Structure Function Studies of *Clostridium perfringens* Alpha-Toxin; A Gas Gangrene Causing Protein.

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Introduction

The alpha-toxin of *Clostridium perfringens* was the first bacterial protein to be shown to possess both enzymatic activity and toxic properties [1]. This protein forms part of a range of toxins secreted by *C. perfringens*, and has been shown to play a major role in the pathogenesis of gas-gangrene in man. Alpha-toxin is a zinc dependent phospholipase C, it consists of two domains; the N-terminal domain and the C-terminal domain. It attacks the outer membrane in phospholipid bilayers and exhibits haemolytic, necrotic, vascular permeabilisation and platelet aggregating properties. Related gram-positive bacteria (*Clostridium bifermentans* and *Bacillus cereus*) secrete phospholipase C enzymes which show homology to alpha-toxin. However, these proteins do not pocess lethal and haemolytic properties to the same degree. Much research has been undertaken in an effort to understand the enzymatic and pathological mechanisms of phospholipase C (PLC) molecules. The ultimate goal is to produce an effective treatment to combat diseases such as gas gangrene. With respect to structural studies, methods of purification of alpha-toxin have been developed and crystallisation and preliminary X-ray diffraction analysis have been completed [2]. We are currently determining the crystal structure of alpha-toxin by X-ray analysis to 1.8 Å. The high resoultion structure of *B. cereus* PLC has been published, using this and sequence homologies to alpha toxin, it has been possible to study the catalytic residues of alpha-toxin by employing site directed mutagenesis. We are undertaking further crystallisation studies including co-crystallisation of alpha-toxin with PLC inhibitor molecules and recombinant variants of alpha-toxin. This work may reveal novel mechanisms by which proteins interact with membrane bound phospholipids and could lead to new membrane targetting technologies.

Biochemical and pathological properties of alpha-toxin.

General properties of alpha-toxin.

At least 12 soluble antigens are synthesised by the bacterium *C. perfringens* [3]. These form the basis of classification into five *C. perfringens* subgroups A, B, C, D and E. It is the type A strains that produce predominantly alpha-toxin.

Alpha-toxin has a molecular weight of approximately 42,500 Dalton [4,5]. It is composed of 370 amino acids [6]. The sequence (see figure 1) can be divided into two domains; the N-terminal domain (residues 1-247), and the C-terminal domain (residues 248-370). The isoelectric point of the protein is approximately 5.6. *C. perfringens* alpha-toxin is thought to bind to 2-3 functionally essential zinc ions, and is reversibly inactivated by metal ion chelators such as EDTA [7]. The *B. cereus* PLC and *C. bifermentans* PLC are also zinc-metalloenzymes.

Table 1 shows a comparison of *C. perfringens, C. bifermentans* and *B. cereus* PLC activities and their abilities to cleave important phospholipids (see figure 2). It can be seen that *C. perfringens* and *C. bifermentans* enzymes possess both PLC and sphingomyelinase activities. The *B. cereus* PLC enzyme (which contains 248 amino acids, and is 27.8% homologous to the N-terminal of alpha-toxin) does not have sphingomyelinase activity. However, *B. cereus* produces a second enzyme separately to hydrolyse sphingomyelin [6]. *C. bifermentans* PLC is 61.9% homologous to the whole of alpha-toxin.

The sphingomyelinase activity is believed to be a function of the extra C-terminal regions found in both *C. perfringens* and *C. bifermentans* enzymes,

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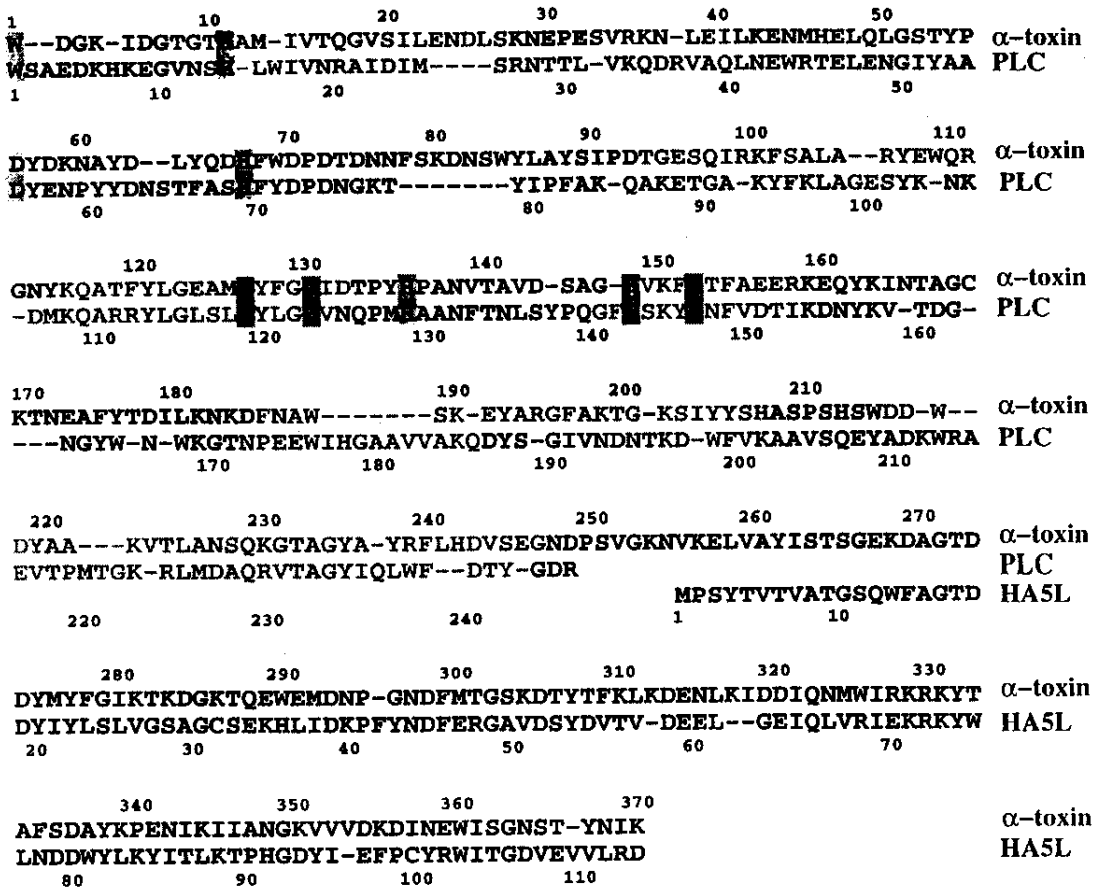
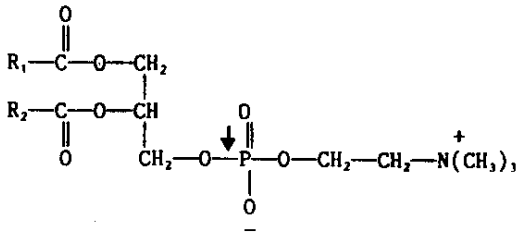


Fig. 1. Aligned sequences of alpha-toxin, *B. cereus* PLC (PLC) and human arachidonate 5-lipoxygenase (HA5L). Grey text represents residues present in alpha helices. Shaded boxes represent conserved residues found in active site of enzymes.

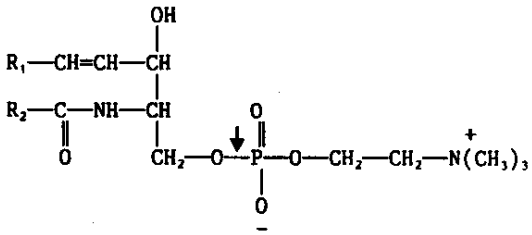
Table 1. Ability of *C. perfringens* PLC (alpha-toxin), *B. cereus* PLC, *B. cereus* sphingomyelinase, and *C. bifermentans* PLC to cleave substrate phospholipids and their various activities.

	Alpha-toxin.	*B.cereus* PLC	*B.cereus* SMase	*C.Bifer.* PLC
Mr (Da)	42,500	28,520	34,233	42,746.
Substrates.	PC, SPM, LPC, PS.	PC, PE, PS.	SPM.	Not reported.
Ion requirement	Zinc, Calcium.	Zinc, Calcium.	Magnesium.	Not reported.
C-terminal domain	Yes	No	NA*1	Yes
PLC activity. (Eyu/mg)*2	252	Yes*3.	NA	5
Sphingo-myelinase activity	Yes	No	Yes	Not reported.
Lethality (μg/mouse)	0.03-0.1	>30	NA	1-5
Hemolytic ability *4	520,000	0	NA	12,000

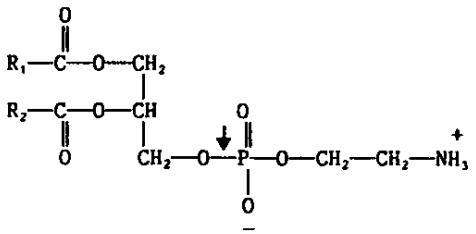
PC- phosphatidylcholine
PE- phosphatidylethanolamine
PS- phosphatidylserine
SPM- sphingomyelin
*1 NA- Not applicable.
*2 Eyu- egg yolk phospholipid-hydrolysing units.
*3 Value not available.
*4 Hemolytic ability measured in hemolytic units / mg / 30 min.
(Table adapted from Titball 1993).



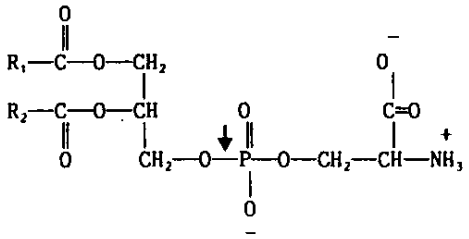
phosphatidylcholine



sphingomyelin



phosphatidylethanolamine



phosphatidylserine

Fig. 2. Site of cleavage of the major phospholipids (arrowed) by phospholipases C. From Titball (1993).

which is not present in the *B. cereus* PLC. If the active site architecture of *B. cereus* PLC is changed by replacing the zinc ions with cobalt ions, it is able to hydrolyse sphingomyelin [8]. Although the C-terminal region of *C. perfringens* alpha-toxin does not exhibit any significant homology with *B. cereus* sphingomyelinase, it does exhibit 30% sequence identity with residues 1-113 of human arachidonate 5-lipoxygenase (HA5L) [9]. Comparisons of the hydropathy profiles [6] suggest that the alpha-toxin C-terminal domain is structurally similar to the N-terminal region of HA5L which produces leukotrienes from arachidonic acid.

C. perfringens alpha-toxin is approximately 10-30 fold more toxic than *C. bifermentans* PLC (Table 1). Although both attack similar phospholipids, *C. perfringens* PLC exhibits a far higher turnover rate. *B. cereus* PLC is capable of hydrolysing phosphatidylglycerol (an important component of the bacterial cell membrane), whereas *C. perfringens* alpha-toxin and *C. bifermentans* PLC are not.

The interaction of alpha-toxin with phospholipids and membranes: Role of the C-terminal region.

Eukaryotic cell membranes are composed of a phospholipid bilayer. It is the outer layer, consisting mainly of sphingomyelin and phosphatidylcholine that is accessible to alpha-toxin. Not all parts of the outer layer can be reached by alpha-toxin. The exposed interfacial area includes the lipid head group and the ester bonds linking the head group to the hydrophobic tail. It is believed that alpha-toxin recognises the head group when binding to target phospholipids [10], though the hydrophobic tail and ester bonds are also important for active-site substrate recognition. In addition to the correct phospholipid composition, the fluidity of the membrane is an essential feature in alpha-toxin binding; less toxin-membrane interaction occurs with a lower cholesterol content, and therefore, a lower fluidity [11].

Calcium ions are also required for the binding of alpha-toxin to phospholipid membranes [12,13]. It is believed that a calcium ion binds to the phosphate head group on the phospholipid, this binding then alters the charge and facilitates enzyme-substrate interactions. Kinetic studies have indicated that a calcium ion actually binds to alpha-toxin before phospholipid binding, suggesting that calcium plays an intrinsic role in the reaction mechanism.

For PLC to effectively hydrolyse phospholipids the enzyme must be able to gain access to the cell membrane and the higher the outward pressure the harder it is to do this. Although some phospholipase enzymes are significantly affected by surface pressure, alpha-toxin is apparently not. It has been reported that hydrolysis can occur at greater than 40 dynes/cm^2 pressure [14], an amount exceeding normal cellular membrane pressure. It is possible that the C-terminal region of alpha-toxin facilitates membrane entry. The C-terminal region contains a high proportion of tyrosine residues, these hydrophobic amino acids could aid protein-membrane interactions. Indeed, if chemical modification of the C-terminal tyrosine residues is undertaken then the haemolytic activity of alpha-toxin is abolished [15].

Diseases and mechanisms of pathology of alpha-toxin.

The most well known disease associated with *C. perfringens* is gas gangrene [16]. Gas gangrene can be seen as the result of the pathological effects of *C. perfringens* toxins (especially alpha-toxin); the main effects being haemolysis, vascular permeablisation, and platelet aggregation. The disease usually results from the proliferation of bacteria in tissues which becomes anoxic; either as a result of traumatic damage or from obliterate arterial diseases in the limbs [17]. How alpha-toxin causes gas gangrene is not yet fully established, but it is thought that it is a combination of toxic mechanisms, the two most important being cell lysis and the inappropriate activation of cell signalling pathways.

Cell lysis by destruction of outer-membrane phospholipids is an obvious method by which alpha-toxin can kill tissue. The haemolytic effects of alpha-toxin may be attributed to this. It is believed that if the rate of cell membrane destruction outpaces the rate of cell membrane production the cell will lyse. Hence *C. bifermentans* PLC does not cause lysis because its turnover rate is too low [18].

The arachidonic acid cascade is an intracellular signalling sequence, which leads to an inflammatory response providing a defence against small-scale host tissue damage. The cascade leads to inflammation, muscle contraction, platelet aggregation, and increased vascular permeability (see figure 3). This pathway, however, can be in-inappropriately stimulated by alpha-toxin, (by the release of diacylglycerol from cleaved phospholipids), and the resulting anoxic conditions then promote further growth of *C. perfringens.*

Additional cellular responses have been reported that may be a product of alpha-toxin: Diacylglycerol activates protein kinase C (PKC) in eukaryotic cells. PKC, a regulator of cell signalling pathways, in turn, leads to genetic modulation of cell proliferation and cell growth. Thus, alpha-toxin can play a role in carcinogenesis, and indeed, it has been shown that alpha-toxin mimics a carcinogen in cell culture lines [19]. Associated with the PKC regulated cascade is the inositol tri-phosphate activated signal pathway. Recent work [20,21] suggests that alpha-toxin can stimulate this cascade by increasing phosphatidic acid production, this stimulation being mediated through a G-protein type reaction.

Other diseases associated with alpha-toxin include acute-pancreatitis, illietis and Crohn's disease, and cot-death syndrome. Holdsworth and Parratt [22] showed that patients with acute pancreatis exhibited levels of serum IgG and IgM (anti alpha-toxin antibodies) that were significantly lower than control patients. This, they suggest, is due to a possible release of alpha-toxin into the blood circulation. The excitation of the arachidonic acid pathway by alpha-toxin may play a role in the abnormal inflammation seen in ileitus and Crohn's disease [23]. Although *C. perfringens* is invariably present in the human gut, an elevated level may produce pathological states. Finally, post-mortem studies of sudden infant death syndrome (SIDS) cases, when compared to infants of the same age that had died in other ways, have revealed abnormally high levels of *C. perfringens* in the faeces of SIDS infants [24].

Structural considerations of alpha-toxin.

Structural insights gained from comparison with Bacillus cereus PLC.

The high resolution crystal structure (1.5 Å) of *B. cereus* PLC has been determined [25]. Therefore, it should be possible to predict important

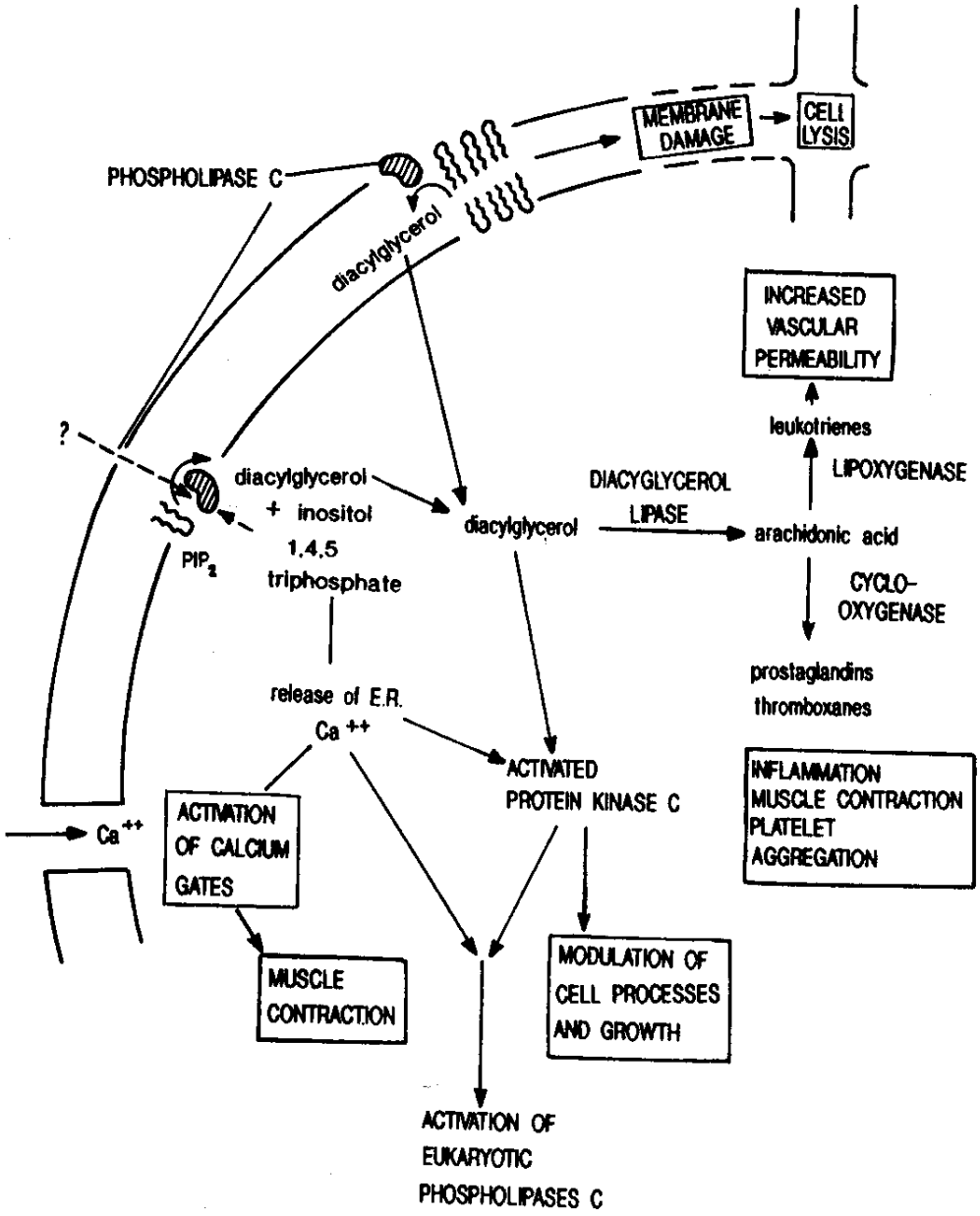


Fig. 3. Effect of alpha-toxin on eukaryotic cell signalling cascades. E.R Endoplasmic reticulum. The bacterial PLC is hatched. Enzymes are shown in capital letters, and possible effects on eukaryotic cells are boxed. From Titball (1993).

structural features of alpha-toxin using the known *B. cereus* structure.

The structure of *B. cereus* PLC is depicted in figure 4 (a). It can be seen that the enzyme is an all helical protein, the most striking feature being the long anti-parallel helix pair. The active site is situated in the centre of the enzyme and is structurally co-ordinated by three zinc ions. The zinc ions interact with the following amino acids: Histidines 14, 69, 118, 128, and 142; Aspartates 55 and 122; Tryptophan 1; and Glutamate 146. It

should be noted that these residues are conserved in both *B. cereus* PLC and *C. perfringens* alpha-toxin. Also reported was a preliminary complex structure[25], showing inorganic phosphate (Pi) bound to the active site. The Pi binds directly to the zinc, interacting with all 3 metal ions (1 Pi binds to zincs 1 and 3, and 1 Pi binds to zinc 2). In the process of inhibitor binding, two water molecules that were previously present were replaced.

The structure of alpha-toxin from *C. perfringens* strain NCTC8237 has been solved by Multiple Isomorphous Replacement (MIR), a high resolution refinement is now being carried out using a second strain, CERL43. The R- and free R- factors are currently 44% and 46% respectively. At this intermediate stage of refinement we cannot make definite conclusions regarding the detailed structure of *C. perfringens* alpha-toxin, however, the overall secondary structural features are clear, and are illustrated in figure 4 (b). The N-terminal domain is similar to *B. cereus* PLC (figure 4 (a)), as predicted by sequence alignment, and three zinc binding sites have been identified at positions homologous to those seen in *B. cereus* PLC. The structure of the C-terminal domain is much less well defined, however, it is clear that this is an all β-structure, consisting of two sheets, each with at least four strands.

Mutagenesis studies of C. perfringens alpha-toxin.

Given the known importance of histidine residues present within the active site of *B. cereus* PLC, the corresponding histidines in alpha-toxin have been the subject of genetic and chemical mutagenesis. This mutagenesis has provided information on the important catalytic and structural residues in alpha-toxin.

Titball and Rubidge [26] incubated alpha-toxin with diethylpyrocarbonate (DEPC), a reagent that preferentially modifies histidines. It was found that alpha-toxin was not inactivated on incubation with DEPC when in its native state, but did undergo complete inactivation if incubated with DEPC and the metal chelating agent EDTA. This suggests that the zinc ions protect susceptible histidine residues from chemical modification, therefore zinc and the histidine residues must be closely associated with each other. The modification was also found to be

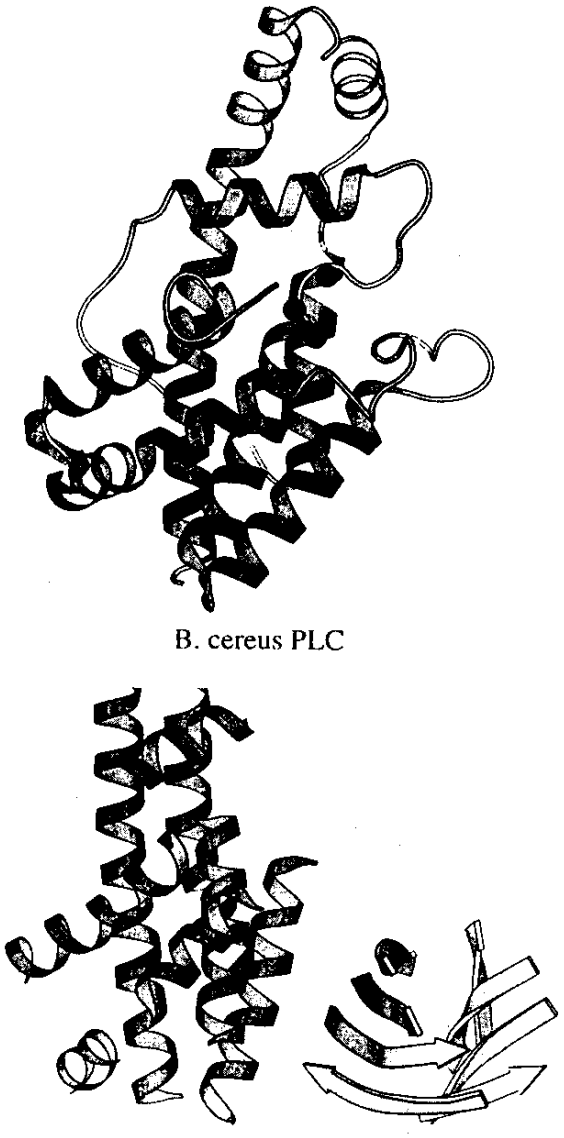


Fig. 4. (a) Ribbon diagram of the high resolution structure of *B. cereus* PLC showing positions of active site zinc atoms. Solved by Hough *et al* 1989. (b) Ribbon diagram showing the secondary structures for the current model of *C. perfringens* alpha-toxin. This structure is currently under refinement. Both diagrams drawn with Molscript, P. Kraulis [35].

reversible; incubation with hydroxylamine, which reverses the action of DEPC, restored enzyme activity.

Nagahama *et al* [27] used site directed mutagenesis of histidine residues to investigate the precise role of the zinc-histidine relationships. It was found that mutagenesis (by conversion of histidine to neutral amino acids such as glycine, alanine, or leucine) of H68 or H148 abolished the catalytic activities of alpha-toxin. Mutagenesis of H126 or H136 decreased the activity by 100 fold. Further experiments showed that H148 toxin variants binds to erythrocytes (in the presence of calcium ions), but the H68, H126, and H136 variants did not. However, on addition of cobalt or manganese ions all variants could bind to erythrocytes. H68, H126, and H136 variants were also found to contain two tightly bind zinc ions, this is the same as wild-type alpha-toxin. If radiolabelled zinc was then added, it was found that wild-type toxin could (loosely) bind a third zinc, but variants H69, H126, and H136 could not. Variant H148 contained only one tightly bound zinc, but could loosely bind the additional radiolabelled zinc. These results are summarised in table 2. These results suggest that H68, H126, and H136 residues loosely bind an exchangeable zinc ion which is important for alpha-toxin membrane interactions, while H148 tightly binds one zinc ion which is essential for a functional alpha-toxin active site.

Table 2.

Toxin variant	Activity	Bind to erythrocytes	No. tightly bound Zn	Ability to bind *Zn.
Wild-type	Yes	Yes	2	Yes
H68, H126, H136	No^	No	2	No
H148	No	Yes	1	Yes

^H126 and H136 showed 100 fold less activity. *Zn indicates radiolabelled zinc added after tightly bound zinc ions have already bound.

Further mutagenesis investigations into alpha-toxin structure were performed by Guillouard *et al*, [28]. By using oligonucleotide-directed mutagenesis to specifically change genetic sites, the function of individual residues was examined. Substitution of aspartate 56 by the similar amino acid asparagine resulted in all biological activities being abolished. The *B. cereus* PLC active site contains a homologous Asp residue (Asp 55). Also, Guillouard suggested that the platelet aggregating properties of alpha-toxin may be due to the interaction of hydrophobic groups between different cells, the hydrophobic groups being left after removal of the phospholipid head groups in the hydrolysis reaction.

Finally, site directed mutagenesis was also used to show that threonine 74 is essential for the activity of alpha-toxin [29]. The T74 mutant toxin was still able to bind three zinc ions, suggesting that T74 plays a catalytic and not a structural role.

Molecular biology of alpha-toxin and the production of anti-toxin vaccines.

C. perfringens alpha-toxin is coded for by a single gene, *cpa*. The *cpa* gene encodes a pre-protein of 399 amino-acids [30] which contains an N-terminal secretory signal which is removed after the toxin has left the bacterial cell. It is thought that for activation of the protein, cleavage of a further N-terminal segment is required; this cleaved fragment binds to the active site in an inhibitory fashion.

How expression of *C. perfringens* PLC is regulated is not fully understood. It is known that other bacterial PLCs are regulated by external phosphate levels [5], however, this does not seem to be the case for alpha-toxin [31]. The fact that *C. perfringens* is a commensal gut bacterium suggests that alpha-toxin production is strictly regulated, as over production of the toxin would not be beneficial for the long term survival of the bacterium. It has been found that an AT nucleotide rich region of DNA, upstream of the *cpa* gene promoter, seems to negatively regulate gene expression. If this region is removed, then gene expression is increased 10 fold.

The level of alpha-toxin production varies significantly between strains of *C. perfringens* [32]. It is known that type A strains produce most alpha-toxin, secreting up to 40 times more toxin than the other *C. perfringens* types. The authors attributed this to extragenic regulation of expression, and not activity of the enzyme itself. The promoter of the *cpa* gene is extremely strong, containing three regions that are not normally associated with housekeeping genes: A TG motif at -12, and an AT block at -43. The variation between strains is likely to be due to mutations in the promoter region, each DNA change affecting the level of messenger RNA production.

The molecular biology of alpha-toxin has been employed to produce a vaccine which provides

protection against *C. perfringens* infection [33]. By using genetic engineering to produce fragments of alpha-toxin, which were then injected into mice, an immunological response against alpha-toxin could be assayed. It was found that a C-terminal region (amino acids 247-370) provided most protection; neutralising the phospholipase, haemolytic, and lethal activities of alpha-toxin. The N-terminal region (1-249) was also a strong immunogen, inducing a high titre of mouse antibody production. However, antibodies against this region did not neutralise alpha-toxin's haemolytic activities, and mice challenged with the toxin died within five hours. The advantages of producing a safe vaccine against the toxic effects of *C. perfringens* are numerous, this is especially the case regarding the elderly and diabetic populations, where the risk of contracting gas gangrene after limb surgery is increased. If a vaccine is produced for clinical use then it would have to remain effective against alpha-toxin gene variation and differing alpha-toxin strains. The ability of the C-terminal vaccine to protect mice against experimental *C. perfringens* alpha-toxin and also against alpha-toxin produced from clinical isolates of *C. perfringens* was tested [34]. The vaccine provided protection against both varieties, indicating that natural genetic variation in the alpha-toxin gene should not be a insurmountable obstacle against the effort to produce an effective vaccine.

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