Effect of bile salts on the interfacial inactivation of pancreatic carboxylester lipase

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Abstract Pig pancreatic carboxylester lipase (cholesterol esterase, E.C. 3.1.1.13) was inactivated at a tributyrin/water interface. The apparent rate constant for inactivation increased with increase in the particle surface area of the tributyrin emulsion. The large energy of activation and entropy change for inactivation (33.7 Kcal · mol^{-1} and 35.8 cal · mol^{-1} · deg^{-1}, using 5 mM sonicated tributyrin at 37°C, respectively) suggest that the observed inactivation reflects denaturation of the enzyme at the tributyrin/water interface. Bile salts protected the enzyme from irreversible inactivation at the tributyrin/water interface. The protection by bile salts was related both to their concentration and to the tributyrin concentration (substrate surface area). The protection by bile salts was not related to their concentration below or above their critical micellar concentration; the binding of bile salts to enzyme was probably the dominant protection factor. Similar stabilization was observed with other detergents such as Brij-35, Triton X-100, and sodium dodecyl sulfate. These results suggest that inactivation of carboxylester lipase occurs at a high-energy lipid-water interface and that an important role of bile salts in vivo is to stabilize carboxylester lipase at interfaces. —**Tsujita, T., and H. Okuda.** Effect of bile salts on the interfacial inactivation of pancreatic carboxylester lipase. *J. Lipid Res.* 1990. **31:** 831–838.

Supplementary key words surface inactivation • tributyrin emulsion

Lipolysis is an example of heterogeneous biocatalysis. Typical substrates are water-insoluble lipids, which are separated from the aqueous medium by the surface phase. In contrast to their substrates, many lipases and phospholipases are water-soluble proteins. Thus, for catalysis, these enzymes must be adsorbed to (or penetrate) the surface of the lipids. Enzymes are sometimes activated or denatured by adsorption to an interface (1, 2). For instance, as we reported previously, carboxylester lipase is denatured by adsorption to a high energy surface (3).

Carboxylester lipase is known to have broad substrate specificity (4), readily acting on triacylglycerol, diacylglycerol, monoacylglycerol, and cholesteryl oleate. It also catalyzes the hydrolysis of water-soluble substrates such as methyl butyrate and *p*-nitrophenyl butyrate. There are many reports suggesting that bile salts are important in regulating the catalytic activity of carboxylester lipase (5–8), but the role of bile salts in regulating the activity of carboxylester lipase is still uncertain. Lombardo et al. (6) postulated the presence of two bile salt binding sites on human pancreatic carboxylester lipase. One site was nonspecific with respect to monomeric primary and secondary bile salts. The interaction of monomeric bile salts with this site leads to activation of the catalytic site for water-soluble substrate. The second site was specific for the 3α- and 7α-hydroxylated positions of primary bile salts. The interaction of primary bile salts with this second site results in dimerization of the enzyme and its ability to act on a water-insoluble (emulsified) substrate. Albro et al. (7) reported that activation of nonspecific lipase activity by bile salts was associated with a conformational change of the enzyme protein.

The present report presents studies on some parameters of enzyme inactivation at a substrate/water interface and the protection effect of bile salts against this surface inactivation.

MATERIALS AND METHODS

Materials

Enzyme substrates and reagents were obtained from the following sources: sodium cholate, sodium deoxycholate, sodium taurocholate, sodium taurodeoxycholate, and *p*-nitrophenyl butyrate from Sigma Chemical Co. (St. Louis, MO); methyl butyrate, Triton X-100, sodium dodecyl sulfate, and rhodamine 6G from Wako Pure Chemical Industries (Osaka, Japan); Brij-35 from Kao Co. (Tokyo, Japan), and soya phosphatidylcholine from Nihon Shoji (Tokyo, Japan). Bovine serum albumin was ob-

Abbreviations: SDS, sodium dodecyl sulfate.

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tained from Wako Pure Chemical Industries and extracted by the method of Chen to remove free fatty acids (9).

Methods

Enzyme purification. Carboxylester lipase was purified from porcine pancreas by the procedure of Rudd, Mizuno, and Brockman (10) with some modifications. The enzyme was extracted from pancreas by the method of Rudd et al. (10) and applied to a Sephadex G-100 column (4.5 × 140 cm) equilibrated with 50 mM sodium phosphate buffer, pH 6.2, containing 0.1 M NaCl, 0.1% 2-mercaptoethanol, 2 mM benzamidine hydrochloride, 2 mM hydrocinnamic acid, and 0.5 mM N-α-benzoyl-D,L-arginine. The fractions of eluate with activity were combined and precipitated by addition of 1.3 volumes of *tert*-butanol–water 95:5 (v/v). The precipitate was dissolved in 50 mM piperazine hydrochloride, pH 4.8, containing 90 mM NaCl, 2 mM benzamindine hydrochloride, 2 mM hydrocinnamic acid, and 0.5 mM N-α-benzoyl-D,L-arginine, and applied to a PBE-94 column (1.0 × 25 cm) equilibrated with the same buffer. The column was washed with the equilibration buffer and the enzyme was eluted by increasing the NaCl concentration to 250 mM. Pooled fractions containing the enzyme were applied to a Blue-Sepharose CL-6B column (2.5 × 14 cm) equilibrated with 10 mM potassium phosphate buffer, pH 6.2 (11). The column was washed with the same buffer and the enzyme was elute with 100 mM Tris-HCl buffer, pH 7.2 containing 0.5 M diethylamine-HCl. The fractions of eluate with activity were pooled, concentrated, and dialyzed against 10 mM potassium phosphate buffer, pH 6.2. The resulting enzyme solution was stored at −80°C.

Enzyme assay. The rates of hydrolysis of tributyrin, triacetin, and methyl butyrate were measured with a Radiometer pH-stat (type HS-2A, TOA Electronics Ltd., Tokyo, Japan) by titration of liberated fatty acid with 0.02 N NaOH, as described previously (12). Unless otherwise indicated, tributyrin was used as a substrate in 0.1 M NaCl with 0.5% gum arabic at pH 7.0 at 37°C in a total volume of 10 ml. Just before use, the substrate mixture was sonicated for 1 min at a power setting of 10 in a Tomy UR 20-P sonicator (Tomy Seiko Co., Tokyo, Japan) (sonicated substrate) or shaken vigorously 100 times by hand (shaken substrate). The reaction mixture was first incubated without enzyme for several minutes, and the rate of nonenzymatic NaOH uptake was recorded. Then the reaction was started by adding the enzyme solution.

In studies on substrate specificity, enzyme activities were assayed with synthetic glyceride or cholesteryl oleate subtrate emulsified with gum arabic. The standard assay system contained the following components in a total volume of 0.2 ml: 0.4 μmol of substrate, 1 mg of gum arabic, 5 mg of bovine serum albumin, and 10 μmol of potassium phosphate. Incubation was carried out at pH 7.0 and 37°C. The free fatty acids produced were extracted and determined as described previously (13).

p-Nitrophenyl butyrate-hydrolyzing activity was determined by measuring the rate of release of *p*-nitrophenol (absorbance at 400 nm at 25°C) as described previously (3).

Other procedures. The critical micellar concentration of bile salts was determined by measuring the shift in the absorbance maximum (λmax) of rhodamine 6G by the method of Carey and Small (14). In cases when it was difficult to obtain a single point corresponding to the critical micellar concentration, the critical micellar range of bile salts was determined as described by Borgström and Erlanson (15). Relative protein concentration was determined with a Bio-Rad protein assay kit using bovine serum albumin as a standard.

RESULTS

The purified enzyme gave two bands on SDS-polyacrylamide gel electrophoresis, from which its molecular weight was estimated to be 80,000 (main band) and 85,000 (minor band), respectively (data not shown). Rudd et al. (10) reported that the low-molecular weight form is a monomer of about 74,000 ± 4,000 and the high-molecular weight form is a dimer with identical subunits of about 83,000 ± 4,000 each. This suggests that our purified enzyme fraction contained monomer and dimer forms. The enzymatic properties of the two forms are nearly indentical (10). The enzyme preparations were found to have specific activities of 700–800 μmol *p*-nitrophenol released/mg protein per min with *p*-nitrophenyl butyrate as a substrate.

The substrate specificity of carboxylester lipase was examined with and without cholate (**Table 1**). The purified enzyme hydrolyzed tri-, di-, and 1-monacylglycerols and cholesteryl oleate with cholate, the relative order of rate of hydrolysis being tri > di > 1-mono. In the absence of cholate, the enzyme could not hydrolyze more hydrophobic substrates, such as triolein, diolein, tricaprin, and cholesteryl oleate. The enzyme activities on less hydrophobic substrates such as monocaprin and triacetin were slightly decreased in the absence of cholate.

Fig. 1 shows the enzymatic activities as functions of tributyrin concentration. With sonicated substrate, the lipase activity decreased abruptly above a concentration of 5 mM tributyrin in the absence of cholate, but not in the presence of cholate (Fig. 1A). With shaken substrate, the lipase activity did not decrease with increase in tributyrin concentration to 20 mM and the activity increased about threefold on addition of cholate (Fig. 1B).

TABLE 1. Substrate specificity of pancreatic carboxylester lipase

Substrate	Substrate Conc.	Activity		Ratio (× 100)
		+ Cholate	− Cholate	− / +
	mM	*μmol/mg protein/min*		
Triolein	20	159 ± 10	0	0
Diolein	50	118 ± 2	0	0
Monolein	50	57 ± 5	11 ± 4	19
Tricaprin	100	2080 ± 18	0	0
Monocaprin	100	81 ± 11	53 ± 13	65
Tributyrin	100	656 ± 10	0	0
Triacetin	500	78 ± 3	70 ± 4	90
Methyl butyrate	300	141 ± 6	5 ± 2	4
Cholesteryl oleate	50	131 ± 7	0	0

Enzyme activities were assayed with (+) (5 mM) or without (−) cholate in the conditions described under Materials and Methods. Values are means ± SE for four separate assays.

In the presence of cholate, the reaction velocity was constant with time (**Fig. 2**, c,d), but in the absence of cholate, the velocity decreased in a time-dependent fashion (Fig. 2, b). The observed decrease of the reaction velocity probably results from inactivation of the enzyme at the tributyrin/water interface with further inactivation on surface denaturation according to a first-order reaction:

$$\ln\left(\frac{V}{V_0}\right) = -k_i \cdot t$$

where k_i is the apparent first order rate constant for inactivation and V_o is the initial velocity of the reaction. The velocity (V) at any time should be proprotional to the moles of active enzyme on the surface with negligible substrate consumption during the course of the reaction (less than 5%). The equation predicts that a plot of log (percent remaining activity) versus t (time) should be linear with a slope of $-k_i/2.30$. **Fig. 3** shows that such plots are linear, indicating that the data are consistent with the proposed model for inactivation. The apparent rate constant for inactivation (k_i) increased with increase in the tributyrin concentration. With sonicated substrate (Fig. 3A), k_i increased about fourfold on increase in the tributyrin concentration from 4 mM ($k_i = 1.03 \times 10^{-3}\ s^{-1}$) to 7 mM ($k_i = 4.48 \times 10^{-3}\ s^{-1}$). With shaken substrate (Fig. 3B), the k_i value was below $1.0 \times 10^{-3}\ s^{-1}$ in 100 mM tributyrin and k_i increased about fourfold on increase in the tributyrin concentration from 100 mM ($k_i = 0.96 \times 10^{-3}\ s^{-1}$) to 300 mM ($k_i = 3.97 \times 10^{-3}\ s^{-1}$). Particles of the tributyrin emulsion were observed under a microscope and the particle size and number were measured with enlarged photographs. The average diameters of sonicated and shaken tributyrin emulsion were 2.90 ± 0.99 μm (mean ± SD) and 41.07 ± 29.77 μm (mean ± SD), re-

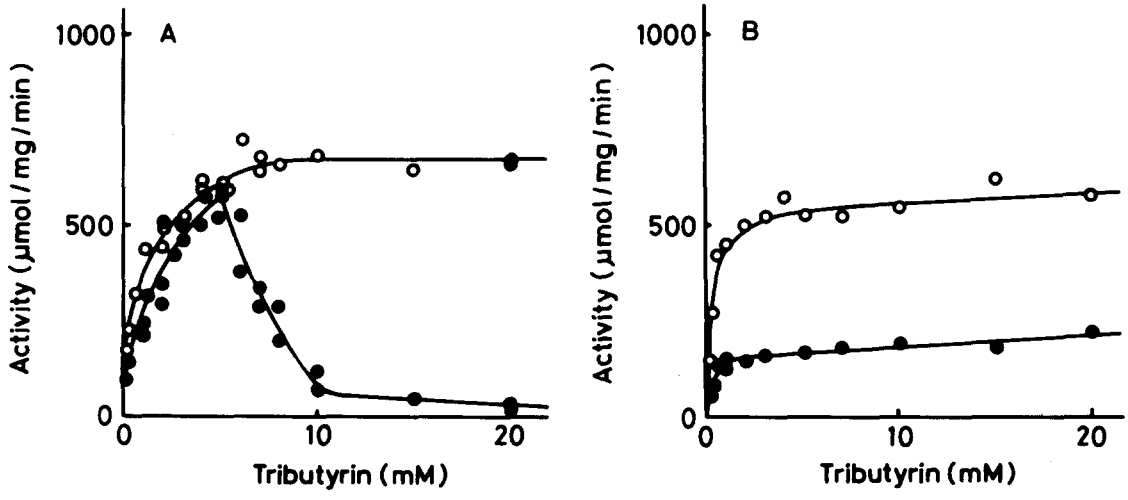

Fig. 1. Effect of tributyrin concentration on hydrolyzing activity of carboxylester lipase. The incubation mixture consisted of the indicated amount of tributyrin, 1.25 μg carboxylester lipase, and cholate ((○), 5 mM and (●), 0 mM) in 10 ml of 0.1 M NaCl containing 0.5% gum arabic. The substrate mixture was sonicated for 1 min (A) or shaken vigorously 100 times by hand (B). The enzyme activity was assayed by measuring the amount of butyric acid released in the first 30 sec.

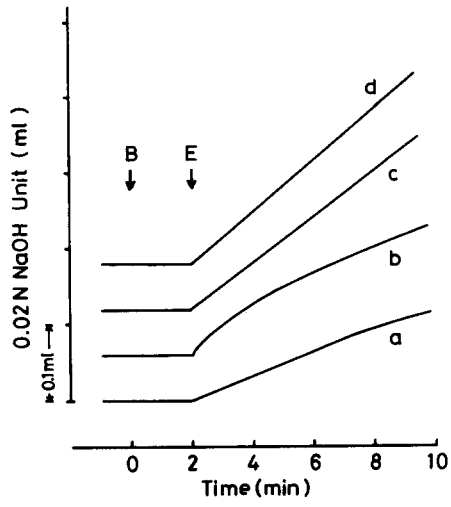

Fig. 2. Effect of cholate on tributyrin hydrolysis by carboxylester lipase. The incubation mixture contained 5 mM tributyrin in a final volume of 10 ml in 0.1 M NaCl containing 0.5% gum arabic. The substrate mixture was sonicated for 1 min (b, d) or shaken vigorously 100 times by hand (a, c). At 0 min cholate (B) (0 mM : a, b and 5 mM (final): c, d) was added and at 2 min 1.25 μg carboxylester lipase (E) was added.

spectively. The average surface areas of sonicated and shaken tributryin were 49.9 ± 12.4 $mm^2/\mu mol$ tributyrin (mean ± SD), and 2.13 ± 3.04 $mm^2/\mu mol$ tributyrin (mean ± SD), respectively. **Fig. 4** shows the effect of substrate surface area on the rate constant for inactivation. The rate constant and the surface area closely correlated with each other.

Cholate protected the enzyme from this inactivation at the tributyrin/water interface. The protective effect of cholate was related to the concentration of both cholate and tributyrin (**Fig. 5**). The concentration of tributyrin at $k_i = 5.0 \times 10^{-3}\ s^{-1}$ increased with increased cholate concentration: 7.2 mM tributyrin without cholate, 9 mM tributyrin with 0.05 mM cholate, 30 mM tributyrin with 0.1 mM cholate, and 150 mM tributyrin with 0.2 mM cholate. In the presence of 1.0 mM cholate, k_i was below $1.0 \times 10^{-3}\ s^{-1}$ into 300 mM tributyrin.

Fig. 6 shows the effect of pH on the rate constant for inactivation. The rate constant increased sharply above pH 7.0, and cholate also caused dose-dependent protection against inactivation. The protection by cholate was greater at an acidic pH than at an alkaline pH. When the enzyme was incubated at various pH values for 1 h at 37°C without tributyrin or cholate, about 95% of the original enzyme activity was retained between pH 5 and pH 7.5 (Fig. 6, insert). Apparent pKa values were changed by addition of cholate (pH 5.4 and 7.4 at 0 mM cholate; pH 5.4 and 8.5 at 1 mM cholate; and pH 5.7 and 9.0 at 2 mM cholate).

As shown in **Fig. 7**, when cholate was added at 2 min to 100 mM sonicated tributyrin and the enzyme was added at 4 min, tributyrin hydrolysis proceeded lineally without any appreciable lag period (Fig. 7, b). When the enzyme was added to the reaction mixture at 2 min and 4 min and cholate was added at 6 min, no tributyrin hydrolysis was observed. But on further addition of enzyme at 8 min, tributyrin hydrolysis occurred at the same rate as in Fig. 7,b (Fig. 7, a). When cholate and enzyme were added at the same time (at 2 min), a time-dependent decrease in velocity was observed (Fig. 7, c). No enzyme activity for *p*-nitrophenyl butyrate was observed after incubation of 100 mM of sonicated tributyrin for 10 min at 37°C without cholate. However, when the enzyme was incubated with 100 mM tributyrin in the presence of 5 mM cholate, 70% of the enzyme activity was retained (data not shown).

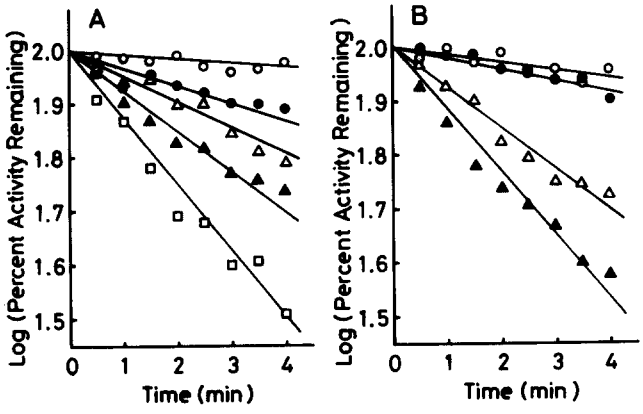

Fig. 3. Effect of tributyrin concentration on the stability of carboxylester lipase. The incubation mixture consisted of the indicated amount of tributyrin and 1.25 μg carboxylester lipase in 10 ml of 0.1 M NaCl containing 0.5% gum arabic. The substrate mixture was sonicated for 1 min (A: tributyrin concentration (○) 2 mM; (●) 4 mM; (△) 5 mM; (▲) 6 mM; and (□) 7 mM) or shaken vigorously 100 times by hand (B: tributyrin concentration (○) 5 mM; (●) 100 mM; (△) 200 mM, and (▲) 300 mM).

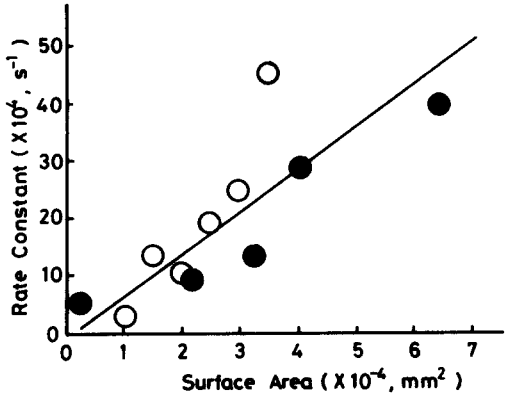

Fig. 4. Effect of surface area on the rate constant for inactivation of carboxylester lipase. Experimental conditions are described in the legend to Fig. 3. (○) Sonicated tributyrin; (●) shaken tributyrin.

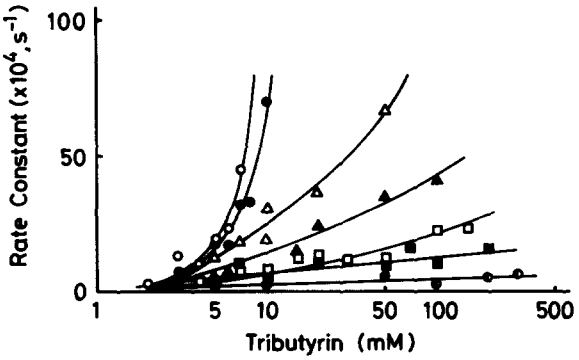

Fig. 5. Effects of cholate and tributyrin concentrations on the apparent rate constant for the inactivation of carboxylester lipase. The incubation mixture consisted of the indicated amount of tributyrin,, 1.25 μg carboxylester lipase and cholate ((○) 0 mM; (●) 0.05 mM; (△) 0.1 mM (▲) 0.2 mM; (□) 0.3 mM; (■) 0.4 mM; and (◐) 0.5 mM) in a final volume of 10 ml in 0.1 M NaCl containing 0.5% gum arabic. The substrate mixture was sonicated for 1 min.

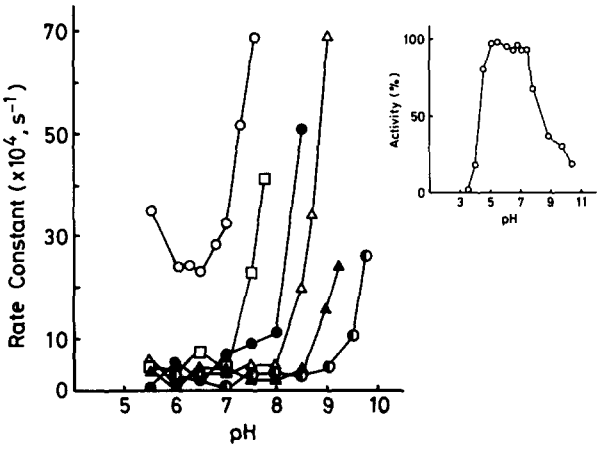

Fig. 6. Effect of pH on the rate constant for inactivation of carboxylester lipase. The incubation mixture consisted of 5 mM tributyrin, 1.25 μg carboxylester lipase and cholate ((○) 0 mM; (□) 0.2 mM; (●) 0.5 mM; (△) 1 mM; (▲) 2 mM; and (◐) 5 mM) in a final volume of 10 ml in 0.1 ml in 0.1 M NaCl containing 0.5% gum arabic. The substrate mixture was sonicated for 1 min. The insert shows the pH stability curve for carboxylester lipase. The enzyme (1.25 μg) was incubated at 37°C in a final volume of 10 ml in 0.1 M NaCl containing 0.5% gum arabic at the indicated pH, and after 1 h incubation, residual enzyme activity was measured with *p*-nitrophenyl butyrate as substrate.

Fig. 8 shows an Arrhenius plot of k_i over the range of 27°C–40°C. From the slope of the line, the energy of activation was calculated to be 33.7 cal · mol^{-1}. Values for $\Delta G^{\ddagger}$, $\Delta H^{\ddagger}$, and $\Delta S^{\ddagger}$ can be calculated assuming that the transition state theory is applicable. At 37°C the values obtained were 22 kcal · mol^{-1}, 33.1 kcal · mol^{-1}, and 35.8 cal · mol^{-1} deg^{-1}.

The effects of different bile salts on surface inactivation of carboxylester lipase are shown in **Fig. 9**. Bile salts caused dose-dependent protection of the enzyme from inactivation at the surface, and the protection effects of 7α-dehydroxy bile salts (deoxycholate and taurodeoxycholate) were greater than those of trihydroxy bile salts (cholate and taurocholate). The protection by bile salts was not related to their critical micellar concentrations. The critical micellar concentrations of bile salts in 0.1 M NaCl at 37°C were estimated as 2–3.5 mM for cholate, 1–2 mM for taurocholate, 0.8–1.0 mM for deoxycholate, and 0.7–1.7 mM for taurocholate. **Fig. 10** shows the effect of different bile salts on the initial rate of tributyrin hydrolysis. The initial rate of hydrolysis was increased 20–30% by an increase in the bile salt concentration.

Other detergents also protected the enzyme from interfacial inactivation (**Table 2**). Various initial rates of hydrolysis were observed by addition of different detergents. but the k_i values remained below 1.0 × 10^{-3} s^{-1}.

DISCUSSION

Carboxylester lipase was rather stable in buffer solution at pH 5–7, but when insoluble fat such as tributyrin emulsified with gum arabic was added, the enzyme activity was rapidly lost. The rate constant for enzyme inactivation increased with increase in tributyrin concentration. An apparent rate constant for inactivation, k_i of 2.38 × 10^{-3} s^{-1} was observed with 6 mM sonicated tributyrin. When the substrate was prepared by shaking the mixture by hand (shaken substrate), a similar k_i (2.88 × 10^{-3} s^{-1}) was observed at 30-fold higher concentration of tributyrin (200 mM) (Fig. 3). However, if the substrate concentration was expressed as the surface area in square millimeters, the rate constant and the surface area closely correlated with each other at both sonicated and shaken emulsions (Fig. 4). These results suggest that the surface inactivation of the enzyme is related to the surface area of substrate and not related to the amount of substrate. The high rate constant for inactivation might explain the absence of activity

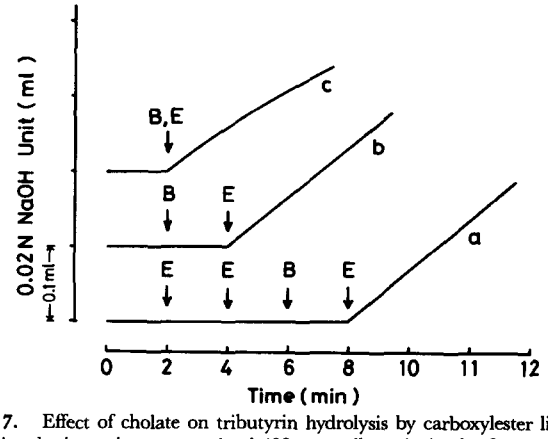

Fig. 7. Effect of cholate on tributyrin hydrolysis by carboxylester lipase. The incubation mixture contained 100 mM tributyrin in the final volume of 10 ml in 0.1 M NaCl containing 0.5% gum arabic. Carboxylester lipase (1.25 μg) (E) and cholate (final 5 mM) (B) were added at the indicated times.

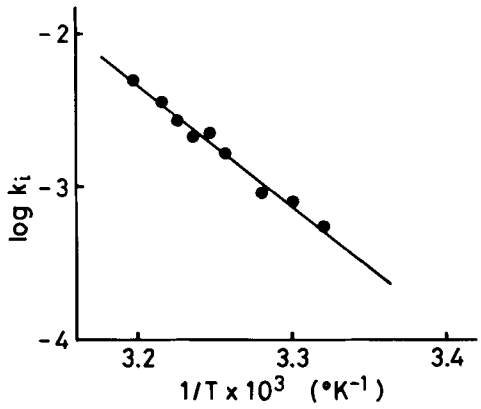

Fig. 8. Arrhenius plot of the rate constant of carboxylester lipase inactivation (k_i). The incubation mixture consisted of 5 mM tributyrin and 1.25 μg carboxylester lipase in 10 ml of 0.1 M NaCl containing 0.5% gum arabic. The substrate mixture was sonicated for 1 min.

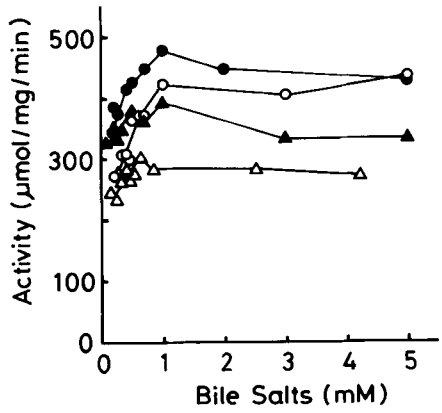

Fig. 10. Effect of bile salts on the initial rate of tributyrin hydrolysis by carboxylester lipase. The conditions of incubation were as described in the legend of Fig. 9 ((○) cholate; (●) taurocholate; (△) deoxycholate; (▲) taurodeoxycholate). The initial rate of tributyrin hydrolysis was calculated by the equation $V_0 = V/e^{(-k_i \cdot t)}$.

of the enzyme on emulsified hydrophobic substrates; no activity was observed with emulsified triolein, diolein, tricaprin, or cholesteryl oleate (Table 1). Human carboxylester lipase and bile salt-activitate lipase are also reported to show no activity with such substrates (16–18): Lombardo, Fauvel, and Guy (16) observed that human carboxylester lipase did not hydrolyze emulsions of olive oil or triolein, and Wang et al. (17, 18) found that long-chain triacylglycerols were not hydrolyzed by bile salt-activated lipase in the absence of bile salt.

Proteins are known to be denatured at a lipid-water interface (19, 20). When a protein in an aqueous phase is adsorbed to an interphase, it may undergo conformational change and, in the case of an enzyme, this change may induce irreversible inactivation. This is the case with carboxylester lipase (Fig. 8). The large energy of activation (33.7 kcal · mol^{-1}) and entropy change (35.8 cal · mol^{-1} · deg^{-1}) for inactivation suggest that the observed inactivation reflects denaturation of the enzyme protein at the surface. The values for energy of inactivation obtained by studies on thermal denaturation of carboxylester lipase are in good agreement with those determined from studies on surface denaturation of pancreatic lipase (21).

Bile salts stabilized carboxylester lipase against the surface inactivation. The rate constant for surface inactivation increased above pH 7.0. Bile salts caused dose-dependent protection of the enzyme from surface inactivation, but their protective effects were greater at acidic pH than at alkaline pH (Fig. 6). These results are consistent with the previous finding that bile salts interacted directly with

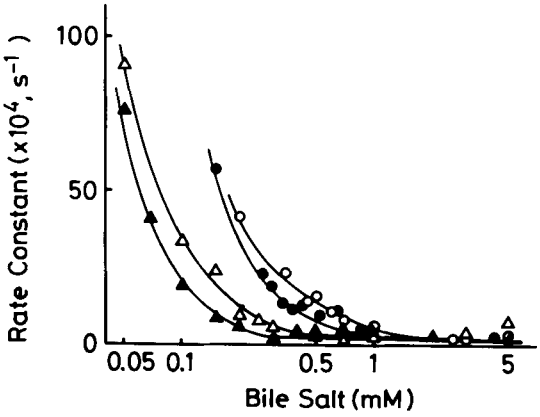

Fig. 9. Effect of bile salts on the rate constant of carboxylester lipase inactivation. The incubation mixture consisted of 100 mM tributyrin, 1.25 μg carboxylester lipase, and the indicated amount of bile salts ((○) cholate; (●) tarocholate; (△) deoxycholate; (▲) taurodeoxycholate) in 10 ml of 0.1 M NaCl containing 0.5% gum arabic. The substrate mixture was sonicated for 1 min.

TABLE 2. Effects of various detergents on the hydrolysis of tributyrin by pancreatic carboxylester lipase

Detergents	Activity	$k_i \times 10^4$
	μmol/mg/min	s^{-1}
None	0	
Cholate	421 ± 7	4.8 ± 1.5
Taurocholate	287 ± 10	5.4 ± 0.7
Deoxycholate	336 ± 13	2.6 ± 1.7
Taurodeoxycholate	477 ± 20	5.5 ± 0.9
Brij-35	387 ± 13	5.3 ± 1.7
Triton X-100	333 ± 2	0.22 ± 0.22
SDS	253 ± 8	10 ± 1
Soya PC[a]	185 ± 4	1.9 ± 1.1

The incubation mixture consisted of 100 mM tributyrin, 1.25 μg carboxylester lipase, and detergent (1 mM) in 10 ml of 0.1 M NaCl containing 0.5% gum arabic. The substrate mixture was sonicated for 1 min. The activity (initial rate of tributyrin hydrolysis) was calculated from the equation $V_0 = V/e^{(-k_i \cdot t)}$. Values are means ± SE for four separate assays.

[a]Phosphatidylcholine

carboxylester lipase by a simple 1:1 interaction far below the critical micellar concentration, and the binding affinity of bile salts to carboxylester lipase is greatly reduced at higher pH (8). The surface excess of cholate did not change over pH 7–9 (data not shown). These results suggest that bile salt binding to enzyme was probably the dominant protection factor. There are reports that bile salts also stabilize pancreatic lipase (15, 22) and bile salt-activated lipase (23), and from these findings it was concluded that bile salts prevent denaturation of enzyme proteins at an oil-water interface. Like bile salts, other detergents such as Triton X-100, Brij-35, and SDS decreased the rate constant for surface inactivation (Table 2).

The protection by bile salts is related both to their concentration and to the tributyrin concentration (Fig. 5). Why is the effective concentration of bile salts related to the tributyrin concentration? Two possibilities may be considered. *1*) Earlier studies suggested that surface pressure (free enrgy) is the dominant factor for surface denaturation (3). In studies using a lipid-film, carboxylester lipase was rapidly denatured at lower surface pressure (below 20 mN/m), but the denaturation was less at higher surface pressure (up to 30 mN/m). The surface pressure of tributyrin emulsion presumably increases when bile salts are adsorbed to the tributyrin-water interface, and the effect of bile salts on the surface pressure might be related to their concentration on the surface. *2*) In the presence of emulsified substrate, bile salts are adsorbed to the substrate and the effective concentration of bile salts for binding to enzyme is decreased.

The bile salt concentrations for protection and the critical micellar concentrations of the bile salts varied in parallel (Fig. 9). The protection effects of bile salts without an hydroxyl at the 7α-position were about three times those of trihydroxy bile salts, and the critical micellar concentrations of dehydroxy bile salts are about two- to three times less than those of trihydroxy bile salts. However, this parallelism does not prove that micelles of bile salts are directly involved in the protective process. The pattern of bile salt protection did not change markedly at their critical micellar concentrations.

Bile salts stimulate the activity of pancreatic lipase either by stablizing lipase at the surface, or by removing the long-chain insoluble fatty acid from the surface, but at high concentration, bile salts inhibit the lipase activity by preventing the adsorption of the enzyme to the surface of its substrate (15, 21). However, the initial rate of hydrolysis of tributyrin by carboxylester lipase was not decreased by addition of high concentrations of bile salts of above the critical micellar concentration (Fig. 10). The effect of bile salts on carboxylester lipase activity is very complex. Lombardo et al. (5, 16, 24) reported the effects of bile salts on the activities of carboxylester lipase on different substrates. They found that bile salts increased the activity of human carboxylester lipase on tributyrin and *p*-nitrophenyl acetate and inhibited its activities on dioctanoylphosphatidylcholine and triacetin. They explained these two opposite effects by supposing that bile salts had a direct effect on the enzyme activity as well as acting as detergents. We also observed similar phenomena. The activity of pig pancreatic carboxylester lipase on a water-soluble ester, *p*-nitropheynl butyrate, was increased about threefold by addition of bile salts (data not shown). Bile salts also increased the activity on tributyrin (Fig. 1B) but inhibited the activity on water-soluble triacetin (20 mM) (data not shown). Wang (25) also reported that the *p*-nitrophenyl acetate hydrolyzing activity of bile salt-activated lipase was increased by addition of bile salts.

In the present investigation, we found that pig carboxylester lipase is denatured at the surface of an emulsified substrate and that bile salts protect the enzyme from surface denaturation. Pancreatic carboxylester lipase is closely related to carboxylester lipase in pancreatic juice and to bile salt-activated lipase in milk (4, 26). The absence of activities of these enzymes on emulsified substrates may be explained by their denaturation at the substrate surface. The data obtained so far suggest that an important role of bile salts in vivo in physiological conditions (in which the bile salt concentration is 2–10 mM and the substrates are long chain fatty acyl glycerols or cholesteryl esters) is to stabilize lipase at lipid-water interfaces.

We would like to thank Prof. Howard L. Brockman (The Hormel Institute, University of Minnesota) for his valuable suggestions.

Manuscript received 17 August 1989 and in revised form 30 November 1989.

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