Use of Site-Directed Mutagenesis To Probe Structure-Function Relationships of Alpha-Toxin from *Clostridium perfringens*

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The NH₂-terminal domain of the alpha-toxin of *Clostridium perfringens* is highly homologous to the complete phospholipase C from *Bacillus cereus* (PC-PLC), for which a high-resolution crystal structure is available. This structural information was used as the basis of a site-directed mutagenesis strategy in which critical amino acid residues of alpha-toxin involved in zinc binding, interaction with substrate, or catalysis were replaced. Biochemical studies with the corresponding toxin variants indicate that there is probably a single active site endowed with lecithinase, sphingomyelinase, and hemolytic activities. By using a highly purified variant in which the catalytic aspartate residue at position 56 was replaced by asparagine, it was shown that phospholipase activity was essential for lethality in vivo and for mediating platelet aggregation in vitro.

The alpha-toxin represents the major toxic factor produced by type A strains of *Clostridium perfringens*. The most devastating *C. perfringens*-mediated pathology in humans is gas gangrene, in which alpha-toxin is believed to play a predominant role (12, 15) even though several other secreted factors could be implicated in the development of the disease (17). Alpha-toxin was the first bacterial toxin for which enzymatic activity was described (11). It is cytolytic, lysing erythrocytes and phagocytes, and it damages fibroblasts and muscle cell membranes (12) and causes platelet aggregation (15). Alpha-toxin contracts the isolated rat aorta and stimulates release of arachidonic acid in the tissue (2). It causes profound hypotension and bradycardia (21). Unlike many other bacterial phospholipases C, the alpha-toxin is readily able to interact with cell membrane phospholipids (24), promoting local cell membrane disruption. It can degrade many phospholipids present in eukaryotic membranes (13), such as phosphatidylcholine (lecithin), which is hydrolyzed to phosphorylcholine and 1,2-diacylglycerol, and sphingomyelin (18). It has frequently been used as a tool in membrane studies (9).

Biochemical studies have indicated that the alpha-toxin is a zinc metalloprotein consisting of a single polypeptide chain with a molecular mass of 43,000 Da (7, 9). Zinc plays an important role in the stability and activity of the enzyme (20, 23, 24). The genes coding for the alpha-toxins of different *C. perfringens* type A strains have been cloned, and the nucleotide sequences and deduced primary structures show only a few differences (9, 16, 18, 23, 27).

There is extensive homology between the primary sequence of the NH₂-terminal domain of the alpha-toxin, comprising 247 residues, and the complete primary sequence of phospholipase C from *Bacillus cereus* (PC-PLC). PC-PLC is a nontoxic protein that is relatively well understood, since its crystal structure has been determined (6). It is also a zinc metalloprotein of 245 amino acids whose preferred substrate is phosphatidylcholine (3). The amino acids implicated in the active site of PC-PLC are all conserved in the primary sequence of the alpha-toxin. Because of this remarkable homology between the two proteins, PC-PLC can be used as a model to study the structure of

the alpha-toxin (Fig. 1). In *B. cereus*, hemolysis results from the synergistic action of PC-PLC and a sphingomyelinase, while in *C. perfringens*, all three activities are contained in a single protein. The alpha-toxin carries an additional COOH-terminal domain of 123 amino acid residues. This region, which has no counterpart in *B. cereus*, is similar to the NH₂-terminal domains of lipoxygenases (24).

Despite intensive interest in the biochemistry of the alpha-toxin, its structure-function relationship has not been fully clarified. Furthermore, it was unclear whether the lethality of alpha-toxin resulted from its enzymatic activities or from an additional, as-yet-unidentified activity such as membrane insertion. Here, site-directed mutagenesis was used to probe the organization of the active site of alpha-toxin and to establish which of its activities was required for toxicity.

MATERIALS AND METHODS

Site-directed mutagenesis. The alpha-toxin gene, *plc*, was previously cloned into the pTZ18R plasmid to produce pTOX5 (18). Codon exchanges, leading to amino acid substitutions, were carried out by site-directed mutagenesis by the method of Kunkel (8). For this technique, 20-base oligonucleotide primers were constructed such that one or two base changes would produce a codon change that resulted in an amino acid substitution (Table 1). The desired nucleotide substitution in the genes encoding the mutant proteins was confirmed by dideoxynucleotide sequencing of the complete gene.

Overproduction of proteins. The T7 polymerase expression system with strain K38/pGP1-2 was employed essentially as described by Tabor and Richardson (22). *Escherichia coli* strains harboring alpha-toxin mutants were grown to an optical density at 600 nm of 1 at 30°C in 50 ml of 2YT medium containing ampicillin (50 µg/ml) and kanamycin (50 µg/ml). After thermal induction of T7 RNA polymerase synthesis during 15 min at 42°C, cultures were treated with rifampin (200 µg/ml) for 30 min to block transcription by the host polymerase. Expression of the mutant genes is achieved after 2 h at 37°C. Cells were then harvested by centrifugation and resuspended in 5 ml of shock buffer (20% sucrose, 0.03 M Tris-HCl [pH 6.8], 1 mM EDTA). After gentle stirring for 10 to 15 min, the cells were centrifuged 20 min at 3,000 rpm at 4°C. The pellet was resuspended in 2 ml of cold double-distilled water and then recentrifuged for 15 min at 4°C at 9,000 rpm.

The supernatant was used to measure the enzymatic activities, analyzed by electrophoresis on sodium dodecyl sulfate (SDS)-polyacrylamide gels (12%), and then stained with Coomassie blue. The protein concentration was determined by the Bradford method (1a). The levels of expression of the alpha-toxin mutants in *E. coli*, and the amounts of recombinant toxin recovered from the periplasm, were essentially the same in all cases.

Purification of proteins. The supernatant obtained by the protocol described above was loaded onto an anion-exchange Hilo Q column (Pharmacia fast protein liquid chromatography) equilibrated with 25 mM Bis-Tris-HCl, pH 6.8. The elution was performed with a gradient of NaCl (0 to 1 M) in Bis-Tris-HCl, pH 6.8. Active fractions were concentrated on a Macrosep system (Filtron) and then loaded onto a gel filtration column.

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FIG. 1. Alignment of phospholipase C sequences. The primary sequences of the phospholipases *C. perfringens* alpha-toxin (alpha) and *B. cereus* PC-PLC are aligned. The positions of α -helices (A to H2) in PC-PLC, established in structural studies (5), are shown. Residues selected for mutation in alpha-toxin are in boldface. Symbols: |, identical residues; :, conservative substitutions.

Radiolabelling and immunoblotting of proteins. To radiolabel the proteins, the above-described protocol was employed except that the culture volume was 5 ml and the cells were transferred to M9-glucose minimal medium, which was supplemented with methionine assay medium (1%) prior to the thermal and chemical induction. Aliquots were labelled for 5 min with [35 S]methionine (15 μ Ci; Amersham plc) and then prepared for SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Immunoblotting was performed as outlined previously (26), with a panel of monoclonal antibodies (E2B5, 7E7C2, 11D9G5, 11D10B6, 11D10F7, 9F3A6, 12G8B11, and 10E3B7) directed against alpha-toxin (kindly provided by Hiroko Sato) (19).

Lecithinase activity determination. Lecithinase activity was measured at 37°C in a reaction mixture (1 ml) containing lecithin (0.75% [wt/vol]), sodium cholate (0.8% [wt/vol]), sodium acetate (5.2 mM), and zinc sulfate (0.005 mM). Hydrolysis of lecithin led to an increase in turbidity as monitored at 420 nm. Arbitrary units were defined as the change in optical density per minute. Specific activities are expressed as enzyme units per milligram of protein.

Sphingomyelinase activity determination. Sphingomyelinase C activity was determined by thin-layer chromatography. After incubation of 0.1 ml of enzyme extract in 1.2 ml of reaction mixture (150 mM Tris-HCl [pH 6.8], 0.02 mM ZnCl₂, and 0.3 mg of sphingomyelin per ml) for 18 h at 37°C, lipids were extracted with chloroform-methanol (9:1, vol/vol) and loaded onto a thin-layer chromatography gel (Silica Gel 60; Merck). Ascending chromatography was carried out for 2 h in chloroform-methanol-water (65:25:4, vol/vol), and the gel was stained with iodine vapor.

Hemolytic activity and platelet aggregation. For qualitative plate assays, transformants were streaked onto agar containing 5% (vol/vol) fresh sheep blood. To monitor platelet aggregation, toxin samples (1 to 10 μ g) were added to a 1-ml solution containing 5×10^8 platelets purified by centrifugation from human blood, and aggregation was measured by monitoring the decrease in A_{590} with a Hitachi U-2000 spectrophotometer.

Lethality assays. Lethal activity was determined by intraperitoneal injection of purified alpha-toxin or mutant derivatives into 4-week-old Swiss-Webster mice. The animals were monitored from 12 h to several days after infection.

RESULTS

In vitro mutagenesis of the *plc* gene of *C. perfringens* and preliminary characterization of products. In order to investigate the roles of specific amino acids in the activity of alpha-toxin, single amino acid substitutions were created in the N-terminal part of the alpha-toxin, which is highly similar to PC-PLC (Fig. 1). Mutations were introduced in vitro by oligonucleotide-assisted, site-directed mutagenesis of the *plc* gene, on plasmid pTOX5 (18), as described in Materials and Methods. After selection of mutated clones, mutant *plc* genes were

TABLE 1. Oligonucleotides used for mutagenesis

Mutation	Oligonucleotide
His11-Ser.....5'	ACA GGA ACT TCT GCT ATG AT 3'
His68-Ser.....5'	TAT CAA GAT TCT TTC TGG GA 3'
His126-Ser.....5'	GAA GCT ATG TAC TAT TTT GGA 3'
His136-Ser.....5'	ACT CCA TAT TCT CCT GCT AA 3'
His148-Ser.....5'	GC GCA GGA TCT GTT AAG TTT 3'
Asp56-Asn.....5'	T ACT TAT CCA GAT TAT GAT A 3'
Asp71-Asn.....5'	TC TAC GCT TCG GAT GGA AAA 3'
Asp130-Asn.....5'	TAT TTT GGA AAT ATA GAT ACT 3'
Glu152-Gln.....5'	GTT AAG TTT CAG ACT TTT GC 3'
Trp1-Ser.....5'	TC TAC GCT TCG GAT GGA AAA 3'
Phe69-Cys.....5'	CAA GAT CAT TGC TGG GAT CC 3'

completely sequenced to verify that only the desired mutations were present and were selectively overexpressed in *E. coli* by using the T7 promoter-polymerase system (22). All mutant proteins were correctly expressed and were exported to the periplasm as efficiently as wild-type alpha-toxin. All of the mutant derivatives appeared to be stable as judged by ³⁵S radiolabelling and pulse-chase experiments (Fig. 2). Furthermore, none of the proteins exhibited gross structural changes, as they were all detected efficiently in immunoblotting experiments using a panel of monoclonal antibodies (data not shown) that recognize conformational epitopes of alpha-toxin (19). This is in marked contrast to the findings with alpha-toxin variants harboring amino acid substitutions in the COOH-terminal domain, as some of these result in loss of recognition by five different monoclonal antibodies (5).

To establish the relationship between biological activities and important amino acids of the alpha-toxin, activities of mutant proteins in periplasmic extracts were compared with those of native alpha-toxin. The abilities of the partially purified proteins to hydrolyze lecithin and sphingomyelin or to lyse erythrocytes were evaluated as described in Materials and Methods and are summarized in Table 2. No biological activity was detected with periplasmic extracts from *E. coli* containing the vector pTZ18R (Table 2).

Substitution of residues involved in zinc binding. Five histidine residues are directly implicated in zinc binding in the active site of PC-PLC and are conserved in alpha-toxin (Fig. 1). The corresponding histidines of the alpha-toxin in positions 11, 68, 126, 136, and 148 were changed independently to serine, and the enzymatic activities of mutant proteins were measured. All five changes greatly reduced or abolished the lecithinase, sphingomyelinase, and hemolytic activities (Table 2). In the case of the protein carrying the His-11→Ser (His11-Ser) sub-

TABLE 2. Enzymatic activities of mutant proteins

Protein	Activity			Production, stability, secretion
	Lecithinase ^a	SMase ^b	Hemolytic ^c	
Alpha-toxin	252.7	+	+	+
His11-Ser	6.2	—	—	+
His68-Ser	0.4	—	—	+
His126-Ser	<0.1	—	—	+
His136-Ser	0.1	—	—	+
His148-Ser	1.5	—	—	+
Glu152-Gln	0.1	—	—	+
Trp1-Ser	2.6	—	—	+
Phe69-Cys	0.9	—	—	+
Asp56-Asn	<0.1	—	—	+
Asp71-Asn	247.1	+	+	+
Asp130-Asn	0.1	—	—	+

^a Activities are expressed as change in optical density per minute per milligram of protein and are the means from five independent assays.

^b SMase, sphingomyelinase. +, hydrolysis of sphingomyelin; —, no hydrolysis of sphingomyelin.

^c +, hemolytic activity; —, no hemolytic activity.

stitution, low but significant levels of lecithinase activity (~2% of the wild-type level) were retained.

The NH₂-terminal tryptophan is buried immediately behind the active site of PC-PLC and also contacts zinc (6). When the corresponding residue in alpha-toxin was mutated to serine, all of the biological activities of the protein decreased considerably (Table 2).

Replacement of acidic amino acids. The active site of PC-PLC consists of an acidic pocket (6). Among the acid residues implicated in this pocket, the aspartates in positions 56 and 130 and the glutamate in position 152 of the alpha-toxin were mutated to asparagines and to glutamine, respectively. These substitutions abolished completely the enzymatic activity of the protein, in the case of the aspartates, or decreased it drastically, in the case of the glutamate (Table 2).

Substitution of residues involved in substrate binding. By analogy with the choline pocket of PC-PLC, the phenylalanine and aspartate residues of alpha-toxin at positions 69 and 71 (labelled 70 and 72 in Fig. 1 and 4), respectively, were believed to constitute the substrate binding site. To test this hypothesis, both residues were replaced. Replacement of phenylalanine 69 by cysteine greatly reduced all activities associated with alpha-toxin, whereas replacement of aspartate 71 by asparagine had no detectable effect (Table 2).

Purification of proteins. Recombinant alpha-toxin and the variant protein carrying the Asp56-Asn substitution (Asp56-Asn protein) were purified from cultures of the corresponding *E. coli* transformants by ion-exchange chromatography and gel filtration. During the purification procedure, the elution profiles of wild-type and variant proteins were identical, and in both cases SDS-PAGE revealed a single band with the same apparent molecular mass (Fig. 3).

In vivo activity of Asp56-Asn protein and platelet aggregation. The replacement of aspartate 56 in alpha-toxin by asparagine leads to no detectable activity in vitro (Table 2). To determine whether this substitution also led to loss of toxicity in vivo, mouse lethality assays were performed. Purified recombinant alpha-toxin provokes death of mice at doses <0.5 µg per animal, whereas the Asp56-Asn protein showed no lethality at doses of >100 µg per animal several days after intraperitoneal injection. Platelet association is also mediated by alpha-toxin, and to determine whether this also required the

FIG. 2. SDS-PAGE analysis of wild-type and variant Asp56-Asn proteins during the purification procedure. Lane 1, molecular mass marker; lane 2, osmotic shock content of pTOX5 *E. coli* transformants; lane 3, purified alpha-toxin; lane 4, osmotic shock content of Asp56-Asn variant *E. coli* transformants; lane 5, purified Asp56-Asn variant protein. About 10 µg of protein was loaded in each case.

FIG. 3. ^{35}S radiolabelling of wild-type and variant proteins. Labelled polypeptides were separated on an SDS-12.5% PAGE gel and visualized by autoradiography. The proteins were from pTZ18R *E. coli* transformants (A), pTOX5 *E. coli* transformants (B), and Asp56-Asn *E. coli* transformants (C). Samples were labelled for 5 min and then chased with unlabelled methionine for 0 min (lanes 1), 2 min (lanes 2), 5 min (lanes 3), or 60 min (lanes 4).

phospholipase activity, human platelets were incubated with samples of recombinant native or Asp56-Asn protein. Aggregation was observed only in the former case.

DISCUSSION

In this study, oligonucleotide-directed mutagenesis was used to construct variants of *C. perfringens* alpha-toxin in which residues from the active site, believed to be involved in zinc binding, catalysis, or substrate binding, were replaced. Each of the modified recombinant toxins was expressed in *E. coli* and assayed for lecithinase, sphingomyelinase, and hemolytic activities (Table 2). Pulse-chase experiments (Fig. 2) revealed all the variant proteins to be stable, and immunoblotting with monoclonal antibodies recognizing conformational epitopes suggested that the general structures of the variant proteins were similar to that of the wild-type protein. Interpretation of our results was facilitated by the availability of a high-resolution structural model (Fig. 4) of the related enzyme PC-PLC (6). Most of the amino acids of the PC-PLC active site are conserved in the alpha-toxin (Fig. 1). Histidine residues were

selected for mutation, since they are required for coordination of zinc atoms and since previous studies, using chemical modification with diethylpyrocarbonate (25), indicated that they are implicated in the active site of the enzyme. Substitution of histidine in positions 11, 68, 128, 136, and 148 of the alpha-toxin was sufficient to inactivate the lecithinase, sphingomyelinase, and hemolytic activities of the protein, thus confirming that all five histidine residues are required for maximal expression of alpha-toxin activity and indicating that the active site is probably organized in the same manner as that of PC-PLC (6).

While this work was in progress, Nagahama et al. (14) described similar studies in which these histidine residues were replaced by glycine, leucine, or alanine. Their findings were essentially in agreement with our own, except that those authors, who used *Bacillus subtilis* as the host, were unable to obtain sufficient protein for study from the alpha-toxin variant in which histidine 11 had been replaced. As predicted by the structural model (Fig. 4), those authors found that the zinc content of the mutant proteins was reduced (14).

Biochemical analysis indicates that both PC-PLC and alpha-toxin contain two tightly bound zinc atoms and a third zinc atom that is more loosely associated (10, 12, 14). It is tempting to suggest, on the basis of the enzymatic activities observed with the mutant derivatives of alpha-toxin (Table 2), that the loosely bound zinc atom is coordinated by tryptophan 1, histidine 11, and aspartate 130 (as predicted by the structure of PC-PLC [Fig. 4]), since two of the variant proteins, in which tryptophan 1 and histidine 11 were changed, display appreciably higher levels of lecithinase activity than the other mutant proteins with alterations at the two other Zn binding sites. The same conclusion was reached for PC-PLC from analysis of heavy-atom derivatives in which Zn^{2+} was replaced by Cd^{2+} . However, additional work is required to confirm this hypothesis. In the third derivative lower activities were seen, but this might be due to the fact that aspartate 130 coordinates two different zinc atoms.

Individual replacements of all amino acid residues directly implicated in Zn binding (Fig. 4) led to nearly complete abolition of the catalytic activities associated with alpha-toxin (Table 2), indicating not only that these conserved residues are critical for the biological activity of alpha-toxin but also that the organization of this domain of the protein must be highly similar to that of PC-PLC. Replacement of two other residues likely to be involved in substrate binding, phenylalanine 69 and aspartate 71, produced distinct functional effects. Whereas the replacement of phenylalanine 69 led to strongly reduced alpha-

FIG. 4. Structural model of the active-site region of PC-PLC, showing the α -carbon atoms of the main chain (grey) and the side chains (black) of the principal amino acids discussed in the text.