Site-directed Mutagenesis of the Basic N-terminal Cluster of Pancreatic Bile Salt-dependent Lipase

FUNCTIONAL SIGNIFICANCE*

Emeline Aubert‡, Véronique Sbarra, Josette Le Petit-Thèvenin, Anne Valette, and Dominique Lombardo‡

From the INSERM U-559, Unité de Recherche de Physiopathologie des Cellules Epithéliales, Faculté de Médecine, 27 blv Jean MOULIN, 13385 Marseille cedex 05, France

Previous studies have postulated the presence of a heparin-binding site on the bile salt-dependent lipase (BSDL), whereas two bile salt-binding sites regulate the enzyme activity. One of these sites may overlap with the tentative heparin-binding site at the level of an N-terminal basic cluster consisting of positive residues Lys336, Lys361, Lys363, Lys429, Arg454, Arg458, Lys462, and Arg503. The present study uses specific site-directed mutagenesis to determine the functional significance of this basic cluster. Mutations in this sequence resulted in recombinant enzymes that were able to bind to immobilized and to cell-associated heparin before moving throughout intestinal cells. Recombinant BSDL was fully active on soluble substrate, but mutants were less active on micellar cholesterol olate in comparison with the wild-type enzyme. Activation studies by primary (sodium taurocholate) and by secondary (sodium taurodeoxycholate) bile salts revealed that the activation of BSDL by sodium taurocholate at concentrations below the critical micellar concentration, and not that evoked by micellar bile salts, was affected by substitutions, suggesting that this N-terminal basic cluster likely represents the specific bile salt-binding site of BSDL. Substitutions also affected the activation of the enzyme promoted by anionic phospholipids, extending the function of this site to that of a cationic regulatory site susceptible to accommodate anionic ligands.

Bile salt-dependent lipase (BSDL, EC 3.1.1.13) is a lipolytic enzyme secreted by the acinar pancreatic cell into the duodenum, where it plays a significant role in dietary lipid digestion. BSDL has been shown to display wide substrate reactivities ranging from the hydrolysis of both long-chain and short-chain fatty acid esters of glycerol, as well as phospholipids, lysophospholipids, and esters of cholesterol and of the fat-soluble vitamins A, E, and D (1). BSDL, unlike other lipases, is characterized by a unique activation mechanism requiring the binding of bile salts. Early studies have proposed that bile salts interact with two sites on the protein (2). One site, specific for primary bile salts, is associated with enzyme dimerization and activation, whereas the second is less specific, is able to bind indistinctly primary and secondary bile salts, and is involved in the enzyme binding to micellar or aggregated substrates (3, 4). More recently, the presence of these two bile salt-binding sites has been detected on the human milk counterpart enzyme referred to as bile salt-stimulated lipase (5). These two bile salt-binding sites have been tentatively localized on each BSDL molecule forming the dimeric BSDL-taurocholate complex crystal (6, 7). The first one, proximal to the catalytic site, could be identified as the specific binding site (8). The second one is located in a depression region on the back side of the catalytic domain of the enzyme. Binding of a monomeric primary bile salt to the specific site leads to the opening of a loop comprised of residues His115 to Tyr125 of the bovine BSDL, a loop that otherwise is in a closed conformation which might hinder substrate binding (6, 7). Using chemical modification approaches, tyrosine and arginine have been identified as key residues for BSDL interaction with bile salts (3, 4). Furthermore, a recent study demonstrated that Arg63 is essential for the enzyme activity on substrates such as cholesteryl olate solubilized by sodium taurocholate (9).

It has been shown that BSDL binds to heparin-like molecules lining the intestinal wall and that this binding is reversed by the addition of soluble heparin (10). Furthermore, BSDL located on the epithelial cell surface may facilitate the uptake of hydrolyzed dietary lipids and cholesterol (11). However, the association of BSDL with membrane-associated heparin of intestinal cells may be a prerequisite for the uptake of BSDL by these cells and the consecutive transcytotic motion of the enzyme throughout the enterocyte (12). Several groups have proposed that a basic cluster in the N-terminal domain of BSDL may be involved in the binding to heparin (6, 8). This N-terminal cluster of positively charged residues forms a cationic protrusion at the surface of the protein (6, 8). The side chains of Lys336, Lys361, and Lys429 lie on one side of a groove, whereas those of Lys454, Lys462, and Arg503 lie on the other side (6). These latter cationic residues are common to all BSDL (13). Another basic cluster consisting of seven positively charged residues (Lys356, Arg358, Lys361, Arg448, Arg454, Lys462, and Lys503) has been localized on the BSDL surface (6). This cluster could facilitate the binding of BSDL (possibly dimers) to micelles (6).
On the surface of the BSDL dimer, these residues form two rows of positively charged residues parallel to each other that could also represent another putative heparin-binding site. From those data, it looks possible that heparin-binding and bile salt-binding surfaces may overlap to some degree.

The close localization of the sequence Lys-Lys-Arg⁶³⁷ forming the putative heparin site with the disulfide bridge formed by Cys⁶⁴ and Cys⁶⁶, leads us to also postulate that this site may be involved in the sensing of the BSDL folding and the secretion of the protein (1). Furthermore, cationic residues of this cluster may not only accommodate the sulfate group of taurine-conjugated bile salts and those of heparin but also the anionic polar head group of phospholipids such as phosphatidylserine, phosphatidylinositol, and phosphatidic acid that have been shown to regulate neutral cholesterol ester hydrolase (14). The aim of the present study is to use a site-directed specific mutagenesis approach to determine the functional significance of the N-terminal basic cluster of BSDL.

EXPERIMENTAL PROCEDURES

Materials—Unless otherwise stated, all A grade chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Bovine serum albumin (BSA), trypsin, and all restriction enzymes were purchased from New England BioLabs (Beverly, MA). 

Plasmid Construction—The pECE-1 cDNA fragment was isolated from a plasmid, pCEP4 containing the wild-type cDNA encoding BSDL. The pCEP4 plasmid was digested with HindIII and NotI restriction enzymes, and the digested plasmid was ligated into the pBluescript SK vector from which the wild-type cDNA encoding BSDL has been excised previously following the same restriction procedure. This eliminates undesired substitution in the vector. Plasmids pECE-1-3M and pECE-1-5M, bearing the desired mutation, were amplified and sequenced as above, and plasmids with the right orientation were transfected into CHO-K1 cells.

Mutagenesis of BSDL N-terminal Basic Cluster

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4-chloro-3-indoyl phosphate (0.5 mM each) in 0.1 M Tris/Cl buffer (pH 9.5), 100 mM NaCl, and 1 mM MgCl₂ and then analyzed by densitometric scanning and quantified using the Image program (National Institutes of Health, Bethesda, MD).

**Northern Blotting**—Total RNAs were extracted from transfected CHO cells using Trizol reagent (Invitrogen). After migration on 1% agarose gel, RNAs (about 20 μg) were transferred onto a nitrocellulose membrane (22). cDNA probes specific for pancreatic BSDL (500 bp) and for β-actin (300 bp) were obtained and used as described previously (13). Probes were radiolabeled by using [α-32P]dCTP (PerkinElmer Life Sciences) and the random primed DNA labeling kit (Roche Molecular Biochemicals). Conditions used for dot-blot dilution and quantification have been already described (22).

**Binding of Wild-type and Recombinant Mutants of BSDL to Heparin-Sepharose**—Heparin-Sepharose (Sigma) column (3 ml wet gel) was equilibrated in a Tris-Cl buffer (10 mM, pH 7.0, 50 mM NaCl, loading buffer). Wild-type or recombinant mutants of BSDL expressed during 16 h in the conditioned medium (without FCS) of transfected CHO-K1 cells were concentrated by centrifugation on Amicon filters (Bedford, MA) up to 3.8–4.0 ml. This medium containing 0.3–0.4 BSDL units as determined on 4-NPC was loaded onto the heparin-Sepharose column and incubated for 16 h at 4 °C under rotation. Unbound material was then eluted (1 ml/min) with the loading buffer, and bound material was further eluted with a linear NaCl concentration gradient from 50 to 500 mM in 10 ml (pH 7.0, Tris-Cl buffer). Two washings were then performed successively with 1 and 2.5 mM NaCl in the Tris-Cl buffer. The elution profile was monitored by recording BSDL activity on 4-NPC.

**RESULTS**

**Expression of Recombinant Mutagenized BSDL**—The expression of BSDL bearing the mutagenized putative heparin-binding site K32I, K56I, R63A and that bearing mutation of the basic N-terminal cluster K32I, K56I, K61I, K62I, and R63A was examined in all clones selected for G418 resistance (i.e. six clones with three mutations and three clones with five mutations) and compared with that of the wild-type 3B clone (17) and with that of the control clone (i.e. transfected with the pMAM-neo plasmid only). For this purpose, all positive clones were cultured to subconfluence, their respective conditioned media were withdrawn for further analyses, and cells were washed with PBS, harvested, and lysed. Alternatively, before lysis, cells expressing recombinant BSDL were washed with PBS containing 0.25 mM NaCl (a salt concentration that should liberate BSDL associated with heparanoids of the outer CHO cell surface, see below). Under these conditions, the amount of BSDL released from membranes never exceeded 5% of that found in the cell lysate (as assessed from the enzyme activity). The cell lysates were cleared and analyzed on SDS-PAGE and by Western blotting. Their activity on 4-NPC in the presence of 4 mM NaTC was also recorded. As shown in Fig. 1, all selected clones (excepted control clones) expressed BSDL albeit at various levels. The expression level of BSDL protein correlated with the activity on 4-NPC recorded in each lysate. When the BSDL activity monitored in each lysate was reported to the corresponding amount of protein determined from Western blotting quantification, similar ratios were obtained (230 ± 56, 222 ± 58 and 180 ± 50 for the wild-type enzyme (clone 3B), K61I/K62I/R63A mutants (clones 3M1-3M7), and K32I/K56I/K61I/K62I/R63A mutants (clones 5M1-5M5), respectively). This means that substitutions do not affect significantly the enzyme activity on 4-NPC. The esterolytic activity recorded in control clone lysates (clones C1-C3) could be due to endogenous esterases (29). The presence of BSDL was also examined in the conditioned medium of selected clones. Western blotting (Fig. 2) showed that BSDL is present in all culture media except those of control clones. Once again, the activity on 4-NPC recorded in these culture media paralleled the amount of BSDL as quantified by Western blotting.

These data indicated that, independently of the number of mutagenized residues of the N-terminal basic cluster, BSDL is normally expressed and secreted by transfected CHO cells. The amount of BSDL secreted by each clone corroborates the amount of enzyme that is expressed by the clone in question. Overall, in the presence of 4 mM NaTC, K61I/K62I/R63A and K32I/K56I/K61I/K62I/R63A mutagenized BSDL had comparable activity on synthetic water-soluble ester substrate such as 4-NPC than the wild-type enzyme.

From this point and besides clone 3B expressing the wild-type BSDL, two clones expressing K61I/K62I/R63A and K32I/K56I/K61I/K62I/R63A mutagenized BSDL (i.e. clones 3M2 and clone 5M5, respectively) expressing an amount of enzyme comparable with that of clone 3B were selected and further analyzed. Dot-blot and Northern blot analyses were used to assess the mRNA abundance and size in selected clones (Fig. 3). Dot-blot quantification indicated that the BSDL mRNA was in similar amounts in stably transfected CHO cell clones expressing wild-type (clone 3B), K61I/K62I/R63A (clone 3M2), and K32I/K56I/K61I/K62I/R63A BSDL (clone 5M5), whereas no mRNA can be detected in control clone C1. The mRNA (2.0 kb) encoding BSDL detected in clone 3B expressing the wild-type enzyme and that detected in clone 3M2 and 5M5 expressing K61I/K62I/R63A and K32I/K56I/K61I/K62I/R63A mutagenized variants of BSDL, respectively, was of the expected size (15). Also, the cDNA probe for β-actin hybridized with a transcript of the right size (13), present in all selected clones. The amount of mRNA encoding BSDL, equivalent in all selected clones, correlated quite well with the amount of BSDL expressed by the corresponding clone. Furthermore, and except for the control clone C1 that does not secrete BSDL, the three selected clones expressing wild-type, K61I/K62I/R63A, and K32I/K56I/K61I/K62I/R63A (i.e. 3B, 3M2 and 5M5 clones) secreted the enzyme at the same rate (19.4 ± 2.1 10⁻³ units/mg of cell proteins/h).

**Binding of Recombinant Wild-type and Mutant BSDL to Immobilized Heparin**—Wild-type BSDL (clone 3B), K61I/K62I/R63A (clone 3M2), and K32I/K56I/K61I/K62I/R63A (clone 5M5) mutagenized recombinant variants of BSDL were characterized based on heparin binding. Heparin-Sepharose chromatography was used to measure the relative affinities of mutants for heparin, and the position of the peak in the heparin-Sepharose chromatogram reflects the affinity of protein for immobilized heparin. Therefore the position of the peak is expected to shift to a lower salt concentration upon mutation of any residue that forms a heparin-binding site in the wild-type enzyme. Accordingly, the same amount of recombinant BSDL was chromatographed on a heparin-Sepharose column, and after elution of unbound material, bound enzyme was eluted with a linear gradient in NaCl. The results showed that mutations of either the putative heparin-binding sequence Lys-Lys-Arg or of this sequence associated with the substitutions of Lys residues to Lys and Lys did not affect the binding of BSDL to Sepharose-immobilized heparin. Wild-type and mutagenized variants of BSDL were eluted at a similar volume of the NaCl gradient (Fig. 4). Such results strongly suggest that amino acids of the N-terminal cationic cluster of BSDL, i.e. Lys-Lys-Arg, Lys, and Lys likely do not participate in binding to immobilized heparin.

**Transcytosis of Recombinant Wild-type and Mutants BSDL through Intestinal Cells**—Heparin-like molecules lining intestinal microvillosities have been implicated in the binding of BSDL to the intestinal wall (10). This binding should precede the transcytotic motion of the enzyme throughout enterocytes (17). Therefore, if mutagenized BSDL is still capable of binding to immobilized heparin molecules, it should a priori also be taken up by and move throughout intestinal cells. We attempted to demonstrate this specific point by examining the transcytosis of BSDL through Int-407 intestinal cells cultured in Transwell inserts to form a tight epithelium (12). As shown
in Fig. 5A, the wild-type (clone 3B), K61I/K62I/R63A (clone 3M2), and K32I/K56I/K61I/K62I/R63A (clone 5M5) recombinant variants of BSDL were allowed to move throughout Int407 cells at the same rate, from the apical reservoir to the lower reservoir of the Transwell insert. No activity on 4-NPC in the presence of 4 mM NaTC can be recorded with time in the lower reservoir when Int407 cells were incubated with the conditioned medium of control clone. Overall, independently of mutations, the transcytosis of recombinant BSDL after 3 h of incubation was inhibited by ~50% when heparin was co-incubated with the enzymes in the apical reservoir (Fig. 5B). At the end of the incubation of Int407 cells with recombinant variants of BSDL, the cell epithelium was exhaustively washed (12), and then the cells were scraped and lysed, and BSDL present in cell lysate was finally determined by recording the enzyme activity on 4-NPC in the presence of 4 mM NaTC (C). Values are means ± S.D. of three independent determinations.

**Catalytic Activity of the Recombinant Variants of BSDL—**
The activity of the wild-type (clone 3B), K61I/K62I/R63A (clone 3M2), and K32I/K56I/K61I/K62I/R63A (clone 5M5) recombinant variants of BSDL was recorded in the presence of 4 mM NaTC as a function of the 4-NPC concentration. The results showed that mutations of either Lys-Lys-Arg63 or of Lys-Lys-Arg63, Lys32, and Lys56 did not alter the hydrolytic activity of recombinant enzymes on this water-soluble substrate (Fig. 6). The double-reciprocal plot indicated that the affinity constant and the maximal velocity for 4-NPC hydrolysis was of the same order of magnitude for each recombinant variant.

The bile salt-dependent hydrolysis of cholesterol [14C]oleate was also recorded, and analysis of the enzyme kinetic data (Fig. 7) revealed that the major difference between the mutants and the wild-type enzyme resided in the maximal velocity, which significantly decreased with the number of mutations, the K32I/K56I/K61I/K62I/R63A (clone 5M5) mutant being much less active than the K61I/K62I/R63A (clone 3M2) mutant, which is itself much less active than the wild-type enzyme (clone 3B). The affinity constant for cholesteryl [14C]oleate showed no significant difference between the wild-type and mutagenized enzymes (approx. 10⁻⁷ M).

Activation of the Esterolytic Activity of Mutagenized and Wild-type BSDL by Bile Salts—We next examined the effect of primary and secondary bile salts on the esterolytic activity of mutagenized and wild-type BSDL. As shown in Fig. 8A, increasing concentrations of the primary bile salt NaTC also
enhanced the activity of recombinant variants of BSDL. Although activation kinetics of the wild-type BSDL and of the K61I/K62I/R63A mutant of BSDL were parallel, that of the K32I/K56I/K61I/K62I/R63A mutant differed a little and appeared biphasic. At NaTC concentrations below 150 μM (Fig. 8A, inset), the K32I/K56I/K61I/K62I/R63A BSDL mutant activity on 4-NPC was low. Increasing the NaTC concentration led to a higher activity, which reached that of the wild-type BSDL and K61I/K62I/R63A BSDL mutant for NaTC concentrations close to 500 μM. Then the activity of all recombinant variants of BSDL remained similar with NaTC concentrations up to 4 mM (Fig. 8A). When examining the effects of a secondary bile salt such as NaTDC, no difference in activation kinetics can be recorded between recombinant variants of BSDL (Fig. 8B) even at concentrations below 150 μM (Fig. 8B, inset). Clearly the maximal velocity of BSDL is reached above the CMC (CMC = 0.5 mM) of NaTDC (Fig. 8B), whereas this maximal activity is obtained below the CMC (CMC = 1.4 mM) of NaTC (Fig. 8A).

These data indicated that the interaction of NaTC with the K32I/K56I/K61I/K62I/R63A BSDL mutant differed from that of K61I/K62I/R63A mutant and wild-type BSDL and suggested that mutagenized residues are implicated in the binding of NaTC to the enzyme.

**Activation of the Esterolytic Activity of Mutagenized and Wild-type BSDL by Acid Phospholipids**—In the light of the cationic nature of the mutagenized residues, we wondered whether the N-terminal basic cluster may not be involved in the binding of acidic lipids to BSDL. We therefore investigated the effect of phosphatidylserine, phosphatidylinositol, and phosphatidic acid, all acid phospholipids, and phosphatidylcholine (a neutral-charged phospholipid) and compared this effect with that of NaTC. For these experiments, the activity of recombinant BSDL was recorded on 4-NPC in the presence of 4 mM NaTC, BSDL expressed by clone 3B; □, BSDL expressed by clone 3M2; ○, BSDL expressed by clone 5M5.
same extent, whereas at 125 μM, this bile salt poorly activated the K32I/K56I/K61I/K62I/R63A BSDL mutant (compare with the activity in the absence of bile salt). Independently of its concentration, NaTDC activated BSDL mutants and the wild-type enzyme to the same extent. When examining the effect of phospholipids on the esterolytic activity of BSDL, anionic phos-

FIG. 5. Transcytosis of recombinant variants of BSDL through human intestinal Int407 cells. Mutagenized and wild-type recombinant variants of BSDL (about 200 × 10⁻³ units of each) were incubated at 37 °C with monolayers of Int407 cells cultured on Transwell filters (12). The BSDL activity representing the amount of enzyme that has moved across Int407 cells was recorded in the lower reservoir (A). ○, wild-type BSDL (clone 3B); △, K61I/K62I/R63A BSDL mutant (clone 3M2); □, K32I/K56I/K61I/K62I/R63A BSDL mutant (clone 5M5); X, serum-free conditioned medium of control clone C1. Values are means ± S.D. of three independent determinations. Int407 cells cultured on Transwell filters were preincubated for 1 h either with 1 mg/ml heparin (~3000 Da, dark hatched columns) or without heparin (simple hatched columns) (B). Then recombinant variants of BSDL (about 200 × 10⁻³ units of each) were added, and the transcytosis of BSDL was determined after 3 h incubation by recording the enzyme activity in the lower reservoir. Values are means ± S.D. of three independent determinations and expressed as percent of values recorded in the absence of heparin. At the end of the incubation of Int407 cells with BSDL, cells were exhaustively washed, harvested, and lysed, and the amount of BSDL taken up by Int407 cells was determined by recording the enzyme activity (C). In cell lysate, the BSDL activity was defined as the difference of activity on 4-NPC recorded in the presence and in the absence of NaTDC (23). Results are averages of two independent determinations.
phospholipids phosphatidylserine, phosphatidylinositol, and phosphatic acid (125/H9262M) significantly enhanced the wild-type BSDL activity on 4-NPC to a significant value approximately half that promoted by NaTC at 4 mM, whereas the effect of zwitterionic phosphatidylcholine (125/H9262M) is much less significant and close to the effect of NaTDC at the same concentration. The activating effect of acid phospholipids on mutagenized variants of BSDL is less pronounced, and interestingly, the more basic charges were substituted, the less the BSDL activity is increased by anionic phospholipids. The effect of phosphatidylcholine on 4-NPC hydrolysis was not different between recombinant mutants and wild-type BSDL.

**DISCUSSION**

Two site-directed mutants of BSDL were constructed to define the functionality of the N-terminal basic cluster or putative heparin-binding site consisting of residues Lys32, Lys56, Lys61, Lys62, and Arg63. Site-directed mutagenesis was used to alter some amino acids involved in binding properties, and a limitation of this method is that there exists a possibility that the amino acid substitution causes a loss of function by altering the structure of the protein. Informative elements such as preservation of enzymatic activity suggest that the overall structure has not been perturbed, but one cannot state with certainty that this is the case. Despite this limitation and in the absence of information such as x-ray crystallography, site-directed mutagenesis is a useful tool for identifying potential structural features of proteins.

In this study, five clones bearing three substitutions (K61I, K62I, and R63A) and three clones with five mutations (K32I, K56I, K61I, K62I, and R63A) were obtained after selection with G418 and compared with the clone 3B expressing the wild-type BSDL (17). The activity of recombinant wild-type and mutant BSDL always paralleled the amount of enzyme detected by Western blotting in corresponding clones, demonstrating that the overall structure of the enzyme was not significantly altered by substitutions. Each selected clone expressing BSDL also secreted BSDL, and among these, clone 3B expressing the wild-type enzyme, clone 3M2 expressing the K61I/K62I/R63A BSDL mutant, and clone 5M5 expressing the K32I/K56I/K61I/K62I/R63A BSDL mutant that displayed similar amounts of BSDL mRNA, of BSDL protein, and of BSDL activity also secreted the enzyme at the same rate. These results demonstrated that BSDL is expressed and secreted by either clone and bring evidence that the substitutions in part of the sequence Lys-Lys-Arg63, which is close to the Cys64 residue that forms a bridge with Cys80 (6), do not interfere with the folding and the degradation of BSDL (1, 24).

The putative heparin-binding domain of BSDL was predicted...
from analyses of the primary amino acid sequence of the enzyme based on comparison with the consensus sequence of other heparin-binding proteins (25). However, once subjected to site-directed mutagenesis, no modification in heparin binding as determined on Sepharose-immobilized heparin can be recorded with the K61I/K62I/R63A BSDL mutant and with the K32I/K66I/K61I/K62I/R63A BSDL mutant as compared with the wild-type recombinant enzyme. This result agrees with that of Liang et al. (9) on the R63A mutant of BSDL. Furthermore, recombinant BSDL mutants were still capable of tran-

et al. (7, 8), another positive area was detected in the C-terminal primary sequence of BSDL. In the human enzyme, this cluster consists of six positively charged residues, five of which are also conserved in all known BSDL (13). This positive area could facilitate the binding of the BSDL dimer to anionic micelles (6). Another possibility is that this cluster helps BSDL (possibly a dimer) to anchor to heparin molecules lining the brush-border membrane. The localization of this cluster, remote from the catalytic site, may leave the enzyme totally functional and agree with the results of Spilburg et al. (26), showing that the interaction of BSDL with membrane-associated heparin enhanced the enzyme activity on micelles of NaToco-cholesteryl oleate. Therefore it could be that the C-ter-

terminal positive cluster of BSDL represents the major functional site for binding to immobilized or membrane-associated heparin. Multiple cationic sites susceptible to bind (or not) to heparin are also present in lipoprotein lipase and hepatic lipase sequences (27–29).

From this point, the functional significance of the N-terminal basic cluster of BSDL remains an open question. The position of the N-terminal basic cluster, proximal to the active site (7), and its possible overlap with a site for bile salt binding lead us to investigate the catalytic properties and activation of muta-
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From this point, the functional significance of the N-terminal basic cluster of BSDL remains an open question. The position of the N-terminal basic cluster, proximal to the active site (7), and its possible overlap with a site for bile salt binding lead us to investigate the catalytic properties and activation of mutagenized BSDL. The activity of the two BSDL mutants on the water-soluble 4-NPC substrate in the presence of various ligands at the indicated concentrations. The activity was reported to
ing the maximal velocity above the CMC of NaTDC (i.e., > 0.5 m). These results demonstrated that the interaction of NaTDC, and not that of micellar bile salts, with BSDL is affected by amino acid substitutions in the N-terminal basic cluster. Consequently, this cluster likely represents the specific bile salt-binding site of BSDL susceptible to bind NaTDC at concentrations below the CMC (3).

BSDL was also detected in blood (30), where it is associated with atherogenic low density lipoproteins (31). BSDL is synthesized to a limited extent by human-monocytes-macrophages (32), endothelial cells (33, 34), eosinophils (35), and the liver (22, 36, 37). BSDL has also been localized in necrotic areas of the pancreas, consecutive to an acute pancreatitis (38). The presence of BSDL in such a wide range of tissues and organs, normal or pathological, suggests a broad physiological function in the body. The underlying question about the physiological function of BSDL outside the duodenum concerns the activation of the enzyme, which in the absence of bile salt cannot hydrolyze lipid substrates (2, 39). We, therefore, have extended the effect of anionic bile salts to acidic phospholipids that have been shown to regulate neutral cholesterol esterase of alveolar macrophages (14). As shown here, anionic phospholipids such as phosphatidylserine, phosphatidylinositol, and phosphatidic acid, contrary to the zwitterionic phosphatidylcholine, are able to enhance the activity of the wild-type BSDL to a value higher than that of NaTC at the same concentration. Furthermore, the two mutants of BSDL used here were less and less activatable with an increasing number of mutagenized amino acids. These data suggest that the N-terminal cationic cluster of BSDL could be, in fact, not only a specific bile salt-binding site but more generally a cationic regulatory site capable of accommodating anionic ligands. Occupancy of this site by soluble hepamin may explain the inhibition promoted by this ligand on the human BSDL activity on NaTDC-cholesteryl oleate micelles (26). The presence of this regulatory site on BSDL could be physiologically relevant as it may be involved in regulating the enzyme activity once, for example, in the atherosclerotic lesions of the vascular endothelium, where both BSDL and acid phospholipids (40) were detected.

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