Critical Role of Micelles in Pancreatic Lipase Activation Revealed by Small Angle Neutron Scattering*

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In the duodenum, pancreatic lipase (PL) develops its activity on triglycerides by binding to the bile-emulsified oil droplets in the presence of its protein cofactor pancreatic colipase (PC). The neutron crystal structure of a PC-PL-micelle complex (Hermoso, J., Pignol, D., Penel, S., Roth, M., Chapus, C., and Fontecilla-Camps, J. C. (1997) EMBO J. 16, 5531–5536) has suggested that the stabilization of the enzyme in its active conformation and its adsorption to the emulsified oil droplets are mediated by a preformed lipase-colipase-micelle complex. Here, we correlate the ability of different amphiphatic compounds to activate PL, with their association with PC-PL in solution. The method of small angle neutron scattering with D2O/H2O contrast variation was used to characterize a solution containing PC-PL complex and taurodeoxycholate micelles. The resulting radius of gyration (56 Å) and the match point of the solution indicate the formation of a ternary complex that is similar to the one observed in the neutron crystal structure. In addition, we show that either bile salts, lyso-phospholipids, or nonionic detergents that form micelles with radii of gyration ranging from 13 to 26 Å are able to bind to the PC-PL complex, whereas smaller micelles or nonmicellar compounds are not. This further supports the notion of a micelle size-dependent affinity process for lipase activation in vivo.

Pancreatic lipase (PL)† hydrolyzes triglycerides at the oil-water interface. PL comes into contact with pure triglycerides only during in vitro experiments. Under these conditions, the enzyme can be completely but reversibly inactivated by bile salts normally found in the duodenum (1). In vivo, the lipase activity on bile salt-emulsified interfaces is possible thanks to the presence of colipase (PC), a 10-kDa nonenzymatic protein found in the pancreatic juice (2, 3). The system lipase-colipase-bile-substrate involves several important interactions, the nature of which are not completely understood.

There is significant structural information concerning the protein components of this system. The x-ray structures of the uncomplexed human (4) and horse (5) pancreatic lipases revealed that the enzyme is divided into two domains. The N-terminal domain contains the active site covered by an amphiphilic flap that prevents the access of the substrate. This observation provides an explanation for the low activity of PL on monomeric substrates. The catalytic triad (Ser153-His264-Asp177) is similar to that found in other serine hydrolases. The noncatalytic C-terminal domain is responsible for colipase binding, as shown by the human lipase-porcine colipase structure (6). This structure showed that colipase is a flattened molecule that consists of a three-finger-shaped region held together by five disulfide bridges (7). The human-porcine complex that was crystallized in the presence of mixed bile salt phospholipid micelles revealed that the flap could move away from the position it occupies in the uncomplexed PL (8). The motion of the flap makes the active site accessible to the substrate and unmask a large hydrophobic region on the surface of the enzyme. This region is thought to be responsible for the binding of the enzyme to the oil droplet-water interface. The structure of the homospecific porcine lipase-colipase complex (9) was solved from crystals obtained in the presence of the nonionic detergent C8E4. Under these conditions, the enzyme also displays an open active site conformation. The activation of the enzyme was attributed, in this case, to the combined effect of pure detergent micelles and colipase in the crystallization medium. This was confirmed by PL inhibition in solution with the serine specific inhibitor diethyl p-nitrophenylphosphate (E600) (9).

Bile salt interactions with PC have been extensively studied in the past. Calorimetry (10), circular dichroism (11), affinity chromatography (12), spectrofluorometry (13, 14), fluorescence (15–17), ultracentrifugation (18), and small-angle neutron scattering studies (19) have been reported. These results suggest that PC does not bind a significant amount of bile salt monomers but interacts specifically with a preformed micelle. More recently, the neutron diffraction low resolution structure of a ternary lipase-colipase-micelle complex confirmed those results and showed for the first time the structural organization of the three partners of the system (20). In this activated complex, the disc-shaped micelle interacts with both PC and the distal tip of the C-terminal domain of PL. It indirectly helps to stabilize the PL open flap configuration. The opening of the flap requires the presence of both PC and micelles and is a reversible phenomenon because upon removal of micelles, PL adopts an inactive closed conformation (9). Based on these findings, it was proposed that in vivo, PL adsorption to the emulsified oil droplets is mediated by a preformed PC-PL-mixed micelle complex (20).
The aim of the work presented here was to better characterize the formation of the lipase-colipase-micelle ternary complex in solution and to establish its dependence as a function of the size of the micelles. We carried out a series of small angle neutron scattering (SANS) experiments on the PC-PL-bile salt system coupled to PL inhibition by E600 to investigate the ability of different lipids to activate the enzyme (E600 inhibition requires an exposed active site and thus is indicative of the opening of the flap). In addition, because the SANS method coupled to contrast variation is very well adapted to obtaining structural information on systems composed of two components of different scattering density (such as lipid-protein associations (21)), we characterized the formation of a PC-PL-taurodeoxycholate micelle complex in solution.

**EXPERIMENTAL PROCEDURES**

**E600 Inhibition**

PL and PC were purified from a delipidated acetonic pancreatic powder. PL was purified according to the method of Lombardo et al. (22). PC was purified according to Chapus et al. (23).

The inhibition experiments were performed in 50 mM sodium acetate buffer, pH 6.0, containing 0.1 mM NaCl. PL, in the presence of PC and different types of surfactants (bile salts, nonionic detergents, and phospholipids) was treated with 2 mM E600. 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 mM emulsified triacylbutyrylglycerol (tributyrin) in 1 mM Tris/HCl buffer containing 0.1 mM NaCl, 5 mM CaCl₂. Control experiments in which PC was omitted were also performed in order to check PL stability.

**SANS Experiments**

**SANS on Different Types of Micelles**

Bile salts (sodium taurodeoxycholate (NaTDC), sodium taurocholate (NaTC)), phospholipids (lecithin and lyssolecithin), and nonionic detergent samples (N,N-dimethyldodecylamine-N-oxide, β-ocetylglucosamide, tetra-ethylene glycolmonooctyl-ether (C₁₈E₄)) were prepared from commercial powders in 100% H₂O solvent, at pH 7.0 (100% H₂O solvent), at submicellar concentrations (between 2 and 10 mg/ml), without further purification.

SANS data were collected on the D11 instrument (24) at the Institut Laue-Langevin (Grenoble, France). The two-dimensional multidetector was placed at a distance of 2.5 m from the sample. Incident neutrons of wavelength 4 Å were used. Samples were contained in quartz cuvettes with a path length of 2 mm thermostatted at 20 °C. A buffer solution was used as the blank in the neutron scattering experiments.

**SANS on Lipase/Colipase in the Presence and Absence of Bile Salt Micelles**

Sample Preparation—PL and PC solutions were freshly prepared from the purified freeze-dried proteins in 10 mM Tris/HCl, pH 7.0, containing either 0, 28, 68, or 100% D₂O. The protein concentrations were measured by UV spectrophotometry at 280 nm (E₅₀₀) = 13.3 and 4 for PL and PC, respectively) and the monodispersity of the solutions was initially checked using dynamic light scattering. Solutions were mixed at pH 7.5 and 25 °C using 0.11M emulsified triacylbutyrylglycerol (tributyrin) in 1 mM Tris/HCl buffer containing 0.1 mM NaCl, 5 mM CaCl₂. Control experiments in which PC was omitted were also performed in order to check PL stability.

**SANS Data Processing—**

SANS experiments were carried out as a function of contrast. Extrapolated I(0) values as determined by the Guinier plots allowed the determination of the experimental match points from linear plots of I(0)/c versus D₂O concentration in the sample. This match point value is defined as the solvent extrapolated zero-angle scattering intensity, I(0).

In the case of PC/PL and PC/PLM solutions, the experiments were carried out as a function of contrast. Extrapolated I(0) values as determined by the Guinier plots allowed the determination of the experimental match points from linear plots of I(0)/c versus D₂O concentration in the sample. This match point value is defined as the solvent extrapolated zero-angle scattering intensity, I(0).

**RESULTS**

**E600 Inhibition—** As reported previously (9), PL was irreversibly inhibited by E600 in the presence of PC and micellar concentration of either NaTDC or nonionic detergents, such as β-ocetyl glucoside or tetra-ethylene glycol mono-octyl-ether. Under those conditions, PL displayed an open active conformation of the protein at the experimental match point of the complex, ρ₀ the scattering length density of the detergent at the experimental match point of the complex, and ρ₅ the scattering length density of the solvent (26).

The SANS results (the radius of gyration and the bile salt mass fraction in the complexes) were modeled using different molecular structures. The x-ray structure of the lipase-colipase complex (9) was used in the case of the PC/PL solution spectrum and the low resolution neutron diffraction PL-PC-C₈E₄ structure (20) was used in the case of the PC/PLM solution. In addition, different lipase/colipase/micelle associations were built using the graphic program O (27).

The SANS data were collected on the D11 instrument (24) at the Institut Laue-Langevin (Grenoble, France). The two-dimensional multidetector was placed at a distance of 2.5 m from the sample. Incident neutrons of wavelength 4 Å were used. Samples were contained in quartz cuvettes with a path length of 2 mm thermostatted at 20 °C. A buffer solution was used as the blank in the neutron scattering experiments.

The two-dimensional multidetector was placed at a distance of either 3 or 1.5 m from the sample. The wavelength was 10 Å. Samples were deposited in quartz cuvettes of either 2 or 1 mm path length, depending on the D₂O content of the sample, and thermostated at 20 °C. The D₂O content of samples and buffer were verified by neutron transmission measurements. Neutron scattering data were collected for about 20 min to obtain statistically significant spectra for samples in H₂O, whereas 10 min were sufficient for samples in D₂O.

**SANS Data Processing—** All spectra were background-subtracted and normalized to the incoherent scattering of a 1-mm-thick sample of water using the standard data reduction programs RNILS, XPOLLY, and RPOUT (25). The corrected scattering curves I(Q) versus Q were interpreted via the linear Guinier approximation for a globular particle, as follows,

\[ I(Q) = I(0) \exp \left( -R_g^2Q^2 \right) \]  

where \( Q = 4\pi \sin(2\theta /\lambda) \), \( 2\theta \) is the scattering angle, and \( \lambda \) is the neutron wavelength. Hence, a plot of ln[I(Q)] versus Q yields two independent parameters: the radius of gyration, \( R_g \), and the extrapolated zero-angle scattering intensity, I(0).

The aim of the work presented here was to better characterize the formation of the lipase-colipase-micelle ternary complex in solution and to establish its dependence as a function of the size of the micelles. We carried out a series of small angle neutron scattering (SANS) experiments on the PC-PL-bile salt system coupled to PL inhibition by E600 to investigate the ability of different lipids to activate the enzyme (E600 inhibition requires an exposed active site and thus is indicative of the opening of the flap). In addition, because the SANS method coupled to contrast variation is very well adapted to obtaining structural information on systems composed of two components of different scattering density (such as lipid-protein associations (21)), we characterized the formation of a PC-PL-taurodeoxycholate micelle complex in solution.
concentration. The same results were observed using either nonionic detergent with unchanged polar groups (n-alkyl-β-thioglucosides or n-alkylmaltosides) or n-alkyl dimethylamin-oxide (N,N-dimethyldodecylamine-N-oxide), which is zwitterionic at pH 6 (data not shown).

By contrast, no inhibition was observed in the presence of pure lecithins (Table I), which do not form micelles but rather vesicular structures or liquid crystals (28, 29). However, when lecithins formed mixed micelles with oleic acid, lyssolecithins, or NaTDC, a significant rate of PL inhibition was restored. The influence of both the NaTDC concentration and the lecithin-to-NaTDC molar ratio on the rate of inhibition by E600 was investigated (Table I). For a NaTDC concentration close to the critical micellar concentration (1 mM), the addition of lecithins at molar ratios ranging from 1 to 5 led to rapid and complete PL inhibition by E600. However, increasing the lecithin to NaTDC molar ratio to 6 resulted in a significant decrease of the rate of inhibition. This can be explained by a transition from micellar to vesicular structures induced by the high lecithin concentration relative to the NaTDC concentration.

It must also be pointed out that the efficiency of mixed micelles depends upon their lecithin content (Table I). This finding, which is quite obvious in the case of NaTDC/lecithin micelles, could be of physiological significance.

SANS on Micelles—In order to relate E600 inhibition to the structural organization of the different hydrophobic compounds, SANS experiments were carried out under the same conditions of pH and salt concentration. The slope of the linear Guinier plots gives the radius of gyration for the different micellar compounds: NaTDC, N,N-dimethyldodecylamine-N-oxide, dodecyl maltoside, and lyssolecithins. The Guinier plots at different D2O concentrations are presented in Fig. 1A. The slope of Rg (radius of gyration) at infinite contrast of 8.25 Å (data not shown) leads to a radius of gyration at infinite contrast of 10 Å, which is in good agreement with that calculated for the PC-PL complex using the x-ray structure coordinates (9) (Rg = 25 Å). The square root intensity at zero angle, normalized to unit concentration, thickness, and transmission, is a linear function of the scattering density of the solvent (Fig. 2). The point at which the fitted straight line crosses the abscissa indicates the match point of the solution. From the observed value of 41.6% as compared with the theoretical value of 43.5% (see “Experimental Procedures”), it can be deduced that 90% of exchangeable protons of the lipase-colipase complex were exchanged with the D2O content of the solvent.

Data were also collected on the free NaTDC micelle solutions at different D2O concentrations. Processing of these data (not shown) gives results similar to those previously described by Charles et al. (19), that is, an Rg of 13 Å and a match point of 20% D2O.

We applied the same procedure to the spectra obtained from the lipase-colipase complex solutions in the presence of NaTDC bile salt micelles. Data collected at different D2O concentrations were processed using the corresponding free NaTDC micelle spectra obtained from the dialyzed solutions as blanks. This allowed us to cancel out the signal coming from uncomplexed micelles. The Guinier plots at different D2O concentrations are presented in Fig. 1B. The resulting radius of gyration of 56 Å clearly shows that in the presence of micelles, a complex larger than one lipase/colipase is formed. The square root intensity at an angle of 0° is a linear function of D2O content (Fig. 2). The resulting match point is 38.6%. Because this value significantly differs from the one obtained for the protein alone (41.6%), we can conclude that bile salt micelles are bound to the lipase/colipase complex. Association of two lipase-colipase complexes to one micelle of detergent (assuming a molecular mass of 12,000 Da for the micelle (19)) would result in a theoretical...
match point of 39.2%, a value in good agreement with the experimental match point.

On the basis of this stoichiometry, different protein-micelle associations were modeled (Fig. 3) using the neutron crystal structure coordinates of the lipase-colipase-micelle complex. In the crystal structure, two lipase-colipase complexes bind to the same micelle, with their longest axes oriented in a parallel fashion (20). This type of association gives a radius of gyration of 55 Å, very close to the experimental value of 56 Å. All of the other models that we built did not fit the experimental results (Fig. 3). In our model, the volume of the cavity defined by the two lipase-colipase complexes and containing the bile salt micelle is around 13,700 Å³, a value close to the volume of a NaTDC micelle, which is 12,200 Å³.

In order to study the influence of the micelle size on the formation of the ternary complex, SANS experiments at a single contrast of 100% D₂O were also carried out on a PL-PC complex solution extensively dialyzed against a NaTDC micelle solution. The signal coming from uncomplexed micelles was

FIG. 1. Small angle neutron scattering curves plotted as Guinier profiles at different D₂O/H₂O contrasts for the lipase-colipase complex solution (A) and the lipase-colipase-NaTDC micelle complex solution (B). The D₂O percentage in the buffer is shown against each curve. C, Guinier plots at 100% D₂O were obtained from lipase-colipase solutions in either the presence or the absence of NaTDC micelles.

FIG. 2. Variation of zero angle scattering with solvent deuteration for the lipase-colipase solution in the absence (red lines) or the presence (blue line) of NaTDC micelles. The I(0) values, obtained from the scattering curves shown in Fig. 1, A and B, have been normalized to unit concentration, thickness, and transmission.

FIG. 3. Different putative lipase-colipase-micelle associations were built with the program O (27). For each model, the open flap lipase is depicted in blue, colipase in cyan, and the bile salt micelle as an orange circle. Models A and B are derived from the neutron crystal structure (20). In model C, the hydrophobic regions of the two activated lipase molecules are facing each other; in model D, they are stabilized by the tip of the colipase fingers region; and in model E, they directly interact with the bile salt micelle. The theoretical radius of gyration and match point percentages for each model are given. Only models A and B can explain the SANS experimental parameters (Rg = 55 Å; match point, 39.3%).
elminated as previously. The Guinier plot (Fig. 1C) is similar to the one obtained from a PC-PL solution in the absence of bile salt micelles and leads to an Rg value of 26 Å. This result, related to the lipase inhibition data, confirms that NaTC micelles are too small to fill in the PL-PC cavity, thus leading to an inefficient ternary complex.

**DISCUSSION**

Pancreatic lipase, colipase, and bile salts act synergistically in the lipid hydrolytic reactions of the upper small intestine. Besides the well-documented interaction between lipase and its protein cofactor (7), little structural information on their interactions with bile salts is available. The relevance of a presumed association between lipase, colipase, and a micelle during lipolysis has been highlighted by the resolution of the neutron crystal structure of a lipase-colipase-nonidet detergent micelle complex (20). This study has led to a model of the activation process that involves the unmasking of the active site of the enzyme in the aqueous phase through the binding of the micelle to the protein complex (20). Here, we have further studied the formation of the protein-micelle ternary complex by a series of experiments carried out in solution with natural surfactants. PL inhibition by the serine hydrolase-specific inhibitor E600 has been combined with small angle neutron scattering studies on pure micelle, protein, and protein micelle solutions.

In the absence of any water-lipid interface, we demonstrated the ability of a series of amphiphilic compounds (NaTDC, long chain fatty acid, nonionic detergents, and egg lysolecithins) to activate PL, although at slightly different rates. These compounds possess different chemical structures but form micelles in solution with radii of gyration that vary from 13 to 27 Å. The direct involvement of a micelle in PL activation through its binding to the protein complex in solution was shown by SANS experiments with $D_2O/H_2O$ contrast variation performed on PL-PC solutions and PL-PC-NaTDC solutions. In the presence of micelles, the radius of gyration of the observed complex and the match point of the solution are 56 Å and 38.6% $D_2O$, respectively. Those values are very close to the one calculated for a model built with a stoichiometry of 2 lipase/colipase molecules per micelle, which corresponds to the one observed in the neutron low resolution crystal structure (20). However, this stoichiometry is likely to result from the very high concentration of PL and PC used in these experiments. In *vivo*, a stoichiometry of 1 lipase/colipase molecule per micelle is expected, based on the physiological concentrations of the proteins.

The requirement of micellar structures for the opening of the PL flap is further illustrated by the results obtained with lecithins. Indeed, pure lecithins, which do not form micelles but rather liquid crystalline bilayers in solution, are unable to stabilize the enzyme active conformation, as shown by inhibition experiments. However, the PL inhibition by E600 is restored when NaTDC is added to lecithin solutions. Addition of bile salts to lecithin solutions has been shown to yield a spontaneous vesicle-to-micelle transition, leading to the coexistence of vesicles and worm-like micelles (30). The vesicle-to-micelle transition is also likely to occur when lecithin solutions are mixed with other micelle-forming compounds, such as long chain fatty acids or lysolecithins, because incubation of PC-PL with those mixtures leads to the opening of the flap. However, increasing the lecithin concentration beyond a critical value in these mixtures induced the reverse transition (micelles to vesicular structures), abolishing the PL activation process.

PL activation by a micelle size-dependent process is further supported by the results obtained in the case of NaTDC solutions. NaTC is poorly efficient in lipase activation (20). Although closely related to NaTDC, NaTC forms smaller aggregates (radius of 8 Å for NaTC, compared to 13 Å for NaTDC). SANS experiments performed on PC-PL NaTC solutions suggest that NaTC micelles are probably too small to maintain PL in its open activated conformation by forming a stable ternary complex.

As a result, the difference observed in the capability of the micelles to activate PL are related to their size (lower limit close to 13 Å) or form and to their chemical nature. It must be pointed out that mixed micelles are more efficient than pure micelles. This can be correlated with the observation that, *in vivo*, mainly mixed micelles enriched in lecithins are present in the duodenum.

Taken together, these results reinforce the hypothesis of a micellar activation of PL in the intestine (20). *In vitro*, in the absence of bile salt, PL can readily bind the triglyceride-water interface and fully display its activity. *In vivo*, the physiological triglyceride vesicles covered by dietary and biliary phospholipids, cholesterol, and bile salts are at equilibrium with smaller mixed aggregates of micellar size in the aqueous phase (32). Our results strongly suggest that those physiological mixed micelles have the suitable size to form a ternary complex with PC-PL in which the enzyme is maintained, in the aqueous phase, in its active conformation. This preformed complex behaves as an entity that determines lipase adsorption to the emulsified substrate.

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