS AND METHODS

Materials. ApoC-II$_{39-62}$ (KTYLPAVDEKLRDLYSKSTA-AMST-NH_2) fully protected at all side chains and deprotected at the α-amino group of the N-terminal lysine was purchased from Chiron Mimotopes (Melbourne, Australia). The peptide was obtained still attached to its synthesis "pin". 4-Fluoro-7-nitrobenz-2-oxa-1,3-diazole (NBD-F) was purchased from Molecular Probes, Inc. (Eugene, OR). Egg yolk phosphatidylcholine (EYPC) was purchased from Lipid Products (Surrey, U.K.). ApoC-II purified from human plasma, gum arabic (GA), and dimyristoylphosphatidylcholine (DMPC) were purchased from Sigma Chemical Co. (St. Louis, MO). Ether-linked 1-*O*-oleyl-2-*O*-palmityl-*rac*-glycero-3-phosphocholine (Et-OPPC) was from Doosan Serdary Research Laboratories. ^{14}C-labeled triolein was purchased from Amersham Life Science (Uppsala, Sweden). DNA primers, *Sca*I forward primer (5′ GTCCAGAGTACTCAACAGC-CCCAGCAAG), and *Pst*I reverse primer (5′ GCCCTCCT-GCAGTTACTCCTCTCCCTTCAGC) were obtained from Pacific Oligos (Toowong, Queensland, Australia). Amylose affinity resin was purchased from New England Biolabs (Beverly, MA). A plasmid, designated pCII-711, containing cDNA for human apoC-II (*11*) was kindly provided by N. S. Shachter (Rockefeller University, New York, NY). Factor Xa protease was purchased from Pharmacia-Biotech (Uppsala, Sweden). Restriction enzymes were purchased from New England Biolabs.

N-Terminal Labeling and Purification of ApoC-II$_{39-62}$. ApoC-II$_{39-62}$, attached to the synthesis pin, was incubated in 99.9% anhydrous dimethylformamide containing a 14-fold molar excess of NBD-F for 24 h in the dark to specifically label the N-terminal amino group. The pin was washed with dichloromethane; the peptide was cleaved from the pin, and the side chains were deprotected by immersion in a stirred mixture of 95% trifluoroacetic acid, 2.5% ethanedithiol, and 2.5% H_2O (1.5 h at room temperature). The trifluoroacetic acid was removed under N_2, and the peptide was precipitated with ice-cold ether. The cleaved peptide was resuspended in water and lyophilized three times to remove the remaining traces of volatile acid.

Unlabeled apoC-II$_{39-62}$ (1 mmol unmodified) was also prepared, in which case the N-terminal α-amino group was acetylated by reaction for 12 h in acetic anhydride (1 mmol), dicyclohexylcarbodiimide (1 mmol), *N*-hydroxybenzotriazole (1 mmol), and dimethylformamide (2 mL) while the peptide was attached to the synthesis pin. The resin-bound peptide was cleaved from the synthesis pin and deprotected as described above.

Crude apoC-II$_{39-62}$ was purified on a semipreparative reverse phase C-18 HPLC column (Brownlee, 10 mm × 250 mm) using a linear gradient of 20 to 40% (0.5%/min) acetonitrile containing 0.1% trifluoroacetic acid for elution. Fractions were analyzed by MALDI-TOF mass spectrometry and fluorescence spectroscopy, and the desired fractions were pooled and lyophilized. The dried product was resuspended in water, and the lyophilization cycle was repeated three times. The peptide was greater than 95% pure as judged by HPLC using an analytical reverse phase C-18 column (Brownlee Spheri-5, 4.6 mm × 220 mm) with gradient elution as described above.

Expression and Purification of Recombinant ApoC-II. ApoC-II cDNA was amplified by PCR from an apoC-II-containing plasmid (*11*) using *Sca*I forward and *Pst*I reverse primers. The PCR product was digested with *Pst*I and *Sca*I restriction endonucleases and ligated directly into pMAL-c2 via the *Pst*I and *Xmn*I restriction sites. The correct sequence of the maltose binding protein−apoC-II hybrid cDNA in pMAL-c2 was confirmed by sequencing with the ABI PRISM Dye Terminator system (Perkin-Elmer, Norwalk, CT). The fusion plasmid was transformed into *Escherichia coli* strain UT5600, and an overnight culture of a freshly transformed colony was grown in LB medium, 0.2% glucose, and 100 μg/mL ampicillin. An aliquot of the overnight culture (6 mL) was subcultured into 3 L of LB medium, 0.2% glucose, and 100 μg/mL ampicillin and grown to an OD_{600} of 0.8 at 37 °C with shaking at 150 rpm. Expression of the maltose binding protein (MBP)−apoC-II fusion protein was induced by the addition of 1 mM isopropyl β-D-thiogalactopyranoside, and the cells were incubated for an additional 2 h. The cells were harvested by centrifugation at 4000*g* for 15 min, resuspended in 100 mL of column buffer [20 mM Tris-HCl, 200 mM NaCl, 0.02% NaN_3, and 1 mM EDTA (pH 7.4)], and frozen overnight at −20 °C. The cells were thawed and then lysed by the addition of hen egg white lysozyme to a final concentration of 0.2 mg/mL for 30 min at 4 °C. The lysate was sonicated for 30 s on medium power using a probe sonicator (Soniprep 150, MSE Scientific Instruments, Sussex, England), and insoluble debris was removed by centrifugation at 15000*g* for 30 min at 4 °C. The lysate was applied to 10 mL of amylose affinity resin packed in a 2.5 cm × 20 cm Econocolumn (Bio-Rad, Hercules, CA) by gravity flow. The resin was washed with 50 mL of chilled column buffer, and the fusion protein eluted with column buffer containing 15 mM maltose. The concentration of MBP−apoC-II was determined spectrophotometrically using an extinction coefficient of 79 300 M^{-1} cm^{-1} calculated from the amino acid composition. The MBP−apoC-II hybrid was cleaved using factor Xa at a ratio of 1 unit of protease to 150 μg of fusion protein for 16 h at room temperature. The cleaved products were lyophilized and resuspended in 4 M guanidine hydrochloride and 20 mM $NaPO_4$ (pH 7.4) at $^1/_{20}$ of the original volume. The solution was applied in 1 mL aliquots to a HR16-60 column (Pharmacia-Biotech) packed with Superdex-75 preparative resin equilibrated in 4 M guanidine

hydrochloride and 20 mM $NaPO_4$ (pH 7.4). The two proteins eluting from the column were identified using Coomassie Blue-stained Tris-tricine SDS−PAGE (*12*). The apoC-II-containing peaks were pooled and dialyzed exhaustively into 10 mM NH_4HCO_3 (pH 7.4) at 4 °C and lyophilized. The protein was resuspended in 2 mL of 4 M guanidine hydrochloride and 20 mM $NaPO_4$ (pH 7.4) and reapplied to the Superdex-75 column, and the apoC-II fraction was dialyzed and lyophilized as described above. A typical yield of 90 mg of MBP−apoC-II fusion protein per 3 L of culture yielded approximately 3.5 mg of pure apoC-II, or 20% of the potential apoC-II. Mass spectrometry confirmed that the recombinant apoC-II had the same molecular weight as the native protein (8915), and Tris-tricine SDS−PAGE visualized with Coomassie brilliant blue R250 confirmed that the purity was >95%.

Preparation of Unilamellar Vesicles. DMPC (or Et-OPPC) stored in chloroform was dried to a thin film under a stream of nitrogen and left under vacuum overnight to remove residual traces of organic solvent. Large unilamellar vesicles (LUVs) were prepared by resuspension of the lipid in 0.15 M NaCl and 20 mM Tris-HCl (pH 7.4). The dispersion was extruded at room temperature 11 times through two polycarbonate 100 nm filters using a LiposoFast hand-held microextruder (Avestin Inc., Ottawa, ON). Small unilamellar vesicles (SUVs) for CD measurements were prepared by sonication at 20 MHz in an MSE Soniprep instrument for 15 min under a constant stream of nitrogen. A water bath was used to maintain the temperature of the lipid solution at 20 °C. After sonication, the vesicles were centrifuged for 5 min at 1000*g* to remove residual titanium particles arising from the sonicator tip.

Purification of LpL from Bovine Milk. Bovine LpL was chosen for the study of the interaction between LpL and human apoC-II due to the natural occurrence of high levels of the enzyme in bovine milk. The sequence of the bovine enzyme is more than 95% homologous with that of the human enzyme. LpL was purified from bovine skim milk by the method of Bengtsson-Olivecrona and Olivecrona (*12*). Briefly, 32 L of skim milk was adjusted to 0.34 M NaCl and 1 mM PMSF. Heparin Sepharose (250 mL) was added successively to 8 L batches of milk, and the mixtures were stirred for 45 min; the resin-bound LpL was isolated using a sintered glass filter. The resin was then successively washed with buffer containing 10 mM Tris-HCl, 0.02% NaN_3 (pH 6.5), and either 0.5 M NaCl, 0.75 M NaCl, or 0.2% Triton-101 and 0.75 M NaCl, in a total wash volume of 11 L. The washed resin was packed into a column and washed further with 10 mM Tris-HCl, 0.02% NaN_3, and 0.75 M NaCl. The LpL was eluted with a 1 to 2 M (250 mL) linear NaCl gradient containing 20 mM Tris-HCl, 1 mM PMSF, and 0.02% NaN_3 (pH 6.5). All procedures were performed at 4 °C. The purity of the eluted fractions was established by SDS−PAGE. Samples were stored at −20 °C until required.

CD Measurements. Circular dichroism (CD) spectra were recorded in a model 62DS AVIV CD spectrometer. The mean residue ellipticity, MRE, was calculated from the equation $MRE = \theta/(cnl/M)$, where l is the path length in millimeters, θ is the observed ellipticity in millidegrees, c is the protein concentration (milligrams per milliliter), n is number of amino acid residues, and M is the molecular weight.

Enzyme Activity Assays. The phospholipase activity of lipoprotein lipase was measured using DMPC LUVs. LpL (8 μM) was dialyzed over 24 h against four changes of lipase buffer [0.15 M NaCl and 0.1 M Tris-HCl (pH 8.4)] containing 0.135 mg/mL heparin. Assays contained 90 nM heparin, 10 mg/mL BSA, and100 μM DMPC in lipase buffer, and were maintained at 30 °C throughout. In the absence of cofactor, the addition of LpL (85 nM) to vesicles caused an abrupt increase in the degree of light scattered by vesicles, which leveled off to a constant value after a period of 10 min. This is attributed to cross-linking of lipid particles by the enzyme (*13*, *14*). The reaction was started by the addition of 0.9 μM cofactor and monitored for 20 min by absorbance spectroscopy at 400 nm, which measures the decrease in light scattering resulting from hydrolysis of phospholipids in LUVs.

The triacylglycerol hydrolase activity of LpL was measured initially using gum arabic-stabilized ^{14}C-labeled triolein (GA-TO) emulsions. Triolein (54 mmol) containing 87 nmol of ^{14}C-labeled triolein was dried down under a stream of N_2 and resuspended in 5% (w/v) gum arabic. The crude suspension was sonicated at 20 MHz under N_2 for 5 min on ice. BSA was added to give a final concentration of 4% (w/v). Assay samples contained 3.6 mM triolein, 1.6% (w/v) BSA, 1.2% (w/v) gum arabic in 0.15 M NaCl, 0.1 M Tris-HCl (pH 8.4), and 85 nM LpL, and a 0−2-fold molar ratio of activator to LpL. The reaction was started by the addition of substrate, and allowed to proceed for 30 min prior to organic extraction of free fatty acids by the method described by Belfrage and Vaughan (*15*).

The hydrolytic activity of LpL was also measured using EYPC-stabilized ^{14}C-labeled triolein (EYPC-TO) emulsions. Triolein (54 mmol) was resuspended in 0.15 M NaCl and 0.1 M Tris-HCl (pH 8.4) containing 27 mmol of EYPC. Crude emulsions were prepared and assayed as described above, except that 1.8 mM EYPC was substituted for gum arabic.

Competitive Titrations Using ApoC-II in Solution. Lipoprotein lipase purified from bovine milk was prepared as a 28 μM stock solution in 0.15 M NaCl and 20 mM Tris-HCl (pH 7.4) containing 40 μM heparin. The initial concentration of NBD−apoC-II_{39-62} was 1.4 μM, and the initial concentration of full-length recombinant unlabeled apoC-II was 6.2 μM in a volume of 400 μL. LpL was added to the peptide in aliquots of 5 μL. Fluorescence measurements were carried out using a SPEX Fluorolog/Tau-2 spectrofluorometer using a 5 mm path length cuvette. The fluorescence anisotropy of the NBD fluorophore was monitored at 530 nm with excitation at 465 nm. Each data point was the average of 10 measurements over an integration time of 2 s. The final concentrations of NBD−apoC-II_{39-62}, full-length apoC-II, and LpL were corrected for dilution due to the addition of LpL. The assay was performed in triplicate. The competition titration was carried out as described above except for the presence of 6.2 or 30.8 μM full-length recombinant unlabeled apoC-II in the solution at the start of the titration.

Competitive Titrations Using ApoC-II in the Presence of Lipids. Et-OPPC LUVs were prepared as a 26 mg/mL stock solution in 0.15 M NaCl and 20 mM Tris-HCl (pH 7.4). Et-OPPC was added to LpL to give a final lipid concentration of 10 mM. The final concentration of LpL was 21 μM. The initial concentrations of NBD−apoC-II_{39-62} and full-length

recombinant apoC-II in 400 μL were 1.4 and 6.2 μM, respectively. Aliquots (5 μL) of the LpL−Et-OPPC complex were added to the peptide, and the anisotropy of the NBD fluorophore was monitored at 530 nm after excitation at 465 nm. Background due to scattered light was subtracted using an appropriate unlabeled control. At the highest lipid concentration that was examined, scattered light contributed <7% to the fluorescence intensity excited with vertically polarized light.

The analysis of competitive binding isotherms was performed as described by Bailey et al. (*16*). Briefly, the concentration of unbound NBD−apoC-II$_{39-62}$ ([L]) was calculated directly from the binding isotherm:

$$[L] = [L]_t\left(1 - \frac{r - r_F}{r_B - r_F}\right) \quad (1)$$

where $[L]_t$ is the total concentration of NBD−apoC-II$_{39-62}$ and r_B is the maximum change in anisotropy, calculated from anisotropy measurements carried out in the absence of competitor. The fluorescence anisotropy of free peptide (r_F) was 0.044. The data were then fitted for K_U (the association constant describing the interaction between apoC-II and LpL) according to

$$r = r_F + \frac{(r_B - r_F)K_L[E]}{1 + K_L[E]} \quad (2)$$

where [E] (the concentration of unbound LpL) is

$$[E] = \frac{[E]_t}{1 + K_L[L] + \dfrac{K_U([U]_tK_L[L])}{K_U[L]_t - [L](K_U - K_L)}} \quad (3)$$

K_L and K_U are the association constants describing the interaction between the NBD−apoC-II$_{39-62}$ or full-length apoC-II with LpL, respectively. $[E]_t$ and $[U]_t$ are the total concentrations of LpL and apoC-II, respectively. K_L was determined in the absence of competitor, and was treated as an experimental parameter.

RESULTS

Lipid Binding Analysis. The capacity of apoC-II$_{39-62}$ to bind to model lipid surfaces was initially investigated by CD spectroscopy. Figure 1 shows the CD spectrum of the peptide in the presence of DMPC small unilamellar vesicles at a lipid to peptide molar ratio of 100:1. The spectrum is typical of a random coil conformation and is essentially identical to the spectra obtained for the peptide in the absence of lipid, suggesting that apoC-II$_{39-62}$ does not interact with the lipid surface. This argues against the interaction of this putative helical binding motif with the lipid surface. By contrast, full-length apoC-II adopts an α-helical conformation in the presence of lipid, as typified by the double minima at 210 and 222 nm, also shown in Figure 1. The lipid binding properties of apoC-II$_{39-62}$ were further assessed by density gradient sedimentation experiments. These experiments, using small unilamellar DMPC vesicles and solution conditions similar to those used in the experiments whose results are depicted in Figure 1, indicated partitioning of the peptide and the lipid to different regions of the density gradient.

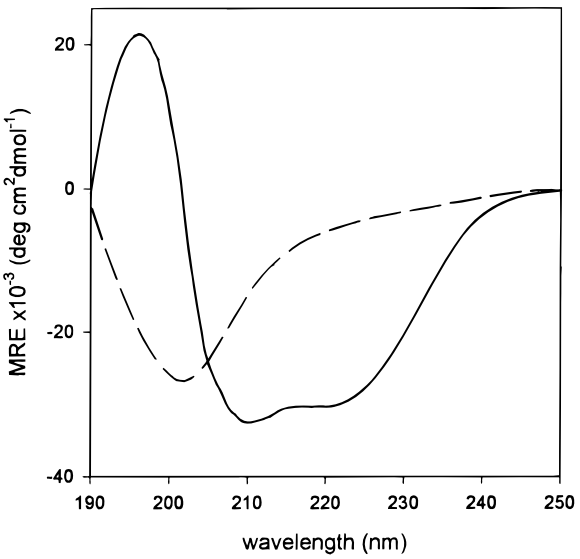

FIGURE 1: Circular dichroism spectra of apoC-II$_{39-62}$ (broken line) and apoC-II (solid line) in the presence of small unilamellar vesicles of DMPC. The apoC-II$_{39-62}$ and apoC-II concentrations were 21 and 16 μM, respectively, and the lipid to protein molar ratio was 100:1 in 0.1 M KF and 20 mM Tris-HCl (pH 7.4).

Differential scanning calorimetry showed no change in the phase transition behavior upon addition of a 1:10 molar ratio of apoC-II$_{39-62}$ to DMPC vesicles. Similarly, the fluorescence properties of a peptide in which the tyrosine residue at position 53 was replaced with a tryptophan were unchanged in the presence and absence of lipid, suggesting little interaction with the hydrophobic surface. Alternative lipids (EYPC, dipalmitoylphosphatidylserine, and dimyristoylphosphatidylethanolamine) and buffer systems (potassium phosphate, containing up to 0.5 M NaCl or KCl) were also investigated, but there was no indication that this peptide binds to model lipid surfaces.

Activation of LpL Phospholipase Activity. In addition to hydrolyzing triacylglycerols, LpL also hydrolyzes phospholipids, although phospholipase activity is low. The activation of LpL by apoC-II$_{39-62}$ was examined initially with clearance assays, which monitor the change in the amount of light scattered by lipid particles on hydrolysis of phospholipids by LpL. In the absence of cofactor, the amount of light scattered by the LpL−LUV complex is constant over the time period of the assay, indicating little or no phospholipase activity. Addition of native apoC-II purified from human plasma causes a decrease in the level of light scattering over time, reflecting the phospholipase activity of LpL (Figure 2). This activation is not due solely to perturbation of the lipid substrate since addition of apoC-II$_{19-39}$ does not activate LpL. ApoC-II$_{39-62}$, at the same molar ratio, causes partial activation of LpL, to a level that is about half of the level of activation induced by the native cofactor (Figure 2). Since apoC-II$_{39-62}$ does not bind to the lipid surface, this result suggests that independent localization of the cofactor to the interface is not required for activation of the phospholipase activity of LpL.

Activation of LpL Triacylglycerol Hydrolase Activity. The ability of apoC-II$_{39-62}$ to increase LpL TGH activity was examined quantitatively by assessing the hydrolysis of ^{14}C-labeled triolein in gum arabic-stabilized (GA-TO) emulsions. LpL is activated more than 3-fold by a 2-fold molar excess

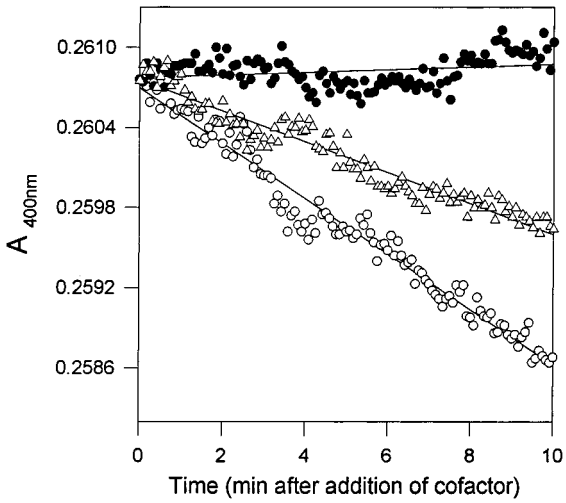

FIGURE 2: Phospholipase activity of lipoprotein lipase. The activation of lipoprotein lipase by apoC-II$_{19-39}$ (●), apoC-II$_{39-62}$ (△), and native apoC-II (○) was assessed with large unilamellar vesicles of DMPC as the substrate and by determining the decrease in optical density at 400 nm. Samples contained 85 nM LpL, 90 nM heparin, 10 mg/mL BSA, 100 μM DMPC, and 0.9 μM cofactor in 0.15 M NaCl and 20 mM Tris-HCl (pH 7.4) at 30 °C.

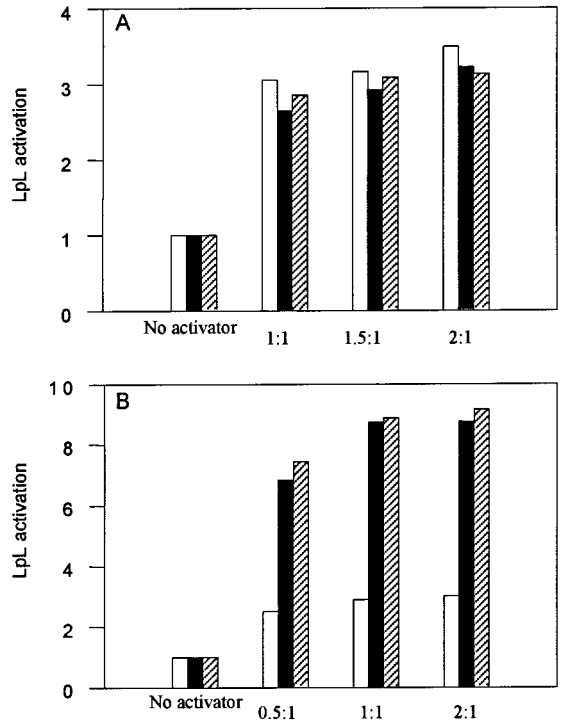

FIGURE 3: Effect of apoC-II$_{39-62}$ (white bars), native apoC-II purified from human plasma (black bars), and recombinant apoC-II, expressed in *E. coli* (hatched bars), on the activity of lipoprotein lipase. The relative level of activation of lipoprotein lipase was determined using gum arabic-stabilized ^{14}C-labeled triolein emulsions (A) or EYPC-stabilized ^{14}C-labeled triolein emulsions (B) as substrates. The molar ratio of cofactor to LpL is displayed on the abscissa. Samples contained 3.6 mM triolein, 1.6% (w/v) BSA, and 85 nM LpL in 0.15 M NaCl and 0.1 M Tris-HCl (pH 8.4). The concentration of gum arabic in panel A was 1.2% (w/v); the concentration of EYPC in panel B was 1.8 mM.

of apoC-II$_{39-62}$ (Figure 3A, white bars). The degree of activation of LpL by apoC-II$_{39-62}$ is comparable to that of native apoC-II (black bars) at all molar ratios of cofactor to enzyme. Also shown in Figure 3A is the degree of activation of LpL by recombinant full-length apoC-II, produced from

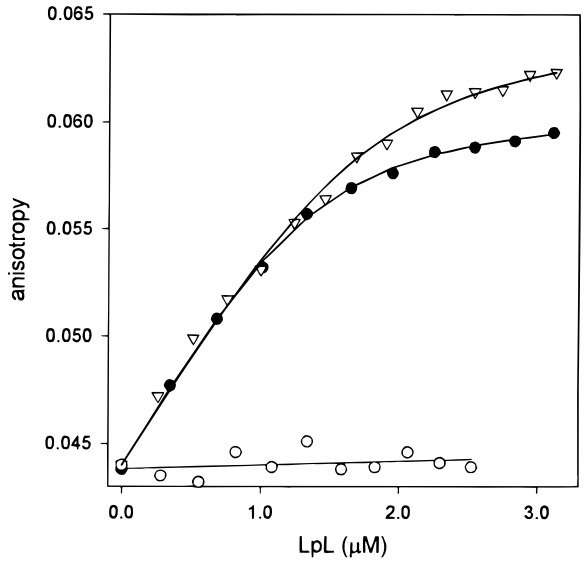

FIGURE 4: Binding of 1.4 μM NBD-labeled apoC-II$_{39-62}$ to LpL monitored by steady-state fluorescence anisotropy at 25 °C. Samples were prepared in 0.15 M NaCl, 4 μM heparin, and 20 mM Tris-HCl (pH 7.4). The binding of NBD−apoC-II$_{39-62}$ to LpL in aqueous solution was monitored in the presence (○) and absence (●) of 2 M NaCl. The binding of NBD−apoC-II$_{39-62}$ to lipid-bound LpL was monitored in the presence of 10 mM nonhydrolyzable Et-OPPC vesicles (▽).

a maltose binding protein fusion protein in *E. coli* (hatched bars). The recombinant apoC-II activates LpL to about the same extent as both the native apoC-II and apoC-II$_{39-62}$.

The activation of LpL by apoC-II$_{39-62}$ was also investigated using phospholipid-stabilized emulsions, in which gum arabic was substituted with EYPC (EYPC-TO; Figure 3B). In the absence of cofactor, the basal level of hydrolysis of ^{14}C-labeled triolein by LpL was similar to that observed in the GA-TO emulsion system. On addition of plasma-purified apoC-II, the enzyme is activated nearly 9-fold. Similarly, recombinant apoC-II increases LpL activity 9.1-fold (hatched bars). By contrast, apoC-II$_{39-62}$ activates LpL ~2.9-fold, and is therefore 3 times less effective at activating LpL than the full-length cofactors in this lipid-based system (white bars). However, the level of activation of LpL by apoC-II$_{39-62}$ is comparable to that observed in the GA-TO emulsion system.

Binding of ApoC-II$_{39-62}$ to LpL in Solution. The peptide-induced activation of LpL strongly suggests complex formation between the enzyme and apoC-II$_{39-62}$. ApoC-II$_{39-62}$ was labeled at the N-terminus with the fluorophore NBD and the interaction between apoC-II$_{39-62}$ and LpL examined by fluorescence anisotropy analysis. The anisotropy of the NBD label increases from 0.044 to 0.060 on titration of apoC-II$_{39-62}$ with LpL (Figure 4), reflecting the decreased rotational mobility of the probe on binding of the peptide to the enzyme. In contrast, the fluorescence intensity of the NBD-labeled apoC-II$_{39-62}$ does not change on addition of LpL. This suggests that the N-terminal portion of apoC-II$_{39-62}$ is not perturbed on binding to LpL, and argues that chemical modification of the peptide does not interfere with the peptide−enzyme interaction. Analysis of the binding data shown in Figure 4 (see Materials and Methods) involved an initial estimate of the maximum change in anisotropy from the ordinate intercept of a plot of 1/r versus 1/[LpL], but this was allowed to vary in the analysis. The value of the dissociation constant that is obtained (0.23 μM) is similar

to the estimated dissociation constant ($1/K_U = 0.2$ μM) for dissociation of full-length apoC-II from LpL (*4*).

Lipoprotein lipase is stored under conditions of high salt (2 M) to prevent loss of enzyme activity over time; under these conditions, the enzyme is not active, although activity can be restored by dilution into medium containing low salt (0.15 M). Clarke and Holbrook (*5*) observed that native apoC-II does not interact with LpL in the presence of 1 M salt. Figure 4 includes results of a control experiment that shows there is essentially no change in the fluorescence anisotropy of NBD−apoC-II$_{39-62}$ upon addition of LpL in the presence of 2 M NaCl.

Binding of ApoC-II$_{39-62}$ to LpL in the Presence of Lipid. ApoC-II undergoes a conformational change in the presence of lipid (*17*). Accompanying this conformational change is an increase in the affinity of the cofactor for lipid-bound LpL (*5*). It has been proposed that the formation of this high-affinity complex induces the active conformation of LpL (*18*, *19*). Since apoC-II$_{39-62}$ does not interact with lipid, it was of interest to determine whether the affinity of the peptide−enzyme complex changes upon binding of the enzyme to the membrane surface. The lipid analogue 1-*O*-oleyl-2-*O*-palmityl-*rac*-glycero-3-phosphocholine (Et-OPPC) contains nonhydrolyzable ether bonds between the glycerol backbone of the phospholipid and the acyl chains. This lipid analogue was used to avoid phospholipase activity (Figure 2) during investigation of the binding event. On the basis of the affinity of LpL for lipid [$K_d = 43$ nM (*13*)], conditions were chosen so that the concentration of lipid vesicles was sufficient to fully bind LpL. Since NBD−apoC-II$_{39-62}$ does not bind to lipid (Figure 1), the interaction that is examined is the binding of NBD−apoC-II$_{39-62}$ to the lipid-bound enzyme. Under these conditions, the fluorescence anisotropy increases from 0.044 to 0.063 (Figure 4) due to the slower rotational mobility of the lipid−enzyme−peptide complex. Analysis of the binding data yields a dissociation constant of 0.28 μM for the formation of the peptide−enzyme complex in the presence of lipid, indicating that there is no significant increase in the affinity of apoC-II$_{39-62}$ for LpL upon binding of the enzyme to a lipid surface.

Competitive Binding Using ApoC-II in Solution. Previous investigations of the affinity of full-length apoC-II for LpL in solution employed fluorescence methods that required an extrinsic probe to be conjugated to apoC-II (*5*, *8*), raising the possibility that the probe may interfere with complex formation. A method for determining the affinity of unlabeled apoC-II for LpL was therefore sought. Figure 5 shows the titration of NBD−apoC-II$_{39-62}$ with LpL in the absence and in the presence of a 4.4- and 22-fold molar excess of full-length apoC-II. Qualitatively, the observed change in the magnitude and shape of the binding isotherm indicates that apoC-II$_{39-62}$ and the full-length cofactor compete for the same site. The data were fitted globally to eqs 1−3 using the association constant for apoC-II (K_U) as a fitting parameter. The association constant for the binding of NBD−apoC-II$_{39-62}$ to LpL (K_L) was constrained to the value obtained by analysis of the data presented in Figure 4. The calculated affinity of full-length apoC-II for LpL ($1/K_U = 0.3$ μM) is similar to that observed for apoC-II$_{39-62}$ ($1/K_L = 0.23$ μM).

Competitive Binding Using ApoC-II in the Presence of Lipid. A similar competitive titration was performed in the presence of nonhydrolyzable lipid. Under these conditions, LpL and apoC-II interact with the lipid surface whereas apoC-II$_{39-62}$ does not. Figure 6 shows the titration of apoC-II$_{39-62}$ with lipid-bound LpL in the presence and absence of a 3.8-fold excess of full-length apoC-II. The binding isotherm indicates competition between the peptide and full-length apoC-II for binding to LpL. The lipid concentrations used in Figure 6 were chosen so that all of the LpL in the system was lipid-bound. Since the affinity of apoC-II for lipid is 2 orders of magnitude lower than that of LpL (*5*, *14*), native apoC-II was not completely lipid-bound in the initial phase of the titration. The requirement to take into account localization of native apoC-II to the lipid surface complicates the analysis of the competitive binding isotherm. Assuming that LpL does not affect the affinity of apoC-II

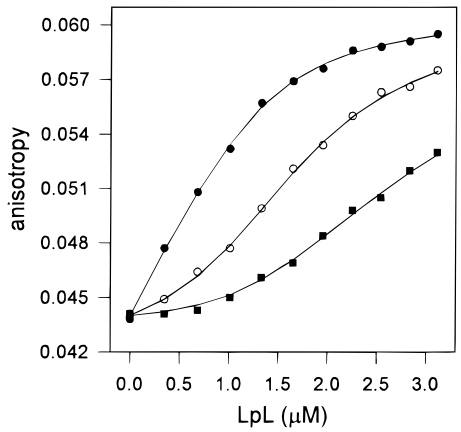

FIGURE 5: Titration of NBD-labeled apoC-II$_{39-62}$ (1.4 μM) with LpL. The binding of peptide to LpL was assessed in the absence (●) and in the presence of a 4.4-fold (○) and 22-fold (■) molar ratio excess of recombinant apoC-II to NBD-labeled apoC-II$_{39-62}$. The lines represent the global fit of the data to a constant of 0.3 μM describing the affinity of full-length apoC-II for LpL. Steady-state fluorescence anisotropy measurements were taken at 25 °C. Samples were prepared in 0.15 M NaCl, 4 μM heparin, and 20 mM Tris-HCl (pH 7.4).

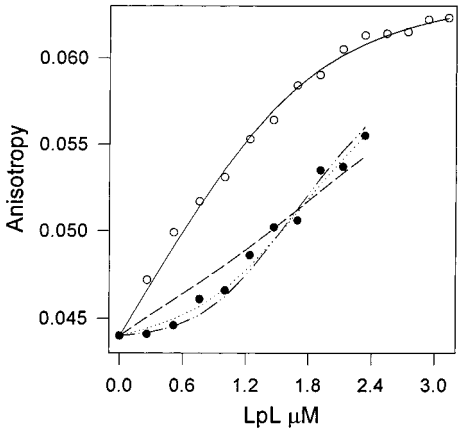

FIGURE 6: Titration of 1.4 μM NBD-labeled apoC-II$_{39-62}$ with a mixture of LpL (21 μM) and 10 mM nonhydrolyzable Et-OPPC LUVs. Titrations were performed in the absence (○) and in the presence (●) of a 4.4-fold molar ratio of recombinant apoC-II. Steady-state fluorescence anisotropy was measured at 25 °C. Samples were prepared in 0.15 M NaCl and 20 mM Tris-HCl (pH 7.4). During data modeling, the dissociation constant ($1/K_L$) for the binding of apoC-II$_{39-62}$ to LpL was constrained to 0.2 μM. The fitted lines correspond to dissociation constants ($1/K_U$) of 0.3 μM (dashed line), 0.03 μM (dashed and dotted line), and 0.003 μM (dotted line) for the interaction of apoC-II with LpL.

for lipid, at the first addition of the LpL−lipid complex only ~55% of apoC-II will be lipid-associated. This value increases to >95% after the first four additions of titrant [assuming a K_d of 3 μM and a stoichiometry of 20 phospholipids/peptide (*14*)]. To examine the effect of this problem on data analysis, the concentration of apoC-II in the system was calculated either as the total concentration of apoC-II in the system (whether lipid-bound or not, $[U]_T$ = [apoC-II]) or as the concentration of apoC-II bound to lipid at any stage in the titration ($[U]_T$ = $[\text{apoC-II}]_L$). Surprisingly, modeling of the data indicates that adjusting $[U]_T$ in this range does not greatly affect the final interpretation of the isotherm, most likely because it is only during the first four additions of lipid that the level of binding of apoC-II to the lipid surface is less than ~95%. This is also the range of the titration where the change in the anisotropy of NBD−apoC-II_{39-62} is smallest and where comparatively large errors in the estimated $[U]_T$ will only produce small errors in the fit to the data.

Due to the large number of parameters required to fit the data in Figure 6, it was not possible to globally fit the data. Instead, three sets of conditions were simulated mathematically using dissociation constants for the LpL−apoC-II interaction of 0.3, 0.03, and 0.003 μM. Simulations taking into account the small amount of lipid-free apoC-II at the start of the titration yielded essentially identical curves. Adequate fits to the data are obtained using K_U values of 0.03 and 0.003 μM. However, the data are not consistent with the dissociation constant describing the affinity of apoC-II for LpL in aqueous solution (0.3 μM). The results suggest that the affinity of apoC-II for LpL increases 1 or 2 orders of magnitude on restriction of both enzyme and cofactor to a lipid surface.

DISCUSSION

There is much variability in the reported degree of activation of LpL by apoC-II, ranging from 2−3-fold (*20*, *21*) to as much as 30−100-fold (*22*, *23*) depending on the assay system that is used. In the study presented here, LpL is activated to different extents depending on whether gum arabic (GA-TO) or egg yolk phosphatidylcholine (EYPC-TO) is used to stabilize the triolein. It is unlikely that this observation is due to a change in the properties of the enzyme, since the basal activity is the same in the two assay systems. A possible explanation for the discrepancy is the orientation of the cofactor at the interface. Gum arabic is a large (240 000 Da) carbohydrate used in the food industry for the emulsification of a variety of hydrophobic compounds. The structure of the particle formed with triolein is unknown. However, a large, charged carbohydrate may be a poor substitute for the lipid−water interface experienced in vivo by both LpL and apolipoproteins. The lower cofactor activity in the GA-TO emulsion system compared to that in the EYPC-TO system may be due to apoC-II binding directly to LpL rather than by interacting and colocalizing via the emulsion surface. A direct interaction between aqueous apoC-II and LpL would also explain why apoC-II and apoC-II_{39-62} activate LpL to the same degree in the GA-TO assay system, given that they have the same affinity for LpL in aqueous solution (K_d = 0.23−0.3 μM). This result highlights the importance of accurately defining the assay system employed for the investigation of LpL activity.

The results presented here indicate that apoC-II_{39-62} does not bind to a model phospholipid surface, suggesting that the putative amphipathic helix between residues 46 and 52 is not sufficient for lipid binding. The interaction between apoC-II_{44-79} and lipid observed by Smith et al. (*8*) implicates a region further toward the C-terminus of the full-length protein as a corequisite in tethering apoC-II_{44-79} to the lipid surface. NMR data in hexafluoro-2-propanol suggest the formation of an additional short helix between residues 67 and 74 (*27*). Given the suggestion that the binding of both apoA-I (*28*) and apoE (*29*) to lipid surfaces is mediated by cooperative interhelical interactions, the driving force behind the binding of apoC-II_{44-79} to lipid may be the creation of a lipid binding region formed via the interaction of two short α-helices. Our studies, demonstrating a strong interaction between an N-terminal human apoC-II_{19-39} peptide and lipid vesicles (*6*), suggest this region may also make up part of the lipid binding surface.

Consistent with the inability of apoC-II_{39-62} to bind lipid is the observation that the affinity of apoC-II_{39-62} for LpL is unchanged in the presence or absence of lipid. This suggests that it is the lipid-binding properties of apoC-II that modulate the increased affinity of the cofactor for LpL at the lipid interface. The conformational change in LpL that produces the active form of the enzyme is thought to involve repositioning of a surface loop that covers the enzyme active site (*30*). A similar loop is found in pancreatic lipase (*31*), and like that of LpL, activation of pancreatic lipase involves the formation of the complex with a small protein cofactor, colipase. The crystal structure of the binary complex of pancreatic lipase and colipase has been determined, with the enzyme in either the "open" (active) or "closed" (inactive) form. Activation of this enzyme by colipase involves the formation of contacts between the cofactor and the loop covering the active site (*32*). Secondary structure prediction suggests that the loop region in LpL consists of a helix−turn−helix motif (*33*). Given that apoC-II_{39-62} is predicted to form a short, amphipathic α-helix, it is possible that this region of apoC-II may not be responsible for tethering the cofactor to the lipid surface. Instead, the region between residues 39 and 62 may facilitate the activation of LpL following the formation of helix−helix interactions between the peptide and the surface loop of LpL.

REFERENCES

1. Shachter, N. S., Hayek, T., Leff, T., Smith, J. D., Rosenberg, D. W., Walsh, A., Ramakrishnan, R., Goldberg, I. J., Ginsberg, H. N., and Breslow, J. L. (1994) *J. Clin. Invest. 93*, 1683−1690.
2. Cox, D. W., Breckenridge, W. C., and Little, J. A. (1978) *N. Engl. J. Med. 299*, 1421−1424.
3. Rapp, D., and Olivecrona, T. (1978) *Eur. J. Biochem. 91*, 379−385.
4. Wittenauer, L. A., Shirai, K., Jackson, R. L., and Johnson, J. D. (1984) *Biochem. Biophys. Res. Commun. 118*, 894−901.
5. Clarke, A. R., and Holbrook, J. J. (1985) *Biochim. Biophys. Acta 827*, 358−368.
6. MacPhee, C. E., Howlett, G. J., Sawyer, W. H., and Clayton, A. H. A. (1999) *Biochemistry 38*, 10878−10884.
7. Catapano, A. L., Kinnunen, P. K., Breckenridge, W. C., Gotto, A. M., Jr., Jackson, R. L., Little, J. A., Smith, L. C., and Sparrow, J. T. (1979) *Biochem. Biophys. Res. Commun. 89*, 951−957.

8. Smith, L. C., Voyta, J. C., Catapano, A. L., Kinnunen, P. K., Gotto, A. M., Jr., and Sparrow, J. T. (1980) *Ann. N.Y. Acad. Sci. 348*, 213−223.
9. Voyta, J. C., Vainio, P., Kinnunen, P. K., Gotto, A. M., Jr., Sparrow, J. T., and Smith, L. C. (1983) *J. Biol. Chem. 258*, 2934−2939.
10. Lambert, D. A., Catapano, A. L., Smith, L. C., Sparrow, J. T., and Gotto, A. M., Jr. (1997) *Clin. Chim. Acta 264*, 75−90.
11. Jackson, C. L, Bruns, G. A. P., and Breslow, J. L. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 2945−2949.
12. Schagger, H., and von Jagow, G. (1987) *Anal. Biochem. 166*, 368−379.
13. Bengtsson-Olivecrona, G., and Olivecrona, T. (1991) *Methods Enzymol. 197*, 345−356.
14. McLean, L. R., and Jackson, R. L. (1985) *Biochemistry 24*, 4196−4201.
15. MacPhee, C. E., Chan, R. Y., Sawyer, W. H., Stafford, W. F., and Howlett, G. J. (1997) *J. Lipid Res. 38*, 1649−1659.
16. Belfrage, P., and Vaughan, M. (1969) *J. Lipid Res. 10*, 341−344.
17. Bailey, M. F., Davidson, B. E., Minton, A. P., Sawyer, W. H., and Howlett, G. J. (1996) *J. Mol. Biol. 263*, 671−684.
18. Tajima, S., Yokoyama, S., Kawai, Y., and Yamamoto, A. (1982) *J. Biochem. 91*, 1273−1279.
19. Shirai, K., Jackson, R. L., and Quinn, D. M. (1982) *J. Biol. Chem. 257*, 10200−10203.
20. Posner, I., Wang, C.-S., and McConathy, W. J. (1983) *Biochemistry 22*, 4041−4047.
21. Fielding, C. J., and Fielding, P. E. (1976) *J. Lipid Res. 17*, 419−423.
22. Schrecker, O., and Greten, H. (1979) *Biochim. Biophys. Acta 572*, 244−256.
23. Chung, J., and Scanu, A. M. (1977) *J. Biol. Chem. 252*, 4202−4209.
24. Olivecrona, G., and Beisiegel, U. (1997) *Arterioscler. Thromb. Vasc. Biol. 17*, 1545−1549.
25. McConathy, W. J., Gesquiere, J. C., Bass, H., Tartar, A., Fruchart, J.-C., and Wang, C.-S. (1992) *J. Lipid Res. 33*, 995−1003.
26. Lambert, D. A., Catapano, A. L., Smith, L. C., Sparrow, J. T., and Gotto, A. M., Jr. (1996) *Atherosclerosis 127*, 205−212.
27. Matsuoka, N., Shirai, K., and Jackson, R. L. (1980) *Biochim. Biophys. Acta 620*, 308−316.
28. Ohman, A., Lycksell, P. O., and Graslund, A. (1993) *Eur. J. Biophys. 22*, 351−357.
29. Palgunachari, M. N., Mishra, V. K., Lund-Katz, S., Phillips, M. C., Adeyeye, S. O., Alluri, S., Anantharamaiah, G. M., and Segrest, J. P. (1996) *Arterioscler. Thromb. Vasc. Biol. 16*, 328−338.
30. Lins, L., Brasseur, R., De Pauw, M., Van Biervliet, J. P., Ruysschaert, J.-M., Rosseneu, M., and Vanloo, B. (1995) *Biochim. Biophys. Acta 1258*, 10−18.
31. Faustinella, F., Smith, L. C., and Chan, L. (1992) *Biochemistry 31*, 7219−7223.
32. Jennens, M. L., and Lowe, M. E. (1994) *J. Biol. Chem. 269*, 25470−25474.
33. Egloff, M. P., Sarda, L., Verger, R., Cambillau, C., and van Tilbeurgh, H. (1995) *Protein Sci. 4*, 44−57.
34. Dugi, K. A., Dichek, H. L., Talley, G. D., Brewer, H. B., Jr., and Santamarina-Fojo, S. (1992) *J. Biol. Chem. 267*, 25086−25091.

BI992523T