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Kinetic and structural characterization of triacylglycerol lipases possessing phospholipase A_1 activity

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ABSTRACT

The pancreatic lipase gene family displays various substrate selectivities for triglycerides and phospholipids. The structural basis for this difference in substrate specificity has not been definitively established. Based on a kinetic comparative study between various pancreatic lipase family members, we showed here that porcine pancreatic lipase (PPL), which was so far classified as "classical lipase", was able to hydrolyze phosphatidylcholine (PC). Amino acid sequence alignments revealed that Val260 residue in PPL lid could be critical for the interaction with lipid substrate. Molecular dynamics was applied to investigate PC binding modes within the catalytic cavity of PPL and human pancreatic lipase (HPL), aiming to explain the difference of specificity of these enzymes towards phospholipids. Results showed that with HPL, the oxyanion hole was not able to accommodate the PC molecule, suggesting that no activity could be obtained. With PPL, the formation of a large pocket involving Val260 allowed the PC molecule to come near the catalytic residues, suggesting that it could be hydrolyzed. One more interesting finding is that human pancreatic lipase related protein 2 could hydrolyze phospholipids through its PLA_1 and PLA_2 activities. Overall, our study shed the light on new structural features of the phospholipase activity of pancreatic lipase family members.

1. Introduction

Triacylglycerol lipases (EC 3.1.1.3) are carboxylester hydrolases that catalyze the hydrolysis of long-chain acylglycerols at oil/water interfaces [1–3]. They are ubiquitous enzymes and have been found in most of the organisms belonging to the microbial, plant, and animal kingdoms. Most cells in the body hydrolyze triacyglycerols (TAGs) through similar pathways, using lipases, generally with a common purpose to provide fatty acids for energy demands [4,5]. Some of these lipases were assumed to be attractive targets for the treatment of dyslipidemias, viral infection and atherosclerosis [4,5].

Classical pancreatic lipases are the main enzymes involved in the digestion of dietary TAGs in the small intestine. Classical pancreatic lipases from human (HPL) [6,7], horse (HoPL) [8], and porcine (PPL) [9] have been characterized extensively at the biochemical and the structural levels. Their three dimensional (3D) structure is composed of two domains (N- and C-terminal domains). The large N-terminal domain belongs to the α/β-hydrolase fold [10] and contains the active site with a catalytic triad formed by Ser, Asp and His. The β-sandwich C-terminal domain is important for colipase binding, a specific lipase-anchoring protein, present in the exocrine secretion of pancreas, that facilitates its adsorption at bile salt-covered lipid/water interfaces [11–13]. Structure–function studies on various lipases have shed light on the interfacial recognition sites present in the molecular structure of these enzymes and the conformational changes occurring in the presence of lipids and amphiphiles [14–16]. In many lipases, access to the active site is controlled by a so-called lid formed by a surface loop. This lid was found to undergo a conformational change in the presence of lipase inhibitors, making the active site accessible to solvent in the 3D structures of several lipases [17–19].

The discovery of novel pancreatic lipase-related proteins (PLRPs) has increased the complexity of structure–function relationships within this family of enzymes. Pancreatic lipase-related proteins 1 and 2 (PLRP1 and PLRP2) belong to the pancreatic lipase gene family, and they share 65–68% amino acid identity with the classical pancreatic lipase [20]. Their structural components such as the catalytic triad (Ser, Asp, His) are highly conserved, and overall, the 3D structures obtained so far are superimposable [7,21,22]. A deletion within the lid domain was however observed in the PLRP2 from guinea pig

Abbreviations: [^{14}C]PAPC, 1-palmitoyl-2-[1-^{14}C]arachidonyl-*sn*-glycero-3-phosphocholine; PPCEH, porcine pancreatic cholesterol ester hydrolase; HoPL, horse pancreaticlipase; HPL, human pancreatic lipase; HPTLC, high performance thin-layer chromatography; PC, phosphatidylcholine; PLA_1, phospholipase A_1; PLA_2, phospholipase A_2; $PPPLA_2$, porcine pancreatic PLA_2; PPL, porcine pancreatic lipase; NaTDC, sodium taurodeoxycholate; TAG, triacylglycerol; HPLRP2, human pancreatic lipase related protein 2

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(GPLRP2), which is able to accommodate more hydrophilic substrates than classical pancreatic lipase, such as phospholipids and galactolipids with large polar heads [23]. Besides this previously determined structural difference, PLRPs also differ from classical pancreatic lipases by their biochemical and physiological properties. PLRP2 showed lipase, phospholipase and galactolipase activities, whereas classical pancreatic lipase only showed lipase activity [23], and PLRP1 is an inactive lipase against all known substrates [22]. PLRP2 is also produced at a high level in species lacking pancreatic phospholipase A_2 (PLA_2), and it was suggested that it might play a significant role in phospholipid digestion [24,25].

The occurrence of pancreatic lipases in the pancreas of various herbivorous, carnivorous, omnivorous and avian species was investigated [24]. Classical pancreatic lipases, PLRP1 and PLRP2 were detected in the pancreatic enzymatic equipment of humans and rats [24,26,27]. However, all attempts to identify porcine PLRP2 in both pure pancreatic juice and a protein fraction isolated from zymogen granules were unsuccessful [24]. Likewise, only classical pancreatic lipase was detected, too, in the case of bird (ostrich and turkey) pancreases [24,28,29].

The regioselectivity of PLRP2s towards phospholipids was not proved yet experimentally, even though amino acid sequence comparisons have shown that PLRP2 might act as a phospholipase A_1 (PLA_1) [30,31]. In contrast to other phospholipases, such as PLA_2, phospholipase C and phospholipase D isozymes, the physiological function(s) of PLA_1 enzymes remain largely unknown. PLA_1 enzymes may have specific roles in producing bioactive lysophospholipids, such as lysophosphatidylserine and lysophosphatidic acid [30]. All mammalian extracellular PLA_1 enzymes belong to the pancreatic lipase gene family which is conserved in a wide range of organisms from insects to mammals [23]. While some extracellular PLA_1s have a broad substrate specificity and hydrolyzing both phospholipids, TAGs and galactolipids, various other PLA_1s such as phosphatidylserine (PS)-specific PLA_1 (PS-PLA_1), membrane-associated phosphatidic acid (PA)-selective $PLA_1\alpha$ (mPA-$PLA_1\alpha$) and mPA-$PLA_1\beta$ show a strict substrate specificity and hydrolyze exclusively PS and PA, respectively [30].

The 3D structures of various lipases revealed that the lid domain covering the active site in the closed form is stabilized *via* van der Waals interactions formed between the lid itself and two other loops called β5 and β9 [7,13]. The lid domain, β5 and β9 loops were hypothesized to undergo a special reorganization in order to facilitate the entrance of the substrate in the active site.

The present study was thus undertaken in order to ascertain whether human PLRP2 (HPLRP2) does hydrolyze ester bonds of phospholipids at the *sn*-1 and/or the *sn*-2 position. Interestingly, a comparative kinetic study of various pancreatic lipases from different origins has revealed that PPL, besides its high TAG hydrolase activity, does possess PLA_1 activity. We used molecular dynamics calculations of lipase–phospholipid transition-state complexes to provide information about molecular interactions which are important for phospholipid breakdown by lipases. Our data suggest that avian pancreatic lipases from ostrich and turkey may act, too, as PLA_1 enzymes. It is therefore suggested that classical pancreatic lipase may fulfill in some cases new biological functions as a PLA_1 enzyme, compensating PLRP2 deficiency in the digestive tract.

2. Materials and methods

2.1. Reagents

Purified egg yolk L-α-phosphatidylcholine (PC), tributyrin, BSA and sodium taurodeoxycholate (NaTDC) were purchased from Sigma-Aldrich-Fluka Chemie (St-Quentin-Fallavier, France). High-performance thin-layer chromatography (HPTLC) plates pre-coated with silica gel 60 were from Merck (Fontenay Sous Bois, France). Molecular mass markers were from Amersham Biosciences (Uppsala, Sweden). 1-Palmitoyl-2-[1-^{14}C]arachidonyl-*sn*-glycero-3-phosphocholine ([^{14}C]PAPC) was from PerkinElmer Life Sciences (Waltham, Massachusetts). All other chemicals and solvents were of reagent or better quality and were obtained from local suppliers.

2.2. Proteins

Recombinant HPL was expressed and purified from insect cells as described by Thirstrup et al. [32]. Recombinant HPLRP2 was produced in the yeast *Pichia pastoris* and purified as described previously [25,33]. Horse pancreatic lipase (HoPL) was purified at the laboratory as described previously [34]. PPL and porcine colipase devoid of phospholipase contamination were purified using methods previously described [35,36]. Porcine pancreatic PLA_2 ($PPPLA_2$) and porcine pancreatic cholesterol ester hydrolase (PPCEH) were purchased from Sigma-Aldrich-Fluka Chemie.

2.3. Lipolytic activity measurements

The release of fatty acids was continuously assayed potentiometrically with a pH-stat apparatus (Metrohm 718 Stat Titrino, Zofingen, Switzerland) under mechanical stirring in a 15-mL reaction vessel at 37 °C, adding 0.1 N NaOH and using either tributyrin or egg yolk PC [37] emulsions as substrates. The pH was adjusted to fixed end point pH 8.0 value and the (phospho)lipase solutions were added at zero time after recording the background level for 5 min. When olive oil was used as substrate, gum arabic was used as an emulsifier as previously described [38].

Tributyrin or olive oil assay: 0.5 mL of tributyrin or 5 mL of olive oil prepared in gum arabic [38] was added and mechanically emulsified in 14.5 mL or 10 mL, respectively, of 2.5 mM Tris–HCl, 150 mM NaCl, 5 mM $CaCl_2$ and 4 mM NaTDC.

Egg PC assay: the assay was performed as previously described [37] with slight modifications. Four grams of egg yolk PC was homogenized by probe sonication in 100 mL of water and filtered through cheesecloth. Five milliliters of the above substrate was added to 9.5 mL of 20 mM NaTDC and 0.5 mL of 0.2 M $CaCl_2$ and mechanically emulsified.

HPL, PPL and HoPL activities were measured in the presence of porcine colipase at a colipase/lipase molar excess of 5. Under the above assay conditions, one international (phospho)lipase unit (U) corresponds to the release of 1 μmol of fatty acid released per minute. The specific activity was expressed in $U \cdot mg^{-1}$ of pure enzyme.

For determination of regioselectivity, the lipase was incubated (100 μL final volume) under continuous stirring for 15 min at room temperature in 25 mM Tris–HCl (pH 8.0), 8 mM $CaCl_2$, 0.4% (w/v) egg yolk PC, 6 mM sodium deoxycholate and [^{14}C]PAPC (3.6 kBq, 4.2 $GBq \cdot mmol^{-1}$). Lipids were extracted immediately after sampling according to Folch's procedure [39] and then separated by HPTLC on silica gel 60. The sample migration was performed with chloroform/methanol/water (65/35/5, v/v/v). The plate was dried and exposed overnight for Phosphor-Imager (PerkinElmer, Waltham, Massachusetts) analysis.

2.4. Protein quantitation and gel electrophoresis

Protein concentrations were determined routinely by using Bradford's procedure [40], with BSA as standard. Alternatively, protein concentrations were determined by amino acid analysis. The homogeneity of the various enzymes was routinely assessed by performing SDS-PAGE on 12% gels using Laemmli's procedure [41]. Electrophoresis in the presence of SDS was carried out on a Mini-Protean II dual vertical slab gel electrophoresis cell (Bio-Rad, Marnes-la-Coquette, France).

2.5. Structural alignment, modeling and docking

The sequence alignment was generated with Clustal Omega [42]. The 3D coordinates of open forms of HPL (1LPB) and PPL (1ETH) were