COMPETITIVE INHIBITORY EFFECT EXERTED BY BILE SALT MICELLES ON THE HYDROLYSIS OF TRIBUTYRIN BY PANCREATIC LIPASE

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SUMMARY. Colipase was readily adsorbed on tributyrin particles emulsified with sodium taurodeoxycholate used above its critical micellar concentration. The ensuing rate of lipase adsorption on tributyrin depended on the amount of colipase already adsorbed at the interface. A molar excess of colipase over lipase was required to observe lipolysis at maximal velocity. Bile salt micelles competitively inhibited this reaction.

High bile salt concentrations markedly reduce the activity of pancreatic lipase (triacylglycerol lipase E.C.3.1.1.3) on triglycerides. According to MAYLIE et al (1) this inhibition corresponds to a sharp decrease of maximum velocity V and this parameter is not entirely restored by the presence of colipase, a small protein cofactor. In addition, the group of DESNUELLE has shown that colipase promotes the formation of a lipase-colipase-bile salt micelle ternary complex (2). This complex was thought to represent a preliminary step for lipolysis in the presence of bile salts (2). The present data support an alternative mechanism. First, our study indicates that colipase and lipase are indeed adsorbed on the substrate in the presence of bile salts. However, this occurs in succession, colipase acting as an anchor for lipase binding. Secondly, the binary colipase-bile salt micelle complex is a competitive inhibitor of the reaction. The binding of lipase to this complex yields an enzyme-inhibitor complex rather than an activated form of the enzyme required for substrate binding.

MATERIALS AND METHODS

Rat pancreatic lipase and bovine colipase were purified as previously described (3). Tributyrin was purchased from Fluka (Buchs, Switzerland). Sodium

taurodeoxycholate (NaTDC) came from Sigma (St. Louis, Missouri, U.S.A.). All the emulsions were prepared with an Ultra-Turrax type TB 18 homogenizer from Janke and Künkel KG (Staufen, Germany) for 1 min at 13,000 rev/min. The emulsion exhibited a slow but perceptible decrease of turbidimetry with time (50 % in 12 hours). However, the standardized emulsification procedure was easily reproducible and care was taken not to use these emulsions for more than one hour after their preparation for quantitative kinetic analysis.

In a first type of experiment the rate of lipase and colipase adsorption on tributyrin emulsified particles was measured. The substrate consisted of a 2 % tributyrin emulsion stabilized with 4 mM taurodeoxycholate and 150 mM NaCl and adjusted to pH 6.0 with a 0.1 M maleic buffer or to pH 7.0 with 0.1 M N-morpholinopropane sulfonic buffer. Incubation media were prepared by mixing 1.7 ml of the emulsion and 400 µl of a solution containing lipase (0.5 or 5.0 µg) together with increasing amounts of colipase. Each mixture was centrifuged immediately or after 1 to 5 min incubation at 25° C. After centrifugation for 5 min at 10° C and 1000 x g, the clear supernatant was decanted for lipase and colipase assays. Lipase was determined at pH 8.0 by an adaptation of the turbidimetric method of SHIHABI and BISHOP (4) using 0.04 % tributyrin instead of triolein and 1 mM taurodeoxycholate as stabilizer. Colipase was added in excess (20 µg/ml) to the substrate emulsion. Colipase was assayed by the same turbidimetric method but the emulsion was adjusted to pH 6.5 and contained 5 mM taurodeoxycholate and 150 mM NaCl. A large excess (10 µg) of pure rat lipase was added per colipase assay.

Kinetic experiments were performed to evaluate the influence of taurodeoxycholate and colipase on lipolysis at pH 6.0 and at 25° C. The activity of rat lipase was measured at seven concentrations of tributyrin (0.4, 0.6, 0.8, 1.0, 1.2, 1.4 and 1.6 %) each at forty different conditions of taurodeoxycholate and colipase concentrations (see below). Tributyrin was emulsified in a mixed buffer

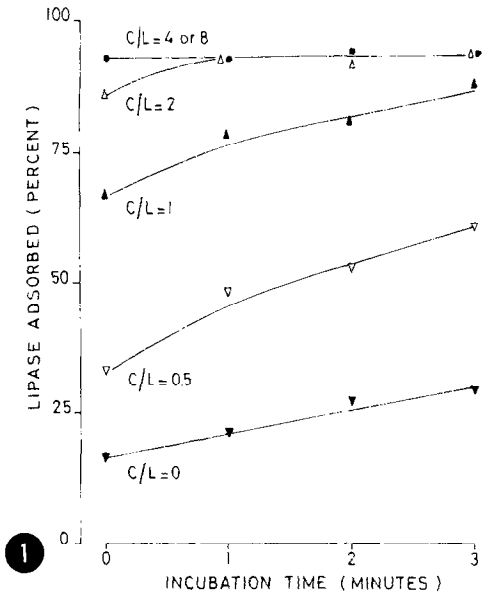
made of 2.4 mM acetic acid, 2.4 mM maleic acid, 2.4 mM N-morpholinopropanesulfonic acid, 2 mM sodium taurodeoxycholate, 150 mM NaCl and NaOH to pH 6.0 . Each incubation medium was made by mixing 1.7 ml emulsion with 400 µl water containing a constant amount of lipase (0.1 µg) plus colipase and taurodeoxycholate in increasing concentrations. Final taurodeoxycholate concentrations were 4, 6, 8, 10 or 12 mM (i.e., they were all above the critical micellar concentration (1). Lipase concentration was 1 nM and colipase concentrations were, respectively, 0.5, 1, 2, 4, 8, 12, 24 or 48 nM. Lipase was assayed by an adaptation of the automated potentiometric method described earlier (5). The incubation time was reduced to 2 min and the rate of analysis was increased to 60 samples per hour. The small pH fall recorded at the end of the incubation to measure lipase activity never exceeded 0.15 pH unit. The results were analysed by the method of WILKINSON (6) by plotting [S]/v versus [S].

RESULTS AND DISCUSSION

ADSORPTION EXPERIMENTS.

Increasing the pH from 6 (Fig. 1) to 7 (Fig. 2) reduced the rate of lipase adsorption on tributyrin emulsified particles coated with sodium taurodeoxycholate. These observations confirm and explain previously obtained pH-adsorption curves (7). It is clear that the rate of lipase adsorption is the limiting step of initial lipolysis at low colipase concentrations. At pH 6.0, however, increasing the colipase/lipase ratio to two was nearly enough to reach immediately a plateau of lipase adsorption, i.e., as soon as the incubation was started.

Fig. 2 also points out that colipase adsorption on the substrate did not parallel that of lipase, and was in fact a more rapid event. The percentage of adsorbed colipase was constant with time while the rate of lipase adsorption was dependent upon the amount of colipase already bound to the interface. This finding strongly suggests that each molecule of bound colipase acted as a binding site for lipase on taurodeoxycholate-coated tributyrin. The analogy



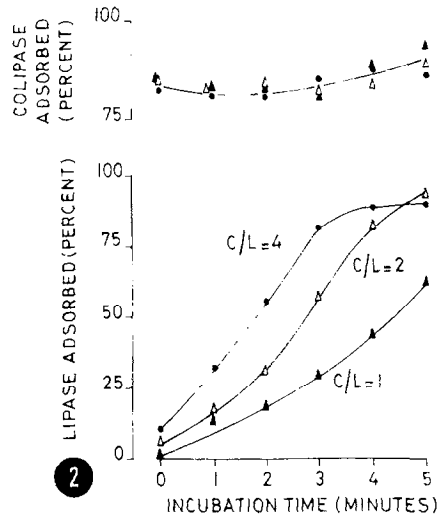


FIGURE 1. Time study of lipase adsorption on tributyrin in the presence of 4 mM taurodeoxycholate at pH 6.0 . The colipase/lipase (C/L) ratios in the incubation mixtures were zero (▼-▼), 0.5 (▽-▽), 1 (▲-▲), 2 (Δ-Δ), 4 and 8 (●-●). The ordinate indicates the percentage of the total lipase (0.5 µg per assay) adsorbed on tributyrin. Lipase adsorption was estimated by a measurement of its residual activity in the aqueous supernatant after centrifugation of the incubation mixture as detailed under Materials and Methods.

FIGURE 2. Time study of lipase and colipase adsorption on tributyrin in the presence of 4 mM taurodeoxycholate at pH 7.0 . Incubation media were prepared by mixing 1.7 ml emulsion with 0.4 ml of a solution containing 5 µg lipase and colipase in a molar ratio (C/L) of 1 (▲-▲), 2 (Δ-Δ) and 4 (●-●). Each mixture was centrifuged after 0 to 5 min incubation as described under Materials and Methods. Lipase and colipase were assayed in the supernatant.

with the ternary complex involving one molecule of colipase, one molecule of lipase and one bile salt micelle, as described by CHARLES et al (2) is obvious. Since colipase did promote lipase binding on tributyrin coated with bile salt (Fig. 2), as well as on bile salt micelles (2), it was logical to expect that taurodeoxycholate used at supramicellar concentration acts as a competitive inhibitor on lipolysis. The following kinetic experiments confirmed this assumption.

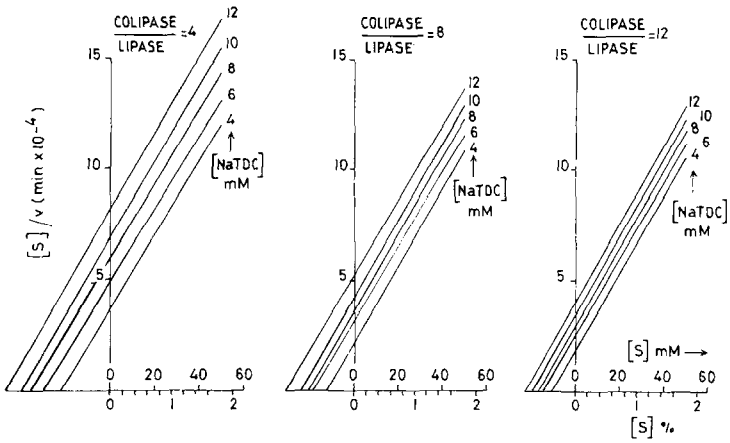


FIGURE 3. WILKINSON plots (6) of tributyrin hydrolysis at pH 6.0 and 25° C. Regression lines for 3 colipase/lipase ratios (C/L) as a function of taurodeoxycholate (NaTDC) concentration. The intercept with the abscissa and the slope of each regression line give the corresponding K_m app and V_o. Each line was calculated from seven points and each point was the mean of two experimental values. Single points are not represented since all correlation coefficients were above 0.99 .

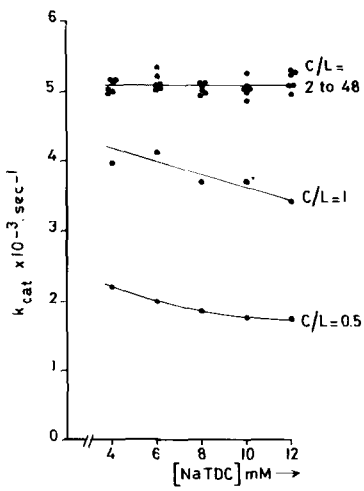


FIGURE 4. Effect of taurodeoxycholate on the apparent catalytic constant at increasing colipase/lipase (C/L) ratios. k_{cat} was estimated from values of V calculated as indicated in Fig. 3 and assuming a molecular weight of 48,000 daltons for rat lipase.

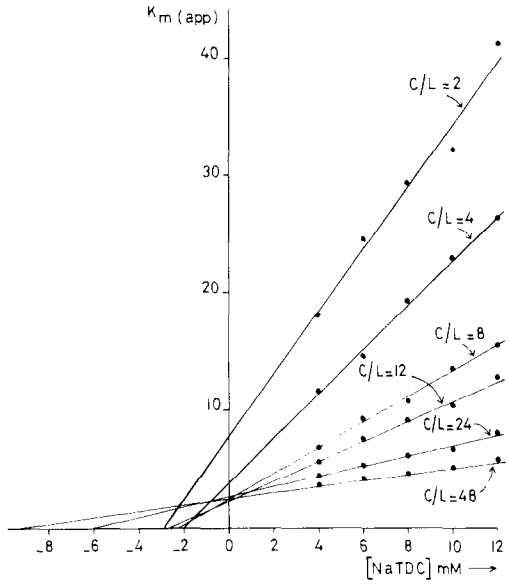


FIGURE 5. Competitive inhibitory effect of taurodeoxycholate on tributyrin hydrolysis for six colipase/lipase ratios (C/L). The values of K_m (app) were calculated as indicated in Fig. 3 and the straight lines are regression lines giving K_m (app) = K_m $(1 + \frac{[I]}{K_i})$.

KINETIC PARAMETERS.

Fig. 3 shows several series of typical linear WILKINSON plots (6) obtained for the action of lipase on tributyrin as a function of taurodeoxycholate concentration for 3 colipase/lipase ratios. These plots gave families of parallel straight lines. The values for K_m app (the intercepts on the abscissa) are valid only for the present kind of tributyrin emulsion utilized at pH 6.0 and at 25° C. This is because the interfacial area per unit volume was playing the role normally devoted to substrate concentration. The presence of taurodeoxycholate clearly increased the K_m app without affecting V (the slopes) inasmuch as all the lines were parallel.

Our results are at variance with those of MAYLIE et al (1) who observed that 4 mM taurodeoxycholate decreases V by 98 % and 52 %, respectively, when the colipase/lipase ratio increases from 0 to 3. On the contrary, Fig. 4 shows

that the relatively high catalytic constant of 5100 sec^{-1} was unaffected by a large (2 to 48) range of colipase/lipase ratios. However k_{cat} apparently decreased when the colipase/lipase ratio was as low as 1.0 or 0.5 . This was at least partly due to the slow rate of lipase adsorption observed under these conditions (Fig. 1) with a reaction system not yet at the steady state when kinetically analysed. It may be concluded that bile salt micelles induce a purely competitive inhibition of lipolysis when tested at steady state. The actual competitive inhibitor increasing the K_m of lipase for the ternary tributyrin-bile salt monolayer-colipase complex (2) was probably the similar binary bile salt micelle-colipase complex.

When the values of K_m app were plotted versus taurodeoxycholate concentration, linear relationships were obtained (Fig. 5). When colipase was increased from 2 to 12 nM, the K_m values (intercepts on the ordinate) decreased from 7.64 to 2.53 mM, while the K_i values of bile salt (intercepts on the abscissa) remained constant around 2.5 mM. When colipase was increased to still higher concentrations (24 or 48mM), the K_m values were not further affected, while the K_i values increased from 2.64 to 9.25 mM. As a result, the K_m/K_i ratios (i.e., the slopes of the straight lines) decreased from 2.75 to 0.27 . More data are necessary to explain why raising the colipase/lipase ratio well above unity still improved the hydrolysis of tributyrin catalyzed by pancreatic lipase. However, it is tempting to postulate that a moderate excess of colipase improved the binding of lipase on its bile salt-coated substrate whereas very high proportions of colipase inhibited the binding of lipase on bile salt micelles.

ACKNOWLEDGMENTS

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