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Lipoprotein lipase cofactor activity of a carboxyl-terminal peptide of apolipoprotein C-II

(triacylglycerol lipase/succinyl-apolipoprotein C-II)

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ABSTRACT **Apolipoprotein C-II (apoC-II) is a small protein found associated with the plasma lipoproteins. It serves a unique function in the activation of the enzyme lipoprotein lipase (triacylglycerol acyl-hydrolase, EC 3.1.1.3). ApoC-II contains a single arginine residue, permitting tryptic cleavage into two peptides after succinylation of the native protein. The succinylated amino-terminal peptide, approximately 50 residues, did not activate lipoprotein lipase. The succinylated carboxyl-terminal peptide, about 29 residues, had significant cofactor activity. Relative to native apoC-II, the maximal activation observed with the succinylated carboxyl-terminal peptide was 50% lower and the concentration required for half-maximal activity was approximately 10 times higher. Mixtures of the carboxyl- and amino-terminal peptides had no more activity than the carboxyl-terminal peptide alone. Localization of functional properties to the carboxyl region is a feature also common to apolipoproteins C-III, A-II, and A-I.**

Lipoprotein lipase (triacylglycerol acyl-hydrolase, EC 3.1.1.3), the enzyme thought to catalyze the hydrolysis of plasma triglycerides, requires a specific apolipoprotein cofactor for the full expression of activity (1–5). This cofactor protein, apolipoprotein C-II (apoC-II), is normally a component of the triglyceride-rich chylomicrons and very low density lipoproteins, as well as the high density lipoproteins. When lipoprotein lipase activity is measured using artificial triglyceride emulsions, the cofactor may be supplied as whole serum, high density lipoprotein (6), or equally well by delipidated and highly purified apoC-II (1–5). Phospholipid is required in addition to apoC-II to achieve maximal activation (7, 8). This cofactor activity also appears to be important *in vivo*. A patient has been described who has severe hypertriglyceridemia, undetectable plasma apoC-II, and normal lipoprotein lipase levels in post-heparin plasma. The hypertriglyceridemia in this patient was corrected by infusion of normal plasma (9).

The nature of the interaction between lipoprotein lipase, apoC-II, and triglyceride has not been defined. We have studied peptide fragments of apoC-II in an effort to identify the region of the protein involved in lipoprotein lipase activation. Because the protein contains only one arginine residue, tryptic digestion of succinylated apoC-II yielded two peptides. The smaller carboxyl-terminal fragment was found to retain significant activating capacity, while the larger amino-terminal peptide was inactive. The properties of the succinyl-apoC-II tryptic peptides are reported here.

MATERIALS AND METHODS

Isolation of ApoC-II. Plasma was obtained from hypertriglyceridemic patients after a 12- to 14-hr fast. Very low density lipoproteins were isolated by ultracentrifugation at plasma density and were recentrifuged once after resuspension in 0.15 M NaCl. The lipoprotein solution was concentrated by dry dialysis and delipidated using a 2:1 mixture of diethyl ether/methanol. ApoC-II was purified by gel and ion-exchange chromatography (10). Homogeneous preparations had a characteristic amino acid composition (10–13), produced a single band on polyacrylamide gel electrophoresis, and did not generate immunoprecipitin lines when reacted with antisera to apoC-I, apoC-III, apoA-I, or apoA-II.

Succinylation, Tryptic Digestion, and Isolation of Peptides. ApoC-II (1–5 mg) was solubilized in 2 ml of 0.01 M NaOH and the pH was adjusted to 7.2 by addition of 0.2 M NaH_2PO_4. An 800-fold molar excess of succinic anhydride was added with continuous stirring (14). The pH was maintained at 7.2 by adding 0.2 M NaOH with an AGLA (Welcome Laboratories) micrometer syringe. Treatment with 1-fluoro-2,4-dinitrobenzene (15) and analysis of unreacted lysine after protein hydrolysis demonstrated that greater than 95% of the lysine residues were acylated under these conditions. The reaction mixture was exhaustively dialyzed against 0.005 M NH_4HCO_3 and lyophilized. Succinylated apoC-II was used directly without additional purification. The homogeneity of succinylated apoC-II was evaluated in one experiment by chromatography on DEAE-cellulose columns eluted with a linear gradient from 0.15 to 0.5 M Tris·HCl/6 M urea, pH 8.0. Greater than 90% of the succinylated protein was recovered in a single peak eluting near the end of the gradient at a conductivity considerably greater than that required to elute the native protein (10).

The succinylated apoC-II was resolubilized in 1 ml of 0.2 M trimethylamine/acetate, pH 8.2, and trypsin treated with tosylphenylalanyl chloromethyl ketone was added (1:75 wt/wt). After incubation at 37° for 1 hr the reaction mixture was lyophilized. The tryptic peptides of succinylated apoC-II were separated by DEAE-cellulose chromatography. The column (0.9 × 15 cm) was equilibrated with 0.01 M NH_4HCO_3 and eluted with a linear gradient to 0.5 NH_4HCO_3. Column fractions were pooled, lyophilized, and resolubilized in 0.1 M NH_4OH.

Amino Acid Analysis and Polyacrylamide Gel Electrophoresis. Protein and peptides were hydrolyzed in 4 M methanesulfonic acid for 20 hr at 115° (16), neutralized with 4 M NaOH, and analyzed on a Beckman 121 amino acid analyzer. Protein and peptide fractions were also analyzed on 12%

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Abbreviations: apoC-II, apolipoprotein C-II; S-apoC-II, succinylated apoC-II; $C\text{-}II_C$, carboxyl-terminal tryptic peptide of succinylated apoC-II; $C\text{-}II_N$, amino-terminal tryptic peptide of succinylated apoC-II.

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polyacrylamide gels using the buffer system of Reisfeld and Small (17), modified so that all solutions contained 8 M urea. In some experiments protein or peptide was eluted from slices of unfixed gels and tested for capacity to activate lipoprotein lipase. The protein bands were localized by comparison with a fixed and stained (18) marker gel.

Preparation of Lipoprotein Lipase. Lipoprotein lipase, partially purified from human post-heparin plasma, was used in the activation studies. Post-heparin plasma was collected from normal subjects by plasmapheresis 10–50 min after the intravenous injection of heparin (Upjohn, prepared from beef lung) at 30 units/kg. Lipoprotein lipase activity was separated from "hepatic lipase" by heparin affinity chromatography (19). An 80- to 100-cm^3 sample of post-heparin plasma was loaded directly on a 1.2- × 15-cm heparin-Sepharose 4B affinity column equilibrated with 0.005 M barbital/0.15 M NaCl/20% (vol/vol) glycerol, pH 7.4. After the column was rinsed with 60 cm^3 of the same buffer containing 0.4 M NaCl, the hepatic lipase was eluted with 75 ml of 0.75 m NaCl and the lipoprotein lipase was eluted with 50 ml of 1.5 M NaCl. Column fractions (3.6 ml) were collected in tubes containing 1.8 ml of 20% (wt/vol) bovine serum albumin. Lipoprotein lipase fractionated in this manner was 95% inhibitable by protamine sulfate, 3 mg/ml, and was stimulated 9- to 12-fold by apoC-II.

Lipoprotein Lipase Assay. Enzyme activity was measured by means of a previously described assay procedure (20). The standard substrate emulsion contained: triolein, 100 mg; glycerol [*1*-^{14}C]trioleate, 5 μCi (60 μCi/μmol); egg lecithin, 0.8 mg; fatty acid-free bovine serum albumin, 600 mg; Triton X-100, 0.6 ml of a 1% aqueous solution; and Tris·HCl, 0.194 M, pH 8.6, containing 0.15 M NaCl to a total volume of 12 ml. The mixture was used immediately after sonication (20). Assays were performed in a total volume of 1 ml of a reaction mixture consisting of 0.9 ml of substrate, 0.025 ml of enzyme capable of releasing 2.5–3.0 μmol of free fatty acid per hr, and 0.075 ml of 0.1 M NH_4OH containing the test protein or peptide. Substrate and activator were mixed at 0°, and the reaction was begun by the addition of enzyme. The assay mixture was incubated for 60 min with agitation at 27°. The reaction was terminated by the addition of acidified isopropanol, free fatty acids were extracted, and the radioactivity of free fatty acids was measured (20). All assays were performed in duplicate and the results were averaged; differences between duplicates were less than 5%.

RESULTS

Characterization of ApoC-II. ApoC-II is a single chain protein that has an apparent molecular weight of about 10,000 on sedimentation equilibrium ultracentrifugation (11). When analyzed by gel chromatography in guanidine·HCl-containing buffer, however, apoC-II coelutes with apoC-III (molecular weight 8751, 79 amino acid residues). Preliminary data from sequence analysis indicate that apoC-II contains 79 or 80 amino acids (Table 1). The protein has been shown to have NH_2-terminal threonine and COOH-terminal glutamic acid, and lacks cysteine, histidine, and covalently bound carbohydrate (10–13, 21).

Characterization of the Tryptic Peptides of Succinylated ApoC-II. The peptides obtained from the tryptic digestion of succinylated apoC-II were purified by chromatography on DEAE-cellulose (Fig. 1). The first major peak (C-II_C) migrated as a single band that was well separated from both native apoC-II and succinylated apoC-II on 12.5% alkaline urea/polyacrylamide gels (Fig. 2). C-II_C contained 29–30 residues, lacked arginine, proline, and tryptophan, and contained the single isoleucine residue in apoC-II (Table 1). The absence of arginine

Table 1. Amino acid composition of apoC-II and the tryptic peptides of succinylated ApoC-II*

Residue	Mol/100 mol amino acids† in ApoC-II	C-II_N	C-II_C
Tryptophan	1.1 (1)	1.8 (1)	0 (0)
Lysine	7.6 (6)	7.5 (4)	6.7 (2)
Histidine	0 (0)	0 (0)	0 (0)
Arginine	1.2 (1)	2.0 (1)	0 (0)
Aspartic acid + asparagine	6.6 (5)	6.2 (3)	5.9 (2)
Threonine	11.0 (9)	9.5 (5)	13.4 (4)
Serine	11.5 (9)	10.4 (5)	13.8 (4)
Glutamic acid + glutamine	17.8 (14–15)	22.3 (11)	11.8 (3–4)
Proline	5.0 (4)	9.4 (4)	0 (0)
Glycine	3.1 (2)	0 (0)	8.1 (2)
Alanine	8.2 (6)	8.0 (4)	7.5 (2)
Valine	5.0 (4)	4.0 (2)	6.6 (2)
Methionine	2.5 (2)	1.7 (1)	3.1 (1)
Isoleucine	1.0 (1)	0 (0)	3.5 (1)
Leucine	10.1 (8)	9.9 (5)	10.5 (3)
Tyrosine	6.2 (5)	6.1 (3)	6.3 (2)
Phenylalanine	2.3 (2)	2.3 (1)	2.7 (1)
Total	79–80	50	29–30

* Methanesulfonic acid hydrolysis. Cysteine is known to be absent and was not separately determined.

† Numbers in parentheses are the calculated residues per mol of protein or peptide.

established its position as the carboxyl-terminal peptide. The second major peak (C-II_N, Fig. 1) eluting from DEAE-cellulose also generated a single band on polyacrylamide gel electrophoresis, with mobility intermediate between succinylated apoC-II and apoC-II (Fig. 2). This peptide consisted of 49 residues without glycine or isoleucine. Carboxypeptidase B digestion released arginine quantitatively from C-II_N, confirming its identity as the amino terminal fragment. The C-II_C and C-II_N peptides together accounted for all of the amino acid residues in native apoC-II (Table 1).

The trailing shoulder of the C-II_N peak had an amino acid composition identical to C-II_N. Polyacrylamide gel electrophoresis of this shoulder revealed two bands, one with the same mobility as C-II_N and another migrating just below C-II_N. The heterogeneity may be due to succinylation of hydroxy-amino acids in addition to the expected reaction with free amino groups (22, 23). In some preparations, the carboxyl-terminal tryptic peptide of succinylated apoC-II also eluted from DEAE-cellulose in two overlapping peaks that had identical amino acid compositions.

A broad third peak (C-II_S, Fig. 1), eluting from DEAE-cellulose after the two tryptic peptides, was found to have an amino acid composition identical to native apoC-II. On polyacrylamide gels it had mobility similar to succinylated apoC-II (Fig. 2) and presumably it represented protein that had not been cleaved by trypsin.

Activation of Lipoprotein Lipase. Crude preparations of succinylated apoC-II produced maximal activation of lipoprotein lipase comparable to that observed with native apoC-II. Succinylated apoC-II, purified by DEAE-cellulose chromatography, also produced an activation curve indistinguishable from that of the native protein. The small amount of material eluting late from DEAE-cellulose (C-II_S, Figs. 1 and 2) which had a composition and electrophoretic mobility similar to succinylated apoC-II, was also tested for activity. It produced

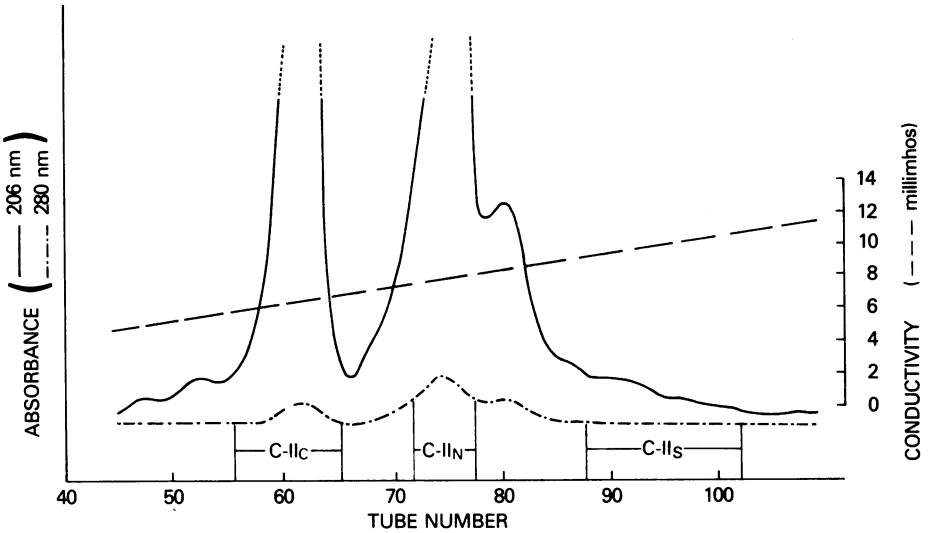

FIG. 1. DEAE-cellulose chromatography of a tryptic digest of 2.7 mg of succinylated apoC-II. The column (0.9 × 15 cm) was eluted with a linear gradient of 0.01–0.5 M NH_4HCO_3. Fractions indicated by the bars were pooled and analzyed.

a maximal stimulation about 60% of that with native apoC-II (Fig. 3), but half-maximal activation was achieved at a concentration (0.1 nmol/ml) similar to apoC-II.

The amino-terminal peptide ($C\text{-}II_N$, Table 1) accounted for more than 60% of the protein molecule but did not activate lipoprotein lipase, even at concentrations of 5 nmol/ml. The carboxyl-terminal fragment ($C\text{-}II_C$), in contrast, produced significant activation (Fig. 4). The maximal activation with $C\text{-}II_C$ was about 50% of that obtained with native apoC-II and the concentration of $C\text{-}II_C$ required for half-maximal activation (1.0 nmol/ml) was considerably greater than that necessary when succinylated apoC-II or native apoC-II was tested.

The possibility that trace contamination with native apoC-II or succinylated apoC-II might account for the activity of the $C\text{-}II_C$ peptide was excluded in other experiments. $C\text{-}II_C$ isolated by DEAE-cellulose chromatography was subjected to polyacrylamide gel electrophoresis and the peptide was eluted from gel slices. $C\text{-}II_C$ is well separated from both the native and succinylated parent protein in the system employed (Fig. 2). $C\text{-}II_C$ so prepared retained the capacity to activate lipoprotein lipase; the activity observed was again approximately half that seen with apoC-II (Table 2, Exp. 1).

Mixtures of apoC-II and $C\text{-}II_C$ at low concentrations produced lipoprotein lipase activation roughly equivalent to the sum of each alone. When the peptide fragment and intact protein were added together in higher concentrations, full activation was achieved (Table 2, Exp. 2). Therefore, the peptide does not appear to inhibit the activity of the native protein and the pattern of activation observed with $C\text{-}II_C$ (Fig. 3) is not readily explained in terms of apoC-II contamination.

Mixtures of apoC-II and $C\text{-}II_N$ were also tested for activation capacity. The addition of 1.5 nmol of $C\text{-}II_N$ to 0.025 nmol of apoC-II did not reduce the activity of the native protein. $C\text{-}II_N$ and $C\text{-}II_C$ were also tested separately and in equimolar mixtures

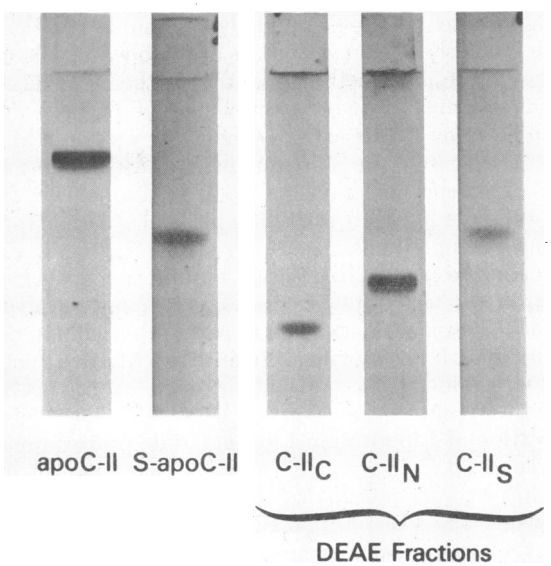

FIG. 2. Polyacrylamide gel electrophoresis in 12.5% acrylamide/8 M urea, pH 9.4 of native apoC-II, succinylated apoC-II (S-apoC-II), and the peptides purified from a tryptic digest of succinylated apoC-II by DEAE-cellulose chromatography (Fig. 1).

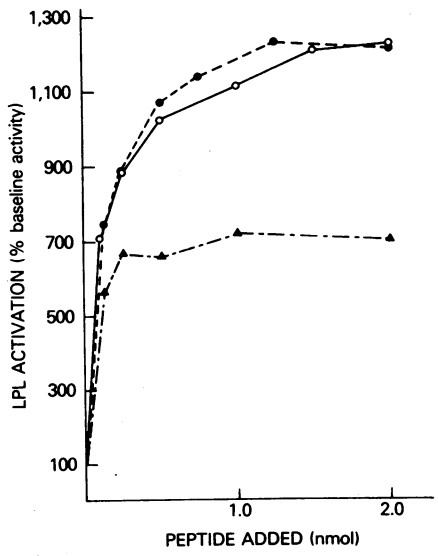

FIG. 3. Activation of lipoprotein lipase (LPL) by apoC-II before and after succinylation. O—O, ApoC-II; ●- - -●, S-apoC-II before purification; ▲ - — - ▲, $C\text{-}II_S$ fraction isolated by DEAE-cellulose chromatography (Fig. 1). The curve for S-apoC-II (not shown) purified by DEAE-cellulose chromatography was indistinguishable from the curves of apoC-II and S-apoC-II.

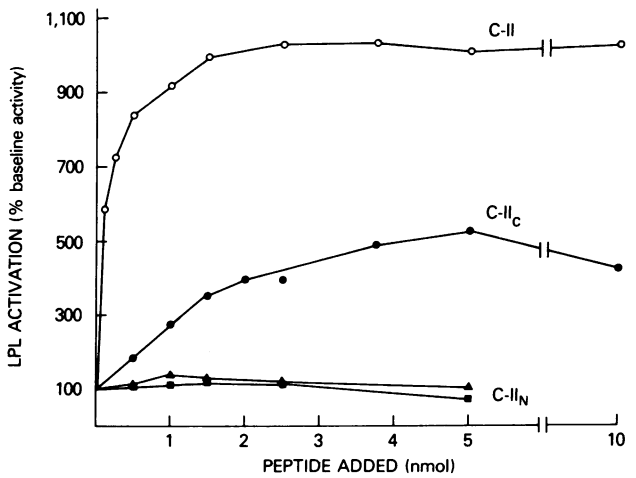

FIG. 4. Activation of lipoprotein lipase (LPL) by apoC-II and the succinylated tryptic peptides. ○, ApoC-II; ●, C-II$_C$; ■, C-II$_N$; ▲, trailing shoulder of C-II$_N$.

(Table 2, Exp. 3). C-II$_N$ failed to enhance the activation produced by C-II$_C$ alone but no suppression of activation was seen.

DISCUSSION

ApoC-II contains a single arginine residue. It was therefore possible to cleave the succinylated protein into two peptides with trypsin. The larger, amino-terminal, peptide did not activate lipoprotein lipase, whereas the smaller, carboxyl-terminal, peptide retained significant cofactor activity. Succinylation of apoC-II without tryptic digestion did not affect the activating properties of the protein. The maximal activation produced by the C-II$_C$ peptide was less than that observed with the succinylated apoC-II. About 10-fold higher concentrations of the C-II$_C$ peptide were required to produce half-maximal activation. This loss of activity is probably not due to the negative charge of the succinylated lysine residues because the succinylated whole protein was fully active. It may be due to conformational constraints related to the loss of the amino-terminal portion of the protein. This hypothesis can be tested by the use of removable acylating agents in the production of the carboxyl-terminal peptide.

The characterization of the C-II$_S$ peptide (Figs. 1 and 2) was limited by the small amount recovered from DEAE-cellulose chromatography. The amino acid composition and electrophoretic mobility of this peptide were similar to succinylated apoC-II but the activation properties were clearly different (Fig. 3). This difference is unexplained. It is possible that C-II$_S$ represents protein that was acylated in one or more hydroxyamino acids (10, 11) and this additional modification may have altered both activating properties and susceptibility to tryptic cleavage.

The localization of lipoprotein lipase cofactor activity to the carboxyl terminus of apoC-II is of particular interest in view of recent studies with other apolipoproteins. Apolipoprotein A-II has been cleaved into two cyanogen bromide fragments (24, 25) and only the carboxyl-terminal peptide retained the capacity to bind lipid. Recombination of apolipoprotein C-III with phospholipid protects the carboxyl terminus but not the amino terminus from tryptic digestion (26). Cyanogen bromide treatment of apoA-I generated four peptides but the 92 residue carboxyl-terminal peptide alone possessed the ability to bind phosphatidylcholine (27). Only in the case of apolipoprotein C-I has lipid binding activity been demonstrated in the amino

Table 2. Activation of lipoprotein lipase by apolipoprotein C-II and its tryptic peptides

Exp.	Peptide added, nmol C-II	C-II$_C$	C-II$_N$	Lipase activity, % baseline
1	—	—	—	100
	0.93*	—	—	827
	—	5.6*	—	400
2	—	—	—	100
	0.025	—	—	319
	—	1.0	—	381
	—	—	1.5	127
	0.025	1.0	—	548
	0.025	—	1.5	390
	1.00	—	—	975
	1.00	2.0	—	1095
3	—	—	—	100
	—	1.0	—	254
	—	—	1.0	98
	—	1.0	1.0	260

Exp. 1. ApoC-II and C-II$_C$ were eluted from homogenized slices of 12.5% polyacrylamide gels (Fig. 2) and tested for capacity to activate lipoprotein lipase. Exp. 2. Activation of lipoprotein lipase by mixtures of apoC-II and various concentrations of its succinylated tryptic peptides. Exp. 3. Activation of lipoprotein lipase by C-II$_C$ and C-II$_N$, individually and in combination.

* Assuming 100% recovery from gels. Typical actual recoveries averaged 40% of sample applied.

as well as carboxyl terminus (28). It may be that the carboxyl termini of the apolipoproteins in general possess the primary and secondary structures requisite for lipid binding. If lipid binding proves to be localized to the carboxyl terminus of apoC-II, the results reported here would suggest that this property is critical to the apoprotein's enzymatic cofactor function.

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