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Kinetics of Bovine Milk Lipoprotein Lipase and the Mechanism of Enzyme Activation by Apolipoprotein C-II†

Israel Posner,‡ Chi-Sun Wang,* and Walter J. McConathy

ABSTRACT: The kinetics of bovine milk lipoprotein lipase (LPL) were studied in order to determine the reaction mechanism of this enzyme. Reaction velocities were determined at varying concentrations of emulsified trioleoylglycerol (TG) and different fixed concentrations of apolipoprotein C-II (C-II) or at varying C-II concentrations and different fixed concentrations of TG. Neither the apparent K_m(TG) nor the apparent K_m(C-II) was affected by varying the concentrations of C-II or TG, respectively. However, C-II increased the apparent V_{max} for the enzyme about 20-fold. The following kinetic parameters were calculated from Lineweaver–Burk plots: K_m(C-II) = 2.5×10^{-8} M and K_m(TG) = 2.5×10^{-3} M. The dissociation constant (K_S) of the enzyme–TG binary complex was determined from Scatchard plots to be 7.6×10^{-8} M. Heparin was found to be a competitive dead-end inhibitor against both TG and C-II. Tricapryloylglycerol represented a competitive inhibitor against TG but a noncompetitive inhibitor against C-II. C-II was shown to interact with dansylated bovine milk LPL, increasing its fluorescent emission by inducing a conformational change in the enzyme. Based on these studies, it was concluded that the LPL-catalyzed reaction follows a random, bireactant, rapid-equilibrium mechanism and the role of C-II in the activation process involves an increase in the catalytic rate constant (k_p) resulting from conformational changes of LPL induced by C-II.

Lipoprotein lipase (LPL,[1] EC 3.1.1.34) is an enzyme which catalyzes the hydrolysis of the triacylglycerols of chylomicrons and very low density lipoproteins (VLDL), thus playing a key role in the metabolism of the plasma lipoproteins. It is well established that LPL is an enzyme which acts at endothelial cell surfaces of various tissues and can be liberated into the

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[1] Abbreviations: LPL, lipoprotein lipase; C-II, apolipoprotein C-II; TG, trioleoylglycerol; FA, fatty acid(s); S_p, pseudosubstrate; DNS-LPL, dansylated lipoprotein lipase; VLDL, very low density lipoprotein(s); DNS-Cl, 5-(dimethylamino)-1-naphthalenesulfonyl chloride; BSA, bovine serum albumin; Tris, tris(hydroxymethyl)aminomethane.

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circulation by heparin. The milk of certain animal species is rich in LPL activity and is a good source of this enzyme (Korn, 1962). In bovine milk, the bulk of the enzyme is associated with the skim milk (Korn, 1962), but in human milk, the enzyme is rather associated with the milk cream (Wang et al., 1982b).

For the full expression of its activity, LPL is dependent on activation by a minor component of plasma lipoproteins, apolipoprotein C-II (C-II), both in vitro (LaRosa et al., 1970) and in vivo (Breckenridge et al., 1978). It was concluded from a recent review of the literature (Posner, 1982) that activation in the LPL system involves enzyme and not substrate activation as was originally proposed (Korn, 1955). Interactions between the enzyme and its specific activator have been demonstrated by monolayer techniques (Miller & Smith, 1973) and by the use of dansylated fragments of C-II (Voyta et al., 1980; Smith et al., 1982). However, no studies have shown that interactions between C-II and the substrate of LPL are relevant to the enzyme reaction mechanism. As a matter of fact, it was shown that C-II fragments devoid of lipid binding regions and which did not bind to human C-II deficient VLDL were still capable of activating the enzyme (Catapano et al., 1979).

From studies which have suggested that LPL activation involves an increased rate of catalysis, two mechanistic models have been proposed. According to one model, the enzyme, when bound to the surface of emulsified triacylglycerol particles, undergoes a spontaneous change of conformation, and the subsequent binding of C-II to the enzyme displaces the equilibrium in favor of the catalytically more active form of the enzyme (Bengtsson & Olivecrona, 1980). Alternatively, it has been proposed that the enzyme which is bound at the surface of emulsified trioleoylglycerol (TG) particles [which were referred to as "pseudosubstrate" (S_p)] is relatively inactive and is activated only through interactions with C-II, which induces a conformational change in the enzyme (Posner, 1980).

In view of the above and the continuing interest in this enzyme (Posner, 1982), we decided to elucidate the nature of the mechanism of LPL activation by C-II. We have shown that C-II has no effect on the apparent K_m(TG) and that the substrate TG has no effect on the apparent K_m(C-II). The effect of the activator, C-II, can be described by an increase in the apparent V_{max} due to an increase in the catalytic rate constant (k_p) caused by conformational changes in LPL induced by the interaction of C-II with LPL.

Materials and Methods

Materials. Trioleoylglycerol (TG), tricapryloylglycerol, fatty acid (FA) free bovine serum albumin (BSA), gel permeation chromatography glass beads, Triton X-100, and 5-(dimethylamino)-1-naphthalenesulfonyl chloride [dansyl chloride (DNS-Cl)] were purchased from Sigma Chemical Co. Heparin was obtained from ICN Nutritional Biochemicals. Sephadex and cyanogen bromide activated Sepharose 4B were obtained from Pharmacia Fine Chemicals, and Celite 545 was from Fisher Scientific Co. All chemicals were reagent grade. Affinity chromatography columns were prepared from microtiter pipets of 0.05-mL delivery (Dynatech Laboratories). Intralipid was a gift of Virtum, Stockholm, Sweden. All solutions were prepared with distilled, deionized water.

Preparation of C-II. The preparation of C-II was described elsewhere in detail (Curry et al., 1981): VLDL was isolated from normal fasting human plasma by ultracentrifugation, and following delipidization, C-II was purified from apoVLDL by a combination of gel filtration and ion-exchange chromatography; the purity of C-II was checked by acidic and basic polyacrylamide gel electrophoresis and by immunological techniques; purified C-II was stored at −20 °C and prior to use was dissolved in 50 mM NH_4OH–HCl buffer, pH 8.5 ("standard buffer"), and its concentration in solution or in sera was determined by a specific electroimmunoassay (Curry et al., 1981).

Preparation of Bovine Milk LPL. Heparin was covalently bound to cyanogen bromide activated Sepharose 4B as described previously (Wang et al., 1981). Fresh whole bovine milk was centrifuged at low speed at 2 °C to remove the cream, and the skim milk was processed immediately or stored at −20 °C. Ten milliliters of Intralipid was added to 210 mL of skim milk, and the contents were subjected to magnetic stirring for 5 min at room temperature. Aliquots of 35 mL were then incubated at 37 °C in a metabolic shaker for 10 min and rapidly cooled in ice. The mixture was centrifuged for 60 min at 25000 rpm in a Beckman swinging-bucket SW27 rotor at 2 °C. The floating fat cakes which contained the LPL were removed with a spatula and resuspended in 0.1 M NaCl–0.02 M sodium citrate at pH 7.5 (12 fat cakes/200 mL). Following a second ultracentrifugation as described above, the washed fat cakes were suspended in 400 mL of cold acetone and filtered on a sintered glass filter of medium porosity. Diethyl ether was added to the filter at room temperature, and the dry acetone–ether powder (approximately 1 mg/5 mL of skim milk) was stored at −20 °C in sealed vials.

Acetone–ether powders were suspended in standard buffer containing 0.1% Triton X-100 (6.25 mg of powder/mL of buffer), and aliquots of 4 mL were applied to small heparin–Sepharose columns containing 1.0 mL of wet gels. The columns were washed with 8 mL of 0.3 M NaCl–0.02 M sodium citrate in ammonium buffer and then with 8 mL of 0.72 M NaCl in standard buffer. The LPL was eluted from the column with 2 mL of 2 M NaCl in ammonium buffer and diluted 20-fold. The purified LPL had a specific activity of 27000 μmol of FA/mg of protein comparable to that reported by others for the purified bovine LPL (Bengtsson & Olivecrona, 1979).

Assay of LPL Activity. The activity of lipoprotein lipase was determined as detailed earlier (Posner & Bermudez, 1977): trioleoylglycerol-coated glass beads were added to 0.2 M Tris-HCl, pH 8.0, containing BSA (32.5 mg/mL), and after being shaken for 15 min at 37 °C, the emulsions [pseudosubstrate (S_p)] were separated from the glass beads by filtration through glass wool. Aliquots of S_p and C-II were added to series of screw-capped tubes to give final concentrations as noted under Results, and the enzymatic reaction was started by the addition of 50 μL of purified bovine milk LPL, and the reactions were allowed to proceed for 20 or 60 min, a period over which the rate of FA release was linear. The reaction was stopped, and FA release was determined following extraction by titration with NaOH (Posner & Bermudez, 1977). Periodically, ^{14}C-labeled TG was employed in the preparation of the S_p, and FA release was determined according to Schotz et al. (1970). Generally, LPL activities determined by the above methodology (Posner & Bermudez, 1977) were identical with those determined by the method of Schotz et al. (1970) (data not shown). Enzyme activities are reported in micromoles of FA released per milliliter of enzyme per hour. In all cases, experiments were initiated immediately after the LPL preparative step to minimize enzyme inactivation. When a series of determinations were made, control samples of purified LPL were included at the beginning and at the end of the series. Experiments were discarded in which a difference of more than 10% in LPL activity between the controls was noted. All experiments were performed in du-

plicate or triplicate, and the results of two or three typical experiments are reported.

Binding Studies. Acetone–ether powders prepared from bovine skim milk were extracted with Triton X-100 in standard buffer (3.25 mg of powder/mL), and the enzyme was purified as described above by applying 4 mL of powder extract per column. Next, aliquots of 10–50 μL of purified LPL were added to three series of 1.5-mL poly(ethylene) microcentrifuge tubes kept at 4 °C and containing 1.75 mM emulsified TG (S_p) and 3.25 mg of BSA in 0.2 M Tris-HCl, pH 8.0 (final volumes were 1.0 mL, and final NaCl concentrations were 0.1 M). In addition to the above, tubes in the second series also contained 2.0 μg of C-II and in the third series, 2.0 μg of C-II and 10.0 μg of heparin. Similarly, LPL was added to three series of paired control tubes which differed from the experimental samples in the sense that they contained no S_p. The tubes were tightly stoppered, incubated for 4 min at 37 °C in a metabolic shaker in a horizontal position to ensure thorough mixing, and immediately cooled in ice. The tubes were then centrifuged for 4 min at 2 °C in a microcentrifuge at 13500*g*. Tubes were punctured with a no. 25 needle, and the infranatant solutions were collected in separate tubes and assayed for LPL activity. Fractional free enzyme ($\bar{F}$) was calculated by dividing the LPL activity remaining in the infranatant solutions of the experimental samples by the LPL activity of the respective control samples. Percent inactivation was calculated by comparing the activity of the control samples with the activity of the enzyme preparation assayed immediately after purification. Enzyme concentrations were calculated from known specific activities in each enzyme preparation. The experimental design for this study was to have minimal lipolysis of S_p. Our criterion was to select an enzyme concentration yielding less than 10% hydrolysis of S_p. Because of high-affinity binding of LPL to substrate, an incubation time of 4 min at 37 °C was found to be satisfactory. Furthermore, at 2 °C, there was no lipolysis exerted by the enzyme.

Fluorescent Labeling of LPL and Enzyme–Activator Interactions. The fluorescent probe dansyl chloride (DNS-Cl) was dispersed on Celite 545 in a 1:100 ratio (DNS-Cl:Celite w/w) (Rinderknecht, 1960; Jonas, 1972), and the DNS-Cl probe was introduced into LPL by a modification of the techniques described by Jonas (1972): 4 mg of Triton X-100, 40 mg of $NaHCO_3$, 30 mg of DNS-Cl-coated Celite, and 100 mg of acetone–ether powder prepared from bovine skim milk were added to a vial containing 4 mL of H_2O. The contents of the vial were gently stirred magnetically for 1 h at 4 °C, the volume was adjusted to 10.0 mL by the addition of 0.1% Triton X-100 in ammonium buffer, and following low-speed centrifugation and filtration through tightly packed glass wool at 4 °C, the DNS-LPL was purified by affinity chromatography (4.0 mL of DNS-LPL solution/column). Enzyme activity was determined as described above and compared with that of a nonreacted control preparation. The DNS-LPL was about 80% as active as the control enzyme. For studying enzyme–activator interactions, 125 μL of DNS-LPL was mixed with 2.5 mL of 0.2 M Tris-HCl, pH 8.0, in a cuvette, the DNS-LPL solution was excited at 340 nm, and the fluorescent emission was read at 500 nm in a Farrand MK-2 spectrophotometer. Aliquots of a known content of C-II were then added stepwise, and the increase in fluorescence was noted. The emission at any given C-II concentration was corrected for volume changes by subtracting the emission noted at the same C-II concentration in the absence of DNS-LPL.

Other Methods. TG concentrations were determined by gas chromatography (Kuksis et al., 1975). Protein concentrations in LPL preparations were determined according to Wang & Smith (1975).

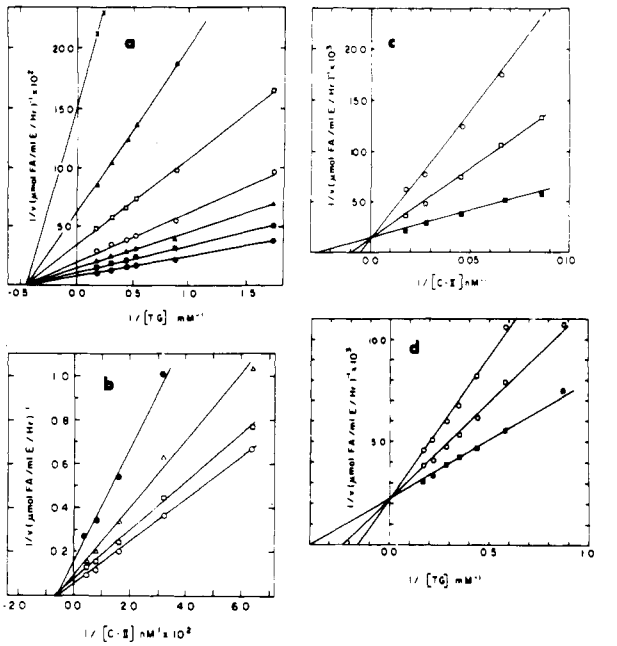


FIGURE 1: Lineweaver–Burk plots of bovine milk LPL. (a) Activities of bovine milk LPL were measured with varying trioleoylglycerol (TG) concentrations. Enzyme activities were measured in a system which contained emulsified trioleoylglycerol, 32.5 mg/mL bovine serum albumin, and one of the following fixed concentrations (micrograms per milliliter) of C-II: (×) 0.03; (△) 0.06; (□) 0.125; (○) 0.25; (▲) 0.50; (■) 1.00; (●) 3.00. The reaction was performed in 0.2 M Tris-HCl, pH 8.0, in a total volume of 1.0 mL. The reaction was initiated by the addition of LPL, and the fatty acid release was determined after 20 min of incubation at 37 °C. (b) Activities of bovine milk LPL were measured with varying C-II concentrations. Enzyme activities were assayed in a system which contained C-II, 32.5 mg/mL bovine serum albumin, and one of the following fixed concentrations (millimolar) of emulsified trioleoylglycerol: (●) 1.14; (△) 2.28; (□) 4.1; (○) 5.7. The reaction was performed in 0.2 M Tris-HCl, pH 8.0, in a total volume of 1.0 mL. The reaction was initiated by the addition of LPL, and fatty acid release was determined after 20 min at 37 °C. (c) Kinetics of heparin inhibition of LPL activity at varying C-II concentrations. Enzyme activities were measured in a system which contained C-II, 32.5 mg/mL bovine serum albumin, and 5.7 mM emulsified trioleoylglycerol. Heparin concentrations were (■) 0, (□) 2.5, and (○) 5.0 μg/mL. The reaction was performed in 0.2 M Tris-HCl, pH 8.0, in a total volume of 1.0 mL. The reaction was initiated by the addition of LPL, and the fatty acid release was determined after 20 min of incubation at 37 °C. (d) Kinetics of heparin inhibition of LPL activity with varying trioleoylglycerol (TG) concentrations. Enzyme activities were measured in a system which contained emulsified trioleoylglycerol, 32.5 mg/mL bovine serum albumin, and 2.5 μg/mL C-II. Heparin concentrations were (■) 0, (□) 2.5, and (○) 5.0 μg/mL. The reaction was performed in 0.2 M Tris-HCl, pH 8.0, in a total volume of 1.0 mL. The reaction was initiated by the addition of LPL, and the fatty acid release was determined after 20 min of incubation at 37 °C.

Analysis of Results. The linear plots were based on least-squares linear regression analysis by assuming a constant variance of reaction velocity (computed with a Compucorp 344 statistician microcomputer). The measured initial rates had a coefficient of variation of less than 5%.

Results

Kinetics of Bovine Milk LPL. The kinetics of purified bovine skim milk LPL were studied under two experimental conditions. First, the effect of varying pseudosubstrate (S_p) concentrations on the reaction velocity was assessed at different fixed concentrations of C-II. As can be seen in Figure 1a, the family of double-reciprocal plots of the data all intercept the

1/[TG] axis at the same point; i.e., C-II has no effect on the apparent K_m(TG) which was calculated as 2.5×10^{-3} M at all of the C-II concentrations studied. The slopes of the plots and consequently the $1/v$ axis intercepts decreased with increasing C-II concentrations; i.e., the apparent V_{max} is directly related to the C-II concentrations in the system. Second, the effect of varying the activator concentrations on the reaction velocity was evaluated at different fixed concentrations of TG. As can be seen in Figure 1b, the family of double-reciprocal plots of the data intercept the 1/[C-II] axis at the same point, from which an apparent K_m(C-II) value of 2.5×10^{-8} M was calculated for any TG concentration studied. The slopes of the plots and consequently the $1/v$ axis intercepts are seen to be dependent on the TG concentrations. It should be pointed out that plots of v vs. [TG] or [C-II] (figures not shown) were typical Michaelis–Menten saturation curves.

Basal LPL Activity. For determination of the basal LPL activity with precision, the apparent V_{max} in the absence of activator was performed with a 20-fold higher amount of LPL in comparison to the activated system. The LPL was purified from bovine milk acetone–ether powders (6.25 mg of powder/mL), and enzyme activities were determined at varying concentrations of TG in the absence and presence of the activator. C-II was added to the system in the form of fasting human serum since with serum the V_{max} was always somewhat higher than with pure C-II. For basal activities, 50-μL aliquots of 2 M NaCl column eluates were assayed directly, whereas in the presence of activators, the LPL preparation was first diluted 20-fold with 2 M NaCl. The final NaCl concentration in the assay system was 0.1 M in these as in all the other kinetic studies. The Lineweaver–Burk plots indicated that both the "basal" and the "plus activator" plots intercepted the 1/[TG] axis at the same point with an apparent K_m(TG) = 2.5×10^{-3} M, whereas in the presence of activator the apparent V_{max} was 19.3 times the apparent basal maximal velocity.

Effect of Dead-End Inhibitors. Because dead-end inhibitors can be used as probes for studying enzyme reaction kinetics, we have examined the effect of heparin on LPL activity. A previous study (Posner & Morrison, 1979) with a crude LPL preparation has shown that heparin is a competitive inhibitor against the activator C-II which we have confirmed in the present study (Figure 1c). We have extended the study by using TG as the variable substrate (Figure 1d), and the results indicated that heparin is also a competitive inhibitor against TG.

Because tricapryloylglycerol represents the best substrate among saturated monoacid triacylglycerols (Wang et al., 1982a), we have also utilized this substrate as a dead-end inhibitor for TG hydrolysis. In this study, we utilized glycerol tri[1-^{14}C]oleate as a substrate for examining the activity of LPL. As expected, Lineweaver–Burk plots indicated that tricapryloylglycerol represents a competitive inhibitor against TG, and no alteration in V_{max} was observed. On the other hand, the double-reciprocal plots maintaining TG concentration at a constant level (10 mM) and with varying C-II concentration indicated that tricapryloylglycerol (10 mM) represents a noncompetitive inhibitor against C-II as seen with the decrease of V_{max} of 2.2-fold.

Binding Studies. Scatchard plots of a typical experiment performed in duplicate under conditions outlined under Materials and Methods are depicted in Figure 2. The equation for the Scatchard plots (Segel, 1975) was

$$\frac{\bar{B}}{\bar{F}} = \frac{1}{K_S}[S]_T - \frac{1}{K_S}[E]_B$$

where $[E]_B$ is the concentration of the enzyme which was

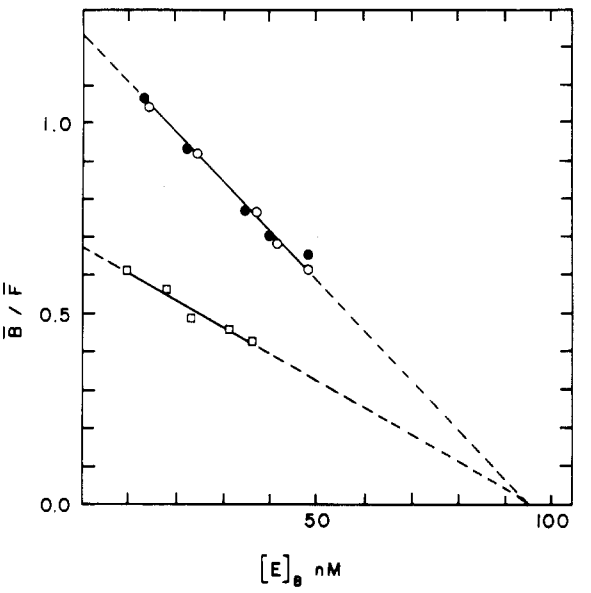


FIGURE 2: Binding of bovine milk LPL to the trioleoylglycerol emulsion (S_p). $\bar{B}$ and $\bar{F}$ represent the fractional bound and free LPL's, respectively. $[E]_B$ is the concentration of the LPL which bound at the S_p surface. (●) LPL; (○) LPL + C-II (2 μg/mL); (□) LPL + C-II (2 μg/mL) + heparin (10 μg/mL). Details are described in the text.

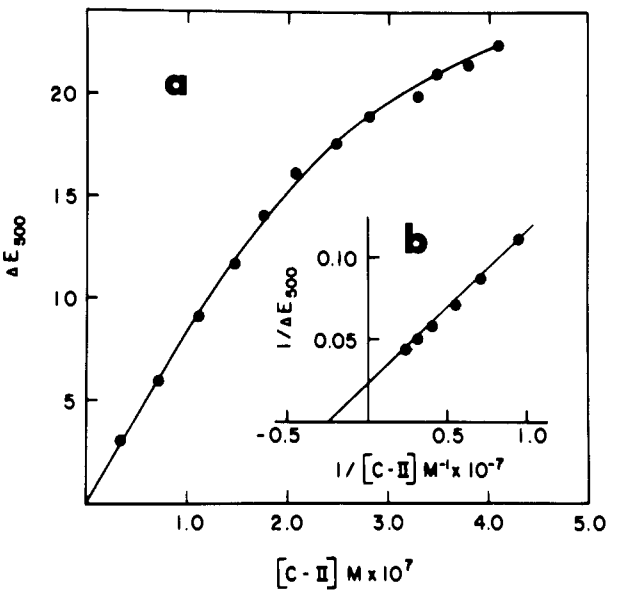


FIGURE 3: Interaction of bovine milk LPL and C-II. (a) Effect of C-II on the 500-nm fluorescent emission of dansylated bovine milk LPL (DNS-LPL) with excitation at 340 nm. (b) Double-reciprocal plots of the data. Details are described in the text.

bound at the S_p surface, $[S]_T$ is the maximum binding capacity of the surface of the S_p, K_S is the LPL–TG dissociation constant, and $\bar{F}$ and $\bar{B}$ are the fractional free and bound LPL's, respectively. The Scatchard plots for the control series and the series which contained C-II were identical; i.e., C-II had no effect on binding. From this plot, a K_S value of 7.6×10^{-8} M was calculated. Heparin (10 μg/mL), however, decreased the slope of the plot, increasing the K_S value 1.8-fold to 1.4×10^{-7} M.

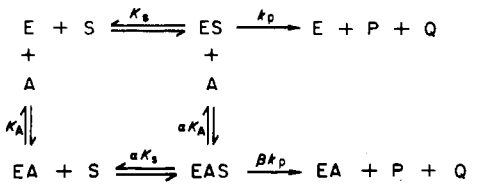
Enzyme–Activator Interactions. The result of a typical experiment (Figure 3a) shows that the fluorescence of DNS-LPL increased with increasing C-II concentrations in the solution. These results are clearly indicative of enzyme–activator interactions, and furthermore, they suggest that as a consequence of the LPL–C-II interactions, the activator induces conformational changes in the enzyme. From the double-re-

ciprocal plot of Figure 3b, an apparent dissociation constant (K_{ia}) value of 4.0×10^{-7} M was calculated.

Discussion

It was suggested (Posner, 1980) that in the LPL system like in other lipase systems (Verger & de Haas, 1977) a first step in the overall enzymatic reaction involves the interaction of the enzyme with and its binding to the pseudosubstrate surface. The second step, a classical Michaelis–Menten reaction, involves the binding of individual triacylglycerol substrate molecules at the active site which is followed by catalysis. Our binding and kinetic studies suggest that the first step in the overall reaction is not rate limiting. The enzyme added to the system in concentrations commonly employed in the assay of LPL activity was quantitatively bound to S_p in less than 1 min at 37 °C (I. Posner, unpublished experiments). Furthermore, as mentioned under Results, the shapes of the substrate and activator saturation curves were perfectly hyperbolic with no indication of a lag in the rate of catalysis, which might have been expected were the enzyme binding to the S_p surface rate limiting. It is reasonable to conclude from the above that under our experimental conditions, the binding of LPL at the S_p surface and the binding of individual triacylglycerol molecules at the active center are simultaneous events.

Judging from the kinetic data of Figure 1, it seems that the LPL system could be treated as a random, bireactant, rapid equilibrium system similar to that previously described for human milk bile salt activated lipase (Wang, 1981). In this reaction system (Segel, 1975), triacylglycerol is the substrate (S) and C-II is the activator (A), and the reaction can be shown as indicated below:



(1)

In the above scheme, E is LPL, P and Q are the alcoholic and carboxylic acid products, respectively, k_p is the rate constant for the breakdown of ES to E + P + Q, βk_p is the rate constant for the breakdown of EAS to EA + P + Q, and K_A, K_S, αK_A, and αK_S are the dissociation constants for each indicated reaction. It is implicitly assumed that the ternary complex (EAS) involves the binding of A at the activator site and S at the active site (Posner, 1980) and that a direct interaction between A and S had no effect on the kinetic pattern obtained. This assumption is based on the observations of Catapano et al. (1979) that C-II fragments lacking in lipid binding regions and which did not bind to C-II-deficient very low density lipoproteins nevertheless activated the enzyme. Accordingly

$$v = k_p[ES] + \beta k_p[EAS] \quad (2)$$

Because the alteration of either the C-II concentration or the TG concentration did not change the K_m(TG) or K_m(C-II), respectively (Figure 1a,b), and the basal LPL and LPL in the presence of a saturating activator concentration had an identical apparent K_m, we have concluded that the parameter $\alpha = 1$. Since bovine milk LPL had a V_{max} of only 5% of that observed in the presence of activator ($\beta = 19.3$), the contribution of basal activity in the observed kinetics of bovine LPL is negligible. In other words, the velocity (eq 2) can be reduced to

$$v \approx \beta k_p[EAS] \quad (3)$$

and the rate equation can be derived as follows:

$$\frac{1}{v} = \frac{1}{\beta V_{max}}\left(1 + \frac{K_A}{[A]} + \frac{K_S}{[S]} + \frac{K_A K_S}{[A][S]}\right) = \frac{1}{\beta V_{max}}\left(1 + \frac{K_A}{[A]}\right)\left(1 + \frac{K_S}{[S]}\right) \quad (4)$$

The kinetic pattern observed in Figure 1a,b which represents families of Lineweaver–Burk plots having a common convergence point at the abscissa is consistent with that of eq 4. Despite the apparent adequacy of eq 4 for describing the kinetic data, due to a possible homeomorphism (different kinetic mechanisms have the same form of kinetic equation), we have studied the dead-end inhibition effect of tricapryloylglycerol and heparin in order to exclude such a possibility. In the present study, we have demonstrated that heparin is a competitive inhibitor against both C-II and TG. For a bireactant enzyme reaction, this led to the conclusion that the enzyme reaction follows a random, rapid-equilibrium mechanism (see below). The symmetry of the rate equation and the fact that heparin was found to be a competitive inhibitor against both C-II and TG do not favor the possibility of a steady-state ordered mechanism. This view is further strengthened by the finding that both C-II and TG could interact directly with the free enzyme. The linearity of the Lineweaver–Burk plots obtained in Figure 1a,b is not compatible with the possibility of a steady-state random mechanism for the enzyme reaction (Segel, 1975). This is supported by the lack of either a substrate inhibition effect by TG or an inhibition effect by the activator C-II (Segel, 1975). However, when the reaction rates of E → ES → EAS and E → EA → EAS do not differ to a great extent, the Lineweaver–Burk plot may appear to be linear (Fromm, 1975). Based on the high-affinity binding of LPL to both C-II and TG, we do not consider the steady-state random mechanism of the enzyme reaction a likely possibility. In reaction scheme 1, when the partial reaction ES + A ⇄ EAS is omitted, and the various enzyme forms are in rapid equilibrium, the resulting reaction represents a homeomorphic form of the reaction scheme 1, because the derived rate equations for these reaction schemes are identical. From the symmetrical consideration, a homeomorphism can also be found with the omission of the partial reaction EA + S ⇄ EAS. The finding of tricapryloylglycerol (I) as a noncompetitive inhibitor against C-II indicated that EAI complex formation is possible. In other words, the EA + S ⇄ EAS reaction cannot be omitted in the reaction scheme. Because C-II has no effect on LPL binding to S_p (Figure 2), we do not consider the omission of ES + A ⇄ EAS of eq 1 to be a likely possibility. However, more conclusive evidence for such a statement will have to await the availability of a classical dead-end inhibitor of C-II for performing direct kinetic analyses to completely exclude this possibility. The LPL binding site for C-II and heparin might be different, but their binding to the enzyme is mutually exclusive; similarly, the binding of TG and heparin to the enzyme is also mutually exclusive. The effect of heparin (I) on the reaction rate can be described as indicated below:

$$\frac{1}{v} = \frac{1}{\beta V_{max}}\left\{1 + \frac{K_A}{[A]} + \frac{K_S}{[S]} + \frac{K_A K_S}{[A][S]}\left(1 + \frac{[I]}{K_i}\right)\right\} \quad (5)$$

where K_i is the dissociation constant for the LPL–heparin binary complex. Because the term $K_A K_S/([A][S])$ is related to the free enzyme concentration in the reaction mixture, this term was multipied by a factor of $(1 + [I]/K_i)$ to account for

the effect of heparin in decreasing the available enzyme concentration. The competitive inhibitory effect of heparin against TG with rat heart LPL has also been described by Ben-Zeev et al. (1981); however, the implications of the inhibitory effect of heparin on the LPL reaction mechanism were not discussed.

Based on the conclusion that the LPL-catalyzed reaction follows a rapid-equilibrium random mechanism, we have further concluded that K_m(C-II) is equivalent to the dissociation constant K_A. The K_A value of 2.5×10^{-8} M compares favorably with the value obtained by Bengtsson & Olivecrona (1979) with values of 3.8×10^{-8} and 3.4×10^{-7} M for bovine milk LPL on the basis of kinetic studies with either phosphatidylcholine-stabilized triacylglycerol emulsions or monoacylglycerol dispersions, respectively. By using DNS-LPL, we have determined a K_{ia} value of 4.0×10^{-7} M which is 1 order of magnitude greater than the value derived from our steady-state kinetic study. The difference can be attributed to the fact that dansylation may have altered the kinetic properties of the enzyme or interfered with the binding of C-II. Chung & Scanu (1977) reported a K_A value of less than 10^{-8} M for rat heart LPL. From experiments where the interactions between dansylated C-II fragments and bovine milk LPL were examined (Voyta et al., 1980; Smith et al., 1982), K_A values ranging from 0.25×10^{-6} to 5.0×10^{-6} M were calculated; the higher values of their dissociation constants might be attributed to the perturbation of the C-II structure by dansylation.

The interpretation of the K_m(TG) is more complex and deserves a more detailed discussion. The major difficulty in kinetic analyses of lipases is how to measure the concentration of an insoluble substrate, an emulsion of oil in water. Early studies by Benzonana & Desnuelle (1965) showed that, for pancreatic lipase, the apparent K_m(TG) for coarse S_p particles was higher than for fine S_p particles when $1/v$ was plotted against the reciprocals of the total triacylglycerol concentrations in the lipolysis mixture. However, no such differences in K_m(TG) were noted when $1/v$ was plotted against the reciprocal of the S_p surface area. Thus, the effective triacylglycerol concentrations are obviously a function of the total S_p surface area, and the latter is directly related to the total triacylglycerol concentration. From the relationship of K_m(TG) and K_S as shown below:

$$K_m(\text{TG}) = \frac{[\text{E}][\text{total TG}]}{[\text{ES}]} \quad (6)$$

$$K_S = \frac{[\text{E}][\text{effective TG}]}{[\text{ES}]} \quad (7)$$

[total TG]/[effective TG] = K_m(TG)/K_S = 2.5×10^{-3} M/7.6×10^{-8} M = 3.3×10^4. In other words, under our experimental conditions, 3.3×10^4 TG molecules correspond to one effective TG molecule in the lipolysis mixture. Because there is a direct relationship between [effective TG] and [total TG], it provides a theoretical basis for the application of Michaelis–Menten kinetics to lipases. However, because the apparent K_m is dependent on the procedures for the emulsification of the substrate, this kinetic parameter cannot be related from one study to another when procedures of emulsification are not the same. On the other hand, a K_S derived from Scatchard plots, which is based solely on the interactions of the enzyme with the S_p surface TG, and not with core TG, should represent a true dissociation constant.

In the binding study, enzyme concentrations were calculated from the known specific activities rather than through the use of radioactively labeled LPL. We preferred this methodological approach as have others (Shirai et al., 1981) since LPL is unstable and a considerable part of the labeled enzyme may be catalytically inactive. Heparin, which has been shown to be a competitive inhibitor of LPL (Posner & Morrison, 1979), increased K_S by interacting with the free enzyme and preventing its binding to S_p surface. C-II had no effect on the binding of the enzyme to substrate in agreement with the conclusions of both Fielding & Havel (1977) and Bengtsson & Olivecrona (1980).

It may be concluded that the above considerations validate our assumption that the kinetic model which best describes the LPL system is that of a random, bireactant, rapid-equilibrium system. Our data have indicated that C-II has no effect on the apparent K_m(TG) nor on K_S and that the only effect of the activator is to increase V_{max} by increasing the catalytic rate constant, k_p. The difference between our conclusions and those reached elsewhere (Schrecker & Greten, 1979; Matsuoka et al., 1981; Fitzharris et al., 1981) that C-II increases K_m(TG) and has little effect on V_{max} is undoubtedly due to differences in experimental design. In the cited papers, reaction velocities were determined at increasing TG concentrations and a constant ratio of C-II:TG concentrations.

The effect of C-II on the catalytic rate constant is most likely a consequence of enzyme–activator interactions. In line with studies of LPL interactions with DNS-C-II fragments (Voyta et al., 1980; Smith et al., 1982), our experimental results with DNS-LPL clearly indicate that protein–protein interactions between LPL and C-II take place as was first shown with monolayer techniques by Miller & Smith (1973). Furthermore, our own studies indicate for the first time that in interacting with LPL, the activator induces conformational change in the enzyme.

Registry No. LPL, 9004-02-8; TG, 122-32-7; heparin, 9005-49-6; tricapryloylglycerol, 538-23-8.

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Redetermination of the Rate-Limiting Step in the Activation of Factor IX by Factor XIa and by Factor VIIa/Tissue Factor. Explanation for Different Electrophoretic Radioactivity Profiles Obtained on Activation of ^{3}H- and ^{125}I-Labeled Factor IX†

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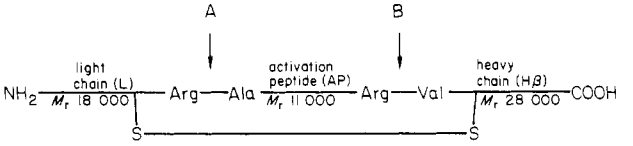
ABSTRACT: During activation of factor IX by factor XIa or by factor VIIa/tissue factor, two peptide bonds are cleaved, an Arg–Ala bond toward the NH_2 terminus and an Arg–Val bond toward the COOH terminus of the molecule. Investigators have disagreed as to the order of bond cleavages and as to which of the peptide bond cleavages constitutes the rate-limiting step. Utilizing sialyl-^{3}H human factor IX and monitoring the activation of the molecule by sodium dodecyl sulfate ($NaDodSO_4$) gel electrophoresis, we demonstrate that activation by each enzyme results initially in the accumulation of the inactive intermediate, factor IXα, which is the product formed by cleavage of the Arg–Ala bond. Subsequently, factor IXα is converted, by the cleavage of the Arg–Val bond, to factor IXaβ. Thus, the cleavage of this second peptide bond is slow and rate limiting when factor IX is activated by either factor XIa or factor VIIa. Furthermore, these data do not support a recent hypothesis, proposed for the bovine molecule, that factor XIa and factor VIIa, by differing in their rate-limiting steps, act synergistically in catalyzing factor IX activation. Steady-state kinetic analysis utilizing purified human factor VIIa in the presence of 5 mM Ca^{2+} and human brain tissue factor sufficient to saturate factor VIIa gave an apparent K_m of about 250 nM and k_{cat} of 13 min^{-1}. The comparable values for human factor IX utilizing XIa as the enzyme are K_m = 2 μM and k_{cat} = 10 min^{-1} [Bajaj, S. P. (1982) *J. Biol. Chem. 257*, 4127–4132]. In additional experiments, we compared the $NaDodSO_4$ gel electrophoretic radioactivity profiles obtained on activation of ^{3}H factor IX and on activation of ^{125}I factor IX. A radioactivity peak corresponding to the "smaller" heavy chain, Hβ, of factor IXaβ was not found upon activation of ^{3}H factor IX. In contrast, a radioactivity peak corresponding to the activation peptide released on formation of factor IXaβ was not found on activation of ^{125}I factor IX. This explains the striking differences in the radioactivity profiles observed with these two labels.

During the activation of single chain human and bovine blood coagulation factor IX by factor XIa and by factor VIIa/tissue factor two peptide bonds are cleaved to yield a smaller molecule and an activation peptide (Fujikawa et al., 1974; Østerud & Rapaport, 1977; Lindquist et al., 1978; DiScipio et al., 1978; Østerud et al., 1978; Zur & Nemerson, 1980). From the data of DiScipio et al. (1978), the following partial structure for human factor IX may be constructed:



human factor IX

Utilizing bovine proteins, Fujikawa et al. (1974) and Lindquist et al. (1978) reported that the first step in the

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