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Unexpected wide substrate specificity of *C. perfringens* α-toxin phospholipase C

Patricia Urbina [a], M. Isabel Collado [b], Alicia Alonso [c], Félix M. Goñi [c], Marietta Flores-Díaz [d,e], Alberto Alape-Girón [d,e,f], Jean-Marie Ruysschaert [a], Marc F. Lensink [g,*]

[a] Centre de Biologie Structurale et de Bioinformatique, Laboratoire de Structure et Fonction des Membranes Biologiques, Faculté des Sciences, Université Libre de Bruxelles, CP 206/2, B-1050 Brussels, Belgium
[b] SGIKER, Facultad de Ciencia y Tecnología, Universidad del País Vasco, 48940, Leioa, Spain
[c] Unidad de Biofísica (Centro Mixto CSIC-UPV/EHU), and Departamento de Bioquímica, Universidad del País Vasco, Aptdo. 644, 48080 Bilbao, Spain
[d] Instituto Clodomiro Picado, Facultad de Microbiología, Universidad de Costa Rica, Costa Rica
[e] Departamento de Bioquímica, Facultad de Medicina, Universidad de Costa Rica, Costa Rica
[f] Centro de Investigaciones en Estructuras Microscópicas (CIEMic), Universidad de Costa Rica, San José 2060, Costa Rica
[g] Biological Nanosystems, Interdisciplinary Research Institute, University of Sciences and Technology Lille1, USR3078 CNRS, 50 Avenue Halley, F-59658 Villeneuve d'Ascq, France

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ABSTRACT

Clostridium perfringens phospholipase C (CpPLC), also called α-toxin, is the main virulence factor for gas gangrene in humans. The lipase activity serves the bacterium to generate lipid signals in the host eukaryotic cell, and ultimately to degrade the host cell membranes. Several previous reports indicated that CpPLC was specific for phosphatidylcholine and sphingomyelin. Molecular docking studies described in this paper predict favorable interactions of the CpPLC active site with other phospholipids, e.g. phosphatidylethanolamine, phosphatidylinositol and, to a lesser extent, phosphatidylglycerol. On the basis of these predictions, we have performed experimental studies showing α-toxin to degrade all the phospholipids mentioned above. The molecular docking data also provide an explanation for the observed lower activity of CpPCL on sphingomyelin as compared to the glycerophospholipids.

1. Introduction

The protein α-toxin (CpPLC) is the key virulence determinant of *Clostridium perfringens* gas gangrene. α-Toxin is a zinc metallophospholipase C [1]. It belongs to a group of related bacterial phospholipases C (*Bacillus cereus* PC-PLC or BcPLC, *Clostridium bifermentans* PLC or CbPLC, *Listeria monocytogenes* PLC-B, and *Clostridium novyi* γ-toxin among others) which contain essential zinc ions and are reversibly inactivated by EDTA or o-phenanthroline [2–7]. From the structural point of view the crystal structure reveals a 370-residue, two-domain protein with the N-terminal domain composed of α-helices and the C-terminal domain consisting mainly of β-sheet [8]. These domains are joined by a flexible linker that favors interactions between the adjacent faces of the domains. Functionally, α-toxin may have at least two modes of action. At high concentrations it degrades eukaryotic cell membranes helping in the spread and growth of the bacterium. At low doses it causes limited phospholipid hydrolysis, which in turn activates DAG- and ceramide-mediated signal transduction pathways, leading to the uncontrolled production of several intracellular mediators [9].

Some of the bacterial phospholipases C (PLC) (sometimes referred to as PC-specific phospholipases C) are active toward PC and show a lower activity toward other phospholipids [2,10–12]. Activity of other phospholipases C (termed PI-specific phospholipases C) is optimal on

Abbreviations: Ala and A, alanine; Asp and D, aspartic acid; BcPLC, *Bacillus cereus* phospholipase C; BSA, bovine serum albumin; CbPLC, *Clostridium bifermentans* phospholipase C; $CDCl_3$-d, deuterated chloroform; Chol, cholesterol; CpPLC, *Clostridium perfringens* phospholipase C; C-terminal, carboxyl-terminal, DAG, diacylglycerol; DHPC, (3)-3,4-di-n-hexanoyloxybutyl-1-phosphocholine; DOPC, dioleoylphosphatidylcholine; DOPG, dioleoylphosphatidylglycerol; D5PC, dipentanoylphosphatidylcholine; D5PE, dipentanoylethanolamine; D5PG, dipentanoylglycerol; D5PI, dipentanoylinositol; D5PS, dipentanoylserine; EDTA-Cs, cesium ethylenediamine-tetra-acetate; EF hand, helix-loop-helix structural domain; Glu and E, glutamic acid; G, glycine; GTP, guanosine triphosphate; His and H, histidine; I, isoleucine; K, lysine; LGA, Lamarckian genetic algorithm; LUV, large unilamellar vesicle; MLV, multilamellar vesicle; N, asparagine; N-domain, amino-terminal domain; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; P-group, phosphate-group; Phe and F, phenilalanine; PI, phosphatidylinositol; PLC-B, *Listeria monocytogenes* phospholipase C; PlcH, *Pseudomonas aeruginosa* phospholipase C; $PlcHR_2$, *Pseudomonas aeruginosa* hetrodimeric complex composed of a phospholipase C and a chaperone protein; P, proline; PS, phosphatidylserine; Rac1, Ras-related C3 botulinum toxin substrate 1; RMSD, root mean square deviation; S, serine; SM, sphingomyelin; TIM barrel, triosephosphateisomerase barrel; Trp and W, tryptophane; Tyr and Y, tyrosine; Zn, zinc; 5SM, pentanoyl sphingomyelin; 5C, five carbon; ^{31}P-NMR, 31-phosphorus nuclear magnetic resonance

* Corresponding author. Tel.: +33 3 62 53 17 33; fax: +33 3 62 53 17 01.
E-mail address: marc.lensink@iri.univ-lille1.fr (M.F. Lensink).

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phosphatidylinositol (PI) [13] and some of the phospholipases C use sphingomyelin as a substrate [14].

Martin and coworkers provided some new insights into the molecular basis for substrate specificity in *B. cereus* PLC (BcPLC). They identified several residues responsible for substrate specificity using mutagenic, kinetic, and crystallographic experiments [15,16]. On the basis of site-directed mutagenesis of α-toxin and the structure–function relationship of BcPLC, it was confirmed that a highly conserved motif, consisting of three zinc atoms that coordinate residues including His, Glu, Asp and Trp, located in similar positions in several members of the bacterial PLC family, is essential for the catalytic activity [17]. Apart from Glu to Asp exchange, from BcPLC to CpPLC respectively, the residues involved in the active site are conserved [18]. The conserved motif in α-toxin is present in or near the active site cleft of the N-domain and is essential for the hydrolytic activity [8]. Despite the active domain of CpPLC showing 29% sequence identity with BcPLC, their hydrolytic activity has been reported to be quite different. While BcPLC is almost non-specific and hydrolyses phospholipids in the order of preference: phosphatidylcholine (PC) >phosphatidylethanolamine (PE) >phosphatidylserine (PS) [15,16], CpPLC is described to be specific for sphingomyelin (SM) and PC. According to these studies α-toxin can bind and disrupt artificial membranes (liposomes) composed of PC or SM but not of PS, PE or phosphatidylglycerol (PG) [4,19].

Molecular docking is an important tool for computer-aided drug design. This *in silico* technique can be used to calculate the three-dimensional structure of a protein–ligand complex starting from the individual structures of the constituent macromolecules. The crystal structure of CpPLC with a lipid ligand is not known, but the structure of BcPLC with a non-hydrolysable phospholipid has been deposited in the protein data bank (PDB Accession No. 1P6D). Moreover there are a number of important similarities with the non-specific BcPLC, namely the CpPLC overall fold similarity (160 ca. atoms can be aligned with a root-mean-square (r.m.s.) deviation of 1.49 Å), the 29% sequence identity they share, and the presence in both PLCs of the highly conserved motif mentioned above, located in a similar position in the bacterial PLC family and essential for the catalytic activity [17]. Thus BcPLC can be considered as a good template for docking experiments in CpPLC (Fig. 1). Our results show that, in addition to PC and SM, other phospholipids e.g. PE, PG and PI are also CpPCL ligands. Subsequent experimental results confirmed that these phospholipids are indeed CpPLC substrates.

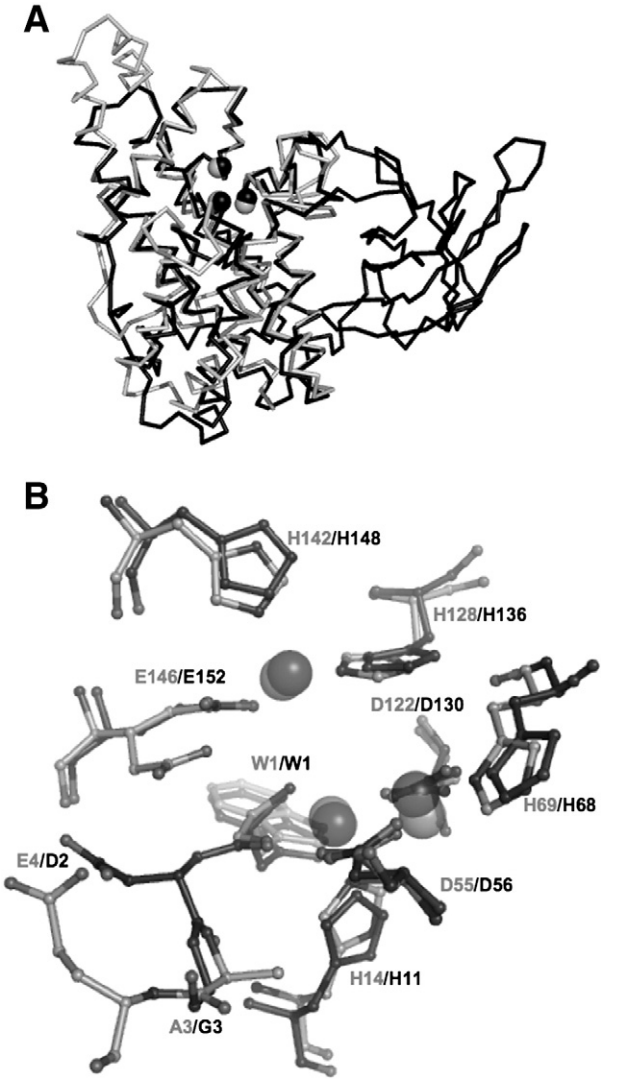

Fig. 1. Stereo picture of *Bacillus cereus* phosphatidylcholine-preferring phospholipase C (gray) and α-toxin (black). (A) Protein whole structure superposition. Spheres represent zinc ions in the active site. (B) Protein active site superposition. Solely aminoacid residues involved in the zinc coordination and phospholipid headgroup interaction were taken into account.

2. Materials and methods

2.1. Materials

Wild type recombinant *C. perfringens* α-toxin from strain 8–6 expressed in *Escherichia coli* was purified as described in Alape-Girón et al. (2000) [20]. Tris ultrapure (Tris) was purchased from Apollo Scientific, NaCl from Fluka and $CaCl_2$ and $ZnSO_4$ from Prolabo. Fatty acid-free bovine serum albumin (BSA) was from Sigma. Egg-yolk phosphatidylethanolamine (PE) was Grade 1 from Lipid Products, egg sphingomyelin (SM), cholesterol (Chol), dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylglycerol (DOPG), dioleoylphosphatidylserine (DOPS) and liver phosphatidylinositol (PI) were supplied by Avanti Polar Lipids (Alabaster, AL). The nature of the fatty acids from egg SM, egg PE or liver PI is the following: egg SM (86% 16:0, 6% 18:0, 3% 22:0, 3% 24:1, 2% unknown), liver PI (46% 18:0, 8% 18:1, 6% 18:2, 13% 20:3, 17% 20:4, 10% unknown) and egg PE (22% 16:0, 37.4% 18:0, 29.4% 18:1, 11.2% 18:2).

Chloroform, methanol, hexane, sulfuric acid, cesium ethylenediaminetetra-acetate (EDTA-Cs) and silicagel plates (20×20 cm), were purchased from Merck. Acetic acid and 37% hydrochloric acid were from Carlo Erba. Deuterated chloroform ($CDCl_3$-d) was purchased from Wilmad LabGlass.

2.2. Methods

2.2.1. Docking computations

The automated docking tool chosen for this purpose was AutoDock. This program allows automated docking of flexible ligands to proteins. It is very fast and provides high quality predictions of ligand conformations. The program is based on a Lamarckian genetic algorithm (LGA). Basically this program determines total interaction energies between random pairs of a ligand and selected portions of a protein to determine docking poses [21,22]. 3D structures used for docking experiments were:

- Template- BcPLC (PDB ID 1P6D).
- Target- CpPLC (PDB ID 1CA1). We removed all water molecules from the crystal structure, including those located in the binding pocket.
- Ligands- PC, PE, PG, PI, PS and SM. We used the natural isomer of the phospholipid (the R form) as the BcPLC-preferred isomer [23]. Since we were only interested in the protein-phospholipid headgroup interaction, 5-carbon acyl chains were used: D5PC, D5PE, D5PG, D5PI, D5PS and 5SM (pentanoyl sphingomyelin).