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Kinetics of Acylglycerol Sequential Hydrolysis by Human Milk Bile Salt Activated Lipase and Effect of Taurocholate as Fatty Acid Acceptor†

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ABSTRACT: The simplest reaction scheme for the conversion of trioleoylglycerol to glycerol catalyzed by human milk bile salt activated lipase can be described by consecutive first-order reactions: triacylglycerol $\xrightarrow{k_1}$ diacylglycerol $\xrightarrow{k_2}$ monoacylglycerol $\xrightarrow{k_3}$ glycerol. In these equations, k_1, k_2, and k_3 represent the pseudo-first-order rate constants for the indicated reactions. The results from this study show that although the relative ratio of k_2/k_1 or k_3/k_1 may change somewhat, depending on the reaction conditions, the enzyme has a reactivity with the order of dioleoylglycerol > trioleoylglycerol > monooleoylglycerol. The incomplete equilibration of the intermediary diacylglycerol and monoacylglycerol with the bulk of the substrate during sequential lipolysis of triacylglycerol provides a means for their efficient lipolysis and minimizes the effect of partial acylglycerol as competitive substrates for intact triacylglycerol lipolysis. Taurocholate functions both as an activator of the enzyme and also as fatty acid acceptor to relieve product inhibition. In the presence of sufficient taurocholate, bovine serum albumin is no longer required as a fatty acid acceptor for the in vitro lipolysis.

Bile salt activated lipase is found in the milk of a limited number of mammals including humans (Hernell & Olivecrona, 1974; Wang, 1981; Olivecrona & Bergtsson, 1984) and a number of primates (Freudenberg, 1966), as well as in dogs and cats, as recently found by Freed et al. (1986). Previously, we have utilized the monoacid long-chain triacylglycerols as substrates of this enzyme to show that, in the absence of the activator, the enzyme can interact only with trioctanoylglycerols and shorter chain monoacid triacylglycerols. The presence of bile salt is required for the lipolysis of longer chain triacylglycerols (Wang & Lee, 1985). The initial interaction

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of the enzyme with activator (taurocholate) leads to an enzyme conformational change which facilitates the binding of a long-chain triacylglycerol substrate to form an enzyme–bile salt–substrate ternary complex.

Because of the functional importance of the enzyme, we have continued our studies of its kinetic properties. In this paper, we describe approaches for determining the rates of sequential lipolysis of triacylglycerols to diacylglycerols (k_1), to monoacylglycerols (k_2), and finally to glycerol (k_3) by measuring the intermediary tri-, di-, and monoacylglycerol concentrations during lipolysis. In the past, kinetic studies of lipase-catalyzed reactions have generally been based on monitoring fatty acid release. Measurement of the individual acylglycerol concentrations allows a more concise description of the reaction kinetics. Moreover, previous kinetic analyses (Foster & Berman, 1981) employing multiple compartments are more difficult to conceptualize than our simpler kinetic model. The role of taurocholate in the activation of the enzyme has been evaluated. For the first time, a kinetic approach has shown that bile salt not only functions as an enzyme activator (Wang & Lee, 1985) but also, in the micellar form, can act as fatty acid acceptor for enhancement of the lipolysis rate.

Materials and Methods

Materials. Unless otherwise stated, all chemicals were purchased from Sigma Chemical Co. Tri-, di-, and monoacylglycerols were supplied by Nu Chek Prep, Inc. The purification of human milk bile salt activated lipase was performed by affinity chromatography on cholate–Sepharose and heparin–Sepharose as previously described (Wang & Johnson, 1983).

Protein Analysis. The protein content of the enzyme preparations was determined by a modification of Lowry's procedure (Wang & Smith, 1975).

Enzyme Activity. The esterase activity of bile salt activated lipase was assayed with *p*-nitrophenyl acetate substrate. The substrate *p*-nitrophenyl acetate (50 μmol/mL) in acetonitrile was diluted 5-fold with distilled water prior to use. The acetonitrile concentration in the assay was 2% (v/v). The esterase activity was assayed at 25 °C in a mixture containing 100 mM sodium phosphate (pH 7.5), 1 mM *p*-nitrophenyl acetate, and 2 mM taurocholate with a final volume of 1 mL. The rate of *p*-nitrophenol production was determined by following the absorbance change at 418 nm using a Beckman Model 25 spectrophotometer equipped with a recorder and a temperature-control unit. The molar extinction coefficient at 418 nm of *p*-nitrophenol (pH 7.5) is 11 500. One unit of the enzyme is defined as the catalytic activity leading to the hydrolysis of 1 μmol of the substrate per minute under these assay conditions. The purified enzyme has a specific activity in the range of 50–60 units/mg. The symbol U[1] is used as a concentration unit; viz., U = units of enzyme per milliliter.

Lipolysis of Acylglycerols. The assay mixture of 10-mL final volume contained an appropriate amount of the enzyme, acylglycerols, bovine serum albumin (60 mg/mL), and taurocholate as described in the figure legends and tables. The lipolysis was performed in 50 mM NH_4OH buffer at pH 8.0. The acylglycerol stock solutions were prepared as 3-fold more concentrated solutions. The substrate was emulsified with Triton X-100 as described by Schotz et al. (1970).

After incubation at 37 °C, the enzyme reaction was terminated at various time intervals by transferring 0.25-mL aliquots, in duplicate, to tubes containing 4 mL of *n*-heptane/2-propanol (3:7 v/v) which also contained 50 μg of cholesterol butyrate as internal standard. Each heptane/2-propanol extract was centrifuged to remove the protein precipitate. The supernatant was transferred to a 25-mL round-bottom flask and evaporated to dryness. The sample was redissolved in 5 mL of acetic anhydride/pyridine (1:1 v/v), incubated at 100 °C in a sand bath for 10 min, and then immediately placed on ice. The reagent was removed by evaporation on a rotary evaporator under vacuum at 30 °C. The sample then was redissolved in 4 mL of *n*-heptane/2-propanol (3:7 v/v) and acidified with 5.0 mL of 0.033 N H_2SO_4. After the solution was mixed for 30 s, the upper phase, which contained the triacylglycerol, acetylated partial acylglycerols, and the internal standard, was transferred to a 3-mL conical tube. After evaporation of the solvent under nitrogen, the residue was redissolved in 100 μL of *n*-hexane. Two-microliter aliquots were injected into the gas–liquid chromatograph (GLC).

The GLC analyses were performed using a Varian 3700 gas chromatograph equipped with a series 8000 autosampler and a SpectraPhysics SP4270 integrator. The separation was performed using 3% OV-1 on 100/120 Supelcoport (Supelco, Inc.) packed on a 50-cm glass column with inner diameter of 2 mm. The initial column temperature was 180 °C with an increase of temperature of 15 °C min^{-1}. When the temperature reached 349 °C, the column was held at this temperature for 7 min. This completed one cycle of the injection. Nitrogen was used as the carrier gas at a flow rate of 25 mL min^{-1}. A flame ionization detector was utilized for analysis of the effluents. Under these chromatographic conditions, the retention times for monooleyol-, dioleoyl-, and trioleoylglycerol were 3.67, 8.87, and 14.55 min, respectively. The positional isomers were not differentiated in these analyses. The retention time for the internal standard, cholesterol butyrate, was 5.95 min.

Analysis of Data. The conversion of trioleoylglycerol to glycerol may be described by a sequence of irreversible pseudo-first-order steps:

$$\mathrm{TG} \xrightarrow{k_1} \mathrm{DG} \xrightarrow{k_2} \mathrm{MG} \xrightarrow{k_3} \text{glycerol} \qquad (1)$$

In this equation, TG, DG, and MG are triacylglycerol, diacylglycerol, and monoacylglycerol, respectively. The pseudo-first-order rate constants k_1, k_2, and k_3 correspond to bile salt activated lipase lipolysis of TG, DG, and MG, respectively. Integration of the rate equations for the reactions shown in eq 1 allows expression of [TG], [DG], and [MG] as a function of time as shown in eq 2–4. In these equations, $[TG]_0$ represents the initial triacylglycerol concentration. The data obtained from analysis of the reaction mixture as a function of time were fit to eq 2–4. The observed $[TG]_0$ was treated as a single data point, and the best value of $[TG]_0$ was determined in the nonlinear least-squares procedure. In all experiments, observed and calculated values of $[TG]_0$ agreed to within 10%. A sequential treatment of the data was employed. The $[TG]_0$ and rate constant k_1 were determined by fitting the [TG] data to eq 2. Then with $[TG]_0$ and k_1 fixed,

$$[\mathrm{TG}] = [\mathrm{TG}]_0 \exp(-k_1 t) \qquad (2)$$

$$[\mathrm{DG}] = \frac{k_1[\mathrm{TG}]_0}{k_2 - k_1}[\exp(-k_1 t) - \exp(-k_2 t)] \qquad (3)$$

$$[\mathrm{MG}] = \frac{k_1 k_2 [\mathrm{TG}]_0}{(k_1 - k_2)(k_2 - k_3)(k_3 - k_1)}[(k_3 - k_2)\exp(-k_1 t) + (k_1 - k_3)\exp(-k_2 t) + (k_2 - k_1)\exp(-k_3 t)] \qquad (4)$$

[1] Abbreviations: BSA, bovine serum albumin; GLC, gas–liquid chromatography; U, concentration unit, viz., units of enzyme per milliliter.

Table I: Pseudo-First-Order Rate Constants for the Sequential Hydrolysis of Trioleoylglycerol by Human Milk Bile Salt Activated Lipase[a]

[enzyme] (U)	[substrate] (mM)	k_1 ($\times 10^{-3}$ min^{-1})	k_2 ($\times 10^{-3}$ min^{-1})	k_3 ($\times 10^{-3}$ min^{-1})	k_2/k_1
0.025 (1)	1	20 ± 4 (1.0)	140 ± 40 (1.0)	31 ± 20 (1.0)	8.1 ± 2
0.05 (2)	1	45 ± 3 (2.2 ± 0.5)	280 ± 30 (2.0 ± 0.6)	24 ± 10 (0.8 ± 0.5)	8.0 ± 1
0.1 (4)	1	75 ± 8 (3.7 ± 0.9)	450 ± 30 (3.1 ± 0.9)	23 ± 5 (0.7 ± 0.4)	6.3 ± 0.7
0.05 (2)	0.5	52 ± 6 (2.6 ± 0.6)	240 ± 30 (1.7 ± 0.5)	16 ± 3 (0.5 ± 0.3)	7.0 ± 1
0.1 (4)	0.5	77 ± 4 (3.8 ± 0.8)	340 ± 50 (2.4 ± 0.7)	22 ± 9 (0.7 ± 0.5)	4.8 ± 0.8

[a] Taurocholate concentration in all experiments was 30 mM. The results are based on eq 2–4. The values of k_1, k_2, and k_3 are derived from four individual experiments and expressed as mean ± SD. The values in parentheses are the ratos of k_1, k_2, and k_3 at various enzyme concentrations with $k_1 = 1.0$, $k_2 = 1.0$, and $k_3 = 1.0$ at the enzyme concentration of 0.025 U. Errors in the ratio are calculated by propagating the errors (Wilson, 1952).

k_2 was found by fitting the [DG] data to eq 3, and similarly, with $[TG]_0$, k_1, and k_2 fixed, k_3 was determined from the fit of the [MG] data to eq 4.

Nonlinear regression analyses used for curve fitting were performed with the SAS programs from the SAS Institute, Inc., Cary, NC. The derivative-free algorithm as described by Ralston and Jennrich (1978) was employed for the nonlinear least-squares curve fitting. Statistically significant difference was evaluated with the Student's t test.

Results

Lipolysis of Trioleoylglycerol. The relative rates of the individual steps in the degradation of triacylglycerol to glycerol have been studied by determining the intermediary tri-, di-, and monoacylglycerol concentrations. Michaelis–Menten reaction kinetics under the condition of $K_m \gg [S] \gg [E]$ predict pseudo-first-order kinetics with rate constants proportional to enzyme concentration and independent of substrate concentration. In a previous study (Wang & Kloer, 1983), we have found that the apparent K_m of the human milk bile salt activated lipase with trioleoylglycerol substrate is 8.3 mM; therefore, the present studies employ an initial trioleoylglycerol concentration equal to or less than 1 mM. The results of the sequential hydrolysis of trioleoylglycerol at different enzyme concentrations and the corresponding rate constants are shown in Figure 1 and Table I. The good agreement between the experimental data and calculated values (Figure 1) based on eq 2–4 indicates that the use of the pseudo-first-order kinetic model (eq 1) is appropriate. When data from the 1 mM trioleoylglycerol in Table I are used, the linear regression of k_1 versus enzyme concentration substantiates the proportionality of these two variables ($R = 0.967$, $p < 0.001$). The best k_1 value is 0.71 min^{-1}·U^{-1}. Furthermore, despite a 2-fold change in concentration of the substrate (1 vs 0.5 mM), the experimentally determined k_1 values at the two enzyme concentrations of 0.05 and 0.1 U were similar (0.045 vs 0.052 min^{-1} and 0.075 vs 0.077 min^{-1}).

Because of the high reaction rate of the enzyme with the diacylglycerol substrate, there is only minimal accumulation of diacylglycerol during the course of the lipolytic reaction. At an initial trioleoylglycerol concentration of 1 mM, the k_2 value is also proportional to enzyme concentration ($R = 0.965$, $p < 0.001$), and the averaged k_2 value from the linear regression is 4.0 ± 0.3 min^{-1}·U^{-1}. However, in contrast to the k_1 value, which is insensitive to substrate concentration, the decrease of substrate concentration from 1 to 0.5 mM led to a condition where k_2 is no longer proportional to enzyme concentration (0.24 min^{-1} at 0.05 U vs 0.34 min^{-1} at 0.1 U).

As is seen in Table I, the k_3 values were not significantly different from each other regardless of enzyme or substrate concentration. Thus, it was concluded that the acyl migration of *sn*-2-monoacyl- to *rac*-1-monoacylglycerol (Mattson & Volpenhein, 1962) probably represents a rate-limiting chemical step for the subsequent enzymic hydrolysis of the primary ester bond of the monoacylglycerol. The averaged k_3 value of 0.022 ± 0.005 min^{-1} corresponds to an acyl migration rate with a half-life of 31.5 min.

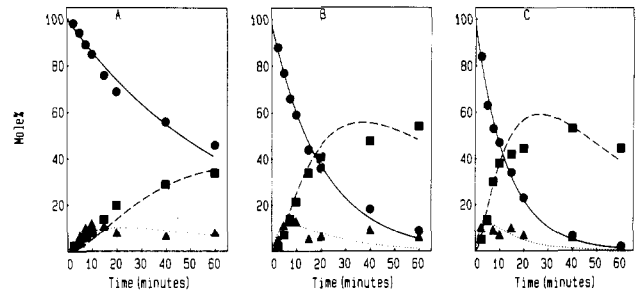

FIGURE 1: Lipolysis of trioleoylglycerol by human milk bile salt activated lipase. The experimentally determined triacyl-, diacyl-, and monoacylglycerol concentrations are denoted as (●), (■), and (▲), respectively. The curves (—), (···), and (- - -) represent the triacyl-, diacyl-, and monoacylglycerol-calculated concentrations, respectively, based on eq 2–4. Bovine serum albumin (60 mg/mL) and taurocholate (30 mM) were incubated in each experiment. Initial trioleoylglycerol concentration was 1 mM. (A), (B), and (C) correspond to experiments containing 0.025, 0.05, and 0.1 U of enzyme, respectively. Calculated rate constants are listed in Table I.

Measurement of k_2 and k_3 from the Sequential Lipolysis of Dioleoylglycerol. In addition to the measurement of k_2 and k_3 from sequential lipolysis of triacylglycerol, these constants can also be measured by using diacylglycerol substrate. In this case, the appropriate integrated rate equations are

$$[DG] = [DG]_0 \exp(-k_2 t) \quad (5)$$

$$[MG] = \frac{k_2[DG]_0}{k_3 - k_2}[\exp(-k_2 t) - \exp(-k_3 t)] \quad (6)$$

where $[DG]_0$ is the initial dioleoylglycerol concentration. As seen in Table II, the k_2 values for *rac*-1,2- and *sn*-1,3-dioleoylglycerol are almost identical and also are proportional to enzyme concentration. The averaged k_2 value is 1.65 min^{-1}·U^{-1}. This k_2 value, obtained from the hydrolysis of dioleoylglycerol, is significantly lower than that derived from the sequential hydrolysis of trioleoylglycerol (4.0 min^{-1}·U^{-1}). For both substrates, k_3 values, not adjusted for enzyme concentration, are greater at 0.1 U than at 0.05 U of enzyme but are not proportional to enzyme concentration. At both enzyme concentrations of 0.05 and 0.1 U, the k_3 values determined from the hydrolysis of *sn*-1,3-dioleoylglycerol are slightly higher than those found with *rac*-1,2-dioleoylglycerol; however, these differences did not reach the significance level. Note that all k_3 values for diglycerol substrate (Table II) are less than the rate-limiting acyl migration rate found with triacylglycerol substrate (Table I).

Direct Measurement of k_3. In addition to the derivation of k_3 from the sequential hydrolysis of tri- or dioleoylglycerol, the k_3 values were directly determined with *rac*-1-monoacylglycerol as substrates (Table III). In this case, the appropriate integrated rate equation is

$$[MG] = [MG]_0 \exp(-k_3 t) \quad (7)$$

At the enzyme concentration of 0.6 U, the apparent k_3 value

Table II: Pseudo-First-Order Rate Constants for the Sequential Hydrolysis of Dioleoylglycerol by Human Milk Bile Salt Activated Lipase[a]

substrate	[enzyme] (U)	no. of expt	k_2 ($\times 10^{-3}$ min^{-1})	k_3 ($\times 10^{-3}$ min^{-1})	k_2/k_1
rac-1,2-dioleoylglycerol	0.05	5	90 ± 10 (1.0)	12 ± 1 (1.0)	2.5 ± 0.4
	0.1	4	170 ± 10 (1.9 ± 0.3)	19 ± 2 (1.6 ± 0.4)	2.4 ± 0.2
sn-1,3-dioleoylglycerol	0.05	3	95 ± 6 (1.0)	14 ± 2 (1.0)	2.7 ± 0.3
	0.1	3	180 ± 10 (1.9 ± 0.3)	22 ± 2 (1.6 ± 0.4)	2.5 ± 0.3

[a] The results are based on eq 5 and 6 and are expressed as mean ± SD. All assay mixtures contained 60 mg/mL bovine serum albumin and 30 mM taurocholate. In these experiments, the initial substrate concentration was 1 mM. The values in parentheses are the ratios of k_2 to k_3 at different enzyme concentrations with $k_2 = 1.0$ and $k_3 = 1.0$ at the enzyme concentration of 0.05 U. Errors in the ratios are calculated by propagating the errors in the rate constants (Wilson, 1952). The k_1 value was derived from Table I.

Table III: Pseudo-First-Order Rate Constant for the Hydrolysis of Monoacylglycerol[a]

substrate	no. of expt	[taurocholate] (mM)	[enzyme] (U)	k_3 ($\times 10^{-3}$ min^{-1})
rac-1-monooleoylglycerol (0.8 mM)	8	0	0.6	26 ± 5
rac-1-monooleoylglycerol (0.8 mM)	4	30	0.6	57 ± 5
rac-1-monooleoylglycerol (0.8 mM) + oleate (2 mM)	4	30	0.6	47 ± 9
monomyristoylglycerol (0.8 mm)	4	30	1.0	17 ± 6

[a] Results are based on eq 7. The data are expressed as mean ± SD. All mixtures contained bovine serum albumin (60 mg/mL).

(0.057 ± 0.005 min^{-1}) was greater than that derived from the third step of the sequential hydrolysis of trioleoylglycerol (0.022 ± 0.004 min^{-1}). This is apparently due to the fact that the substrate can be attacked directly by the enzyme without the necessity of acyl migration.

In the absence of the activator (taurocholate), we have found low but detectable basal activity for monoacylglycerol substrate. This is in contrast to tri- and dioleoylglycerol, where no detectable basal activity was found (data not shown) even at a high enzyme concentration (50 U).

In this study, we have also utilized monomyristoylglycerol as substrate. The results indicate that this monoacylglycerol had an apparent reactivity with bile salt activated lipase approximately one-third that found for monooleoylglycerol.

In the sequential lipolysis of triacylglycerol to glycerol, the hydrolysis of the monoacylglycerol ester bonds occurred during the late phase of the reaction. For this reason, the effect of the presence of the lipolytic products on the monoacylglycerol lipolysis rate has been evaluated. The studies using monooleoylglycerol, and in the absence and presence of added 2 mM oleate, indicate a slight but insignificant decrease of k_3 (0.057 ± 0.005 vs 0.047 ± 0.009 min^{-1}), which suggests an adequate fatty acid acceptor capacity in our assay mixture.

Effect of Taurocholate and BSA. Because BSA has binding affinity to both fatty acids and bile salts, BSA might function both in the enhancement and in the inhibition of the lipolytic reaction. The former is due to the well-known effect of BSA in binding fatty acids to relieve product inhibition on the lipolytic reaction. The latter is due to its high-affinity binding to bile salts, thereby reducing the effective bile salt concentration available for activation of the enzyme. For a better understanding of the role of BSA and bile salt, the effect of taurocholate concentration both in the absence and in the presence of BSA has been examined.

As shown in Figure 2, at a fixed enzyme concentration (0.05 U) and both in the absence and in the presence of BSA and in the presence of 20 mM taurocholate, the degradation of trioleoylglycerol follows an apparent biphasic reaction. The retardation of the lipolytic reaction during the later stages of the reaction is probably due to the accumulation of fatty acid.

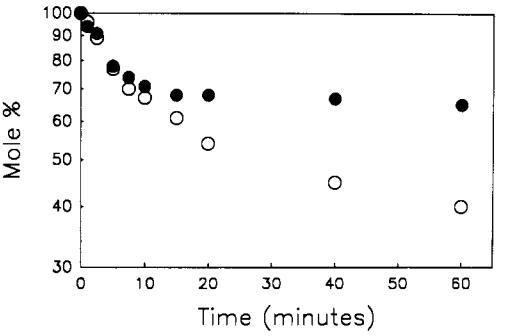

FIGURE 2: Semilogarithmic plot of trioleoylglycerol hydrolysis in the presence of 20 mM taurocholate. Enzyme and substrate concentrations are the same as in Table IV. Open circles are from experiments with no bovine serum albumin, and closed circles are from experiments with 60 mg/mL bovine serum albumin.

Table IV: Effect of Taurocholate and Bovine Serum Albumin on the Pseudo-First-Order Rates for the Sequential Hydrolysis of Trioleoylglycerol[a]

[taurocholate] (mM)	[bovine serum albumin] (mg/mL)	no. of expt	k_1 ($\times 10^{-3}$ min^{-1})	k_2 ($\times 10^{-3}$ min^{-1})	k_3 ($\times 10^{-3}$ min^{-1})
30	0	4	29 ± 3	140 ± 30	18 ± 3
30	60	4	45 ± 3	280 ± 40	24 ± 10
50	0	3	62 ± 6	270 ± 30	23 ± 1
50	60	3	59 ± 4	240 ± 30	22 ± 2
75	0	3	91 ± 20	220 ± 60	6 ± 2
75	60	3	97 ± 3	260 ± 10	10 ± 2
100	0	3	61 ± 2	135 ± 2	16 ± 2
100	60	3	56 ± 3	139 ± 3	11 ± 3

[a] The results are based on eq 2–4. All experiments were performed with the enzyme concentration of 0.05 U and 1 mM trioleoylglycerol. For the sake of comparison, the data using 30 mM taurocholate and 60 mg/mL BSA as shown in Table I are also included in this table.

Consequently, taurocholate may well be serving as fatty acid acceptor. Because of the biphasic behavior of the trioleoylglycerol degradation, the time courses of di- and monoacylglycerol concentrations were not analyzed in experiments with low taurocholate concentration. At 20 mM taurocholate concentration, the degradation of trioleoylglycerol is noticeably slower in the presence of BSA than in its absence; this might relate to a diminished amount of taurocholate available to serve as enzyme activator and as primary fatty acid acceptor when BSA is present to interact with the taurocholate.

We have further increased the taurocholate concentration in the lipolysis mixture from 30 to 100 mM in the presence and absence of BSA at a fixed enzyme concentration (0.05 U). In these ranges of taurocholate concentrations, semilogarithmic plots of trioleoylglycerol concentration versus time were linear and satisfied the criterion of having the calculated and observed initial triacylglycerol concentrations agree to within 10%. In contrast to the finding of lower reactivity of the enzyme at 20 mM taurocholate in the presence of BSA than in the corresponding lipolytic reaction in the absence of BSA, we found the k_1 value at 30 mM taurocholate was higher

when the reaction was proceeding in the presence (0.045 ± 0.003 min^{-1}) than in the absence of BSA (0.029 ± 0.003 min^{-1}) (Table IV). This result indicates BSA is important as a fatty acid acceptor at this taurocholate concentration. Further increase of taurocholate concentration from 50 to 100 mM led to the k_1 value becoming insensitive to the presence of BSA. Consequently, at sufficiently high concentration, taurocholate is capable of acting as fatty acid acceptor without the requirement of the presence of BSA. The k_1 value reaches a maximum at 75 mM, and the further increase of taurocholate concentration to 100 mM results in the reduction of the k_1 values. The increase in taurocholate concentration to 100 mM could lead to a partial inactivation of the enzyme. With *p*-nitrophenyl acetate substrate, a 35% inhibition of the enzyme is observed when 100 mM taurocholate is included in the usual assay used for enzyme activity.

In a manner similar to that found for k_1 values, at 30 mM taurocholate, the k_2 value is also higher in the presence than in the absence of BSA. The further increase of taurocholate concentration also led to the insensitivity of k_2 values to the presence or absence of BSA.

At 30–100 mM taurocholate, k_3 values are insensitive to the presence or absence of BSA. The lowest k_3 value occurs at 75 mM taurocholate which is the taurocholate concentration where the highest k_1 value was found.

Discussion

In general, Michaelis–Menten kinetics apply only to systems of homogeneous catalysis. Usually, insoluble, emulsified substrates require a more complicated description. Since the simpler scheme seems adequate for the present system, we deduce that the analytical and effective substrate concentrations are proportional to one another. Several additional microscopic factors could alter the reactivity of the enzyme for complete lipolysis of all three ester bonds of intact triacylglycerol. Readily identifiable among these factors are the following: (1) The bile salt in the assay mixture may have a different capability to emulsify each partial acylglycerol. (2) Product inhibition by fatty acids may increase during the lipolytic reaction. (3) Acyl migration can occur during lipolysis. (4) Ester bonds in the three positions of the glycerol backbone may have different reactivity with the enzyme. (5) Differences in polarity of the acylglycerols, mono- > di- > triacylglycerol, could affect availability of the substrate to the enzyme (substrate surface activity) in this complex system. Nevertheless, it is apparent from our kinetic study that the determined k_1, k_2, and k_3 values represent the final additive macroscopic expression of these microscopic kinetic effects.

As shown in Figure 1, the fidelity of the trioleoylglycerol degradation in following the first-order reaction kinetics indicates minimal interference of the partial acylglycerols and fatty acids in the cleavage of the three ester bonds of triacylglycerol. Since k_1 is proportional to enzyme concentration and is independent of the substrate concentration, the requirement of $K_m \gg [S] \gg [E]$ for observing pseudo-first-order kinetics of the sequential reaction apparently has been fulfilled. The present kinetic scheme does not require explicit specification of the substrate involvement in interfacial phenomena or of the effect of supramolecular aggregates on the enzyme. However, for a more detailed understanding of the mechanism, it also may be necessary to consider interfacial phenomena. For example, during the lipolysis reaction, the degradation of triacylglycerol could result either in a reduced number of emulsion particles or in a diminished number of triacylglycerol molecules per particle. The analytical determination of intermediary acylglycerol concentration does not distinguish between these possibilities. To understand the mechanism in detail, further studies defining the interfacial aspects of the enzyme–substrate interactions would be necessary (Brockman, 1984).

The k_2 values are much more variable under different lipolytic conditions. During the sequential hydrolysis of trioleoylglycerol, k_2 is proportional to enzyme concentration and independent of substrate concentration only for enzyme concentrations of 0.05 U or less and for 1 mM initial substrate concentrations. When these requirements are met, the ratio k_2/k_1 is 8. As seen in Table I, if [S] is decreased or [E] is increased beyond these limits, the k_2/k_1 ratio is lower. Probably in the presence of an increased turnover, [DG] is not large enough compared to [E] to satisfy the requirement of $[S] \gg [E]$.

When *sn*-1,3- and *rac*-1,2-dioleoylglycerol were used as substrates, the proportionality to [E] and independence of [S] were preserved for the k_2 values. Moreover, surprisingly, these substrates had a similar reactivity with the enzyme (Table II). Thus, as long as there is one primary ester bond in the dioleoylglycerol, the reactivity of the substrate appears not to be affected by the position of the other fatty acyl chain on the glycerol backbone. The previously observed 15% acyl migration of 1,2-diacylglycerol to 1,3-diacylglycerol could contribute in part to the similar reactivity of the dioleoylglycerol isomers with the enzyme (Wang et al., 1983). The similar reactivity of the enzyme with *sn*-1,3- and *rac*-1,2-dioleoylglycerol is in contrast with that found for human milk lipoprotein lipase. In our previous study (Wang et al., 1985), human milk lipoprotein lipase has a 3-fold higher reactivity with the former substrate than with the latter substrate.

In contrast with the finding of proportionality of k_1 or k_2 values to enzyme concentration, we have found that the k_3 values were insensitive to the enzyme concentration (Table I). One of the most probable causes of the insensitivity of the k_3 value to enzyme concentration is the requirement for the chemical reaction involving the acyl migration in converting the *sn*-2 to the *rac*-1 ester of the monoacylglycerol. An average k_3 value as determined from Table I is 0.022 ± 0.005 min^{-1}, with a corresponding half-life of 31.5 min. This half-life value is much longer than that found for acyl migration of 2-monooleoylglycerol (10 min) at pH 8.0 and 40 °C (Mattson & Volpenhein, 1962). Because k_3 is obtained from sequential lipolysis of trioleoylglycerol at a nonequilibrium state, the value also reflects in part the active-site influences by the enzyme. The low k_3 values shown in Table I could be an indication that the microenvironment of the enzyme active site is unfavorable for the acyl migration so that the monoacylglycerol may have to reequilibrate with the bulk environment for acyl migration followed by enzyme-catalyzed lipolysis. Previously, we have found that human milk lipoprotein lipase also exhibits a rate-limiting step by acyl migration in the hydrolysis of the monoacylglycerol ester bond during sequential lipolysis of triacylglycerol. However, the fact that the k_3 value is higher than that found for human milk bile salt activated lipase probably indicates the active-site microenvironment of human milk lipoprotein lipase may be more favorable for the acyl migration.

When the k_3 value is directly measured from the lipolysis of *rac*-1-monooleoylglycerol, the monooleoylglycerol in the bulk of the reaction mixture had a low reactivity. Thus, the lipolysis of monooleoylglycerol was studied at a high concentration (0.6 U). In the absence of activator (taurocholate), the human milk bile salt activated lipase did not exhibit any detectable activity with tri- or dioleoylglycerol, even at a high enzyme concen-

tration. However, a low, but detectable, activity of the enzyme with monooleoylglycerol as substrate in the absence in the absence of the activator is found. Thus, on the basis of the nomenclature of Segel (1975), taurocholate would represent an essential activator with tri- or dioleoylglycerol as substrate while it represents a nonessential activator with monooleoylglycerol as substrate in human milk bile salt activated lipase-catalyzed reaction. The apparent k_3 value as shown in Table III of 0.057 min^{-1} is higher than the 0.022 min^{-1} of the presumed acyl migration rate. The hydrolysis of the *rac*-1 ester bond is not and should not be restricted with the rate-limiting acyl migration step. However, this k_3 value is much smaller if it is adjusted for the enzyme concentration comparable to that derived from the sequential lipolysis of either tri- or dioleoylglycerol. Thus, monooleoylglycerol generated from the sequential lipolysis of trioleoylglycerol might partially be degraded directly without the prior reequilibration with the bulk of the substrate in the reaction mixture if acyl migration were not rate limiting.

Human milk bile salt activated lipase was found previously to attack the secondary esters of trioleoylglycerol (Wang et al., 1983) and 2,3-dioleate butanediol (Jensen et al., 1985). The absence of direct reaction with *sn*-2-monoleoylglycerol, as shown in this work, can be explained by the lack of an inductive effect (Brockerhoff & Jensen, 1974) for enhancing the reactivity of the secondary esters by the adjacent primary esters. O'Connor and Wallace (1985) have shown the lack of an inductive effect in the human milk bile salt activated lipase-catalyzed esterolytic reaction. The absence of direct reaction could also be related to the acyl chain length of the ester (Wang et al., 1983) and the resulting difference in the rate-limiting step of the lipolysis of secondary esters.

After validation of the kinetic scheme as described in eq 1, the effect of taurocholate and BSA on the lipolytic process was studied. One of the most striking findings was the use of low taurocholate concentration (e.g., 20 mM) in the lipolysis mixture is the apparent retardation in the trioleoylglycerol hydrolysis during the late phase of the enzyme reaction. Since this slow down in the lipolysis of the intact triacylglycerol occurs late in the reaction, an obvious explanation for the retardation is product inhibition. Because the increase of taurocholate concentration to 30 mM and above abolishes the biphasic kinetic behavior, taurocholate probably behaves as a fatty acid acceptor in the lipolytic reaction. It has been known for more than a decade that bile salt activated lipase activity shows an unusual sigmoidal response to the increase in taurocholate concentration during the hydrolysis of long-chain triacylglycerol. While part of the effect may be explained by the role of the micellar bile salts emulsifying the substrate (Wang & Lee, 1985; O'Connor, 1986), the more important explanation for this kinetic behavior may be that bile salt micelles could form mixed micelles and thereby drive forward the lipolytic reaction. This finding could also reconcile the different findings regarding taurocholate's role in activating the enzyme. With a water-soluble substrate such as *p*-nitrophenyl acetate, the enzyme requires only a low concentration of taurocholate. The enzyme–taurocholate dissociation constant is 0.37 mM, which is well below the critical micellar concentration of this bile salt (Hofmann & Small, 1967). On the other hand, as revealed from this study, the enzyme requires taurocholate at a concentration above its critical micellar concentration for the hydrolysis of long-chain triacylglycerol. Thus, taurocholate has a dual role in driving the enzyme reaction both in the direct activation of the enzyme and in behaving as a fatty acid acceptor.

At a 20 mM taurocholate concentration, BSA actually reduces the lipolytic rate (Figure 2). A possible explanation for this phenomenon is because BSA has a binding affinity for bile salt, it actually reduces the effective taurocholate concentration (Figure 2) and consequently reduces the reactivity of the enzyme. A further increase of taurocholate concentration to 30 mM provides additional fatty acid acceptor capacity, and the lipolysis follows pseudo-first-order kinetics in the presence or absence of BSA. Moreover, the extra fatty acid binding capacity provided by BSA becomes insignificant upon further increase of the taurocholate concentration in the range tested between 50 and 100 mM (Table III).

Under conditions where the pseudo-first-order kinetics are maintained, the k_2 and k_3 values have been determined at various taurocholate concentrations. The lower k_1 value at 100 mM taurocholate than at 75 mM taurocholate suggests that the extremely high bile salt concentration may lead to a partial inactivation of the enzyme. For this reason, we only discuss the bile salt effect on the acylglycerol reactivity in the range of 30–75 mM taurocholate. The increase of k_1 appears proportional to taurocholate concentration for the 30, 50, and 75 mM taurocholate experiments. In the same experiments, k_2 is relatively constant, and k_3 is minimal at 75 mM taurocholate. Several mechanisms for the influence of bile salt concentration on lipolytic rate have been considered. Emulsification of the triacylglycerol substrate to enhance its binding with the enzyme would be consistent with an increasing k_1 but a constant k_2. However, Table I shows insensitivity to alteration of the ratio of substrate to taurocholate concentration. Taurocholate itself might alter the conformation of the enzyme (activation). Such enhancement of k_{cat} for the triacylglycerol substrate but not the diacylglycerol substrate seems unlikely, and the bile salt concentration is far above that required for activation when *p*-nitrophenyl acetate is substrate. Taurocholate might accelerate the efflux of free fatty acid from the enzyme's active site as suggested by the apparent proportionality of k_1 to taurocholate concentration. Since in the sequential lipolysis of triacylglycerol, the intermediary partial acylglycerol is partially in equilibrium with the bulk substrate, an increase in k_1 would increase the rate of partial acylglycerol formation and subsequently enhance a more complete equilibration of the newly formed partial acylglycerol with the bulk solvent. The result of this increase in partitioning of equilibrium over nonequilibrium lipolysis of partial acylglycerols would be a decrease in the k_2/k_1 and k_3/k_1 ratios as observed in Table IV.

In conclusion, the present studies indicate the preference of human milk bile salt activated lipase for diacylglycerol substrate and demonstrate the suitability of a simple, consecutive first-order kinetic scheme for the lipolysis of triacylglycerol. Although human milk bile salt activated lipase can attack directly the *sn*-2 ester bond of triacylglycerol (Wang et al., 1983), the enzyme cannot attack the *sn*-2 ester of long-chain monoacylglycerol without the prior acyl migration. Finally, micellar taurocholate can behave as fatty acid acceptor, and at high concentration of taurocholate, bovine serum albumin is not necessary for the complete lipolysis of trioleoylglycerol under our assay conditions.

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^{1}H Nuclear Magnetic Resonance Studies of the Conformation of an ATP Analogue at the Active Site of Na,K-ATPase from Kidney Medulla†

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ABSTRACT: ^{1}H nuclear magnetic relaxation measurements have been used to determine the three-dimensional conformation of an ATP analogue, $Co(NH_3)_4ATP$, at the active site of sheep kidney Na,K-ATPase. Previous studies have shown that $Co(NH_3)_4ATP$ is a competitive inhibitor with respect to MnATP for the Na,K-ATPase [Klevickis, C., & Grisham, C. M. (1982) *Biochemistry 21*, 6979; Gantzer, M. L., Klevickis, C., & Grisham, C. M. (1982) *Biochemistry 21*, 4083] and that Mn^{2+} bound to a single, high-affinity site on the ATPase can be an effective paramagnetic probe for nuclear relaxation studies of the Na,K-ATPase [O'Connor, S. E., & Grisham, C. M. (1979) *Biochemistry 18*, 2315]. From the paramagnetic effect of Mn^{2+} bound to the ATPase on the longitudinal relaxation rates of the protons of $Co(NH_3)_4ATP$ at the substrate site (at 300 and 361 MHz), Mn-H distances to seven protons on the bound nucleotide were determined. Taken together with previous ^{31}P nuclear relaxation data, these measurements are consistent with a single nucleotide conformation at the active site. The nucleotide adopts a bent configuration, in which the triphosphate chain lies nearly parallel to the adenine moiety. The glycosidic torsion angle is 35°, and the conformation of the ribose ring is slightly N-type ($C_{2'}$-exo, $C_{3'}$-endo). The δ and γ torsional angles in this conformation are 100° and 178°, respectively. The bound Mn^{2+} lies above and in the plane of the adenine ring. The distances from Mn^{2+} to N_6 and N_7 are too large for first coordination sphere complexes but are appropriate for second-sphere complexes involving, for example, intervening hydrogen-bonded water molecules. The NMR data also indicate that the structure of the bound ATP analogue is independent of the conformational state of the enzyme.

Sodium and potassium ion activated adenosinetriphosphatase, or Na,K-ATPase[1] (EC 3.6.1.3, ATP phosphohydrolase), is an integral membrane protein that couples the hydrolysis of ATP to the vectorial transport of sodium and potassium ions across the plasma membrane. It is important to determine the mechanism of energy coupling in this system. Our approach to this problem has been to attempt to determine

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[1] Abbreviations: Na,K-ATPase, sodium and potassium ion activated adenosinetriphosphatase; CoATP, β,γ-bidentate $Co(NH_3)_4ATP$; TMA, tetramethylammonium; Tes, 2-[[tris(hydroxymethyl)methyl]amino]-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate.

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