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# *Clostridium absonum* α-Toxin: New Insights into Clostridial Phospholipase C Substrate Binding and Specificity

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*Clostridium absonum* phospholipase C (Caa) is a 42.7 kDa protein, which shows 60% amino acid sequence identity with the *Clostridium perfringens* phospholipase C, or α-toxin (Cpa), and has been isolated from patients suffering from gas gangrene. We report the cloning and sequencing, purification, characterisation and crystal structure of the Caa enzyme. Caa had twice the phospholipid-hydrolysing (lecithinase) activity, 1.5 times the haemolytic activity and over seven times the activity towards phosphatidylcholine-based liposomes when compared with Cpa. However, the Caa enzyme had a lower activity than Cpa to the free (i.e. not in lipid bilayer) substrate *para*-nitrophenylphosphorylcholine, towards sphingomyelin-based liposomes and showed half the cytotoxicity. The lethal dose ($LD_{50}$) of Caa in mice was approximately twice that of Cpa. The crystal structure of Caa shows that the 72–93 residue loop is in a conformation different from those of previously determined open-form α-toxin structures. This conformational change suggests a role for W84 in membrane binding and a possible route of entry into the active site along a hydrophobic channel created by the re-arrangement of this loop. Overall, the properties of Caa are compatible with a role as a virulence-determinant in gas gangrene caused by *C. absonum*.

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*Keywords: Clostridium absonum* alpha-toxin; phospholipase C; characterisation; X-ray crystallography; gas gangrene

## Introduction

*Clostridium perfringens* alpha-toxin (Cpa) has been shown to be a key virulence factor required for the development of gas gangrene.[1] The toxin is a phospholipase C (PLC); hydrolysing the phosphodiester bonds that link the polar head groups to diacylglycerol and ceramide in phosphatidylcholine and sphingomyelin, respectively.[2] These are the main phospholipids found in mammalian cell membranes. As a zinc metallophospholipase C, Cpa requires the presence of zinc for its production by *C. perfringens*,[3] and for the activity of the enzyme towards both a synthetic substrate,[4] and phospholipids.[5] However, Cpa is distinguished from other non-toxic zinc metallophospholipase C (e.g. *Bacillus cereus* PC-PLC) by its haemolytic, cytotoxic and myotoxic activities.[6–8]

Elucidation of the crystal structure of Cpa showed that the protein exists as two domains;

G.C.C. & D.C.B. contributed equally to this work.

Abbreviations used: Caa, *Clostridium absonum* alpha-toxin; Cpa, *Clostridium perfringens* alpha-toxin; Cbp, *Clostridium bifermentans* phospholipase C; MTT, 3-(4,5-dimethylthiozolzyl)-2,5-diphenyltetrazolium bromide; PC-PLC, phosphatidylcholine-preferring phospholipase C; pNPPC, *para*-nitrophenylphosphorylcholine; PLC, phospholipase C; SM, sphingomyelin; SMase, sphingomyelinase.

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an α-helical amino-terminal (N) domain (residues 1–246) and a β-sheet-containing carboxy-terminal (C) domain (residues 256–370). The N-terminal domain has 30% sequence identity over the 250 residues of the N-terminal domain and structural similarities to the PC-PLC from *B. cereus*, suggesting a common evolutionary ancestry.[9,10] Site-directed mutagenesis of residues within the N-terminal domain revealed that W1, H11, H68, H126, H136, H148 and E152 were essential for catalytic activity.[11–15] These residues co-ordinate zinc atoms in the putative active site of the toxin. Whilst the N-domain has been shown to possess PLC activity, the C-domain of Cpa is thought to confer sphingomyelinase, haemolytic and lethal activities on α-toxin.[16,17] The C-terminal domain is thought to confer membrane-binding properties on Cpa, facilitating binding in a calcium-dependent manner to the polar head groups of phospholipids, whereupon they are hydrolysed by the catalytic N-domain.[10,13] Substitution of key calcium-binding residues (D269) and putative membrane interaction residues (Y331 and F334) within the C-domain resulted either in a complete loss or a significant reduction in the haemolytic, cytotoxic and myotoxic activity of Cpa, whilst the toxin still retained its PLC activity towards free substrates.[7,8,18]

Other Clostridial species, such as *Clostridium absonum* and *Clostridium bifermentans*, have been shown to produce PLCs.[19,20] The *C. bifermentans* PLC (Cbp) has an overall amino acid sequence identity of 51% with Cpa and is thought to possess a two-domain structure;[21,22] however, Cbp is not toxic and *C. bifermentans* is not associated directly with gas gangrene in man. Like *C. perfringens*, *C. absonum* has been identified as a causative agent of gas gangrene.[23] It is possible that the PLC from *C. absonum*, like the Cpa of *C. perfringens*, is a key virulence determinant. However, the *C. absonum* PLC has not been characterised previously. Here, we describe the isolation, cloning, biological and structural characterisation of the two-domain PLC from *C. absonum*. The activity of the *C. absonum* PLC (Caa) towards a range of substrates was compared with *C. bifermentans* PLC (Cbp) and *C. perfringens* α-toxin (Cpa). The crystal structure of *C. absonum* PLC gives new insights into the mechanism of membrane and substrate binding, and here we correlate structural and biochemical data resulting in a new model for substrate-PLC interactions.

## Results

### Isolation and analysis of the *caa* gene

To isolate the *caa* gene from *C. absonum* strain ATCC 27555 we first cloned *Hin*dIII-digested genomic DNA into plasmid pCR®II-TOPO and transformed the recombinant plasmids into *Escherichia coli* TOP10F′. Several colonies were identified that showed a lecithinase reaction, characterised by opacity around a colony on egg-yolk agar plates. A colony that showed lecithinase activity was selected and used for subsequent experiments. On agar plates containing egg-yolk emulsion, the lecithinase reaction around colonies of this transformant was partially neutralised with an anti-serum that completely neutralised the lecithinase reaction caused by Cpa (data not shown). This indicates that the *E. coli* transformant produces a protein that is immunologically cross-reactive with Cpa. A 2.7 kb DNA fragment was found to be inserted into the plasmid (pCAA1) isolated from the transformant. The nucleotide sequence revealed that this insert contained a 1197 bp open reading frame together with its putative promoter region. The open reading frame would encode a 398 amino acid residue protein with a molecular mass of approximately 45.7 kDa. The encoded protein (Caa) was predicted to possess an N-terminal 28 residue signal peptide. An alignment of the amino acid sequence of the mature form of Caa with the mature forms of other Clostridial PLCs revealed 60% sequence identity with Cpa protein and 54% sequence identity with Cbp (Figure 1). The sequence homology across the proposed catalytic N-terminal domain of the Clostridial PLC's was found to be 60% for Caa and Cpa as compared to 55% for Cbp and Cpa. Comparison of the C-terminal domains of the proteins revealed that Caa and Cpa shared 57% sequence identity (Cbp and Cpa sharing only 45% sequence identity).

Previous workers have shown that the Cpa from most strains of *C. perfringens* are closely related at the nucleotide and amino acid sequence level, and form a monophyletic group.[24] However, we have since shown that Cpa from an avian isolate (strain SWCP) of *C. perfringens* shows a greater degree of sequence divergence from other Cpas.[25] We aligned the deduced amino acid sequences of Cpa from the type strain of *C. perfringens* (strain NCTC8237) with Cpa from *C. perfringens* strain SWCP, the Cbp from *C. bifermentans*, and the Caa from *C. absonum*. This analysis revealed that Caa is clearly divergent from the PLCs produced by *C. perfringens* and *C. bifermentans*. When this analysis was carried out with the *in silico*-generated amino and C-terminal domains of these proteins separately, a similar relationship was observed.

### Characterisation of the Caa protein

Approximately 20 mg of Caa was purified from a five litre culture of the recombinant *E. coli* TOP10F′ carrying the pCR®II-TOPO-*caa* vector. After analysis by SDS-PAGE, the protein was estimated to have a molecular mass of approximately 43 kDa. Using electrospray mass spectrometry, the purified protein was found to have a molecular mass of 42,712 Da. The N-terminal sequencing of the first ten N-terminal residues of the purified protein indicated the absence of a signal peptide from the purified product.

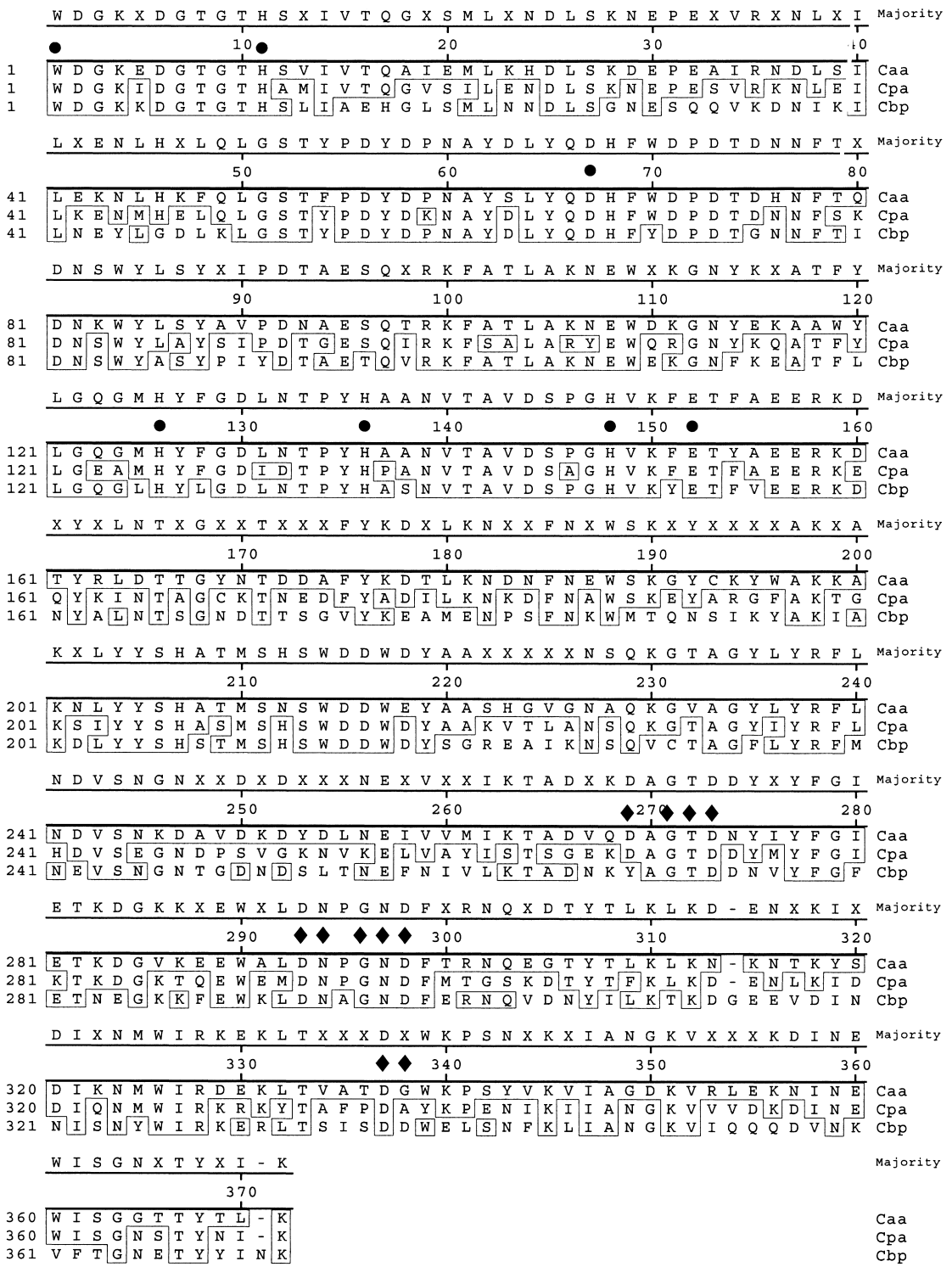


**Figure 1**. Alignment of the deduced amino acid sequences of *C. absonum* PLC (Caa), *C. perfringens* alpha-toxin (Cpa) and *C. bifermentans* PLC (Cbp). All the aligned proteins are in a mature form, lacking the signal peptide found at the N terminus of each enzyme. The residues involved in the coordination of zinc (●) and calcium (◆) ions in Cpa are labelled.

## Caa, Cpa and Cbp have different PLC, SMase and haemolytic activities

The relative activities of the Cpa, Cbp or Caa towards substrates that were either in solution, in micellular form, in synthetic lipid bilayers or present naturally in cells was determined. Measurement of activity using egg-yolk emulsion or *para*-nitrophenylphosphorylcholine (pNPPC) provided an indication of the activities of each enzyme towards substrates in micelles or in solution, respectively. The results showed that Caa

**Table 1.** Comparison of the activities of clostridial PLCs towards various substrates

| PLC | Specific activities (units $mg^{-1}$ $min^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | Egg yolk | ρNPPC | Haemolysis | PC liposomes | SM liposomes | Cytotoxicity BPAOEC |
| Caa | 2664 ± 551 | 69 ± 4 | 32747 ± 4987 | 1282 ± 51 | 34 ± 13 | 6.3 ± 0.35 |
| Cbp | 288 ± 40 | 4 ± 2 | 368 ± 80 | 60 ± 9 | 61 ± 16 | – |
| Cpa | 1296 ± 221 | 101 ± 8 | 21321 ± 3708 | 171 ± 11 | 171 ± 58 | 12.3 ± 0.85 |

Each data set is the mean of six determinations (± standard deviation). The results have been standardised into specific measurable units per milligram of PLC per minute (units $mg^{-1}$ $min^{-1}$). BPAOEC, bovine pulmonary aortal endothelial cells; –, undetectable.

had twice the specific activity of Cpa and over nine times the activity of Cbp towards egg-yolk lipoprotein (Table 1). The specific activity of Caa towards pNPPC was less than the activity of Cpa (~70%) but 17 times that of Cbp towards this substrate.

Using unilamellar liposomes containing an entrapped fluorophore, it was possible to examine the ability of the Caa, Cpa or Cbp enzymes to degrade synthetic membrane bilayers. When phosphatidyl choline (PC) liposomes were tested, Caa had over seven times the specific activity of Cpa and over 21 times the specific activity of Cbp (Table 1). In contrast, Caa had only 20% and 60% the activity of Cpa and Cbp, respectively, towards sphingomyelin (SM) liposomes.

## Comparison of Clostridial PLCs cytotoxicity and *in vivo* lethality

Cultured cell-line monolayers can be used as models of eukaryotic endothelia, allowing the effects of toxins on the cells of these tissues to be predicted *in vitro*. Bovine lung endothelial (BPAOEC) cell monolayers were examined for their susceptibility to Caa, Cbp or Cpa. Under the experimental conditions tested, Cbp was not cytotoxic. In contrast, both Caa and Cpa showed activity towards the cultured cells, with Cpa having twice the specific activity of Caa.

Groups of six female Balb\c mice were challenged intraperitoneally with either Caa or Cpa. The mice were monitored for 22 hours and culled if showing symptoms of discomfort or distress (i.e. hunched and/or ruffled). A 5 μg dose of Caa resulted in the loss of all subjects in a group (Table 2). Our results indicate that Caa has a median lethal dose of 1 μg. As a consequence of the toxicity of Caa, we have renamed *C. absonum* PLC, *C. absonum* α-toxin.

**Table 2.** Comparison of the lethality of *C. perfringens* alpha-toxin (Cpa) and *C. absonum* PLC (Caa)

| Toxin | Dose (μg) | Survival/group size |
|---|---|---|
| Cpa | 1.0 | 0/6 |
| | 0.1 | 6/6 |
| Caa | 5.0 | 0/6 |
| | 1.0 | 3/6 |
| | 0.1 | 6/6 |

Toxins were administered to Balb\c mice by the intraperitoneal route and monitored for 22 hours.

## Crystal structure of the Caa

The final model of the Caa contains four molecules in the asymmetric unit; each monomer consists of 3024 protein atoms (370 amino acid residues) three zinc atoms and two calcium atoms bound to the structure (Figure 2). The third calcium ion observed in the $Ca^{2+}$-bound structure of Cpa[26] is not present in the Caa structure. The asymmetric unit contains 234 well-ordered water molecules. The final $R_{cryst}$ and $R_{free}$ values were 16.9% and 23.3%, respectively. Whilst the majority of the residues had well defined main-chain and side-chain density, residues 245 to 254 (the inter-domain linker) and residues 331 to 335 (a flexible loop in the C-terminal domain) were not as well ordered, which is reflected in the magnitude of the *B*-factors of these residues. PROCHECK[27] indicated that the final model had 91.3% of residues in most favourable areas of the Ramachandran plot and only one residue (Thr335 C) is in a disallowed region; there are five residues within the "generously" allowed regions and these are primarily from the disordered loop regions mentioned above. The average realspace *R*-factor for the four molecules within the asymmetric unit is 7.8%; however, the average realspace *R*-factor for molecule D is 9.1%. This increase in disorder of molecule D may explain why the molecular replacement programs used failed to locate the fourth monomer.

The overall structure of Caa is similar to that of Cpa.[10] The Caa structure has an N-terminal domain consisting of an α-helical bundle and the C-terminal domain consists of an eight-stranded anti-parallel β-sandwich motif. This model of Caa is in the form previously identified as open in Cpa;[10] there were three zinc ions within the active site pocket (in contrast to the "closed" form of Cpa, which contained two zinc ions[28]), and the 138–145 residue loop (involved in co-ordination of the third zinc ion) is in a conformation identical with that described by Naylor *et al*. for open form Cpa.[10] The residues involved in co-ordinating the zinc ions within the active site (W1, H11, D56, H68, H126, D130, H136, H148 and E152 are conserved,[11–15] and in the crystal structure these

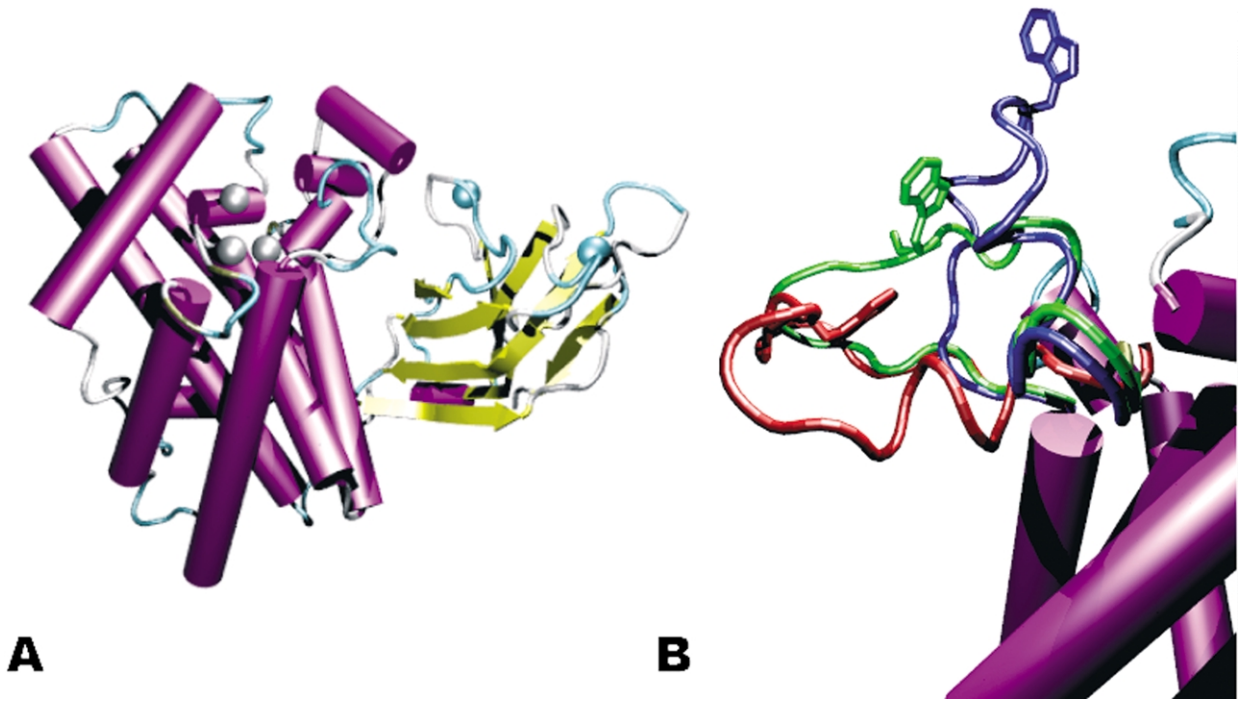


**Figure 2**. A, Caa monomer with the catalytically important zinc cluster in silver and the two calcium ions in cyan. B, The arrangement of the 72–93 loop as seen in the crystal structures of Caa (blue), the Cpa open (green) (PDB number 1CA1) and the closed (red) (PDB number 1QMD) forms. Residue W84, thought to be involved in membrane binding, is highlighted. This Figure was produced in VMD[56] and rendered in PovRay (Pov-Ray: http://www.povray.org).

residues appear to perform an identical function in the structure of Caa.

Three calcium-binding sites have been identified in the carboxyl-terminal domain of Cpa with bound $Ca^{2+}$.[26] All of the residues involved in binding these ions were conserved in Caa, except for residue A337, which is involved in binding Ca1. The glycine substitution in Caa at this position is unlikely to affect calcium ion binding, and indeed Ca1 was bound to the expected site in our structure. We were able to identify a bound calcium ion in the Ca2 position. However, in spite of the presence, and appropriate spatial organisation, of all of the residues required for Ca3 binding, we were unable to identify the third $Ca^{2+}$ in our structure. The absence of this calcium ion is thought to be a consequence of the absence of calcium ions in the crystallisation buffer.

### Conformation of the 72–93 residue loop

From the comparison of the crystal structures of the Caa and open form of Cpa, a prominent conformational difference was found in the loop encompassing residues 72–93. Previous Cpa structures showed that this loop changed conformation between open and closed forms.[28] The 72–93 residue loop in the Caa structure is in a conformation hitherto unseen in Cpa (Figure 3). This loop now points out into a region identified as the membrane-binding surface; this conformational change brings W84 (conserved in Caa, Cpa and Cbp) into a position where it is likely to play a role in membrane binding. The conformation of the 72–93 residue loop is less restrained by crystal packing when compared to previous α-toxin structures (see later). The conformation of this loop is identical in all four molecules of Caa within the asymmetric unit, and these residues have an average *B*-factor 26.3 Å$^2$. The 72–93 residue loop in the Caa structure is more ordered than previously published α-toxin structures. In the open structure,[10] the 72–93 residue loops have average *B*-factors of 50.6 Å$^2$ and the loop is constrained by tight packing of residues 80–86 with a symmetry-related molecule. This crystallographic dimer is clearly not biologically relevant as the active sites are occluded in both molecules. The 72–93 residue loop within the SWCP strain α-toxin structure[25] is more similar to the Caa structure and has an average *B*-factor within the loop of 40.6 Å$^2$. The 72–93 residue loop of Caa contains the short $3_{10}$ helix (residues 83–88) found previously in the SWCP α-toxin structure, but held in a different conformation.

## Discussion

This is the first reported isolation, characterisation and analysis of a gene from *C. absonum*, a Gram-positive pathogen that has been associated with gas gangrene in humans. The amino acid sequence alignments and a phylogenetic analysis

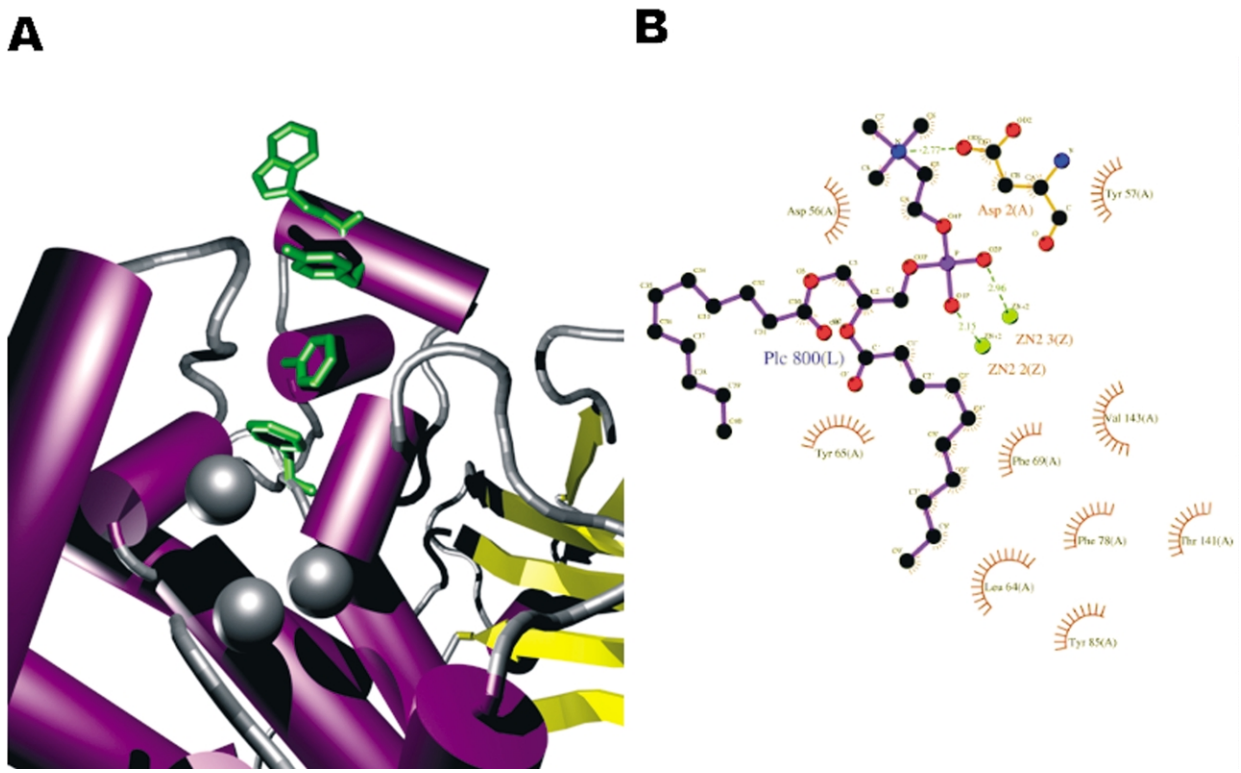


**Figure 3**. A, The Caa active site with the hydrophobic "slide" leading from the membrane surface to the active site shown, and B, a LIGPLOT diagram showing the residues interacting with the phospholipid substrate.[57]

suggest that the *C. absonum* PLC appears to be distinct from the other Clostridial PLCs.

Caa was shown to have lower sphingomyelinase activity when compared to Cpa and Cbp, but increased lecithinase activity compared to both Cpa and Cbp. The haemolytic activity of Caa was higher than that of either Cbp or Cpa. In the case of other bacterial PLCs, such as those produced by *Pseudomonas aeruginosa*[29] or *B. cereus*,[30] haemolytic activity has been attributed to the ability of the enzyme to hydrolyse both phosphatidylcholine and sphingomyelin, the main components of the outer leaflet of eukaryotic cells. Our results with Caa support the suggestion that both lecithinase and sphingomyelinase activity are required for haemolytic activity.

In addition to hydrolysing both phosphatidylcholine and sphingomyelin, it is thought that the haemolytic Clostridial PLCs possess features that allow them to interact with membrane packed phospholipids. For example, the weakly haemolytic Cbp has lecithinase and sphingomyelinase activities but hydrolyses membrane packed phospholipids poorly.[22] In contrast, the activity of Cbp towards pNPPC, a substrate that is water-soluble and lacks fatty acyl chains, is close to that of Cpa.[22] Caa showed a relatively low activity towards pNPPC but high activities towards phosphatidyl choline (PC) liposome bilayers or the micellular egg-yolk lipoprotein. These findings indicate that Caa has a preference for PC substrates in a membrane packed form.

The crystal structure of Caa, in a comparison with the known crystal structures of Cpa[10,26,28] has allowed us to explore the molecular basis for the differences in activity of these two PLCs. For Cpa, two forms of the protein have been described in which the active site is either accessible to substrate (the open form)[10] or occluded (the closed form) by two loops (60–90 and 130–150).[28] The structure and position of loop 130–150 in Caa mirrors that observed for the "open" form Cpa structure.[10] However, in Caa the loop corresponding to residues 60–90 in Cpa was in a conformation not seen previously. This loop is located at the proposed membrane-interfacing surface of the protein.[10] In Caa, the loop projects from the membrane-binding surface, exposing residue W84 (Figure 2B). In contrast, this loop projects towards the C-terminal domain of the Cpa open form structure.[10] The consequence of this in Caa is the formation of a hydrophobic "slide" (Figure 3A) that is lined by aromatic residues F69, F78 and Y85, and that extends from residue W84 at the putative membrane-binding surface into the active site of the enzyme. These aromatic residues are conserved in Cpa, Caa and Cbp; however, in the SWCP strain structure (PDB code 1KH0), the conformation of the 72–93 residue loop removes F78 from the hydrophobic slide, whilst W84 is displaced in the open form structure (PDB code 1CA1). This structural motif could stabilise the hydrophobic tail groups of bound membrane phospholipids in the active site of the enzyme.

The formation of this hydrophobic channel allows us to infer a possible method of phospholipid entry into the active site from the cell membrane (Figure 3B). We proposed previously that the primary site of membrane attachment is *via* $Ca^{2+}$ bound to the C-terminal domain.[26] This brings the N-terminal domain of the toxin into contact with the membrane and the 72–93 residue loop is able to insert partially into the membrane, using W84 as a "hook". The phospholipid is then drawn into the active site by the zinc ions *via* the hydrophobic channel described above. We have, at present, not found any evidence of a second crystal form of the Caa structure similar to that observed by Eaton *et al*.[28] and which corresponds to the closed form of the enzyme.

The enzymatic mode of action of the α-toxin family of phospholipases C is assumed to be broadly similar to that of *B. cereus* PLC. Martin and co-workers have characterised extensively the enzymatic properties of this non-toxic PLC,[31–34] which bears significant sequence identity to the N-terminal (catalytic) domain of α-toxin like proteins. Martin and co-workers postulate that a water molecule associated with D55 (D56 in the Cpa and Caa) is responsible for the nucleophilic attack on the scissile phosphorus–oxygen bond. Whilst there is a water molecule associated with D56 in Caa, it is not in the position observed in *B. cereus* PLC.[33] A higher-resolution structure with a substrate (analogue) bound would be required for more in-depth enzymatic studies of the α-toxin family of PLCs.

Using the substrate bound *B. cereus* PLC structure,[31] we have modelled a phosphatidylcholine molecule into the active site of Caa (Figure 3B) The C′ fatty acyl tail is positioned within the hydrophobic slide outlined earlier, whilst the C″ fatty acyl tail interacts with Y57 and Y65. The model suggests that the stabilisation of these hydrophobic tails whilst the substrate is within the active site is important for the efficiency and potency of the toxin.

In comparison with Cpa, one of the most significant features of Caa is the increased ability to recognise membrane-packed substrates containing PC and reduced ability to recognise membrane-packed substrates containing SM. The head groups of PC and SM are identical and our structural analysis indicates that the active sites of Caa and Cpa are similar. However, it is known that increasing the proportion of sphingomyelin relative to phosphatidylcholine in lipid vesicles increases the phase transition temperature and the overall rigidity of a bilayer,[35] and existing evidence points to differences in the way PC and SM pack in membrane bilayers. Therefore we believe that the differences in activity towards these two substrates reflect differences in the interfacial recognition regions of Caa and Cpa.

The C-terminal domain of Cpa appears to play a key role in the recognition of membrane-packed phospholipids.[10,13,17] Membrane binding is thought to occur partly as a consequence of calcium-mediated interactions between the $Ca^{2+}$ bound in the C-terminal domain of Cpa and the polar head groups of phospholipids.[10,17,26] With the exception of A337, all of the residues in Cpa that play a role in calcium-binding are conserved in Caa. Therefore, we do not believe the differences in activity of Caa and Cpa are due to differences in calcium-mediated recognition of membrane packed phospholipids. A number of other residues in the C-terminal domain of Cpa have been shown to play a role in haemolytic, cytotoxic and myotoxic properties. For example, the residues Y275, Y307 and K330 have been linked to these activities,[7,8] and are conserved in Cpa and Caa.

There are three regions of structural difference between Caa and Cpa that might explain the differences in activities of these proteins (i.e. the loop 72–93, the hydrophobic slide and the loop 330–335). In Cpa, the region corresponding to loop 72–93 is believed to become inserted into the membrane bilayer, and this loop is more prominent in Caa. In comparison with PC bilayers, the increased rigidity of SM bilayers could be an obstacle to the insertion of this loop into the membrane. A number of rapid turnover phospholipases including cytosolic phospholipase $A_2$ and phosphoinositide 3′-hydroxykinase have been shown to processively hydrolyse many phospholipid molecules following a single membrane-binding event (the so-called Scooting mode).[36] Were this to be the case for Caa, then following insertion into the bilayer, Caa may actually be more effective at channelling phospholipids into the active site because of the hydrophobic slide, which is not present in previous Cpa structures. The hydrophobicity of the 330–335 residue loop in Cpa is thought to be important for membrane binding.[10] This loop includes residues Y331 and F334, which are thought to play a role in sphingomyelinase (SMase), haemolytic, and cytotoxic activity towards lung fibroblasts in Cpa; these residues are not conserved in Cbp. Indeed in Cpa, the substitution of Y331 for leucine, or the substitution of F334 with isoleucine (residues that are found naturally in Cbp) resulted in a significant reduction in cytotoxic, haemolytic and SMase activities.[18] However, in Caa these substitutions are found naturally at these positions, with Y331 replaced with leucine and F334 replaced with alanine. Clearly these findings suggest that the effects of these substitutions in Cpa are context-dependent. In this respect, it may be significant that in Cbp there are substitutions in five of the six residues in the 330–335 loop. The significance of other substitutions in the C-terminal domains of Cpa, Cbp and Caa for the properties of these proteins are less easy to reconcile.

Overall, our findings indicate clearly that the *C. absonum* α-toxin has properties that implicate this enzyme as a virulence determinant in gas gangrene caused by this pathogen. The availability of the crystal structure of this protein should aid

ongoing work to design inhibitors that modulate the activity of both the *C. perfringens* and the *C. absonum* α-toxins. Equally important, this work has provided new insight into the mechanisms by which related proteins interact differently with the various substrates.

## Materials and Methods

### Materials

Unless otherwise stated, all chemicals and enzymes were obtained from Sigma-Aldrich (Poole, UK) or from Roche Molecular Biochemicals (Lewes, UK).

### Cloning and expression of the PLC genes

Recombinant *E. coli* clones containing the plasmids pKSα3 and pCBPLC3, used for expression of the *C. perfringens* NCTC8237 or *C. bifermentans* ATCC638 phospholipases C respectively, were cultured as described.[6,22] DNA isolated from *C. absonum* strain ATCC 27555[19] was used for isolation of the *caa* gene. *C. absonum* was grown in GAM medium (Nissui Pharmaceutical Co. Ltd, Tokyo, Japan) in anaerobic conditions at 37 °C. The DNA was extracted from the cultured cells as described.[37] A shotgun method was used for cloning the *caa* gene; the DNA was digested with a variety of restriction endonucleases. Each pool of 2–10 kb DNA fragments was ligated into pCR®II-TOPO vector (Invitrogen Ltd, Paisley, UK) and transformed into *E. coli* TOP10F′ (Invitrogen Ltd). The transformants were screened for phospholipase activity on 10% (v/v) egg-yolk 2 × YT agar plates. Expression of the *caa* gene was controlled by the gene promoter and under the culture conditions used for the production of Cbp and Cpa.

### Nucleotide sequencing

Nucleotide sequencing was carried out using an ABI Prism 310 Genetic Analyser (Applied Biosystems, Warrington, UK) with the BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems).

The deduced amino acid sequences of Clostridial PLCs from *C. perfringens* strain NCTC8237 (Cpa), *C. perfringens* strain SWCP, *C. bifermentans* strain ATCC638 (Cbp) and *C. absonum* strain ATCC 27555 (Caa) were aligned using ClustalW (version 1.75) and the FAST algorithm.

### Purification of Clostridial PLC

The *C. absonum* PLC (Caa), *C. bifermentans* PLC (Cbp) or *C. perfringens* α-toxin (Cpa) were purified from recombinant *E. coli* containing the cloned genes.[6] The proteins were purified using the method as described;[8] which involves two stages of ion-exchange chromatography, a Q-Sepharose column (Amersham-Biosciences Ltd, Little Chalfont, UK), followed by a MonoQ column (Amersham-Biosciences). Size-exclusion chromatography using a HiLoad 16/60 Superdex 200 gel-filtration column (Amersham-Biosciences) was used as a final purification step. The bicinchoninic acid (BCA) protein assay method (Pierce & Warriner Ltd, Chester, UK) and the Bradford method (Bio-Rad Ltd, Hemel Hempstead, Herts, UK) were used for estimation of protein concentration. The mean of both assay methods was taken as the protein concentration. The concentration of each toxin was adjusted according to their percentage purity, which was estimated prior to the activity assays.

### Lecithinase neutralisation test

A lecithinase neutralisation test was performed using *C. perfringens* type A anti-toxin (formerly available from Wellcome Research). The PLC activity towards egg-yolk lipoprotein of Caa was measured in the presence of anti-toxin using the method as described.[38]

### Characterisation of Caa

The first ten residues of the N terminus of purified Caa were determined using the Edman degradation method.[39] The N-terminal sequencing was performed by M-Scan Ltd (Ascot, Berks, UK) using an Applied Biosystems (ABI) 477A automatic protein sequencer. Mass spectroscopy was carried out by M-Scan Ltd using the Sciex API 150 EX single quadrupole mass spectrometer.

### Measurement of phospholipase C and haemolytic activities

Phospholipase C activity was measured using a microtitre assay with egg-yolk phospholipids, phosphatidylcholine liposomes, sphingomyelin liposomes, or *para*-nitrophenylphosphorylcholine (pNPPC) as substrates. Sigmoidal activity curves were generated for each PLC in each assay by the serial dilution of the enzymes in assay buffer across a 96-well microtitre plate followed by the addition of substrate. This allowed the concentration of each enzyme that produced no measurable substrate hydrolysis (0%) or complete hydrolysis (100%) to be established after incubation at 37 °C for one hour. The endpoint for each assay was determined as the reciprocal of the dilution of enzyme that resulted in the turnover of 50% of the substrate involved, i.e. where the rate of hydrolysis is linear with enzyme concentration. This specific unit value allowed the standardisation of assays into specific units of activity per milligram of toxin per minute (unit $mg^{-1}\,min^{-1}$). The egg-yolk assay used the supernatant (diluted 1:10 (v/v) in saline) from egg-yolk emulsion (Oxoid Ltd, Basingstoke, UK), with changes in the turbidity caused by PLC activity measured at 492 nm. A 50% activity endpoint in egg-yolk units related to the toxin dilution that achieved an $A_{492\,nm}$ half that found for the highest concentration of PLC that achieved complete hydrolysis of egg-yolk with the absorbance of the negative control (egg-yolk and buffer; 20 mM 2,2-dimethylglutaric acid, pH7.2) removed prior to calculation. Carboxyfluorescein (CF; 20 mM in $dH_2O$) containing phosphatidylcholine (PC) and sphingomyelin (SM) liposomes were prepared using the procedure reported by Nagahama *et al.*,[40] with the lipids in 1:1 molar ratio with cholesterol. An additional probe sonication step was included after drying and hydration of the liposomes. After incubation, the activity of a PLC was quantified by measuring the amount of released CF from disrupted liposomes using Fluoroskan Ascent fluorimeter (excitation wavelength of 485 nm and emission at 520 nm; ThermoLife Sciences). The activity of each enzyme was expressed in lysis units $mg^{-1}\,min^{-1}$ and as the reciprocal of the dilution of toxin

that caused 50% of entrapped CF to be released from liposomes relative to the controls. Triton X-100 was used as a positive (100% release) control and borate-buffered saline (BBS) pH 7.6 as a negative (0% release) control. The cleavage of pNPPC (40 mM in $dH_2O$) by toxin (in 20 mM 2,2-DMG pH7.2) results in the release of *p*-nitrophenol, which can be measured at 414 nm.[4] The concentration of toxin that resulted in an $A_{414\,nm}$ half that achieved by the highest concentrations of each PLC (i.e. 100% substrate hydrolysis) was taken as the reaction (50%) endpoint represented as hydrolytic units $mg^{-1}\,min^{-1}$. Haemolytic activity was measured in haemolytic units $mg^{-1}\,min^{-1}$ using a microtitre tray assay with 5% (v/v) in BBS (pH 7.6) washed mouse red blood cells and the 50% haemolysis endpoint was determined as described.[6] The results of each assay for each toxin are the mean of six determinations.

### Cytotoxicity of Clostridial PLC

Cytotoxicity assays with bovine lung endothelial cell monolayers were performed. Bovine lung endothelial cells (ECACC ref no. 86123102) were cultured in Dulbecco's modified Eagle's medium containing 2 mM glutamine, 15% (v/v) foetal calf serum (FCS) and 0.5% (v/v) penicillin–streptomycin, and at 37 °C with 5% (v/v) $CO_2$. A seeding density of 30,000 cells $cm^{-2}$ (100 μl/well) was used in 96-well microtitre plates. A confluent monolayer of cells was established (after about three days incubation), and the medium removed. Toxins were diluted serially (1:1, v/v) in FCS-free medium across a 96-well plate in hexuplicate wells. Control wells (six per plate) received FCS-free medium only. Cytotoxic activity was determined on separate plates, in triplicate.

Cultured cells were incubated with each toxin for 23 hours at 37 °C before 3-(4,5-dimethylthiozolzyl)-2,5-diphenyltetrazolium bromide (MTT; 20 μl at 2 mg/ml in FCS-free medium) was added to each well. After incubation for one hour at 37 °C, the fluid content in the wells was removed. Dimethyl sulphoxide (100 μl/well) was added to solubilise the MTT formazan derivative, before each plate was read at 540 nm in a spectrophotometer. The $A_{540\,nm}$ readings of 0.8 were selected as representing 50% toxin activity (or 50% cell death) for the cell density used in the assays. The activity of each enzyme was calculated (cytotoxic units $mg^{-1}\,min^{-1}$) and an average taken of the activity values generated for replicate plates involving the same enzyme.

### Lethality in mice

The comparison of the lethality of Caa and Cpa involved five groups of six Balb\c female mice (~12 months old). Both proteins were administered in PBS (pH 7.2) by intraperitoneal injections at 0.1 μg or 1 μg of Cpa, or 0.1 μg, 1 μg or 5 μg of Caa per mouse. The mice were monitored for 22 hours.

### Crystallisation

Small (~40 μm) flat plate crystals of the Caa were obtained using the hanging-drop, vapour–diffusion method. Each drop contained 1 μl of protein (20 mg/ml in 20 mM Tris-HCl, pH 8.0) and 1 μl of the well buffer. The crystals grew in the presence of a well solution containing 100 mM sodium citrate buffer (pH 5.6), 30% (w/v) PEG 4000 and 200 mM ammonium acetate at 20 °C.

### X-ray data collection

Data were collected from these crystals, (cryo-cooled using 28% (v/v) glycerol as a cryo-protectant) using an ADSC Q4 CCD detector at ID14-2 at ESRF, Grenoble, France. The crystals diffracted to 2.5 Å. Autoindexing with the HKL program suite[41] showed that the crystals had a Bravais lattice of type primitive orthorhombic, unit cell dimensions 92.9 Å × 193.6 Å × 92.7 Å. In total, 623,268 reflections were recorded (resolution range 30–2.5 Å), 56,134 of these being unique. Scaling and merging was performed using the CCP4 program SCALA,[42] giving $R_{merge}$ (2.63–2.5 Å) of 0.062 (0.195) and $I/\sigma I$ (2.63–2.5 Å) 8.0 (2.0). Analysis of the systematic absences revealed that the space group was $P2_12_12$. Subsequent data manipulation was done with the CCP4 program package.[43] The number of molecules within the asymmetric at this point was ambiguous, as the solvent contents for three and four molecules were 62.5% and 50% (v/v), respectively.

### Molecular replacement

Molecular replacement was carried out using the open-form structure of the Cpa (PDB code 1CA1),[10] with loop regions removed and non-conserved residues converted to alanine. The AMoRe,[44] MolRep,[45] COMO[46] and EPMR[47] programs all yielded three molecules in the asymmetric unit (corresponding to a solvent content of 62.5%, v/v). Though initial rigid body refinement of the MolRep model (*CC* = 28.4%; *R*-factor = 54.7%) using the CNS package,[48] the $R_{free}$ dropped to 51% ($R_{cryst}$ = 47%); subsequent refinement was unsuccessful. Inspection of a difference map, contoured at 5σ revealed a cluster of three peaks in open space, corresponding to three zinc ions in an as yet undetected monomer. This monomer was placed into the density using O[49] and following rigid body refinement, the $R_{free}$ and $R_{cryst}$ values dropped to 47% and 37%, respectively. The inclusion of this fourth monomer reduced the solvent content to 50% (v/v).

### Model building and refinement

Rounds of (initially) rigid body refinement, simulated annealing and *B*-factor refinement were carried out using the CNS program package.[48] Phase modification was done using Resolve.[50,51] Model building was performed in O[49] and the Xfit[52] package. Towards the end of refinement, REFMAC5[53] was used in conjunction with ARP/wARP[54] to add and refine ordered solvent atoms within the model. NCS restraints were initially imposed within CNS, but these were removed at the end of refinement (within REFMAC5) as differences between the four monomers within the asymmetric unit became apparent. Refinement was concluded when $R_{cryst}$ and $R_{free}$ converged at 16.9% and 23.3%, respectively.

### Construction of the active-site model

The co-ordinates for a single monomer (with associated $Zn^{2+}$ and $Ca^{2+}$) were superimposed with the crystal structure of *B. cereus* PC-PLC complexed with a substrate analogue structure using LSQKAB.[55] The co-ordinates for the substrate analogue were then copied to the Caa monomer, and substituted for PC. This structure was then subjected to 200 rounds of energy minimisation refinement using the CNS package.

### Data bank accession codes

The nucleotide sequence of the *caa* gene has been deposited within GenBank under the accession number AY159815.

Coordinates and structure factors have been deposited in the PDB: accession codes 1olp and r1olpsf.

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