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Structural studies examining the substrate specificity profiles of PC-PLC$_{Bc}$ protein variants

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Abstract

The phosphatidylcholine preferring phospholipase C from *Bacillus cereus* (PC-PLC$_{Bc}$) catalyzes the hydrolysis of phospholipids in the following order of preference: phosphatidylcholine (PC) > phosphatidylethanolamine (PE) > phosphatidylserine (PS). In previous work, mutagenic, kinetic, and crystallographic experiments suggested that varying the amino acids at the 4th, 56th, and 66th positions had a significant influence upon the substrate specificity profile of PC-PLC$_{Bc}$. Here, we report the crystal structures of the native form of several PC-PLC$_{Bc}$ variants that exhibited altered substrate specificities for PC, PE, and PS at maximum resolutions of 1.90–2.05 Å. Comparing the structures of these variants to the structure of the wild-type enzyme reveals only minor differences with respect to the number and location of active site water molecules and the side chain conformations of residues at the 4th and 56th positions. These results suggest that subtle changes in steric and electronic properties in the substrate binding site of PC-PLC$_{Bc}$ are responsible for the significant changes in substrate selectivity.

Keywords: Phosphatidylcholine; Phospholipase C; *Bacillus cereus*; Substrate specificity; Crystallography

Phosphatidylcholine preferring phospholipase C from *Bacillus cereus* (PC-PLC$_{Bc}$)[1] is a monomeric 28.5 kDa phosphodiesterase that contains three zinc ions in its active site. PC-PLC$_{Bc}$ hydrolyzes phospholipids to give diacylglycerol and an alkyl phosphate (Fig. 1) in the following order of preference: phosphatidylcholine (PC) > phosphatidylethanolamine (PE) > phosphatidylserine (PS) [1]. While there is no significant sequence homology to known eukaryotic gene data bases, antibodies raised towards PC-PLC$_{Bc}$ cross-react with proteins in mammalian cells that hydrolyze PC at considerable rates [2]. Investigations focusing on PC-PLC$_{Bc}$ are thus of interest as they might lead to information regarding the general structure, function, mechanism, and substrate preference of the phospholipase C class of enzymes. Moreover, inasmuch as the headgroups of PC, PE, and PS each contain an ammonium moiety, structural studies of PC-PLC$_{Bc}$ and its variants with these substrates might provide some useful insights regarding the interactions of substituted ammonium ions with proteins [3–6]. Such information would be of general importance because ammonium groups are found in a number of biologically and medicinally active molecules, including histamine, catecholamines, acetylcholine, creatine, and various peptides [7,8].

Studies characterizing the substrate specificity profile of PC-PLC$_{Bc}$ have revealed that the spatial orientation of the glycerol side chains on the phosphatidylcholine moiety is an important contributing factor for binding and catalysis; substrates having the *S*-configuration at the *sn*-2 center are

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[1] *Abbreviations used:* PC-PLC$_{Bc}$, phosphatidylcholine preferring phospholipase C of *Bacillus cereus*; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; CMC, critical micelle concentration; C6PC, 1,2-dihexanoyl-*sn*-glycero-3-phosphatidylcholine; C6PE, 1,2-dihexanoyl-*sn*-glycero-3-phosphatidylethanolamine; C6PS, 1,2-dihexanoyl-*sn*-glycero-3-phospho-L-serine.

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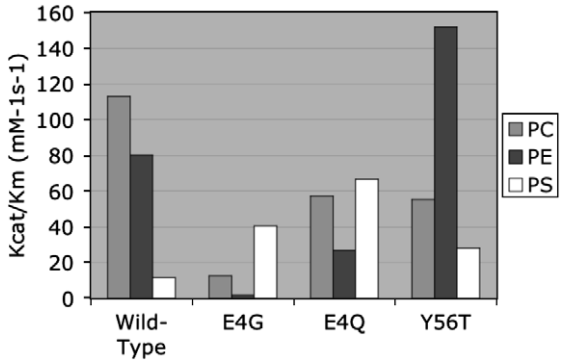

Fig. 1. Catalytic efficiency (k_{cat}/K_m) ($mM^{-1}\ s^{-1}$) of wild-type PC-PLC$_{Bc}$ and protein variants, E4G, E4Q, and Y56T for substrates C6PC, C6PE, and C6PC. The substrates were at concentrations well below their respective CMC [13,27].

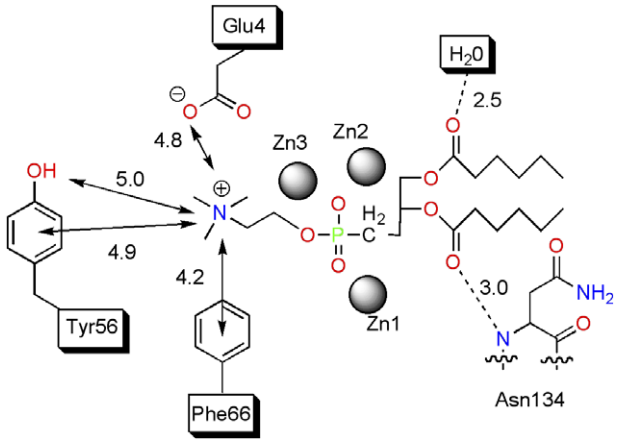

Fig. 2. Residues Glu4, Tyr56, and Phe66 are within close proximity to the choline head group in the structure of wild-type PC-PLC$_{Bc}$ complexed to a non-hydrolyzable PC analogue [15]. The distances (in Å) between the binding pocket residues and the PC-derived inhibitor are shown. For Tyr56 and Phe66, the distances displayed are from the centroids of their aromatic rings to the nitrogen atom the PC analogue.

hydrolyzed 40 times more slowly than the natural *R*-isomers [9]. The *sn*-2 ester linkage is also important as replacing it with an ether linkage yields a substrate that is processed 1000 times more slowly [10,11]. The length of the fatty acid side chains of the glycerol moiety has a modest effect on hydrolysis rates. Namely, PC with six carbon atoms in each of the acyl side chains is processed with slightly greater catalytic efficiency (k_{cat}/K_m) than PC with two–four carbon atoms [10,12]. More relevant to the present work are the different activities of PC-PLC$_{Bc}$ toward the three phospholipid substrates PC, PE and PS, which only differ in the structure of the head group. For example, PC-PLC$_{Bc}$ hydrolyzes PC, PE, and PS with specificity constants (k_{cat}/K_m) in the approximate ratio of 10:7:1, respectively [13].

Crystal structures of native wild-type PC-PLC$_{Bc}$ (PDB 1AH7) [14] and its complex with a non-hydrolyzable phosphatidylcholine analogue [15] have been reported. Active site residues Glu4, Tyr56, and Phe66 comprise the substrate head group binding pocket. An oxygen atom of the carboxyl group on the side chain of Glu4 is located 4.8 Å from the nitrogen atom on the choline head group, thus making a polar and/or ionic interaction that stabilizes the net positive charge on the phosphatidylcholine head group (Fig. 2). The interaction of Phe66 with the choline head group appears to be mediated by a cation-π interaction as the centroid of the aromatic ring of Phe66 and the ammonium group nitrogen atom are separated by 4.2 Å [8,16]. The proximity of the centroid of the aromatic ring and the phenolic hydroxyl group of Tyr56 from the nitrogen atom on the choline head group suggests this residue might help stabilize the positive charge on the inhibitor or substrate. However, this simple analysis does not explain the role of residue 56 as a determinant of specificity (*vide infra*). The phosphodiester group of the inhibitor binds all three zinc ions, whereas the diacylglycerol moiety binds partially within a hydrophobic cleft of the active site. The oxygen atom of the *sn*-2 carbonyl group forms a hydrogen bond with the backbone nitrogen atom of Asn134, whereas the *sn*-1 carbonyl oxygen atom is involved in a hydrogen bond network with surrounding water molecules.

In previous work by our laboratory, a combinatorial library of mutants was recently generated that contained random permutations of Glu4, Tyr56, and Phe66 to identify PC-PLC$_{Bc}$ variants with different specificity profiles for PC, PE, and PS [1,13,15]. These studies suggested replacing Glu4 with a neutral or positively charged amino acid increased the specificity constant for PS and substantially reduced the specificity constants toward PE and PC. Substitution of Tyr56 by a non-aromatic residue lowered the specificity constant for PC, but increased it for PE and PS. For example, the PC-PLC$_{Bc}$ variants E4Q, E4G, and Y56T exhibited a preference for either PS or PE instead of PC, the favored substrate of the wild-type enzyme. Although these variants had significantly different substrate specificity profiles from wild-type, they retained their catalytic activities and were found to have approximately two to 6-fold *higher* specificity constants for their preferred phospholipid substrates compared to that of wild-type (Fig. 1). In order to investigate the structural origin of these changes in substrate selectivity on a molecular level, the crystal structures of the E4Q, E4G, and Y56T variants of PC-PLC$_{Bc}$ were determined and compared with that of wild-type. These results are presented herein.

Materials and methods

Chemicals

Trypsin, trypsin inhibitor, and ampicillin were purchased from Sigma (St. Louis, MO). Amylose resin was purchased from New England Biolabs (Beverly, MA). *Q*-Sepharose was obtained from GE Healthcare Bio-Sciences (Little Chalfont, UK). Unless otherwise noted, all chemicals were purchased from Fisher Scientific (Hampton NH).