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Bile salt-stimulated lipase in human milk

Evidence that bile salt induces lipid binding and activation via binding to different sites

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Human milk bile salt-stimulated lipase ensures efficient triacylglycerol utilization in breast-fed newborns. For activity against long-chain triacylglycerol, primary bile salts are a prerequisite. Bile salts also protect the enzyme from inactivation by intestinal proteases. We have studied the effect of different bile salts on activation, protease protection, lipid binding, and enzyme inactivation, caused by an arginine modifying agent. Based on the results we propose a model involving two bile salt binding sites; one activation-site specific for primary bile salt, and another, less specific, lipid binding promoting site at which also secondary bile salt binds. Binding to this latter site induces binding of enzyme to emulsified substrates but without subsequent lipolysis.

Bile salt-stimulated lipase; Human milk; Bile salt; Lipase; Enzyme activation

1. INTRODUCTION

The bile salt-stimulated lipase, a constituent of human milk, has a recognized function in milk lipid digestion in the breast-fed infant [1,2]. Cloning and sequencing of the respective cDNA from human mammary gland and pancreas has shown that the peptide chain of the milk enzyme is identical to that of the carboxyl ester hydrolase secreted from the pancreas [3–5]. There may, however, be minor molecular differences, presumably with regard to glycosylation, revealed by a slight difference in molecular size [6].

Bile salts have several effects on the enzyme(s). Activation against emulsified triacylglycerol or cholesteryl ester is achieved exclusively with primary bile salts, i.e. bile salt containing the 7α-hydroxyl group [7,8]. For activity against other substrates, e.g. *p*-nitrophenyl acetate, such activation is not required, although enhanced activity is obtained with various bile salts [7–9] but also other detergents [10]. For the milk enzyme it has been shown that protection against intestinal proteolysis is obtained by primary as well as secondary bile salts [11]. Kinetics of the bile salt effect has been studied using different substrates [7,9,10,12,13]. Models for the interaction of the milk enzyme with different substrates have also been proposed [14,15]. As yet, however, the mechanism of activation is largely unknown. Wang and Lee suggested that activation is caused merely by bile salt-induced binding of the enzyme to the emulsion particle surface [13]. For the pancreatic enzyme Lombardo et al. [16] have proposed a model according to which the peptide chain has two different bile salt binding sites; one that is non-specific as to type of bile salt and another that is specific for primary bile salts. They give evidence that primary, but not secondary bile salt, cause dimerization of the enzyme [17], presumably via interaction at the second site. Their model does not include any suggestions as to how the catalytical efficiency is affected by interaction at the different binding sites. The present study shows that activation of the milk enzyme cannot be fully explained by either of these two proposed mechanisms.

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2. MATERIALS AND METHODS

Bile salt-stimulated lipase was purified from human milk as described [18]. Sodium taurocholate was prepared by Dr L. Krabisch, Lund, Sweden. Sodium taurodeoxycholate, gum arabic, bovine serum albumin and butanedione (diacetyl) were from Sigma Chemical Co., St. Louis, MO, USA, and cholamidopropyl dimethylammonio propanesulphate (CHAPS) from Pierce Chemical Co., Rockford, IL, USA. Bovine trypsin (Trypsin TPCK) was from Worthington Diagnostic Systems Inc., Freehold, NJ, USA.

Lipase activity was determined as previously described using long-chain triacylglycerol emulsified in gum arabic as substrate [18].

2.1. *Binding to lipid emulsion*

Purified bile salt-stimulated lipase was labeled with ^{125}I by the lactoperoxidase/glucosoxidase method [19]. Iodination did not interfere with the specific activity of the enzyme. Labeled lipase was added to 4.5 ml of a mixture of 0.1 M NaCl, 40 mM Tris-Cl buffer, pH 9.0, and

contained an emulsion of 2 mg long-chain triacylglycerol in gum arabic and 9 mg bovine serum albumin per ml. After 15 min incubation at 20°C this mixture was transferred to ultracentrifuge tubes (13 × 51 mm, Beckman Instruments, Palo Alto, CA, USA). After centrifugation at 60,000 × *g* for 20 min the tubes were sliced below the fatty top phase This phase, as well as the aqueous sub-phase, was collected, the radioactivity counted and used as an estimate of the amount of enzyme present in the respective phase.

2 2. *Agarose gel electrophoresis*

15 ml of agarose solution, 1% (w/v) in 0.05 M sodium barbital buffer, pH 8.5, was poured onto glass plates (83.5 × 93.5 mm^2). Wells (1 × 7 mm^2) were punched 1 cm from the cathodal end. Samples (10–15 μl with a protein concentration of approximately 0.5 mg/ml) were applied and electrophoresis run for 2 h on a Multiphore (LKB, Sweden) at 4°C with a current of 4 V/cm. Bovine serum albumin and transferrin were included as references in all runs. 0 05 M sodium barbital, pH 8.5, was used as electrode buffer. When electrophoresis was run in the presence of bile salts these were included, at the concentrations given in Table I, in the cathode buffer as well as in the agarose solution. CHAPS was added only to the agarose solution. Protein was fixed with saturated picric acid in 15% acetic acid prior to pressing and drying of the plates. Proteins were stained with Coomassie brilliant blue.

3. RESULTS AND DISCUSSION

To explore the underlying structural requirements of a bile salt, or analogue, for various effects ascribed to

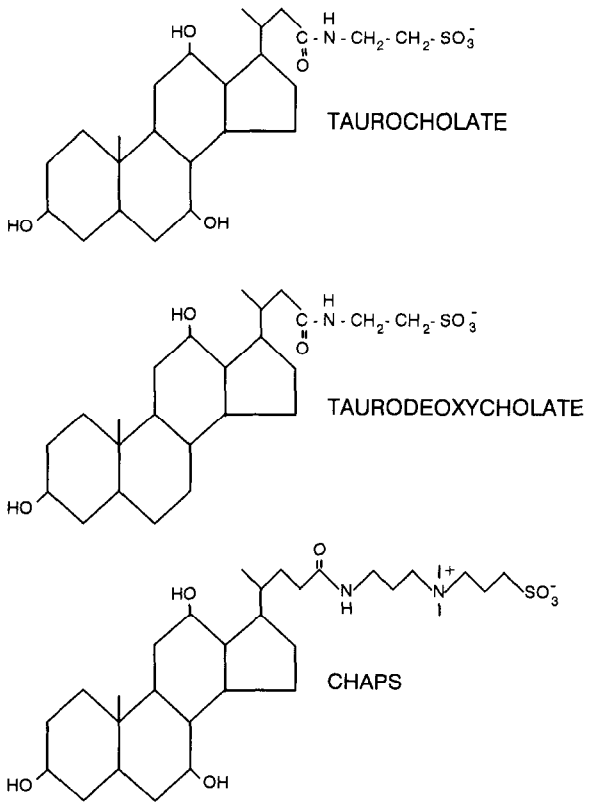

Fig. 1 Structures of the bile salts and bile salt analogue used in the present study: 2-[(3α,7α,12α-trihydroxy-24-oxo-5β-cholan-24-yl)-amino]ethane sulfonate (taurocholate), 2-[(3α,12α-dihydroxy-24-oxo-5β-cholan-24-yl)amino]ethane sulfonate (taurodeoxycholate), and 3α,7α,12α-trihydroxy-24-cholanamidopropyl dimethylammonio propanesulphate (CHAPS).

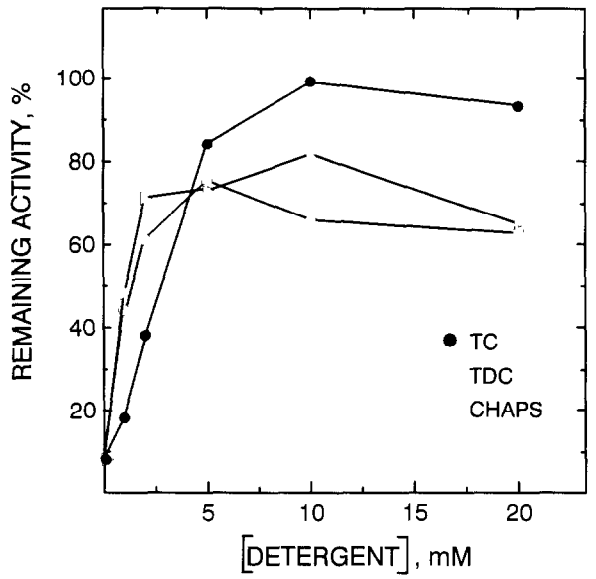

Fig. 2. Sensitivity of bile salt-stimulated lipase to inactivation by trypsin in the presence of bile salt or CHAPS. Purified bile salt-stimulated lipase was incubated with 10-fold molar excess of trypsin in 5 mM sodium barbital buffer, pH 7.4, containing 0.05 M NaCl. Taurocholate (TC), taurodeoxycholate (TDC) or CHAPS were included at the concentrations indicated. After 30 min incubation at 37°C aliquots were withdrawn and the remaining enzyme activity determined. Values are expressed as percent of parallel incubations in absence of trypsin.

bile salts we chose the following: cholate, which contains three hydroxyl groups (3α, 7α, 12α) and is classified as a primary bile salt; deoxycholate, which lacks the 7α-hydroxyl group and is classified as a secondary bile salt; and CHAPS, which is not classified as a bile salt but has an identical steroid backbone to cholate, including the 3α, 7α, 12α hydroxyl groups. For detailed structures see Fig. 1. In duodenal contents the major part of the bile salts are present as conjugates with taurine or glycine. CHAPS differs by having a permanent side chain of different structure. Thus, while the bile salts, conjugated or not, are negatively charged CHAPS has no net charge. Previously we have found that primary bile salts are a prerequisite for activity of bile salt-stimulated lipase with long-chain triacylglycerol, including those of human milk, as substrate [6,7,20]. Since conjugated, as well as non-conjugated cholate, is effective it is evident that the side chain is dispensable with regard to activation, however, since CHAPS was unable to activate the enzyme (data not shown) it is obvious that a structurally different side chain can interfere with the activation mechanism.

Another previously recognized physiologically important function of bile salts is to protect the enzyme from inactivation by trypsin and other proteolytic enzymes present in intestinal contents [11,21]. In contrast to activation, both types of bile salts, as well as CHAPS, have a protective effect (Fig. 2). This might suggest that protection is due to a non-specific detergent effect, but

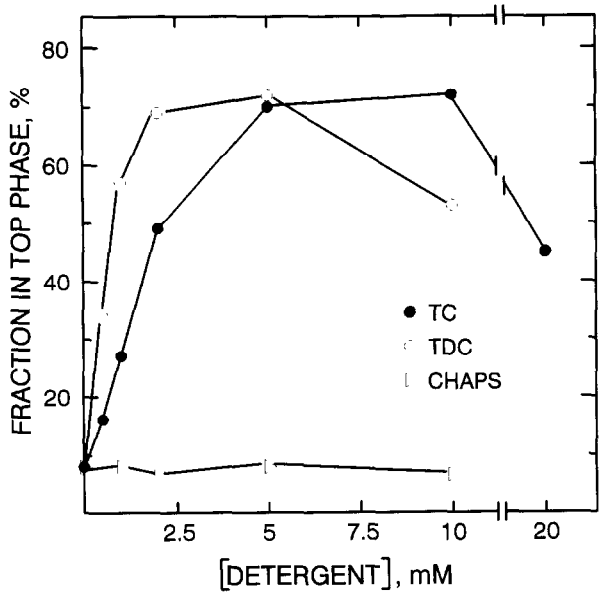

Fig. 3. Effect of bile salts and CHAPS on the binding of bile salt-stimulated lipase to a lipid emulsion. Purified and ^{125}I-labeled bile salt-stimulated lipase was incubated with a lipid emulsion as described in section 2. Taurocholate (TC), taurodeoxycholate (TDC) or CHAPS were included at the concentrations given. The fatty top phase and aqueous sub-phase were recovered by centrifugation and their radioactivity counted.

Triton X-100, a potent detergent with completely different structure, caused no protection (data not shown).

Except for the requirement of primary bile salts the molecular events behind activation are unknown. Recently it was shown that taurocholate increased the binding of enzyme to a lipid–water interface, and this was proposed as the essential event in activation [17]. Therefore we studied whether bile salt-induced binding had the same specificity as activation.

Taurodeoxycholate, which does not activate, was at least as effective as taurocholate in binding the enzyme to emulsion particles (Fig. 3). At a 5 mM concentration, which is above that required for activity, about 70% of the enzyme was bound both with taurocholate and taurodeoxycholate. In contrast, CHAPS, irrespective of concentration, did not induce binding, demonstrating that the side chain, possibly due to its lack of net charge, had a decisive effect. From these results it is evident that the activation mechanism is more complex than previously suggested. Primary as well as secondary bile salts affected the mobility of purified lipase in charge-shift electrophoresis (Table I), demonstrating that both bile salts bind to the enzyme. This is in concert with previous studies with immobilized bile salt [22]. That the lipase is affected by primary as well as secondary bile salts is further supported by similar spectral changes observed for both types of bile salt when studied by fluorescence spectroscopy [23].

Table I
Charge-shift electrophoresis of bile salt-stimulated lipase

Addition	Relative migration
None	1.00
5 mM taurocholate	1.37
5 mM taurodeoxycholate	1.69
5 mM taurocholate and 5 mM CHAPS	1.19
5 mM taurodeoxycholate and 5 mM CHAPS	1.72

Since CHAPS did not induce binding to emulsion particles (Fig. 3) one would expect no interference of CHAPS with binding between enzyme and the two bile salts. Because CHAPS is devoid of a net charge this could be tested by use of charge-shift electrophoresis. Interestingly, binding of deoxycholate was unaffected while that of cholate was significantly reduced (Table I). This observation would be compatible with two binding sites for bile salts, one that is less specific and mediated via charge and another that is specifically dependent on the 7α-hydroxyl group of primary bile salts at which CHAPS competes.

Further studies of the interaction of bile salts with the enzyme protein were carried out by use of butanedione. This amino acid modifying agent binds covalently to arginine residues. When enzyme was mixed with butanedione, activity was lost in a time- and dose-dependent manner (data not shown). This is in agreement with observations made with carboxylic ester hydrolase [16], the pancreatic counterpart of the milk enzyme [3,5]. Amino acid analysis revealed that, at the highest con-

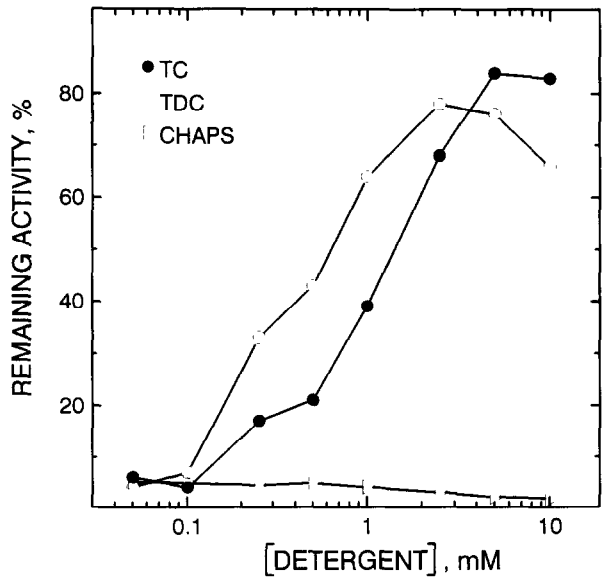

Fig. 4. Effect of bile salt and CHAPS on the inhibition of lipase activity by butanedione. Purified bile salt-stimulated lipase was incubated at 37°C in 10 mM butanedione in 0.1 M sodium borate buffer, pH 8.5. Prior to the addition of butanedione taurocholate (TC), taurodeoxycholate (TDC) or CHAPS were added to the concentrations indicated. After 30 min incubation aliquots were withdrawn and their enzyme activity assayed. Values are expressed as percent of a control incubated in parallel in the absence of butanedione.

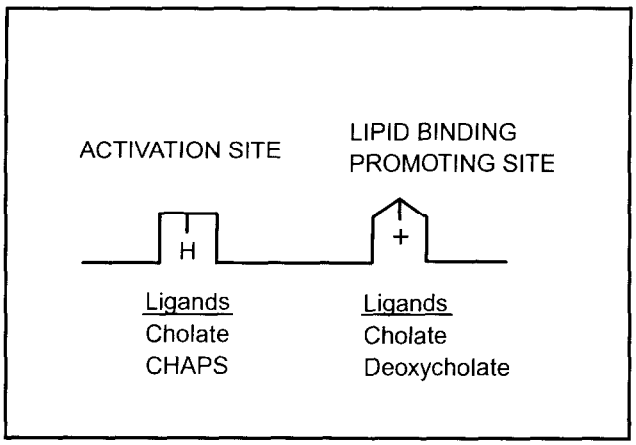

Fig. 5. Schematic presentation of the tentative bile salt binding sites on bile salt-stimulated lipase. The proposed nature of the binding to the respective sites are indicated by H for hydrogen bonding and by + for charge-dependent interaction The potential ligands, used in this study, to the sites are indicated

centration used, about two thirds of the arginine residues were modified (data not shown). If the enzyme was mixed with bile salts prior to incubation with butanedione the results were different. Increasing concentrations of cholate, or of deoxycholate, led to a protection (Fig. 4), i.e. the higher the bile salt concentration the more enzyme activity was retained up to a maximum of 80% at 2 mM bile salt. These data strongly suggest that arginine residue(s) are involved in bile salt binding. That CHAPS did not have a protective effect (Fig. 4) implies that this binding is dependent on the charge of the side chain of the ligand.

From the presented data a tentative model for the bile salt interaction with bile salt-stimulated lipase can be drawn (Fig. 5). Bile salt interacts with at least two sites on the enzyme protein. The first site is less specific and may be designated the 'lipid binding promoting site'. At this site one or several arginine residues on the enzyme interact with negatively charged side chains of bile salts, regardless of the hydroxylation pattern of the backbone. Hence, both primary and secondary bile salts are potential ligands to this site. Binding of bile salt to this site is a prerequisite for binding of the enzyme to a triacylglycerol emulsion surface. In contrast to previous suggestions [13], however, merely binding does not cause activation. If it did, there should be no obligatory requirement for primary bile salts for activation. Thus, a second site must be present which is specific for bile salts containing a 7α-hydroxyl group. This is evident from the fact that deoxycholate, differing from cholate only by its lack of a 7α-hydroxyl group (Fig. 1), is unable to activate the enzyme. Most reasonably, interaction at this site occurs specifically via this hydroxyl group.

This model is compatible with our previous observation that the presence of secondary bile salt (taurodeoxycholate) reduces the concentration of primary bile salt (taurocholate) needed for activation [21]. Two binding sites with different specificity is also in agreement with what has been proposed for carboxylic ester hydrolase [16]. For this enzyme binding at the specific site causes dimerization of the enzyme which is considered as the essential step in activation. This does not occur with bile salt-stimulated lipase (unpublished observation). A specific conformational change induced by binding of primary bile salt seems a likely prerequisite but needs to be verified.

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