Site-directed Mutagenesis of Apolipoprotein CII to Probe the Role of Its Secondary Structure for Activation of Lipoprotein Lipase

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Apolipoprotein CII (apoCII) is a necessary activator for lipoprotein lipase (LPL). We have identified four residues (Tyr-63, Ile-66, Asp-69, and Gln-70), presumably contained in an α-helix, as a potential binding site for LPL. We have now used structure prediction, mutagenesis, and functional assays to explore the functional role of the secondary structure in this part of apoCII. First, mutants were generated by replacements with proline residues to disturb the helical structure. Activation by mutant G65P was reduced by 30%, whereas mutant S54P retained activation ability. Mutants V71P and L72P should be located outside the LPL-binding site, but V71P was totally inactive, whereas activation by L72P was reduced by 65%. Insertion of alanine after Tyr-63, changing the position of the putative LPL-binding site in relation to the hydrophobic face of the α-helix, also severely impaired the activation ability, and a double mutant (Y63A/I66A) was completely inactive. Next, to investigate the importance of conserved hydrophobic residues in the C-terminal end of apoCII, Phe-67, Val-71, Leu-72, and Leu-75 were exchanged for polar residues. Only F67S showed dramatic loss of function. Finally, fragment 39–62, previously claimed to activate LPL, was found to be completely inactive. Our data support the view that the helical structure close to the C-terminal end of apoCII is important for activation of LPL, probably by placing residues 63, 66, 69, and 70 in an optimal steric position. The structural requirements for the hydrophobic face on the back side of this helix and further out toward the C terminus were less stringent.

Apolipoprotein CII (apoCII) plays an important role in the metabolism of blood lipids as an activator of lipoprotein lipase (LPL) (1). Human apoCII is a 79-amino acid protein that is mainly expressed in liver and intestine (2). ApoCII appears in blood as a surface component of chylomicrons, very low density lipoproteins, and high density lipoproteins (3, 4). The mechanism for the activation has not been resolved in molecular detail, but the structures necessary for the activation reside in the C-terminal one-third of the apoCII sequence (5).

Previous studies of the three-dimensional structure of apoCII by NMR using full-length apoCII or an active fragment spanning residues 44–79 in the presence of lipid mimetics like micelles of SDS or dodecylphosphatidylcholine have revealed that apoCII contains three α-helices spanning approximately residues 16–38, 45–57, and 65–74 (6–8). From earlier studies using synthetic peptide fragments of apoCII and secondary structure predictions it is known that the molecule is organized in two separate domains with different functions. The N-terminal part is involved in lipid binding by an amphipathic α-helix of the type found in all exchangeable apolipoproteins, whereas the C-terminal part (residues 56–79) is responsible for activation of LPL (5, 9–12). In several in vitro systems, the activation by the C-terminal fragments is comparable with the full-length apoCII, demonstrating that the lipid binding property of apoCII is not crucial for the activation (5). However, with natural lipoproteins, like chylomicrons from an apoCII-deficient patient, full-length apoCII was required for efficient activation of LPL (13). For most lipases the catalytic activity is strongly dependent on the quality of the lipid/water interface of the substrate emulsions (14). This is the case also for LPL. With substrate emulsions that are less accessible to LPL alone, the activity is more dependent on apoCII, and the activity is, therefore, larger than with simpler substrates (15).

MacPhee et al. (16) reported that the synthetic apoCII fragment 39–62 did not bind to lipids but could activate LPL to the same extent as full-length apoCII. Fragment 39–62 lacks the main part of the domain that had previously been identified to be important for activation of LPL. Therefore, the binding by MacPhee et al. (16) challenged all previous data but had not been reinvestigated until now.

In a previous study using mutagenesis of apoCII, we had identified four fully conserved residues in the C-terminal domain that appeared to be particularly important for the activation of LPL (Tyr-63, Ile-66, Asp-69, and Gln-70) (17). The side chains of these residues were all located on the same side of a putative α-helix. This arrangement was later confirmed in the structure determined by NMR, where the critical residues are related to the most C-terminal (third) α-helix (6, 7). It was proposed that the functionally important four residues probably form a binding site for LPL (6, 17). On the opposite side of this
α-helix and further out toward the C-terminal end there are several hydrophobic residues that are functionally conserved between apoCII sequences (Phe-67, Val-71, Leu-72, Leu-74, and Leu-75 [residue numbers from the human sequence]). From previous calculations of hydrophobic interaction energies, the rotational freedom with regard to lipid binding was predicted to be somewhat larger for the third amphipathic helix in apoCII than for helix 1 and 2 (6). We have now by site-directed mutagenesis explored the role of the secondary structure in the C-terminal end of apoCII and the role of the hydrophobic residues in this part of the molecule for the activation of LPL. For this we created mutants that were predicted to change either the backbone conformation or the hydrophobicity. In addition we have reinvestigated activation of LPL by the synthetic apoCII fragment 39–62.

**EXPERIMENTAL PROCEDURES**

**Secondary Structure Predication**—The secondary structure predications for wild-type apoCII and for the mutants were carried out at the NPS@dNetwork Protein Sequence analysis web sites. Eight different predictive methods were used to obtain the consensus predication: SOPMA, PHD, Predator, GOIV, DPM, DSC, SIMPA96, and HNNC. These were the same as those used for apoCII by Dahim et al. (18).

**Plasmid Constructions and Site-directed Mutagenesis of Human ApoCII**—Human apoCII cDNA was in the plasmid pET-histag-hapoCII as described before (17). All mutants were generated using the QuickChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA), according to the instructions from the manufacturer. The primers used are listed in supplementary Table S1. All mutants were verified using an ABI 337 DNA sequencer and the Dye Terminator Cycle Sequencing Ready Reaction kit (PerkinElmer Life Sciences). Among the 15 mutants in this study, the mutant -64A insertion was made with addition of an alanine residue after residue Tyr-63. Mutant 63A/66A was a double-point mutation with replacement with alanine at both residue Tyr-63 and Ile-66. The other 13 mutants were single-point mutations. The alanine codons utilized for the mutagenesis were GCC, GCA, and GCT. The proline codon used was CCC.

**Expression and Purification of Wild-type and Mutated ApoCII Proteins**—The pET-histag-hapoCII and the mutated plasmids (P43A, S54A, S54P, T57A, G65P, F67A, F67S, V71A, V71P, L72P, L72Y, L72S, L75A, -64A insertion, and 63A/-66A) were transformed into Epicurian Coli® BL21-codonPlus(DE3)-RIL competent cells (Stratagene). The expression and purification procedures were the same as previously described (17). Fusion proteins were eluted between pH 6.3 and 4.5 from the nickel-nitritoltriacetic acid-agarose and were further purified by high performance ion exchange chromatography on an ÄKTA purifier 900 (Amersham Biosciences) using a Mini Q PE 4.6/50. Fractions were combined and analyzed by electrophoresis on SDS-Tricine gels and stained by 0.025% Serva-blue G. Western blotting was made using a rabbit anti-human apoCII serum as antibody. SDS-Tricine gels and stained by 0.025% Serva-blue G. Western Analysis was performed using a rabbit anti-human apoCII serum as indicator.

**Lipase Assays**—For analysis of activation of LPL, an emulsion of soy bean triacylglycerols in egg yolk phosphatidylcholine (same composition as Intralipid 10%) containing a trace amount of [3H]oleic acid-labeled trioleoylglycerol (kindly prepared by KABI-Fresenius, Uppsala, Sweden) was used as previously described (17). ApoCII, the mutants, and the fragment 39–62 were dissolved in 5 mM urea, 10 mM Tris, pH 8.5. 5 μl of the stock solutions or serial dilutions in 5 mM urea, 10 mM Tris, pH 8.5, were added to the incubation mixtures. The total volume was 200 μl. Control experiments showed that the final concentration of urea (125 mM) did not interfere with the activation. The reactions were stopped after 15 min of incubation at 25 °C by the addition of organic solvents followed by extraction of the fatty acids and counting of radioactivity. Lipase activity is expressed in units or milliunits, where 1 unit corresponds to release of 1 μmol of fatty acid/min. For measurement of the activation ability of peptide 39–62 compared with that of peptide 50–79, three substrates with different properties were used to explore the activation ability of the apoCII fragments. One was the same as that described above for the mutants, whereas the second one was a gum-arabic-stabilized emulsion of 3H-labeled trioleoylglycerol prepared as described (15). The conditions were otherwise the same for both systems. The third system detected activation of phospholipase activity of LPL by using dimyristoylphosphatidylcholine (DMPC) as substrate (Sigma). In this system the basal activity of LPL is very low, and the activation by apoCII is, therefore, much more pronounced than in the other assay systems. For preparation of liposomes of DMPC, 20 mg of DMPC was suspended in 4.1 ml of 20 mM Tris, 0.1 mM NaCl, pH 8.5, and the sample was sonicated at room temperature (with a MSE Soniprep 150 equipped with a 5-mm probe) for 10 min with 4 s on and 4 s off. The liposome vesicles were then kept for 2–3 h at room temperature before the experiment. The activity of LPL was measured in a 1-ml mixture containing 1.46 mg of DMPC in the same buffer as that used for the triacylglycerol emulsions. To each incubation was added 10 μl of stock solution of apoCII or dilutions in 5 mM urea, 10 mM Tris, pH 8.5. The incubation temperature was 30 °C, which is well above the transition temperature for DMPC. The reactions were stopped after 10 min by the addition of organic solvents. In this case the fatty acids released were extracted and titrated manually by 0.1 M NaOH using bromphenol blue as pH indicator.

**Determination of Kinetic Parameters from Activation Curves**—Calculations of Kd and β were performed according to the equation,
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where \( L \) corresponds to the concentration of LPL, and \( C \) corresponds to the concentration of apoCII as previously described (17). \( K_d \) characterizes the apparent affinity of apoCII for LPL, and \( \beta \) indicates how much more active the LPL-apoCII complex is compared with LPL alone (-fold activation). When \( \beta \) is equal to 1, apoCII does not activate LPL.

ApoCII-deficient Human Plasma as Substrate for LPL—Plasma from an apoCII-deficient patient with the apoCII-Hamburg mutation (13) was a kind gift from Prof. Ulrike Beisiegel, Hamburg, Germany. For studies of lipolysis, the incubation mixture contained in a total volume of 1 ml the following: patient plasma corresponding to 1.5 mg triacylglycerol, 60 mg of bovine serum albumin (Sigma fraction V), 1.5 IU heparin, 0.1 M NaCl, and 0.15 M Tris-Cl, pH 8.5. Ten \( \mu \)l of apoCII stock solutions in 5 M urea was added to the incubation mixtures, which were preincubated at 25 °C for at least 15 min before LPL (bovine) was added. The reactions were stopped by addition of organic solvents, and the fatty acids released were extracted and titrated manually as described above.

Conformational Studies by Measurements of Circular Dichroism (CD)—Possible global changes of the secondary structure by some of the mutations were studied by measurements of CD in the presence of liposomes made from egg yolk phosphatidylcholine (EYPC) prepared as previously described (17). Measurements were carried out at 25 °C on a Jasco J-700 spectropolarimeter in the far UV region (195–250 nm) using a 1-mm path length cell. The concentration of phospholipids for the CD experiments was 240 \( \mu \)M in 0.1 M phosphate buffer, pH 7.4, at 25 °C. The concentration of apoCII or mutants was 6.0 \( \mu \)M. For all spectra, a reference sample containing the corresponding buffer was subtracted from the CD signal. The \( \alpha \)-helical content was calculated from the spectra using DichroWeb and the K2d algorithm (20).

Binding of ApoCII to Liposomes—Binding of apoCII to EYPC liposomes was measured as the change of fluorescence of the single Tyr-26 in apoCII as previously described (17). The increase of fluorescence was attributed to binding of apoCII to the liposomes. Surface plasmon resonance measurements were performed on a BIAcore 2000 instrument using L1 sensor chips (Biacore, Uppsala, Sweden). This sensor chip provides a dextran matrix to which hydrophobic residues are covalently linked. Liposomes can be easily captured on such a matrix. In the present study, EYPC liposomes were immobilized. The immobilization increased the surface plasmon resonance signal by 520–5400 response units. Binding studies were performed by injection of 20 \( \mu \)l of solutions of apoCII or apoCII mutants (4.4 \( \mu \)M, diluted in running buffer) into the flow cells of sensor chips with immobilized liposomes. The running buffer was 20 mM Hepes, 0.15 M NaCl, pH 7.4, and the temperature was 25 °C. After each measurement the surface was regenerated by a wash with 50 mM octyl-D-glucoside and 30% ethanol.

RESULTS

Use of Secondary Structure Predictions for Construction of Mutants—The consensus structure obtained by secondary structure prediction agreed reasonably well with the structures determined by NMR (6, 7). Structure prediction was used to evaluate the consequences of mutations in apoCII (Fig. 1). Replacement of Pro-43 by alanine was predicted to cause fusion of the first and second helices to a longer \( \alpha \)-helix spanning 41 residues (Fig. 1). Replacements of Ser-54 or Thr-57 by alanine were predicted to cause prolongation of the second \( \alpha \)-helix, whereas replacement of Ser-54 by proline should make the second helix somewhat shorter. Replacement of Gly-65 with proline was predicted to have little influence on the coiled struc-
ture in that region. Replacement of Phe-67 by alanine should not disturb the overall helical structure, whereas replacement of Phe-67 by serine was predicted to reduce the length of the third α-helix. Replacements of Val-71 or Leu-72 with proline were predicted to seriously disturb the third α-helix, resulting in a random coil structure. The global α-helical content of these mutants should be about 17% lower than that of wild-type apoCII. Replacement of Val-71 by alanine should not influence the third helix but was predicted to increase the tendency to form α-helix in the region 58–60. Replacements of Leu-72 by tyrosine or serine and Leu-75 by alanine were predicted to have minor conformational effects. The double mutant 63A/-66A, in which two important residues for LPL activation were replaced, as well as the insertion mutant with alanine inserted in position 64 (-64Ainsertion) were predicted to have largely intact structure in the third helix. The region 57–63 was predicted to have an even higher tendency to form α-helix in both of these mutants compared with the wild type (Fig. 1). With regard to the insertion mutant, the previously predicted LPL-binding site should be seriously disturbed by shifting the face for potential interaction formed by residues Ile-66, Asp-69, and Gln-70 away from Tyr-63 by about 100 degrees.

Activation of LPL by ApoCII Mutants Using the Intralipid-like Emulsion—All mutants described above and in Fig. 1 were expressed and purified on nickel-nitrilotriacetic acid-agarose (supplemental Fig. S1). In a previous study we had found that a His tag at the N terminus of apoCII did not interfere with the activation (17). Therefore, in the present study recombinant proteins were used for the functional studies without removal of the His tag. Replacements of Ser-54, Thr-57, or Pro-43 by alanine did not have any major influence on the activation of LPL (Fig. 2A). Because apoCII from all animal species, except apoCII from rat and trout, have proline residues between helix 1 and 2, and because trout apoCII appeared to be more efficient than human apoCII in activating LPL at low temperature (21), the P43A mutant was also tested at a lower incubation temperature (10 °C). The activation was, however, comparable with that of wild-type apoCII at this temperature (data not shown).

Replacement of Ser-54 by Pro had essentially no effect on the maximal activation, but it increased the apparent affinity for LPL. The Kd was 0.037 ± 0.014 μM compared with 0.16 ± 0.03 μM for wild-type apoCII (Table 1, Fig. 2B). In contrast, the mutant G65P had similar affinity for LPL as wild-type apoCII (Kd = 0.15 ± 0.03 μM), but the maximal activation was reduced by 30% compared with that of wild-type apoCII (Fig. 2B). Replacement of Leu-72 by proline caused a dramatic decrease in the activation ability (Fig. 2B), although this residue should be outside of the LPL-binding site (17). The maximal activation reached was only 35% that for wild-type apoCII, and the apparent affinity of L72P was decreased about 9-fold (Kd = 1.5 ± 0.5 μM) compared with wild-type apoCII. Similarly, replacement of Val-71 by alanine had no effect on the activation (curve not shown, Kd = 0.37 ± 0.07 μM, Table 1), but replacement at that position with proline made apoCII almost inactive (Fig. 2C).

**FIGURE 2. Activation of lipoprotein lipase by apoCII and mutants.** ApoCII was mixed with the lipid substrate in a total volume of 200 μl. The reactions were started by the addition of bovine lipoprotein lipase (5 ng) and carried on for 15 min. Panel A, WT, P43A, S54A, and T57A. Panel B, WT, S54P, G65P, and L72P. Panel C, WT, V71P, L72Y, and L72S. Panel D, WT, F67S, F67A, and L75A. Panel E, WT, 63A/-66A double mutant and -64Ainsertion mutant. The curve for mutant V71A is not shown. Data points from duplicate measurements of parallel samples with each apoCII variant are shown, and the curves are fitted to all data for each variant. Kinetic constants (Kd and β) are shown in Table 1.
Thus, mutants predicted to interfere with the proximal part of the third α-helix (Fig. 1, G65P, V71P, and L72P) had reduced activation ability.

To further probe the importance of the function of hydrophobic, presumably lipid binding residues in or close to the third helix, we replaced Phe-67 and Leu-75 by alanine, but as for V71A, little effect was found (Fig. 2D), whereas replacement of Phe-67 with serine significantly reduced the apparent affinity for LPL (Fig. 2D and Table 1). The activation curve for this mutant was essentially linear up to 3.75 μM, and the $K_d$ value, therefore, deviated a lot ($K_d = 5.62 ± 2.63 \mu M$). Also this mutant was predicted to interfere with the proximal part of the third α-helix (Fig. 1).

The insertion of an alanine residue at position 64, which should result in a repositioning of the downstream residues in the third helix, caused a major loss of activation ability (Fig. 2E). This mutant had one of the lowest apparent affinities for LPL ($K_d = 4.02 ± 2.04 \mu M$) and reached about the same level of activation as the L72P mutant. As expected from our previous results with single mutations (17), the double mutant 63A/-66A was completely inactive (Fig. 2E).

Activation of LPL by ApoCII Mutants Using Chylomicrons from an ApoCII-deficient Patient as Substrate for the Enzyme—

In this system the maximal -fold activation was higher than with the Intralipid-like substrate (about 10-fold compared with about 6-fold). Previous studies had shown that for activation in this system the amphipathic α-helices in the N-terminal two-thirds of apoCII are crucial, because apoCII fragment 50–79 was almost inactive (13). We performed incubations with some of the apoCII mutants in which hydrophobic, and presumably lipid-binding residues in the C-terminal part had been replaced by less hydrophobic residues or by polar residues. Incubations were made at only one concentration of apoCII or mutants (1.7 μM). All mutants, except F67S, were able to stimulate LPL activity to about the same level as wild-type apoCII (Table 2), demonstrating that details in the hydrophobicity properties in the third helix did not matter much, but a change at residue Phe-67 to the polar serine residue was about as harmful with natural chylomicrons as with the Intralipid-like substrate.

CD Measurements—To investigate whether the markedly reduced activation ability of some of the apoCII mutants was due to a major change of secondary structure, mutants G65P, F67S, V71P, and L72P were analyzed by measurements of circular dichroism in the presence of EYPC-liposomes. The concentration of apoCII was in all cases 4.2 μM, and the concentration of phospholipids was 240 μM. The experiment was performed in 0.1 M urea, 20 mM phosphate buffer, pH 7.4, at 25 °C.

![FIGURE 3. Circular dichroism spectra of mutants F67S, G65P, V71P, and L72P and wild-type apoCII in the presence of EYPC-liposomes.](image-url)

**TABLE 1**

| ApoCII variant | $K_d$ (μM) | $\beta$ |
|----------------|-----------|---------|
| WT            | 0.16 ± 0.03 | 5.6 ± 0.4 |
| P43A          | 0.26 ± 0.03 | 5.7 ± 0.3 |
| S54A          | 0.32 ± 0.06 | 6.1 ± 0.4 |
| S54P          | 0.037 ± 0.014 | 5.1 ± 1.2 |
| T57A          | 0.13 ± 0.05 | 6.1 ± 0.4 |
| G65P          | 0.15 ± 0.03 | 4.1 ± 0.8 |
| F67A          | 0.14 ± 0.04 | 5.8 ± 0.8 |
| F67S          | 5.62 ± 263  | 5.5 ± 1.1 |
| V71A          | 0.37 ± 0.07 | 4.7 ± 0.7 |
| V71P          | No activation | |
| L72P          | 1.50 ± 0.50 | 2.0 ± 0.4 |
| L72S          | 0.26 ± 0.07 | 4.8 ± 0.7 |
| L72Y          | 0.10 ± 0.03 | 4.6 ± 0.6 |
| L75A          | 0.37 ± 0.07 | 6.3 ± 0.8 |
| -63A/-66A     | No activation | |
| -64Ainsertion | 4.02 ± 2.04 | 2.4 ± 0.7 |

**TABLE 2**

| ApoCII variant | LPL activity (milliunits/μg) |
|----------------|-----------------------------|
| WT            | 390 ± 19 |
| F67A          | 403.1 ± 39 |
| F67S          | 495.3 ± 46 |
| V71A          | 160.8 ± 70 |
| L72P          | 474.0 ± 41 |
| L72S          | 443.3 ± 73 |
| L72Y          | 483.5 ± 27 |
| L75A          | 413.1 ± 28 |

FIGURE 3. Circular dichroism spectra of mutants F67S, G65P, V71P, and L72P and wild-type apoCII in the presence of EYPC-liposomes. The concentration of apoCII was in all cases 4.2 μM, and the concentration of phospholipids was 240 μM. The experiment was performed in 0.1 M urea, 20 mM phosphate buffer, pH 7.4, at 25 °C.
EYPC liposomes, and the Trp fluorescence was recorded as previously described (17). Although the maximal intrinsic fluorescence differed slightly, mutants L72P and G65P showed similar lipid binding affinities as the fully active S54P mutant and the wild-type apoCII (Fig. 4A). These results were confirmed by measurements of surface plasmon resonance with liposomes of EYPC immobilized on the sensor chip surfaces. Injections of the same concentrations of wild-type apoCII or mutants caused a similar increase of response units. This demonstrated that all variants of apoCII bound to the immobilized liposomes with an affinity that was comparable with, or even higher than that of wild-type apoCII (Fig. 4B). Thus, the overall lipid interaction was not much disturbed in the mutants with lower activation ability (G65P, V71P, and L72P) compared with wild-type apoCII or to mutant S54P that had full activation ability.

Fragment 39–62—The effects of the synthetic apoCII fragment 39–62 were studied in three different substrate systems comparable with those previously used by MacPhee et al. (12); that is, a phosphatidylcholine-stabilized emulsion of triacylglycerols (Fig. 5A), a gum-arabic-stabilized emulsion of trioleylglycerols (Fig. 5B), and liposomes of DMPC (Fig. 5C). With DMPC liposomes the activity of LPL in the absence of apoCII is almost zero, and the enzyme is, therefore, more dependent on the activator than in any of the other two systems. The activation factor ($\beta$) for wild-type apoCII with DMPC was 31, which was 5-fold higher than that in the assays with triacylglycerol. In all three systems the effect of the fragment 39–62 was compared with that of apoCII purified from human plasma, to recombinant His-tagged (wild-type) apoCII, or to the synthetic apoCII fragment 50–79. Although both variants of full-length apoCII activated LPL to a similar extent in all three systems, the peptide 39–62 had no effect on LPL activity with any of the substrates. In contrast, with fragment 50–79, almost the same activation level was reached as with full-length apoCII in the two systems with triacylglycerols. With the more challenging DMPC, only about half-maximal stimulation of LPL activity was reached with fragment 50–79.

**DISCUSSION**

Previously, four residues (Tyr-63, Ile-66, Asp-69, and Gln-70) related to the C-terminal third $\alpha$-helix of apoCII had been identified to be of particular importance for the activation of LPL (6, 17). They could be modeled to occupy one face of an $\alpha$-helix spanning the region 59–75, forming a putative LPL-binding site (Fig. 6A). We have now used site-directed mutagenesis to explore the role of the secondary structure in
this region for the activation of LPL. We made proline replacements at residues 54, 65, 71, and 72 with the aim to disturb the helical arrangement and, thereby, the interaction of apoCII with LPL. We found that the mutant S54P had the same ability to activate LPL as wild-type apoCII, indicating that the predicted shortening of the second $\alpha$-helix has no direct influence on the ability of apoCII to bind to and activate LPL. Replacement of Val-71 and Leu-72 with proline was predicted to totally break the third $\alpha$-helix and form a random coil structure. Mutant V71P was totally inactive, whereas mutant L72P showed low apparent affinity for LPL, and the maximal activating ability was reduced to one-third compared with wild-type apoCII. CD measurement in the presence of EYPC liposomes showed that both mutants had a lower degree of helical structure than wild-type apoCII. Sequence alignment of all known apoCII sequences showed that Leu-72 is not a conserved residue and was previously concluded to be located outside the LPL-binding site, because mutant L72A had full activity (17). This was confirmed in the present study by replacement of Leu-72 with polar residues (tyrosine and serine). It was recently reported by Lam et al. (22) that patients with the missense mutation L72P in apoCII developed chylomicronemia. Taken together all available data indicate that the secondary structure of the third $\alpha$-helix is very important for the activation of LPL.

Residue Gly-65 was replaced by proline to target the extended $\alpha$-helix spanning from residue 63, according to NMR data for wild-type apoCII (7). Structure prediction for the mutant G65P showed, however, that this mutant could still have a secondary structure very similar to that of wild-type apoCII. The interaction between this mutant and LPL appeared intact ($K_d$ was similar to that for wild-type apoCII), but the maximal level of activation was 30% lower with the G65P

FIGURE 5. Activation of lipoprotein lipase by apoCII fragment 39–62 compared with activation by wild-type apoCII and apoCII fragment 50–79. The activation of bovine LPL by apoCII fragment 39–62 (open squares), apoCII fragment 50–79 (solid squares), apoCII purified from human plasma (solid circles), and recombinant His-tagged wild-type apoCII (open circles) was determined by using three different substrates as described under “Experimental Procedures.” Panel A, EYPC-stabilized emulsion of soy bean triacylglycerols is shown. Panel B, gum-arabic-stabilized emulsion of trioleoelyglycerol is shown. Panel C, liposomes made from DMPC are shown. Data points from duplicate measurements of parallel samples with each apoCII variant are shown, and the curves are fitted to all data for each variant. U, units.

FIGURE 6. Comparison of a model $\alpha$-helix of the region 59–70 of apoCII and of the same region in the -64Ainsertion mutant. The positions of the $C_{\alpha}$ atoms of the seven residues that are fully conserved in apoCII from all sequenced animal species are shown as red spheres. Panel A, shown is a model for wild-type apoCII. Panel B, shown is a model for -64Ainsertion mutant. Here the positions of the residues affected by the insertion are indicated with the old residue number but with a prime sign (green spheres).
mutant than that with wild-type apoCII. In a previous study we had shown that replacement of Gly-65 with alanine did not change the activation properties of apoCII (17). Therefore, the loss of activation by the G65P mutant was probably not due to loss of important structures in the side chain but to changed backbone conformation around this residue.

The importance of the overall steric arrangement in the third α-helix was also investigated by insertion of an alanine residue at position 64 (-64Ainsertion). This mutant was designed to change the steric relation between residue Tyr-63 and the other three most important residues for activation (Ile-66, Asp-69, and Gln-70) (Fig. 6B). The consensus secondary structure prediction for the -64Ainsertion mutant showed a pattern similar to that of wild-type apoCII. The insertion mutant had the highest $K_d$ value of all apoCII variants tested, indicating low affinity for LPL. The calculated level for maximal stimulation was reduced to about one-third that for wild-type apoCII. Thus, the insertion had a more severe effect on the function of apoCII than the replacement with proline at residue 65. This indicated that residue Tyr-63 had to point in the same direction as the other three most important residues and probably act in concert with them for efficient activation of LPL by apoCII. The mutants S54P, G65P, L72P showed similar lipid binding ability as wild-type apoCII, demonstrating, as expected, that the reduced activation with mutants G65P and L72P was not due to reduced ability to bind to the lipid substrate.

There are several reports on the structure of human apoCII or on the C-terminal fragment as determined by NMR spectroscopy (6–8, 19, 23). The results show a high level of similarity when compared with each other. Three major α-helices are formed approximately by residues 16–38 (helix1), 45–57 (helix 2), and 65–74 (helix3). The transition region between helix 2 and helix 3 (from residue 58–64) was reported by Zdunek et al. (6) to form a less perfect α-helical conformation, which had to form an angle of ~30° between helix 2 relative to helix 3. MacRaid et al. (7) found instead that the helical structure was disrupted around residue 63 and proposed that this residue was projected away from the lipoprotein surface to enable interaction with LPL. If this was the case, both the G65P and the -64Ainsertion mutants should retain their activating ability. In clear contrast, our data demonstrate that the steric arrangement around residue Tyr-63 was very important for the function of apoCII and that this was severely disturbed by replacing Tyr-63 with a proline residue.

We previously concluded that no single amino acid was absolutely crucial for the apoCII/LPL interaction, indicating that there are several cooperating structures involved in the interaction that can partly compensate for each other (17). To test this we made a double mutant at positions 63 and 66 (63A/66A). This totally wiped out the activation ability, whereas the corresponding single mutants each retained about 30% of the maximal activation ability (17). It is likely that other pairs of double mutations affecting the seven fully conserved residues in apoCII would have similar effects. This was, however, not further explored. Together with the -64Ainsertion mutant, the double mutant represented the best proof for the role of the secondary structure of apoCII for the activation.

To investigate if there are regions in apoCII in addition to those related to the third α-helix that are important for the interaction with LPL, three additional residues toward the N terminus were mutated. Residue Ser-54 was selected because it is conserved in all presently known apoCII sequences, with the exception for chicken apoCII, which has a glutamine residue in this position. Residue Thr-57 was selected because it is functionally conserved in all known apoCII sequences. 7 of 10 animal species have threonine residues in this position, whereas the others have serine instead. In all apoCII sequences except in those from rat and rainbow trout, there is a proline residue at position 42 or 43 (human numbering). In this region there is a break between helix 1 and helix 2. Replacement by alanine at positions 43, 54, and 57 did not, however, change the activation properties of apoCII. It is possible that with other types of lipid substrates presenting more curved lipid/water interfaces, the break between helix 1 and helix 2 due to Pro-43 might allow more flexibility in the lipid interaction. For other exchangeable apolipoproteins like apoE and LpIII, which form helix bundles in the absence of lipids, it has been suggested that the hinge regions are involved in the initial anchoring of the lipid-free protein to the lipid surface (24). For apoCII, the hinge region might be involved in the ability to transfer between different lipid particles. If so, it might influence on the distribution of apoCII between very low density lipoproteins and high density lipoproteins.

Residues Pro-43, Ser-54, and Thr-57 are all contained within fragment 39–62, previously reported to have similar ability to activate LPL as wild-type apoCII (16). In this region, only residues 59 and 62 are fully conserved (21). We had previously found that substitution of those two residues by alanine had little influence on the activation (17). A natural point mutation, K55Q, was reported to retain normal function (25). Lys-55 is conserved in apoCII from all animal species except for apoCII from rainbow trout, which has aspartic acid in this position, demonstrating that the charge of this residue is not important for the function. A synthetic apoCII fragment 56–79 in which serine at position 61 was substituted with glycine was shown to activate LPL activity as efficiently as wild-type fragment 56–79 (26). This ruled out the possibility that the hydroxyl group at position 61 might form an acylated intermediate during LPL activation, as had previously been proposed by Kinnunen et al. (27).

We were not surprised to see that the apoCII fragment 39–62 did not activate LPL with any of the three different substrates used. This fragment lacks the main part of the domain that had previously been identified by others and by us to be critical for the activation (17). We are unable to explain the difference between our present results and those previously reported (16). The substrate systems used for the activity assays are closely similar in both studies. In the systems with triacylglycerols, fragment 50–79 stimulated LPL almost as efficiently as full-length apoCII. However, with the more challenging DMPC substrate, fragment 50–79 fragment did not reach full activation.

Taken together our data confirm the importance of the C-terminal part of apoCII for activation of LPL. The α-helix in this region is crucial in addition to the individual contributions
of the four fully conserved residues (Thr-63, Ile-66, Asp-69, and Gln-70). The structural requirements for the functionally conserved hydrophobic residues on the opposite side of the third helix were less stringent.

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