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Ion Pairing between Lipase and Colipase Plays a Critical Role in Catalysis*

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Laurence Ayvazian, Isabelle Crenon, Juan Hermoso‡, David Pignol§, Catherine Chapus, and Brigitte Kerfelec¶

From the Unité de Bioénergétique et Ingénierie des Protéines, UPR 9036 CNRS, 31 Chemin Joseph Aiguier, 13402 Marseille Cedex 9, France, the ‡Departamento Cristalografia, Instituto Quimica-Fisica "Rocasolano" CSIC, Serrano 119, 28006 Madrid, Spain, and the §Laboratoire de Cristallographie et Cristallogénèse des Protéines, Institut de Biologie Structurale Jean-Pierre Ebel, CEA-CNRS, 41 Avenue des Martyrs, 38027 Grenoble Cedex 1, France

Among the polar interactions occurring in pancreatic lipase/colipase binding, only one ion pair involving lysine 400 on lipase and glutamic acid 45 on colipase has been described. These residues are strictly conserved among species, suggesting that the ion pair is likely to play an important role. Therefore, in order to prevent this interaction, mutations intended to neutralize or inverse the charge of these residues have been introduced in the cDNAs encoding horse lipase and colipase. The recombinant proteins have been expressed in insect cells, and their catalytic properties have been investigated. In all cases, preventing the formation of the correct ion pair Lys^{400}/Glu^{45} leads to lipase-colipase complexes of reduced affinity unable to perform an efficient catalysis, notably in the presence of bile salt micelles. Diethyl *p*-nitrophenyl phosphate inhibition experiments with either mutant lipase or mutant colipase indicate a poor stabilization of the lipase flap. These results suggest that the ion pair plays a critical role in the active conformation of the lipase-colipase-micelle ternary complex by contributing to a correct orientation of colipase relative to lipase resulting in a proper opening of the flap.

The pancreatic lipase-colipase complex fulfills a key function in dietary fat digestion by converting insoluble long chain triacylglycerols into more polar products able to cross the brush border membrane of enterocytes. As confirmed by structural data (1, 2), pancreatic lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) possesses a two-domain organization, the N-terminal domain bearing the active site and the C-terminal domain being devoted to colipase binding (3–6). The enzyme has been shown to exhibit two conformations, an inactive closed conformation in solution, in which a special loop (the flap) masks the active site, and an active open conformation resulting from the motion of the flap and the exposure of the active site to the substrate interface (7). This conformation change has been thought to account for the "interfacial activation" of pancreatic lipase. However, recently, it was shown that detergent micelles together with colipase are able to induce the opening of the flap (or stabilize the open lipase conformation) in the absence of any substrate interface (8). This finding was further supported by the observation of a ternary complex associating pancreatic lipase in its open flap state, colipase, and a well characterized detergent micelle (9). The micellar binding site is distinct from the interfacial binding site of the lipase-colipase complex, thus shedding new light on lipase activation under physiological conditions.

In solution, in the absence of detergent micelles, colipase exclusively binds to the C-terminal domain of lipase. The binding involves the formation of an ion pair between a glutamic acid residue (Glu^{45}) of colipase and a lysine residue (Lys^{400}) of lipase, these residues being strictly conserved among species. In the presence of micelles, a secondary colipase-binding site involving the lipase flap is generated 25 Å apart from the C-terminal domain binding site. This extra binding site, smaller than the first one, only involves polar interactions and van der Waals contacts.

To understand better the pathway of pancreatic lipase activation *in vivo*, we have investigated the importance of the sole ion pair existing between lipase and colipase. In this respect, we have mutated lysine 400 to glutamic acid or glutamine in lipase and glutamic acid 45 to lysine or glutamine in colipase, and we looked at the impact of these mutations on the lipase/colipase binding and catalytic efficiency of the resulting lipase-colipase complexes either in the absence or in the presence of bile salt micelles. The results clearly indicate that the ion pair Lys^{400}/Glu^{45} plays a key role in controlling the activity of lipase in the presence of bile salt micelles.

EXPERIMENTAL PROCEDURES

The BaculoGold starter package, baculovirus pVL1393 transfer vector, was from PharMingen (San Diego, CA). The culture media X-Press were from Bioproducts (France). Fetal calf serum and antibiotics were purchased from Dutscher (France) and Life Technologies, Inc. (Copenhagen, Denmark). Alkaline phosphatase-labeled goat anti-rabbit IgG was from Sigma. The Transformer™ Site-Directed Mutagenesis Kit was from CLONTECH. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (UK).

Purification of Native Horse Lipase and Colipase—Both proteins were obtained from a horse pancreatic acetonic powder. Horse lipase and colipase were purified according to the methods of Lombardo *et al.* (10) and Chapus *et al.* (11), respectively. The protein concentrations were determined at 280 nm using $E^{1\%} = 13.3$ for lipase and 8.0 for colipase.

Site-directed Mutagenesis—Mutations were introduced into the pVL1393HoPL and pVL1393HCol plasmids using the Transformer Site-directed Mutagenesis Kit according to the manufacturer's instructions. After selection by *Nde*I restriction analysis, the presence of the desired mutation was ascertained by sequencing using the dideoxy chain termination method (12), and the subsequent plasmids were purified by CsCl gradient ultracentrifugation.

Expression of the Mutated Proteins in Sf-21 Cells and Purification—Transfections were performed as described in the Baculovirus Expression Vector System Manual from PharMingen. Recombinant baculoviruses were produced and purified as described previously (6). Five to six days postinfection, the cultures of infected Sf-21 cells were harvested

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¶ To whom correspondence should be addressed. Tel.: (33) 04 91 16 41 57; Fax: (33) 04 91 16 45 78; E-mail: kerfelec@ibsm.cnrs-mrs.fr.

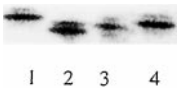


FIG. 1. **Analysis of LipK400Q and LipK400E by polyacrylamide gel electrophoresis.** Electrophoresis was performed in non-denaturating conditions: *lanes 1* and *4*, native horse lipase; *lane 2*, LipK400E; and *lane 3*, LipK400Q.

and centrifuged to remove cells and debris. The supernatants were dialyzed overnight against distilled water and freeze-dried. The dry material was then resuspended in distilled water and submitted to molecular sieving on an Ultrogel AcA 54 column (4 × 200 cm) equilibrated in a 50 mM Tris/HCl buffer, pH 7.5, containing 0.2 M NaCl and 1 mM benzamidine. After an overnight dialysis at 4 °C against a 50 mM Tris/HCl buffer, pH 7.5, containing 10 mM NaCl and 1 mM benzamidine, the recombinant lipases were further purified on a Q Fast Flow column. Elution was performed using a linear NaCl gradient (10–200 mM) in the same buffer. The recombinant colipases were dialyzed overnight at 4 °C against a 20 mM Tris/HCl buffer, pH 7.5, containing 1 mM benzamidine and loaded onto a HiTrap blue column (5 ml, Amersham Pharmacia Biotech). Colipase was selectively eluted in the pass through. The protein elution profiles were followed spectrophotometrically at 280 nm, and the presence of lipase or colipase was investigated by activity measurements and/or Western blotting.

Gel Electrophoresis and Western Blotting—Electrophoresis on 10–15% polyacrylamide gels was carried out either in the absence or in the presence of SDS as described by Laemmli (13). Western blots were performed according to Burnette (14). The membranes were incubated for 1 h at room temperature with specific polyclonal anti-lipase or anti-colipase antibodies from rabbit, and immunodetection was carried out using alkaline phosphatase-labeled goat anti-rabbit IgG.

Protein Blotting/Protein Overlay Assay—Native horse colipase (1 μg) or mutant ColE45K or ColE45Q (1–3 μg) was loaded onto a 15% anionic polyacrylamide gel prior to electroblotting on a polyvinylidene difluoride membrane in the same conditions as mentioned above. The protein blotting/protein overlay assay was performed as described (6). Immunodetection was carried out as mentioned above.

Activity Measurements—Lipase and colipase activities were potentiometrically determined at pH 7.5 and 25 °C using 0.11 M emulsified triacylbutyrylglycerol (tributyrin) in 1 mM Tris/HCl buffer containing 0.1 M NaCl, 5 mM $CaCl_2$ in the presence of various NaTDC[1] concentrations. One unit corresponds to the release of 1.0 μmol of fatty acid per min.

E600 Inhibition of Pancreatic Lipase—The inhibition experiments were performed at pH 6.0 with 4 mM E600 in the presence of supramicellar concentrations of NaTDC as reported by Hermoso *et al*. (8). The mixtures were incubated at 20 °C, and aliquots were withdrawn from the mixture at various time intervals. The remaining lipase activity was measured as mentioned above in the presence of 1 mM NaTDC and an excess of native colipase.

Modeling—Models of the different mutant lipases and colipases were built on the basis of the crystal structure of the porcine lipase-colipase complex (8). Amino acid changes were introduced using the graphic program O (15) running on a Silicon Graphics workstation. Side chain rotamers were chosen from the data base of more common conformers (16). Models were first energy minimized using the Powell minimizer algorithm implemented in X-PLOR (17). The Engh and Huber (18) force field was used in all energy minimization and molecular dynamic simulations. Subsequently, the slow-cooling molecular dynamic protocol of Brunger *et al.* (19) was applied by using the weak temperature coupling method of Berendsen *et al*. (20). The target temperature of 1000 K was decreased by 25 K every 100 steps to reach the final temperature of 300 K. The time step was set to 0.05 fs during the molecular dynamic stages. Finally, the conformation of the different mutants trapped at 300 K was subjected to 500 additional steps of energy minimization.

RESULTS

Effect of Lys^{400} Mutations on Lipase Activity—The mutant lipases, LipK400E and LipK400Q, were purified to homogeneity as described under "Experimental Procedures." The presence of both mutations was confirmed from the electrophoretic behavior of the mutant lipases on polyacrylamide gel in the absence of SDS (Fig. 1).

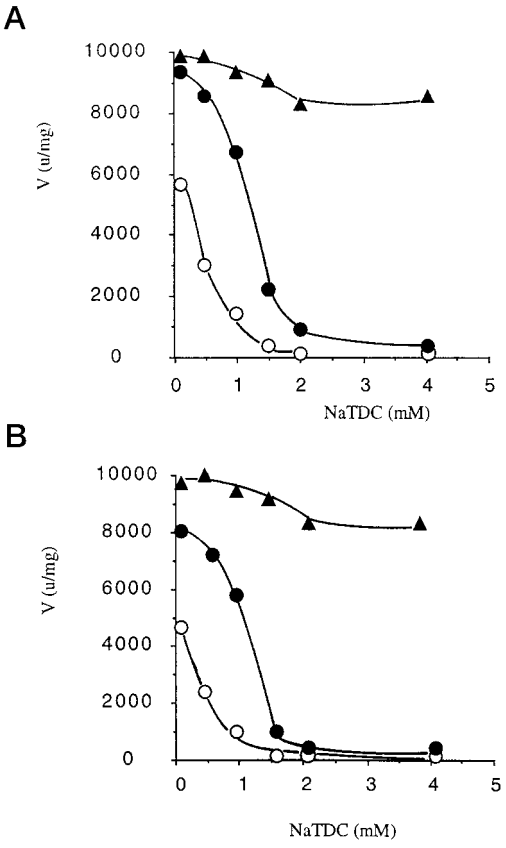


FIG. 2. **Influence of NaTDC concentration on the rate of hydrolysis of emulsified tributyrin (V_S) by LipK400Q and LipK400E either in the absence or presence of colipase.** The values of V_S were determined as indicated in the text at pH 7.5 and 25 °C using 0.11 M tributyrin. The influence of NaTDC concentration on V_S was measured for LipK400Q (*A*) and LipK400E (*B*) either in the absence (○) or presence of a large excess ($\gg K_{d(app)}$) of native colipase (●). In each case, the rate of hydrolysis of the substrate by the native horse lipase in the presence of colipase (▲) has been reported. The values of V_S are expressed as units (μmol of released fatty acids/min)/mg of either native or mutant lipases.

The two mutant lipases were shown to retain at least 80% of the activity of the native or recombinant lipase on emulsified tributyrin in the presence of a non-inhibitory NaTDC concentration (0.1 mM) (Fig. 2). As native lipase, LipK400E and LipK400Q were inhibited by increasing amounts of NaTDC in the absence of colipase. However, in contrast to the native enzyme, they were not fully reactivated by native colipase (Fig. 2).

To investigate this effect in more detail, we looked at the impact of the mutations on both the apparent affinity between the mutant lipases and native colipase and the catalytic efficiency of the mutant lipase-colipase complexes. Therefore, the rate of hydrolysis of 0.11 M emulsified tributyrin (V_S) against native colipase concentration was determined at different NaTDC concentrations (Table I). The selected tributyrin concentration was close to the substrate saturating concentration; thus the experimental value of V_S approached the value of the limiting rate $V = k_{cat}[E_o]$. For each bile salt concentration, the experimental points (V_S *versus* colipase concentration) were fitted to rectangular hyperbolic curves corresponding to a 1:1 stoichiometry between lipase and colipase. The values of $K_{d(app)}$ (lipase/colipase apparent dissociation constant in the presence of a water/lipid interface) and the limiting values of V_S (corre-

[1] The abbreviations used are: NaTDC, sodium taurodeoxycholate; E600, diethyl *p*-nitrophenyl phosphate; CMC, critical micellar concentration.

TABLE I

Influence of NaTDC concentration on the lipolytic activity of the lipase mutants in the presence of native colipase

The rate of hydrolysis (V_S) was measured using a saturating concentration of emulsified tributyrin. For each NaTDC concentration, the value of $K_{d(app)}$ and the limiting value of V_S were determined by plotting V_S *versus* colipase concentration. The limiting values of V_S are expressed in units (μmol of released fatty acids/min) per mg of lipase mutant. ND, not determined. The corresponding values for the native horse lipase are 0.32 ± 0.11 nM and 10500 ± 460 units/mg for $K_{d(app)}$ and V_S, respectively.

NaTDC	LipK400Q		LipK400E	
	$K_{d(app)}$	V_S	$K_{d(app)}$	V_S
mM	*nM*	*units/mg*	*nM*	*units/mg*
0.1	ND	9413 ± 460	ND	8090 ± 320
0.5	1.21 ± 0.14	8663 ± 259	3.1 ± 0.5	7580 ± 279
1	2.04 ± 0.29	6634 ± 159	6.5 ± 0.39	5990 ± 230
1.5	10.7 ± 0.6	2183 ± 230	34.5 ± 5.21	848 ± 53
2	14 ± 1.8	960 ± 43	24.2 ± 3.26	397 ± 33
4	9.82 ± 2.8	375 ± 21	39.7 ± 6.8	116 ± 15

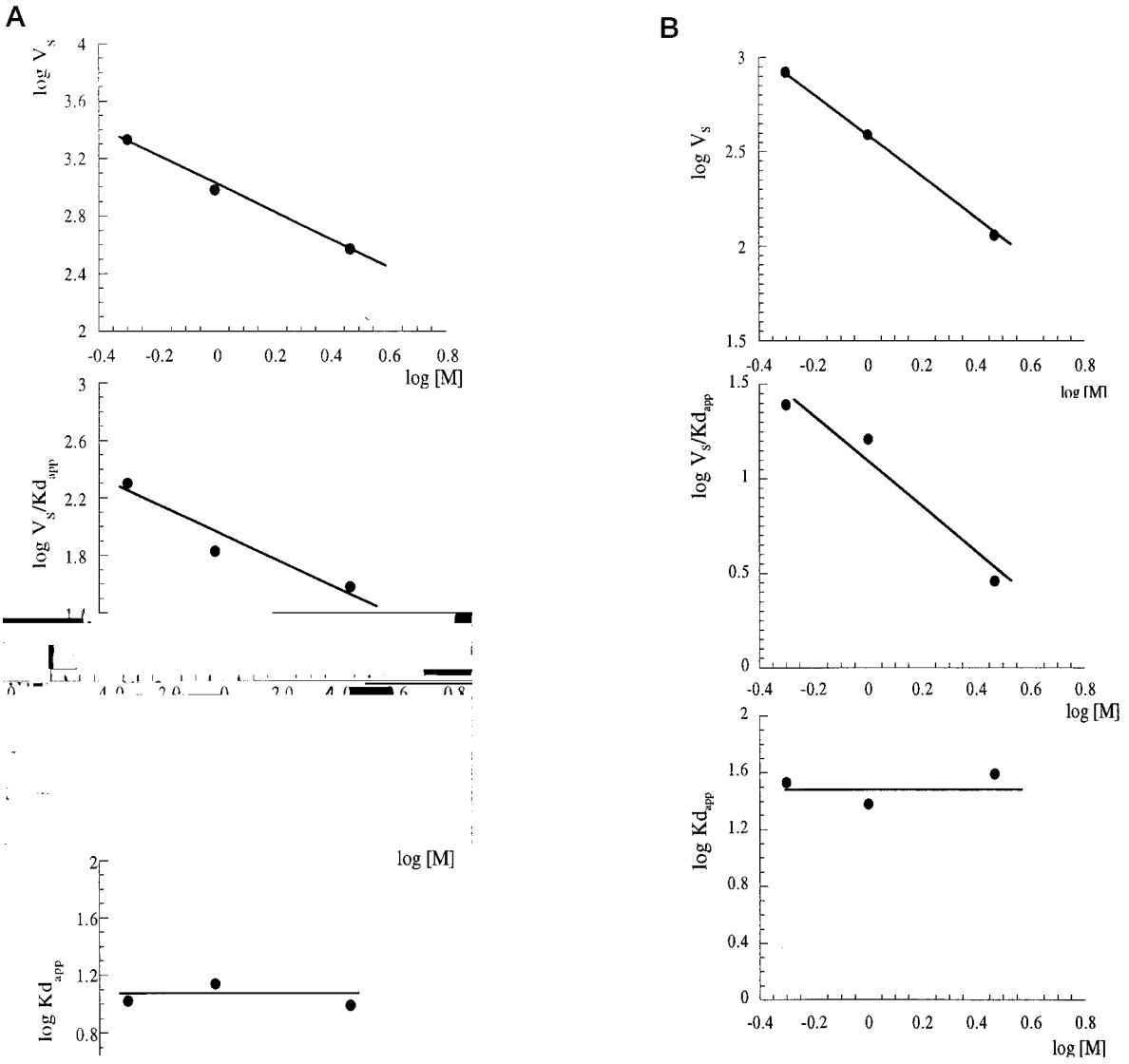


FIG. 3. **Plots of log V_S, log $V_S/K_{d(app)}$, and log $K_{d(app)}$ *versus* log [*M*].** Both V_S and $V_S/K_{d(app)}$ vary with the NaTDC micelle concentration ([*M*]) according to the following equations: $K_{d(app)} = V/(1 + [M]/K'_{mic})$ and $V_S/K_{d(app)} = V/K_d\ (1 + [M]/K_{mic})$ derived from the schema presented under "Results." *A,* LipK400Q; *B,* LipK400E. For details, see text.

sponding to 100% lipase complexed with colipase) were determined from the curves and reported in Table I.

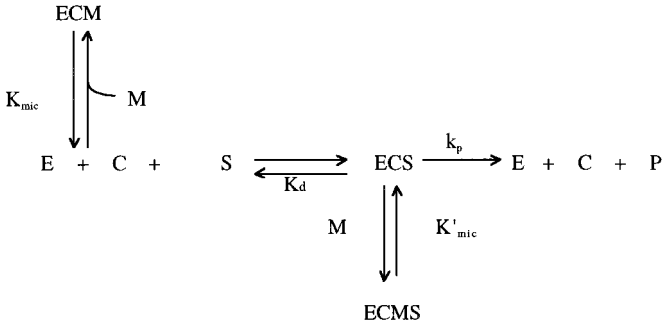
For NaTDC concentrations below the CMC (<1 mM), a moderate effect of NaTDC was observed on the LipK400Q activity on emulsified tributyrin, whereas above this concentration a noticeable change occurred on both the apparent affinity of the mutant lipase for colipase and the catalytic efficiency. However, although $K_{d(app)}$, after a sharp increase, reached a plateau value, V_S kept on decreasing, when increasing NaTDC concentration.

A similar behavior with a somewhat sharper effect on $K_{d(app)}$ was observed with the LipK400E mutant, as expected from the greater negative charge repulsion induced by the mutation.

The plots V_S *versus* NaTDC concentration (Fig. 2) show that

colipase fails to efficiently reverse the inhibitory effect of bile salts for the two mutant lipases, mainly in the presence of micelles. The recombinant wild type horse lipase, as well as native horse lipase, was fully reactivated by native colipase, thus ensuring that the sensitivity of LipK400E and LipK400Q to supramicellar concentrations of NaTDC in the presence of colipase has to be only correlated to the introduced mutations.

The finding of linear plots with a negative slope of −1 when plotting either log V_S or log $V_S/K_{d(app)}$ *versus* log [NaTDC micelles] (Fig. 3) indicates that there is no micelle cooperativity. The simplest schema fitting the experimental results corresponds to Scheme 1, where *M* represents the concentration of NaTDC micelles; *E* represents free lipase; *C* represents free colipase; ECS represents the lipase-colipase complex linked to the substrate; ECM and ECMS represent the lipase-colipase-micelle complex free or linked to the substrate, k_p represents the specific rate constant of product formation; and K_d, K_{mic}, and K'_{mic} represent the dissociation constants of the ECS, ECM, and ECMS complexes, respectively. In Scheme 1, ECMS is considered as a nonproductive complex. The mutant lipase-native colipase complexes, although able to bind to the water/lipid interface in the presence of bile salt micelles, can no longer perform an efficient catalysis suggesting a behavior toward NaTDC similar to that of native lipase alone.



SCHEME 1.

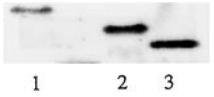


FIG. 4. **Immunoblotting of ColE45Q and ColE45K.** After electrophoresis on polyacrylamide gel performed in non-denaturating conditions, the colipase mutants were transferred on polyvinylidene difluoride membranes, and immunodetection using anti-colipase antibodies was carried out as described under "Experimental Procedures." *Lane 1*, ColE45K; *lane 2*, ColE45Q, *lane 3*, native horse colipase.

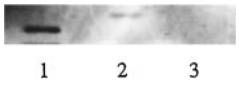


FIG. 5. **Binding of native horse lipase to colipase mutants.** The protein blotting/protein overlay assays were performed as described under "Experimental Procedures." 1–3 μg of native colipase (*lane 1*), ColE45Q (*lane 2*) or ColE45K (*lane 3*) were electroblotted on a polyvinylidene difluoride membrane. Then the membrane was soaked in a native horse lipase solution (1 μM), and immunodetection was carried out using anti-horse lipase antibodies.

Effect of Glu^{45} Mutations on Colipase Properties—Purification of the recombinant wild type or mutant colipase was achieved as reported under "Experimental Procedures." Recombinant wild type (data not shown) and colipase mutants possess the expected molecular mass and the expected charge as estimated from their electrophoretic behavior on polyacrylamide gel in the absence of SDS (Fig. 4).

The lipase binding property of both mutants was first investigated by the protein blotting/protein overlay technique (Fig. 5). A faint band was revealed by anti-lipase antibodies for ColE45Q, whereas no binding could be detected with ColE45K suggesting a lower affinity of these mutant colipases for lipase. Thus, the ability of colipase mutants to reactivate native lipase was kinetically investigated using a saturating concentration of emulsified tributyrin as mentioned above. The values of $K_{d(app)}$ and V_S were determined for various NaTDC concentrations (Table II). The two mutants are poorly efficient in counteracting the inhibitory effect of bile salts, notably in the presence of micelles. The E45K mutation led to a particularly severe change in the properties of colipase which became very ineffective in restoring lipase activity even at low NaTDC concentration since at 0.75 mM NaTDC, only 25% of the lipase activity could be recovered. The $K_{d(app)}$ value was moderately affected for NaTDC concentrations below 1 mM but dramatically increased for NaTDC concentrations above 1 mM. The kinetic analysis of the data could not be performed due to the difficulty in obtaining accurate measurements at high NaTDC concentration.

The E45Q mutation resulted in a less drastic effect, ColE45Q being able to partly counteract the inhibitory effect of NaTDC for somewhat higher bile salt concentrations.

Effect of ColE45K on the Catalytic Properties of LipK400E—In order to see if the E45K mutation on colipase could compensate the K400E mutation on lipase, we investigated the capability of ColE45K to restore the activity of LipK400E in the presence of various NaTDC concentrations. As shown in Table III, even at low NaTDC concentrations, a sharp increase of $K_{d(app)}$ is observed in the LipK400E·ColE45K complex as compared with the native lipase-colipase complex, indicative of a lower apparent affinity between lipase and colipase in the former complex. As well, analysis of the V_S values indicates that ColE45K failed to

TABLE II

Influence of NaTDC concentration on the lipolytic activity of native lipase in the presence of colipase mutants

The rate of hydrolysis (V_S) was measured using a saturating concentration of emulsified tributyrin. For each NaTDC concentration, the value of $K_{d(app)}$ and the limiting value of V_S were determined by plotting V_S *versus* the colipase mutant concentration. The corresponding values for the native horse lipase are 0.32 ± 0.11 nM and 10500 ± 460 units/mg (100%) for $K_{d(app)}$ and V_S, respectively. The limiting values of V_S thus determined are expressed in percent of the value obtained with the native lipase-colipase complex. ND, not determined.

NaTDC	ColE45Q		ColE45K	
	$K_{d(app)}$	V_S	$K_{d(app)}$	V_S
mM	*nM*	*%*	*nM*	*%*
0.75	ND	89.6 ± 3.8	3.85 ± 0.39	27.8 ± 3.2
1	3.3 ± 0.4	82.3 ± 5.1	10.1 ± 3.8	11.2 ± 1.7
1.5	15.6 ± 1.3	60.2 ± 3.4	248 ± 74	2.6 ± 0.5
2	44.7 ± 3.8	50.6 ± 4.3	ND	ND
4	62.5 ± 27.7	23.5 ± 2.1	ND	ND

correctly abolish the inhibitory effect of NaTDC on LipK400E activity. Moreover, as compared with the LipK400E-native colipase or native lipase-ColE45K complexes, there was no significant increase in the apparent affinity in the LipK400E·ColE45K complex. Therefore there is no compensatory effect of the mutations in the LipK400E·ColE45K complex.

E600 Inhibition of Native and Mutant Lipase in the Presence of Mutant or Native Colipase—E600 inhibition experiments were performed on both native and K400E lipase using either native or E45K colipase in the presence of NaTDC micelles. In all cases a complete inhibition of the native or recombinant lipase was observed. Blank experiments performed in the absence of colipase clearly indicated that lipase activity was not affected by E600 and remained stable for at least 72 h incubation. Thus, the observed inhibition is indicative of the unmasking of the lipase active site.

As previously observed with the native partners (8), at high bile salt micelle concentration the E600 rate of inhibition is increased (Table IV). However, it must be pointed out that the time required to achieve 50% lipase inhibition was considerably longer with the mutant proteins.

Finally, as already observed in the kinetic studies, no compensatory effect could be detected when E600 experiments were performed in the presence of both LipK400E and ColE400K.

Modeling Data—In order to better understand the functional properties of the mutants, structural models of the various mutant lipases and colipases were built on the basis of the crystal structure of the porcine lipase-colipase complex (8). Careful investigation of the models shows that in the case of ColE45K, the mutant displaying the more drastic effects, the location of the Lys^{45} side chain of the mutant colipase is likely to decrease the stability of the resulting native lipase-mutant colipase complex since this residue faces two lysine side chains of lipase (Lys^{398} and Lys^{400}), which is energetically unfavorable (Fig. 6*A*).

In the LipK400E-native colipase complex, a repulsive effect, although less severe, can also occur between the two negatively charged side chains, Glu^{400} and Glu^{45}. However, it must be pointed out that in LipK400E, an intramolecular ion pair is likely to be formed between Glu^{400} and Lys^{398} (Fig. 6*B*) thus stabilizing the negative charge of the mutation.

TABLE III
Influence of NaTDC concentration on the lipolytic activity of LipK400E in the presence of ColE45K

The rate of hydrolysis (V_S) was measured using a saturating concentration of emulsified tributyrin. For each NaTDC concentration, the value of $K_{d(app)}$ and the limiting value of V_S were determined by plotting V_S *versus* ColE45K concentration. The limiting values of V_S are expressed in units (μmol of released fatty acids/min) per mg of LipK400E. The corresponding values for the native horse lipase are 0.32 ± 0.11 nM and 10500 ± 460 units/mg for $K_{d(app)}$ and V_S, respectively.

NaTDC	$K_{d(app)}$	V_S
mM	*nM*	*units/mg*
0.75	90 ± 15	3070 ± 280
1	128 ± 45	1879 ± 128
1.5	60 ± 7.8	185 ± 34

TABLE IV
Native lipase and LipK400E inhibition by E600

Native horse lipase or LipK400E (8 μM) was incubated at pH 6.0 at room temperature with 4 mM E600 in the presence of NaTDC and either 15 μM native colipase or 16 μM ColE45K. At different times, aliquots were withdrawn from the incubation mixtures, and the remaining lipase activity was determined at pH 7.5 and 25 °C as described under "Experimental Procedures." *t* 50% is the time required to reach 50% lipase inhibition. ND, not determined. No inhibition was observed in the absence of colipase.

	t 50% (h)	
	NaTDC, 1.5 mM	NaTDC, 4 mM
Native lipase, native colipase	3.1 ± 0.2	<0.15
Native lipase, ColE45K	16 ± 2	4 ± 0.3
LipK400E, native colipase	35 ± 5	4.2 ± 0.4
LipK400E, ColE45K	ND	5.5 ± 0.3

DISCUSSION

In the presence of amphiphilic compounds, lipolysis occurs through lipase/colipase interactions. Although our basic knowledge on pancreatic lipase/colipase has advanced in the last decade, many of the molecular events occurring at the amphiphile-coated water/lipid interface remain obscure. To obtain more information on the lipase/colipase mechanism of action, we decided to look at the role of the sole ion pair linking lipase to colipase.

As expected, impeding the formation of the Lys^{400}/Glu^{45} ion pair between lipase and colipase affects the apparent affinity between the mutant protein and its native partner. It must be emphasized that this effect depends upon the form of presen-

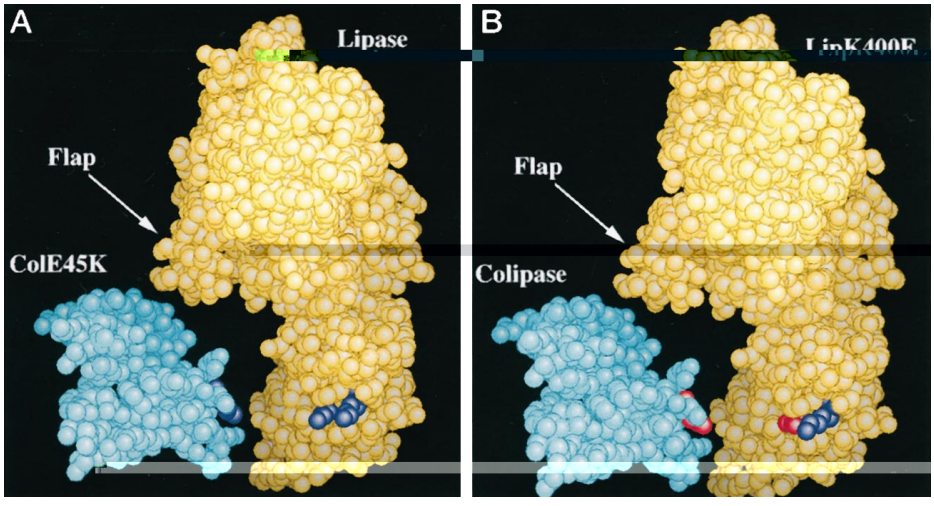


FIG. 6. **MOLSCRIPT (21) representations of the native lipase-ColE45K complex (*A*) and of the LipK400E-native colipase complex (*B*).** The models were obtained as described in the text. Colipase is represented in *cyan* and lipase in *gold*. For clarity, colipase has been horizontally shifted. Only the side chains of the residues in positions 398 and 400 in lipase and in position 45 in colipase are represented, in *blue* for lysine and *red* for glutamic acid residues. *A,* Lys^{400} and Lys^{398} in native lipase and Lys^{45} in ColE45K. *B,* Lys^{398} and Glu^{400} in LipK400E and Glu^{45} in native colipase.

tation of the bile salt. When NaTDC is spread at the water/lipid interface (below the CMC), the apparent affinity is only moderately decreased. By contrast, above the CMC, when bile salt micelles are formed in the bulk, the apparent affinity is further reduced.

For all the mutants, the charge inversion results in a more severe damage than the charge neutralization, particularly for the colipase mutants. Based on modeling experiments, a likely explanation for this finding is that the E45K mutation in colipase gives rise to an electrostatic repulsion between native lipase and the mutant colipase, the Lys^{45} side chain of colipase facing two lysyl side chains of lipase in the resulting complex. In the LipK400E model, an intramolecular ion pair can occur between Glu^{400} and Lys^{398} thus stabilizing the introduced negative charge.

The most striking result concerns the effect of the mutations on the catalytic efficiency of the various lipase-colipase complexes (mutant lipase-native colipase or native lipase-mutant colipase). Impeding the formation of the ion pair between lipase and colipase results in a loss of activity against emulsified substrates depending on the bile salt concentration. Increasing the bile salt micelle concentration leads to quite inactive complexes.

Two hypotheses could be proposed to explain this phenomenom as follows: (i) a cooperative effect of bile salts micelles resulting in the binding of more than one micelle to the lipase-colipase complex, or (ii) an uncorrect conformation of the lipase-colipase mutant complex worsened by the presence of the micelle. From the analysis of the kinetic results the first hypothesis can be discarded. Thus, although not directly involved in micelle binding, the ion pair Lys^{400}/Glu^{45} is likely to control the correct orientation of colipase relative to lipase in the ternary complex resulting in a proper opening of the flap.

This hypothesis is further supported by experiments of lipase inhibition by E600 in the presence of NaTDC micelles and ColE45K. These experiments, performed in the absence of any water/lipid interface, are representative of the combined effect of bile salt micelles and colipase on the opening of the lipase flap. As compared with native colipase, a very slow inhibition is observed indicative of a poorly active conformation of lipase likely due to a poor stabilization of the lipase flap by the mutant colipase. Preventing the interaction between colipase and the flap of lipase led to the same observation (22).

It must be emphasized that, in the absence of micelles, the more the bile salts accumulate on the water/lipid interface, the less colipase is able to restore the lipase activity, although the apparent affinity of the lipase-colipase mutant complex remains moderately affected. This finding suggests that impairing the formation of the ion pair could also result in an uncorrect positioning of colipase toward the water/lipid interface and further supports the possible role of colipase in the rearrangement of the amphiphilic compounds coating the water/lipid interface during catalysis, as postulated by Momsen *et al*. (23).

Attempts to compensate the K400E mutation on lipase by the E45K mutation on colipase failed. From modeling investigation on mutant lipase, it is proposed that Glu^{400} is involved in an intramolecular ion pair with Lys^{398} rather than in an intermolecular ion pair with Lys^{45} of colipase, thus impeding a proper orientation of the two partners in the complex. Another explanation could be that the lipase/colipase binding is (partly) driven by polar forces and that inverting the charges, notably in colipase, results in an uncorrect association.

In conclusion, this work demonstrates that the sole ion pair linking lipase to colipase plays an essential role in the formation of the active conformation of the lipase-colipase complex at the water/lipid interface in the physiological conditions. This ion pair is likely to maintain a correct orientation of colipase relative to lipase, particularly in the presence of bile salt micelles. Moreover, the finding that mutant colipase is unable to correctly abolish the inhibitory effect of bile salts spread at the water/lipid interface in the absence of micelles suggests that colipase might play a complex function during catalysis. Besides its involvement in anchoring lipase at the substrate interface, stabilization of the open lipase conformation, and micelle bind-ing, colipase might participate in the rearrangement of the amphiphilic compounds coating the water/lipid interface.

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REFERENCES

1. Winkler, F. K., D'Arcy, A., and Hunziker, W. (1990) *Nature* **343,** 771–774
2. Bourne, Y., Martinez, C., Kerfelec, B., Lombardo, D., Chapus, C., and Cambillau, C. (1994) *J. Mol. Biol.* **238,** 709–732
3. Mahé-Gouhier, N., and Léger, C. (1988) *Biochim. Biophys. Acta* **229,** 286–289
4. Aboushalam, A., Chaillan, C., Kerfelec, B., Foglizzo, E., and Chapus, C. (1992) *Protein Eng.* **5,** 105–111
5. van Tilbeurgh, H., Egloff, M. P., Martinez, C., Rugani, N., Verger, R., and Cambillau, C. (1993) *Nature* **362,** 814–820
6. Ayvazian, L., Crenon, I., Granon, S., Chapus, C., and Kerfelec, B. (1996) *Protein Eng.* **9,** 707–711
7. van Tilbeurgh, H., Sarda, L., Verger, R., and Cambillau, C. (1992) *Nature* **359,** 159–162
8. Hermoso, J., Pignol, D., Kerfelec, B., Crenon, I., Chapus, C., and Fontecilla-Camps, J. C. (1996) *J. Biol. Chem.* **271,** 18007–18016
9. Hermoso, J., Pignol, D., Penel, S., Roth, M., Chapus, C., and Fontecilla-Camps, J. C. (1997) *EMBO J.* **16,** 5531–5536
10. Lombardo, D., Chapus, C., Bourne, Y., and Cambillau, C. (1989) *J. Mol. Biol.* **205,** 259–261
11. Chapus, C., Desnuelle, P., and Foglizzo, E. (1981) *Eur. J. Biochem.* **115,** 99–105
12. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 546 3–5467
13. Laemmli, U. K. (1970) *Nature* **227,** 680–685
14. Burnette, W. N. (1981) *Anal. Biochem.* **112,** 195–203
15. Jones, T. A., Zou, J. Y., Cowan, S. W., and Kjeldgaard, M. (1991) *Acta Crystallogr. B* **47,** 110–119
16. Ponder, J., and Richards, F. (1987) *J. Mol. Biol.* **193,** 775–779
17. Brunger, A. T. (1988) in *Crystallographic Computing 4: Techniques and Technologies* (Isaac, N. W., and Taylor, M. R., eds) pp. 126–140, Oxford University Press, New York
18. Engh, R. A. and Huber, R. (1991) *Acta Crystallogr. A* **47,** 392–400
19. Brunger, A. T., Krukowski, A., and Erickson, J. W. (1990) *Acta Crystallogr. A* **46,** 585–593
20. Berendsen, H. J. C., Postma, J. P., van Gunstern, W. F., DiNola, A., and Haak, J. R. (1984) *J. Chem. Phys.* **81,** 3684–3690
21. Kraulis, P. J. (1991) *J. Appl. Crystallogr.* **24,** 946–950
22. Lowe, M. E. (1997) *J. Biol. Chem.* **272,** 9–12
23. Momsen, W. E., Momsen, M. M., and Brockman, H. L. (1995) *Biochemistry* **34,** 7271–7281