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C-Terminal domain of apolipoprotein CII as both activator and competitive inhibitor of lipoprotein lipase

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In this study we have prepared peptides of the *C*-terminal domain of apolipoprotein CII (ApoCII) by a solid-peptide-synthesis technique and demonstrated that the *C*-terminal tetrapeptide, Lys-Gly-Glu-Glu, represents an inhibitor of lipoprotein lipase. The tetrapeptide not only inhibits the basal activity of lipoprotein lipase, but also blocks the activation effect of native ApoCII. The lengthening of this tetrapeptide resulted in a corresponding increase in affinity for lipoprotein lipase. This suggested that amino acids other than those of the *C*-terminal tetrapeptide also contribute to the binding affinity of ApoCII for lipoprotein lipase. On the basis of an essential requirement of the ApoCII terminal domain for binding to lipoprotein lipase, we suggest that the initial interaction of ApoCII, mediated via the *C*-terminal tetrapeptide, promotes the proper alignment of ApoCII with lipoprotein lipase, followed by the weak interaction of the ApoCII activator domain with the lipoprotein lipase activator site, enhancing the lipolysis process.

INTRODUCTION

Lipoprotein lipase (LPL) catalyses the hydrolysis of long-chain triacylglycerols (TG) at maximal rates in the presence of the activator, apolipoprotein CII (ApoCII). The physiological site of action of LPL is at the luminal surface of the capillary endothelium, and hydrolysis of blood TG facilitates fatty acid uptake by extrahepatic tissues (Nilsson-Ehle *et al.*, 1980; Cryer, 1981). Because of its key role in controlling the flux of TG through the plasma compartment, LPL activity is tightly regulated by nutrients and hormones to permit the tissues to respond to energy requirements and storage needs.

Owing to the small size of ApoCII (79 amino acid residues), this apolipoprotein has been previously studied by the systematic examination of a number of synthetic peptides of ApoCII for their respective kinetic effects on the activation of LPL (Kinnunen *et al.*, 1977; Smith *et al.*, 1981; Quinn *et al.*, 1982; Smith & Pownall, 1984; Balasubramaniam *et al.*, 1986; Jackson *et al.*, 1986). We initiated our study on the basis of a demonstration that the ApoCII-(56–79)-peptide domain represents the region for the activation of LPL (for reviews, see Quinn *et al.*, 1982; Smith & Pownall, 1984). Our particular focus on the *C*-terminal domain was based on the deduction that the peptide residues near the *C*-terminal amino acid represent an ionic binding site of ApoCII to LPL (Quinn *et al.*, 1982). We have further hypothesized that it may be possible to construct a LPL inhibitor corresponding to a portion of this domain which has the functional property of binding to LPL without activating LPL, and thus could behave as a competitive inhibitor of LPL. The availability of such a competitive inhibitor of LPL would provide a reagent for the perturbation of LPL activity necessary for more refined studies on the physiological function of LPL. If an ApoCII fragment were a competitive inhibitor of LPL, this would provide evidence for the kinetic mechanism previously described (Posner *et al.*, 1983*a*). In addition, it may also provide probes for further studies of the mechanism of activation of LPL and ApoCII interaction. Here we describe the properties of several *C*-terminal peptides, prepared by the solid-peptide-synthesis technique, which exhibit such an inhibitor effect on LPL activity, and report their respective kinetic parameters.

EXPERIMENTAL

Materials

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

Preparation of ApoCII peptides

The ApoCII peptides were synthesized on an Applied Biosystem Synthesizer (model 430A) by the Molecular Biology Resource Facility at the Oklahoma University Health Science Center according to the t-butyloxycarbonyl methodology described by the manufacturer, and were purified by reversed-phase h.p.l.c.; each peptide used in the present study had the expected amino acid composition. The amino acid sequence for ApoCII contains 79 rather than 78 residues (Smith & Pownall, 1984); the amino acid sequence numbers reported in previous publications should be increased by 1 after residue 25 for purposes of comparison. The longest peptide used in this study, ApoCII-(56–79)-peptide, has the following sequence:

Ser-Thr-Ala-Ala-Met-Ser-Thr-Tyr-Thr-Gly-Ile-Phe-Thr-Asp-Gln-Val-Leu-Ser-Val-Leu-Lys-Gly-Glu-Glu

Other synthetic ApoCII peptides prepared for this study included those corresponding to residues 61–79, 73–79, 76–79, 76–78 and 73–78.

Preparation of LPL

Bovine milk LPL was used in these studies. The purification of bovine milk LPL using heparin–Sepharose affinity chromatography was performed as described by Posner *et al.* (1983*b*).

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 twice-concentrated substrate was prepared as described by Eckel *et al.* (1988). The assay mixture contained 1.4 mM-tri-

Abbreviations used: LPL, lipoprotein lipase; TG, triacylglycerols; ApoCII, apolipoprotein CII.

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oleoylglycerol, BSA (60 mg/ml) and an appropriate volume of 100-fold diluted enzyme (10–20 μl). 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 on 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, by vol.) and 1 ml of 0.2 M-NaOH. After centrifugation, 1.2 ml of the top layer is 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. One unit of enzyme activity is defined as 1 nmol of fatty acid released/h at 37 °C.

Data analysis

Kinetic analysis was performed by using the LOTUS 1-2-3 spreadsheet program and an IBM-AT computer. The approach for performance of least-squares non-linear curve-fitting was based on the approach described by Bevington (1969).

RESULTS

On the basis of our previous study (Posner *et al.*, 1983*a*) we have suggested that the mechanism of lipolysis in the LPL-catalysed reaction can be described by a rapid-equilibrium random mechanism illustrated below:

$$\begin{array}{ccccc} E+S & \overset{K_S}{\rightleftharpoons} & ES & \xrightarrow{k_p} & E+P+Q \\ + & & + & & \\ A & & A & & \\ \Big\Downarrow{\scriptstyle K_A} & & \Big\Downarrow{\scriptstyle \alpha K_A} & & \\ EA+S & \underset{\alpha K_S}{\rightleftharpoons} & ESA & \xrightarrow{\beta k_p} & EA+P+Q \end{array} \qquad (1)$$

In the reaction scheme, E is the enzyme (LPL), S is the substrate (TG), A is the activator or inhibitor (ApoCII or ApoCII peptides), P and Q are the product alcohols and carboxylic acids respectively, k_p is the rate constant for breakdown of ES to E+P+Q, βk_p is the rate constant for breakdown of ESA to EA+P+Q, and K_A, K_S, αK_A and αK_S are Michaelis–Menten constants for each of the indicated reactions. Because the effective and analytical substrate concentrations are not the same for the emulsified TG substrate, the derived K_S is related to the 'true' dissociation constant by a constant factor which corresponds to the ratio of the 'effective' to the 'analytical' substrate concentrations (Posner *et al.*, 1983*a*). On the other hand, the derived K_A values should represent the dissociation constants of the LPL and ApoCII interaction. On the basis of the kinetic scheme described above (eqn. 1), the velocity equation is shown below (Segel, 1975):

$$v = \frac{V_{max.}\dfrac{[S]}{K_S} + \beta V_{max.}\dfrac{[A][S]}{\alpha K_A K_S}}{1 + \dfrac{[S]}{K_S} + \dfrac{[A]}{K_A} + \dfrac{[A][S]}{\alpha K_A K_S}} \qquad (2)$$

For the determination of the kinetic parameters of the various synthetic peptides of the *C*-terminal domain of ApoCII with respect to their effect on LPL activity, we first examined the kinetic parameters of the basal activity, K_S and $V_{max.}$. This then permitted the determination of the effect of ApoCII peptides on these kinetic parameters, as indicated by α and β respectively. The kinetics of the reaction velocity of the basal activity as a function of TG concentration is shown in Fig. 1. On the basis of setting [A] = 0 for eqn. (2), we have deduced the parameters K_S and $V_{max.}$ by non-linear curve-fitting of the basal LPL activity, as shown in Table 1. In addition, we have also performed the lipolysis reaction with varying concentrations of TG, but in the presence of a fixed concentration of ApoCII peptide, which allows the determination of $K_{S,app.}$ and $V_{max.,app.}$. The data of these experiments are also shown in Table 1. Although we have also observed inhibitory effect of ApoCII-(76–78)-peptide on LPL activity, we did not further pursue the study of its kinetic effect because the estimated K_A was much greater than 5 mM. In Table 1 we define the ratio $K_{S,app.}/K_S$ as $\alpha_{app.}$. Since $K_{S,app.}$ was not determined at the saturating concentration of ApoCII peptides, the relationship of α to $\alpha_{app.}$ has to be further deduced. The relationship between $\alpha_{app.}$ and the ApoCII-peptide concentration or α at a fixed [S] (1.4 mM) can be deduced from eqn. (2), as shown below (Segel, 1975):

$$\alpha_{app.} = \frac{(1+[A]/K_A)}{(1+[A]/\alpha K_A)} \qquad (3)$$

For the determination of the α and β values of the ApoCII peptides, we also performed the lipolysis reaction at a fixed TG concentration (1.4 mM) and with varying concentrations of ApoCII peptides. A typical experiment exhibiting the effect of varying ApoCII-(1–79)-peptide or ApoCII-(76–79)-peptide on

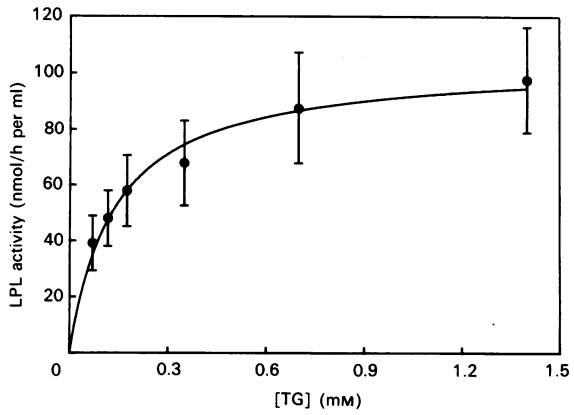

Fig. 1. Effect of substrate concentration on basal activity of LPL

The LPL concentration in the assay mixture was 87 ng/ml. The curve was obtained from eqn. (2) by setting [A] = 0 and by least-squares non-linear curve-fitting. The derived $V_{max.}$ and K_S values are shown in Table 1. The error bars represent S.E.M. from five separate experiments.

Table 1. Synthetic ApoCII peptides as either activators or inhibitors of LPL: effect on apparent K_S and $V_{max.}$

Results are means ± S.E.M.

ApoCII peptide (residue nos.)	*n*	[A]	$K_{S,app.}$ (mM)	$\alpha_{app.}$	$V_{max.,app.}$ (nmol/h per ml)
Control*	5	0	0.16±0.01	–	106±21
1–79	4	0.56 μM	0.22±0.05	1.38	927±108
56–79	6	39 μM	0.17±0.01	1.06	498±51
61–79	4	60 μM	0.09±0.01	0.58	230±61
73–79	4	1.31 mM	0.12±0.05	0.75	34±9
73–78	4	1.58 mM	0.12±0.01	0.75	32±5
76–79	6	1.45 mM	0.11±0.02	0.69	32±1

*Basal activity.

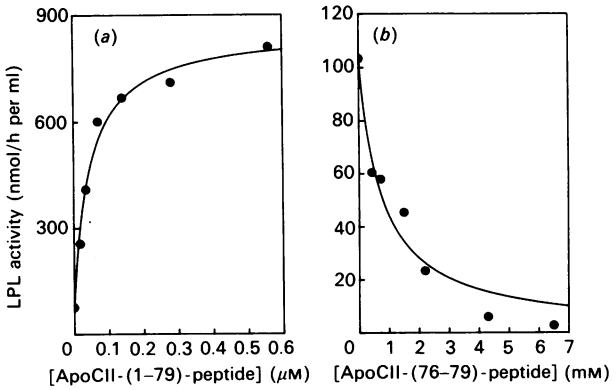

Fig. 2. (*a*) Effect of ApoCII-(1–79)-peptide on basal LPL activity and (*b*) effect of ApoCII-(76–79)-peptide on basal LPL activity

The substrate concentration was 1.4 mM. The curve was derived on the basis of eqn. (2); kinetic parameters are shown in Table 2.

LPL activity is shown in Fig. 2. It was found that ApoCII-(1–79)-peptide behaved as activator, whereas ApoCII-(76–79)-peptide behaved as inhibitor of basal LPL activity. To derive α and β, we performed the non-linear curve-fitting, based on eqn. (2), on data of the kind presented in Fig. 2. Since the kinetic parameters K_S and $V_{max.}$ for the basal activity have already been determined, as shown in Table 1, there are still three parameters (α, β and K_A) remaining to be determined for each of the synthetic ApoCII peptides. The calculation of SS (sum of the square of the error) is indicated below:

$$SS = \Sigma(v - v_c)^2 \quad (4)$$

where v represents the experimentally determined, and v_c represents the calculated, reaction velocity, as based on eqn. (2). We can obtain the best β value by minimizing SS when α and K_A are known, with eqn. (5), as shown below:

$$\beta = \frac{\Sigma\left[\dfrac{v\left(V_{max.}\dfrac{[A][S]}{\alpha K_A K_S}\right)}{\left(1+\dfrac{[S]}{K_S}+\dfrac{[A]}{K_A}+\dfrac{[A][S]}{\alpha K_A K_S}\right)} \cdot \dfrac{\left(V_{max.}\dfrac{[S]}{K_S}\right)\left(V_{max.}\dfrac{[A][S]}{\alpha K_A K_S}\right)}{\left(1+\dfrac{[S]}{K_S}+\dfrac{[A]}{K_A}+\dfrac{[A][S]}{\alpha K_A K_S}\right)^2}\right]}{\Sigma\left[\dfrac{\left(V_{max.}\dfrac{[A][S]}{\alpha K_A K_S}\right)^2}{\left(1+\dfrac{[S]}{K_S}+\dfrac{[A]}{K_A}+\dfrac{[A][S]}{\alpha K_A K_S}\right)^2}\right]} \quad (5)$$

Thus, for any pair of α and K_A values, β can be derived. In addition, since the relationship of α and K_A is constrained as indicated in eqn. (3) and Table 1, there is only one independent parameter remaining to be determined. By a series of iterative calculations of the α value, we have obtained the corresponding K_A and β values for the calculation of corresponding SS. By the criterion of the least SS, we obtained the best α, K_A and β values, which are shown in Table 2. The goodness-of-fit for the data to the curve, as based on eqn. (2), can be seen in Figs. 2(*a*) and 2(*b*). The derived kinetic data are also shown in Table 2. On the basis of these results it is concluded that the effect of ApoCII peptides on LPL activity is mainly reflected in the β values. When $\beta > 1$, as in the cases of ApoCII-(1–79)-peptide, ApoCII-(56–79)-peptide and ApoCII-(61–79)-peptide, the peptides represent activators for the LPL reaction. On the other hand, ApoCII-(73–79)-peptide, ApoCII-(76–79)-peptide and ApoCII-(73–78)-peptide act as inhibitors with $\beta = 0$. It appears that the parameter α plays little role in the activation property of intact ApoCII peptides. Since $\alpha > 1$ for ApoCII-(1–79)-peptide, this indicates that the intact apolipoprotein actually caused a reduced binding affinity of the enzyme for substrate. For the shorter ApoCII peptides, the finding of $\alpha < 1$ indicated the binding of the enzyme to the substrate enhances further binding of the enzyme to the inhibitor, and the reverse is also true: the binding of the enzyme to the inhibitor enhances further binding to the substrate. The ApoCII-(76–79)-peptide can inhibit not only the basal activity of LPL, but can also inhibit the ApoCII-activated LPL activity, as shown in Fig. 3.

Table 2. Kinetic parameters of ApoCII synthetic peptides

Results are means ± S.E.M.

ApoCII peptide (residue nos.)	n	K_A	α	β
1–79	6	24±3 nM	1.40±0.01	7.8±1.1
56–79	6	4.8±2 μM	1.07±0.01	6.4±1.1
61–79	4	20±6 μM	0.50±0.02	2.1±0.1
73–79	4	0.6±0.2 mM	0.68±0.02	0
73–78	4	1.5±0.2 mM	0.61±0.01	0
76–79	5	1.8±0.4 mM	0.51±0.03	0

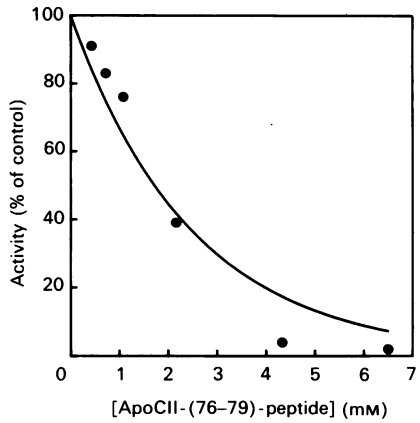

Fig. 3. Effect of ApoCII-(76–79)-peptide on LPL activity in the presence of a fixed concentration of ApoCII-(1–79)-peptide (0.28 μM) and substrate (1.4 mM) of TG

The data represent averages for four separate experiments. The enzyme concentration in the assay mixture was 87 ng/ml.

Table 3. Difference in free energy of binding between the ApoCII peptides and intact ApoCII in their interaction with LPL

ApoCII peptide (residue nos.)	ΔG [kJ (kcal)/mol]
56–79	13.8 (3.3)
61–79	17.2 (4.1)
73–79	25.9 (6.2)
73–78	28.5 (6.8)
76–79	28.9 (6.9)

On the basis of the deduced K_A values, we have also calculated, by using eqn. (6) below, the difference of the free energy of binding between the various peptides and that of intact ApoCII in the interaction with LPL (Table 3):

$$\Delta G = -\mathbf{RT} \ln\left(\frac{K_{A,\text{ApoCII-(1-79)-peptide}}}{K_{A,\text{intact peptide}}}\right) \quad (6)$$

DISCUSSION

The physiological function of ApoCII in the activation of LPL has been well established. The ability of the chemically synthesized ApoCII (Kinnunen *et al.*, 1977; Fairwell *et al.*, 1987) to activate LPL provides the most conclusive evidence for the functional role of ApoCII in the activation of LPL. The possibility of the presence of a highly active but trace quantity of contaminant in the ApoCII preparation from tissue sources was conclusively ruled out by the use of chemically synthesized ApoCII as activator (Fairwell *et al.*, 1987). In addition, the availability of a chemically synthesized ApoCII fragment provides the probe for a more detailed understanding of the reaction mechanism of LPL–ApoCII interaction.

Previously we have suggested that the availability of a competitive inhibitor of LPL against ApoCII would provide an additional tool for the further confirmation of the theory that the LPL reaction follows the kinetic scheme depicted in eqn. (1). On the basis of the study by Kinnunen *et al.* (1977), we hypothesized that there must be a structure region in the *C*-terminal end of the ApoCII peptide which exhibited binding, but was devoid of activation effect, and thus behaved as a competitive inhibitor. In the present study we demonstrate the presence of such a structural region in the *C*-terminal domain of ApoCII and determined the binding energy contributed by various domains of ApoCII. The proposed functions of ApoCII structural domains in their interaction with lipoprotein lipase are summarized in Fig. 4.

Results obtained from the present study for LPL reaction kinetics for ApoCII-(1–79)-peptide, ApoCII-(56–79)-peptide and ApoCII-(61–79)-peptide are comparable with those described by Kinnunen *et al.* (1977). The deduced β values as described by these workers for ApoCII-(1–79)-peptide, ApoCII-(56–79)-peptide and ApoCII-(61–79)-peptide are 13-, 12- and 4-fold activation effects, which are of similar magnitude to, but not identical with, those described in Table 2. However, since the study by Kinnunen *et al.* (1977) utilized gum arabic rather than phosphatidylcholine as the emulsifier for the substrate, a difference in the kinetic parameters of LPL between the two studies is anticipated. Our previously determined kinetic parameters (Posner *et al.*, 1983*a*) for ApoCII-(1–79)-peptide of α (= 1.0) and β (= 20) are also different from the results obtained in the present study. This could be due to the fact that the TG substrate used was prepared in the absence of emulsifier, and BSA was used as the stabilizer of the emulsion. On the other hand, the K_A value for ApoCII-(1–79)-peptide is essentially the same in the two studies, which was anticipated, since the parameter K_A should be independent of the substrate utilized, as depicted in the scheme of eqn. (1).

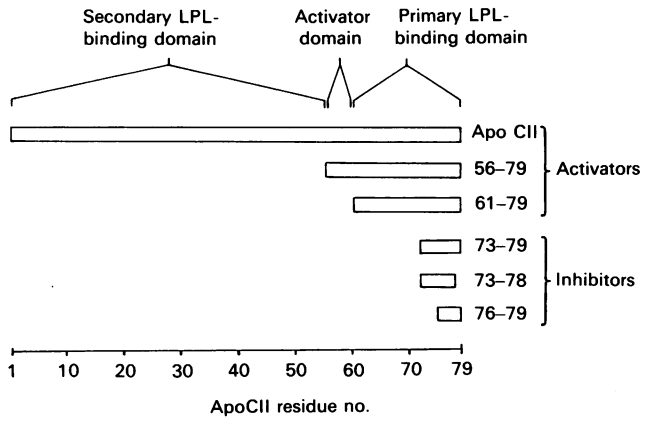

Fig. 4. Role of ApoCII structural domains in the interaction of ApoCII with lipoprotein lipase

The region labelled 'Activator domain' is not precisely defined and probably extends slightly into the primary LPL-binding domain.

Our initial tests indicated that the short peptides [ApoCII-(73–79)-peptide, ApoCII-(73–78)-peptide, ApoCII-(76–79)-peptide and ApoCII-(76–78)-peptide] all exhibited a detectable inhibitory effect on LPL activity. Although the tripeptide ApoCII-(76–78)-peptide exhibited inhibition activity, because of the high estimated K_A value ($\gg$ 5 mM), we did not perform a more detailed study on the kinetic effect of this tripeptide. Our observation of ApoCII-(73–79)-peptide, ApoCII-(73–78)-peptide and ApoCII-(76–79)-peptide as non-competitive inhibitors (I) of LPL against the substrate (S) demonstrated that the formation of ESI ternary complex is possible. Since with all these inhibitor peptides the α values are less than 1, it also demonstrated that the inhibitor actually had higher affinity for the ES complex than for E. As the reaction follows a random sequence, the conclusion for the reverse is also true, i.e. the substrate had a higher affinity for EI complex than for E. The study by Kinnunen *et al.* (1977) did not detect any effect of ApoCII-(67–79)-peptide. We believe the reason for this is the fact that their tested concentration was in the micromolar range, which was too low to allow observation of its inhibitory effect on LPL activity.

Because our deduced K_A also represents the dissociation constants, we have calculated that the difference of the free energy of binding for intact ApoCII and ApoCII-(56–79)-peptide to LPL was 13.8 kJ (3.3 kcal)/mol. The data suggest that the difference of these interactions is due to the binding energy contributed by the *N*-terminal portion of ApoCII in its interaction with LPL. Thus we conclude that the *N*-terminal domain of the ApoCII peptide must also contribute to the binding energy of ApoCII–LPL interaction. However, additional studies are necessary to pinpoint the location of the minimum amino acid sequence of this binding domain. The difference of the free energy of binding between ApoCII-(56–79)-peptide and ApoCII-(61–79)-peptide was 3.3 kJ (0.8 kcal)/mol. This difference in free energy is not large, yet ApoCII-(56–79)-peptide is a significantly better activator than ApoCII-(61–79)-peptide. We conclude that residues 56–60 contribute little to the overall strength of binding in the LPL–ApoCII interaction; however, this region may be crucial for triggering the conformational change of the enzyme and for enhancing the cleavage of the substrate ester bond by increasing the parameter β, consistent with this being the core of the activator region (Kinnunen *et al.*, 1977). Since the binding energies for ApoCII-(76–79)-peptide and ApoCII-(73–78)-peptide are similar, this would indicate that the *C*-terminal glutamic acid is not an essential element for the interaction with LPL, but it does contribute to the binding affinity in the ApoCII–LPL interaction. This is based on the finding of low inhibitory effect of ApoCII-(76–78)-peptide as compared with ApoCII-(76–79)-peptide. Thus, on the basis of the present study, we have concluded that the *C*-terminal Lys-Gly-Glu-Glu tetrapeptide provides a key region for the ionic interaction with LPL. The inhibition of lipoprotein lipase basal activity by the tetrapeptide indicates that the enzyme undergoes a conformational change that is not favourable for catalysis.

Since the binding affinity of the *C*-terminal peptides increases with lengthening of the peptide beyond this tetrapeptide, we conclude that the other amino acids extending from the *C*-terminal tetrapeptide also contribute to the binding energy for LPL and ApoCII interaction. The recent finding of a mutation which caused the alteration of ApoCII-peptide sequence starting from residue 69, due to frameshift mutation ($ApoCII_{Toronto}$), and its subsequent inactivity as an activator of LPL (Connelly *et al.*, 1987), also pointed out the absolute requirement of *C*-terminal sequence for the LPL and ApoCII interaction. Thus the initial ionic interaction of *C*-terminal ApoCII tetrapeptide with LPL may promote the proper alignment and allow the weak interaction to occur between the activator domain of ApoCII and the LPL-activator site.

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