Kinetics of Product Inhibition and Mechanisms of Lipoprotein Lipase Activation by Apolipoprotein C-II†

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ABSTRACT: The kinetics of product inhibition of bovine milk lipoprotein lipase (LPL) were studied in a system of emulsified trioleoylglycerol (TG) at different fixed initial concentrations of oleic acid ($[OA]_0$) without a fatty acid (FA) acceptor. In the absence of apolipoprotein C-II (C-II), the apparent V_{max} and the n_H(TG) (the slope of the corresponding Hill plot for TG) of 1.82 decreased by about 52% and $[TG]_{0.5}$ increased 13-fold by raising the $[OA]_0$ to 0.3 mM. At low $[OA]_0$, product inhibition was competitive with respect to TG: the n_H(OA) averaged 1.1, and $[OA]_{0.5}$ was increased about 2-fold by TG. At the higher $[OA]_0$, n_H(OA) was 3.5, and TG had no effect on $[OA]_{0.5}$. In the presence of 3 μg/mL C-II, the apparent V_{max} was 4.3–7.1-fold higher than in its absence, and the n_H(TG) was 2.45. Both parameters decreased by only 20–25%, and $[TG]_{0.5}$ increased only 3-fold at an $[OA]_0$ of 0.3 mM. Conversely, n_H(OA) decreased by 35% and $[OA]_{0.5}$ increased 6-fold by increasing TG concentrations. Similar kinetics were observed with very low density lipoproteins (VLDL). At saturating TG and varying C-II concentrations, n_H(C-II) was 1.78, and product inhibition was found to be competitive with respect to C-II. At the $[OA]_0$ employed, the FA had no effect on enzyme binding to TG emulsions, and there was no evidence that LPL catalyzes the reverse reaction. It is concluded that (a) the LPL kinetics are those of a multisite enzyme that probably has three high-affinity binding sites for TG, two for C-II, and four for OA, (b) product inhibition being competitive with respect to both TG and C-II supports earlier conclusions that the LPL-catalyzed reaction follows a random, bireactant, rapid equilibrium mechanism, and (c) C-II activates LPL by enabling the displacement of the product from the active center by the substrate.

Since its discovery, lipoprotein lipase (LPL,[1] EC 3.1.1.34) has been known to require a fatty acid (FA) acceptor such as bovine serum albumin (BSA) or calcium ions for catalyzing the in vitro hydrolysis of the triacylglycerols (TG) of chylomicrons, very low density lipoproteins (VLDL), or artificial emulsions (Gordon, 1953; Korn, 1955). In the absence of an FA acceptor, enzymatic activity against long-chain TG is severely curtailed but can be readily restored by the addition of BSA to the incubation medium (Posner & Morales, 1972; Bengtsson & Olivecrona, 1980). As the fatty acid binding sites on BSA become filled with product, the rate of triacylglycerol hydrolysis slows down (Scow & Olivecrona, 1977; Posner & DeSanctis, 1987a). Bengtsson and Olivecrona (1979, 1980) found that LPL can form complexes with FA and suggested that the product might act as a competitive inhibitor and that BSA might prevent the formation of LPL:FA complexes because it has a higher affinity for FA than the enzyme. At oleic acid concentrations of 0.5 mM, the binding of LPL to triacylglycerol–phosphatidylcholine droplets was impaired (Olivecrona & Bengtsson, 1985). Aside from requiring an FA acceptor, LPL is specifically activated by apolipoprotein C-II (C-II) (Posner, 1982). In the absence of an FA acceptor, 2 mM oleate was found to abolish the effect of C-II on the enzyme even though greater FA concentrations had no effect on the basal LPL activity (Bengtsson & Olivecrona, 1979). Product inhibition of LPL is unique to this enzyme since another triacylglycerol hydrolase, pancreatic lipase, was fully active in the presence of relatively high concentrations of oleic acid (OA) in emulsified trioleoylglycerol particles ["pseudosubstrate" (S_P)] (Posner & DeSanctis, 1987a).

A potential physiological role for product inhibition as a feedback control of the activity of lipoprotein lipase, a key enzyme in lipoprotein metabolism, has been proposed (Bengtsson & Olivecrona, 1979; Olivecrona & Bengtsson, 1985). Nevertheless, the kinetics of product inhibition have not been explored, nor has the mechanism of product inhibition in the LPL system been defined experimentally. These kinetic studies were carried out in the absence of a fatty acid acceptor and have led to the following conclusions: (a) the effects of OA on LPL binding or on the reverse reaction play no role in the mechanism of product inhibition, (b) OA inhibits LPL competitively with respect to both TG and C-II, (c) the product inhibition kinetics observed in these studies are consistent with earlier conclusions (Posner & DeSanctis, 1987b) that LPL is an enzyme with multiple catalytic sites (Segel, 1975), and (d) C-II activates the enzyme by enabling the displacement of the product from the active site by the substrate.

Materials and Methods

Materials. The following were purchased from Sigma Chemical Co.: trioleoylglycerol (>99% pure), monoolein (90% α-isomer, 99% pure), oleic acid (99% pure), heparin from porcine intestinal mucosa, permeation chromatography glass beads, Sepharose 4B, tetramethylurea (TMU), DEAE-cellulose, cyanogen bromide, BSA (Cohn fraction V, 96–99% pure), and Triton X-100. Celite 545 and dialysis tubing with a

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[1] Abbreviations: LPL, lipoprotein lipase; FA, fatty acid(s); BSA, bovine serum albumin; TG, triacylglycerol(s); VLDL, very low density lipoprotein(s); C-II, apolipoprotein C-II; OA, oleic acid; S_P, pseudosubstrate; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; $[OA]_0$, initial oleic acid concentration; n_H(TG), n_H(C-II), and n_H(OA), apparent number of binding sites for TG, C-II, and OA, respectively, i.e., slopes of the respective Hill plots.

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3500-dalton cutoff were purchased from Fisher Scientific. Intralipid was obtained from the University Hospital pharmacy. All chemicals used were of reagent grade, and all solutions were prepared in deionized, distilled water.

Enzyme Preparation. Bovine milk lipoprotein lipase was purified as described elsewhere (Posner et al., 1983a). Briefly, skim milk was incubated with Intralipid (20:1 v/v) for 10 min at 37 °C, and after being cooled to 4 °C, the mixture was centrifuged at $10^5 g_{max}$ for 60 min at 2 °C, and acetone–ether powder was prepared of the floating fat cakes. The powder was stored at −20 °C until use. For the preparation of pure enzyme solutions, acetone–ether powder was extracted with 0.1% Triton X-100 in 0.05 M NH_4OH–HCl, pH 8.5, and the extract (10 mg/mL acetone powder) was added to a heparin–Sepharose column. The enzyme was eluted from the column with 2 M NaCl in ammonia buffer after the column was washed, first with 0.3 and then with 0.72 M NaCl in ammonia buffer. The average specific activity of the purified LPL was 25 000 μmol of FA (mg of LPL)$^{-1}$ h^{-1}. The enzyme was used for the kinetic studies immediately after preparation.

Preparation of VLDL and ApoVLDL. Fresh, normal or type IV, fasting human plasma was adjusted to 1 mM EDTA, 0.02% NaN_3, and 0.05% Thimerosal and centrifuged at $65000 g_{min}$ at 4 °C for 18 h. The floating VLDL fractions were pooled, and their background density was raised to 1.025 g/mL. The isolated VLDL were further purified by layering under 0.154 M NaCl, 1 mM EDTA, 0.02% NaN_3, and 0.05% Thimerosal and refloating by ultracentrifugation as above; this procedure was repeated twice. The purified VLDL fractions were pooled, dialyzed at 4 °C against a number of changes of 50 mM NH_4HCO_3, pH 8.5, containing EDTA, sodium azide, and Thimerosal as above and finally against ammonium bicarbonate, and lyophilized. Freeze-dried VLDL was delipidized by extraction with chloroform–methanol (2:1 v/v), addition of diethyl ether, and low-speed centrifugation (Shulman et al., 1974). The precipitated proteins were dried in vacuo and kept at −20 °C until use. The soluble apolipoproteins were dissolved in 0.05 M NH_4Cl–HCl, pH 8.5, to a final concentration of about 400 μg/mL.

Preparation of C-II. Apolipoprotein C-II was isolated and purified as recently described (Posner & DeSanctis, 1986). Dehydrated VLDL (320 mg) were suspended at room temperature in 5 mL of 1 mM phosphate buffer, pH 7.4, and the suspension was mixed with 10 mL of TMU. After low-speed centrifugation, the infranate was passed through a 2.5 × 4 cm DEAE-cellulose column. The adsorbed TMU-soluble VLDL apoproteins were eluted with a 250-mL NaCl gradient (20–70 mM) in 1 mM phosphate buffer, pH 7.4, containing 6 M deionized urea at a flow rate of 120 mL/h. The eluted C-II peak was exhaustively dialyzed against 5 mM sodium bicarbonate, pH 8.2, at 4 °C and lyophilized. The dried powder recovered from four columns was dissolved in 10 mL of 1 mM phosphate buffer, pH 7.4, containing 6 M deionized urea and rechromatographed as above. The purified material under the C-II peak was dialyzed as before and lyophilized. The purified C-II was readily dissolved in dilute buffer solutions to concentrations as high as 15 mg/mL and showed up as one band on basic polyacrylamide gel electrophoresis (PAGE) in 6 M urea.

Enzyme Assay. Trioleoylglycerol-coated glass beads (0.15 mg of TG/mg of beads) and pseudosubstrates (S_P) were prepared as described earlier (Posner & Bermudez, 1977). For the kinetic studies, TG-coated beads were added to 0.2 M Tris-HCl, pH 8.0, which contained 1 mg/mL BSA (45 mg of beads/mL of medium), and after being shaken for 15–30 min at 37 °C in order to release the TG from the beads and form the pseudosubstrate, the beads were removed by filtration through glass wool. Aliquots of the S_P and other additions were pipetted to series of Erlenmeyer flasks to final volumes of 9.5 mL. After equilibration to 37 °C, the enzymatic reactions were initiated by the addition of 500 μL of purified LPL. In order to determine the linear rate of fatty acid (FA) release, aliquots of 1 mL were removed at 12-min intervals (0–72 min), and their FA content was analyzed. For the assay of enzymatic activity, S_P was prepared in 0.2 M Tris-HCl, pH 8.0, which contained 60 mg/mL BSA and 3 μg/mL C-II, and 0.95-mL aliquots were pipetted into screw-capped tubes. An aliquot of 50 μL of LPL was added to each tube, and FA release was determined after the reactions were stopped at time intervals over which FA release was linear (5–60 min). Monoolein emulsions were prepared as described for S_P except that the monoolein was coated on Celite instead of glass beads (0.1 mg of monoolein/mg of Celite).

Binding Studies. Enzyme binding was performed as before (Posner, 1980; Posner et al., 1983b): samples of 0.3 mL of enzyme solution were added to 5.7 mL of S_P, and after being shaken for 4 min at 37 °C, the media were rapidly cooled to 4 °C and centrifuged at $10^5 g$ for 60 min at 2 °C. Enzyme activities in the infranatant solutions were determined by incubating 0.5-mL aliquots with 0.5 mL of S_P that contained 13.4 mM trioleoylglycerol and 60 mg/mL fatty acid free BSA (prepared as described above by the addition of 90 mg of beads/mL of buffer). For 100% control samples, enzyme solutions were incubated with media that contained no emulsified trioleoylglycerol, and aliquots were processed as mentioned for the experimental samples. Fractional free enzyme was calculated by dividing the enzyme activities in the infranates by the corresponding control activities.

Preparation of Oleic Acid Suspensions. Two grams of Celite 545 was coated with 200 mg of oleic acid according to Spector et al. (1969) in a manner similar to that described for coating Celite with trioleoylglycerol (Posner & Morales, 1972). Oleic acid coated Celite was added to a solution of 1 mg/mL BSA in 0.2 M Tris-HCl, pH 8.0, which was previously equilibrated to 37 °C. After 30 min of vigorous shaking, the Celite was removed by filtration through glass wool. The filtrates were centrifuged at $10^5 g$ for 30 min at 2 °C, and the FA content in the clear supernates was analyzed. The fatty acid was found to be uniformly distributed throughout the supernates in concentrations of up to 3.5 mM. About 72% of the oleic acid that had been coated on the Celite was found to be in suspension.

Incorporation of Oleic Acid into S_P. Oleic acid (OA) was incorporated into S_P in one of three ways: (a) S_P (6.7 mM trioleoylglycerol, 1 mg/mL BSA) was first prepared as above and then incubated with oleic acid coated Celite for 30 min at 37 °C, and the Celite was removed by filtration; (b) trioleoylglycerol-coated beads were added to oleic acid suspensions previously equilibrated to 37 °C, and after being shaken for 15–30 min, the glass beads were removed by filtration; or (c) S_P preparations containing 1 mg/mL BSA and oleic acid suspensions with desired FA concentrations were mixed and incubated for 15 min at 37 °C. The FA that was present in the system prior to the incorporation of OA into S_P plus the BSA-bound FA [assuming seven high-affinity FA binding sites on BSA (Spector, 1969; Scow & Olivecrona, 1977)] was subtracted from the total FA present in the system after the incorporation of OA into the S_P. The corrected oleic acid concentrations are referred to as "initial OA concentrations" ($[OA]_0$).

Other Methods. Bovine serum albumin was freed of fatty acids according to Chen (1967). Protein concentrations were determined by the method of Wang and Smith (1975), and triacylglycerols were determined by the method of Van Handel and Zilversmith (1957). Fatty acids in reaction mixtures were determined according to a modified (Posner & Bermudez, 1977) method of Dole (1956): media (1.0 mL) were extracted with 5.0 mL of 2-propanol–heptane–2 N H_2SO_4 (40:10:1 v/v/v); following the addition of 3.0 mL of water and 3.0 mL of heptane, shaking, and phase separation, the lower phase was titrated with 0.0185 N NaOH and thymol blue as indicator.

All data reported in this paper are representative of four to five similar experiments done in duplicate. Curves were computer fitted by weighted least-squares analysis (Segel, 1975).

Results

Preliminary Experiments. Oleic acid was incorporated into S_P by either one of the methods described above, and the media were centrifuged at 10^5g for 60 min at 2 °C. The floating fat cakes were resuspended in 0.2 M Tris-HCl, pH 8.0, containing 1 mg/mL BSA, and the distribution of oleic acid between the infranates and the resuspended S_P was determined. It was found that about 75% of the oleic acid originally coated on the Celite and 90% of the OA that had been in suspension were incorporated into the S_p. Over 90% of the S_P-associated oleic acid could be transferred to the aqueous phase by the addition of sufficient FA-free albumin to the medium. In the presence of 1 mg/mL BSA, lipoprotein lipase activity was 2.2% of the activity observed in the presence of 30 mg/mL BSA. In a system that contained 0.35 μmol/mL oleic acid and 1 mg of BSA/mL of medium, LPL activity dropped to 0.5%. Hence, the kinetic studies described below were carried out at relatively high LPL concentrations (0.4–4 μg/mL). At saturating [TG], plots of v vs. [LPL] were linear (not shown).

Binding Studies. LPL binding to S_P was studied in the presence of varying initial OA concentrations (0–0.3 μmol/mL). At this range of OA concentrations the fatty acid was found to have no effect on LPL binding. At 2 μmol/mL OA, LPL binding to S_P decreased by 50%. Hence, the kinetic studies described below were carried out at an $[OA]_0$ that did not affect enzyme binding to S_P.

Kinetics of Product Inhibition of the Basal LPL Activity. Reaction velocities were determined by incubating LPL with increasing concentrations of S_P at four different fixed $[OA]_0$. As the latter were increased, the reaction velocities observed at any given TG concentration decreased. The double-reciprocal plots $1/v$ vs. $1/[TG]$ as well as the Dixon plots of the data (figures not shown) were all parabolic, suggesting that there might be multiple binding sites for the product on the enzyme (Segel 1975). The apparent V_{max} values, estimated by extrapolating the Lineweaver–Burk plots to zero $1/[TG]$, decreased by OA. These changes were nearly linear: 0.29 mM $[OA]_0$ decreased the apparent V_{max} by 54% (Figure 1A). The experimental results, computed and plotted according to Hill (Segel 1975), are shown in Figure 2A. At $[OA]_0 = 0.0$, the n_H(TG), i.e., the slope of the Hill plot, was 1.82, and as the $[OA]_0$ was increased to 0.29 mM, it declined to 0.86; concomitantly, the $[TG]_{0.5}$ increased parabolically from 2.0 to 23.4 mM (legend to Figure 2A). Thus, there was an inverse relation between n_H(TG) and $[TG]_{0.5}$. The Hill plots for the inhibidor are presented in Figure 2B. By increasing the TG concentrations in the system from 1.34 to 6.7 mM at initial oleic concentrations of 0.06–0.18 mM, the n_H(OA) increased 1.8-fold while the $[OA]_{0.5}$ increased about 2-fold. At higher initial oleic acid concentrations, the n_H(OA) increased sharply

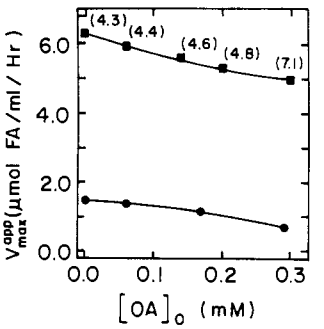

FIGURE 1: Kinetic parameters of LPL. Enzyme activities were measured in a system that contained varying concentrations of emulsified trioleoylglycerol, 1 mg/mL BSA, and different fixed $[OA]_0$ in the absence of C-II (●) or in the presence of 3 μg/mL C-II (■). The reaction was performed in 0.2 M Tris-HCl, pH 8.0, in a total volume of 10.0 mL. The reaction was initiated by the addition of LPL, and FA release over a period of 72 min of incubation at 37 °C was determined (seven 1.0-mL samples were taken for assay at 12-min intervals). The apparent V_{max} values were estimated from Lineweaver–Burk plots (the numbers in parentheses represent ratios of plus-activator to basal apparent V_{max}).

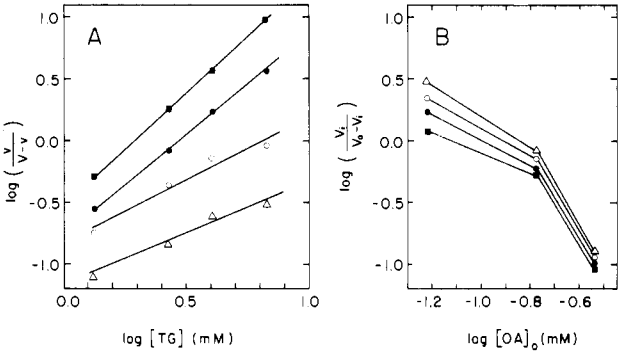

FIGURE 2: Product inhibition of basal LPL activity. The experimental conditions were as described in the legend to Figure 1; the system contained no C-II, and the reaction was initiated by the addition of 4 μg/mL LPL. (A) Hill plots for the substrate at the following fixed millimolar $[OA]_0$: (■) 0.0; (●) 0.06; (○) 0.17; (△) 0.29. The slopes of the respective plots $[n_H(TG)]$ are 1.82, 1.62, 0.99, and 0.86 with corresponding $[TG]_{0.5}$ millimolar values of 2.0, 3.0, 6.6, and 23.4. (B) Hill plots for the product at the following millimolar concentrations of TG: (■) 1.34; (●) 2.68; (○) 4.02; (△) 6.70. The slopes of the respective plots $[n_H(OA)]$ at the lower $[OA]_0$ are 0.70, 1.07, 1.09, and 1.27 with corresponding $[OA]_{0.5}$ millimolar values of 0.071, 0.100, 0.123, and 0.145. At the higher $[OA]_0$ the n_H(OA) values average 3.5.

to 3.5, and TG had no appreciable effect on $[OA]_{0.5}$. It may be tentatively concluded from these data that (a) the LPL kinetics are those of a multisite enzyme with two high-affinity sites for TG and four for OA and (b) at the lower $[OA]_0$ the product inhibited LPL competitively with respect to TG.

Kinetics of Product Inhibition of the "Plus-Activator" LPL Activity. (A) Saturating C-II Concentrations. The experiments described in the previous section were repeated as detailed except that all incubation media contained "saturating" concentrations of C-II (3 μg/mL). Under these conditions Lineweaver–Burk and Dixon plots were again parabolic, and the apparent V_{max}, estimated by extrapolating the double-reciprocal plots to $1/[TG] = 0$ (figures not shown), decreased linearly, Figure 1A. At 0.3 mM $[OA]_0$, the apparent V_{max} was 80% that of the uninhibited reaction. It should be noted that at any given initial OA concentration the "plus-activator" apparent V_{max} was 4.3–7.1-fold greater than the basal apparent V_{max}, Figure 1A. The Hill plots of the data are shown in Figure 3A. In the presence of C-II, the initial n_H(TG) was higher, and the value of this parameter decreased to a lesser degree than it did in the absence of the activator, while $[TG]_{0.5}$

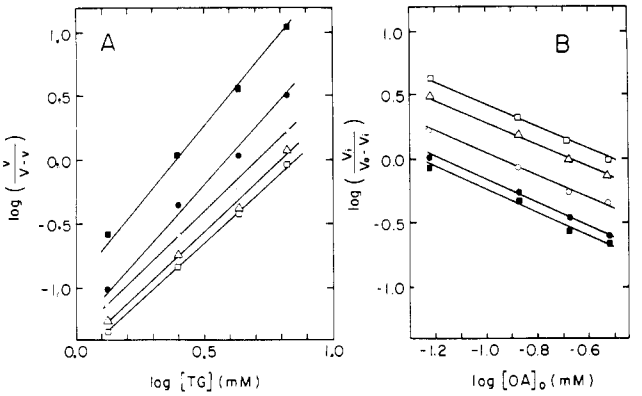

FIGURE 3: Product inhibition of the plus-activator LPL activity. The experimental conditions were as described in the legend to Figure 1; the system also contained constant saturating concentrations of C-II (3 μg/mL), and the reaction was initiated by the addition of 0.4 μg/mL LPL. (A) Hill plots for the substrate at the following fixed millimolar $[OA]_0$: (■) 0.0; (●) 0.061; (○) 0.135; (△) 0.200; (▲) 0.300. The slopes of the respective plots [n_H(TG)] are 2.45, 2.22, 1.93, 1.90, and 1.80 with corresponding $[TG]_{0.5}$ millimolar values of 2.5, 4.0, 5.1, 6.3, and 7.2. (B) Hill plots for the product at the following millimolar concentrations of TG: (■) 1.34; (●) 2.68; (○) 4.36; (△) 6.70; (□) 8.0. The slopes of the respective plots [n_H(OA)] are 1.06, 1.01, 1.00, 0.900, and 0.790 with corresponding $[OA]_{0.5}$ millimolar values of 0.056, 0.070, 0.112, 0.250, and 0.320.

increased only about 3-fold (legend to Figure 3A). The Hill plots for the inhibitor, Figure 3B, show that, unlike the plots in Figure 2B, n_H(OA) decreased from 1.06 at 1.34 mM TG to 0.80 at 8.0 mM TG, whereas the $[OA]_{0.5}$ values increased 5.7-fold by increasing the [TG]. Thus, C-II activated LPL over the entire range of $[OA]_0$ employed in these studies. In the presence of the activator, the effects of the product on n_H(TG) and on $[TG]_{0.5}$ were far less pronounced than in its absence.

(B) Varying C-II Concentrations. In these experiments the inhibitory effect of OA was assessed by varying the C-II concentrations in the system at four different fixed $[OA]_0$ and constant TG concentrations of 13.4 mM. The Lineweaver–Burk plots, $1/v$ vs. $1/[C\text{-}II]$, as well as the Dixon plots of the data all curved upward. The apparent V_{max} values, estimated by extrapolating the double-reciprocal plots to $1/[C\text{-}II] = 0$, decreased linearly to about 60% of the uninhibited apparent V_{max} (figures not shown). The Hill plots of the data, shown in Figure 4A, suggest that C-II might have two high-affinity binding sites on the enzyme. Increasing the $[OA]_0$ to 0.3 mM decreased n_H(C-II) from 1.78 to 1.23 and concomitantly increased $[C\text{-}II]_{0.5}$ more than 2.1-fold. Conversely, C-II decreased n_H(OA) from 1.44 to 1.01 and increased $[OA]_{0.5}$ 2.2-fold, as can be seen in Figure 4B. Hence, OA inhibited LPL competitively with respect to C-II.

Kinetics of Product Inhibition of the Hydrolysis of VLDL–TG by LPL. The kinetics of LPL hydrolysis of VLDL–TG was studied as follows: VLDL was isolated by ultracentrifugation as described under Materials and Methods and dialyzed against three changes of 0.2 M Tris-HCl, pH 8.0 (1:1000 v/v), at 4 °C. Aliquots of VLDL were incubated with OA suspensions or with OA-coated Celite as described above for S_P. ApoVLDL apoproteins soluble in 50 mM NH_4OH–HCl (about 400 μg/mL) were similarly dialyzed against 0.2 M Tris-HCl, pH 8.0. Next, aliquots of VLDL with increasing concentrations of VLDL–TG varying from 1.02 to 5.26 mM and aliquots of the soluble apoVLDL necessary for maintaining the total final concentrations of soluble apolipoproteins in the system constant were pipetted into series of tubes. The rate of FA release at 37 °C was determined after

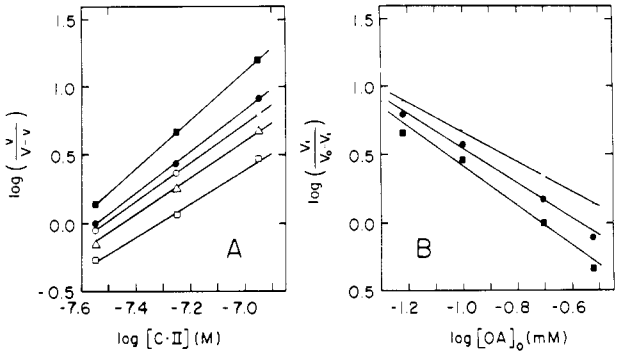

FIGURE 4: Product inhibition of the plus-activator LPL activity. Enzyme activities were measured in a system that contained varying concentrations of C-II, 13.4 mM TG, 2 mg/mL BSA, different fixed initial concentrations of OA, and 2 μg/mL LPL. Otherwise, the reaction was performed as in the legend to Figure 1. For other details see the text. (A) Hill plots for the activator at the following millimolar $[OA]_0$: (■) 0.00; (●) 0.60; (○) 0.10; (△) 0.20; (□) 0.30. The slopes of the respective plots [n_H(C-II)] are 1.78, 1.51, 1.40, 1.33, and 1.23 with corresponding $[C\text{-}II]_{0.5}$ nanomolar values of 23.4, 29.2, 31.0, 36.0, and 50.0. (B) Hill plots for the product at the following nanomolar concentrations of C-II: (■) 28; (●) 56; (○) 112. The respective n_H(OA) values are 1.43, 1.26, and 1.01 with corresponding $[OA]_{0.5}$ millimolar values of 0.19, 0.27, and 0.42.

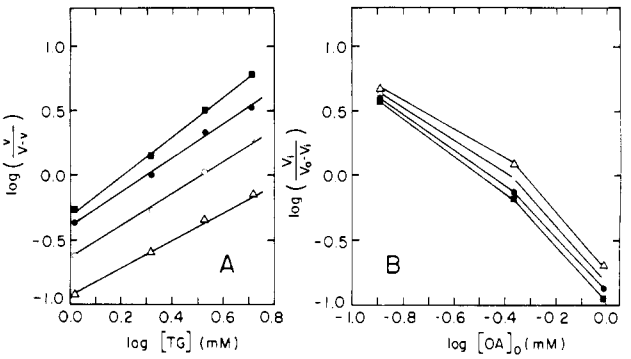

FIGURE 5: Product inhibition of the LPL activity against VLDL–TG. Enzyme activities were measured in a system that contained varying concentrations of VLDL–TG, constant total concentrations of soluble apoVLDL proteins, different fixed initial concentrations of OA, and 4 μg/mL LPL. Otherwise, the reaction was performed as in the legend to Figure 2. (A) Hill plot for the substrate at the following fixed millimolar $[OA]_0$: (■) 0.0; (●) 0.131; (○) 0.433; (△) 0.965. The respective n_H(TG) values are 1.56, 1.44, 1.25, and 1.07 with corresponding $[TG]_{0.5}$ millimolar values of 1.60, 2.0, 3.2, and 7.6. (B) Hill plots for the product at the following millimolar concentrations of TG: (■) 1.04; (●) 2.08; (○) 3.30; (△) 5.26. The respective n_H(OA) values at the lower $[OA]_0$ are 1.43, 1.38, 1.19, and 1.10 with corresponding $[OA]_{0.5}$ millimolar values of 0.32, 0.35, 0.41, and 0.55.

the addition of LPL. As before, the apparent V_{max} values were estimated by extrapolating the double-reciprocal plots to $1/[TG] = 0$ (not shown). It was found that the hydrolysis of VLDL–TG was not very susceptible to product inhibition; i.e., at 0.43 mM $[OA]_0$ the apparent V_{max} was 73%, and at 1.0 mM $[OA]_0$ the apparent V_{max} was 34.3% that of the uninhibited reaction. The results of these experiments, plotted according to Hill, are depicted in Figure 5. It can be seen that OA decreased n_H(TG) from 1.6 to 1.1 and concomitantly increased $[TG]_{0.5}$ about 5-fold by increasing the $[OA]_0$ to 1.0 mM, Figure 5A. As can be seen in Figure 5B, the Hill plots for the product increased in slope at the higher $[OA]_0$. At $[OA]_0$ below 0.43 mM, the n_H(OA) decreased from 1.43 to 1.1, and $[OA]_{0.5}$ increased 1.7-fold with increasing [TG]. At $[OA]_0$ higher than 0.43 mM, the n_H(OA) averaged 2.2 although $[OA]_{0.5}$ was increased slightly by TG.

Studies of the Possible Reversibility of the LPL Reaction. Lipoprotein lipase (4 μg/mL) was added to OA suspensions

(3.5 μmol/mL of OA in 0.2 M Tris-HCl–1 mg/mL BSA, pH 8.0), which contained none or 3 μg/mL C-II and in addition contained one of the following combinations of components: (1) 1 μmol/mL glycerol, (2) 5.3 μmol/mL emulsified monoolein, (3) 1 μmol/mL glycerol and 5.3 μmol/mL emulsified monoolein, or (4) the same combination of components as in (1) through (3), but in each case the system also contained 5.3 mM S_P. The changes in fatty acid concentrations in any of these assay systems were determined after incubations at 37 °C for 60 or 180 min. In no case could a decrease in OA concentration be detected; where changes were observed, these represented small increments in [OA] that were always about 4-fold greater in the presence of C-II. Thus, no evidence was obtained from these experiments that LPL catalyzes the reverse reaction, i.e., from hydrolytic products to higher acylglycerols.

Discussion

It is clear from a number of studies (Gordon et al., 1953; Korn, 1955; Posner & Morales, 1972; Scow & Olivecrona, 1977; Posner & DeSanctis, 1987a) that LPL activity cannot be expressed unless an FA acceptor is present in the system. Although these observations indicate that the accumulation of hydrolytic products at the site of lipolysis, i.e., the S_P surface (Posner, 1982), slows down the reaction rate, they shed no light on possible mechanisms for product inhibition. Scow and Olivecrona (1977) proposed that polar fatty acids, which are more surface active than TG, might displace the latter from the oil–water interface. We have shown earlier (Posner & DeSanctis, 197a) that the capacity of the S_P to accomodate OA is quite large. It is possible that high OA concentrations in the system may change the nature of the interface as well as displace sufficient TG from the surface. However, Bengtsson and Olivecrona (1980) considered that a decrease in TG concentration at the interface is not per se the main factor in product inhibition. In the same work it was found that relatively high OA concentrations (>0.5 mM) interfere with enzyme binding at the interface. The present studies confirm these findings except that lower $[OA]_0$ were found to have no effect on LPL binding to S_P. Since our kinetic experiments were carried out at $[OA]_0 < 0.3$ mM, interference with LPL binding can be ignored as a factor in the inhibition mechanism.

Bengtsson and Olivecrona (1980) observed that LPL can catalyze the reverse reaction at pH 7.3. At pH 8.5 there was little esterification of OA to diacylglycerol and none to monoacyl- and triacylglycerols. No acylglycerols were formed when the enzyme was incubated with OA and glycerol in these studies. Hence, the authors concluded that the reverse reaction was not a major factor in product inhibition. Our own experiments lead to the same conclusion. It should be pointed out, however, that even at extremely high $[OA]_0$, i.e., 3.5 mM, and sufficiently high LPL concentrations there was always some forward reaction with emulsified TG and monoolein. Furthermore, FA release was always 4-fold greater in the presence of 3 μg/mL C-II than in its absence. Bengtsson and Olivecrona (1979, 1980) reported that, in a system of monoolein and Triton X-100, oleate concentrations greater than 0.5 mM abolished the activation of LPL by C-II. In our kinetic studies, which were carried out under differing experimental conditions, the reaction velocities and the apparent V_{max} decreased with increasing $[OA]_0$. However, the "plus activator" reaction velocities at any given [TG] (not shown) as well as the apparent V_{max} values were higher than under basal conditions, Figure 1A. Thus, the three potential factors that might contribute to product inhibition, namely, reverse reaction, interference with binding, and decreased activation by C-II, do not seem to be involved in the mechanism of product inhibition in the present system.

Various physicochemical studies suggest that LPL is a multisite enzyme. Sedimentation velocity data (Iverius & Östlund-Linqvist, 1976) and sedimentation equilibrium analysis (Osborne et al., 1985) of bovine milk LPL as well as target size analysis of rat heart and adipose tissue LPL (Garfinkel et al., 1983) and of bovine milk LPL (Olivecrona et al., 1985) have led to the conclusion that the dimer is the predominantly active species of the enzyme. Higher oligomeric forms of LPL were also shown to exist and the monomeric form of the enzyme was assumed to be inactive (Olivecrona et al., 1985; Osborne et al., 1985). It was found by Matsuoka et al. (1980) that C-II could bind to LPL, which was covalently immobilized on CH-Sepharose with C-II:LPL molar ratios of 1.5–2.7:1.0 (Posner, 1982).

When enzyme kinetics were evaluated at saturating TG or C-II concentrations in the presence of a commercial preparation of FA-free BSA (Posner et al., 1983a,b, 1985), Henri–Michaelis–Menten kinetics were observed, suggesting that under the experimental conditions employed in those studies substrate binding was not cooperative, since one molecule of an n-site nonregulatory enzyme is known to yield the same velocity curve as n molecules of a singe-site enzyme (Segel, 1975). Recently, we conducted similar experiments (Posner & DeSanctis, 1987b) but employed a wider range of TG concentrations and twice the concentration of BSA (the latter was freed of FA in our own laboratory). In these experiments the velocity curves were sigmoidal, and n_H(TG) and n_H(C-II) greater than 2.00 were computed from Hill plots. C-II had no effect on n_H(TG) or $[TG]_{0.5}$, and TG had no effect on n_H(C-II) or $[C\text{-}II]_{0.5}$. Heparin (0.3 μg/mL) inhibited the basal lipase activity of LPL competitively: it decreased n_H(TG) from 2.94 to 1.98 and increased $[TG]_{0.5}$, while 13.4 mM TG decreased n_H(heparin) from 2.14 to 0.95 and increased $[heparin]_{0.5}$ a fewfold. At constant inhibitor concentrations, C-II increased the sigmoidicity of the velocity curve; i.e., it increased n_H(TG) from 1.78 to 2.52 and decreased $[TG]_{0.5}$ about 10-fold; it also increased the apparent V_{max}. We have concluded from these kinetic studies that LPL is a multisite enzyme, possibly a tetramer, with three high-affinity catalytic sites and an equal number of sites for C-II and heparin per oligomer and that BSA can affect the cooperativity of substrate or activator binding.

In line with the above, the present studies similarly suggest the possibility that LPL is a multisite enzyme with three high-affinity sites for TG, four for oleic acid, and two for C-II. These conclusions have been reached on the basis of Hill plots of the kinetic data and remain to be confirmed by binding studies. In this regard, Gatt and Bartfai (1977) considered systems similar to ours and derived rate equations for models in which the substrate interacts with the enzyme at sites other than the active center or with a contaminating protein. Simulated kinetic curves were presented to show that under these conditions sigmoidal velocity curves may be obtained even with single-site enzymes. Criteria were established for discriminating between these and other kinetic models. According to these criteria, the sigmoidicity of the velocity curves in our own studies is probably due to cooperativity between multiple interacting sites. We have consistently found (unpublished data) that under the present and previous experimental conditions (Posner & DeSanctis, 1987b) (a) identical Hill plots are obtained at different enzyme concentrations, (b) the v vs. [LPL] curves at saturating TG concentrations are linear both

in the absence and in the presence of C-II, and (c) biphasic v vs. [TG] curves are never observed, even at very high [TG]. Furthermore, in these as well as in earlier studies, LPL was found to bind to S_P quantitatively in less than 1 min (I. Posner, unpublished results). The rate of FA release was found to be linear with no lag period in FA release (not shown). Consequently, LPL binding to S_P and the binding of individual TG molecules at the active center are probably simultaneous events, neither of which is rate limiting (Posner et al., 1983b). Finally, it may be recalled that rapid equilibrium velocity equations are really equilibrium ligand binding equations that become velocity equations when Y_S, the fraction of occupied sites, is equated to v/V_{max} (Segel, 1975). In this regard, it had been previously shown that the fractional S_P-bound enzyme and fractional reaction velocities in our system are directly related (Posner & Morrison, 1979a,b).

Shinomiya et al. (1984) have found that C-II causes a chain length dependent increase in the entropy of activation of the LPL-catalyzed reaction and concluded that C-II causes a change in the reaction pathway that enables the transfer of fatty acyl chains of the substrate or the product to a more hydrophobic environment in the transition-state complex. Implicit in this model for LPL activation by C-II is the fact that C-II affects a catalytic event that occurs after the E:A:S (or A:E:S) ternary complex had formed. This mechanism is in keeping with the concept that the role of C-II in the activation process is to increase the catalytic rate constant (k_p) as a result of conformational changes that the activator induces in the enzyme (Posner et al., 1983b). Accordingly, C-II would be expected to increase the apparent V_{max} of the LPL reaction as found in the present and in previous studies (Posner et al., 1983a,b, 1985; Posner & DeSanctis, 1987b) and not to affect K_m(TG) or K_S (Posner et al., 1983b). The results of the present studies on the mechanism of product inhibition suggest the possibility that C-II enables the displacement of FA from the active center by the substrate.

In the absence of C-II, oleic acid had a profound effect on LPL activity. At 0.3 mM $[OA]_0$, the apparent basal V_{max} and n_H(TG) were both 52% lower and $[TG]_{0.5}$ was 13-fold greater than at zero $[OA]_0$ (Figure 1A and legend to Figure 2A). At low $[OA]_0$, increasing the TG concentrations resulted in only a 2-fold increase in $[OA]_{0.5}$ and an actual increase in n_H(OA), Figure 2B. It would seem that increasing the TG concentrations in the system leads to an atenuated effect on the reaction velocity, apparently due to the fact that as more OA is produced at the higher [TG] it is dumped into the S_P surface and is added to preexisting fatty acid, thus tending to further slow down the reaction. At the higher oleic acid concentrations the predominance of product inhibition was obvious: n_H(OA) increased sharply to about 3.5, $[TG]_{0.5}$ increased from 6.6 to 23.4 mM, and the apparent V_{max} declined from 81 to 48% of the uninhibited reaction. Furthermore, TG had no measurable effect on n_H(OA) or $[OA]_{0.5}$.

In contrast to the above, at saturating C-II concentrations (Figure 1A and legend to Figure 3A), the apparent V_{max} declined by only 20%. The n_H(TG) was 2.45 at zero $[OA]_0$, suggesting three high-affinity binding sites for TG, and only 26% lower at 0.3 mM $[OA]_0$. At the latter $[OA]_0$, $[TG]_{0.5}$ increased only 3-fold. Moreover, as the TG concentrations were increased, n_H(OA) decreased by 30% and $[OA]_{0.5}$ increased almost 6-fold, Figure 3B, despite the fact that the reaction velocities and the apparent V_{max} at corresponding initial oleic acid concentrations were higher than in the absence of C-II; i.e., more product was added to preexisting fatty acid in the S_P. Similar results were obtained by varying the concentrations of VLDL–TG at constant saturating C-II concentrations, Figure 5. Relatively high $[OA]_0$ were required to produce the inhibitory effects that were seen under basal conditions with S_P. These kinetic studies clearly show that in the presence of the activator the substrate can more readily compete with the product for binding to the active center. The LPL kinetics observed at varying [C-II] and constant saturating [TG], Figure 4, further support this conclusion. The product inhibited LPL competitively with regard to C-II: OA lowered the apparent V_{max} and n_H(C-II) and increased $[C\text{-}II]_{0.5}$, whereas C-II lowered n_H(OA) and increased $[OA]_{0.5}$.

The fact that OA inhibited lipoprotein lipase competitively with respect to both TG and C-II is in agreement with the proposal that the LPL system follows a random, bireactant, rapid equilibrium model (Posner et al., 1983a,b, 1985). Whether the decrease in n_H(C-II) by oleic acid means that the product physically displaces the activator from binding sites on the enzyme or whether it merely reflects the working of opposing conformational effects needs to be evaluated by direct binding experiments. There is no doubt that oleic acid and TG compete for the same site on the enzyme, i.e., the active center. Hence, the comparative affinities of substrate and product for the active center also need to be checked by direct binding studies. It was concluded earlier that of 3.3×10^4 TG molecules in S_P one molecule represents an effective TG molecule (Posner et al., 1983b). If all of the FA that was initially present in S_P plus that produced during lipolysis was located at the pseudosubstrate surface (Scow & Olivecrona, 1977), then the affinity of the substrate for the active center would seem to be about 3 orders of magnitude greater than that of the product: $[TG]_{0.5}$ was derived from total and not from effective TG concentrations. On the other hand, it should be pointed out that it is quite likely that the total FA present in the system at any given moment was distributed between the S_P surface and the S_P core, and if so, then the exchange between core OA and surface OA must have been quite fast. There is no reason to assume that the oleic acid initially present in the S_P and that produced during lipolysis were distributed in different pools. As pointed out above, the enzymatic activity against long-chain TG, which is severely curtailed in the absence of an FA acceptor, can be quickly restored by the addition of BSA to the assay medium (Posner & Morales, 1972; Bengtsson & Olivecrona, 1980). Furthermore, the present studies have shown that BSA can readily transfer practically all of the S_P-bound oleic acid into the aqueous phase.

In light of the present studies and those of others (Scow & Olivecrona, 1977; Bengtsson & Olivecrona, 1979, 1980), it is hard to ascribe a physiological role to product inhibition. In the studies with human VLDL in the total absence of albumin in the system, 0.5 and 1.0 mM $[OA]_0$ inhibited 23 and 65% of the LPL activity, respectively. In man, the FA:albumin molar ratio is between 0.5 and 2.0 and rarely exceeds 4 (Fredrickson & Gordon, 1958), whereas human plasma albumin has at least seven high-affinity FA-binding sites (Ashbrook et al., 1975). In other words, at concentrations of about 0.53 mM, circulating human albumin has the capacity for binding more than 3.5 μmol/mL long-chain FA, concentrations not seen even in pathological cases.

In conclusion, it should be stated that LPL differs from classical allosteric enzymes in that its specific activator C-II does not act by increasing the cooperativity of substrate binding but instead has a profound effect on the catalytic step of the enzymatic reaction. As mentioned above, we have found that C-II has no effect on either n_H(TG) or $[TG]_{0.5}$ in the presence of BSA (Posner & DeSanctis, 1987b). The activator rather

affects the catalytic step of the LPL reaction (Posner, 1980) by inducing conformational changes in the enzyme (Posner et al., 1983b). The latter enable the transfer of the fatty acyl chains of the substrate or product to a more hydrophobic environment in the transition-state complex (Shinomiya et al., 1984) and/or increase the rate of product removal from the active site. Kinetically, the effects of C-II are reflected in an increase in the catalytic rate constant (k_p), i.e., the turnover number or molecular activity of the enzyme (Posner et al., 1983b, 1985), and hence in increased V_{max}.

Registry No. Lipoprotein lipase, 9004-02-8; trioleoylglycerol, 122-32-7; oleic acid, 112-80-1.

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