Biochemical and structural comparative study between bird and mammal pancreatic colipases

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Abstract Three colipases were purified from pancreas of two birds (ostrich and turkey) and one mammal (dromedary). After acidic and/or heat treatment and precipitation by sulfate ammonium and then ethanol, cofactors were purified by Sephadex G-50 gel filtration followed by ion-exchange chromatography first on Mono S and then on Mono Q. One molecular form was obtained from each species with a molecular mass of ~10 kDa. Cofactors were not glycosylated. The N-terminal sequences of the three purified cofactors showed high sequence homology. A 90 amino acid sequence of the ostrich cofactor was established based on peptide sequences from four different digests of the denaturated protein using trypsin, chymotrypsin, thermolysin, or staphylococcal protease. This sequence exhibited a high degree of homology with chicken and mammal cofactors. Bile salt-inhibited pancreatic lipases from five species were activated to variable extents by colipases from bird and mammal origins. The bird pancreatic lipase-colipase system appears to be functionally similar to homologous lipolytic systems from higher mammals. Our comparative study showed that mammal colipase presents a lower activation level toward bird lipases than the bird counterpart. Three-dimensional modeling of ostrich colipase suggested a structural explanation of this fact.—Bacha, A. B., F. Frikha, I. Djemal, A. Fendri, N. Miled, Y. Gargouri, and H. Mejdoub. **Biochemical and structural comparative study between bird and mammal pancreatic colipases. *J. Lipid Res.* 2006.** 47: **2701–2711.**

Supplementary key words pancreatic lipase • inhibition • reactivation • amino acid sequence • modeling

In mammals, the duodenal digestion of dietary triacylglycerols is accomplished essentially by the action of pancreatic lipase in the presence of colipase and bile salts. Colipase is a low molecular mass protein secreted by the exocrine pancreas that counteracts (in vitro) the inhibiting action of bile salts on lipase activity (1) via the formation of a specific lipase-colipase complex (2). Colipase has been purified from the pancreatic glands of several species (1).

The first indication of the existence of a cofactor for pancreatic lipases was reported in 1910 by Rosenheim (3). Colipase, which has been isolated from the pancreas of many mammals (human, dog, horse, ox, pig, rat, and sheep) (4–10) and from chicken (11), was found in the pancreatic juice of higher mammals in a proform (procolipase). Procolipase is converted to its physiologically more active form by a tryptic cleavage of a single bond, arginine 5-glycine 6 (12). The removal of the N-terminal pentapeptide of native colipase, conserved in all mammal colipases studied to date, was found to highly increase the capacity of colipase to anchor the lipase to a lipid-water interface (13–15).

The three-dimensional structure of the colipase was determined in complex with the pancreatic lipase by X-ray crystallography (16) and alone in solution by NMR (17). Colipase belongs to a family of small cysteine-rich proteins. It is a fairly flat molecule of dimensions 25 × 30 × 35 Å with a three-finger topology comparable to that of snake toxins (16, 18). The tips of the fingers contain the binding site to a lipolytic interface. Colipase binds to the noncatalytic C-terminal domain of pancreatic lipase and exposes the hydrophobic tips of its fingers at the opposite side of its hydrophilic lipase binding site. These hydrophobic tips probably help to bring the catalytic N-terminal domain of pancreatic lipase into close contact with the interface, where a drastic change occurs in the conformation of the lid domain, a surface loop controlling the access of the substrate to the active site, which pops open. As a result of this structural reorganization, the N-terminal part of colipase binds to the lid domain, forming a second lipase-colipase interaction site. The open lid and the extremities of the colipase fingers, as well as the β9 loop (19), form an impressive continuous hydrophobic plateau

Manuscript received 5 June 2006 and in revised form 17 July 2006 and in re-revised form 14 August 2006 and in re-re-revised form 5 September 2006.

Published, JLR Papers in Press, September 6, 2006.
DOI 10.1194/jlr.M600242-JLR200

Abbreviations: HPL, recombinant human pancreatic lipase; K_{cat}, catalytic constant; K_d, dissociation constant; NaDC, sodium deoxycholate; OPL, ostrich pancreatic lipase; TC_4, tributyrin; TPL, turkey pancreatic lipase.

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extending over >50 Å that might be able to interact strongly with a lipid-water interface. The interaction with the open lid suggested a second role of colipase: stabilization of an active (open) lipase conformation (20). Other studies have investigated the interaction between colipase and the C-terminal domain of lipase and the relative importance of the putative lipase binding residues in the hydrophilic surface of colipase. Jennens and Lowe (21) demonstrated that deletion of the C-terminal domain greatly decreased lipase activity, but the truncated lipase still required colipase for activity in the presence of bile acids. These results raised questions about the importance of the interactions between colipase and the C-terminal domain of lipase. Later, Ayvazian et al. (22) demonstrated that disruption of the interaction between glutamate 45 of colipase and lysine 400 of the lipase C-terminal domain impaired lipolytic activity secondary to an inability of the colipase mutant to bind lipase. Furthermore, Crandall and Lowe (23) have suggested that the hydrophilic surface of colipase interacts with the lipase in solution to form an active lipase-colipase complex. This complex formation was shown to be influenced by bile salt micelles and to require the glutamate 64-arginine 65 colipase binding region.

Several studies have provided evidence that no difference can be observed among mammals in the activation of a pancreatic lipase from one species by colipase from another species when emulsified triolein or tributyrin (TC_4) is used as a substrate (24, 25). Pancreatic lipase and colipase were purified from chicken (11, 26, 27) and their biochemical properties studied. Recently, turkey and ostrich pancreatic lipases (TPL and OPL) were purified in our laboratory (28, 29). The biochemical properties of these enzymes were reported to be similar to those of mammals and chicken. However, the main difference observed was the capacity of TPL to hydrolyze the long-chain triacylglycerols more efficiently than the short-chain triacylglycerols. On the other hand, in contrast to TPL and to mammal pancreatic lipases, OPL hydrolyzes both short- and long-chain triacylglycerols at comparable rates. To gain more information about the bird pancreatic lipase-colipase function, we report here the purification and some biochemical properties of one mammal and two bird colipases. The complete primary structure of ostrich colipase allowed its comparison with the known sequences of mammal colipases. This work also addressed the question of the specificity of interaction of bird colipases with lipases of different origins.

MATERIALS AND METHODS

Materials

Chymotrypsin (treated with L-1-tosylamido 2-lysyl chloromethyketone), trypsin (treated with L-1-tosylamido 2-phenylethyl chloromethyketone), thermolysin, proteinase V8, TC_4 (99% pure), and benzamidine were from Fluka (Buchs, Switzerland). BSA, sodium deoxycholate (NaDC), sodium taurodeoxycholate, and Triton X-100 were from Sigma Chemical (St. Louis, MO). Gum arabic was from Mayaud Baker, Ltd. (Dagenham, UK). Acrylamide and bis-acrylamide (electrophoresis grade) were from BDH (Poole, UK). Marker proteins and supports of chromatography used for colipase purification, Sephadex G-50, Mono S, and Mono Q, were from Pharmacia (Uppsala, Sweden). Polyvinylidene difluoride membranes and the Procise 492 protein sequencer equipped with the 140 C HPLC system were purchased from Applied Biosystems (Roissy, France). The C-8 reverse-phase Eurospher 100 column was from Knauer. pH-stat was from Metrohm (Herisau, Switzerland).

Pancreas collection

Pancreases from different species were collected immediately after slaughter and kept at −20°C. All pancreases were collected from a local slaughterhouse (Sfax, Tunisia) except ostrich pancreases (Nabeul, Tunisia).

Delipidation of pancreas

After decongelation, pancreases were cut into small pieces (1–2 cm^2) and delipidated according to the method described previously (30). After delipidation, ~15 g of delipidated powder of each pancreas was obtained from 100 g of fresh tissue.

Determination of lipase activity and colipase activity

Lipase activity was measured titrimetrically at pH 8.5 and 37°C with a pH-stat, under the standard assay conditions described previously, using olive oil emulsion (31) or TC_4 (0.25 ml) in 30 ml of 2.5 mM Tris-HCl and 1 mM $CaCl_2$, pH 8.5 (32), as substrate. Some lipase assays were performed in the presence or absence of NaDC and colipase. One lipase unit corresponds to 1 μmol of fatty acid liberated per minute.

Colipase activity was measured at pH 8.5 and 37°C as described by Rathelot et al. (32). One colipase unit corresponds to the amount of cofactor that increases bile salt-inhibited pancreatic lipase activity by 1 enzyme unit.

Enzymes and proteins

OPL (25), dromedary pancreatic lipase (33), and colipase from pig (8) were prepared in our laboratory as described previously. Chicken pancreatic lipase and TPL were purified according to previous works (28, 34). Recombinant human pancreatic lipase (HPL) was a generous gift from Dr. R. Verger (Centre National de la Recherche Scientifique, Marseille, France). The activated form of the pig cofactor was obtained by limited trypsinolysis under controlled conditions (14).

Determination of protein concentration

Protein concentration was determined as described by Bradford (35) using BSA ($E^{1\%}_{1cm} = 6.7$) as a reference.

Purification of pancreatic colipases from various species

Ostrich, turkey, and dromedary pancreatic colipases were purified under the same conditions. Twenty grams of delipidated powder of ostrich, turkey, or dromedary pancreas was suspended in 300 ml of water containing 2 mM benzamidine, 150 mM NaCl, and 0.2% Triton X-100 (v/v) and ground mechanically twice for 30 s at 4°C using the Waring Blendor system. The mixture was stirred with a magnetic bar for 30 min at 4°C and then centrifuged for 30 min at 12,000 rpm.

Heat and/or acidic treatment. To inactivate the lipase, the supernatant was incubated for 5 min at 70°C in the case of ostrich and dromedary colipases. After rapid cooling, insoluble material was removed by centrifugation for 30 min at 12,000 rpm. Afterward, the pH of the previous supernatant was brought to 3.0 by adding

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6 N HCl under gentle stirring at 0°C. After centrifugation (30 min at 12,000 rpm), the clear supernatant was adjusted to pH 7 with 6 N NaOH. In the case of turkey colipase, the pH of the homogenate was brought to 1.5 by adding 6 N HCl under gentle stirring at 0°C and incubated for 5 min. We obtained a clear supernatant after centrifugation for 20 min at 12,000 rpm, which contained 20,000 colipase units per gram of delipidated pancreatic tissue. The results show that the bird pancreases presented higher colipase content than the mammal pancreases. The highest level was observed with turkey (20,000 colipase units per gram of delipidated pancreas). Ostrich presented only 10,000 colipase units per gram of delipidated powder, and dromedary presented the lowest level: 1,100 colipase units.

Ammonium sulfate precipitation. Pancreatic extracts from ostrich, turkey, and dromedary pancreases containing 200,000, 400,000, and 22,000 colipase units, respectively, were brought to 60% saturation with solid ammonium sulfate under stirring conditions and maintained for 30 min at 4°C. After centrifugation (30 min, 12,000 rpm), precipitates were resuspended in a minimum of extraction solution (water containing 2 mM benzamidine, 150 mM NaCl, and 0.2% Triton X-100). Insoluble proteins were discarded by centrifugation (15 min, 12,000 rpm). Preparations of colipases contained between 70% and 80% of the starting amount of colipase.

Ethanol fractionation. Supernatants issued from ammonium sulfate precipitation were subjected to fractionation using ethanol. We added an equal volume of ethanol at 0°C. Insoluble proteins were removed by centrifugation, and the ethanol (4 v/v) was added slowly to the supernatant, bringing the solvent concentration to 90% (v/v) at 0°C. Precipitated proteins, which contained ~60% of the starting amount of colipase, were collected and solubilized in minimum of 10 mM acetate buffer, pH 4.5, containing 0.05% Triton X-100 and 2 mM benzamidine (buffer A). In this study, we found this step critical to eliminate the last traces of lipids, facilitating the filtration chromatography step.

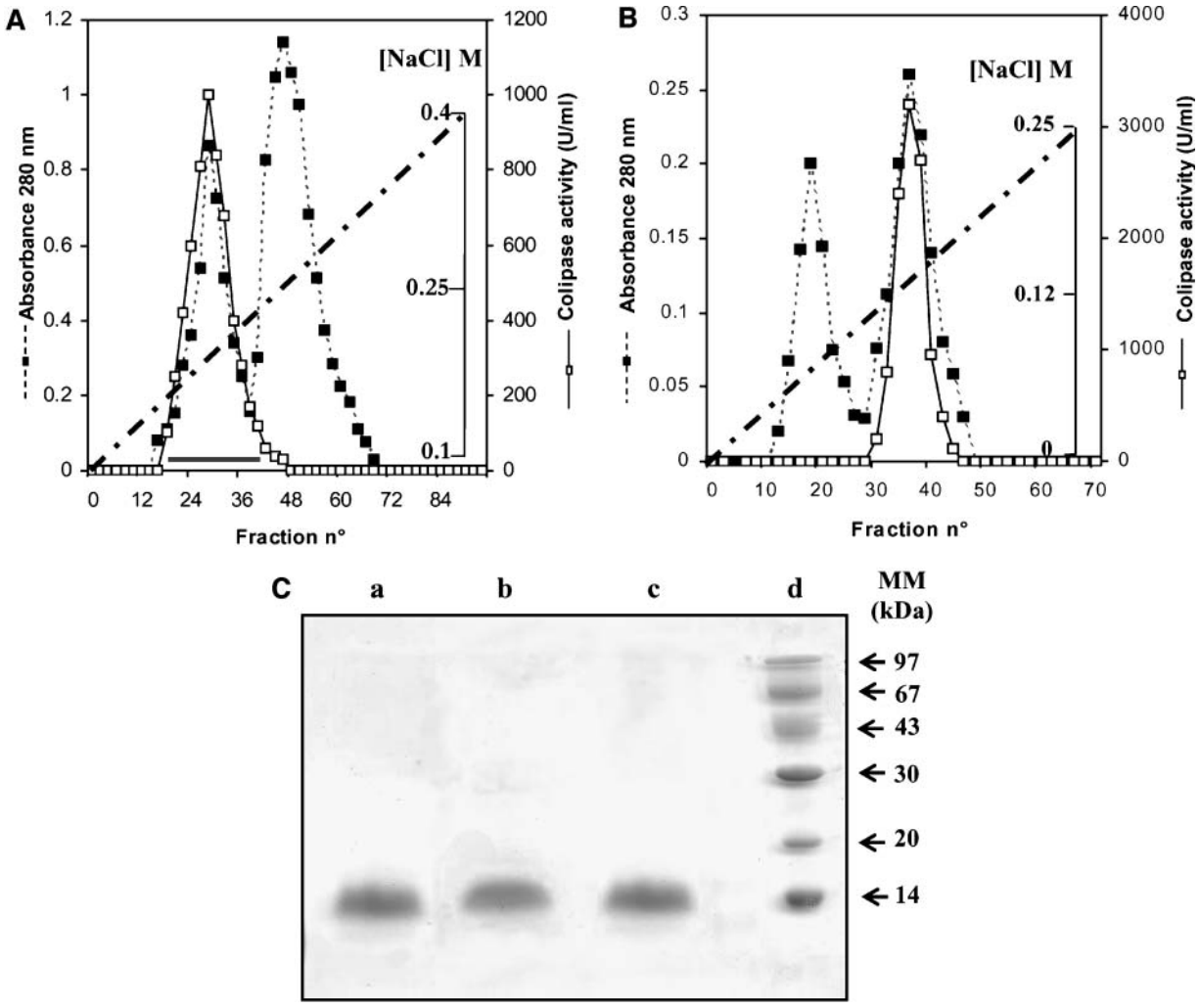

Fig. 1. A: Chromatography of ostrich pancreatic colipase on Mono S Sepharose. The column (2.6 cm × 20 cm) was equilibrated with 10 mM acetate buffer, pH 4.5, containing 0.05% Triton X-100; a linear salt gradient (0.1–0.4 M NaCl) in buffer B was applied to the column. The gradient chamber was 200 ml, the fraction was 4.2 ml, and the flow rate was 30 ml/h. B: Chromatography of ostrich pancreatic colipase on Mono Q Sepharose. The column (1.5 cm × 10 cm) was equilibrated with 10 mM Tris-HCl buffer, pH 8.2, containing 10 mM NaCl; a linear salt gradient (0.01–0.25 M NaCl) in buffer C was applied to the column. The gradient chamber was 75 ml, the fraction was 2 ml, and the flow rate was 40 ml/h. C: SDS-PAGE (15%) of pure cofactors. Lanes a, b, and c, 15 μg of purified turkey, dromedary, and ostrich pancreatic colipases, respectively, obtained after Mono Q chromatography; lane d, molecular mass (MM) markers (Pharmacia).

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Filtration on Sephadex G-50. The colipase sample was submitted to gel filtration through a Sephadex G-50 column (95 cm × 2.6 cm) equilibrated with buffer A. Elution of proteins was performed with the same buffer at 30 ml/h. The fractions containing the colipase activity eluted between 1.4 and 2 void volumes were pooled.

Cation-exchange chromatography. Active fractions eluted from Sephadex G-50 were subjected to cation-exchange chromatography using a Mono S column (2.6 cm × 20 cm) equilibrated with 10 mM acetate buffer, pH 4.5, containing 0.05% Triton X-100 (buffer B). Nonbound proteins were washed out with 200 ml of buffer B. After a wash with 100 ml of 0.1 M NaCl in buffer B, elution was performed with a linear gradient of NaCl (0.1–0.4 M). Ostrich colipase activity emerged in a single peak at a NaCl concentration of ~180 mM (**Fig. 1A**). Similar results were obtained with turkey and dromedary cofactors (data not shown). The colipase-containing fractions were pooled. Benzamidine was added at a final concentration of 2 mM, and then the fractions were lyophilized for the purpose of concentration. The recovery of colipase activity after the Mono S step was ~75% for all purified colipases.

Second gel filtration and Mono Q chromatography. The purified colipases were dissolved in ~10 ml of 10 mM Tris-HCl buffer, pH 8.2, containing 10 mM NaCl (buffer C). Each preparation was filtered through a column (2.6 cm × 100 cm) of Sephadex G-50 equilibrated in buffer C. The active fractions were pooled and then deposited on a Mono Q Sepharose column (1.5 cm × 10 cm) equilibrated in the same buffer. After washing with buffer C until the eluent was free of proteins, elution of fixed proteins was carried out using a linear gradient of NaCl from 10 to 250 mM in buffer C. Each colipase was eluted in a single peak corresponding to a NaCl concentration of ~100 mM (Fig. 1B). Pure colipases were lyophilized and conserved at −20°C.

Oligosaccharide content

The presence of glycan chains in the purified cofactors was checked by the anthrone-sulfuric acid method using glucose as a standard (36).

Alkylation of Cys residues

The alkylation of Cys residues of colipase was performed using the technique of Okazaki, Yamada, and Imoto (37). One milligram of cofactor in 1 ml of 10 mM Tris-HCl and 10 mM NaCl, pH 8.2, was denaturated in 375 μl of 8 M guanidine hydrochloride, 125 μl of 1 M Tris-HCl, 4 mM EDTA, pH 8.5, and 80 mM DTT during 30 min at 60°C. *S*-Pyridylethylation of cysteine residues of protein was performed by adding 4 μl of vinyl pyridine during 3 h at 25°C. The modified colipase was dialyzed against water for N-terminal sequencing.

Enzymatic cleavage

Purified ostrich pancreatic colipase was denaturated, reduced, and carboxymethylated as described previously and dialyzed against 50 mM Tris-HCl buffer, pH 8.5, without benzamidine. The cofactor solution (1 mg/ml) was digested at 37°C with the selected endopeptidase. The endopeptidase/cofactor varied from 1% to 10% (w/w). The samples (20 μl) were withdrawn from the incubation mixture at various times. The reaction was stopped by the addition of acetic acid (20% final concentration). For V8 digestion, some assays were performed with the denaturated cofactor solution (1 mg/ml) in 50 mM sodium acetate buffer, pH 5.2.

Purification of peptides

The resulting peptides from enzymatic cleavage were then separated by chromatography on a C-8 reverse-phase column (250 mm × 4.6 mm). Elution was carried out with a gradient from 0 to 80% solvent B for 30 min at a flow rate of 0.6 ml/min (solvent A, 0.01% trifluoroacetic acid in water; solvent B, acetonitrile).

Analytical methods

Analytical SDS-PAGE was performed by the method of Laemmli (38). The proteins were stained with either Coomassie Brilliant Blue or silver nitrate. Samples for sequencing were electroblotted according to Bergman and Jörnvall (39). Protein transfer was performed during 1 h at 1 mA/cm^2 at room temperature. The molecular masses of purified colipases were determined by matrix-assisted laser desorption ionization-time of flight mass spectrometry.

Amino acid sequencing

The N-terminal sequences of colipases and peptides were determined by automated Edman degradation, using an Applied Biosystems Procise 492 protein sequencer equipped with the 140 C HPLC system (40).

TABLE 1. Flow sheet of ostrich, turkey, and dromedary pancreatic colipase purification

Purification Step	Species	Total Activity (*units*)	Protein (*mg*)	Specific Activity (*U/mg*)	Activity Recovery (%)	Purification Factor
Heat and/or acidic treatment	Ostrich	200,000	1,091	183.33	100	1
	Turkey	400,000	2,046.04	195.5		
	Dromedary	22,000	2,000	11		
$(NH_4)_2SO_4$ precipitation (60%)	Ostrich	165,000	600	275	82.5	1.5
	Turkey	336,000	1,518.3	221.3	84	1.13
	Dromedary	16,500	1,270	13	75	1.18
Ethanol fractionation (50–90%)	Ostrich	144,000	500	288	72	1.57
	Turkey	200,000	685.92	291.6	50	1.49
	Dromedary	13,200	700	18.85	60	1.71
G-50 chromatography	Ostrich	108,000	230	469.56	54	2.56
	Turkey	166,800	353.8	471.4	42	2.41
	Dromedary	9,900	170	58.23	45	5.29
Mono S Sepharose	Ostrich	70,560	25	2,822.4	35.28	15.4
	Turkey	120,000	91.18	1,316	30	6.73
	Dromedary	7,425	40	185.62	33.75	16.87
Mono Q Sepharose	Ostrich	38,500	3.5	11,000	19.25	60
	Turkey	85,000	6.54	13,000	21	66.5
	Dromedary	3,638	0.36	10,000	16.5	909

For total activity, 1 colipase unit corresponds to the amount of the cofactor that increases bile salt-inhibited pancreatic lipase activity by 1 enzyme unit. Proteins were estimated by the Bradford method (35). The experiments were conducted three times.

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Three-dimensional structure prediction

The structure of the ostrich colipase was modeled using the three-dimensional coordinates of the open form of the HPL/colipase complex (Protein Data Base code 1lpa) as template using the structure-modeling program Deep View/Swiss-PDB Viewer version 3.7 (SP5) (http://www.expasy.org/spdbv/). The model was then subjected to energy minimization using the GROMOS96 software implementation of Swiss-Pdb Viewer http://iqc.ethz.ch/gromos). Five cycles of minimization were performed (5,000 steps of steepest descent, 5,000 steps of conjugate gradients, 5,000 steps of steepest descent, cutoff at 30 Å) using a harmonic constraint and a cutoff of 30 Å.

The geometry of the final models was stereochemically analyzed using the PROCHECK program (41). The accessible surfaces of the two models were calculated with Surface Racer, a computer program for fast calculation of accessible and molecular surface areas (42).

RESULTS AND DISCUSSION

Purification of colipases

Three colipases were purified from the pancreases of two birds (ostrich and turkey) and one mammal (dromedary) according to the procedure described in Materials and Methods. The results of SDS-PAGE analysis of the colipases eluted from the Mono Q column (Fig. 1C) showed that each of the three purified cofactors exhibited one band corresponding to a molecular mass of ~10 kDa. This was in agreement with molecular mass estimation using a gel filtration Superose 12 column by fast-protein liquid chromatography. On the other hand, molecular masses of 9,593.03, 9,138.12, and 9,245.23 Da were determined by mass spectrometry for ostrich, turkey, and dromedary pancreatic colipases, respectively (data not shown). Altogether, these results suggest that bird colipases are monomeric proteins, as was found for mammal colipases.

The presence of glycan chains in pure colipase molecules was checked. Our results showed that the three purified proteins are not glycosylated (data not shown). The purification flow given in **Table 1** shows that the specific activity of pure proteins reached 13,000, 11,000, and 10,000 U/mg for turkey, ostrich, and dromedary colipases, respectively, when olive oil emulsion was used as a substrate at pH 8.5 and 37°C and in the presence of 6 mM NaDC.

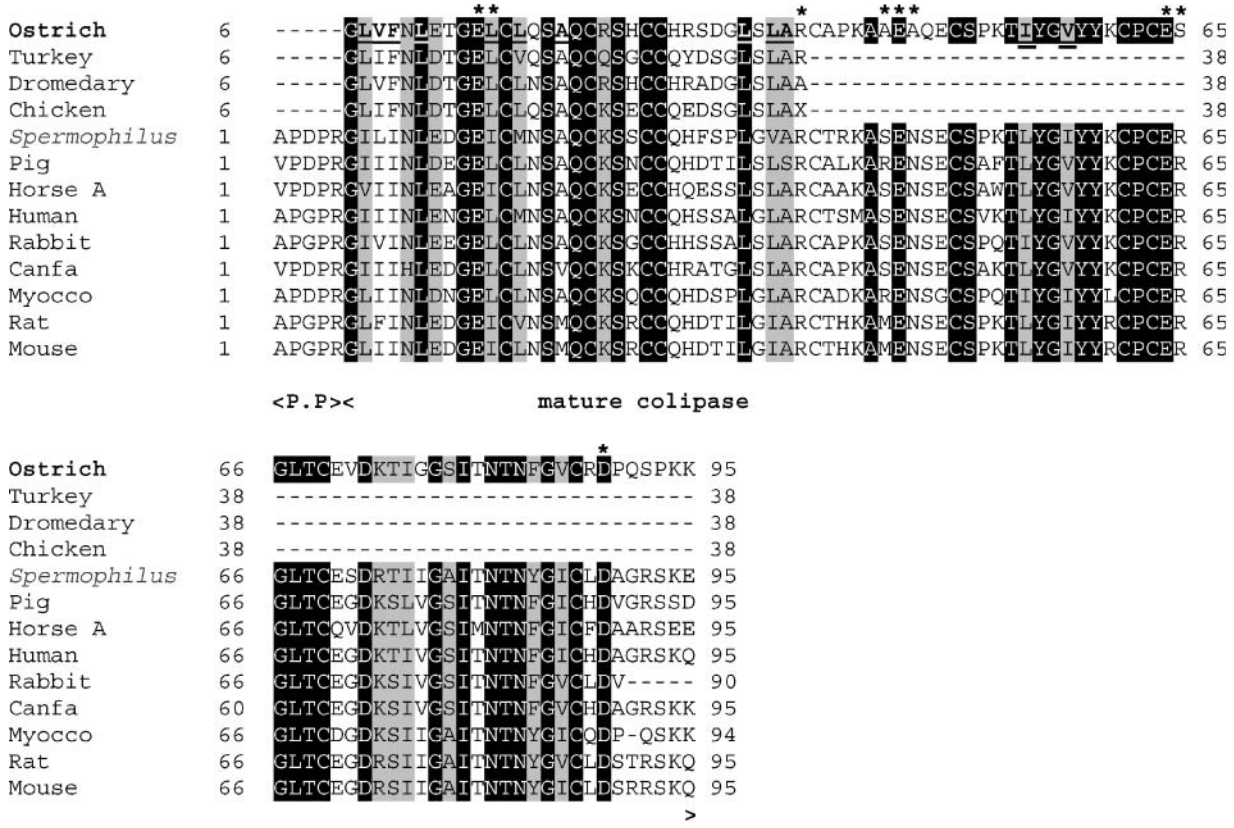

Fig. 2. Alignment of sequences of ostrich, turkey, and dromedary colipases (this study) with those of chicken (11), *Spermophilus tridecemlineatus* (GenBank database number AAH17897), pig (8, 14), horse A (43), human (44), rabbit (GenBank database number AAH17897), canfa (GenBank database number P19090), myocco (GenBank database number P42889), rat (GenBank database number P17084), and mouse (GenBank database number Q9CQC2) pancreatic colipases. Black boxes indicate the positions of identical amino acids, and gray boxes indicate the locations of similar residues. The stars at top indicate the positions of residues involved in lipase-colipase interactions. Hydrophobic residues involved in the interaction with the interface are shown in boldface and underlined. Dashes represent gaps introduced during the alignment process. P.P corresponds to the propeptide and refers to the N-terminal pentapeptide cleaved by trypsin. This alignment was generated by BioEdit.

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N-terminal analysis

Purified cofactors were alkylated as described in Materials and Methods and dialyzed against distilled water. The N-terminal sequencing of colipases allowed the unambiguous identification of 39 residues. The same sequences were obtained when the pure cofactors were transferred without alkylation on a polyvinylidene difluoride membrane. **Figure 2** shows the sequence alignment of ostrich, turkey, and dromedary pancreatic colipases (this study) together with those of chicken (11), *Spermophilus tridecemlineatus* (GenBank database number AAH17897) pig (8, 14), horse A (43), human (44), rabbit (GenBank database number AAH17897), canfa (GenBank database number P19090), myocco (GenBank database number P42889), rat (GenBank database number P17084), and mouse (GenBank database number Q9CQC2). N-terminal sequences of bird colipases exhibit a high degree of homology with N-terminal sequences of mammal colipases. As found in the human and chicken cofactors, the three purified protein sequences start at an N-terminal glycine residue and lack the N-terminal pentapeptide valine-proline-aspartate-proline-arginine found in the pig and horse procolipases. As was shown previously (15), this peptide may be lost by proteolytic cleavage at the arginine 5-glycine 6 bond. Although, in this study, cofactors were purified from delipidated tissue prepared in 0.2% Triton X-100 under similar conditions to those described by Julien et al. (7) and Erlanson-Albertsson (9) for pig and horse colipases, we cannot exclude the cleavage of the arginine 5-glycine 6 bond during purification.

In both pure bird and mammal colipases, we noted the presence of five half-cysteine residues at positions 17, 23, 27, 28, and 39. This corroborates previous findings in the cases of pig and horse pancreatic colipases (7, 9). It is noteworthy that all three species possess free carboxylic groups at positions 12 and 15. It has been well established that the residue glutamate 15 from colipase interacts with the lipase lid domain. The Glu15Arg colipase mutant has a 175-fold lower activity compared with the wild-type colipase, but it is as effective as the wild type in anchoring lipase to mixed emulsions (20). One can say that mammal and bird cofactors show extensive homology suggesting similar functional properties.

Effect of colipase on the rate of TC_4 hydrolysis by pancreatic lipase

It has been established that some mammal pancreatic lipases lack enzymatic activity when TC_4 is used as a substrate in the absence of bile salts and colipase. The high energy existing at the TC_4-water interface is responsible for their irreversible denaturation (45). In contrast to dromedary pancreatic lipase (**Fig. 3A**) and TPL (data not shown), which are able to efficiently hydrolyze triacylglycerols at high interfacial energy (TC_4) without any denaturation, OPL failed to catalyze the hydrolysis of pure TC_4 (Fig. 3B). When colipase was added to the lipolytic system, bird pancreatic lipases were protected against surface denaturation. Nevertheless, cofactors cannot fully protect enzymes from interfacial inactivation. The combined effect of colipase and bile salts is most effective at preventing this denaturation (Fig. 3). The curve representative of the hydrolysis rate of TC_4 remained linear for >30 min when bile salts and colipase were added together before the lipase in the lipolytic medium independent of the colipase origin (Fig. 3). Our findings confirm the idea that in the presence of bile salts, colipase helps to keep lipase at the interface at high energy and linearizes its kinetics.

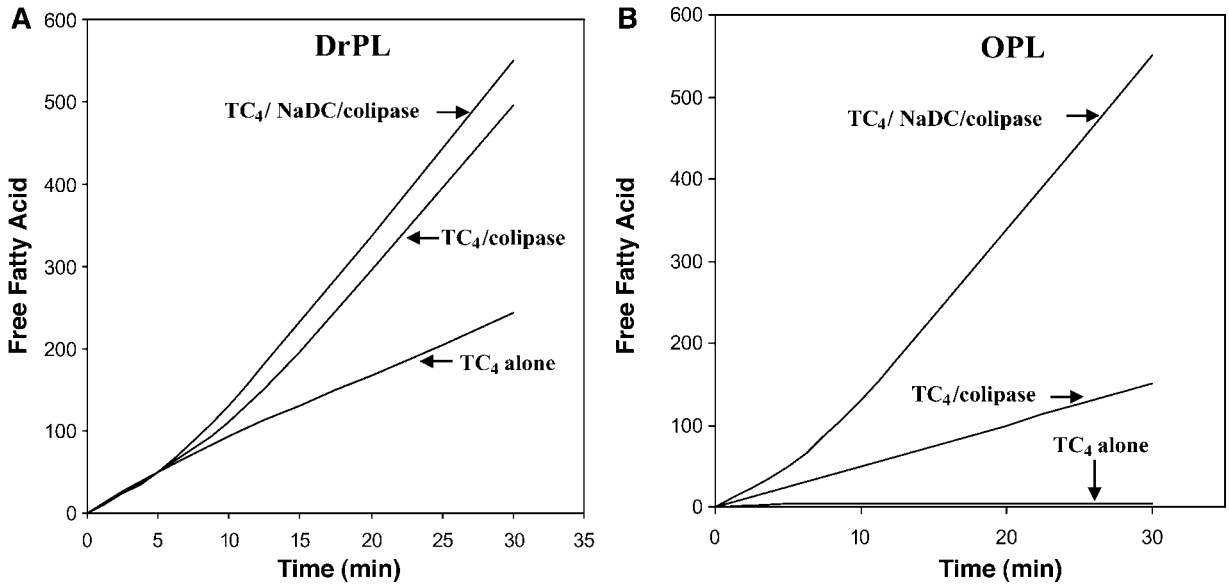

Fig. 3. Kinetics of the hydrolysis of tributyrin (TC_4) emulsion by dromedary pancreatic lipase (DrPL; 22 units) (A) and by ostrich pancreatic lipase (OPL; 22 units) (B). Lipolytic activity was followed at pH 8.5 and 37°C in the absence or presence of a molar excess of ostrich pancreatic colipase (5) and 4 sodium deoxycholate mM (NaDC).

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Activation of bile salts inhibits bird and mammal pancreatic lipases by colipases from various species

It is well established that bile salts are strong inhibitors of all pure pancreatic lipases independent of their origins (46, 47). At low concentration (below critical micellar concentration), bile salts stabilize lipase (48). With an increase in bile salt concentration, this effect can no longer be seen as the lipase desorbs from the substrate interface. This study investigated the activation of the bile salt-inhibited lipases from various species (dromedary, human, ostrich, turkey, and chicken) by bird and mammal pure colipases. The activity of pure pancreatic lipases against emulsified olive oil was determined at increasing concentrations of bile salts in the absence or presence of a molar excess of colipase (data not shown). Sodium taurodeoxycholate was shown to act as a strong inhibitor of pancreatic lipases. Inhibition was reversed after the addition of ostrich colipase to the assay system. No difference was observed in the ability of pure colipase from dromedary or turkey to activate bile salt-inhibited lipase from various species (data not shown).

To check the affinity between lipases and colipases isolated from different species, enzymatic activity was measured using emulsified olive oil as a substrate under standard conditions in the presence of 6 mM NaDC at increasing concentrations of purified colipase from ostrich, turkey, dromedary, and pig tissue (**Fig. 4**). Under our experimental conditions, the maximal lipase activity was obtained with a lipase-colipase molar ratio of ~1:2. All pure pancreatic lipases tested, independent of their origins, were found to be activated by colipase from bird and mammal as by their homologous colipase. However, dromedary colipase was a less effective activator of bird enzymes (Fig. 4A) than the bird cofactors (Fig. 4B). A similar result was obtained with an activated form of the pig cofactor (Fig. 4C). To determine the apparent dissociation constant (K_d) of

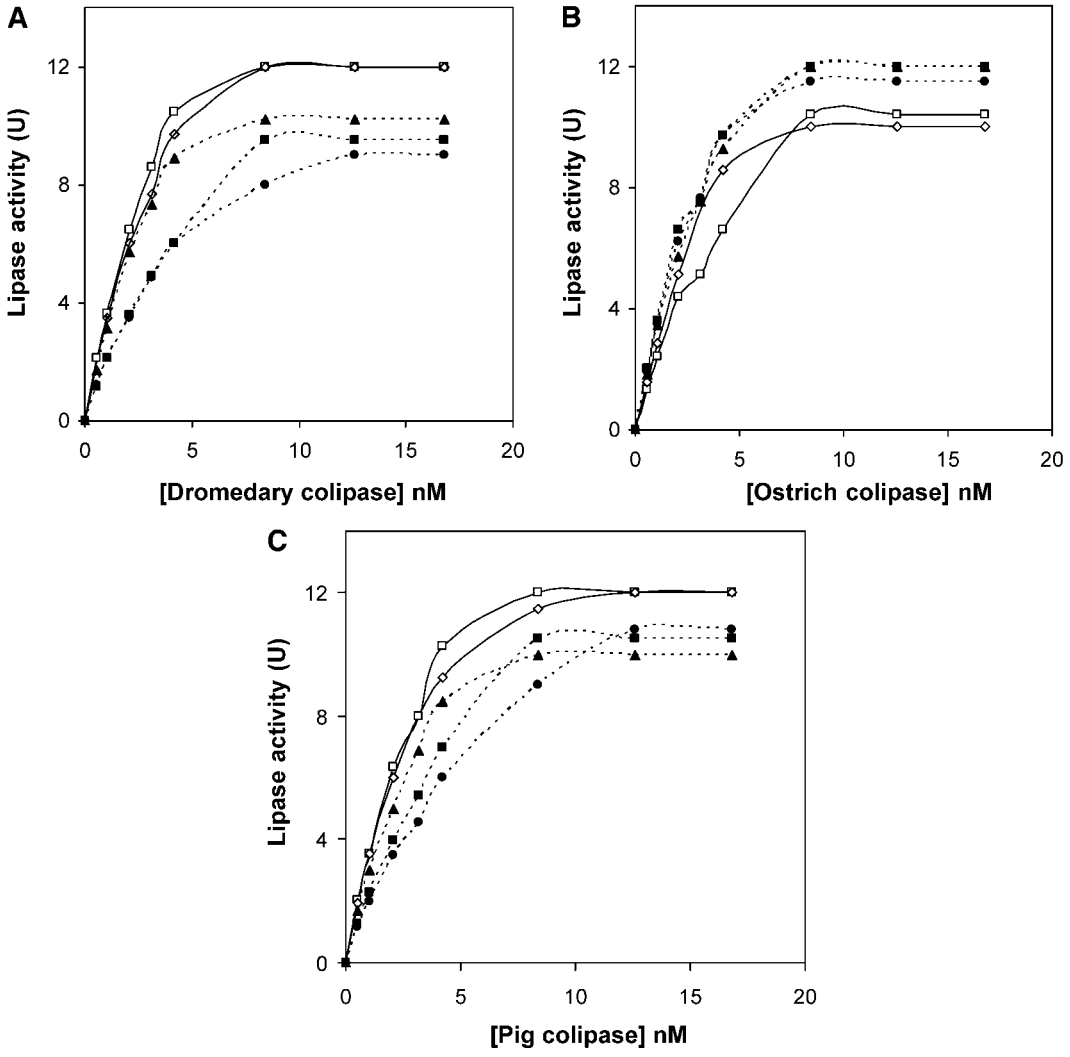

Fig. 4. Effects of varying amounts of dromedary (A), ostrich (B), and pig (C) pancreatic colipases on the activity of pure lipases from various species in the presence of bile salts at pH 8.5 and 37°C. The enzymatic activity of lipase (2.1 nM) was assayed in the presence of 6 mM NaDC with increasing amounts of colipase. Closed circles, chicken pancreatic lipase; open squares, dromedary pancreatic lipase; closed squares, OPL; closed triangles, turkey pancreatic lipase; open diamonds, recombinant human pancreatic lipase.

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TABLE 2. Kinetic parameters between colipases and lipases of various species deduced from Fig. 4

Colipases/Lipases	Apparent K_d (10^{-9} M)			Apparent V_{max} (μmol min^{-1} mg^{-1})			k_{cat} (S^{-1})			k_{cat}/K_d (S^{-1} M^{-1} 10^{-9})		
	Ostrich	Dromedary	Porcine	Ostrich	Dromedary	Porcine	Ostrich	Dromedary	Porcine	Ostrich	Dromedary	Porcine
Ostrich pancreatic lipase	1.2 ± 0.1	3.4 ± 0.1	3.7 ± 0.1	4,300 ± 150	3,399 ± 300	3,761 ± 125	3,225 ± 225	2,549 ± 50	2,821 ± 120	2,687 ± 414	749 ± 7	762 ± 29
Turkey pancreatic lipase	1.5 ± 0.15	2 ± 0.1	1.8 ± 0.07	4,300 ± 100	3,655 ± 235	3,581 ± 111	3,225 ± 115	2,741 ± 129	2,686 ± 256	2,150 ± 191	1,370 ± 128	1,492 ± 81
Chicken pancreatic lipase	1.4 ± 0.12	2.5 ± 0.07	3.4 ± 0.05	3,828 ± 170	2,990 ± 185	3,000 ± 100	3,067 ± 36	2,395 ± 105	2,403 ± 201	2,190 ± 167	958 ± 14	707 ± 70
Recombinant human pancreatic lipase	2.6 ± 0.06	1.5 ± 0.08	1.2 ± 0.1	3,333 ± 135	4,000 ± 200	3,832 ± 172	2,777 ± 206	3,333 ± 167	3,193 ± 100	1,068 ± 65	2,222 ± 218	2,661 ± 169
Dromedary pancreatic lipase	2.5 ± 0.07	1.2 ± 0.07	1.3 ± 0.05	3,116 ± 115	3,600 ± 264	3,600 ± 200	2,596 ± 100	3,000 ± 200	3,000 ± 100	1,038 ± 70	2,500 ± 182	2,307 ± 59

Apparent dissociation constant (K_d) values were deduced from the slope of the linear curves of the Scatchard plots.

the lipase-colipase complex, the linear curves corresponding to the Scatchard representation were plotted (data not shown) from the results in Fig. 4 as described by Rathelot et al. (24). The K_d values of different lipase-colipase complexes were determined from the slope of the linear curves of the Scatchard plots. The apparent V_{max} values were also deduced from Fig. 4. Then, the ratio representing the catalytic efficiency [k_{cat}(catalytic constant)/K_d] was calculated for each lipase (**Table 2**). From these values, it can be concluded that the ratio k_{cat}/K_d between colipase and lipase from the same class (varying from 2,150 to 2,687) is higher than that across two classes (varying from 707 to 1,492). This might reflect the higher ability of the bird enzymes to interact with colipase from the same species than with the mammal enzymes when olive oil was used as a substrate. This result suggests that this difference in the affinity toward bird pancreatic lipases might be related to the structural differences between the dromedary and bird colipases.

Previous works established the fact that cofactors from different mammal species are interchangeable and fully activate mammal pancreatic lipases (32, 49, 50). The same K_d value (1.1×10^{-9} M) was obtained by Rathelot et al. (24) when horse lipase was used, in presence of substrate, to form a complex with colipases from horse, pig, or ox. Our calculated K_d values for bird lipase-colipase complexes were found to be comparable to those reported for the mammal complexes by Rathelot et al. (24). Our findings provide evidence that bird pancreas contains cofactors with molecular properties very similar to those of porcine, bovine, and dromedary cofactors. Thus, bird and mammal pancreatic systems appear to be functionally similar.

Determination of internal peptide sequences of ostrich pancreatic colipase by proteolysis

To gain insights into the bird pancreatic cofactor primary structure, we performed a limited proteolysis experiment on ostrich pancreatic colipase to determine the complete sequence of the protein. The procedure used for this experiment included: the denaturation and alkylation of the native cofactor; the hydrolysis of the alkylated colipase by trypsin, chymotrypsin, thermolysin, or V8; the separation and isolation of the resulting peptides by chromatography on a C-8 reverse-phase column; and finally, sequencing of the proteolytic fragment by automated Edman degradation.

Native or denaturated cofactor (1 mg/ml) of 50 mM Tris-HCl, pH 8.5, was incubated with proteolytic enzymes in various conditions: temperatures of 25, 30, and 37°C; incubation times varying from 5 min to 72 h; and enzyme substrate ratios from 0.01 to 1 (w/w). Very long incubation times were required for the hydrolysis of the native cofactor by different proteases, whereas the denaturated colipase cleavage occurred after short incubation times. The best conditions for the proteolysis appeared to be 1 h at 37°C with a protease-denaturated cofactor ratio of 0.05, except for thermolysin, for which the incubation time was 2 h (data not shown).

The deduced polypeptide sequence comprises 90 amino acids with a calculated molecular mass of 9,675.56 Da and

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an isolectric point of 7.4. In comparison with the homologous sequence of the pancreatic cofactor described above, the primary structure of this bird pancreatic cofactor showed a high degree of similarity with all mammal pancreatic colipases (Fig. 2). The highest degree of homology (64%) was obtained with myocco colipase.

As mentioned above, porcine, myocco, and horse colipases start with the N-terminal pentapeptide valine/alanine-proline-aspartate/glycine-proline-arginine, whereas the N-terminal amino acid in ostrich is glycine followed by a hydrophobic region with the sequence isoleucine-valine-phenylalanine. The protein, which contains 10 half-cysteines, no methionines, and no tryptophans, has a high content of hydroxylated and acidic amino acid residues. The importance of some of the most prominent hydrophobic residues in the putative middle finger of colipase (residues 47–63) was tested by site-directed mutagenesis (51). One interesting part of this region, which is conserved in ostrich cofactor, contains three tyrosine residues situated close to one another: tyrosine 55-glycine-valine-tyrosine 58-tyrosine 59-lysine. Residues tyrosine 55 and tyrosine 59 were found to influence the catalytic activity of the lipase-colipase complex: their mutation to aspartate decreased the activity toward long-chain substrates without changing their ability to anchor the lipase to the lipid-water interface (51). Moreover, the comparison of these sequences allowed us to identify conserved hydrophobic amino acids involved in interactions with the lipidic substrate (boldfaced and underlined in Fig. 2): residues 7, 8, 9, 11, 16, 18, 21, 34, 36, 37, 54, and 57 in ostrich colipase. Despite this conservation, the involved hydrophobic lipid-interacting surface was lower in ostrich compared with that in pig. Given the fact that the hydrophobic surface of colipase is important in interacting with the substrate, this result might explain the higher activity of the mammal lipase-colipase complex compared with the mammal lipase-bird colipase complex. Other residues (stars in Fig. 2) involved in the interaction with lipase are also identified (residues 15, 16, 38, 43, 45, 46, 64, 65, and 89). In particular, residues 15, 16, 45, 64, and 89 are strictly conserved among all known colipases. Amino acid 39 displays the lowest conservation (serine for *Spermophilus*, horse, human, rabbit, and canfa colipases, arginine for pig and myocco, methionine for mouse and rat, and alanine for ostrich). Residues arginine 38 and arginine 65 are conserved in all colipases except dromedary (alanine 38) and ostrich (serine 65). These variations might account for the specificity of interaction of the lipase-colipase complex.

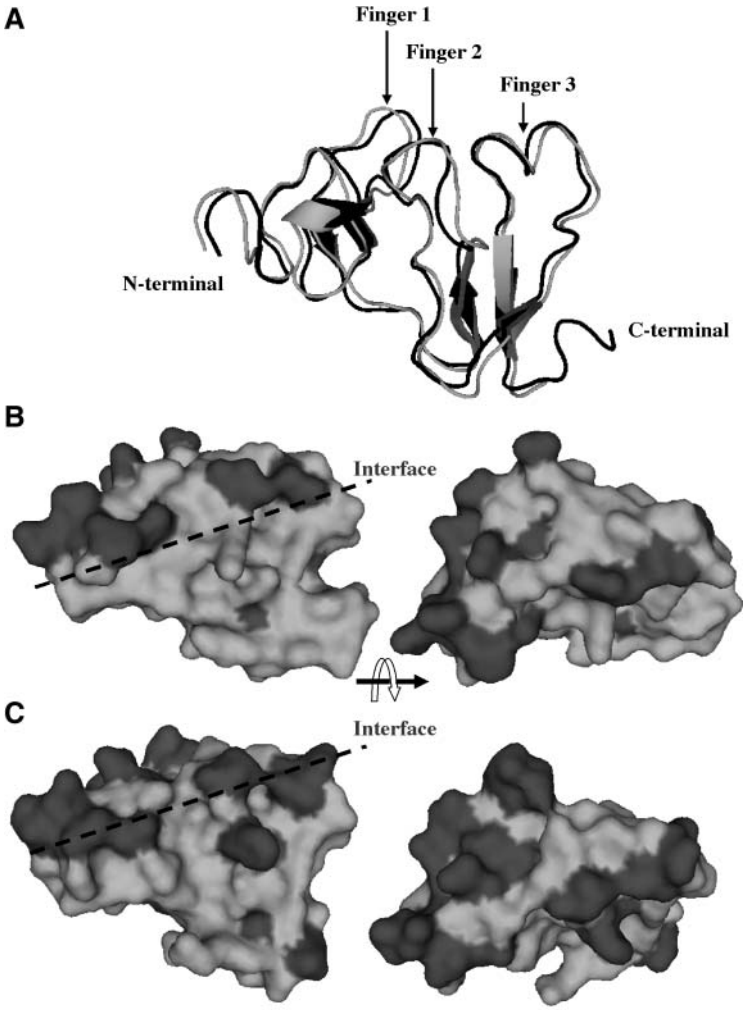

Fig. 5. A: Cartoon superposition of the procolipase structures of pig (in gray) and ostrich (in black). B, C: Surface GRASP representations using GRASP of an ostrich colipase model (B) and pig colipase (C). The hydrophobic amino acids are shown in dark gray. This figure was generated by PyMol.

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Three-dimensional structure models

The three-dimensional structure of the pig colipase from the HPL-colipase complex (Protein Data Base code 1lpa) was used as a starting template to build pig and ostrich colipases models using the Swiss-Pdb Viewer program. The resulting three-dimensional models were then subjected to energy minimization. The root mean squared deviation using carbon α only between colipases from pig and ostrich was 1.07 Å (85 carbon αs involved) (**Fig. 5A**). Ramachandran plot statistics of the pig and ostrich colipases were determined using PROCHECK and showed that ~97% of the residues were either in the most favored regions or in the additional allowed regions.

The total accessible surfaces of ostrich and pig colipases were 5,247 and 5,354 $Å^2$, respectively. The analysis of this surface showed that ostrich colipase exposes a less hydrophobic surface (1,662 vs. 2,180 $Å^2$) and a more positively charged surface (1,159 vs. 907 $Å^2$) compared with pig colipase.

Van Tilbeurgh et al. (52) have emphasized the overall amphipathic character of the procolipase molecule, whose most hydrophobic residues are located at the tips of the fingers, whereas hydrophilic residues are found at the opposite site and form the binding site for the C-terminal domain of the lipase. The colipase was found to interact also with the open lid of the lipase (52). Those authors reported that the interacting residues of the lid (valine 246, serine 243, and asparagine 240) and of the colipase (arginine 38, leucine 16, and glutamate 15) are strictly conserved in all known pancreatic lipase and colipase sequences.

Van Tilbeurgh et al. (52) also reported that the colipase peptide (residues 6–9) forms a hook exposing three successive isoleucine residues (7–9) in the same direction as the fingers, and the peptide cleavage shows that these isoleucines are important for the function of colipase. They concluded that this N-terminal region may be stabilized by interaction with the open flap. The alignment of the primary sequences of ostrich and pig colipases showed that these three isoleucines correspond to leucine, valine, and phenylalanine in ostrich colipase.

By analyzing the amino acids situated in the tips of the fingers, we noticed that in ostrich, the hydrophobic residues potentially involved in the lipid binding site differed from those in pig colipase. Indeed, in the former, we counted 12 hydrophobic amino acids making up the lipid binding region of the ostrich colipase (one isoleucine, six leucines, two valines, one phenylalanine, and two alanines) (Fig. 2), corresponding to a surface of 1,248 $Å^2$, whereas in the latter, we counted 14 hydrophobic amino acids (five isoleucines, six leucines, one alanine, and two valines) that represent a surface of 1,472 $Å^2$. This discrepancy may explain in part the fact that ostrich colipase is less effective at activating mammal pancreatic lipase (Fig. 5B, C). The fact that bird and mammal lipases are more activated by their own colipases can be attributable to a higher specificity of the colipase-lipase interaction.

In conclusion, despite a few differences, the structural features involved in interactions with the lipase and the substrate are conserved among bird and mammal colipases. The fact that colipase is a universal lipase cofactor might be explained by a conservation of the colipase-lipase interaction interface.

The authors thank Mr. A. Hajji from the Engineering School of Sfax for his help with English. The authors are very grateful to M. Saâdaoui (Faculté des Sciences de Sfax) for his technical assistance. This work received financial support from the Direction Générale de la Recherche Scientifique et Technologique granted to the Laboratoire de Biochimie et de Génie Enzymatique des Lipases.

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