Activation of Horse PLRP2 by Bile Salts Does Not Require Colipase†

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ABSTRACT: Although structurally similar to pancreatic lipase (PL), the key enzyme of intestinal fat digestion, pancreatic lipase-related protein type 2 (PLRP2) differs from PL in certain functional properties. Notably, PLRP2 has a broader substrate specificity than PL, and unlike that of PL, its activity is not restored by colipase in the presence of bile salts. In the studies presented here, the activation mechanism of horse PLRP2 was studied through active site-directed inhibition experiments, and the results demonstrate fundamental differences with that of PL. The opening of the horse PLRP2 flap occurs as soon as bile salt monomers are present, is accelerated in the presence of micelles, and does not require the presence of colipase. Moreover, in contrast to PL, horse PLRP2 is able to directly interact with a bile salt micelle to form an active binary complex, without the micelle being presented by colipase, as evidenced by molecular sieving experiments. These findings, together with the sensitivity of the horse PLRP2 flap to partial proteolysis, are indicative of a higher flexibility of the flap of horse PLRP2 relative to PL. From these results, it can be concluded that PLRP2 can adopt an active conformation in the intestine, which could be important for the further understanding of the physiological role of PLRP2. Finally, this work emphasizes the essential role of colipase in lipase catalysis at the lipid−water interface in the presence of bile.

Although very similar to pancreatic lipase (PL),[1] the key enzyme of intestinal fat digestion, the pancreatic lipase-related proteins (PLRP1 and PLRP2) possess specific features and have been proposed to belong to different lipase subfamilies. Nevertheless, PL and PLRP2 have a high degree of sequence identity (65−70%) and the same two-domain structural organization (*1*, *2*), an N-terminal domain bearing the catalytic site and a C-terminal domain that, in PL, is devoted to colipase binding. Except for guinea pig PLRP2 which possesses a "miniflap" (*3*), the catalytic site of PLRP2 is buried inside the molecule due to the presence of a surface loop (called a flap) which has been shown to be displaced during PL activation (*4*, *5*).

Despite these structural similarities, PLRP2 exhibits functional properties different from those of PL. Besides their lipase activity, PLRP2 displays a phospholipase activity (*3*, *6*−*8*). The protein has also been described as a galactolipase in some species (*9*). Interestingly, the behavior of most PLRP2s toward colipase is different from that of PL, even though the residues involved in colipase binding are conserved in PLRP2. In general, the lipase activity of the protein is inhibited by bile salts, but unlike that of PL, its activity is not restored by colipase. This implies that PLRP2 could hardly display a lipase activity under physiological conditions and would rather act as a phospholipase in most cases.

On the basis of the functional differences between PLRP2 and PL, we decided to determine whether PLRP2 obeys the same mechanism of activation as PL. It is now well-established that the opening of the flap in PL occurs through the formation of a ternary complex involving lipase, colipase, and a bile lipid micelle, the micelle being located in the cavity delineated by colipase and the C-terminal domain of lipase (*5*). The micelle, which mainly interacts with colipase, establishes only a few contacts with lipase. This finding is in agreement with previous works indicating that lipase is unable to bind a bile micelle in the absence of colipase (*10*). The ternary complex represents a functional entity, and this lipase "micellar" activation process is believed to prevail in the intestine.

To determine the activation mechanism for PLRP2, we investigated the effect of bile salts and/or colipase on the opening of the horse PLRP2 flap, as well as the ability of the enzyme to interact directly with bile salt micelles in the absence of colipase. Moreover, we studied the sensitivity of the horse PLRP2 flap to limited proteolysis. Our results demonstrate that the mechanism of activation of horse PLRP2 is different from that of PL. Further, they indicate a higher flexibility of the flap of horse PLRP2 relative to that of PL, and raise the question of the location of the micelle in the PLRP2−micelle complex. These findings establish the activation mechanisms underlying the functional differences between PLRP2 and PL and could be important for the understanding of the physiological role of PLRP2 in the digestive tract.

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[1] Abbreviations: PL, pancreatic lipase; PLRP2, pancreatic lipase-related protein 2; rPLRP2, recombinant pancreatic lipase-related protein 2; NaTDC, sodium taurodeoxycholate; E600, diethyl *p*-nitrophenyl phosphate; CMC, critical micellar concentration.

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EXPERIMENTAL PROCEDURES

Tributyrin and sodium taurodeoxycholate were purchased from Sigma (St. Louis, MO).

Purification of Horse Pancreatic Lipase, Colipase, and Native and Recombinant PLRP2. Horse PL, PLRP2, and colipase were purified from a pancreatic powder as previously described in refs *11*, *8*, and *12*, respectively. Recombinant horse PLRP2 was expressed in insect cells and purified as indicated in ref *8*. The recombinant protein bears a three-residue N-terminal extension (Ala-Asp-Leu) as compared to native PLRP2, due to the strategy used for cloning. Protein concentrations were determined at 280 nm using molecular extinction coefficients (ϵ) of 6.65×10^4 M^{-1} cm^{-1} for PL and PLRP2 and 0.4×10^4 M^{-1} cm^{-1} for colipase.

Gel Electrophoresis and Western Blotting. Electrophoresis on 12% polyacrylamide gels was carried out in the presence of SDS as described by Laemmli (*13*). Western blots were performed according to the method of Burnette (*14*).

N-Terminal Sequence Analysis. N-Terminal sequence analyses were performed by Stepwise Edman degradation using a gas-phase sequencer (Applied Biosystems, model 470A). The resulting phenylthiohydantoins were analyzed by HPLC using a C18 column [Brownlee, 5 μm, 220 mm $\times$ 2.1 mm (inside diameter)]. They were eluted using a gradient from 10 to 46% methanol in 7 mM sodium acetate buffer (pH 4.84).

Lipase and Colipase Activity Measurements. Horse PLRP2 activity was titrimetrically measured at 25 °C using emulsified 0.11 M tributyrin in a 1 mM Tris-HCl buffer (pH 7.5) containing 0.1 M NaCl and 5 mM $CaCl_2$. The assays were performed either in the absence or in the presence of bile salts and colipase. Horse pancreatic lipase (PL) activity was measured under the same conditions in the presence of 1 mM NaTDC and a 2−5-fold molar excess of colipase. One unit corresponds to the release of 1 μmol of fatty acid per minute. Colipase activity was determined through the ability of the protein to restore PL activity in the presence of 4 mM NaTDC. One colipase unit corresponds to the amount of colipase able to restore one lipase unit.

Limited Proteolysis of Horse PLRP2. Horse PL or recombinant horse PLRP2 (1−2 mg) was dissolved in 1 mL of 10 mM Tris-HCl (pH 7.5) containing 0.1 M NaCl. The lipase solutions were incubated at 25 °C with elastase at an endoprotease/lipase ratio of 1/60 (w/w). Samples (50 μL) were withdrawn from the incubation mixtures at various intervals of time, and the reaction was stopped by addition of 0.2 mM PMSF. To ensure a total inhibition of the endoproteases, the pH of the samples was then lowered to 2−3 by addition of HCl. The samples were then analyzed by SDS electrophoresis on 12% polyacrylamide gels.

Horse PLRP2 Inhibition by E600. The inhibition experiments were performed in 50 mM sodium acetate buffer (pH 6.0) containing 0.1 M NaCl. PLRP2 (1 mg) was treated with E600 (0.5 or 0.05 mM) either in the absence or in the presence of bile salt and/or colipase (large molar excess). The mixture was incubated at 25 °C, and aliquots were withdrawn from the mixture at various time intervals. The remaining lipase activity was titrimetrically determined at pH 7.5 and 25 °C using 0.11 M emulsified tributyrin in 1 mM Tris-HCl buffer containing 0.1 M NaCl and 5 mM $CaCl_2$. Control experiments were also performed to check PLRP2 stability.

Fluorescence Measurements. Horse PLRP2 (10^{-6} M) and NaTDC solutions were prepared in 10 mM Tris-HCl (pH 7.5) containing 0.1 M NaCl. Fluorescence spectra were recorded at 25 °C using a Kontron spectrofluorimeter equipped with a thermostated cell and a magnetic stirring device. The excitation wavelength was 295 nm. Titration of horse PLRP2 with NaTDC was performed by successive addition of 1 μL of an 80 mM stock solution of NaTDC to 2 mL of a horse PLRP2 solution.

Fractionation by Gel Filtration. Molecular sieving experiments were performed on an Ultrogel AcA 54 column (90 cm $\times$ 1 cm) equilibrated in 20 mM Tris-HCl buffer (pH 7.5) containing 0.1 M NaCl either without NaTDC or with 0.5 or 4 mM NaTDC. Blue dextran (1 mg/mL) was used to determine the void volume of the column. The flow rate was 8 mL/h. The volume of the fractions was 0.6 mL. The fractions were analyzed by 12% SDS−PAGE according to the method of Laemmli (*13*). To normalize the data, the K_{av} values were calculated according to the following equation:

$$K_{av} = (V_e - V_o)/(V_t - V_o)$$

where V_e, V_o, and V_t correspond to the elution volume, the void volume, and the total volume of the column, respectively.

Molecular Modeling. Models of the horse PLRP2 molecule in both closed and open conformations were built on the basis of the crystal structure of the highly homologous rat PLRP2 (*2*). Structural modifications associated with the active state were built on the basis of the crystal structure of the porcine lipase−colipase complex (*15*). Amino acid changes were introduced using the graphics program O (*16*) running on a Silicon Graphics workstation. Side chain rotamers were chosen from the database of more common conformers (*17*). Models were first energy minimized using the Powell minimizer algorithm implemented in X-PLOR (*18*). The Engh and Huber (*19*) force field was used in all energy minimization and molecular dynamics simulations. Subsequently, the slow-cooling molecular dynamic protocol of Brünger et al. (*20*) was applied by using the weak temperature coupling method of Berendsen et al. (*21*). The target temperature of 2000 K was decreased by 25 K every 50 steps to reach the final temperature of 300 K. Finally, the conformation of the different models trapped at 300 K was subjected to 120 additional steps of energy minimization.

RESULTS

Limited Proteolysis of Horse PLRP2. The sensitivity of horse PLRP2 to partial proteolysis has been observed during purification of the protein from pancreatic extracts. As shown in Figure 1, a partial proteolysis of the protein generating two fragments of 27.5 and 22.5 kDa could be observed. N-Terminal sequencing yielded the following sequences: Lys-Glu-Val-Cys-Tyr-Thr- and Thr-Phe-Ile-Asp-Ile-Asn- for the 27.5 and 22.5 kDa fragments, respectively, indicating that the 27.5 kDa fragment corresponds to the N-terminal sequence of the protein extending from residue 1 to residue 244, while the 22.5 kDa fragment corresponds to the sequence extending from residue 245 to the C-terminus of the chain.

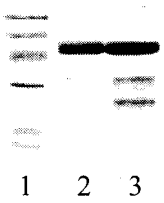

FIGURE 1: SDS−PAGE of purified native horse PLRP2. Electrophoresis was performed using 12% polyacrylamide gels: lane 1, molecular mass markers (90, 64, 44, and 30 kDa); lane 2, intact native PLRP2; and lane 3, native horse PLRP2 purified in the presence of small amounts of protease inhibitors.

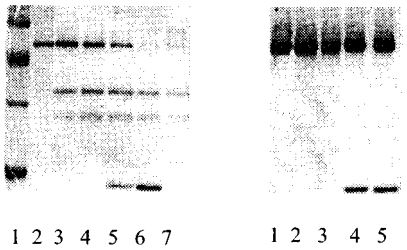

FIGURE 2: Limited proteolysis by elastase of recombinant horse PLRP2 and horse PL. Horse recombinant PLRP2 (1 mg/mL) (A) and horse PL (1 mg/mL) (B) were incubated for 3 h with elastase (60/1, w/w) at pH 7.5 and 25 °C, as indicated in Experimental Procedures. Samples were then withdrawn from the incubation mixtures and analyzed on 12% SDS−polyacrylamide gels. (A) Molecular mass markers (64, 44, 30, and 20 kDa) (lane 1), horse rPLRP2 in the absence of elastase (lane 2), horse rPLRP2 incubated with elastase in the absence of colipase without NaTDC (lane 3) or with 0.1 mM NaTDC (lane 4) or 4 mM NaTDC (lane 7), and horse rPLRP2 incubated with elastase in the presence of a large molar excess of colipase without NaTDC (lane 5) or with 4 mM NaTDC (lane 6). (B) Horse PL in the absence of elastase (lane 1), horse PL incubated with elastase in the absence of colipase without NaTDC (lane 2) or with 4 mM NaTDC (lane 3), and horse PL incubated with elastase in the presence of a large molar excess of colipase without NaTDC (lane 4) or with 4 mM NaTDC (lane 5).

Thus, the cleavage of the horse PLRP2 chain occurs at the Ser_{244}−Thr_{245} bond which is located in the flap sequence. Because this cleavage is likely to result from an elastase attack, limited proteolysis experiments with elastase were performed using the recombinant horse PLRP2 to avoid any contamination with pancreatic proteases. The influence of the presence of colipase and/or NaTDC as monomer or micelle was also investigated. As shown by SDS−PAGE analysis of the various incubation mixtures (Figure 2A), the cleavage of the recombinant PLRP2 chain, insensitive to the presence of colipase, is promoted by the presence of NaTDC micelles. This cleavage has no influence on the activity of horse rPLRP2. The same site of cleavage was identified in the native and recombinant forms of horse PLRP2. In contrast, similar experiments performed on horse PL clearly showed that this bond, in which Thr_{245} is replaced with Gln, is quite resistant to elastase whether colipase and bile salts are present (Figure 2B).

Influence of NaTDC and Colipase on Horse PLRP2 Inhibition by E600. To obtain more information about the motion of the flap of horse PLRP2 and the resulting unmasking of the catalytic triad of the enzyme, E600 inhibition experiments were carried out in the absence or presence of various NaTDC concentrations, with or without colipase. E600 has been shown to covalently block the active site serine of PL, provided the lipase adopts an open flap conformation. In the absence of NaTDC and colipase, a noticeable inhibition of horse PLRP2 by E600 could be observed. In the presence of 5 mM E600, 80% inhibition was obtained within 6 h (data not shown). When the E600 concentration was decreased to 0.5 mM, the rate of inhibition was slower and 24 h was required to obtain 70−80% PLRP2 inhibition. This result indicates that the flap of horse PLRP2 is likely to be not completely closed in solution, allowing partial access to the active site. Addition of colipase had no influence on horse PLRP2 inhibition by E600. In contrast, as shown in Figure 3, the rate of inhibition was significantly increased in the presence of NaTDC monomers. The rate of inhibition increased with NaTDC monomer concentration, and addition of colipase still had no influence. To better visualize the effect of monomer concentration, only part of the inhibition curve without NaTDC and colipase has been represented in Figure 3.

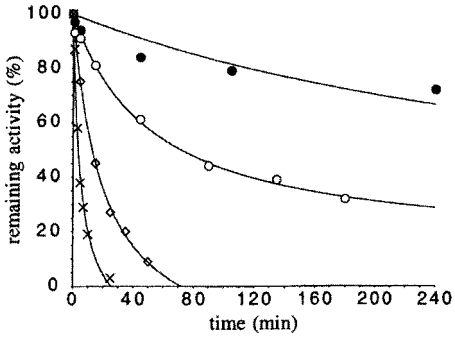

FIGURE 3: Influence of NaTDC monomers on horse PLRP2 inhibition by E600. PLRP2 (2×10^{-5} M) was incubated at 25 °C and pH 6.0 [50 mM sodium acetate buffer (pH 6.0) containing 0.1 M NaCl] with 0.5 mM E600 either in the absence of NaTDC (●) or in the presence of 0.1 (○), 0.5 (◇), or 1 mM NaTDC (×), in the absence of colipase. At different time intervals, aliquots were withdrawn from the incubation mixture and the remaining lipase activity was determined as described in Experimental Procedures. The remaining activity was expressed as a percentage of the total lipase activity.

In this set of experiments, once the CMC was reached, the rate of inhibition was too fast to accurately evaluate the influence of the presence of micelles. Therefore, to check the effect of micelles on the rate of E600 inhibition, inhibition experiments were performed using a 10-fold lower E600 concentration (0.05 mM). As shown in Figure 4, increasing the NaTDC concentration beyond the CMC leads to a significant increase of the rate of inhibition of horse PLRP2 by E600. Since the monomer concentration remains constant once the CMC is reached, this result indicates that NaTDC micelles are efficient in PLRP2 activation. It must be noted, yet, that mixed NaTDC/phospholipid micelles had a lower efficiency in PLRP2 activation than pure NaTDC micelles.

Blank experiments performed in the absence of E600 showed that in any case, horse PLRP2 retains its full activity for at least 48 h, indicating that the enzyme was stable under the conditions used for this study.

Interactions between NaTDC and Horse PLRP2. The opening of the horse PLRP2 flap is promoted by NaTDC monomers as well as NaTDC micelles. To obtain more information about the interactions of NaTDC monomers or micelles with horse PLRP2, a spectrofluorometric study as well as molecular sieving experiments in the presence of

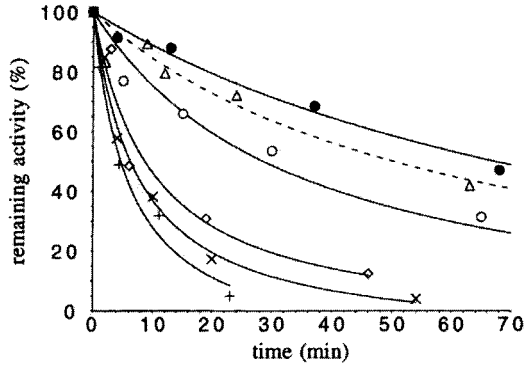

FIGURE 4: Influence of NaTDC micelles on horse PLRP2 inhibition by E600. PLRP2 (2×10^{-5} M) was incubated at 25 °C and pH 6.0 [50 mM sodium acetate buffer (pH 6.0) containing 0.1 M NaCl] with 0.05 mM E600, in the absence of colipase, in the presence of 0.5 (●), 1 (○), 2 (◇), 5 (×), or 7.5 mM NaTDC (+) or with mixed micelles (△) containing phosphatidylcholine (2 mM NaTDC and 0.27 mM phosphatidylcholine). At different time intervals, aliquots were withdrawn from the incubation mixture and the remaining lipase activity was determined as described in Experimental Procedures. The remaining activity was expressed as a percentage of the total lipase activity.

Table 1: K_{av} Values for Horse PLRP2 in the Presence of NaTDC and/or Colipase[a]

molecular species	K_{av}
horse PLRP2	0.35−0.36
horse PLRP2 and 0.5 mM NaTDC	0.35−0.36
horse PLRP2 and 4 mM NaTDC	0.27
horse colipase	0.64−0.66
horse colipase and 4 mM NaTDC	0.42
horse PLRP2 in the presence of colipase	0.35−0.36
colipase in the presence of horse PLRP2	0.66
horse PLRP2 and 4 mM NaTDC in the presence of colipase	0.27−0.28
colipase and 4 mM NaTDC in the presence of horse PLRP2	0.38

[a] The K_{av} values were calculated from the peak elution volumes using Ultrogel AcA 54 columns (90 cm × 1 cm). The columns were equilibrated and eluted at 4 °C with 20 mM Tris-HCl buffer (pH 7.5) containing 0.1 M NaCl, either in the absence or in the presence of NaTDC (0.5 or 4 mM NaTDC) without or with 2×10^{-4} M colipase. The protein concentration of the horse PLRP2 solution loaded on the column was 10^{-5} M (volume of 0.5 mL).

either NaTDC monomers or micelles was performed. No significant changes in the fluorescence intrinsic spectrum of PLRP2 could be observed upon addition of NaTDC monomers or micelles. Molecular sieving experiments on Ultrogel AcA54 were performed on horse PLRP2 in the absence of colipase. In the presence of 0.5 mM NaTDC, no change in the elution volume of PLRP2 was observed, as expected if only a few NaTDC monomers bind to the protein. In contrast, the elution volume of PLRP2 was significantly modified in the presence of NaTDC micelles. Analysis of the K_{av} values reported in Table 1 shows a significant decrease in the PLRP2 K_{av} value in the presence of 4 mM NaTDC, while the K_{av} value of PLRP2 is not affected by the presence of NaTDC monomers as mentioned above. The K_{av} value obtained in the presence of NaTDC micelles is consistent with the formation of a complex in which horse PLRP2 associates with at least one NaTDC micelle.

Molecular sieving experiments were also performed in the presence of a large excess of colipase (2×10^{-4} M) either without NaTDC or in the presence of 0.5 or 4 mM NaTDC.

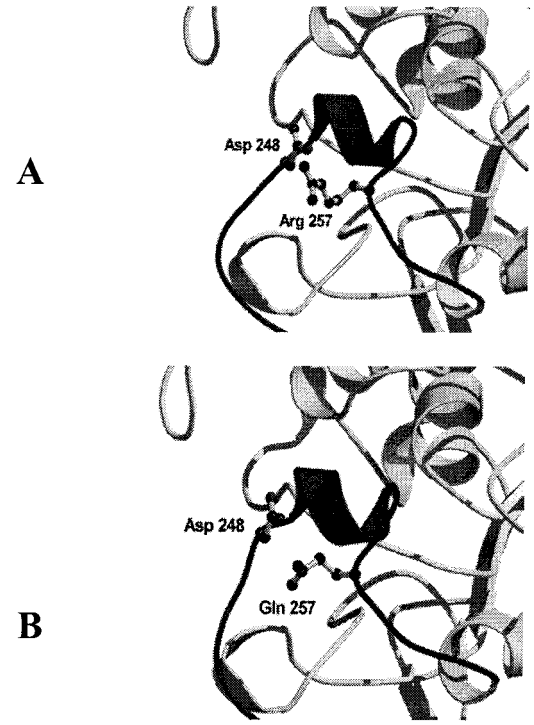

FIGURE 5: Flap stabilization in horse PL and PLRP2. Molscript (*28*) representation of horse PL showing the Asp_{248}−Arg_{257} ion pair involved in the flap stabilization in the closed conformation (dark gray) (A). This stabilizing interaction cannot be produced in the horse PLRP2 closed conformation as Arg_{257} is not conserved but replaced by Gln in PLRP2 (B).

Analysis of the K_{av} values, as well as colipase activity measurements for detecting traces of colipase, indicates that, in all cases, neither horse PLRP2−colipase nor horse PLRP2−colipase−micelle complexes could be detected. Only complexes associating either colipase or horse PLRP2 with a micelle could be observed (Table 1).

Modeling Experiments. Modeling experiments were performed on horse PLRP2 to investigate the structural bases of the mobility of the flap and the absence of the colipase effect. The three-dimensional structure model of horse PLRP2 has been built on the basis of the crystal structure of the closed conformation of rat PLRP2 (2) and compared to the three-dimensional structure of the closed conformation of horse PL (*22*). As shown in Figure 5, an ion pair (Asp_{248}−Arg_{257}) plays an important role in the stabilization of the horse PL flap in the closed conformation. The same ion pair, which has also been observed in closed human PL, is likely to occur in all PLs based on the strict conservation of these residues. When the flap opens, this ion pair is broken, each residue establishing new interactions. The presence in horse PLRP2 of a glutamine residue in position 257 hinders the formation of this ion pair and could explain the lower-level stabilization of the flap in the closed conformation.

Despite the presence of most residues involved in colipase binding in horse PLRP2, colipase has no effect on lipase activity. A detailed investigation of the horse PLRP2 three-dimensional model revealed that Lys_{400}, which, in PL, plays an essential role by forming an ion pair with Glu_{45} in colipase, is likely to be involved in an intramolecular ion pair with Glu_{443} (Figure 6), thus impairing PLRP2−colipase binding.

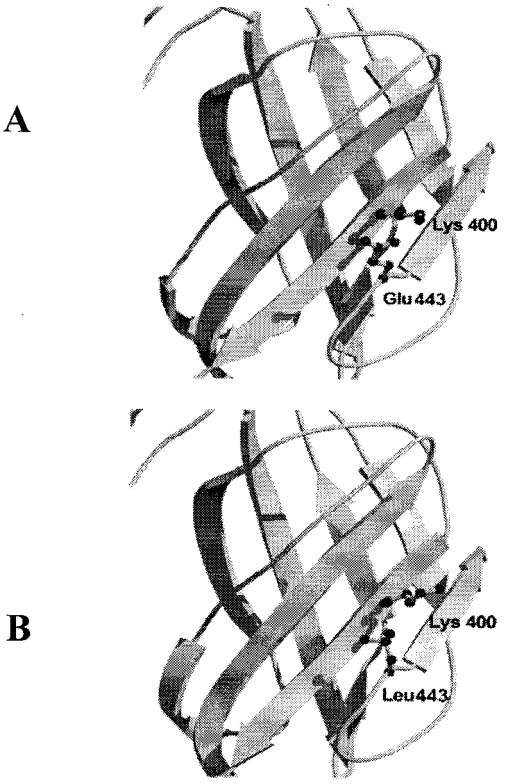

FIGURE 6: $Lys_{400}-Glu_{443}$ intramolecular ion pairing in horse PLRP2. Lys_{400} and Glu_{443} are likely to be involved in ion pairing in horse PLRP2 (A). Due to the presence of a leucine residue in position 443, this intramolecular interaction does not exist in PL (B). The figure was created using Molscript (*28*).

DISCUSSION

The PLRP2s form a subfamily clearly distinct from the classical lipase subfamily, notably concerning their pattern of expression and functional properties. Moreover, a considerable variability is observed among the members of the PLRP2 subfamily which could explain why defining a general physiological role for this protein remains a difficult challenge.

In contrast to PL which is expressed only after birth, PLRP2 would be expressed at a significant level within a short period of time around birth (*23*, *24*), although large amounts of PLRP2 were found in adults of some species (*6*, *8*). From a functional point of view, PLRP2 displays a broader substrate specificity than PL. Yet the main difference between both proteins concerns the absence of a colipase effect [or the limited colipase effect (*7*, *25*)] in the case of PLRP2. Indeed, colipase is unable to counteract the inhibitory effect of bile salts on the lipase activity of most PLRP2s. These findings raise the question of the mechanism of action of PLRP2 relative to PL. Since colipase and bile salts play an essential role not only in the expression of the lipolytic activity of PL but also in the activation of the enzyme, we decided to investigate the mechanism of activation of PLRP2.

In PLRP2, as well as in PL, the active site has to be unmasked by the displacement of the flap. The motion of the flap of PL has been shown to result from the combined effect of colipase and micelles through the formation of a ternary complex associating lipase, colipase, and a bile salt micelle (*5*, *15*). Under these conditions, PL was irreversibly inhibited by E600 which reacts with the serine residue (Ser_{153}) of the catalytic triad. In this paper, we demonstrate that, in contrast to PL, horse PLRP2 was sensitive to E600 even in the absence of both bile salts and colipase, indicating that the flap of the protein in solution does not adopt a fully closed conformation. Interestingly, not only bile salt micelles but bile salt monomers alone are able to promote an efficient motion of the flap. These results suggest a higher mobility of the flap of horse PLRP2 as compared to that of PL and emphasize the important role of bile salts in the activation process of horse PLRP2. Molecular modeling suggests that the higher mobility of the horse PLRP2 flap is likely to result from the absence of an ion pair between residues 248 and 257. In PL, Arg_{257} and Asp_{248} stabilize the flap of the enzyme in the closed conformation (*4*, *15*). It is noteworthy that, except for PLRP2 found in the mouse cytotoxic lymphocytes (*26*), Arg_{257} is substituted in all PLRP2s with a nonbasic residue, thus impairing the formation of this ion pair in PLRP2. Taken together, we propose that, compared to that of PL, a higher mobility of the flap is a specific feature of PLRP2. Further, another striking difference between horse PLRP2 and PL activation is that the motion of the flap of horse PLRP2 does not require the presence of colipase, either in the presence or in the absence of bile salts.

Limited proteolysis experiments revealed a high sensitivity of the $Ser_{244}-Thr_{245}$ bond to elastase in horse PLRP2, while the corresponding $Ser_{244}-Gln_{245}$ bond in horse PL is resistant. Molecular modeling of horse PLRP2 shows that the $Ser_{244}-Thr_{245}$ bond is exposed at the surface of the molecule and that Thr_{245} is fully accessible, while in PL, Gln_{245} is involved in a hydrogen bond with Gly_{237}. The finding that the $Ser_{244}-Thr_{245}$ bond is cleaved by elastase in horse PLRP2, and that the rate of cleavage is significantly increased by NaTDC, in the absence of colipase, supports the hypothesis of a different conformation of the horse PLRP2 flap compared to the PL flap, and emphasizes the involvement of NaTDC in the motion of the horse PLRP2 flap.

To obtain more information about the role of NaTDC in horse PLRP2 activation, the interactions between the protein and NaTDC monomers or micelles have been investigated using spectrofluorometry and molecular sieving. No significant change in the intrinsic fluorescence spectrum of horse PLRP2 was observed upon addition of either monomers or NaTDC micelles, indicating no sensitivity change in the environment of the tryptophan residues of the protein. From this result, it can be proposed that Trp_{253}, which is located at the top of the flap, remains exposed to the solvent in native horse PLRP2. In contrast, on the basis of crystallographic data, this residue is likely to be partly buried in the closed conformation of PL and becomes more exposed to the solvent after the motion of the flap toward an open conformation. This observation further supports a different conformation of the PLRP2 flap as compared to the PL flap in solution.

Neither spectrofluorometry nor molecular sieving allowed us to study the binding of bile salt monomers to horse PLRP2. Nevertheless, it can be concluded from the molecular sieving experiments that no PLRP2 dimerization occurs in the presence of NaTDC monomers. Therefore, the activation of horse PLRP2 by NaTDC monomers does not proceed via the formation of a PLRP2 dimer. Another striking difference between horse PL and PLRP2 concerns the interaction of the latter protein with NaTDC micelles. Indeed, it clearly

appears that PLRP2 is able to form a binary complex with a bile salt micelle in the absence of colipase.

The finding that activation of horse PLRP2 does not require colipase and is promoted by bile salt monomers and reinforced by micelles raises the question of the exact role of monomers and micelles in this process. Whether bile salt monomers and micelles act independently or in synergy remains to be elucidated. Moreover, the micelle binding site in PLRP2 has not yet been identified. It must be noted that, in the ternary PL complex (*5*), the micelle which is located in the cavity delineated by colipase and the C-terminal domain of lipase mainly interacts with colipase and establishes only a few contacts with the C-terminal domain of PL. Since the micelle is not presented by colipase in the horse PLRP2 complex, it is doubtful that the same site would prevail for this lipase.

It must be emphasized that as already observed with classical lipase (*5*), bile salts exhibit opposite effects according to their mode of presentation. Spread at the water−lipid interface, they act as PLRP2 inhibitors, whereas in solution they act as PLRP2 activators. These finding further support the complex function of bile in intestinal lipolysis.

In contrast to PL, horse PLRP2 has a very low affinity for colipase (*8*), resulting either from the inability of the protein to form the essential $Lys_{400}-Glu_{45}$ ion pair with colipase or from a particular conformation of the flap which impedes colipase binding. This very low affinity implies that, in vivo, no PLRP2−colipase complex is likely to be formed, either in solution or at the lipid−water interface. This provides a simple explanation for the observation that colipase is unable to reverse the inhibitory effect of bile salts at the lipid−water interface. In solution, the lack of complex formation between PLRP2 and colipase has no effect on the activation of the enzyme. In contrast, the inability of PLRP2 to bind colipase impedes the expression of the PLRP2 lipase activity at the lipid−water interface in the presence of bile. This finding emphasizes the crucial role of colipase in PL catalysis at the lipid−water interface under physiological conditions, notably concerning lipase anchoring, orientation effects, and displacement of molecules at the lipid−water interface.

In conclusion, these studies indicate that horse PL and PLRP2 have quite different mechanisms of activation, corresponding to different specific behaviors toward bile salts and colipase. In this work, we demonstrate that, although likely unable to display a lipase activity on dietary triglyceride droplets, horse PLRP2 may adopt an open active conformation under physiological conditions mainly due to the presence of bile salt micelles. Yet the finding that horse PLRP2 could also be partly activated by bile salt monomers could be physiologically relevant since PLRP2 is likely to be expressed around birth in humans and rats (*23*, *24*), at a period of age where bile has a specific composition, notably, a lower bile salt concentration (*27*), and PL expression is absent. Nevertheless, due to the intrinsic variability of PLRP2, detailed studies of the functional properties of PLRP2 from various species have to be performed to clearly define the specific features of the PLRP2 subfamily.

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