Inhibition of lipoprotein lipase activity by synthetic peptides of apolipoprotein C-III

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Abstract In this study we have examined effects of synthetic polypeptide fragments of apoC-III on the kinetic properties of lipoprotein lipase (LPL) activity. Based on the loss of 79% of LPL-inhibitory activity after CNBr cleavage at the N-terminal portion of apoC-III and a systematic search for synthetic peptides with LPL-inhibitory activity spanning the apoC-III sequence, we concluded that the N-terminal domain is the most important in the modulation of LPL activity. In addition, there are multiple attachment sites in apoC-III for its interaction with LPL and these sites reside in the hydrophilic sequences of apoC-III. Probably for this reason the intact apo-CIII exhibited higher inhibitory potential than its peptide components. Based on the deduced inhibition constants derived for the synthetic apoC-III_{1-79} we concluded that apoC-III is likely to exhibit a physiological role in regulating LPL activity since the derived dissociation constants for the LPL-apoC-III interaction are within the physiological concentration range of plasma apoC-III. In addition, as the synthetic apoC-III_{1-79} lacks the carbohydrate moiety, we also concluded that the presence of the oligosaccharide in native apoC-III is not essential for its inhibitory activity on LPL. The fact that the I_{50} (concentration for inhibition of LPL at 50% activity) decreases for apoC-III-1 when assayed in the presence of apoC-II indicated that the activator actually caused an increased affinity between LPL and apoC-III and demonstrated that apoC-III does not compete for the activator site of apoC-II. —**McConathy, W. J., J. C. Gesquiere, H. Bass, A. Tartar, J-C. Fruchart, and C-S. Wang.** Inhibition of lipoprotein lipase activity by synthetic peptides of apolipoprotein C-III. *J. Lipid Res.* 1992. **33:** 995–1003.

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One of the major functions of plasma lipoproteins is to transport and distribute triacylglycerols (TG) to peripheral tissues to meet their energy and storage requirements. An important means of delivery of TG to tissues is a rate-limiting hydrolysis step catalyzed by lipoprotein lipase (LPL) (1–4). Because of its importance in controlling energy utilization, the LPL level is under tight hormonal control. Previous studies have shown that apoC-III represents an important physiological LPL inhibitor (5–7). In a previous study (5), we found that plasma from a subpopulation of hypertriglyceridemic patients had a normal level of LPL but with a plasma inhibition activity. This suggested that regulation of LPL by plasma inhibitors may represent one of the mechanisms that lead to the pathogenesis of hypertriglyceridemia. The correlation analyses (5) of plasma LPL-inhibition activity with plasma level led to the identification of apoC-III as one of the most important plasma factors involved in the regulation of LPL activity. Recently, studies by Ito et al. (8) have shown in a transgenic model that the overexpression of human apoC-III in mice can be a primary cause of hypertriglyceridemia. This observation further substantiated the suggestion of a direct relationship between apoC-III levels and hypertriglyceridemia.

Because of the importance of apoC-III in modulating LPL activity, we have pursued a kinetic analysis of the inhibition effect with apoC-III and its fragments to understand the LPL-apoC-III interaction. Structural analyses demonstrated that the gene for apoC-III contains 4 exons (9). While the first and second exon code untranslated nucleotides, the initiation codon and signal peptide sequence, the third exon codes for residues 1–40 and the fourth exon codes for residues 41–79. The products of exon 3 and 4 code for sequences predicted to form lipid-binding amphipathic helices (10, 11). By thrombin cleavage (12), plasma apoC-III is cleaved into two polypeptides, apoC-III_{1-40} and apoC-III_{41-79}, corresponding to the products of exons 3 and 4, respectively. Thrombin cleavage products have experimentally defined the lipid-binding domain of apoC-III as the C-terminal domain (12) as expressed by exon 4. In order to define the molecular domain of apoC-III required for the inhibition of LPL

Abbreviations: VLDL, very low density lipoproteins; LPL, lipoprotein lipase; apoC-III, apolipoprotein C-III; TG, triacylglycerol.

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and the molecular mechanism of apoC-III's effect on LPL activity, we report detailed kinetic analyses using CNBr-fragmented plasma apoC-III, synthetic apoC-III, and selected synthetic polypeptide fragments of apoC-III. These studies indicated that the product of exon 3, the N-terminal domain of apoC-III, is primarily responsible for modulation of LPL activity.

MATERIALS AND METHODS

Materials

Unless otherwise stated, all chemicals were purchased from Sigma Chemical Co. Glycerol tri[9,10-^{3}H]oleate was obtained from Amersham International.

Isolation of plasma apoC-III

Plasma from hypertriglyceridemic donors was used as a source of VLDL isolated as previously described (13). ApoC-III-1 and apoC-III-2 were isolated from delipidized VLDL as previously described (5, 14). ApoC-III exists in plasma in three forms (apoC-III-0, apoC-III-1, and apoC-III-2) differing in content of sialic acid with the Arabic numeral indicating the moles of sialic acid (15).

Fragmentation of apoC-III

The fragmentation of apoC-III was performed by the incubation of apoC-III-1 (2 mg) with cyanogen bromide (10 mg) in 70% formic acid solution (1 ml) as described by Steers et al. (16). After incubation at room temperature for 20 h, the reaction mixture was diluted with distilled water (25 ml) and cyanogen bromide was removed by lyophilization.

Preparation of synthetic apoC-III and its peptides

Synthesis of the peptide chains of apoC-III$_{1-79}$, apoC-III$_{1-41}$, apoC-III$_{1-17}$, apoC-III$_{5-17}$, apoC-III$_{12-35}$, apoC-III$_{29-45}$, and apoC-III$_{58-79}$ was carried out by solid phase methodology (17) using an Applied Biosystems 430A peptide synthesizer. Syntheses were started from C-terminal amino acids linked on (phenylacetamido)methyl (Pam) resins. The side chains of Boc amino acids were protected as follows: tosyl (Arg), formyl (Trp), 2,4-dinitrophenyl (His), (2-bromobenzyl)oxycarbonyl (Lys), (2-chlorobenzyl) oxycarbonyl (Tyr), and benzyl (Ser, Thr, Glu, Asp). When involved in an Asp–Gly sequence, Asp was protected by a cyclohexyl group in order to minimize β migration and aspartimide formation (18). The Boc group was deprotected by 50% trifluoroacetic acid in CH_2Cl_2. After coupling of a tryptophan or a methionine, 2% dimethylsulfide was added in all deprotection steps until the end of the synthesis. Couplings were carried out twice for each residue using symmetrical anhydrides of amino acids in DMF for the first coupling and HOBT esters in CH_2Cl_2 for the second coupling. Activation leading to symmetrical anhydrides or HOBT esters was performed by dicyclohexylcarbodiimide. At the end of the synthesis, His(Dnp) was deprotected by thiolysis using β-mercaptoethanol. The terminal Boc group was then removed and the peptidyl-resin was submitted to the classical Low-High HF treatment (19) with the addition of *p*-thiocresol in order to remove the formyl groups from tryptophan residues. Peptides were purified by gel filtration using Fractogel TSK HW 40(S) followed by reverse phase HPLC using a C18 column. Purity was checked by analytical HPLC, amino acid analysis, and plasma desorption mass spectrometry. ApoC-III$_{1-41}$ was further purified by gel filtration using Sephadex G10 in 10 mM ammonium acetate, pH 4.5, followed by ion exchange chromatography using Trisacryl M CM with a gradient from 10 to 100 mM ammonium acetate, pH 4.5.

Synthesis of complete apoC-III was carried out following similar procedures except that each double-coupling step was followed by capping with acetic anhydride in order to prevent the growth of peptides containing deletions. After the Low-High HF treatment (19), removal of protecting group for tryptophan residues was checked by monitoring the UV spectrum. The overall yield of the synthesis was estimated according to the following procedure. Taking advantage of the fact that isoleucine is not present in the sequence of apoC-III, at the end of the synthesis, an additional Boc Ile was incorporated onto a small sample of the peptidyl resin. Amino acid analysis after acidic hydrolysis of the extended peptidyl-resin allowed the determination of a ratio Ile/Pro of 0.12 relative to the two proline residues that were incorporated during cycles 6 and 10 at the beginning of the synthesis, indicating that about 20% of the peptidic chains had gone to completion. Crude apoC-III was submitted to a gel filtration using Fractogel TSK HW-55(S) in 4 M guanidine-HCl. Fractions from gel filtration were checked by amino acid analysis, thin-layer chromatography, and SDS-PAGE. The amino acid sequence of apoC-III is shown in **Fig. 1**.

In our initial studies, we tested the synthetic peptides in aqueous buffer without urea. However, some of the polypeptides were difficult to solubilize and in some cases the solution appeared slightly opaque or turbid. To ensure complete solubilization and a known effective concentration of synthetic polypeptide, we used 6 M urea for all of the polypeptides. The urea solutions (6 M) were prepared with ultrapure grade urea and the stock solutions were kept at 4°C. The addition of urea gave a clear solution in each case. This treatment allowed the comparison of the inhibition potential of these various peptides using the same treatment and ensured maximum solubility. The urea concentration was adjusted to 0.15 M in the final LPL assay mixture. To validate this approach using urea as a solubilizing agent, we compared the inhibition potential of one synthetic polypeptide solubilized with and without urea. We determined the I_{50} value of 110 μM for

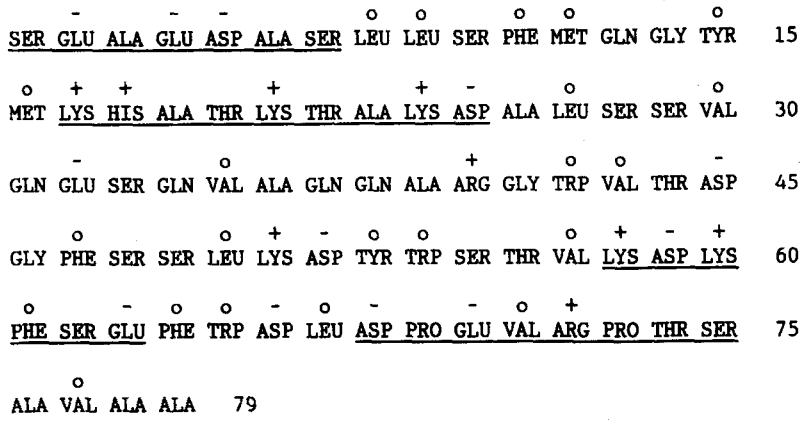


Fig. 1. Amino acid sequence of human apoC-III (20). The positive charge (+), negative charge (−) and hydrophobic amino acid residues (O) are marked in the sequence. The hydrophilic amino acid sequences with a high probability of being located on the surface of the apoC-III molecule are underlined.

apoC-III$_{1-41}$ when assayed in the absence of urea, which was similar to that obtained for the same polypeptide solubilized in the presence of urea (I_{50} value of 95 ± 33 μM), as shown in footnote of Table 1. Thus, we concluded that the presence of urea does not interfere with the I_{50} determination.

Based on these observations, all peptides were solubilized in 6 M urea and diluted 40-fold in the final assay mixture. Although the low concentration of urea had little effect on activity, in order to ascertain that the inhibition effect is due to the peptide alone, we also included 0.15 M urea in the control LPL assay mixture. In this study we operationally defined an apoC-III peptide with I_{50} (concentration for 50% inhibition of LPL activity) values greater than 5 mM as noninhibitory.

Preparation of LPL

Bovine milk LPL was used in this study. LPL was first purified from bovine skim milk by flotation of the enzyme with added Intralipid, followed by removal of fat from the isolated fat cake with acetone–ether treatment, as described previously (21). Acetone–ether powder represents a readily available and enriched source of bovine LPL and was utilized for the purification of LPL. Acetone–ether powder was suspended (5 mg/ml) in 50 mM NH_4Cl buffer, pH 8.5, containing 0.1% Triton X-100. Aliquots of 4 ml of the suspension (5 mg/ml) were applied to a small heparin-Sepharose column calibrated to contain 1 ml of wet gel. The column was eluted with 4 ml of 0.3 M NaCl and 4 ml of 0.72 M NaCl. The purified enzyme was eluted with 3 ml of heparin (10 mg/ml) and diluted 50-fold prior to use for the assay. The 0.3 M NaCl, 0.72 M NaCl, and heparin solutions were prepared with 50 mM NH_4Cl, pH 8.5. LPL eluted with heparin was stable for several days when kept at 0°C.

Assay of LPL

The assay of LPL activity was performed with dioleoyl phosphatidylcholine–emulsified glycerol tri[9,10-^{3}H]oleate as substrate, with a specific radioactivity of 2 μCi/μmol. The molar ratio of phosphatidylcholine to trioleoylglycerol was 1:10. The substrate was prepared as described previously (22). The assay mixture contained the amount of substrate described in the text, bovine serum albumin (60 mg/ml), and an appropriate volume of 50-fold diluted enzyme (10–20 μl). When activator was required, we added serum (3%, v/v) to the assay mixture. This serum concentration was selected because it gave similar I_{50} values (1.6 vs. 1.9 μM) in comparison to the data when apoC-II (1 μg/per assay) was utilized as LPL activator. The final volume of the assay mixture was adjusted to 100 μl with the addition of 50 mM NH_4Cl, pH 8.5. The mixture was shaken in a water bath at 37°C for 1 h. The reaction was terminated by adding 3.2 ml of chloroform–heptane–methanol 25:20:23 (v/v/v) and 1 ml of 0.2 M NaOH. After centrifugation, 1.2 ml of the top layer was mixed with 10 ml of Instagel (Packard) and the radioactivity was measured in a Packard liquid-scintillation counter. In all assays the substrate utilization was less than 10%, and under this condition the product release is linear with respect to the time of incubation.

Determination of the rate of lipolysis of VLDL catalyzed by LPL

The measurement of the rate of lipolysis of human plasma VLDL was based on the measurement of the first order kinetic rate, k_1, according to the previously described procedure (23). All incubations were carried out in a shaking water bath for up to 60 min at 37°C at pH 8.0. Incubation mixtures (final volume 6 ml) con-

tained 0.5 mM lipoprotein-triacylglycerol, bovine milk LPL (1 μg/ml), and bovine serum albumin (60 mg/ml). The final volume was adjusted by the addition of 50 mM of NH_4Cl, pH 8.5. The extent of lipolysis was estimated from the decrease in triacylglycerol concentration as determined by a gas-liquid chromatography procedure (22) originally described by Kuksis et al. (24). At various time intervals, (2.5, 5, 7.5, 10, 15, 10, 40, and 60 min) duplicate samples (0.25 ml) were removed and added to 4 ml of n-heptane–isopropanol 3:7 (v/v) which contained 25 μg of cholesterol butyrate as internal standard. After acidification with 2.5 ml of 0.033 N H_2SO_4, the mixture was vortexed for 30 sec, the upper phase containing triacylglycerols and the internal standard was transferred to a 3-ml conical tube, and the solvent was evaporated under nitrogen. The residue was redissolved in 100 μl of n-hexane and 2-μl aliquots were injected, with the use of an autosampler, into the gas chromatograph for triacylglycerol analysis. The pseudo-first order rate (k_1) of the rate of lipolysis was determined by least square non-linear curve-fitting (21).

Data analysis

Kinetic analysis was performed using a LOTUS 1-2-3 spreadsheet program and an IBM-AT computer. The performance of least-square non-linear curve-fitting was based on the approach described by Bevington (25). For the determination of sum of square of errors with two input variable parameters, we used the "table" function of the spreadsheet program for the calculation.

RESULTS

Effect of cyanogen bromide treatment on the inhibition activity of apoC-III

In order to understand whether the structural integrity of apoC-III is important for its inhibition effect, we examined the inhibitory effect of intact and CNBr-treated apoC-III isolated from plasma on LPL activity. ApoC-III contains two methionine residues: one resides at position 12 and the other at position 16 (Fig. 1). From the sequence data, treatment of apoC-III with CNBr will yield peptide fragments, apoC-III_{1-12}, apoC-III_{13-16}, and apoC-III_{17-79}. In our studies, CNBr fragmentation of apoC-III yielded only a single band of lower mobility than either intact apoC-III or apoC-II as assessed by basic polyacrylamide gel electrophoresis. Based on the use of human VLDL as substrate with purified bovine milk LPL, we have observed that the intact apoC-III (20 μM) exhibited a 52% inhibition effect on the lipolysis rate. The lipolysis experiment including the same concentration of the unfractionated CNBr peptides of apoC-III exhibited only an 11% inhibition effect (**Fig. 2**). We concluded that there is a 79% loss of inhibition potential of apoC-III after treatment with cyanogen bromide. Based on this finding, we concluded that the structural integrity of apoC-III is important in the observed apoC-III inhibitory effect. Since the two methionine residues of apoC-III are clustered in the N-terminal region, the finding also pointed to the probability that the functional domain of apoC-III inhibiting LPL may reside primarily in the N-terminal region. The results with cyanogen bromide cleavage products led us to focus our studies on the search for the apoC-III inhibitory domain in the N-terminal structural region.

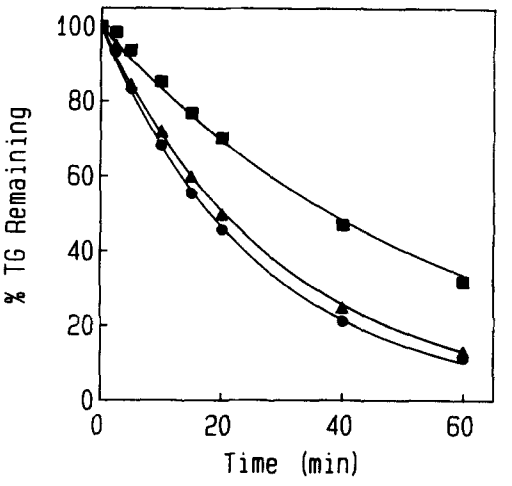


Fig. 2. Effect of CNBr cleavage on apoC-III inhibition of LPL-mediated lipolysis of VLDL. The kinetics of VLDL lipolysis as catalyzed by purified bovine milk LPL. The lipolysis was performed in the absence (●) and presence of 20 μM of apoC-III (■) and 20 μM apoC-III which was fragmented by CNBr (▲). The respective least-square fitted k_1 values of pseudo-first order rates are 0.038 ± 0.001 min^{-1}, 0.018 ± 0.001 min^{-1}, and 0.034 ± 0.001 min^{-1}, respectively.

Effect of apoC-III synthetic peptides on LPL activity

In order to determine the LPL inhibition functional domain of apoC-III, we prepared a number of synthetic peptides of apoC-III for this kinetic study (**Table 1**). Since

TABLE 1. I_{50} values for apoC-III-1 and apoC-III synthetic peptides for the inhibition of LPL

ApoC-III Peptide (residue numbers)	Basal Activity	Serum-Activated Activity[a]
	μM	
ApoC-III-1	10.0 ± 0.7	1.9 ± 0.2
1-79	2.7 ± 1.3	4.3 ± 1.1
1-17	330 ± 40	430 ± 130
1-41	95[b] ± 33	76 ± 14
5-17	noninhibitory	noninhibitory
12-35	470 ± 50	440 ± 140
29-45	noninhibitory	noninhibitory
58-79	2340 ± 620	780 ± 330

Results are expressed as mean ± SD (n = 3).
[a]The final serum concentration in the assay mixture was 3% (v/v).
[b]Polypeptide solubilized without urea gave a similar I_{50} (110 μM).

LPL exhibited basal activity, and because of the simplicity of the kinetic treatment, we initially examined the effect of these peptides on basal LPL activity. Among these peptides, as anticipated, apoC-III_{1-79} exhibited the most potent inhibitory effect with an I_{50} value (concentration for exhibiting 50% inhibition) of 2.7 ± 1.3 μM (**Fig. 3**, Table 1). The I_{50} value of apoC-III-1 is of the same magnitude but not identical to that of apoC-III_{1-79} (Table 1). Among the peptide fragments, apoC-III_{1-41} was the next most potent inhibitor. However, the I_{50} value is about 35-fold higher than apoC-III_{1-79} which indicated that the C-terminal domain of apoC-III could also contribute to the inhibitory effect of apoC-III. ApoC-III_{1-17} also exhibited inhibitory effect. However, the I_{50} is about 3-fold higher than that of apoC-III_{1-41} which would indicate that the peptide residues between 18 and 41 play a role in the interaction with LPL. The removal of the N-terminal four hydrophilic residues, H_2N-Ser-Glu-Ala-Glu, renders the peptide (apoC-III_{5-17}) inactive in inhibiting LPL; however, the presence of these four residues is not an absolute requirement since we have detected the inhibitory effect of apoC-III_{12-35} (Table 1). In addition to apoC-III_{5-17}, we have also observed the lack of inhibition effect of apoC-III_{29-45}. By further extending the study to the C-terminal region, we have also observed the inhibitory effect of apoC-III_{58-79} but with a high I_{50} value (2340 ± 620 μM).

When assaying the effects of apoC-III peptides on LPL activity and in the presence of serum as a source of activator, the results indicated a comparable I_{50} value, except with apoC-III_{58-79} which exhibited a 3-fold lower I_{50} value (Table 1).

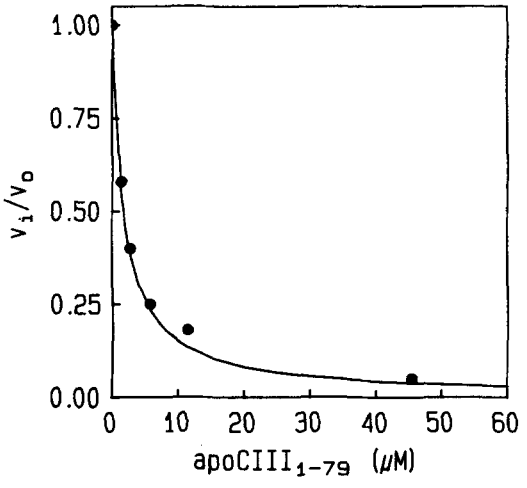
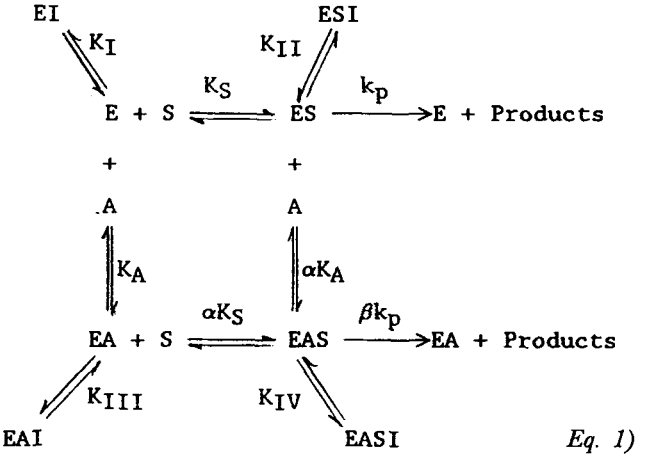


Fig. 3. Effect of apoC-III_{1-79} on basal activity of LPL with varying of inhibitor concentration. The substrate concentration (TG) was 1.4 mM. The curve was generated using equation 4. The parameters utilized for the calculation were K_S = 0.075 mM, K_I = 0.15 μM, and K_{II} = 4.3 μM.

Kinetic study with apoC-III_{1-79}

In order to understand the mechanism of LPL-apoC-III interaction, and having available a sufficient quantity of synthetic apoC-III_{1-79}, we performed detailed kinetic analyses to study the mechanism of apoC-III inhibition effect. When we considered the reaction scheme as indicated below, there were four inhibition constants to be determined, including K_I, K_{II}, K_{III}, and K_{IV}, as indicated in equation 1. On the basis of our previous studies (26), we have suggested that the mechanism of lipolysis in the LPL-catalyzed reaction can be described by a rapid equilibrium-random mechanism, as indicated below:



Eq. 1)

In the kinetic scheme, E is the enzyme (LPL), S is the substrate (TG), A is the activator, I is the inhibitor apoC-III, k_p is the rate constant for the breakdown of ES to E + products, βk_p is the rate constant for the breakdown of EAS to EA + products, and K_I, K_{II}, K_{III}, and K_{IV} are the dissociation constants for each of the indicated reactions of apoC-III with various enzyme forms. The kinetic parameters K_A, K_S, αK_A, and αK_S are the Michaelis-Menten constants for each of the indicated reactions. Because the effective substrate concentration is not the same for the emulsified substrate, the derived K_S is related to the "true" dissociation constant by a constant factor that corresponds to the ratio of the "effective" to the "analytical" substrate concentration (26). On the other hand, the derived K_A value should represent the dissociation constant of the LPL and apoC-II interaction. For the kinetic scheme described above, the velocity of the enzyme reaction as affected by apoC-III can be described by the following equation:

$$v = \frac{V_{max}\left(\frac{[S]}{K_S}\right) + \beta V_{max}\left(\frac{[A]\,[S]}{\alpha K_A K_S}\right)}{\left(1 + \frac{[I]}{K_I}\right) + \frac{[S]}{K_S}\left(1 + \frac{[I]}{K_{II}}\right) + \frac{[A]}{K_A}\left(1 + \frac{[I]}{K_{III}}\right) + \frac{[A]\,[S]}{\alpha K_A K_S}\left(1 + \frac{[I]}{K_{IV}}\right)}$$

Eq. 2)

In order to determine the dissociation constants K_I and K_{II}, we examined the inhibition effect of apoC-III on the basal activity of LPL. In this experimental condition, [A] = 0 and therefore the equation can be reduced to the following:

$$v = \frac{V_{max}\left(\frac{[S]}{K_S}\right)}{\left(1 + \frac{[I]}{K_I}\right) + \frac{[S]}{K_S}\left(1 + \frac{[I]}{K_{II}}\right)} \quad \textit{Eq. 3)}$$

The kinetic pattern of the apoC-III inhibition effect under the experimental conditions, with varying substrate concentrations is shown in **Fig. 4**. Based on the effect of apoC-III on the apparent V_{max} and K_s compared with that of control and based on equation 3, we have deduced the K_I and K_{II} values as shown in **Table 2**. When we utilized these two kinetic parameters based on equation 4, below, we obtained an I_{50} value for apoC-III_{1-79} of 1.7 μM (Fig. 3), which is in close agreement with the 2.7 μM shown in Table 1. In equation 4 below,

$$\frac{v_i}{v_o} = \frac{1 + \frac{K_S}{[S]}}{1 + \frac{[I]}{K_{II}} + \frac{K_S}{[S]}\left(1 + \frac{[I]}{K_I}\right)} \quad \textit{Eq. 4)}$$

v_o is the control basal activity of LPL in the absence of inhibitor, while v_i represents the basal activity of LPL in the presence of inhibitor apoC-III.

We further proceeded to examine the kinetic effect of apoC-III with varying apoC-II concentrations (**Fig. 5**). By using non-linear curve-fitting and varying the two parameters K_{III} and K_{IV} systematically, we have deduced the best values of K_{III} and K_{IV}, which are shown in Table 2. Based on the finding that K_I versus K_{III}, and K_{II} versus K_{IV} are of the same magnitude, we concluded that the binding of apoC-II to LPL has little effect on the subsequent binding of LPL with apoC-III. On the other hand, the binding of substrate to the enzyme apparently hampers the further binding of the enzyme to the inhibitor, as can be seen by the deduced value of approximately 29 for K_{II}/K_I and the ratio of 10 for K_{IV}/K_{III}.

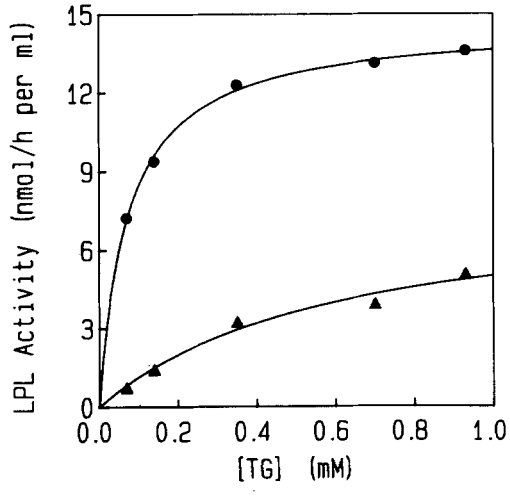


Fig. 4. Effect of apoC-III on LPL basal activity. The inhibitor apoC-III concentrations were 0 μM (●) and 1.4 μM (▲).

TABLE 2. Inhibition constants of apoC-III_{1-79}

K_I	K_{II}	K_{III}	K_{IV}
	μM		
0.15 ± 0.05 (n = 4)	4.3 ± 3.7 (n = 4)	0.44 ± 0.20 (n = 8)	4.5 ± 4.0 (n = 8)

The inhibition constants are defined in equations 1 and 2. Results are mean ± SD.

DISCUSSION

In this study we have examined synthetic apoC-III and a number of its peptides with the aim of defining the functional domain of apoC-III responsible for its inhibitory effect on LPL activity. Among the apoC-III peptides, apoC-III_{1-79} had the lowest I_{50} values for both the basal and the serum-activated LPL activities. One of the reasons for this finding may be that only apoC-III_{1-79} can refold to acquire the proper conformation for exhibiting full inhibition activity; alternatively, the other possibility

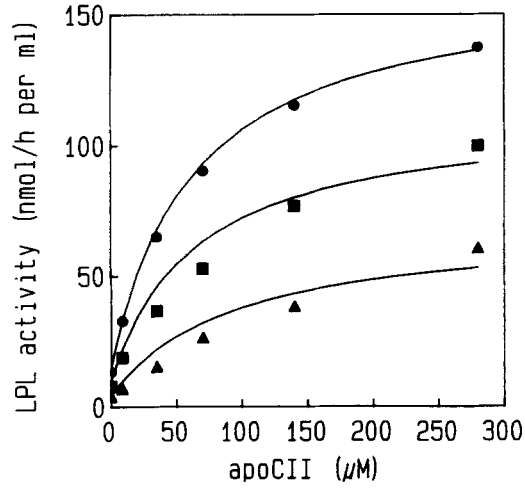


Fig. 5. Effect of apoC-III_{1-79} on LPL activity with various activator apoC-II concentrations. ApoC-III_{1-79} concentrations were 0, (●) 0.35 μM, (■), and 1.4 μM (▲). The curves were generated using equation 2. The parameters utilized for these sets of experiments were: α = 1.4, β = 12, V_{max} = 17.8 nmol/ml; K_A = 48 nM, K_S = 0.075 mM, K_I = 0.15 μM, and K_{II} = 4.3 μM. The best K_{III} and K_{IV} values as derived from this plot are shown in Table 2. The parameters utilized are similar but not identical to those previously reported (22).

is that there are multiple attachment points for apoC-III to interact with LPL. Therefore, intact apoC-III can bind more tightly with LPL than apoC-III peptide fragments because the intact apoC-III contains more attachment sites. Since the peptides from different regions of apoC-III exhibited detectable LPL-inhibitory activities, we therefore favor the latter possibility. As our synthetic apoC-III does not contain carbohydrate, we also concluded that the carbohydrate moiety of apoC-III is not an absolute requirement for exhibiting inhibitory activity. ApoC-III-1 that was isolated from human plasma has an I_{50} value of the same magnitude as that of apoC-III_{1-79} (Table 1), suggesting that the carbohydrate moiety may modulate, but is not essential for, the inhibition activity of apoC-III. Our data suggest that the basal form of LPL has a higher affinity for apoC-III, while the activated form of the enzyme has a higher affinity for the glycosylated form of apoC-III. This can explain the 5-fold lower I_{50} value of apoC-III-1 when serum is present. Consistent with such an assessment, the 3-fold lower I_{50} value of apoC-III_{1-79} than that of apoC-III-1 for LPL basal activity can be explained by the lack of carbohydrate in the synthetic apoC-III_{1-79}.

Depending on their sequence, some of the apoC-III peptides are hydrophobic and are difficult to solubilize in aqueous medium. In order to reproducibly solubilize these peptides, we used 6 M urea as the solubilizing reagent. As all of the apoC-III peptides were initially solubilized in 6 M urea, and the effect of urea was essentially eliminated by dilution to 0.15 M, the observed kinetic effect would indicate that these peptides may be initially unfolded in 6 M urea and can rapidly refold to a conformation that can exhibit the inhibition effect on LPL activity. This conclusion was supported by the similarity of the I_{50} of apoC-III_{1-41} in the presence and absence of urea (Table 1).

The two methionine residues of apoC-III are clustered in the N-terminal region and the cleavage of apoC-III at these sites by cyanogen bromide led to a large reduction of its inhibitory potential and led us to focus our initial studies on the search for the apoC-III inhibitory domain in the N-terminal structural region. When the inhibition effects of apoC-III_{1-17} and apoC-III_{5-17} were compared, the former exhibited detectable inhibitory effect while the latter did not. Thus, we have concluded that the N-terminal four amino acid residues must be important for interaction with LPL. As the N-terminal seven residues of apoC-III are highly hydrophilic and also contain three negatively charged amino acid residues, they are likely to be on the surface of the apoC-III molecule. Based on the sequence shown in Fig. 1, the N-terminal seven amino acid residues may constitute one of the important structural domains for interaction with LPL. Even though apoC-III_{5-17} does not inhibit LPL, we have found that apoC-III_{12-35} had a detectable inhibitory activity. In this peptide, there is a stretch of hydrophilic amino acids in the region between residue 17 and residue 26 (with five charged amino acids and two hydroxyl-amino acids) and this could represent a second putative binding domain of apoC-III. We also prepared a short peptide of apoC-III_{29-45}, which did not contain such hydrophilic peptides and did not exhibit LPL inhibitory activity. By extending the structure of apoC-III_{1-17}, we also prepared apoC-III_{1-41}. With this extension apoC-III_{1-41} is about 3-fold more potent than apoC-III_{1-17} in inhibiting LPL activity (Table 1). However, the inhibition potential of apoC-III_{1-79} is still about 35-fold more potent than apoC-III_{1-41}. This finding would indicate that the C-terminal region of apoC-III must also contribute to the apoC-III-LPL interaction. We therefore prepared the apoC-III_{58-79} to investigate its inhibition effect on LPL activity. The finding of a low but detectable inhibitory activity of this peptide would suggest that this C-terminal region of apoC-III could also interact with LPL. There are two stretches of hydrophilic amino acids in this region, with one located between 58–63 and the other between 68–76, both with a high probability of exposure to the surface. Whether the interaction of apoC-III with LPL involves one or both of these structural regions will require future fine mapping studies.

The lipid-binding domain residues have been demonstrated experimentally in the domain as expressed by exon 4 (12), rather than in the apoC-III_{1-41} segment of the polypeptide chain coded by exon 3 (9). Although residues 1–40 contain a predicted amphipathic helical segment, "the failure to isolate a lipid/protein complex indicated that the total hydrophobicity of this peptide is insufficient to permit stable complex formation" (27). Furthermore, the charge distribution of this domain (residues 16–33) has been suggested to be only weakly amphipathic by Segrest (28). This is also supported by the circular dichroism studies of Sparrow and Gotto (27) which demonstrated, in contrast to the apoC-III_{41-79} domain, apoC-III_{1-40}'s lack of change in molar ellipicity in the presence of dimyristoyl phosphatidyl choline. Because apoC-III_{1-41} does not experimentally contain a demonstrated lipid-binding domain, its interaction with lipid was not considered to be of importance. In fact, based on the conserved nature of the exon 3 product and its lack of experimentally documented lipid-binding properties, our findings suggest that the modulation of LPL activity may be an important property of apoC-III that has been evolutionarily conserved in exon 3. This is supported by the N-terminal sequence from several species (29) and swine (V. Trieu, personal communication) which indicates that the N-terminal 1–40 amino acid sequence is well conserved (**Table 3**) in comparison to the C-terminal domain.

As apoC-III_{1-41} does not contain the experimentally determined phospholipid-binding domain (12) and still exhibits an inhibition effect, we therefore concluded that

TABLE 3. Homology of exon 3 and exon 4 products of the apoC-III gene of several species compared to human apoC-III

	ApoC-III	Exon 3 Residues 1-40	Exon 4 Residues 41-79
		% identical residues[a]	
Bovine[b]	73	84	59
Canine	67	75	62
Swine[c]	68	71	65
Rat	52	58	41

[a] Based on number of identical residues in corresponding sequences when compared to human sequence.
[b] Based on reported sequence to residue 69.
[c] Sequence of swine apoC-III (V. Trieu, personal communication).

the protein:protein domain interaction is the most important aspect for the inhibition effect of apoC-III. Moreover, it is not likely that such a peptide will displace apoC-II from the substrate interface. In view of the fact that activation of LPL requires the direct and specific interaction between LPL and apoC-II, as well as the fact that an apoC-II domain lacking a phospholipid-binding domain can activate LPL (30), we believe the displacement of apoC-II from the substrate by apoC-III (31) is not kinetically relevant to the inhibition of LPL activity. For apoC-III to represent a signal of plasma triacylglycerol (TG) concentration, the inhibitory domain should be exposed and reside on the surface of TG-rich lipoproteins, thereby permitting a direct protein:protein interaction between LPL and apoC-III. If this is the case, it would provide an efficient control of the lipolysis reaction.

If we consider that I_{50} value is proportional to the dissociation constant (K_D) between the inhibitor and the enzyme, the following discussion is appropriate concerning the consequence of the increase of molecular size of apoC-III synthetic polypeptides on the apoC-III-LPL interaction where K_D would correspond to K_I, K_{II}, K_{III}, K_{IV} under the different conditions in the absence and presence of other ligands, including the substrate and apoC-II. The dissociation constant (K_D) of the enzyme-inhibitor interaction represents the ratio of the rate constant of dissociation (k_2) and association (k_1):

$$K_D = \frac{k_2}{k_1} \qquad \textit{Eq. 5)}$$

Increasing the molecular size of the inhibitor will cause a decrease in the diffusion rate, and consequently decrease the k_1 value. Therefore, it is easily deduced from equation 5 that the increase of molecular size will have an unfavorable effect on reducing K_D. On the other hand, the increase of molecular size from the peptide fragment to intact apoC-III provides the complete attachment sites and therefore can effectively reduce the k_2 and consequently favor the reduction of K_D. Based on this analysis, the effect of increase of peptide length of apoC-III has more effect on k_2 than on k_1. Experimental determination of the k_1 and k_2 values will have to await fast-kinetic analyses.

In order to further define the physical meaning of the I_{50} value, we have examined the inhibition effect of apoC-III under experimental conditions of varying substrate and activator concentration. One of the surprising findings from this study is that the I_{50} value of apoC-III_{1-79} is about 19-fold that of the K_I value. Thus, it is very important to note that I_{50} values provide a convenient index for comparing the affinity of homologous inhibitors in their interaction with the enzyme, but these values can be very different in magnitude from the corresponding dissociation constants. The apoC-III concentration in normolipidemic plasma is about 10 μM (14) which is much greater than the K_I or K_{III}; thus, it can be deduced that one of the important functions of apoC-III is to maintain the homeostasis of plasma TG by retarding the hydrolysis of TG when the plasma TG concentration is low. On the other hand, when plasma TG concentration is high, the apoC-III-LPL dissociation constant will switch to the high dissociation constants, as indicated for K_{II} and K_{IV}. As apoC-III represents one of the components of TG-rich lipoproteins, the accumulation of TG is associated with increased apoC-III levels (5, 6). Therefore, the increase of TG will inhibit LPL activity due to the increased apoC-III level, as indicated by the inhibition constant of K_{II} and K_{IV}. The physiological function of apoC-III inhibition of LPL activity when plasma TG is high is to prevent the LPL reaction from occurring under V_{max} conditions. Without such an inhibition mechanism the serum albumin could become saturated with fatty acids. The presence of high plasma free fatty acid levels could be more harmful than hypertriglyceridemia per se.

In conclusion, we have demonstrated in the present study that there are multiple binding regions of apoC-III for its interaction with LPL and for this reason the intact apoC-III can bind more tightly than the constitutive apoC-III peptide fragments. These sites are located primarily in the N-terminal domain and are likely to represent hydrophilic domains of apoC-III, which are most likely located on the surface of the apoC-III molecule. Because the dissociation constant derived for LPL-apoC-III is comparable to or below the physiological concentration level of plasma apoC-III, this indicates that apoC-III can function as LPL inhibitor in vivo.

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