Biochimica et Biophysica Acta, 611 (1980) 147—155

BBA 68877

STUDIES ON THE SUBSTRATE SPECIFICITY OF A CARBOXYL ESTER HYDROLASE FROM HUMAN PANCREATIC JUICE

II. ACTION ON CHOLESTEROL ESTERS AND LIPID-SOLUBLE VITAMIN ESTERS

DOMINIQUE LOMBARDO and ODETTE GUY

Unité de Recherches de Pathologie Digestive U 31 INSERM 46, Boulevard de la Gaye, 13009 Marseille (France)

(Received March 18th, 1979)

Key words: Carboxyl ester hydrolase; Substrate specificity; Cholesterol ester; Vitamin ester; (Human pancreatic juice)

Summary

Evidence is presented that human carboxyl ester hydrolase (carboxylic-ester hydrolase, EC 3.1.1.1) is able to hydrolyze cholesterol esters and lipid-soluble vitamins A, D-3 and E esters. Those activities require the presence of bile salts and the 3α,7α-dihydroxylated bile salts have been found the most efficient activators.

The results presented in this paper and in the preceding one suggest the existence of two sites of bile salts recognition. One site, specific of the 3α,7α-hydroxyl group of cholanic acid would induce dimerization and activation of the enzyme. The other site, unspecific towards bile salts hydroxylation would be located at the active center and would be implicated in substrate recognition.

Introduction

Human carboxyl ester hydrolase (carboxylic-ester hydrolase, EC 3.1.1.1) purified from pancreatic juice [1] exhibits a very broad substrate specificity. As demonstrated in the preceding paper, the enzyme hydrolyzes various carboxyl esters such as triacetin, tributyrin and *p*-nitrophenylacetate and also some of the specific substrates of lysophospholipase and nonspecific lipase [2]. In this paper, evidence is presented that human carboxyl ester hydrolase is responsible for the cholesterol ester hydrolase activity which has been characterized in human pancreatic juice [3,4].

Cholesterol ester hydrolase from porcine pancreas has been recently purified and some molecular properties have been determined [5]. The rat enzyme, earlier isolated, has pointed out an important bile salts dependence of the activity and molecular association [6—9]. Preliminary results on the enzyme present in human pancreatic juice have shown an absolute requirement of sodium taurocholate for cholesterol ester hydrolase activity [3]. The effects of different bile salts on the purified human enzyme are reported here.

We also demonstrate that human carboxyl ester hydrolase is responsible for the vitamin A esterase activity characterized in rat pancreatic juice [10] and is able to split the ester bonds of other lipid-soluble vitamins like vitamins D-3 and E, in the presence of bile salts. A hypothesis on the important role played by bile salts in the activity of carboxyl ester hydrolase is discussed.

Material and Methods

Materials. Human carboxyl ester hydrolase was purified as described in the preceding paper [2]. Sodium deoxycholate and sodium cholate came from Fluka. Sodium taurocholate, glycocholate, taurodeoxycholate, taurochenodeoxycholate and tauroursodeoxycholate were A grade products from Calbiochem. Vitamin A palmitate and *p*-nitrophenylacetate were obtained from Sigma, Brij 35 from Pierce, vitamin E acetate and vitamin A acetate from Serva. Vitamin D-3 was a generous gift of Hofmann-LaRoche and vitamin D-3 acetate was synthesized in our laboratory as follows: 1 mmol of vitamin D-3 was dissolved in 1 ml of the mixture chloroform/pyridin (50 : 50, v/v) and incubated 24 h at 4°C in the presence of 25 mM acetic anhydride. 5 ml of cold distilled water were then added twice to remove acetic acid and pyridin. The chloroformic phase was evaporated under nitrogen and the remaining product was vitamin D-3 acetate. The product was homogeneous by thin-layer chromatography. F-1500 plates from Schleicher and Shull (F.R.G.) were used for thin-layer chromatography.

Enzyme assays. The hydrolysis of *p*-nitrophenylacetate was measured according to Erlanson [11].

Cholesterol ester hydrolase activity was determined according to the method of Lombardo and Guy [3].

The hydrolysis of vitamins A, D-3 and E acetate was measured titrimetrically at constant pH 7.5 with 20 mM NaOH. Substrates were prepared in 5-mM solutions with 0.5% Brij 35, 10 mM bile salts and 200 mM NaCl. The assays were performed at 25°C under nitrogen.

Specific activities were expressed as μmol of substrate hydrolyzed/h and per mg of proteins for the hydrolysis of cholesterol esters and vitamin esters.

The qualitative hydrolysis of vitamin A palmitate was performed with solubilized or emulsified substrate using the method of Erlanson and Borgstrom [10]. After 2 h of incubation at 37°C, the mixture was extracted with 1 ml of petroleum ether and evaporated under nitrogen. The oily pellet was then solubilized by 50 μl of chloroform and 5 μl were submitted to thin-layer chromatography in cyclohexane-ether (80 : 20, v/v). Spots were stained by reaction with 50% sulfuric acid. The R_F values were 0.13 and 0.84, respectively, for vitamin A and vitamin A palmitate. The qualitative hydrolysis of vitamin E

acetate was carried out in the same conditions. The R_F values were, respectively, 0.50 and 0.70 for vitamin E and vitamin E acetate.

Molecular weight determination. Molecular weights have been determined by ultracentrifugation according to Yphantis [12]. The protein was in solution (0.5—0.8 mg/ml) in a 5 mM Tris-HCl buffer (pH 7.5) containing 100 mM NaCl and 0.5 mM bile salt.

Results

Activity on cholesterol esters

Human carboxyl ester hydrolase hydrolyzed cholesterol esters in the presence of bile salts. By filtration of proteins of human pancreatic juice on Sephadex G-100, besides fractions containing carboxyl ester hydrolase, no other protein fraction was found active on cholesterol esters. Moreover, the ratio of cholesterol esterase to *p*-nitrophenylacetate esterase activity was nearly constant thoughout purification steps (Table I). Those results demonstrate that carboxyl ester hydrolase and cholesterol ester hydrolase are a single enzyme.

In the absence of bile salts, human carboxyl ester hydrolase was unable to hydrolyze cholesterol esters. Table II summarizes the effects of different bile salts on cholesterol ester hydrolase activity and gives the specific activities obtained in the presence of 10 mM bile salt, concentration corresponding to the maximal activity. The trihydroxylated bile salts activate cholesterol ester hydrolase. Taurocholate and glycocholate gave similar activities, respectively, 50 and 40 and cholate was the best activator since specific activity increased to 120. In contrast, in the presence of the 3α,12α-dihydroxylated bile salts, deoxycholate and taurodeoxycholate, cholesterol ester hydrolase activity was very low. But in the presence of taurochenodeoxycholate, 3α,7α-dihydroxylated bile salt, the activity was similar to that obtained with glycocholate, whereas in the presence of tauroursodeoxycholate (3α, 7β) the activity was negligible. These results show clearly the importance of the 7α-hydroxylated group for the activity.

Fig. 1 represents the bile salts effects on cholesterol ester hydrolase activity in different conditions. As shown on Fig. 1A, in the presence of very low quantities of sodium taurocholate (below 2.5 mM), the activity was very low (below 10%), then the activity increased linearly and rapidly to reach the maximal activity at 8 mM. In the presence of cholate, the effect was similar. In

TABLE I

SPECIFIC ACTIVITIES TOWARDS *p*-NITROPHENYLACETATE AND CHOLESTEROL ESTERS DURING PURIFICATION OF CARBOXYL ESTER HYDROLASE

Purification step	Specific activity on		Ratio
	p-Nitrophenylacetate (μmol/min per mg)	Cholesterol esters (μmol/h per mg)	
Pancreatic juice	2	2.3	0.87
Sephadex G-100	26	26.3	0.99
CM-Sepharose	40	45.4	0.88

TABLE II

EFFECT OF VARIOUS BILE SALTS ON CHOLESTEROL ESTER HYDROLASE AND VITAMIN E ACETATE HYDROLASE ACTIVITIES OF HUMAN CARBOXYL ESTER HYDROLASE

The experiments were performed in the presence of 10 mM sodium bile salt concentration. n.d., not determined.

Bile salt	Hydroxylation	Specific activity on:	
		Cholesterol esters (μmol/h per mg)	Vitamin E acetate (μmol/h per mg)
None	—	0	0
Cholate	3α, 7α, 12α	120	2500
Taurocholate	3α, 7α, 12α	50	1500
Glycocholate	3α, 7α, 12α	40	700
Deoxycholate	3α, , 12α	4	84
Taurodeoxycholate	3α, , 12α	4	96
Taurochenodeoxycholate	3α, 7α,	35	n.d.
Tauroursodeoxycholate	3α, 7β,	5	n.d.

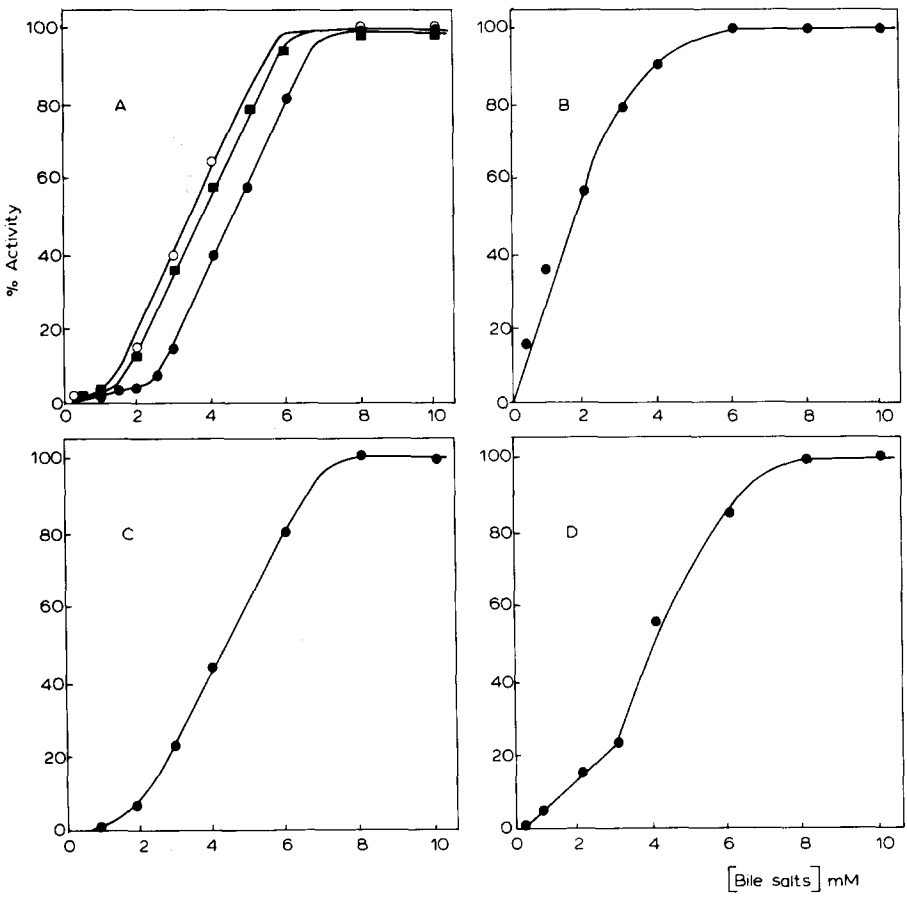

Fig. 1. Effect of bile salts concentration on cholesterol ester hydrolase activity. (A) Effect of sodium taurocholate (●), cholate (○) and taurocholate/taurodeoxycholate mixture (■). (B) Effect of sodium taurocholate after incubation of substrate with 10 mM sodium taurodeoxycholate. (C) Effect of sodium taurocholate after incubation of enzyme with 5 mM sodium taurodeoxycholate. (D) Effect of sodium taurocholate after incubation of enzyme with 5 mM sodium taurocholate.

the presence of 10 mM sodium taurodeoxycholate, we have noticed a very low specific activity (Table II). However, Fig. 1A shows that the mixture sodium taurocholate/sodium taurodeoxycholate (50/50 in molarity) had nearly the same activator effect as sodium taurocholate alone. This surprising effect led us to study separately the action of the two bile salts on substrate and on enzyme by incubation of each component of the reaction with each bile salt. When the substrate was incubated 1 h at 37°C in the presence of 10 mM sodium taurodeoxycholate, the activator effect of sodium taurocholate started immediately (Fig. 1B) and maximal activity was obtained at a slightly lower concentration (6 mM) than in the experiment without preincubation. By contrast, when the enzyme was incubated 1 h at 0°C with 5 mM sodium taurodeoxycholate, cholesterol ester hydrolase activity related to sodium taurocholate concentration was identical to the activity of the enzyme without preincubation (Fig. 1C), which demonstrates that sodium taurodeoxycholate has no effect on enzyme but acts on substrate conformation. Fig. 1D shows that preincubation of the enzyme with 5 mM sodium taurocholate 1 h at 0°C led to an increase of the activity for the low sodium taurocholate concentrations (less than 3 mM). Above 3 mM, the activity increased progressively but not linearly to reach the maximal value at 8 mM. The enzyme conformation would be different after incubation with sodium taurocholate and the enzyme would be more active. The plotting of Fig. 1D points out a transition state at 3 mM sodium taurocholate. This transition which was less obvious but present in all kinetics of activation except in Fig. 1B, was located between 2 and 3 mM, close to sodium taurocholate critical micellar concentration. It seems to correspond to a physicochemical modification of cholesterol esters which become a better substrate for the enzyme.

Activity on lipid-soluble vitamin esters

The hydrolysis of vitamin A palmitate and vitamin E acetate have been demonstrated by thin-layer chromatography (Fig. 2). In the presence of sodium taurodeoxycholate or sodium taurocholate free vitamin A and free vitamin E were released by human carboxyl ester hydrolase. In the absence of bile salts, the hydrolysis did not occur. The hydrolysis of vitamin E acetate has been studied titrimetrically. We have found that neutral detergent like Brij 35 did not inactivate the enzyme and this detergent has been used for substrate solubilization. Whatever Brij concentration, the addition of sodium taurocholate increased the activity of carboxyl ester hydrolase on vitamin E acetate. Fig. 3 represents the enzyme activity related to sodium taurocholate concentration for different quantities of Brij. The inset in Fig. 3 represents the linear relationship between Brij and sodium taurocholate concentrations corresponding to 50% of the maximal activity. This result demonstrates that the two compounds play the same role of solubilizer by their detergent property. The intercept of the line with the ordinate gives the Brij concentration necessary to obtain 50% of activity in the absence of bile salt. The extrapolated number (2.5%) has been verified experimentally. The activities of carboxyl ester hydrolase on vitamin E acetate in the presence of 0.5% Brij 35 and different bile salts are reported on Table II. In the presence of cholate, taurocholate and glycocholate the specific activities were very high, whereas in the presence of deoxycholate and tauro-

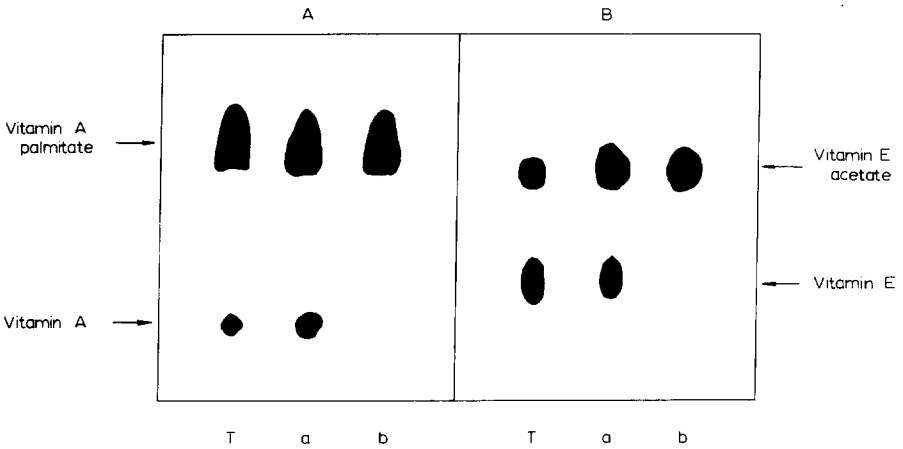

Fig. 2. Thin-layer chromatography of vitamin esters hydrolysates. (A) Hydrolysis of vitamin A palmitate. (B) Hydrolysis of vitamin E acetate. Hydrolysis have been performed in the presence of 6 mM sodium taurocholate. T, references; a, incubation mixture with enzyme; b, incubation mixture without enzyme.

deoxycholate both activities were low and represented approximately 6% of the activity measured with sodium taurocholate. Those results indicate that besides their detergent effect, trihydroxylated bile salts activate the enzyme.

Carboxyl ester hydrolase also hydrolyzed vitamin D-3 acetate and vitamin A

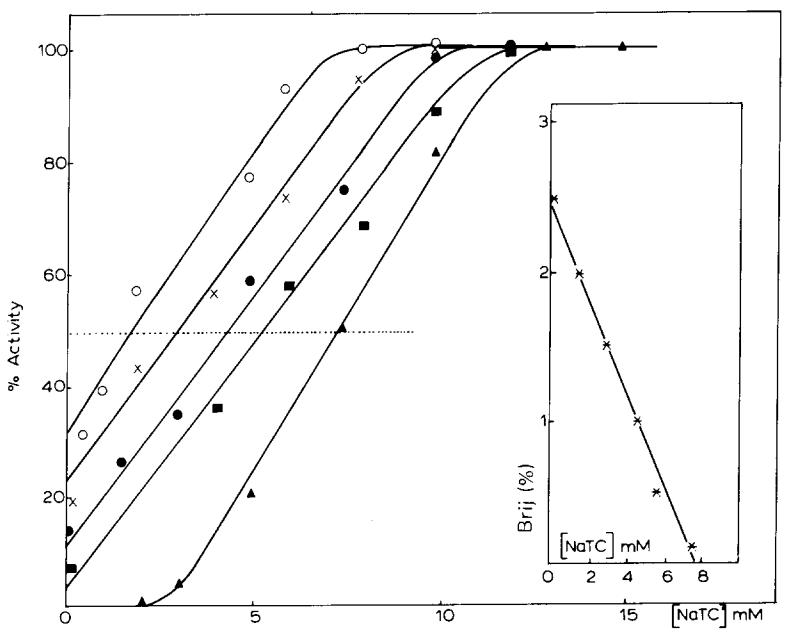

Fig. 3. Activity of carboxyl ester hydrolase on vitamin E acetate in relation to sodium taurocholate concentration (NaTC). The experiments have been performed in the presence of different quantities of Brij 35 (○, 2%; X, 1.5%; ●, 1%; ■, 0.5%; ▲, 0.12%). ------, 50% of the maximal activity. The inset gives the relationship between Brij and sodium taurocholate concentrations for 50% of maximal activity.

TABLE III

MOLECULAR WEIGHT OF CARBOXYL ESTER HYDROLASE IN THE PRESENCE OF BILE SALTS

Ble salt (0.5 mM)	Hydroxylation	Molecular weight
None	—	100 600
Taurocholate	3α, 7α, 12α	185 700
Taurodeoxycholate	3α, 12α	108 000
Taurochenodeoxycholate	3α, 7α	172 000

acetate dispersed in 0.5% Brij in the presence of 10 mM sodium cholate with specific activities of 250 and 140, respectively. In the presence of sodium deoxycholate no detectable hydrolysis appeared.

Molecular weight of carboxyl ester hydrolase in the presence of bile salts

On Table III are summarized the molecular weight of carboxyl ester hydrolase determined by ultracentrifugation in the presence of different bile salts, in concentration below their critical micellar concentration. In the presence of sodium taurocholate or taurochenodeoxycholate we have obtained a major component corresponding to a dimer, whereas in the presence of taurodeoxycholate the enzyme behaved like a monomer, as in the absence of bile salt.

Discussion

The results presented here have shown that in human pancreatic juice, carboxyl ester hydrolase and cholesterol ester hydrolase activities are properties of the same enzyme. This identity had been suggested by Erlanson for the rat enzyme [13]. In this report the constant ratio between the two activities during the different purification steps demonstrates clearly that the two enzyme activities belong to the same protein. It is interesting to compare the molecular properties of the human enzyme with cholesterol ester hydrolases purified from porcine pancreas [5] and rat pancreatic juice [6,9]. The molecular weights are different (100 000 for human, 80 000 for porcine and 65—69 000 for rat enzyme) and the amino acid compositions do not reveal a great homology between the rat and human proteins. Moreover the human enzyme is the only protein characterized as a glycoprotein [1]. However, if it is difficult to compare specific activities because of different assay methods, all these enzymes require the presence of bile salts for their activities.

Human carboxyl ester hydrolase is also responsible for the hydrolysis of lipid-soluble vitamin esters present in the diet. The identity with vitamin A esterase activity characterized in rat pancreatic juice is demonstrated. Moreover the human enzyme catalyzes the ester bond hydrolysis of vitamin D-3 and E. These activities show the lack of specificity towards the nature of the alcohol implicated in the ester bond since vitamin E, cholesterol or vitamin D-3 and vitamin A are, respectively, tertiary, secondary and primary alcohols. This activity is also bile salts dependent.

The important part played by bile salts on the activity of carboxyl ester

hydrolase needs to be discussed. Human cholesterol ester hydrolase did not hydrolyze cholesterol esters in the absence of bile salts and bile salts appeared to have a cofactor function. However, the results presented here have shown that this activator effect is dependent of bile salt hydroxylation. In the presence of 3α,12α-dihydroxylated bile salts (sodium deoxycholate and taurodeoxycholate) the activity was very low. It increased 30 times in the presence of sodium cholate and ten times in the presence of its taurine and glycine conjugates. Similar results had been obtained by Vahouny et al. [8] with cholesterol ester hydrolase activity present in rat pancreatic juice but the same activator effect had been observed for the three trihydroxylated bile salts. Additional experiments with sodium taurochenodeoxycholate and tauroursodeoxycholate have shown the specific activator effect of the 3α,7α-dihydroxylated bile salt and demonstrated the importance of the 7α-hydroxyl group for the activity of human cholesterol ester hydrolase. Similarly, the activity of carboxyl ester hydrolase on vitamin E acetate in the presence of di- (3α,12α) and trihydroxylated bile salts point out a specific activator effect of trihydroxylated bile salts. This activator effect is associated to a molecular interaction between bile salt and enzyme. Evidence is presented by the enzyme dimerization observed in the presence of the same bile salts as those which increased cholesterol ester hydrolase or vitamin ester hydrolase activities (taurocholate and taurochenodeoxycholate). This binding of specific bile salts would induce in the same time dimerization and activation of enzyme, which explains why the enzyme preincubated with sodium taurocholate was more active towards cholesterol esters than the enzyme preincubated with sodium taurodeoxycholate or the native enzyme. Similar bile salt-enzyme complexes have been reported on other cholesterol ester hydrolases. The porcine enzyme has been found to dimerize [5] and the rat enzyme was shown to form a polymer with a molecular weight of 400 000 in the presence of sodium taurocholate [9,14]. Additional data on human enzyme have demonstrated that this binding site specifically recognized the 7α-hydroxyl group and probably also the 3α-hydroxyl group. From a conformational point of view the 7α group must be necessarily in axial position while the 3α group would be equatorial, all extending to the same side of the molecule, opposite to the two methyl groups of bile salt.

If this cofactor function of bile salt is related to bile salt hydroxylation, other effects of bile salts on carboxyl ester hydrolase activity have been found independent of the nature of bile salt. As shown in the preceding papers [1,2] sodium taurocholate and taurodeoxycholate increase the activity on *p*-nitrophenylacetate and tributyrin. Both bile salts also decrease the activity on triacetin and phospholipids and in addition, our experiments have shown a competitive inhibition of triacetin hydrolysis by those bile salts. All those results suggest the existence of a second site of bile salts recognition which would be located at (or near) the catalytic site and would recognize bile salts as well as substrates. This effect of bile salt on the enzyme active site cannot be dissociated from the detergent effect on substrate since the three components are responsible for the catalytic action, enzyme, substrate and bile salt. In some cases, bile salt can form mixed micelles with substrate (phospholipids) in other cases and especially in the case of insoluble substrates (tributyrin, cholesterol or vitamin esters) bile salts surrounding or complexing substrate would play the

role of anchorage for the enzyme. This effect of bile salts on enzyme and substrate by the intermediary of the second site of recognition would explain the marked increase of cholesterol esters hydrolysis obtained with sodium cholate or taurocholate above 3 mM concentration, independently of the critical micellar concentration of each bile salt. It would be responsible for the augmentation of activity on tributyrin [2] and the ten-fold increase of affinity of human carboxyl ester hydrolase for *p*-nitrophenylacetate in the presence of bile salts [1]. Nevertheless, the direct detergent effect of bile salts on substrate cannot be eliminated. It is obviously demonstrated by the compensatory effect of Brij 35 in the absence of bile salt on vitamin E acetate hydrolysis.

The conjugated effect of bile salts on the two binding sites would explain some results obtained on cholesterol ester hydrolase activity: the high activity observed for low sodium taurocholate concentration after substrate preincubation with sodium taurodeoxycholate and the identical activator effects observed with sodium taurocholate alone and the mixture sodium taurocholate/taurodeoxycholate.

In conclusion, it seems evident that carboxyl ester hydrolase can bind bile salts by the intermediary of two sites. The first one would be specific of the 3α- and 7α-hydroxyl groups of cholanic acid; it would promote dimerization and consequently the activation of enzyme. The second site different from the first one would be unspecific towards the hydroxylation position of bile salts and would be located at (or near) the active center of human carboxyl ester hydrolase.

Acknowledgements

The authors are grateful to Dr. C. Figarella for fruitful discussion throughout this work. They express gratitude to Professor H. Sarles for his interest and encouragement. It is a pleasure to acknowledge Miss J. Savary (Laboratoire de Chimie Biologique, Faculté de Pharmacie, Marseille) for kindly performing the ultracentrifugation assays. The authors are glad to thank Mrs. P. Deprez for her skillful technical assistance and Mrs. D. Michel for the careful preparation of the manuscript.

References

1 Lombardo, D., Guy, O. and Figarella, C. (1978) Biochim. Biophys. Acta 527, 142—149
2 Lombardo, D., Fauvel, J. and Guy, O. (1979) Biochim. Biophys. Acta 611, 136—146
3 Lombardo, D. and Guy, O. (1979) Biochimie 61, 415—417
4 Erlanson, C. and Borgstrom, B. (1970) Scand. J. Gastroenterol. 5, 395—400
5 Momsen, W.E. and Brockman, H.L. (1977) Biochim. Biophys. Acta 286, 103—113
6 Hyun, J., Kothary, H., Hern, E., Mortenson, J., Treadwell, C.R. and Vahouny, G.V. (1969) J. Biol. Chem. 244, 1937—1945
7 Calame, K.B., Gallo, L., Cheriathundam, E., Vahouny, G.V. and Treadwell, C.R. (1975) Arch. Biochem. Biophys. 168, 57—65
8 Vahouny, G.V., Weersing, S. and Treadwell, C.R. (1965) Biochim. Biophys. Acta 98, 607—616
9 Hyun, J., Treadwell, C.R. and Vahouny, G.V. (1972) Arch. Biochem. Biophys. 152, 233—242
10 Erlanson, C. and Borgstrom, B. (1968) Biochim. Biophys. Acta, 167, 629—631
11 Erlanson, C. (1970) Scand. J. Gastroenterol. 5, 333—336
12 Yphantis, D.A. (1964) Biochemistry 3, 297—317
13 Erlanson, C. (1975) Scand. J. Gastroenterol. 10, 401—408
14 Hyun, J., Steinberg, M., Treadwell, C.R. and Vahouny, G.V. (1971) Biochem. Biophys. Res. Commun. 44, 819—825