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BINDING OF HUMAN PANCREATIC CARBOXYLIC ESTER HYDROLASE TO LIPID INTERFACES

DOMINIQUE LOMBARDO * and ODETTE GUY

Unité de Recherches de Pathologie Digestive de l'INSERM, 46 Boulevard de la Gaye, 13009 Marseille (France)

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Summary

Human pancreatic carboxylic ester hydrolase (EC 3.1.1.1), usually characterized by its activity on water-soluble substrates, is shown to catalyze reactions taking place at a lipid/water interface. The inhibition of tributyrin hydrolysis by 1-alcohols follows the pattern of a Langmuir adsorption isotherm. Experiments peformed with siliconized glass beads show that human pancreatic carboxylic ester hydrolase is adsorbed on this artificial (or substitute) interface with a dissociation constant for the enzyme-glass beads complex equal to $1.5 \cdot 10^{-8}$ M. The average molecular area at saturation is 4375 $Å^2$. Tripropionin hydrolysis is increased by the presence of glass beads. These results are strong arguments in favor of the interfacial activity of pancreatic carboxylic ester hydrolase. The activation of the enzyme bound to the interface is very weak. Bile salts do not prevent the adsorption of carboxylic ester hydrolase on siliconized glass beads and increase strongly the hydrolysis rate of emulsified tributyrin.

Introduction

The presence in pancreas of a carboxylic ester hydrolase activity different from lipase was reported many years ago [1,2]. This enzyme, able to hydrolyse water-soluble esters and thus characterized by its activity on *p*-nitrophenyl acetate [3], was considered to be a lipolytic enzyme. A carboxylic ester hydrolase (EC 3.1.1.1) has been purified from human pancreatic juice [4].

The enzyme exhibits a very broad substrate specificity. It catalyzes the hydrolysis of water-soluble carboxyl esters and insoluble esters such as cholesterol and lipid-soluble vitamins A, D-3 and E esters when solubilized by bile

salts [5]. But in contrast to pancreatic lipase and phospholipase, human carboxylic ester hydrolase has a poor activity on most of the substrates presented as emulsions or monomolecular films. However, it is able to catalyse emulsified tributyrin hydrolysis. The rate of hydrolysis, maximal in the presence of bile salts, is not negligible in the absence of the latter since it is similar to the rate of hydrolysis of soluble substrates such as triacetin or methyl butyrate [6]. We have studied this reaction, which takes place at an interface, using the same approaches as those described for pancreatic lipase [7,8]. The inhibition of tributyrin hydrolysis by alcohols associated with the adsorption of the enzyme on glass beads has permitted us to demonstrate an interfacial activity of pancreatic carboxylic ester hydrolase which had never been described before. Moreover, the enzyme adsorbed to siliconized glass beads catalyzes the hydrolysis of tripropionin more efficiently than it does in solutions. Nevertheless, the activation of the enzyme bound to the interface is negligible with respect to lipase or phospholipase interfacial activation.

Materials and Methods

Materials

Tributyrin (glycerol tributyrate) was a 'puriss.' product (≥99%) from Fluka. Only freshly opened bottles were used. Tripropionin (glycerol tripropionate), a technical product from Fluka, was purified by adsorption on an alumina column eluted with a petroleum ether/hexane mixture (90 : 10, by volume). The product obtained shows a single spot by chromatography on F1500 Silicagel G plates (from Schleicher and Schull) developed in diethyl ether/hexane (50 : 50, by volume). Spots were stained with Usui reagent [9]. *p*-Nitrophenyl acetate was a Sigma product (U.S.A.). Sodium taurocholate and taurodeoxycholate (A grade) came from Calbiochem (U.S.A.). Siliconized glass beads came from Serva (F.R.G.). Methanol, 1-butanol, 2-butanol, 1-propanol and 1-octanol were at least 99% pure products from Carlo Erba (Italy) while 3-butanol (2-methyl-2-propanol) was a ≥99.7% puriss. product from Fluka. All organic compounds were the best available commercial products. Deionized water was distilled from alkaline permanganate to remove organic compounds.

Human pancreatic carboxylic ester hydrolase (EC 3.1.1.1) was obtained homogeneous from pancreatic juice according to the method previously described [4]. The sample was checked for the absence of any contaminant lipase by two methods. No precipitin line was detected by immunodiffusion against an antiserum to pure human lipase according to the method of Ouchterlony [10]. Moreover, when the enzyme activity was measured on a monomolecular film of 1,2-dilaurin, 330 μg of enzyme were necessary to obtain traces of activity which would correspond to less than 0.2% of lipase activity. In these conditions of assay it is difficult to discriminate between a contaminant and an intrinsic activity.

Enzyme assays

The rates of hydrolysis were measured titrimetrically at 25°C using a Radiometer titrator with a standard mechanical stirring device and 100 mM or 20 mM NaOH as titrant according to the enzyme activity. A gentle stream of

nitrogen was passed over the incubation surface. Tributyrin hydrolysis was followed at pH 8.0 in 10 ml reaction mixture containing 100 mM substrate, 150 mM NaCl and 0.5 mM sodium taurocholate. The spontaneous hydrolysis of substrate in the presence or in the absence of alcohol was monitored and then the enzyme (20—100 μl) was added. The hydrolysis rates were recorded for at least 5 min and corrected for spontaneous hydrolysis of substrate. The formation of bubbles due to stirring was carefully avoided. Tripropionin hydrolysis was measured at pH 7.5 in 9 ml reaction mixture containing 5 mM substrate, 100 mM NaCl and 1 mM potassium phosphate buffer according to the method described by Brockman et al. [11]. The hydrolysis of *p*-nitrophenyl acetate was measured spectrophotometrically at 400 nm according to the method described by Erlanson [3].

Adsorption experiments

Experiments were performed with spherical siliconized glass beads with a mean diameter of 167 μm, corresponding to a surface of 164 cm^2/g of beads, according to the method of Chapus and Semeriva [12]. The assays were performed by adding 500 mg of beads to several silicone-coated glass tubes containing increasing quantities of enzyme in solution in 1 ml of 0.1 M Tris-HCl buffer, pH 7.5. Siliconized test tubes were used to have the same interface quality in contact with the enzyme solution. The surface of the tube in contact with this solution represented less than 7% of the glass beads surface and was negligible. One assay without beads was performed as control for each quantity of enzyme. After shaking for 1 min with a Vortex apparatus, beads were allowed to settle for about 15 s and the protein content of the supernatant was estimated by the measure of absorbance at 220 nm or by the activity on *p*-nitrophenyl acetate.

Protein concentration

The concentration of carboxylic ester hydrolase was determined from the absorbance at 280 nm using $E^{1\%}_{1cm} = 14.5$ and a molecular weight of 100 000 [4].

Langmuir treatment

In an heterogenous system containing water-insoluble substrate such as tributyrin, added alcohol is adsorbed at the interface. The amount of adsorbed surfactant is a function of the amount of alcohol present and is related to the desorption and adsorption of alcohol depending upon the surfactant itself and upon the interface nature. According to Mattson et al. [7], the hydrolysis of an enzyme which acts at an interface is inhibited by added alcohols and the rate of hydrolysis should be proportional to the fraction of surface not covered by alcohol, since the adsorbed alcohol promotes a steric hindrance for the enzyme adsorption. In these conditions the same authors used the approach of Langmuir to describe the reaction, and according to this treatment the data follows the equation:

$$\frac{I}{v_0 - v_i} = \frac{K + I}{v_0}$$

Where I is the absolute amount of alcohol, v_0 is the rate of hydrolysis in the absence of alcohol, v_i is the rate of hydrolysis in the presence of alcohol and $K = k_d/k_a$, k_d and k_a being proportionality constants related to desorption and adsorption.

It follows that $(v_0 - v_i)$ is proportional to the amount of surface that is covered by alcohol. A plot of $I/(v_0 - v_i)$ against I will yield a straight line and the intercept with the ordinate will be K/v_0. Further, if the Langmuir equation is obeyed, $K = I_{50}$, I_{50} being the amount of alcohol causing 50% inhibition.

Results

(I) Inhibition of tributyrin hydrolysis by alcohols

The addition of alcohols inhibited tributyrin hydrolysis by carboxylic ester hydrolase and Table I summarizes the effects of various alcohols with different chain lengths. The alcohol concentration necessary to inhibit 50% of activity (I_{50}) decreased with the alcohol solubility in water, the less soluble alcohol being the more potent inhibitor. 1-Butanol and 1-octanol, the most active inhibitors, were used to investigate the effects of alcohol on this reaction which takes place at an interface. Fig. 1 shows the inhibitory effect of butanol and octanol on tributyrin hydrolysis. The I_{50} values listed in Table I were deduced from these graphs. If we follow the approach of Langmuir for the treatment of these data, as performed by Mattson et al. [7], we can plot $I/(v_0 - v_i)$ versus I. The diagram shown in Fig. 2 (A and B) gives straight lines and the intercept with the ordinate gives K/v_0 and permits the calculation of K. These K values, respectively 2000 and 320 μmol for 1-butanol and 1-octanol, were similar to the I_{50} values obtained with each alcohol (see Table I), which is in accordance with the Langmuir theory of adsorption of alcohol at the interface.

(II) Interfacial activity of carboxylic ester hydrolase

(1) Adsorption of carboxylic ester hydrolase on siliconized glass beads. Fig. 3 shows that human carboxylic ester hydrolase is adsorbed at the artificial interface formed by siliconized glass beads. Little enzyme activity can be detected in the supernatant after the first additions of enzyme. Then, after a breakpoint, the plotting of the activity versus total enzyme becomes linear. The extrapolation of this line gives on the abcissa the amount of carboxylic ester

TABLE I

INHIBITORY EFFECTS OF VARIOUS ALCOHOLS ON TRIBUTYRIN HYDROLYSIS BY CARBOXYLIC ESTER HYDROLASE

I_{50} is expressed in μmol for 10-ml incubation mixture. Enzyme in the assay, $3.5 \cdot 10^{-11}$ mol.

Alcohol	I_{50} (μmol)	Solubility at 20°C (%)
Methanol	19 000	∞
1-Propanol	5 000	∞
1-Butanol	1 700	7.9
1-Octanol	330	~0
2-Butanol	6 100	12.5
3-Butanol	13 000	∞

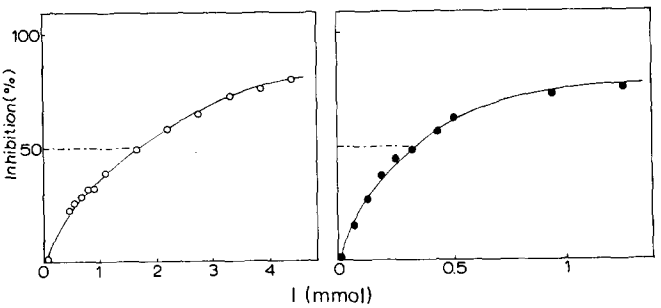


Fig. 1. Inhibition of carboxylic ester hydrolase activity by alcohols. Effect of 1-butanol (○) and 1-octanol (●) on emulsified tributyrin hydrolysis by $3.5 \cdot 10^{-11}$ mol of carboxylic ester hydrolase.

hydrolase necessary to saturate the interface. This amount ($2.8 \cdot 10^{-10}$ mol) corresponds to an average density of one molecule of carboxylic ester hydrolase per 4870 Å^2 of interface. This density can also be calculated from the saturation curve of the enzyme bound to the interface of glass beads. This plotting leads to 4130 Å^2 per enzyme molecule. The equilibrium constant, $K' = k_d/k_a$, for the dissociation of the enzyme-surface complex has been calculated from the equation of equilibrium state [11].

$$E \underset{k_a}{\overset{k_d}{\leftrightharpoons}} E^*$$

giving the relation:

$$\frac{E}{E^*} = \left(\frac{a_0}{A_0}\right) K' v + \left(\frac{a_0}{A_0}\right) E$$

where E is free enzyme (mol), E^* bound enzyme (mol), k_d and k_a the rate constants related to desorption and adsorption, respectively, A_0 total interface

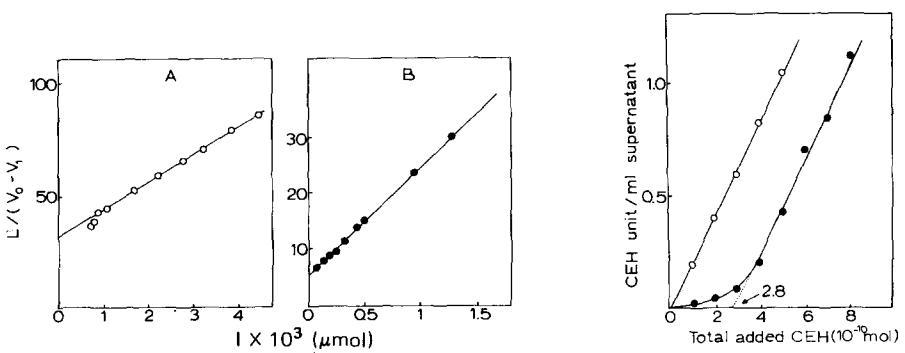


Fig. 2. Inhibition of tributyrin hydrolysis by alcohols. $I/(v_0 - v_i)$ versus alcohol concentration. v_0 and v_i are the rates of hydrolysis in the absence and in the presence of added 1-butanol (○) and 1-octanol (●). Hydrolysis by $3.5 \cdot 10^{-11}$ mol of carboxylic ester hydrolase.

Fig. 3. Adsorption of carboxylic ester hydrolase (CEH) on siliconized glass beads. The amount of enzyme present in supernatant was measured by the activity on *p*-nitrophenyl acetate. Experiments were performed without (○) and with (●) glass beads (interface area: 82 cm^2).

area (cm^2), a_0 molar area (cm^2/mol) of the protein-binding sites on the surface and v the volume of the solution (cm^3). The disappearance of the enzyme from solution was determined after incubation with a coated surface of 82 cm^2 by measuring the decrease of the enzyme activity on *p*-nitrophenyl acetate. Thus, according to Brockman et al. [11], by plotting E/E^* as a function of E, a straight line is obtained, the slope and intercept of which should yield a_0 and K'. The calculated values are 2.45×10^{11} cm^2 per mol for a_0, which corresponds to a molecular area of 4100 $Å^2$ and $1.50 \cdot 10^{-11}$ mol/ml for K'. These values were determined from a linear regression of experimental data with a correlation coefficient of 0.99.

(2) Interfacial activation of carboxylic ester hydrolase. Carboxylic ester hydrolase catalyzes the hydrolysis of tripropionin and we have shown that the addition of siliconized glass beads prior to the enzyme increased the proton release. As shown in Fig. 4A, a short period (at most 2 min) of acceleration of proton (P) release is observed before the linear steady-state of hydrolysis (V_{ss}). This can indicate a time-dependent interaction of the enzyme with the interface, resulting in the formation of an enzyme species with increased catalytic activity. This short period of acceleration can also be interpreted as the time needed for penetration of the enzyme into the substrate interface. We have found a latency period (τ) of 30 s within experimental errors, this lag-time seems to be independent upon enzyme concentration, even if the steady-state rate of hydrolysis is proportional to the quantity of enzyme in the assay. If we study the kinetics of acceleration according to Brockman et al. [11], we can show that the acceleration is first-order with respect to the enzyme. According to the equation $\ln (P—P') + \ln C_2 = -k_{exp} \cdot t$ where P' was the ordinate obtained by extrapolation of V_{ss} to zero time, C_2 was a derivation constant equal to $P—P'$ when $t = 0$, and k_{exp} the apparent first-order constant for acceleration. The plotting of ln $(P—P')$ versus t should be linear. Such a plot is shown in Fig. 4B. An excellent fit with a correlation coefficient of 0.99 is obtained all over the range of the acceleration process. The increase of V_{ss} with the interface area measured at constant enzyme concentration (Fig. 5) reaches

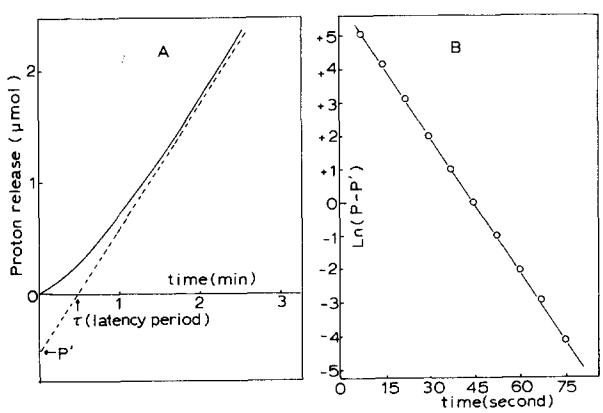
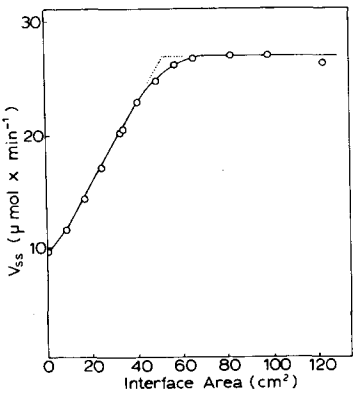


Fig. 4. Hydrolysis of tripropionin by carboxylic ester hydrolase in the presence of glass beads. Interface area of glass beads: 82 cm^2; quantity of enzyme in the assay: $2 \cdot 10^{-10}$ mol. (A) Proton release (P) as a function of time (min). (B) Plotting of ln $(P—P')$ versus time (s) according to Brockman et al. [11].



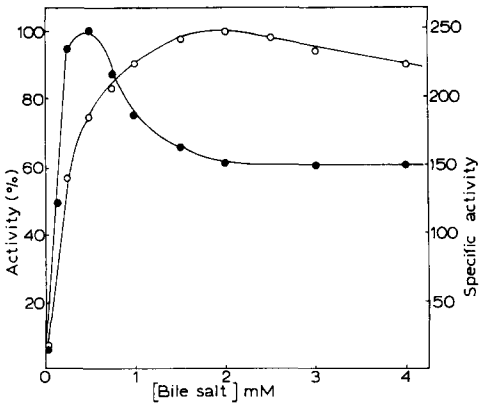


Fig. 5. Dependence of the steady-state rate of tripropionin hydrolysis (V_{ss}) upon interface area. Quantity of enzyme in the assay: $2 \cdot 10^{-10}$ mol.

Fig. 6. Effect of bile salt concentration on the rate of tributyrin hydrolysis. The activities are expressed as % of the maximal activity, and specific activities (μmol $\cdot$ min^{-1} $\cdot$ mg^{-1} protein). Effect of sodium taurocholate, •; effect of sodium taurodeoxycholate, ○.

a plateau at saturation of the interface by enzyme. This point of saturation gives a molecular area of 4400 Å^2 and an increase of V_{ss} equal to 2.7.

(3) Effect of bile salts at the interface. The addition of 1 mM sodium taurocholate did not change the adsorption of carboxylic ester hydrolase on the artificial interface formed by siliconized glass beads. A pattern similar to that shown in Fig. 3 was obtained in the experiments performed in the presence of bile salts, and the amount of carboxylic ester hydrolase necessary to saturate the interface was $1.45 \cdot 10^{-10}$ mol instead of $2.8 \cdot 10^{-10}$ mol in the absence of bile salts. We have shown that in the presence of sodium taurocholate, human carboxylic ester hydrolase dimerizes [5]. If we assume a molecular weight of 200 000 for the dimer, the molar area a_0 deduced from the saturation curve becomes equal to $5.13 \cdot 10^{11}$ cm^2, which gives an interfacial area of 8530 Å^2 per molecule of dimer. K' is not significantly modified.

When the interface was formed by an emulsion of tributyrin, bile salts produce an increase of more than 15 times of the activity of carboxylic ester hydrolase. Fig. 6 shows the different effects of sodium taurocholate and taurodeoxycholate. The fact that sodium taurocholate, which has a higher critical micellar concentration than sodium taurodeoxycholate, appears to be activator at a lower concentration than sodium taurodeoxycholate shows that a substrate solubilization must be ruled out but that a modification of the interface properties due to the presence of bile salts can be postulated. The decrease of activity obtained with each bile salt at different concentration may be due to competition between substrate and bile salts, which are both recognized by the enzyme [5]. Such a competitive type of inhibition has been observed with triacetin hydrolysis in the presence of these two bile salts [6]. An interfacial steric hindrance may be also involved in this inhibitory effect.

(4) pH-dependency of surface reaction. The pH-dependency of the enzyme-catalyzed surface reaction was determined with tripropionin-adsorbed on glass beads. Tripropionin hydrolysis was followed at various pH values in 0.1 M

NaCl using a surface area of 82 cm^2 in the presence of $1.2 \cdot 10^{-10}$ mol of enzyme. A simple sigmoïdal relationship was observed between the steady-state rate of substrate hydrolysis and pH. The curve shows an inflection at pH 5.9, indicating that the interface substrate hydrolysis requires the presence of a group with an apparent pK of 5.9.

The same experiment performed with emulsified tributyrin showed that the surface reaction depends upon a group with an apparent pK of 5.6.

Discussion

Specificity studies have shown that human carboxylic ester hydrolase was unable to hydrolyze most of the substrates presented as emulsion or monomolecular films [6]. However, an activity was detectable during the hydrolysis of a monomolecular film of tricaprylin and tributyrin emulsion. The specific activities were, respectively, 0.5 and about 20 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ [6]. If the activity on a monomolecular film of tricaprylin was weak, the activity on emulsified tributyrin was comparable to that obtained with soluble substrates such as triacetin or methyl butyrate [6]. These results have pointed out a possible interfacial activity of the enzyme. The data presented here give evidence that carboxylic ester hydrolase is able to bind and to hydrolyze substrates at an interface. The inhibition of tributyrin hydrolysis by 1-alcohols has shown that the effect of alcohols increases while the solubility of the alcohol decreases. In an heterotropic medium containing a water/lipid interface, the alcohol partitions between the two phases and the fraction of interface covered by alcohol is not accessible to the enzyme. Using the approach of Langmuir to treat the kinetics of interfacial reactions with tributyrin as substrate, we obtained similar values for K and I_{50}, which demonstrates that the inhibition by alcohols is proportional to the surface covered by alcohols.

The experiments performed with an artificial interface consisting of siliconized glass beads demonstrate clearly the binding of human carboxylic ester hydrolase. The dissociation constant of the complex, $K' = 1.5 \cdot 10^{-11}$ mol/ml, shows that carboxylic ester hydrolase is strongly adsorbed to the interface.

The pH-dependency of surface reactions has shown that a group with an apparent pK of 5.6—5.9 was implicated in the reaction. These values of pK are fully consistent with the deionization of an imidazolium ion of histidine. In pancreatic lipase, it has been shown that an histidine residue with a pK of 5.8 was involved in the surface reaction catalyzed by this enzyme [11,12]. The chemical modification of histidine residues did not prevent the binding of pancreatic lipase to an interface while the enzyme lost its activity on monomeric substrates. This result suggests that an essential histidine residue is situated at the active center and is responsible for the hydrolysis of substrate but not for the binding to emulsified substrate [13]. Some recent experiments have shown that in the case of human pancreatic carboxylic ester hydrolase, like in lipase, histidine residues are not implicated in the binding of the protein to the interface (unpublished data).

The average area occupied by one molecule of carboxylic ester hydrolase at saturation of the interface is 4375 $Å^2$. This area, much smaller than the area

occupied by the completely denaturated molecule (17 000 Å^2) [14], demonstrates that the enzyme is not denaturated by the adsorption at the interface but remains fully active. Taking into account the molecular interfacial area of carboxylic ester hydrolase and its dissociation constant with the interface, it is surprising to find the same results as those obtained with pancreatic lipase (i.e. a molecular area of 4180 Å^2 and $K' = 1 \cdot 10^{-11}$ mol/ml [15]). Thus it appears possible that the interfacial binding sites of both enzymes are quite similar, even if the molecular weight of carboxylic ester hydrolase is twice that of lipase. However, if we extend the comparison with lipase, some important differences appear. While the catalytic efficiency of surface-bound lipase is about 2 or 3 orders of magnitude greater than that of the free enzyme [11,16] the accelerating effect of the interface on carboxylic ester hydrolase activity is very weak. Tripropionin hydrolysis is increased less than 3-times and we have observed only a 10-fold increase of *p*-nitrophenyl acetate hydrolysis when assayed titrimetrically compared to the spectrophotometric measurements (unpublished data). In contrast to lipase [15], the adsorption of carboxylic ester hydrolase on glass beads is not affected by the presence of bile salts. The association constant is not modified and the twice higher molecular area is in agreement with an even adsorption of each molecule of monomer associated to form dimer in the presence of taurocholate. Bile salts do not prevent the adsorption of carboxylic ester hydrolase on siliconized glass beads but they greatly increase the hydrolysis rates of tributyrin. This activator effect of bile salts has been extensively studied in a previous work [5].

One activator effect is specific for the 3α, 7α-hydroxylated bile salts and is associated with a dimerization of the enzyme. The other activator effect is unspecific for bile salts hydroxylation and associated with a detergent effect on the substrate. In the case of tributyrin hydrolysis which increases with sodium taurocholate as well as sodium taurodeoxycholate, it is probable that bile salts modify the interface properties [17]. This modification of interface properties can lead to an increase of the affinity of carboxylic ester hydrolase for the emulsified substrate, as has been demonstrated for soluble substrates [4]. Therefore, even if we observed an increase in interfacial activity in the presence of bile salts, this increase does not seem to be a 'surface-like' activation.

In conclusion, human carboxylic ester hydrolase is able to bind to artificial or lipid interfaces. This adsorption to lipid interfaces explains the macromolecular form earlier found associated with lipids and lipase in human intestinal content [18]. The accelerating effect of the interface on substrate hydrolysis is very weak, if not negligible. Therefore, if carboxylic ester hydrolase is a lipolytic enzyme able to adsorb to interfaces, the distinction with lipase still holds because lipase is strongly activated by interfaces while carboxylic ester hydrolase is not. All these results have considerable physiological importance since carboxylic ester hydrolase is responsible for the intra-luminal hydrolysis of dietary cholesterol esters and lipid-soluble vitamin esters. These substrates are present in the non-polar core of mixed micelles probably formed with apolar cholesterol or vitamins esters, surrounded by acylglycerol, phospholipids and bile salts. The enzyme, which recognizes bile salts and is activated by bile salts present in the intestinal lumen, would be able to reach the hydrophobic core inside the mixed micelle where it could be adsorbed and could then catalyze substrate hydrolysis.

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