Acyl-chain specificity of human milk bile-salt-activated lipase

Chi-Sun WANG

Protein Studies Program, Oklahoma Medical Research Foundation and Department of Biochemistry and Molecular Biology, Oklahoma University Health Sciences Center, 825 N.E. 13th Street, Oklahoma City, OK 73104, U.S.A.

In order to probe the active-site structure of human milk bile-salt-activated lipase (BAL), the kinetics of the BAL-catalysed reaction were studied using monoesters as substrates. Among the fatty acyl chains, ranging from C₈ to C₁₆ of monoacylglycerols in a single equimolar assay mixture, there was a consistent trend of increased reactivity with decreased fatty-acyl-chain length for both the basal and taurocholate-stimulated activities of BAL. In addition, the detection of hydrolysis of long-chain monoacylglycerols in the absence of bile salt indicates that it is possible for the long-chain fatty acid monoester to form an enzyme–substrate complex with the basal form of BAL. I further examined the reaction kinetics of BAL with water-soluble short-chain esters of *p*-nitrophenol. The results indicated that there is a consistent trend towards a decreased Michaelis–Menten constant with increased acyl-chain length. Therefore it was concluded that the decreased reactivity with increased acyl-chain length of acylglycerols is probably not a consequence of the lowered affinity of the substrate for the enzyme. The fact that butyrate ester has the optimum acyl chain to be a substrate of BAL can be attributed to its acyl-chain length being long enough for interaction with the active centre of BAL and short enough to provide adequate positioning of the ester bond for transition state complex formation. The calculated free energy of BAL catalysis based on the derived kinetic parameters provides additional insight into the effect on the enzyme–substrate interaction of increasing the number of methylene groups in the acyl chain of substrates.

INTRODUCTION

Human milk contains a bile-salt-activated lipase (BAL) at a very high level, representing about 0.5–1% of the total milk protein (Wang & Johnson, 1983; Olivecrona & Bengtsson, 1984). The primary amino acid sequence of BAL has been deduced recently (Nilsson *et al.*, 1990; Baba *et al.*, 1991). Ser-194 has been identified as the active-site serine, which has been found to be modified with di-isopropylfluorophosphate (Baba *et al.*, 1991). Based on *N*-terminal sequence comparison (Wang, 1988; Abouakil *et al.*, 1988), it has become clear that human milk BAL and pancreatic carboxyl ester lipase have the same amino acid sequence and are likely to be the products of the same gene. The minor difference in the kinetic properties of the two enzymes is possibly due to a difference in the post-translational modification of the enzymes in mammary gland and pancreas. We have shown previously that bile salt can introduce a conformational change in the enzyme (Singh *et al.*, 1987) to allow interaction with the bulky triacylglycerol substrate (Wang & Lee, 1985). In order to further probe the active-site structure of BAL, the reactivity of BAL with monoesters as substrates, including monoacylglycerol and *p*-nitrophenyl esters, has been examined. This provides information on the interaction between the acyl chain of the substrate and the enzyme active-site centre, and eliminates the secondary interaction with the enzyme of the second or third acyl chains present in diacyl- or triacyl-glycerols. Based on the present kinetic study, I have concluded that the chemical step of the formation of the transition state complex is the likely rate-limiting step for the observed preferential acyl-chain reactivity of BAL. The main effect of taurocholate in activating BAL is to increase the affinity of the enzyme for the substrates, thereby increasing the probability of committing the resulting enzyme–substrate complex to form the transition state complex and subsequently to yield the lipolytic products. The calculated free energy of BAL catalysis based on the kinetic parameters provides additional insight into the effects of additional methylene groups in the acyl chain of substrates on the enzyme–substrate interaction.

EXPERIMENTAL

Materials

Unless otherwise stated, all chemicals were purchased from Sigma Chemical Co. Purification of BAL was performed as described previously (Wang & Johnson, 1983).

Lipolysis of monoacylglycerols

The assay mixture (10 ml final volume) contained appropriate amounts of the purified enzyme, monoacylglycerols and BSA in the absence or presence of 20 mM-taurocholate (Wang *et al.*, 1988). Lipolysis was performed in a buffer solution containing 50 mM-NH₄OH which was titrated to pH 8.0 with HCl. The *rac*-1-monoacylglycerols stock solution (12 ml) was prepared as a 3-fold-concentrated solution. The substrate was emulsified with Triton X-100 (0.15%, v/v) using a Biosonic IV sonicator (Brownwill Inc.) at 60% of the high setting for 30 s in an ice bath, and after cooling down was sonicated for an additional 30 s. After incubation at 37 °C, the enzyme reaction was terminated after various time intervals by transferring 0.25 ml aliquots in duplicate to tubes containing 4 ml of *n*-heptane/propan-2-ol (3:7, v/v) and 25 µg of cholesteryl butyrate as internal standard. The tube contents were then centrifuged to remove the protein precipitate. The supernatant was transferred to a 25 ml round-bottom flask and evaporated to dryness. The samples were 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 rotatory evaporator under vacuum at 30 °C. The samples were then redissolved in 4 ml of *n*-heptane/propan-2-ol (3:7, v/v) and acidified with 5 ml of 16.5 mM-H₂SO₄. After the solution was mixed for 30 s, the upper phase, which contained the acetylated monoacylglycerols 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. Aliquots (2 µl) were injected into the g.l.c. apparatus.

The g.l.c. analyses were performed using a Varian 3700 gas chromatograph equipped with a series 8000 autosampler and a

Abbreviation used: BAL, bile-salt-activated lipase.

SpectroPhysics SP4270 integrator. The separation was performed using 3% OV-1 on 100/120 Supelcoport (Supelco, Inc.) packed in a 50 cm glass column with an inner diameter of 2 mm. The initial column temperature was 180 °C, with an increase in temperature of 15 °C/min. When the temperature reached 347 °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. A flame ionization detector was utilized for analysis of the effluents. The retention times of the monoacylglycerols were as follows: C₈, 1.83 min; C₁₀, 2.87 min; C₁₂, 3.94 min; C₁₄, 4.96 min; C₁₆, 6.83 min; cholesteryl butyrate (internal standard), 9.03 min.

Esterase assay

The assay of BAL activity with *p*-nitrophenyl esters as substrates was performed using *p*-nitrophenyl esters with acyl-chain lengths ranging from C₂ to C₆. The reaction mixture contained 2.5 µg of BAL/ml, an appropriate concentration of *p*-nitrophenyl esters in 3% (v/v) acetonitrile, taurocholate, and buffer to a total volume of 1 ml. Because prolonged mixing is necessary to solubilize *p*-nitrophenyl esters, and in order to achieve a rapid solubilization of the substrates, the esters were initially dissolved in acetonitrile followed by dilution with distilled water to obtain a 10-fold-concentrated stock substrate solution in 30% (v/v) acetonitrile. A 0.1 ml aliquot of the stock substrate solution was then used in each assay. The BAL reactivity was measured, and the results indicated that a concentration of up to 5% (v/v) acetonitrile had no significant effect on BAL activity. The ranges of substrate concentration in the final assay mixture were: *p*-nitrophenyl acetate, 0.4–2.0 mM; *p*-nitrophenyl propionate, 0.2–1.0 mM; *p*-nitrophenyl butyrate, 0.1–0.5 mM; *p*-nitrophenyl valerate, 0.05–0.25 mM; *p*-nitrophenyl caproate, 0.01–0.1 mM. These concentration ranges were optimum for deducing the respective kinetic constants. The time course of the reactions was followed by measuring the release of *p*-nitrophenol product at 418 nm in a 1 cm path-length cuvette. The enzyme reaction was performed at 25 °C rather than 37 °C to minimize the non-enzymic hydrolysis of the substrate. The rate of *p*-nitrophenol production was determined from the initial portion of the absorbance change at 418 nm using a Beckman model 25 spectrophotometer equipped with a recorder and temperature unit. The extinction coefficient at 418 nm of *p*-nitrophenol (pH 7.5) is $11.5 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Heller & Hanahan, 1972).

Analysis of results

The linearity of double-reciprocal plots was checked graphically. Kinetic analysis was performed using the LOTUS 1-2-3 spreadsheet program. The performance of linear and non-linear curve-fitting was based on the approach described by Bevington (1969).

RESULTS

Reaction with monoacylglycerols

In this study I first examined the substrate specificity of a BAL-catalysed reaction by using a monoacylglycerol as substrate. The monoacylglycerol concentrations in the lipolysis reaction mixture were determined by g.l.c. With this fractionation technique, it was possible to determine the monoacylglycerol concentrations of various acyl-chain lengths from C₈ to C₁₆ in a single competitive assay mixture. Because of the short retention time of shorter-chain monoacylglycerols with the g.l.c. analysis, I did not include them as substrates in the experiment. One of the unexpected observations of the present study was the detection of basal activity of BAL with long-chain monoacylglycerols as

substrate. In a previous study (Wang & Lee, 1985) it was demonstrated that assay at a high concentration (1 mg/ml) of BAL failed to detect the possible presence of a low level of basal activity with long-chain triacylglycerol substrates, and it was concluded that BAL has an absolute requirement for bile-salt activator to catalyse the hydrolysis of these substrates. Thus, based on the present study, it can be deduced that, in the non-activated form, the active site of BAL is accessible to long-chain fatty acid esters such as monoacylglycerols, but not to the bulky long-chain triacylglycerols. As shown in Fig. 1(a), the trend of the lipolysis rate of BAL in the absence of activator was inversely related to acyl-chain length, i.e. C₈ > C₁₀ > C₁₂ > C₁₄ > C₁₆. The inclusion of taurocholate (20 mM) as activator increased the lipolysis rate; however, the trend of preferential reactivity of acyl-chain length was the same as that found for the basal activity of BAL. The apparent pseudo-first-order rate constants of the basal and taurocholate-activated BAL activity are shown in Table 1. Since the pseudo-first-order rate is derived from the lipolysis of various monoacylglycerols in a single assay mixture, the parameter is the expression of the relative reactivity due to the acyl-chain length effect of the substrates for basal and taurocholate-activated BAL activities.

Reaction with *p*-nitrophenyl esters

In order to gain insight into the preferential reactivity of BAL with medium-chain over long-chain monoacylglycerols, the study was extended to include chromogenic short-chain *p*-nitrophenyl esters as substrates. One advantage of using these substrates is that the short-chain fatty acyl esters are soluble in aqueous solution. Therefore the effective substrate concentration and the analytical substrate concentration can be considered to be the same. The necessity to define effective substrate concentration, as encountered with emulsified substrates, can be avoided. I have shown previously (Wang, 1981) that the BAL-catalysed reaction with water-soluble substrates can be considered as a rapid-equilibrium reaction, as indicated in the following reaction scheme:

In the equation, E is the enzyme (BAL); S is the substrate; A is the activator (taurocholate); P and Q are the alcoholic and carboxylic acid products respectively; k_{cat} is the rate constant for the breakdown of ES to E + P + Q, and βk_{cat} is the rate constant for the breakdown of EAS to EA + P + Q. The derived Michaelis–Menten constants K_s , K_A , αK_s and αK_A are also the dissociation constants of each indicated reaction. The K_A value, which is the dissociation constant for enzyme–activator interaction, was previously determined to be 0.37 mM for the BAL–taurocholate interaction (Wang, 1981). On the basis of the kinetic scheme described above (eqn. 1), the velocity equation is shown below (Segel, 1975):

$$v = \frac{V_{\text{max}} \frac{[\text{S}]}{K_s} + \beta V_{\text{max}} \frac{[\text{A}][\text{S}]}{\alpha K_A K_s}}{1 + \frac{[\text{A}]}{K_A} + \frac{[\text{S}]}{K_s} + \frac{[\text{A}][\text{S}]}{\alpha K_A K_s}} \quad (2)$$

The Lineweaver–Burk plots with *p*-nitrophenyl butyrate as substrate, in the absence and in the presence of a low (0.5 mM-taurocholate) and a saturating concentration (2 mM-taurocholate) of activator, are shown in Fig. 2.

Fig. 1. Hydrolysis of *rac*-1-monoacylglycerol mixture by human milk BAL

Incubation of the mixture containing each monoacylglycerol (0.1 mM) was performed at 37 °C in duplicate and in the presence of 60 mg of BSA/ml. The enzyme concentration was 0.1 mg/ml. The numbers of acyl-carbon in the substrates were: C₈ (●), C₁₀ (■), C₁₂ (▲), C₁₄ (○) and C₁₆ (□). The lipolysis was performed in the absence (a) and in the presence (b) of 20 mM-taurocholate.

The determined values of K_s , αK_s , k_{cat} , and βk_{cat} for a number of *p*-nitrophenyl esters are shown in Table 2. From the kinetic data it can be deduced that the major effect of taurocholate is to lead to an increased affinity of BAL for the substrates. In contrast with this finding of a consistent trend of decreased K_s and αK_s with increased acyl-chain length, I have found that butyrate ester has the highest k_{cat} and βk_{cat} values. Based on the magnitude of the parameters of $1/\alpha$, ranging from 4.3 to 11.2, and β , ranging from 0.7 to 1.6, I concluded that the major effect of taurocholate in activating BAL is to increase the affinity of the enzyme for the substrate, with a secondary effect of increasing

Fig. 2. Double-reciprocal plot with *p*-nitrophenyl butyrate as the variable substrate

The taurocholate concentration was held constant at 2 mM (●), 0.5 mM (▲) or 0 mM (○). The enzyme concentration was 2.5 μg/ml.

the parameter β , except for butyrate ester, which actually has a resulting β value of less than 1.

Free energy of the BAL-catalysed reaction

The energetics of an enzyme–substrate interaction involving the transition state intermediate in catalysis can be seen in Fig. 3(a). The free energy of activation, $\Delta G^\ddagger$, is composed of two terms: an ‘energy-unfavourable’ term, $\Delta G^\ddagger$, due to the activation energy of the chemical step of bond making and breaking, and a compensating ‘energy-favourable’ term, ΔG_s , due to the realization of the binding energy. The effect of incremental increases in the numbers of methylene groups in the acyl chain of the *p*-

Table 1. Pseudo-first-order rate constants of the lipolysis of monoacylglycerols

The results are from Figs. 1(a) and 1(b). Values are means $\pm$ S.E.M. derived from computer non-linear curve-fitting. Values in parentheses indicate activation (fold), for which the propagation of errors of ratios was performed as described by Bevington (1969).

	Rate constant (min ⁻¹)				
	C ₈	C ₁₀	C ₁₂	C ₁₄	C ₁₆
Without bile salt	1.12 $\pm$ 0.09	0.021 $\pm$ 0.002	0.013 $\pm$ 0.001	0.0020 $\pm$ 0.0003	0.0013 $\pm$ 0.0002
With 20 mM-taurocholate	2.18 $\pm$ 0.02 (1.9 $\pm$ 0.2)	0.041 $\pm$ 0.001 (2.0 $\pm$ 0.2)	0.028 $\pm$ 0.002 (2.2 $\pm$ 0.2)	0.010 $\pm$ 0.001 (5.0 $\pm$ 0.9)	0.0071 $\pm$ 0.0006 (5.5 $\pm$ 0.9)

Table 2. Kinetic parameters of human milk BAL with short-chain esters of *p*-nitrophenol as substrates

The kinetic parameters are defined in eqns. (1) and (2). Values are means $\pm$ S.E.M. ($n = 3$). Propagation of errors of ratios for β and $1/\alpha$ was performed as described by Bevington (1969).

		C ₂	C ₃	C ₄	C ₅	C ₆
Basal activity	$10^{-3} \times k_{cat}$ (s ⁻¹)	0.63 $\pm$ 0.006	1.07 $\pm$ 0.007	3.12 $\pm$ 0.44	1.42 $\pm$ 0.52	0.86 $\pm$ 0.23
	K_s (mM)	10.3 $\pm$ 0.9	4.59 $\pm$ 0.31	4.25 $\pm$ 0.83	0.95 $\pm$ 0.39	0.13 $\pm$ 0.03
Taurocholate-activated activity	$10^{-3} \times \beta k_{cat}$ (s ⁻¹)	1.02 $\pm$ 0.04	1.19 $\pm$ 0.09	2.21 $\pm$ 0.02	1.75 $\pm$ 0.24	0.89 $\pm$ 0.03
	αK_s (mM)	2.40 $\pm$ 0.12	0.87 $\pm$ 0.10	0.38 $\pm$ 0.01	0.21 $\pm$ 0.04	0.03 $\pm$ 0.02
	β	1.6 $\pm$ 0.2	1.1 $\pm$ 0.1	0.7 $\pm$ 0.1	1.2 $\pm$ 0.5	1.0 $\pm$ 0.3
	$1/\alpha$	4.3 $\pm$ 0.5	5.3 $\pm$ 0.8	11.2 $\pm$ 2.2	4.5 $\pm$ 2.0	5.0 $\pm$ 1.4

Fig. 3. Free energy diagram for catalysis involving ground state and transition state enzyme-substrate complex transition

$\Delta G_s^\ddagger$ represents the energy of activation from $E + S$ to $ES^\ddagger$. ΔG_s is the energy realized for ground state ES complex formation. $\Delta G^\ddagger$ is the free energy required for the chemical step of bond making or breaking. The energy level in (a) describes that of *p*-nitrophenyl ester of C_n acyl chain and that in (b) describes the effect of adding a methylene group to the acyl chain of substrate (C_{n+1}) on the free energy level. The calculation of $\Delta\Delta G_s^\ddagger$, $\Delta\Delta G_s$ and $\Delta\Delta G^\ddagger$ are described in eqns. (3)–(5).

p-nitrophenyl esters in causing the incremental changes, $\Delta\Delta G_s^\ddagger$ and $\Delta\Delta G_s$, for basal activity of BAL catalysis can be expressed based on the following equations:

$$\Delta\Delta G_s^\ddagger = -RT \ln [(k_{cat}/K_s)_{n+1}/(k_{cat}/K_s)_n] \quad (3)$$

$$\Delta\Delta G_s = -RT \ln [(K_s)_n/(K_s)_{n+1}] \quad (4)$$

where R is the gas constant, T is the absolute temperature and the subscripts n and $n+1$ are utilized to denote the parameters referring to the respective acyl carbons. From Figs. 3(a) and 3(b), it can be further deduced that:

$$\Delta\Delta G^\ddagger = \Delta\Delta G_s^\ddagger - \Delta\Delta G_s \quad (5)$$

where $\Delta\Delta G^\ddagger$ is the incremental change of $\Delta G^\ddagger$ due to the addition of a methylene group. The above equations are also applicable to the calculation of free energy involving taurocholate-activated BAL activity by including the parameters α and β as shown in eqns. (6) and (7):

$$\Delta\Delta G_s^\ddagger = -RT \ln [(\beta k_{cat}/\alpha K_s)_{n+1}/(\beta k_{cat}/\alpha K_s)_n] \quad (6)$$

$$\Delta\Delta G_s = -RT \ln [(\alpha K_s)_n/(\alpha K_s)_{n+1}] \quad (7)$$

The incremental free energy, $\Delta\Delta G^\ddagger$, can be calculated as shown in eqn. (5). The calculated values of $\Delta\Delta G_s^\ddagger$, $\Delta\Delta G_s$ and $\Delta\Delta G^\ddagger$ for both basal and taurocholate-activated BAL catalysis are shown in Table 3. Since all $\Delta\Delta G_s^\ddagger$ and $\Delta\Delta G_s$ values are numerically negative, the data imply that the incremental increases in

Table 4. Effect of taurocholate on the free energy of BAL catalysis with *p*-nitrophenyl esters as substrate

The calculations are based on data from Table 2. 1 kcal = 4.184 kJ.

Substrate	$\Delta\Delta G_s^\ddagger$ (kJ/mol)	$\Delta\Delta G_s$ (kJ/mol)	$\Delta\Delta G^\ddagger$ (kJ/mol)
C_2	−4.849	−20.752	15.067
C_3	−4.368	−21.673	16.895
C_4	−5.134	−23.129	17.995
C_5	−4.272	−20.887	16.610
C_6	−4.071	−21.133	17.029

methylene group numbers in the acyl chain of the substrate caused the decrease of both transition state and ground state energy. On the other hand, the deduced $\Delta\Delta G^\ddagger$ value implies that butyrate ester has the lowest $\Delta G^\ddagger$.

Free energy of the BAL–taurocholate interaction

Similar calculations, based on the assumption that BAL maintains the same conformation upon interaction with taurocholate, yield $\Delta\Delta G^\ddagger$ values of 15.1, 16.7, 18.0, 16.7 and 17.2 kJ for the *p*-nitrophenyl esters with acyl-chain lengths of C_2 , C_3 , C_4 , C_5 and C_6 respectively (Table 4). Since $\Delta\Delta G^\ddagger$ was calculated based on the specific taurocholate effect, as anticipated, the values obtained did not vary significantly with the substrate utilized for the calculation. In view of the taurocholate activation effect, the large activation energy needed for BAL catalysis must have been overcome through a taurocholate-induced conformational change of the enzyme, as previously shown by fluorescence probe of BAL conformation (Singh *et al.*, 1987).

DISCUSSION

Because of the presence of only one acyl chain in the monoacylglycerol molecule, it is more straightforward to use this acylglycerol than to use triacylglycerol as a substrate for deducing the acyl-chain effect of an enzyme–substrate interaction. One of the surprising findings of this study is the presence of basal activity (in the absence of bile-salt activator) of BAL with a long-chain monoacylglycerol as substrate. This is in contrast with the finding of the absence of basal activity of the enzyme with long-chain triacylglycerols (Wang & Lee, 1985). We have suggested previously that the presence of more than one acyl chain in the long-chain triacylglycerol molecule can cause steric hindrance, as its interaction with the unactivated BAL and the major role of bile salt in activating BAL is to induce the conformational change of the enzyme (Singh *et al.*, 1987) to facilitate the interaction of the active site with the bulky long-chain triacyl-

Table 3. Effect of numbers of methylene groups on the free energy of reaction for taurocholate-activated and basal activities of BAL

The calculations are based on eqns. (3)–(7) and data from Table 2. 1 kcal = 4.184 kJ.

	Taurocholate-activated (kJ/mol)			Basal (kJ/mol)		
	$\Delta\Delta G_s^\ddagger$	$\Delta\Delta G_s$	$\Delta\Delta G^\ddagger$	$\Delta\Delta G_s^\ddagger$	$\Delta\Delta G_s$	$\Delta\Delta G^\ddagger$
C_3 versus C_2	−2.887	−2.519	−0.368	−3.699	−2.004	−1.360
C_4 versus C_3	−3.590	−2.054	−1.536	−2.824	−0.192	−2.632
C_5 versus C_4	−0.895	−1.473	0.577	−1.757	−3.715	1.962
C_6 versus C_5	−3.481	−5.184	1.703	−3.686	−4.933	1.251

glycerols. In contrast with BAL, although lipoprotein lipase also has an activator requirement (apolipoprotein CII) (Posner *et al.*, 1983; Cheng *et al.*, 1990), this enzyme was found to have low but detectable basal activity with long-chain triacylglycerols. This would imply that the active site of the basal form of lipoprotein lipase can accommodate the bulky long-chain triacylglycerols. The probable physiological reason for the absence of basal activity of BAL with long-chain triacylglycerols is to allow expression of its activity, not in the milk, but in the infant's intestinal lumen (Wang *et al.*, 1989; Alemi *et al.*, 1981) where bile salt becomes accessible to the enzyme for digestion of milk fat.

Because *p*-nitrophenyl esters are highly chromogenic substrates and short-chain fatty acid esters of these substrates are water-soluble, this allowed me to examine the acyl-chain effect on the steady-state kinetic profile of BAL. In a previous study, I have shown that the reaction kinetics of BAL-catalysed hydrolysis of water-soluble substrates can be described as 'rapid equilibrium-random' (Wang, 1981) in the reaction steps of substrate and activator binding, which implies that the formation of activated ES complex is much slower than the dissociation of ES to E and S, and points out that k_{cat} and βk_{cat} are the likely rate-limiting kinetic parameters for the observed acyl-chain effect on BAL activity. Furthermore, the derived Michaelis-Menten constants for the various substrates can be considered to be essentially equivalent to the corresponding dissociation constants. From the findings in Table 2, it can readily be seen that there is an increased affinity of the enzyme for the substrate with an increase in the acyl-chain length. However, it is apparent that the increase in affinity for the substrate is not always advantageous for promoting a higher catalytic rate, as butyrate ester actually had the highest k_{cat} and βk_{cat} values but not the lowest K_s and αK_s values. For a better understanding of BAL reaction kinetics, I have further calculated the effect of incrementally increasing the numbers of methylene groups in the substrate on the free energy of the BAL catalytic reaction. Based on the enzyme-substrate complementarity, Fersht (1984) concluded that enzymes in general have evolved to bind the transition state of a substrate more strongly than the substrates themselves. This principle contradicts the widely held belief that strong substrate binding, or a low Michaelis-Menten constant, is always an important component of efficient enzymic catalysis. The energy diagram demonstrating this point is shown in Fig. 3. Based on the transition state theory, the activation energy $\Delta G^\ddagger$ of the transition state complex formation is composed of two terms, an energetically unfavourable term, $\Delta G^\ddagger$, due to the activation steps of bond making and breaking, and a compensating energetically favourable term, ΔG_s , due to the realization of the binding energy. If the enzyme has a high affinity for the undistorted substrate, the low Michaelis-Menten constant can lead to a thermodynamic 'pit' into which the reaction 'falls' and has to 'climb out'. By inspection of Table 3, it can be readily concluded that incremental increases in methylene group number in the acyl chain of the substrate led to the stabilization of both the ground state and the transition state of enzyme-substrate complexes. However, there was a higher degree of stabilization of the transition state than of the ground state from C_2 to C_4 , as can be seen with $\Delta\Delta G^\ddagger$ values being numerically negative (Table 3). In contrast, there was a higher degree of stabilization of the ground state than of the transition state complex from C_4 to C_6 , as the corresponding $\Delta\Delta G^\ddagger$ values are numerically positive (Table 3). From this calculation, it can be reasoned that the decrease in dissociation constant of an enzyme-substrate interaction is not always advantageous in improving the catalysis rate, as can be seen from the fact (eqn. 6) that this can cause the increase of free energy $\Delta G^\ddagger$ for the catalytic step. Based on the energy diagram in Fig. 3, the likely explanation for the highest k_{cat} and βk_{cat}

values being for butyrate ester in comparison with the other related substrates is that this substrate has acyl-chain length adequate to provide a sufficient hydrophobic binding with the enzyme active site centre, and yet short enough to provide freedom of the acyl chain to distort the ester bond for the formation of the transition state complex.

Based on the finding of preferential reactivity of BAL in the order $C_4 > C_6 > C_8 > C_{10} > C_{12} > C_{14} > C_{16}$ of monoacid triacylglycerols (Wang *et al.*, 1983) and $C_8 > C_{10} > C_{12} > C_{14} > C_{16}$ of monoacylglycerols (Table 1), I conclude that the ability of long-chain acylglycerols to form a lipid interface is not in itself advantageous, or an obligatory requirement for promoting lipolysis. It is likely that the decreased water-solubility of long-chain acylglycerols could in fact lead to a lower effective substrate concentration and may, in part, contribute to the lower reactivity with BAL. It is possible that the observed increased affinity of the enzyme with the increased acyl-chain length can be considered to be a necessary evolutionary consequence, at least in part, to overcome the lower effective substrate concentration of long-chain acylglycerols. Because of the high affinity of lipases for long-chain acylglycerols, there is a tendency for this class of enzymes to be adsorbed to the lipid interface. However, this phenomenon does not imply, in general, that the longer the acyl chain of acylglycerol, the higher the reactivity of the lipases. In fact, as shown by Desnuelle and his co-workers (Entressangles *et al.*, 1961; Desnuelle & Savary, 1963), pig pancreatic lipase has the maximum reaction rate with tributyrorylglycerol among a series of monoacid triacylglycerol substrates. Furthermore, the presence of a lipid interface does not necessarily lead to the interfacial activation of lipases, as recently shown by Scott *et al.* (1990), who found from the X-ray crystal structure of phospholipase A_2 that there is no evidence for allosteric changes in the enzyme in the presence of a lipid interface. Compatible with our findings, Bonelli & Jonas (1989) also utilized the short-chain esters of *p*-nitrophenol as alternative substrates for lecithin:cholesterol acyltransferase to probe the catalytic step of hydrolysis, and came to the conclusion that short fatty-acyl esters are the preferred substrate despite the fact that long-chain fatty acid esters represent the physiological substrate of the enzyme. These findings can be summarized based on the free energy diagram (Fig. 3a) showing that excessively tight substrate binding can lead to a higher $\Delta G^\ddagger$ and, consequently, a lower catalysis rate.

Previously, I have provided evidence that the preferential reactivity of BAL for butyrate ester represents an intramolecular phenomenon. It was demonstrated that when *rac*-1,2-dipalmitoyl-3-butyroylglycerol and *sn*-1,3-dipalmitoyl-2-butyroylglycerol were utilized as substrates of BAL, only dipalmitoylglycerol was detected as the intermediary diacylglycerol product (Wang *et al.*, 1983). This would indicate that there is a preferential reactivity of BAL with butyrate ester, even though the butyrate and palmitate acyl chains reside in the same acylglycerol molecule. The intramolecular preferential reactivity for butyrate ester has also been observed with pig pancreatic lipase by Desnuelle & Savary (1963), who found that when 1-butyroyl-3-palmitoylglycerol is hydrolysed to an extent of 10%, the released non-esterified fatty acids consist of 72 mol% butyrate and 28 mol% palmitate.

In conclusion, with the use of a series of monoesters as substrates of BAL, I have deduced that butyrate ester represents the optimum substrate for interaction with BAL in forming the transition state complex. The role of taurocholate as an activator is mainly to increase the affinity of the enzyme for the substrate. The obligatory offsetting effect of decreasing the catalytic rate due to tighter substrate binding is overcome by the conformational change of the enzyme. A further in-depth under-

standing of the molecular events of the enzyme-substrate interaction will have to await the availability of the crystallographic structure of the enzyme in the presence of ligands for deducing the important interactions of the enzyme and the substrate and the effect on BAL reaction kinetics.

I thank Dr. J. Hartsuck, Dr. W. J. McConathy and Dr. J. Tang for their valuable suggestions, D. Downs and Randy Whitmer for their skilful technical assistance, and J. Pilcher and the OASIS staff for help in preparation of the manuscript. This work was supported in part by grant HD-23472 from the Public Health Service, grant HR0-017/3924 from the Oklahoma Center for the Advancement of Science and Technology, and funds from the Oklahoma Medical Research Foundation.

REFERENCES

- Abouakil, N., Rogalska, E., Bonicel, J. & Lombardo, D. (1988) *Biochim. Biophys. Acta* **961**, 299–308
- Alemi, B., Hamosh, M., Scanlon, J. W., Salzman-Mann, C. & Hamosh, P. (1981) *Pediatrics* **68**, 484–489
- Baba, T., Downs, D., Jackson, K., Tang, J. & Wang, C.-S. (1991) *Biochemistry* **30**, 500–510
- Bevington, P. R. (1969) *Data Reduction and Error Analysis for the Physical Sciences*, pp. 56–65 and 204–246, McGraw-Hill, New York
- Bonelli, F. S. & Jonas, A. (1989) *J. Biol. Chem.* **264**, 14723–14728
- Cheng, Q., Blackett, P., Jackson, K. W., McConathy, W. J. & Wang, C.-S. (1990) *Biochem. J.* **269**, 403–407
- Desnuelle, P. & Savary, P. (1963) *J. Lipid Res.* **4**, 369–384
- Entressangles, B., Paséro, L., Savary, P., Sarda, L. & Desnuelle, P. (1961) *Bull. Soc. Chim. Biol.* **43**, 581–591
- Fersht, A. (1984) *Enzyme Structure and Mechanism* (2nd edn.) pp. 311–346, W. H. Freeman & Co., New York
- Heller, M. & Hanahan, D. J. (1972) *Biochim. Biophys. Acta* **255**, 239–250
- Nilsson, J., Bläckberg, L., Carlsson, P., Enerbäck, S., Hernell, O. & Bjursell, G. (1990) *Eur. J. Biochem.* **192**, 543–550
- Olivecrona, T. & Bengtsson, G. (1984) in *Lipases* (Borgström, B. & Brookman, H. L., eds.), pp. 205–261, Elsevier, Amsterdam
- Posner, I., Wang, C.-S. & McConathy, W. J. (1983) *Biochemistry* **22**, 4041–4047
- Scott, D. L., White, S. P., Otwinowski, Z., Yuan, W., Gelb, M. H. & Sigler, P. B. (1990) *Science* **250**, 1541–1546
- Segel, I. H. (1975) *Enzyme Kinetics*, pp. 227–231, John Wiley and Sons, New York
- Singh, S., Lee, D. M. & Wang, C.-S. (1987) *Int. J. Biol. Macromol.* **9**, 294–296
- Wang, C.-S. (1981) *J. Biol. Chem.* **256**, 10198–10202
- Wang, C.-S. (1988) *Biochem. Biophys. Res. Commun.* **155**, 950–955
- Wang, C.-S. & Johnson, K. (1983) *Anal. Biochem.* **133**, 457–461
- Wang, C.-S. & Lee, D. M. (1985) *J. Lipid Res.* **26**, 824–830
- Wang, C.-S., Kuksis, A., Manganaro, F., Myher, J. J., Downs, D. & Bass, H. B. (1983) *J. Biol. Chem.* **258**, 9197–9202
- Wang, C.-S., Hartsuck, J. A. & Downs, D. (1988) *Biochemistry* **27**, 4834–4840
- Wang, C.-S., Martindale, M. E., King, M. M. & Tang, J. (1989) *Am. J. Clin. Nutr.* **49**, 457–463

Received 12 March 1991/8 May 1991; accepted 21 May 1991