Nonspecific high affinity binding of bile salts to carboxylester lipases

T. Tsujita, N. K. Mizuno, and H. L. Brockman[1]

The Hormel Institute, University of Minnesota, Austin, MN 55912

Abstract The interactions with bile salts of carboxylester lipases (EC 3.1.1.13) from human pancreatic juice and pig pancreas were characterized by physical methods. Bile salts cause a decrease in the fluorescence intensity of the proteins at the emission maximum of 333–335 nm. The concentration dependence of this decrease shows saturation behavior, is relatively nonspecific with respect to bile salt conjugation or the presence of the 7α-hydroxyl group, and is consistent with a 1:1 interaction between enzyme and bile salt. Direct measurement of the binding of [³H]cholate by equilibrium dialysis supports the stoichiometry. Other detergents also bind, causing fluorescence changes, but with much lower affinities. Binding of taurocholate to the monomeric pig enzyme is enhanced by increasing ionic strength, indicating the predominance of hydrophobic interactions. In the range of pH 5.5–6.8, binding is pH-independent with dissociation constants of 3–20 μM. At higher pH, affinity is greatly reduced and the fluorescence spectrum changes, indicating the importance of a protonated group for efficient interaction. Occupancy of the bile salt binding site partially stabilizes the enzyme against inactivation by heat but not trypsin. However, circular dichroism spectra do not indicate that bile salt binding is accompanied by any change in secondary structure. The monomeric pig enzyme binds to the argon/water interface in the presence of bile salts and binding of taurocholate to diisopropylphosphoryl-enzyme is similar to that measured with native enzyme. ■■ These results suggest that surface binding and catalysis occur at sites distinct from the bile salt binding site of the enzyme. Stabilization of the monomeric pig enzyme against denaturation at high energy surfaces occurs concomitantly with occupancy of the bile salt binding site. Overall, the data suggest that an important role of bile salts in vivo is to stabilize these enzymes at lipid–water interfaces.— Tsujita, T., N. K. Mizuno, and H. L. Brockman. Nonspecific high affinity binding of bile salts to carboxylester lipases. *J. Lipid Res.* 1987. **28:** 1434–1443.

Supplementary key words circular dichroism • equilibrium dialysis • fluorescence spectra • porcine pancreatic cholesterol esterase • human pancreatic juice cholesterol esterase

In the gut, lipolytic enzymes function on emulsified substrates in the presence of bile salts. Functionally, the bile salts may contribute little to substrate emulsification (1), their primary role being to facilitate the diffusion of lipolysis products to the intestinal wall (2). Bile salts are also implicated in the regulation of lipolysis itself. At micellar concentrations they bind to pancreatic colipase

in vitro (3) and stabilize pancreatic lipase against inactivation at high energy surfaces (4). There is also a body of literature suggesting that bile salts are important in regulating the activity of pancreatic carboxylester lipase (cholesterol esterase EC 3.1.1.13). Although the bile salts may, in part, be acting through their effects on substrate properties (5), some data indicate a specificity with respect to the presence of the 7α-hydroxyl group on the bile salt molecule. This implies that they are acting not simply as detergents but as specific ligands.

The specificity of interaction of human pancreatic carboxylester lipases with bile salts has been deduced primarily from their effects on catalysis (6, 7) and protein modification (8, 9). From this work a complex, two-site hypothesis has been proposed. Briefly, monomeric primary and secondary bile salts can interact with an unspecific or premicellar site, thereby activating the catalytic site toward water-soluble substrates. For sodium taurodeoxycholate and cholate this occurs with dissociation constants of 0.5 and 3.5 mM, respectively (9). Interaction of the enzyme with monomers of bile salts with 7α-hydroxylation, i.e., taurocholate or taurochenodeoxycholate, at 0.5 mM induces enzyme dimerization (7) which, it is postulated, converts the premicellar site to a micellar binding site. This in turn activates the enzyme toward the hydrolysis of substrates dispersed in bile salt micelles (9). In contrast, another study (10) suggests that taurocholate is simply a nonessential activator with a dissociation constant of 0.2 mM. Similarly, studies of the immunologically related and kinetically similar (11) bile salt-stimulated lipase of human milk suggest a single binding site for taurocholate with a dissociation constant of 0.37 mM (12). Although equilibrium dialysis measurements were performed, binding stoichiometry was not given but is presumed to be 1:1. Other kinetic studies of

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Abbreviations: CHAPS, 3-[3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate; Zwittergent 3-12, N-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate; octylglucoside, n-octyl-β-D-glucopyranoside.

[1]To whom reprint requests should be addressed at: The Hormel Institute, University of Minnesota, 801 16th Avenue NE, Austin, MN 55912.

the milk lipase support the lack of specificity for bile salt binding (13) but do not indicate a direct role for bile salts as activators at the catalytic site (14).

In all of the studies noted above, the half maximal effects of bile salts occurred at concentrations ≥ 0.2 mM. In contrast, earlier studies of the activation of hydrolysis of soluble substrates by bile salts showed marked stimulation at far lower concentrations (15, Fig. 4; 16, Fig. 3). For example, data abstracted from the latter reference show classical saturation behavior when replotted using a linear abscissa. Fitting the data sets to an equation of the Michaelis-Menten form using the method described herein gives apparent activation constants of 21 μM for taurodeoxycholate and 8 μM for taurocholate. Moreover, the monomeric form of rat pancreatic juice carboxylester lipase, the first such enzyme to be isolated, reportedly binds [24-^{14}C]cholic acid in stoichiometric amounts at 1 μM (17). A source of the disagreement among all but the last of the previous studies is the requirement for the presence of substrates or chemical modifiers to detect enzyme–bile salt interactions. That many of the reported effects occur at concentrations near the critical micelle concentrations of the bile salts further complicates interpretation. This occurs because of the tendency of bile salts to aggregate in a step-wise fashion (18) and because of the possible formation of mixed micelles with the substrates and protein-modifying reagents. To better understand the role of bile salts in regulating carboxylester lipases, we have examined enzyme–bile salt interactions in the absence of substrates and other amphipathic compounds. The results for the human and pig enzymes reveal a stoichiometric interaction occurring at micromolar concentrations, as implied by the earlier literature. The data further suggest that an important consequence of this relatively nonspecific binding is to stabilize the enzyme at high energy surfaces.

MATERIALS AND METHODS

Materials

Benzamidine hydrochloride, 3-phenylpropionic acid (hydrocinnamic acid), N-α-D, L-arginine, p-nitrophenyl acetate, bovine trypsin, piperazine, sodium azide, 3-[(3-cholamidopropyl)-dimethylammoniol]-1-propanesulfonate (CHAPS), and sodium taurocholate were from Sigma Chemical Company (St. Louis, MO). N-dodecyl-N,N-di-methyl-3-ammonio-1-propanesulfonate (Zwittergent 3-12), and n-octyl-β-D-glucopyranoside (octylglucoside), diisopropylfluorophosphate, sodium cholate, sodium deoxycholate, and sodium taurodeoxycholate were obtained from Calbiochem (La Jolla, CA). Tert-butanol was from MCB (Germany). Decanoic acid was from Nu Chek Prep, Inc. (Elysian, MN). Precast

PAA 4/30 gradient polyacrylamide gels, and Mono P chromatofocusing column were from Pharmacia, Inc. (Piscataway, NJ). Dye-binding protein assay reagent, bovine serum albumin standard, and sodium dodecylsulfate molecular weight standards were from Bio-Rad Laboratories (Richmond, CA). [1,3-^{3}H]Diisopropylfluorophosphate, ^{32}P (as orthophosphoric acid in HCl-free water solution), and [2,4-^{3}H(N)]cholic acid were from NEN (Boston, MA). Human pancreatic juice collected over dry ice was a generous gift from Drs. James Meyer and Nabeel Adham of the Veterans Administration Medical Center, Sepulveda, CA.

Methods

Carboxylester lipase activity. Activity was monitored by measuring spectrophotometrically the release of p-nitrophenolate ion from its acetate ester as previously described (19).

Protein determination. The dye-binding procedure of Bradford (20) was used with bovine serum albumin as a standard. As noted earlier the results of this assay must be multiplied by 1.75 or 2.04 to obtain an equivalent dry weight for the porcine monomeric and dimeric species, respectively (21). A factor of 1.75 was assumed for the human pancreatic juice enzyme in reporting weights of proteins used in this study.

Enzyme purification. Pig pancreatic carboxylester lipase was purified to homogeneity as previously described. The procedure yields two distinct forms, a monomer of molecular weight 74,000 and a dimer of 167,000 (21). The carboxylester lipase of human pancreatic juice was purified by a modification of the method used for the pig enzyme. A 23-ml sample of pancreatic juice was lyophilized and redissolved with 9 ml/g of powder in 50 mM potassium phosphate buffer, pH 6.0, containing 100 mM potassium chloride, 5 mM sodium taurocholate, 2 mM benzamidine hydrochloride, 2.0 mM hydrocinnamic acid, and 0.5 mM N-α-benzoyl-DL-arginine. After centrifugation at 19,000 g for 30 min to remove insoluble material, the sample was applied to a 2.15 × 30 cm TSK 3000SW column equilibrated in the same buffer. Following elution at 3.0 ml/min, the active fractions were pooled, cooled to 4°C, and the enzyme was precipitated by the addition of 1.5 volumes of tert-butanol–water 95:5 (v/v) at 4°C. Following the centrifugation as above, the resulting precipitate was dissolved in 50 mM piperazine, 100 mM sodium acetate to pH 6.0 with acetic acid. After centrifugation to remove insoluble material, the sample was applied to a 0.5 × 20 cm Mono P column (Pharmacia) equilibrated with the same buffer. The column was washed with 4 ml of the buffer followed by a 20-ml, linear sodium acetate gradient to 1.0 M. The active fraction obtained contained 60% of the activity in the reconstituted juice and was homogeneous as judged by sodium dodecylsulfate-polyacrylamide gel electrophoresis (21) using Pharmacia PAA 4/30 gels. The

specific activity, based on the colorimetric assay procedure and the dye-binding protein assay, was 6,500 μmol/hr per mg of protein. The enzyme was stable for several months at $-70°C$.

Equilibrium dialysis. Binding of [³H]cholate to monomeric pig carboxylester lipase was measured in a dialysis cell (Bolab model 287) consisting of two 1-ml chambers separated by a dialysis membrane of 12,000–14,000 molecular weight cutoff (Spectrum Medical Industries, Inc.). One ml of enzyme solution [5.94 mg in 50 mM sodium phosphate, pH 6.8, containing 0.10 M sodium chloride and 0.02% sodium azide (w/v)] was placed in one compartment and 1.0 ml of the buffer solution containing 367 μM [³H]cholate (6 Ci/mol) in the other. The apparatus was gently shaken at 130 cycles/min at 25°C and 0.01-ml aliquots were withdrawn at intervals for determination of [³H]cholate by scintilla tion counting. Measurements of activity demonstrated that the enzyme was stable and remained in one compartment.

Modification of carboxylester lipase. The monomeric pig enzyme was converted to its catalytically inactive form using [³H]diisopropylfluorophosphate as previously described (22).

Trypsin inactivation of carboxylester lipase. Inactivation was carried out at 37°C by the procedure of Bläckberg and Hernell (23) with the exception that 50 mM phosphate buffer containing 0.10 M sodium chloride was used at a pH of 7.4. Carboxylester lipase concentration in all incubations was 0.24 mg/ml. At intervals, aliquots were withdrawn for determination of activity remaining. From data describing the loss of 80% of the initial activity, pseudo-first order rate constants were calculated from plots of log of activity remaining versus time. Control experiments showed that even after the loss of 80% of carboxylester lipase activity, trypsin accounted for $\leq 2\%$ of the measured activity toward p-nitrophenylacetate.

Thermal inactivation of carboxylester lipase. Samples of the human and pig enzymes were incubated at the desired temperature in 50 mM sodium phosphate buffer, pH 6.8, containing 0.10 M sodium chloride, 0.02% sodium azide (w/v), and bile salt. Inactivation rate constants were determined as described above.

Fluorescence measurements. Fluorescence spectra were obtained with an SLM 8000DS spectrofluorimeter at 25°C unless otherwise indicated. Enzyme at 0.12–0.19 mg/ml in 50 mM sodium phosphate was stirred and fluorescence spectra were recorded using an excitation wavelength of 277 nm. Successive aliquots of concentrated bile salt in the same buffer were added and a spectrum was recorded after each addition. For data analysis fluorescence intensity was corrected for the dilution due to addition of bile salt solution. Unless otherwise indicated, sodium chloride was present at 0.10 M and the buffer was 50 mM potassium phosphate at pH 6.8.

Analysis of fluorescence data. Changes in fluorescence intensity of the carboxylester lipase (E) induced by bile salts and other detergents (D) were fitted to a simple interaction model of the form

$$E + D \overset{K_d}{\rightleftharpoons} E - D$$
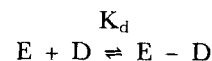
where $K_d = [E-D]/[E][D]$. When the total concentrations of enzyme and detergent are denoted by $[E_o]$ and $[D_o]$, the corrected fluorescence intensity decrease by $\triangle F$, and the fluorescence intensity in the absence of detergent by F_o, it is readily shown that the fractional fluorescence decrease, $\triangle F/F_o$, is related to enzyme and detergent concentrations by the quadratic relationship

$$\left(\frac{\triangle F}{F_o}\right) = \left(\frac{\triangle F}{F_o}\right)_{max}$$

$$\frac{([D_o] + [E_o] + K_d \pm \sqrt{([D_o] + [E_o] + K_d)^2 - 4[E_o][D_o]})}{2[E_o]}$$

The parameters $(\triangle F/F_o)_{max}$ and K_d were obtained for measured values of $\triangle F/F_o$, $[E_o]$, and $[D_o]$ using a fitting program, SSQMIN, kindly provided by Dr. S. Bryant of the University of Cincinnati. The sign of the square root term was determined by the condition that $0 \leq [E-D] \leq [E_o]$. The program uses a finite difference Levenberg-Marquardt least squares algorithm (24, 25) and standard deviations of optimum parameters are calculated from a variance/covariance matrix by the method of Hamilton (26).

Adsorption of carboxylester lipase to interfaces. Rates of enzyme adsorption to the argon/buffer interface at 24°C were measured using a multiprocessor, interfacial monitor/controller as previously described (22). Briefly, the enzyme was injected into an aqueous subphase to give a concentration of 6 nM and stirred at 60 rpm for 10 min after which the surface phase, containing adsorbed enzyme, was collected using hydrophobic paper. For experiments with native enzyme, the paper was eluted with detergent solution and enzyme activity was measured colorimetrically. With the modified radioactive enzyme, the paper was cut into pieces, placed in a vial, and radioactivity was determined by scintillation counting. Carryover of enzyme in the aqueous subphase recovered with the paper was corrected for by inclusion of [³²P]phosphate in the aqueous phase.

RESULTS

In an earlier study (10), it was stated that supramicellar taurocholate could induce a small decrease in the
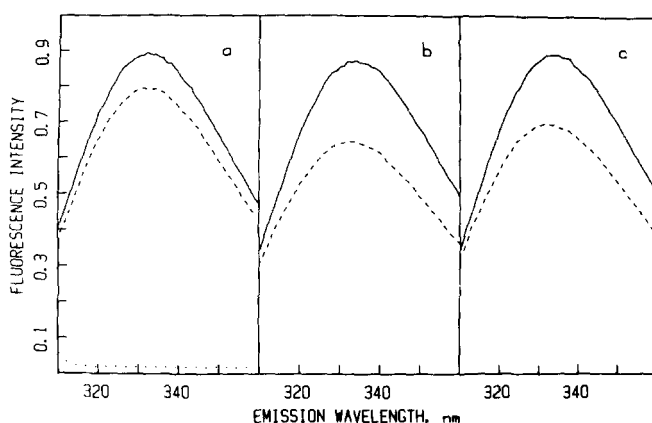
fluorescence of human pancreatic and milk carboxylester lipases. Because this change occurs in the absence of substrates or other effectors of enzyme activity, the data suggested that intrinsic protein fluorescence could be useful for monitoring the nature and specificity of interactions of the juice enzyme with bile salts. Also of interest were the monomeric and dimeric pig carboxylester lipases. In contrast to the human enzymes, to which they are related, the two distinct porcine forms are well-behaved proteins with low carbohydrate contents (21). In the presence of taurocholate at >200 μM, each of the three enzymes exhibited a decrease in intrinsic fluorescence which was not accompanied by a change in the wavelength of the emission maximum, 333–335 nm (**Fig. 1**). Fluorescence was not decreased by further addition of taurocholate up to millimolar concentrations; bile salt alone showed no fluorescence in this spectral region (Fig. 1); and addition of buffer alone caused no change beyond that expected from dilution. The decreases ranged from 12 to 26% of total fluorescence intensity after correction for dilution due to addition of the bile salt (**Table 1**).

When measured as a function of bile salt concentration, the volume-corrected fluorescence decreases, expressed as a fraction of the fluorescence intensity in the absence of bile salt, showed saturation behavior as exemplified in **Fig. 2**. Fitting such data to a simple 1:1 interaction model yielded the binding parameters listed in Table 1. For all three enzyme species the dissociation constants were 12–19 μM. Because of reports in the literature suggesting the specificity of bile salt–carboxylester lipase interactions, similar experiments were carried out using sodium cholate, sodium deoxycholate, and sodium taurodeoxycholate. All three enzyme species interacted with all four bile salts, yielding dissociation constants between 2 and 20 μM and fractional fluorescence decreases of 0.11–0.26.

Fig. 1. Effects of taurocholate on fluorescence spectra of carboxylester lipases. Excitation wavelength 277 nm; (——), enzyme alone; (------) enzyme plus taurocholate; (·····), taurocholate alone. a) Human, 227 μM taurocholate; b) pig monomer, 242 μM taurocholate; c) pig dimer, 208 μM taurocholate.

TABLE 1. Binding parameters for the interaction of carboxylester lipases with bile salts

Enzyme	Taurocholate		Taurodeoxycholate		Cholate		Deoxycholate	
	K_d[a]	$\left(\dfrac{\Delta F}{F_o}\right)_{max}$[a,b]	K_d[a]	$\left(\dfrac{\Delta F}{F_o}\right)_{max}$[a,b]	K_d[a]	$\left(\dfrac{\Delta F}{F_o}\right)$[a,b]	K_d[a]	$\left(\dfrac{\Delta F}{F_o}\right)_{max}$[a,b]
	μM		μM		μM		μM	
Pig pancreas, monomer	12 ± 0.60[c]	0.26 ± 0.004	6.2 ± 0.33	0.22 ± 0.0027	8.4 ± 0.8	0.24 ± 0.0064	2.6 ± 0.15	0.22 ± 0.0029
Pig pancreas, dimer	14 ± 0.42	0.24 ± 0.002	8.7 ± 0.41	0.21 ± 0.0028	9.3 ± 0.4	0.23 ± 0.0027	2.5 ± 0.11	0.23 ± 0.0023
Human juice	19 ± 1.00	0.12 ± 0.002	4.6 ± 0.96	0.13 ± 0.0050	18.0 ± 1.4	0.11 ± 0.0026	14.0 ± 1.20	0.13 ± 0.0027

[a]Conditions were as described in Fig. 2.
[b]Maximum fractional decrease in fluorescence intensity.
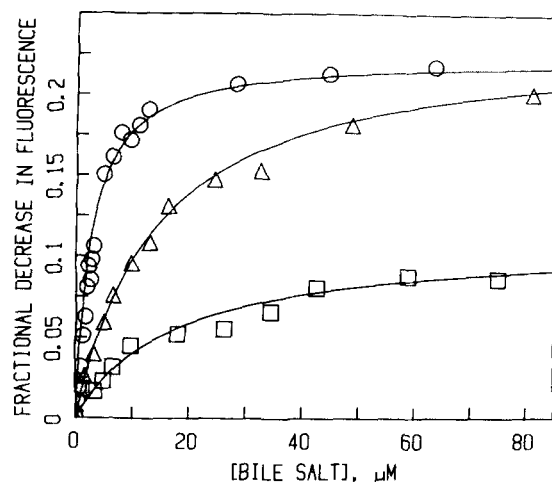[c]Standard deviation calculated as described in Methods.

Fig. 2. Examples of the dependence on bile salt concentration of the fractional decrease in intrinsic fluorescence intensity of carboxylester lipase. Pig monomer with deoxycholate, (O); human with cholic acid, (□); pig dimer with taurocholate, (△).

As shown in Table 1 the variation in dissociation constants indicates some specificity for the interaction with each enzyme species. However, the constancy of the maximal fluorescence change for each enzyme species suggests a common site of interaction. To test this hypothesis, taurocholate was added to a sample of the monomeric pig enzyme to a concentration of 1.17 mM. This concentration is 90 times the dissociation constant for the bile salt and produced a fractional fluorescence decrease of 0.22. To this, cholate was added at 1.05 mM, a level 120 times its dissociation constant. In this case the fractional fluorescence decrease was only 0.007. This experiment

was also performed using taurocholate at 0.9 mM followed by taurodeoxycholate at 0.83 mM, yielding a similar result. Thus, both conjugated and unconjugated, dihydroxy and trihydroxy bile salts do not appear to interact at separate sites on the enzyme, but at the same site.

The specificity of the interaction with respect to non-bile salts was investigated using octylglucoside, decanoic acid, Zwittergent 3-12, and CHAPS. All interacted with the porcine enzyme to produce fluorescence decreases that were saturable and were maximal at the same emission wavelength as when bile salts were used. However, **Table 2** shows that, except for decanoate, the values of the maximal changes were about half that observed with bile salts and the dissociation constants were one to two orders of magnitude higher. Thus, the interaction is not unique to bile salts, although they are preferred.

The effects of experimental conditions on the binding of bile salts to the monomeric pig enzyme are shown in Table 2. As ionic strength is increased the maximal change in fluorescence decreases, whereas the affinity between enzyme and bile salt is increased about 20-fold relative to buffer alone. The temperature dependence of binding was determined between 15° and 30°C. As shown in Table 2, both binding parameters are relatively independent of temperature in this range. The pH dependence of the interaction was studied from pH 5.5 to 8.4. From pH 5.5 to 6.8 the binding parameters are invariant with either cholate or taurocholate (Tables 1 and 2). At pH values of 7.1 and 7.4 the data were well described by the simple binding model but showed an apparent increase in the dissociation constant and decrease in max-

TABLE 2. Effect of experimental conditions on binding parameters for the interaction of surfactants with monomeric pig carboxylester lipase

[NaCl]	pH	T, °C	Surfactant	K_d	$\left(\dfrac{\Delta F}{F_0}\right)_{max}$[a]
				μM	
0.10	6.8	25	octylglucoside	640 ± 70[b]	0.096 ± 0.0033[b]
0.10	6.8	25	CHAPS	110 ± 13	0.10 ± 0.0020
0.10	6.8	25	decanoate	1600 ± 130	0.19 ± 0.0054
0.10	6.8	25	Zwittergent 3-12	400 ± 93	0.091 ± 0.0064
2.00	6.8	25	taurocholate	5.0 ± 0.57	0.13 ± 0.0032
1.00	6.8	25	taurocholate	15 ± 0.92	0.13 ± 0.0024
0.50	6.8	25	taurocholate	24 ± 0.85	0.20 ± 0.0024
0.00	6.8	25	taurocholate	110 ± 3.5	0.28 ± 0.0032
0.10	6.8	15.1	taurocholate	16 ± 0.60	0.18 ± 0.0018
0.10	6.8	19.8	taurocholate	21 ± 1.4	0.23 ± 0.0045
0.10	6.8	29.5	taurocholate	16 ± 0.66	0.20 ± 0.0021
0.10	5.5	25	taurodeoxycholate	6.4 ± 0.39	0.24 ± 0.0034
0.10	6.0	25	taurodeoxycholate	7.5 ± 0.59	0.24 ± 0.0051
0.10	7.1	25	taurodeoxycholate	25 ± 1.2	0.20 ± 0.0029
0.10	7.4	25	taurodeoxycholate	70 ± 3.2	0.24 ± 0.0051
0.10	5.5	25	cholate	7.2 ± 0.45	0.26 ± 0.0041

[a] Maximum fractional decrease in fluorescence intensity.
[b] Standard deviation calculated as described in Methods.

imal fluorescence change with pH (Table 2). At pH 7.8 and 8.4 the data became increasingly sigmoidal and, therefore, could not be described by the simple binding model. Moreover, the fluorescence maximum of the enzyme alone shifted to 338 nm at pH 8.4, suggesting ionization of a group on the protein. At pH 8.4, the fluorescence decrease was saturable and the half maximal change required a concentration above 200 μM taurodeoxycholate. Thus, above pH 7.4 the affinity of the bile salt for the enzyme is greatly diminished.

Because bile salts are not only micelle-forming surfactants but tend to form premicellar aggregates (18) that could interact with the enzyme, the stoichiometry of the interaction was determined by equilibrium dialysis. The monomeric pig enzyme was used and the bile salt was [^{3}H]cholate at a concentration of 367 μM. This level was at least an order of magnitude below its critical micelle concentration (18) but 41 times its dissociation constant for interaction with the enzyme (Table 1). Equilibration was virtually complete in 6 hr and distribution of [^{3}H]cholate was not altered by continued incubation up to 19 hr (data not shown). Even after 19 hr, 95% of the enzyme activity remained. Under these conditions the number of cholate molecules per enzyme was 1.2, supporting the notion of a simple 1:1 interaction between enzyme and bile salt.

The relationship of the bile salt binding site to the catalytic site was probed using the [^{3}H]diisopropylphosphoryl derivative of the monomeric pig enzyme. This species is catalytically inactive due to the incorporation of one reagent molecule per molecule of enzyme (21). The fluorescence spectrum of this species exhibited a maximum at 335 nM and fluorescence was decreased by addition of taurocholate (data not shown). This decrease was saturable at 0.15 fractional decrease and occurred with a dissociation constant of 31 μM. The comparable values for native enzyme are 0.26 and 12, respectively. Thus, modification of the active site perturbs, but does not block, binding of bile salt to the enzyme.

It is reported that at millimolar concentrations particular bile salts alter the aggregation state (7) and the conformation (10) of carboxylester lipases. To determine whether the binding of monomeric bile salt could induce changes in enzyme conformation, circular dichroism spectra of the monomeric pig enzyme were obtained in the presence of saturating cholate and deoxycholate. As shown in **Fig. 3**, the bile salt caused no significant perturbation of the spectrum between 200 and 260 nm.

At millimolar concentrations, bile salts protect carboxylester lipases from proteolytic and thermal inactivation (21, 23). The activity of each of the three enzymes in the presence of a 10-fold molar excess of trypsin and the four representative bile salts at concentrations 10 times the dissociation constants given in Table 1 were measured
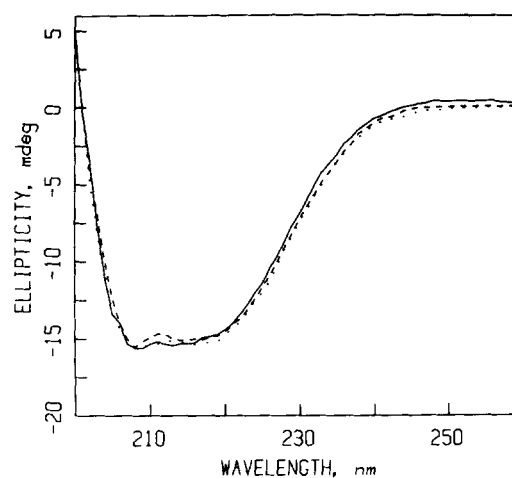

Fig. 3. Effects of cholate and deoxycholate on the circular dichroism spectra of pig monomeric carboxylester lipase; (------) cholate, 200 μM; (·····) deoxycholate, 100 μM; (——) enzyme alone. Spectra are corrected for the contribution from the bile salt and buffer.

as a function of time. At the pH and temperature used, 7.4 and 37°C, $\geq$80% saturation of the binding site should be achieved provided the dissociation constant does not change markedly between 30 and 37°C (Table 2). As shown in **Fig. 4a**, none of the bile salts afforded any protection to the monomeric pig enzyme. Similar results were obtained with the other enzyme species (not shown) and approximate rate constants for the inactivation were determined. These are listed in **Table 3**. None of the enzymes are significantly protected by monomeric bile salt binding.

Inactivation of the enzymes can also be induced thermally. For the pig enzymes measurable rates of inactivation are observed at 48°C in the absence of bile salt (Fig. 4b), but for the human enzyme the apparent first order rate constant was only one-tenth that of the monomeric pig form. At 53°C, however, the human enzyme was inactivated at rates comparable to pig enzyme at 48°C. As exemplified in Fig. 4b, the presence of bile salts offers some protection against inactivation, again assuming that dissociation constants are not greatly altered at the higher temperature. Data for the other enzyme species (Table 3) show similar protection of the dimeric pig enzyme but a much smaller protective effect of bile salts for the human enzyme.

Because carboxylester lipases normally function at interfaces, it was of interest to examine the possible role of monomeric bile salt binding in regulating their interfacial adsorption and stability. These processes were examined using the monomeric pig species because its adsorption to lipid–water interfaces in the absence of bile salts has been previously characterized (22). As described, the adsorption of active enzyme versus adsorption of active enzyme followed by its denaturation can be compared by
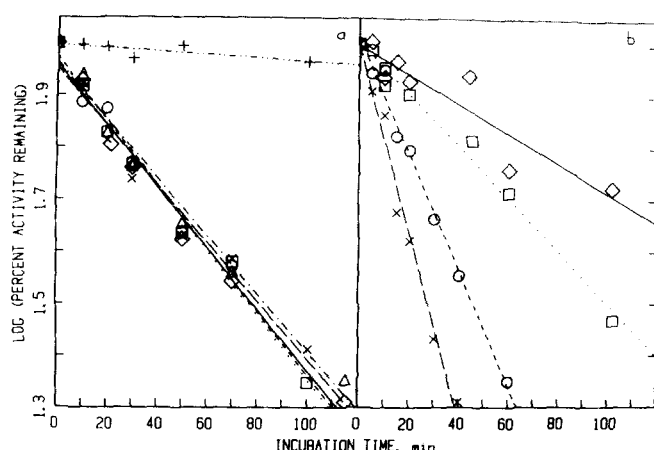
Fig. 4. Effect of bile salts on the stability of pig monomeric carboxylester lipase ; (---O---) taurocholate at 130 μM; (···□···) taurodeoxycholate at 69 μM; (— · — △ — · —) cholate at 89 μM; (— ◇ —) deoxycholate at 35 μM; (— —X— —) no bile salt; (— ·· + ·· —) no bile salt or trypsin (panel a only). a) Trypsin inactivation, 37°C; b) thermal inactivation, 48°C.

measuring the catalytic activity of native enzyme recovered at the interface versus the adsorption of the [³H]diisopropylphosphoryl-enzyme. These two quantities, expressed as apparent rate constants for enzyme adsorption under initial rate conditions, are shown as a function of taurocholate concentration in **Fig. 5**. As the figure shows, recovered activity increases with bile salt concentration, nearing maximal values at 100 μM. In contrast, binding of modified enzyme occurs at all concentrations but decreases about 10% between 5 and 100 μM cholate. These data indicate that the bile salt does not

interfere with the adsorption process and that protection, as measured by the recovery of active enzyme relative to protein, occurs over the same concentration range as binding of monomeric bile salt (Table 1).

The presence of bile salt in the aqueous phase is accompanied by its adsorption to the interface, giving rise to an increase in the surface pressure (Fig. 5). At 100 μM bile salt, where protection is approaching maximal, this change is about 10 mN/m. It is possible that the protection is not bile salt-specific but due to the change in surface pressure. To test this, rate constants for adsorption of native enzyme were determined in the presence of bile salt or octylglucoside concentrations sufficient to produce a surface pressure of 10–13 mN/m **(Table 4)**. As shown in the table, all the bile salts caused similar rate constants for enzyme adsorption, but with octylglucoside the rate was only one-third as great. In control experiments the adsorption of native and [³H]enzymes was compared in the presence of taurocholate or octylglucoside sufficient to produce a surface pressure of 10 mN/m. These showed that the lower apparent rate constant for adsorption in the presence of octylglucoside was due to denaturation of adsorbed enzyme, not slower adsorption.

DISCUSSION

The decrease in fluorescence induced by bile salts (Fig. 1) suggests a direct interaction between carboxylester lipases and the surfactants. The bile salt concentration dependencies exhibited classical saturation behavior (Fig. 2) which suggested a simple 1:1 interaction and, within er-

TABLE 3. Rate constants for trypsin- and thermally induced inactivation of carboxylester lipases

| Enzyme | Bile Salt | | Rate Constant × 10⁵, s⁻¹ | |
	Species	Concentration	Trypsin[a]	Thermal[b]
		μM		
Human juice	taurocholate	130	23.0 ± 0.7[c]	37.0 ± 0.7[c]
Human juice	taurodeoxycholate	69	24.0 ± 1.1	29.0 ± 2.0
Human juice	cholate	89	18.0 ± 0.7	32.0 ± 2.5
Human juice	deoxycholate	35	21.0 ± 0.7	34.0 ± 3.0
Human juice	none		17.0 ± 1.1	38.0 ± 2.1
Pig, monomer	taurocholate	130	10.0 ± 0.9	19.0 ± 0.8
Pig, monomer	taurodeoxycholate	69	10.0 ± 0.6	8.4 ± 0.4
Pig, monomer	cholate	89	9.3 ± 0.6	9.2 ± 0.4
Pig, monomer	deoxycholate	35	9.8 ± 0.6	4.8 ± 0.8
Pig, monomer	none		9.4 ± 0.8	30.0 ± 1.7
Pig, dimer	taurocholate	130	6.8 ± 0.3	11.0 ± 2.2
Pig, dimer	taurodeoxycholate	69	7.5 ± 0.6	6.7 ± 0.8
Pig, dimer	cholate	89	8.3 ± 0.6	8.9 ± 8.7
Pig, dimer	deoxycholate	35	7.4 ± 0.5	4.2 ± 0.7
Pig, dimer	none		8.3 ± 0.2	24.0 ± 1.2

[a] Enzyme at 0.24 mg/ml with trypsin at a 10-fold molar excess, 37°C.
[b] For the human enzyme, inactivation temperature was 53°C and for the pig enzymes, 48°C; enzyme concentrations were 0.44–0.95 mg/ml.
[c] Standard deviation of the slope of the first order plot.
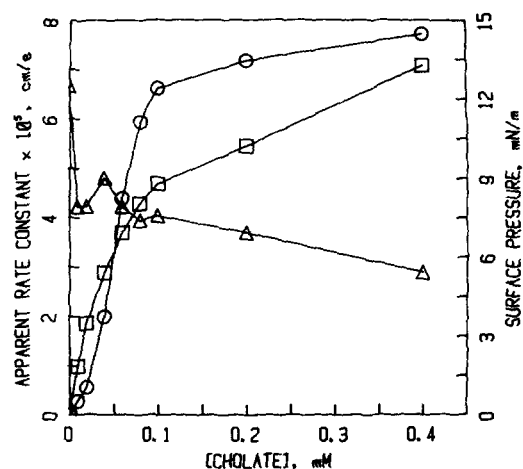
Fig. 5. Effect of cholate on the adsorption of native and [³H]diisopropylphosphoryl monomeric pig carboxylester lipases to the argon/buffer interface; (○) recovered native enzyme; (△) recovered [³H]enzyme; (□) surface pressure changes in mNewtons/m (= dyne/cm) caused by bile salt addition.

ror, this was confirmed by a direct measurement of binding stoichiometry. Such a simple stoichiometry is reasonable because saturation occurs far below the critical micelle concentrations of the bile salts and below the range of formation of premicellar aggregates (18). The lack of specificity in bile salt binding is consistent with the postulated existence of a nonspecific bile salt binding site on human pancreatic juice carboxylester lipase (9) and of nonspecific activation of human milk bile salt-stimulated lipase (13). Thus, the major distinction between the present results and previous indirect studies of bile salt-enzyme interaction is the magnitude of the dissociation constants. Those constants (Table 1) do, however, correspond to the concentration range over which bile salts activate the hydrolysis of *p*-nitrophenylacetate in homogeneous solution (15, 16). The reasons for the higher dissociation constants reported in other studies are suggested by data from Table 2 and related experiments. Although the salt and temperature dependencies of the dissociation constants are unremarkable, the pH-dependent increase above 6.8 and the dramatic inhibition of binding at higher pH values suggest that deprotonation of an ionizable group on the enzyme regulates bile salt binding. Unfortunately, this effect occurs in the same range of pH, 7–8.5, in which most earlier measurements of bile salt effects were made (e.g., 8, 9), thereby complicating their interpretation. Thus, the data show that the affinity of carboxylester lipase for bile salts, in the physiological range of 6.5 and below (27), is far greater than previously reported.

The increased affinity of the enzymes for bile salts at lower pH values suggested that some of the consequences of bile salt binding reported to occur at millimolar concentrations might be evident at lower bile salt concentrations at pH 6.8. As shown in Table 3, this was not true for

thermal and trypsin inactivation of the enzyme. For the related human milk bile salt-stimulated lipase, 5 mM taurocholate affords complete protection (23) and 3 mM taurocholate stabilizes the pig enzymes at 53–55°C (21). Nor was it true for the conformational change postulated simply on the basis that bile salts decrease fluorescence (10). As shown in Fig. 4, there is no measurable effect of bile salt binding on enzyme secondary structure. Because high levels of bile salts do cause aggregation of human and pig carboxylester lipases (7, 19) and protection against thermal and trypsin inactivation, the data support the postulated existence of a second site for bile salt-enzyme interactions (9) or an interaction of the monomer binding site described herein with micelles. The latter is suggested by the postulated alteration of the unspecific site to a micelle-binding site (9).

As noted above, early reports showed stimulation by bile salts of the hydrolysis of *p*-nitrophenylacetate in homogeneous solution. This led to the concept that the nonspecific binding site was also an activator site. That catalysis and bile salt binding occur at topographically distinct sites is supported by the ability of the catalytically inactive diisopropylphosphoryl-enzyme to bind bile salts. However, the affinity of the modified enzyme for bile salts is decreased twofold, suggesting the proximity or coupling of the sites.

As recently demonstrated (22), the surface binding site of monomeric pig carboxylester lipase is also functionally, if not topographically, distinct from the catalytic site. Its relation to the bile salt binding site was explored by measuring rates of adsorption of the bile salt-saturated enzyme to the argon/buffer interface. These measurements showed that bile salt only weakly inhibits adsorption of the enzyme protein at submicellar concentrations (Fig. 5). This is consistent with an earlier report that bile salts do not block adsorption of the human enzyme to siliconized glass beads (28). More importantly, however, the presence of saturating bile salts protected the enzyme against surface denaturation. This could occur through several mechanisms. One is that the ability of bile salts to

TABLE 4. Adsorption of monomeric pig carboxylester lipases to interfaces in the presence of surfactants

Surfactant	Concentration	Surface Pressure	Apparent Rate Constant × 10⁵
	μM	*mN/m*[a]	*cm/s*
None	0	0.0	0.4
Taurocholate	200	13.1	7.4
Taurodeoxycholate	72	12.2	8.1
Cholate	200	11.0	8.1
Deoxycholate	40	11.7	8.6
Octylglucoside	520	10.0	2.8

[a]mNewtons/m = dyne/cm.

change the interfacial tension could protect against denaturation. That this was not the case is indicated by the data of Table 4 and related controls. At surface pressures of 10-13 mN/m, the bulk concentrations of the bile salts were saturating with respect to enzyme binding whereas octylglucoside was near its dissociation constant. However, retention of activity by adsorbed enzyme was complete with bile salts but only one-third as great with octylglucoside. When, instead of a soluble surfactant, an insoluble monolayer of fatty acid at 10 mN/m is used, denaturation is nearly complete (22). Thus, the fact that any activity remained at all with octylglucoside supports the hypothesis that bile salt binding to the enzyme, itself, contributes to stabilization. From present data, however, we cannot rule out that the presence of a surface excess of surfactant at the interface decreases enzyme denaturation in a manner not dependent on direct interaction with the protein or the lowering of interfacial tension. Whether a direct or indirect mechanism is operative, the results clearly show that bile salts stabilize the carboxylester lipase at high energy surfaces. Thus, their presence likely contributes to the stability of the enzyme in vivo. ∎

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