Site-directed Mutagenesis of the Distal Basic Cluster of Pancreatic Bile-dependent Lipase*  

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Received for publication, July 8, 2004, and in revised form, July 19, 2004  
Published, JBC Papers in Press, July 20, 2004, DOI 10.1074/jbc.M407646200  

Previous studies have postulated the presence of two bile salt-binding sites regulating the activity of the pancreatic bile-dependent lipase. One of these sites, located in an N-terminal basic cluster, has been identified as the specific bile salt-binding site. Interaction of primary bile salts with this proximal site induces the formation of a micellar binding site from a pre-existing nonspecific or pre-micellar bile salt-binding site. Here we have investigated the functional significance of another basic cluster comprised of amino acid residues Arg454, Lys458, Arg462, and Lys465, distal from the catalytic site. For this purpose these residues were mutated in Ile or Ala residues. The mutagenized enzyme lost activity on both soluble and emulsified substrates in the presence of bile salts. However, in the absence of bile salts, the mutagenized enzyme displayed the same activity on soluble substrate as the wild-type recombinant enzyme. Consequently, the distal basic cluster may represent the nonspecific (or pre-micellar) bile salt-binding site susceptible to accommodate primary and secondary bile salts. According to the literature, tyrosine residue(s) should participate in this site. Therefore, two tyrosine residues, Tyr452 and Tyr453, associated with the distal basic cluster were also mutagenized. Each tyrosine substitution to serine did not inhibit the enzyme activity on soluble substrate, independently of the presence of primary or secondary bile salts. However, the enzyme activity on cholesteryl oleate solubilized by sodium taurocholate, primary bile salt micelles, was decreased by mutations substantiating that these residues are part of the nonspecific bile salt-binding site.

Bile salt-dependent lipase (BSDL, EC 3.1.1.13), also referred to as cholesteryl ester hydrolase or carboxyl ester lipase, 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 (2) have proposed that bile salts interact with two sites on the protein. One site, specific for primary bile salts, is associated with enzyme dimerization and activation, whereas the second site is less specific, 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 distal to the active site locates in the region on the back side of the catalytic domain of the enzyme. For instance, binding of a monomeric primary bile salt to the specific site leads to the opening of an active site loop comprised of residues His115 to Tyr120 of the bovine BSDL, a loop that otherwise is in a closed conformation that might hinder substrate binding (6, 7). This loop is flexible when bile salts are absent (6), giving access to water-soluble substrates. Conflicting results were obtained with the truncated human BSDL crystal suggesting that the opening of the loop does not require bile salts (8). However, analysis of the full-length glycosylated native structure of human BSDL shows that the active site loop is in a closed conformation (8). By using chemical modification approaches, tyrosine and arginine have been identified as key residues for BSDL interaction with bile salts (3–5). Furthermore, a recent study (9) demonstrated that Arg63 is essential for the enzyme activity on substrate such as cholesteryl oleate solubilized by sodium taurocholate.

Crystal structure analysis shows that the N-terminal cluster of positively charged residues forms a cationic protrusion at the surface of the protein (6, 8). The side chains of Lys31, Lys56, and Lys58 lie on one side of a groove, whereas those of Lys61, Lys62, and Arg63 lie on the other side (6). These latter cationic residues are common to all BSDL (10). We have recently shown that this N-terminal basic cluster likely represents the so-called specific (or proximal) bile salt-binding site of BSDL also susceptible to accommodating anionic ligands such as acidic phospholipids (11). Functionally, this site may be involved in regulating the enzyme activity (11). Another basic cluster (referred to as the C-terminal or distal basic cluster) consisting of seven positively charged residues (Lys326, Arg328, Lys329, Arg454, Arg458, Lys462, and Lys503) has been localized on the BSDL surface at the back side of the N-terminal basic cluster.

* This work was supported in part by a grant-in-aid from the Conseil General des Bouches-du-Rhône (Marseille, France) and by institutional funding from INSERM (Paris, France) and the Université de la Méditerranée (Marseille, France). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

† Recipient of a fellowship awarded by INSERM and the Conseil Régional PACA (Marseille, France).

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§ The abbreviations used are: BSDL, bile salt-dependent lipase; 4-NPC, 4-nitrophenyl hexanoate; CMC, critical micellar concentration; NaTC, sodium taurocholate; NaTDC, sodium taurodeoxycholate; CHO, Chinese hamster ovary; PA, phosphatidic acid; Mes, 4-morpholineethanesulfonic acid; FCS, fetal calf serum.

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Mutagenesis of BSDL Distal Basic Cluster

EXPERIMENTAL PROCEDURES

Materials—Unless otherwise stated, all chemicals were purchased from Sigma. Ham's F-12 culture medium was from Invitrogen. Taq polymerase was purchased from Clontech and was a part of the GC-rich PCR kit. Polyclonal antibodies (pAbL10) against a peptide sequence representing the distal basic cluster of BSDL (10) were used to detect any misincorporation of bases by the polymerase in the amplification reaction. The amount of the initial substrate was maintained below 100 ng. Independently of the pair of primers used, the amplification was performed on a PerkinElmer Life Sciences 2400 GeneAmp PCR system using a pre-run cycle (94 °C, 140 s), and 12 reaction cycles program as follows: denaturation (94 °C, 20 s), annealing (60 °C, 30 s), and extension (68 °C, 4 min). A treatment with DpnI endonuclease was used to eliminate the methylated parental DNA template. About 10 μl of the digested material were used to transform competent E. coli cells (Top10 F' strain) that were spread on agar plates containing ampicillin and incubated at 37 °C overnight. Colonies were picked up and cultured in Luria-Bertani medium supplemented with 50 μg/ml ampicillin. Cells were pelleted, and plasmid cDNA was isolated by using a miniprep kit method (NucleoSpin Plus, Macherey-Nagel). The first mutagenized plasmid was obtained using the first pair of primers (Table I) allowing the mutagenesis of Arg423 and Lys429 into Ala and Ile residues. This latter plasmid, including sequences encoding Ala and Ile residues at positions 423 and 429, was totally sequenced and used as template to substitute Arg454, Arg458, and Lys462 residues by Ala454, Ala458, and Ile462. At the end one plasmid was obtained bearing mutations on a codon encoding residues Arg454, Lys459, Arg458, Arg454, and Lys462, representing the distal basic cluster of BSDL. The final plasmid was completely sequenced by using specific primers for the BSDL sequence (10) to detect any misincorporation of bases by the polymerase in the BSDL cDNA. Mutagenized cDNA of BSDL was then digested using EcoRI restriction enzymes and ligated into the pECE-1 vector from which the wild-type cDNA encoding BSDL had been excised previously by following the same restrictive digestion procedure. This eliminates undesired substitution in the vector. The plasmid pECE-1-SMC was thus obtained and, bearing the desired mutations, was amplified and sequenced as above. The same protocol was used to obtain pECE-1-E and pECE-1-I, plasmids, which include mutagenized cDNA encoding Y427S and Y455S recombinant mutants of BSDL. Plasmids with the right orientation were transfected into CHO-K1 cells.

Co-transfection—pECE-1-SMC plasmid encoding mutagenized BSDL on the basic C-terminal cluster (referred to as R423A/R429I/R454A/R458A/K462I BSDL) was co-transfected with pMAM-neo vector.
Mutagenesis of BSDL Distal Basic Cluster

TABLE I

Oligonucleotide primer used for site-directed mutagenesis

| Mutagenesis of BSDL Distal Basic Cluster | 39699 |
|-----------------------------------------|-------|

| I. R423A/K429I mutations               |       |
|-----------------------------------------|-------|
| DNA sequence 5-TAC TCT TAC CGT TTT TCC CAC CCT TCA CGA ATG ACT ATC TAC CCA AAA TGG ATG GGG cDNA 3-AGG AGA GTG ACC AGT GCC ATG AGT GTG TTT ACC TAC CCC GGT GTG ATG Primer 5-CAT CGA ATG ACT ATC TCC CCA AAA TGG ATG GGG GCA GAC CAC- P-RT-Met-Gly- |       |
| Amino acids Gly-Lys-Pro-Pha-Ala-Thr-Pro-Leu-Gly-Tyr-Arg-Ala-Gln-Asp-Arg-Thr-Val-Ser-Asp-Ala-Thr-Val-Ser-Ile-Ala-Met |       |

| II. R454A/R458A/K462I mutations         |       |
|-----------------------------------------|-------|
| DNA sequence 5-GGG AGG GTG GGA AGT GCC ACC CGG TAC GGG GCC CAA GAC ACT GTC TCC cDNA 3-CGC AAA GGG TGG GTG GAC CGG GTG ACC GTT CTG TAC CTG GAG- P-RT-Met-Gly- |       |
| Amino acids Phe-Ser-Glu-Pro-Pha-Ala-Thr-Pro-Leu-Gly-Tyr-Arg-Ala-Gln-Asp-Arg-Thr-Val-Ser-Asp-Ala-Thr-Val-Ser-Ile-Ala-Met |       |

| III. Y427S mutation                    |       |
|-----------------------------------------|-------|
| DNA sequence 5-GCC CCG AAC CCA CTG GGC TAC CGG GCC CAA GAC ACT GTC TCC cDNA 3-GGC CAC ACC CCA CTG GGC TAC CGG GCC CAA GAC ACT GTC TCC- P-RT-Met-Gly- |       |
| Amino acids Phe-Ser-Arg-Pro-Pha-Ala-Thr-Pro-Leu-Gly-Tyr-Arg-Ala-Gln-Asp-Arg-Thr-Val-Ser-Asp-Ala-Thr-Val-Ser-Ile-Ala-Met |       |

| IV. Y453S mutation                    |       |
|-----------------------------------------|-------|
| DNA sequence 5-GCC CCA CGG GGA AGT GCC ACC CGG TAC GGG GCC CAA GAC ACT GTC TCC cDNA 3-GCC CCA CGG GGA AGT GCC ACC CGG TAC GGG GCC CAA GAC ACT GTC TCC- P-RT-Met-Gly- |       |
| Amino acids Gly-Lys-Pro-Pha-Ala-Thr-Pro-Leu-Gly-Tyr-Arg-Ala-Gln-Asp-Arg-Thr-Val-Ser- |       |

The DNA codon and the resulting amino acid substitutions are underlined.

Expression of R423A/K429I/R454A/R458A/K462I Recombinant Mutagenized BSDL—The expression of BSDL bearing mutations of the distal basic cluster R423A, K429I, R454A, R458A, and K462I was examined in all clones selected for G418 resistance and compared with that of the wild-type 3B clone (14) to that of the control clone (i.e. transfected with the empty pMAM-neo plasmid). For this purpose all positive clones were cultured to subconfluence; their respective conditioned media were withdrawn for further analyses, and cells were washed with phosphate-buffered saline, harvested, and lysed.

First, dot blot and Northern blot analyses were used to assess the mRNA abundance and size in selected clones. Dot blot quantification (18) indicated that the BSDL mRNA can be detected in stably transfected CHO cell clones expressing wild-type (clone 3B) or R423A/K429I/R454A/R458A/K462I BSDL, whereas mRNA encoding BSDL cannot be detected in control
media did not parallel the amount of BSDL as quantified from Western blotting. Obviously, no activity can be recorded in the culture medium of MC9 cells. Those data suggest that substitutions significantly affect the enzyme activity on 4-NPC when recorded in the presence of activating concentrations of bile salt (4 mM NaTC). We have shown that unfolded BSDL cannot be secreted and instead is degraded by the proteasome (20, 21). No degradation product can be detected on Western blotting performed on MC9 clone lysates (not shown). Furthermore, the secretion of mutagenized BSDL suggests that mutations did not affect the overall structure of the enzyme. We thus have hypothesized that mutagenized residues could be involved in BSDL activation by bile salts.

Activation of Esterolytic Activities of R423A/K429I/R454A/R458A/K462I Mutagenized and Wild-type BSDL by Bile Salts—We then examined the effect of primary and secondary bile salts on the esterolytic activity of mutagenized and wild-type BSDL. As shown in Fig. 3A, the activities of wild-type and R423A/K429I/R454A/R458A/K462I BSDL, albeit low, are identical (see also Fig. 3B) when recorded in the absence of bile salt (without bile salt, see Fig. 3B, inset). These activities were corrected for the background esterolytic activity detected in conditioned medium of control clone C1. However, wild-type BSDL activity on 4-NPC largely increased in the presence of 4 mM NaTC, whereas NaTDC (125 μM) or NaTDC (125 μM or 4 mM) activates BSDL to a relatively lesser extent (50%) compared with NaTC. The activity of mutagenized R423A/K429I/R454A/R458A/K462I BSDL cannot be activated by NaTC or by NaTDC when used at lower (125 μM) or higher (4 mM) concentrations than their respective critical micellar concentration (CMC) determined under BSDL assay conditions that were 1.4 and 0.5 mM for NaTC and NaTDC, respectively (11).

In previous studies (11) we have shown that the bile salt-binding site specific for primary bile salts, which is constituted by the basic N-terminal cluster of BSDL (11), may accommodate acidic phospholipids. These acidic phospholipids have a similar activating effect than a primary bile salt such as NaTC at comparable concentrations. Therefore, we have tested the effects of phosphatidylserine, phosphatidylinositol, and phosphatidic acid (PA) on BSDL esterolytic activity on 4-NPC. As shown on Fig. 3B, whereas PA, phosphatidylinositol, and phosphatidylserine are activators of the wild-type enzyme on 4-NPC, they are more or less inefficient in activating the mutagenized BSDL. Note that zwitterionic phosphatidylcholine did not promote the increase in activity of both the wild-type and mutagenized recombinant BSDL. Therefore, the distal basic cluster of BSDL could also be able to recognize acidic phospholipids, and mutations of basic residues involved in this cluster impaired this recognition. These results were advanced by measuring the cholesterol ester hydrolase activity of wild-type BSDL and of mutagenized R423A/K429I/R454A/R458A/K462I BSDL by using cholesterol [14C]oleate as substrate. As shown in Fig. 3C, the wild type enzyme gains significant activity on cholesterol oleate when solubilized by primary bile salt such as NaTC, in part at a concentration above its CMC (i.e. >1.4 mM). Other ligands susceptible to activating the esterolytic activity of BSDL on 4-NPC such as PA or NaTDC (Fig. 3A) (11) failed to increase the activity of the wild-type enzyme on cholesterol oleate even when solubilized in micelles of secondary bile salt (for example, compare the enzyme activity on cholesterol oleate solubilized by 14.5 mM NaTDC with and without 0.5 mM PA). Preincubation of BSDL (15 min) with NaTC or PA (0.5 mM) prior to assay on cholesterol oleate solubilized by micellar NaTDC (14.5 mM) does not modify the activation profile (not shown). Independently of the conditions

Fig. 2. Secretion of mutagenized R423A/K429I/R454A/R458A/K462I BSDL by transfected CHO-K1 cells. Clones C1, MC9, and 3B were allowed to stand in the culture medium without FCS for 16–24 h. At the end, cell-conditioned medium of each clone was analyzed for BSDL expression by Western blotting using pAbL10 (Δ, 25 μl of cell-conditioned medium/lane). The kinetics of secretion (B) was performed by removing samples of the conditioned medium with time. Samples (50 μl) were analyzed by Western blotting with pAbL10 followed by quantification and by recording the BSDL activity on 4-NPC in the presence of 4 mM NaTC. Values are means ± S.D. of three independent determinations (C, 3B; Δ, MC9).

clone C1. Among transfected clones, clone MC9 expressed the same amount of mRNA encoding mutagenized BSDL than that of clone 3B expressing mRNA of wild-type BSDL. The mRNA encoding BSDL detected in clone 3B expressing the wild-type enzyme and that detected in clone MC9 expressing R423A/K429I/R454A/R458A/K462I mutagenized variant of BSDL respectively, was of the expected size (i.e. 2.0 kb) (12). Also, the cDNA probe for β-actin hybridized with a transcript of the right size (10), present in selected clones (data not shown).

The presence of BSDL was examined in conditioned medium of selected clones. Western blotting (Fig. 2) showed that BSDL is present in culture media of 3B and MC9 clone excepted that of control clone C1. However, clone 3B expressed and secreted two glycoforms of the wild-type BSDL migrating at 74 and 70 kDa, respectively (11, 14). These two glycoforms are also detected in rat pancreatic AR4–2J cells expressing BSDL (19). Clone MC9 only secreted the BSDL glycoform associated with the highest migration (see Fig. 2A). The rates of secretion of mutagenized and wild-type variants of BSDL are identical (Fig. 2B); nevertheless, the activity on 4-NPC recorded in culture
used, no activity can be recorded by using the mutagenized R423A/K429I/R454A/R458A/K462I BSDL.

These data indicated that the R423A/K429I/R454A/R458A/K462I BSDL mutant is active on soluble ester substrates but not on emulsified cholesterol oleate. Furthermore, this mutagenized variant of BSDL, contrary to the wild-type enzyme, cannot be activated by primary (NaTC) and secondary (NaTDC) bile salts nor by anionic phospholipids. Consequently, the basic C-terminal cluster of BSDL may represent the so-called nonspecific bile salt-binding site susceptible to accommodate primary and secondary bile salts (3, 4).

Expression of Y427S and Y453S Mutagenized BSDL—At least one tyrosine residue has been implicated in the nonspecific bile salt-binding site of BSDL (4). As shown on Fig. 1, two tyrosine residues, Tyr427 and Tyr453, are exposed at the surface of the enzyme as part of the distal basic cluster. Thus one (or both) of these Tyr residues can actually be essential to the interaction of BSDL with lipid substrates or micelles. Therefore, these two Tyr residues have been mutagenized separately into Ser residues. The pECE-1-E and pECE-1-L plasmids, including the mutagenized Y427S and Y453S cDNA of BSDL, were then transfected into CHO-K1 cells, and among clones resistant to G418, two clones were selected as they express comparable amounts of mutagenized BSDL than the 3B clone (Fig. 4A), although the secretion level of the Y453S mutant looks a little lower after 24 h. These clones referred to as E2 and L8 clones expressed Y427S BSDL and Y453S BSDL, respectively. Both mutagenized E2 and L8 and wild-type 3B clones secreted comparable amounts of enzyme (Fig. 4B).

Activation of Esterolytic Activities of Y427S and Y453S Mutagenized and Wild-type BSDL—In the absence of bile salt, E2 and L8 clones present a low activity on 4-NPC that is not significantly different from that of 3B clone (Fig. 4A, inset, without bile salt). However, mutagenized BSDL expressed by E2 (Y427S) and L8 (Y453S) clones is activable by bile salts. NaTC and NaTDC at a concentration above CMC (4 mM) or at a submicellar concentration (125 μM) are able to activate the esterolytic activity of Y427S and Y453S BSDL on 4-NPC.

FIG. 3. Activity of recombinant R423A/K429I/R454A/R458A/K462I variants of BSDL. A, the activity of recombinant wild-type BSDL and mutagenized R423A/K429I/R454A/R458A/K462I BSDL was recorded by using 4-NPC as soluble substrate in the absence of bile salt (inset, without bile salt, 100% represents the activity of the wild-type recombinant enzyme expressed by clone 3B) or in the presence of NaTC or of NaTDC at the indicated concentration. Empty column, clone 3B; filled column, MC9 clone. Values are means ± S.D. of three independent determinations. B, the activity of recombinant wild-type and mutagenized R423A/K429I/R454A/R458A/K462I BSDL was recorded using 4-NPC as in A in the absence (inset, without bile salt, 100% represents the activity of the wild-type recombinant enzyme expressed by clone 3B) or in the presence of 125 μM phospholipids as indicated. C, the activity of recombinant wild-type and mutagenized R423A/K429I/R454A/R458A/K462I BSDL was recorded using [1-14C]oleyl cholesterol (20 μM) emulsified in various conditions as indicated.

FIG. 4. Secretion of Y427S and Y453S mutagenized BSDL by transfected CHO-K1 cells. Clones C1, E2, and L8 were allowed to stand in the culture medium without FCS for 16–24 h. At the end, the cell-conditioned medium of each clone was analyzed for BSDL by Western blotting using pAbL10 (A, 25 μl of cell-conditioned medium/lane). B, the kinetics of secretion was performed by removing samples of the conditioned medium over time. Samples (25 μl) were analyzed by Western blotting with pAbL10. Results are representative of at least three independent experiments (C, 3B; □, E2; and △, L8).
activate both Y427S (E2) and Y453S (L8) mutants of BSDL. Micellar concentration of each bile salt has a more effective activating effect on the Y427S mutant than on the wild-type recombinant enzyme. This effect was lower when the activity of recombinant enzymes was recorded on soluble 4-NPC in the presence of monomeric bile salts. This result suggests that the interaction of bile salts with bile salt-binding sites affects the catalytic properties of the enzyme. Furthermore, the similar effect of primary and secondary bile salts on BSDL activity on the soluble substrate supports the hypothesis that tyrosine residues at positions 427 and/or 453 belong to the nonspecific bile salt-binding site. However, the nonspecific bile salt-binding site transforms into a micellar binding site upon the interaction of primary bile salts with the specific site (3). Therefore, we have recorded the activity of Y427S BSDL and Y453S BSDL on cholesterol oleate in the presence of NaTC micelles. This activity was decreased by some 30% for the E2 mutant and by 70% for the L8 mutant compared with that of the wild-type enzyme (Fig. 5B), meaning that the Y427S BSDL and Y453S BSDL are less susceptible to recognize the micellar substrate. Consequently, these Tyr residues in part Tyr^{427} could be actually part of the nonspecific bile salt-binding site. The apparent discrepancy observed between the effect of bile salts, in part at a concentration over the CMC, on 4-NPC and on cholesteryl oleate activities can be explained by the fact that the mutagenesis of one Tyr residue is not efficient enough to abrogate the binding of the enzyme to bile salt micelles; however, this binding seems sufficiently correct to allow the hydrolysis of soluble substrate and/or to affect the catalytic site topology by opening the catalytic site loop (6, 8, 9). This binding could be incorrect concerning the position of the open catalytic site vis-à-vis the lipid-soluble substrate within the micelle of bile salts. Another possibility is that tyrosine residues, in part Tyr^{453}, are involved in a hydrophobic interaction with the lipid-soluble substrate within the micelle. This latter point agrees with chemical modifications of Tyr residues on BSDL (4) and supports the idea that the micellar bile salt-binding site (4) could be in fact a lipid binding-promoting site (5).

**DISCUSSION**

The mechanism of BSDL activation by bile salt has been the focus of many studies for 20 years (3–5). The conclusions from these studies indicated the possible presence of two bile salt-binding sites, regulating the enzyme activity both on water and lipid-soluble substrates.

The first site that is specific for the 7α-hydroxylated position of primary bile salts would induce the dimerization (or any other structural modification) of the enzyme and is responsible for the enzyme activity on emulsified substrates. The second site is less specific with respect to the bile salt hydroxylation and could be a premicellar binding site that transforms into a micellar binding site (2) or into a lipid-binding promoting site (5) upon the binding of primary bile salts to the specific site. These two sites, the presence of which on pancreatic bile salts-dependent lipase has been hypothesized in the earliest studies (2) and the latter on the milk counterpart (5), were characterized on BSDL crystal as proximal vis-à-vis the catalytic site) and distal bile salt-binding sites (6). We have recently shown that the proximal bile salt-binding site was specific toward bile salt hydroxylation and was constituted by the basic N-terminal cluster including Lys^{32}, Lys^{46}, Lys^{81}, Lys^{82}, and Arg^{63} (11).

In this paper, we present evidence showing that the distal basic cluster (6) of BSDL, also called the C-terminal (1) bile salt-binding site, could be the nonspecific bile salt-binding site, i.e. the premicellar (2) or lipid-binding promoting (5) site. For this purpose a site-directed mutant of BSDL was constructed to define the functionality of the distal basic cluster consisting of residues Arg^{425}, Lys^{429}, Arg^{454}, Arg^{458}, and Lys^{462} that were substituted by Ala or Ile residues. Such methodology is limited by the possibility that observed effects consecutive to amino acid substitution could be the result of an altered enzyme structure leading to a loss of function. This may not be the case as the mutagenized enzyme is normally secreted, whereas we have shown previously (20, 21) that the unfolded or incorrectly folded BSDL is directed toward the ubiquitination and proteasome degradation pathway instead of the secretion route. Furthermore, the wild-type and mutagenized enzymes are still active to the same level on soluble substrates in the absence of activating bile salts. Among the five clones expressing mutagenized BSDL, the MC9 clone was selected because it expressed the same amount of mRNA and BSDL protein as the clone 3B expressing the wild-type enzyme. Also the kinetics of enzyme secretion by these clones are superimposable. Both primary and secondary bile salts are unable to enhance the
activity of the R423A/K429I/R454A/R458A/K462I mutagenized BSDL on the soluble substrates such as 4-NPC. This inefficiency is independent of the bile salt concentration, and in part NaTC at concentrations below its CMC, which does bind to the specific bile salt-binding site (2, 4), also does not activate the enzyme. This result means that the binding of (monomeric) primary bile salts to the specific site is not sufficient to increase the enzyme activity even on soluble substrates.

Furthermore, neither primary (NaTC) nor secondary (NaTDC) bile salts with the nonspecific or pre-micellar bile salt-binding site induces the formation of a more effective catalytic site by opening the catalytic site loop (residues His115 to Tyr125). The C-terminal domain of the enzyme may also move away during this step. Step 2, the interaction of monomeric primary bile salts (NaTC) transforms the nonspecific bile salt-binding site or pre-micellar binding site into a micellar binding site (or lipid-promoting binding site). Step 3, the binding of the micelle (in which the hydrophobic substrate is solubilized) to the micellar binding site promotes the opening of the catalytic site (see step 1) that becomes susceptible to hydrolyze the bulky hydrophobic substrate.

Therefore, the distal basic cluster including Arg423, Lys429, Arg454, Arg458, and Lys462 as well as Tyr427 and Tyr453 may represent the so-called nonspecific bile salt-binding site (2) that is transformed into a micellar (4) or a lipid binding-promoting (5) site upon interaction of primary bile salt with the specific site located at the N-terminal basic cluster of BSDL (11). Most interestingly, these residues are closely located to the V3-like loop of BSDL (see Fig. 1) which is involved in (glyco)sphingolipids binding (22), supporting that the surface domain of BSDL associating with the distal basic cluster, Tyr427 and/or Tyr453 and the V3-like loop, could constitute a large lipid binding area.

The present results along with those found in the literature can be integrated into a coherent (but still hypothetical) mechanistic scheme as follows (Fig. 6). First, BSDL is capable of hydrolyzing soluble ester substrates in the absence of bile salts or activators such as phospholipids (11), suggesting the presence of a pre-formed or partially accessible catalytic site constituted of Ser194, Asp320, and His435 (23–25). Second, monomers of primary and secondary bile salts are susceptible to binding with the specific (the N-terminal basic cluster or regulatory site (11) including Arg residues (3) in part Arg63 (9))
and with the nonspecific (the distal basic cluster (6) or pre-micellar binding site (5)) associating Tyr residues (4), respectively.

On the one hand, the binding of primary or secondary bile salts to the nonspecific site induces the opening of the catalytic site loop from residues 115 to 126 (6, 8, 9) and/or the displacement of the C-terminal repeated sequences (6, 26) giving full access to the catalytic site (step 1). This is compatible with observed variations in catalytic properties of the enzyme acting on soluble substrates in the presence of bile salts (2, 4, 27).

On the other hand, primary (monomeric) bile salts once interacting with the N-terminal basic cluster or proximal or specific bile salt-binding site (2, 6, 11) induce the transformation of the pre-micellar nonspecific bile salt-binding site (step 2) into a micellar or lipid-binding promoting site (2, 5) located at the distal basic cluster and involving Tyr 453 and possibly Tyr 427 residues. This transformation allows the binding of voluminous micelles (8) including lipid-soluble substrates and their correct presentation into the catalytic site (step 3), which becomes susceptible to accommodate bulky lipid substrate (26).

Binding to micellar substrate or to the lipid interface may also stabilize the enzyme structure (9, 26) and possibly the catalytic site in its open form (8). A corollary of this model is that the occupancy of each bile salt-binding site is a sine qua non condition for BSDL to gain full activity on bulky hydrophobic substrates (i.e., physiological substrates). Although dimerization has been presented as a key step in enzyme activation by primary bile salts (2, 3, 6), the model as presented here does not necessitate such dimerization as the functional role of the BSDL dimer may be questionable in many aspects (6, 28). This mechanism correlates with the following: (i) the residual activity of BSDL on soluble substrates in the absence of bile salt (recognized by the pre-formed catalytic site); (ii) the partial activation of the enzyme promoted by monomeric primary or secondary bile salt on soluble substrate; (iii) the full activation of the enzyme acting on soluble substrate in the presence of micelles of primary bile salt, whereas micelles of secondary bile salts are only seldom activating ligands, although this could be due to different affinities for primary and secondary bile salt micelles; and (iv) the absence of activity of BSDL on a lipid-soluble substrate solubilized by secondary bile salt and the full gain in activity when the substrate is solubilized in primary bile salt micelles or in mixed micelles.

As clearly shown here, R423A/K429I/R454A/R458A/K462I BSDL cannot be activated by acidic phospholipids contrary to the wild-type enzyme. Such a result was expected as the nonspecific bile salt-binding site has been described as a lipid-binding promoting site (3, 6–8). Binding of bile salts to this site has been presented as a prerequisite for binding of the enzyme to the surface of an emulsion (3, 6). Consequently, it could be that acidic phospholipids play the same role as that primarily attributed to primary and secondary bile salts. Binding of acidic phospholipids to the specific and to the nonspecific bile salt-binding sites may result in the enzyme activation as observed with primary and secondary bile salts (2). This could be of physiological relevance as BSDL, which has been detected in monocytes-macrophages, endothelial cells, eosinophils (for a review see Ref. 29), and in the atheroma plaque where it is involved in smooth muscle cells proliferation (30), cells in which the amount of bile salts likely cannot be high enough to help the enzyme gain full activity. Under those conditions acidic phospholipids may be essential to the enzyme activity on natural substrates. Accordingly, the activity of the wild-type recombinant enzyme on hydrophobic substrates should be increased by PA, and this is obviously not the case. However, it is possible that in PA liposomes as well as in mixed micelles made of PA and NaTDC, the cholesteryl olate, used as substrate, may not be fully accessible to the wild-type and mutagenized enzyme. Another possibility is that binding of PA to the specific site (i.e., the N-terminal basic cluster of BSDL (11)) is specific unable to transform the nonspecific bile salt-binding site (the distal basic cluster) into a micellar binding site or lipid-binding promoting site (3, 6–8). Finally, it is also possible that BSDL may hydrolyze PA (or other acidic phospholipids) at a faster rate than phosphatidylcholine (31), and this hydrolysis could be competitive with that of cholesteryl olate. This point needs additional studies for clarification.

REFERENCES

1. Lombardo, D. (2001) Biochim. Biophys. Acta 1533, 1–28
2. Lombardo, D., and Guy, O. (1980) Biochim. Biophys. Acta 611, 147–155
3. Lombardo, D., Campé, D., Maltugin, L., Lafont, H., and De Caro, A. (1983) Eur. J. Biochem. 133, 327–333
4. Campé, D., Lombardo, D., Maltugin, L., Lafont, H., and De Caro, A. (1984) Biochim. Biophys. Acta 784, 147–157
5. Blackberg, L., and Hernell, O. (1993) FEBS Lett. 323, 207–210
6. Wang, X., Wang, C.-S., Tang, J., Dyda, F., and Zhang, X.-C. (1997) Structure (Lond.) 5, 1209–1218
7. Terzyan, S., Wang, C.-S., Downs, D., Hunter, B., and Zhang, X.-C. (2000) Proc. Natl. Acad. Sci. USA 97, 1783–1790
8. Moore, S. A., Kingston, R. L., Loomes, K. M., Hernell, O., Blackburn, L., Baker, K. E. M., and Baker, K. E. M. (2001) J. Mol. Biol. 312, 511–523
9. Liang, Y., Medhekar, R., Brockman, H. L., Quinn, D. M., and Hui, D. Y. (2000) J. Biol. Chem. 275, 24040–24046
10. Sharra, V., Bruneau, N., Mas, E., Hamosh, M., Lombardo, D., and Hamosh, P. (1998) Biochim. Biophys. Acta 1390, 80–89
11. Aurbet, E., Sharra, V., Le Petit-Thévenin, J., Valette, A., and Lombardo, D. (2002) J. Biol. Chem. 277, 34987–34996
12. Han, J. H., Stratowa, C., and Butler, W. J. (1987) Biochemistry 26, 1617–1625
13. Ansald, M., Lepelletier, M., and Mejean, V. (1996) Anal. Biochem. 234, 110–111
14. Bruneau, N., Nganga, A., Fisher, E. A., and Lombardo, D. (1997) J. Biol. Chem. 272, 27353–27361
15. Gjellesvik, D. R., Lombardo, D., and Walther, B. T. (1992) Biochim. Biophys. Acta 1124, 123–134
16. Vérine, A., Saling, P., and Boyer, J. (1982) Am. J. Physiol. 243, E175–E181
17. Laemmli, U. K. (1970) Nature 227, 680–685
18. Vérine, A., Bruneau, N., Valette, A., Le Petit-Thévenin, J., Pasqualini, E., and Lombardo, D. (1999) J. Biol. Chem. 274, 179–187
19. Aboussalih, N., Mas, E., Bruneau, N., Benaïba, A., and Lombardo, D. (1993) J. Biol. Chem. 268, 25755–25763
20. Le Petit-Thévenin, J., Vérine, A., Nganga, A., Nohli, O., Lombardo, D., and Bruneau, N. (2001) Biochim. Biophys. Acta 1530, 184–198
21. Nganga, A., Bruneau, N., Sharra, V., Lombardo, D., and Le Petit-Thévenin, J. (2000) Biochem. J. 352, 865–874
22. Aurbet-Jousset, E., Garmy, N., Sharra, V., Fantini, J., Sadolet, M.-O., and Lombardo, D. (2004) Structure (Camb.) 12, 1437–1447
23. Dipersio, L. P., Fontaine, R. N., and Hui, D. Y. (1990) J. Biol. Chem. 265, 16801–16806
24. Dipersio, L. P., and Hui, D. Y. (1993) J. Biol. Chem. 268, 300–304
25. Dipersio, L. P., Fontaine, R. N., and Hui, D. Y. (1991) J. Biol. Chem. 266, 14033–14036
26. Chen, J. C., Miercke, L. J., Krucinski, J., Starr, J. R., Saenz, G., Wang, X., Spilburg, C. A., Lange, L. G., Ellsworth, J. L., and Stroud, R. M. (1998) Biochemistry 37, 5307–5317
27. Wang, C.-S. (1981) J. Biol. Chem. 256, 10198–10202
28. Loomes, K. M., and Senior, H. E. (1997) FEBS Lett. 405, 369–372
29. Hui, D. Y., and Howles, P. N. (2002) J. Lipid Res. 43, 2017–2030
30. Auge, D. Y., and Howles, P. N. (2002) J. Lipid Res. 43, 10198–10202
31. Lombardo, D., Fauvel, J., and Guy, O. (1980) Biochim. Biophys. Acta 611, 136–146
Site-directed Mutagenesis of the Distal Basic Cluster of Pancreatic Bile Salt-dependent Lipase
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J. Biol. Chem. 2004, 279:39697-39704.
doi: 10.1074/jbc.M407646200 originally published online July 20, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M407646200

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