Distinct sites for myotoxic and membrane-damaging activities in the C-terminal region of a Lys⁴⁹-phospholipase A₂

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Bothropstoxin-I (BthTx-I) is a Lys⁴⁹-phospholipase A₂ from the venom of *Bothrops jararacussu* which demonstrates both myotoxic and Ca²⁺-independent membrane-damaging activities. The structural determinants of these activities are poorly defined, therefore site-directed mutagenesis has been used to substitute all cationic and aromatic residues between positions 115 and 129 in the C-terminal loop region of the protein. Substitution of lysine and arginine residues with alanine in the region 117–122 resulted in a significant reduction of myotoxic activity of the recombinant BthTx-I. With the exception of Lys¹²², these same substitutions did not significantly alter the Ca²⁺-independent membrane-damaging activity. In contrast, substitution of the positively-charged residues at positions 115, 116 and 122 resulted in reduced Ca²⁺-independent membrane-damaging activity but, with the exception of Lys¹²², had no effect on myotoxicity. These

results indicate that the two activities are independent and are determined by discrete yet partially overlapping motifs in the C-terminal loop. Results from site-directed mutagenesis of the aromatic residues in the same part of the protein suggest that a region including residues 115–119 interacts superficially with the membrane interface and that the residues around position 125 partially insert into the lipid membrane. These results represent the first detailed mapping of a myotoxic site in a phospholipase A₂, and support a model of a Ca²⁺-independent membrane-damaging mechanism in which the C-terminal region of BthTx-I interacts with and contributes to the perturbation of the phospholipid bilayer.

Key words: bothropstoxin-I, calcium-independent activity, site-directed mutagenesis.

INTRODUCTION

Phospholipase A₂s (PLA₂s; EC 3.1.1.4) catalyse the hydrolysis of the *sn*-2 acyl bonds of *sn*-3 phospholipids [1], and are extensively studied as a paradigm for interfacial catalysis. A model for catalytic activity has been proposed whereby the His⁴⁸/Asp⁹⁹ pair abstract a proton from a structurally conserved water molecule which acts as a nucleophile in the attack on the *sn*-2 position of the phospholipid [2]. The oxyanion reaction intermediate is stabilized by a Ca²⁺ co-factor bound to the main-chain carbonyl oxygens of the Ca²⁺-binding loop and the carboxyl oxygens of Asp⁴⁹ [2]. A group of PLA₂s in which the Asp⁴⁹ is substituted by lysine has been isolated from viperid snake venoms [3], and it has been suggested that the loss of Ca²⁺-binding results in the lack of detectable hydrolytic activity against both synthetic and natural phospholipids [4]. Despite their lack of catalytic activity, the Lys⁴⁹-PLA₂s demonstrate membrane-damaging activity via a Ca²⁺-independent, non-hydrolytic mechanism [5,6]. Bothropstoxin-I (BthTx-I) is a Lys⁴⁹-PLA₂ isolated from the venom of *Bothrops jararacussu*, which forms dimers both in solution and in the crystalline state. Conformational flexibility at the dimer interface permits a quaternary structure transition between 'open' and 'closed' forms, and it has been proposed that in the membrane-bound form this transition may disrupt the packing of the bilayer lipid, thereby resulting in disruption of the membrane [7]. The model predicts the association between the C-terminal loop region and the outer membrane lipid leaflet, and suggests that the C-terminal loop is involved in the Ca²⁺-independent membrane-damaging activity of BthTx-I.

Snake venom PLA₂s demonstrate a wide range of pharmacological effects, which include myotoxic, neurotoxic and anti-coagulant activities [8]. All Lys⁴⁹-PLA₂s characterized to date are myotoxic [3], and although a wealth of amino-acid sequence data is available from snake venom PLA₂s, sequence comparison studies of Lys⁴⁹ and Asp⁴⁹ PLA₂s have not yielded any clear correlation between protein structure and pharmacological function [9,10]. In the case of myotoxin II, a Lys⁴⁹-PLA₂ isolated from the venom of *Bothrops asper*, binding of monoclonal antibody-specific C-terminal epitopes [11], together with functional studies of synthetic C-terminal peptides [12], have highlighted the involvement of the C-terminal region of the Lys⁴⁹-PLA₂s as a determinant of myotoxic activity. The C-terminal loop of all Lys⁴⁹-PLA₂s is rich in cationic and aromatic residues, and it has been previously suggested that this cationic/aromatic residue combination in the C-terminal loop region may be important for myotoxic activity [13]. Although chemical modification of lysine, tyrosine and tryptophan result in reduced myotoxic activity [14], it is unclear from these studies as to which regions of the protein are modified and whether the observed effects are secondary in nature, and the specific role of these residue types is unknown. Furthermore, it remains unclear as to whether the membrane-damaging and myotoxic activities are correlated.

Recently an expression system for BthTx-I has been described [15], and in the present study we describe a site-directed mutagenesis study in which all cationic residues in the region Lys¹¹⁵ to Cys¹²⁹ were substituted for alanines, and all aromatic residues in the same region were substituted for tryptophan. The myotoxic

Abbreviations used: (Asp⁴⁹/Lys⁴⁹)-PLA₂s, phospholipase A₂s with either aspartate or lysine at residue position 49; BthTx-I, bothropstoxin-I; CK, creatine kinase; DMPA, dimyristoylphosphatidic acid; EYPC, egg yolk phosphatidylcholine; i-face, interfacial binding face; rBthTx-I, recombinant bothropstoxin-I produced in *E. coli*.

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and membrane-damaging activities of the recombinant mutant proteins were compared with the native BthTx-I purified from snake venom, and the results demonstrate that although both activities have specific structural determinants in the C-terminal region, these motifs are distinct.

EXPERIMENTAL

Site-directed mutagenesis

A complete cDNA encoding BthTx-I has been isolated previously from venom gland cDNA by reverse transcriptase-PCR [16] (GenBank[®] accession No. X78599), and sub-cloned into the expression vector pET3-d [15]. Nucleotide sequencing has confirmed the construct in which Ser¹ of BthTx-I is preceded by a methionine, and that a stop codon immediately follows Cys¹³³ (homology numbering as reported previously [17] is used throughout this report). After linearization of this construct with *ScaI*, site-directed mutagenesis of BthTx-I was performed by PCR mutagenesis [18] to introduce the following single mutations (the changed bases are shown underlined): Lys¹¹⁵ → Ala (5'-CCTATATTTTIGCGTTGTACCT-3'), Lys¹¹⁶ → Ala (5'-CTATATIGCTTTGTTGTA-3'), Tyr¹¹⁷ → Trp (5'-GTGATACCTCCATTTTTTTGTT-3'), Arg¹¹⁸ → Ala (5'-CAGGTGATACGCATATTTTTTT-3'), Tyr¹¹⁹ → Trp (5'-TTTCAGGTGCCA-CCCTATATTT-3'), Lys¹²² → Ala (5'-GCAAAAAGGTGCCA-GGTGATA-3'), Phe¹²⁵ → Trp (5'-CTTTTGCACCAAGGTTTCAG-3'), Lys¹²⁷ → Ala (5'-GTCGGCCTTTTCGCGCAAAAAGG-3'), and Lys¹²⁹ → Ala (5'-GGCGTCGGCCCGCTTTGCAAA-3'). The PCR reactions were performed using oligonucleotides complementary to the vector sequences flanking the BthTx-I insert which contained restriction sites for *XbaI* and *BamHI* at the 5' and 3' extremities respectively. After digestion with these enzymes, the amplified fragments were sub-cloned into the equivalent sites in the expression vector pET3-d and fully sequenced.

Recombinant protein expression and purification

A 150 ml volume of growth medium (2.5 g yeast extract, 10 mM MgSO₄, 15 µg · l⁻¹ chloramphenicol and 150 µg · l⁻¹ ampicillin, pH 7.5) was inoculated with *E. coli* strain BL21(DE3)pLysS transformed with native or mutant constructs, and grown at 37 °C to a *D*₆₀₀ of 0.6. Recombinant protein expression was induced by addition of 0.6 mM isopropylthiogalactoside ('IPTG'), and the culture was grown for an additional 5 h. Inclusion bodies were isolated from bacterial pellets by repeated rounds of sonication in 20 ml of lysis buffer [50 mM Tris-HCl (pH 8.0)/1 mM EDTA/1% (v/v) Triton X-100] followed by centrifugation at 12000 g. The protocol for the solubilization, refolding and purification by reverse-phase chromatography of recombinant BthTx-I (rBthTx-I) was performed as described previously [15].

CD spectroscopy

All measurements were performed in 20 mM HEPES (pH 7.0) containing 150 mM NaCl at 25 °C. Far UV CD spectra (200–250 nm) were measured with a JASCO 810 spectrometer (JASCO Inc., Tokyo, Japan) using 0.1 mm pathlength cuvettes and protein concentrations of 600 µg · ml⁻¹ of BthTx-I. In each case, a total of nine spectra were collected, averaged and corrected by subtraction of a buffer blank.

Release of entrapped fluorescent markers from liposomes

Membrane-damaging activity was evaluated by the release of the liposome-entrapped self-quenching fluorescent dye 2',7'-bis(carboxymethyl)-aminomethylfluorescein (calcein). The loss of liposome membrane-integrity results in dilution of the fluorophore with a consequent increase in the fluorescence signal [6,19]. Liposomes composed of a 9:1 molar ratio of egg yolk phosphatidylcholine (EYPC)/dimyristoyl phosphatidic acid (DMPA), were prepared by reverse-phase evaporation in buffer [25 mM HEPES (pH 7.0)/150 mM NaCl] containing 25 mM calcein (Sigma). The liposomes were passed through a 400 nm polycarbonate filter, and applied to a Sephadex G50 column (1 cm × 8 cm) to separate the liposomes from the free calcein. Proteins and liposomes were mixed to a final protein:lipid molar ratio of 1:400, and the kinetics of membrane damage was monitored by the increase in fluorescence emission at 520 nm with excitation at 490 nm. The fluorescence signal was expressed as the percentage of total calcein liberation on addition of 5 mM Triton X-100.

Myotoxic activity

Protein (30 µg) in a total volume of 50 µl of PBS was injected into the gastrocnemius muscle of male Swiss mice (body weight 18–22 g). After 3 h, a blood sample was collected from the tail in a heparinized capillary tube and the plasma separated by centrifugation. The plasma creatine kinase (CK) activity was determined using 4 µl of plasma with the CK-UV kinetic kit (Sigma Chemical Co.), following the manufacturer's instructions. CK activity was expressed in units · l⁻¹, where one unit is defined as the amount of enzyme which produces 1 mM of NADH · min⁻¹ under the standard conditions used in the assay. Animals used as negative controls received 50 µl of PBS, and all results were expressed as the mean plasma CK activity from a minimum of five experiments.

RESULTS

Native and recombinant BthTx-I, together with all C-terminal mutants, were successfully expressed and purified to yield single bands on SDS/PAGE, and Figure 1 presents the far ultraviolet CD spectra of the purified recombinant proteins as compared with the native protein purified from the snake venom. Figure 1 demonstrates that all protein samples show essentially identical CD spectra, indicating that site-directed mutagenesis of the surface residues in the C-terminal region and the subsequent purification procedures do not significantly perturb the protein secondary structure. These results are similar to those previously observed for the recombinant BthTX-I [15,20], and correlate well with the identical dynamic light-scattering and intrinsic fluorescence emission properties of the native and recombinant proteins which have previously been reported [20]. The effects of these single mutations in the C-terminal region on the myotoxic and Ca²⁺-independent membrane-damaging activities were evaluated *in vivo* and *in vitro* respectively.

The results of the myotoxicity assays for the rBthTx-I and C-terminal mutants were compared with the native protein, and are presented in Figure 2. Injection of both native BthTx-I and rBthTx-I resulted in similar levels of CK release, which provides additional functional evidence that the native and recombinant proteins are structurally conserved. All mutant proteins showed significantly higher levels (*P* ≤ 0.01) of myotoxicity with respect to injections of PBS alone; however, amino acid substitutions in a discrete region between Tyr¹¹⁷ and Lys¹²² demonstrated a reduced myotoxic activity, as compared with native and recombinant BthTx-I. Within this limited region, both the conservative

Figure 1 Far UV CD spectroscopy confirms the native secondary structure for all C-terminal mutants

Comparison of far UV CD spectra of native BthTx-I (bold continuous line), rBthTx-I (continuous line), Lys¹¹⁵ → Ala (dotted line), Lys¹¹⁶ → Ala (bold dotted line), Tyr¹¹⁷ → Trp (dashed line), Arg¹¹⁸ → Ala (dot-dashed line), Tyr¹¹⁹ → Trp (short dotted line), Lys¹²² → Ala (bold dot-dashed line), Phe¹²⁵ → Trp (bold dashed line), Lys¹²⁷ → Ala (double-dot dashed line), and Lys¹²⁹ → Ala (bold double-dot dashed line). See experimental procedures for details of spectra measurement.

Figure 2 Myotoxicity assays comparing the native and mutant BthTx-I

Plasma levels of CK activity 3 h after injection of 30 µg of BthTx-I and C-terminal mutants (as indicated on graph) into the mouse gastrocnemius muscle of mice. The control experiment used the same volume of PBS in the absence of protein. The results show the mean ± S.D. for at least five independent experiments.

mutation of an aromatic residue (Tyr¹¹⁷ → Trp) and the elimination of positively charged side chains (Arg¹¹⁸ → Ala, Lys¹²² → Ala) resulted in a significant reduction ($P < 0.01$) in activity, which suggests that both these residue-types are involved in defining the structural motif which determines the myotoxic function of BthTx-I.

The Ca²⁺-independent membrane-damaging activity was monitored by the liberation of the liposome-entrapped self-quenching

Figure 3 Ca²⁺-independent membrane-damaging activities of the native and mutant BthTx-I

Release of entrapped calcein from liposomes (composed of a 9:1 molar ratio of EYPC and DMPA) 150 s after mixing with BthTx-I and C-terminal mutants at a protein:lipid ratio of 1:400. The final protein concentration in all experiments was 3 µg/ml. The results are presented as the mean percentage ± S.D. of total calcein-release after addition of 5 mM Triton X-100 for at least four independent experiments.

fluorophore, calcein. In these experiments, the loss of liposome membrane-integrity results in dilution of the fluorophore with a consequent increase in the fluorescence signal [6,19]. Figure 3 presents the percentage of calcein liberation 140 s after exposure of the liposome membrane to native BthTx-I, rBthTx-I and all C-terminus mutants. Native BthTx, rBthTx-I and mutants R118A, Y119W and Lys¹²⁹ → Ala demonstrated similar levels of membrane-damaging activity, whereas in a paired *t*-test, the mutants Lys¹¹⁵ → Ala, Lys¹¹⁶ → Ala, Lys¹²² → Ala and Phe¹²⁵ → Trp showed reduced ($P < 0.01$) membrane-damaging activity as compared with the native protein. This result suggests that the determinants of the Ca²⁺-independent membrane-damaging activity in the C-terminal loop may be functionally segregated into discrete regions. Furthermore, in the region between residues 115–122, which was identified as a determinant in myotoxic activity, the Tyr¹¹⁷ → Trp mutant displays a significant increase in membrane-damaging activity. The differing distributions of residues involved in the myotoxic and Ca²⁺-independent membrane-damaging activities, together with the contrary effects of the Tyr¹¹⁷ → Trp mutant, demonstrate that these two functions are not correlated.

The C-terminal loop of PLA₂s extends either from residue 111 to residue 127 in Class I, or from residue 111 to residue 133 in Class II, and runs in a peripheral path enclosing the Ca²⁺-binding loop, as shown in Figure 4. Within the C-terminal loop, the polypeptide main chain adopts a single turn of α -helix between residues 115 and 118 and is a highly conserved feature in both Class I and Class II PLA₂s. Although elevated B-factors in many crystal structures indicate a flexible conformation, the presence of the conserved Cys²⁷–Cys¹²⁶ disulphide bridge constrains the position and orientation of the loop region. A characteristic feature of Class II PLA₂s is a 5–6 residue C-terminal extension, which is covalently linked to the body of the protein by the Cys⁵⁰–Cys¹³³ disulphide bridge. As shown in Figure 4(A), the polypeptide backbone of this extended C-terminal loop in Class

Figure 4 Structural localization and surface mapping of the mutants affecting myotoxic and membrane damaging activities

Ribbon representation of BthTx-I dimer crystal structure indicating the positions of mutated residues in the C-terminal region. The positions of the N- and C-termini in the foremost molecule are indicated in italics (A). Solid surface representation of the BthTx-I monomer in which residues identified as determinants of myotoxic (B) and Ca^{2+} -independent membrane-damaging activity (C) activity are shown in grey. The figure was prepared using SwissPDB protein visualization software [36].

II PLA_2 adopts a kinked semi-circular conformation between residues 118 and 133. Residues altering the myotoxic activity are clustered mainly around the single α -helical turn (residues 117–119), whereas residues influencing the Ca^{2+} -independent membrane-damaging activity are located at the entrance to the short α -helix (residues 115 and 116) and at the kink in the C-terminal loop (residues 122 and 125). Figures 4(B) and 4(C) further illustrate the molecular surface of the BthTx-I crystal structure, in which residues identified as determinants of the myotoxic and membrane-damaging effects are shaded. It can be seen that the structural determinants for both activities are residues within the C-terminal region and that, with the exception of Lys¹²², the residues involved in the determination of myotoxic activity are distinct from those which determine Ca^{2+} -independent membrane-damage.

DISCUSSION

The contributions by individual amino acids towards the definition of the myotoxic site in the C-terminal loop region of Lys⁴⁹- PLA_2 s are poorly understood. Therefore we have used site-directed mutagenesis to evaluate the role of the positively charged and aromatic amino acids in the C-terminal loop region on both the myotoxic and Ca^{2+} -independent membrane-damaging activities of BthTx-I, a Lys⁴⁹- PLA_2 from the venom of *B. jararacussu*. We have identified a linear sequence of amino acids between residues Tyr¹¹⁷ and Lys¹²², in which both the elimination

of positively charged side chains and the conservative substitution of aromatic residues decreases the myotoxic activity. It has previously been reported that chemical modification of either lysine or tyrosine residues in BthTx-I results in decreased myotoxic activity [14], and although this technique is non-selective with respect to the residue positions which are modified, these previous results are in broad agreement with the present site-directed mutagenesis study. Additionally, heparin has been shown to decrease the myotoxic activity of several Lys⁴⁹- PLA_2 s [21], and all members of this protein family, including BthTx-I, present a consensus heparin-binding motif (115–Lys–Lys–x–Arg–118, where x is a tyrosine or a histidine residue) located in the C-terminal loop region. Charge-elimination mutagenesis of the Tyr¹¹⁷ and Arg¹¹⁸ residues within the heparin-consensus repeat of BthTx-I results in a decreased myotoxic activity, and due to the highly conserved nature of this motif, we suggest that this region contributes residues to a core sequence in the determination of myotoxic activity in the Lys⁴⁹- PLA_2 s. The observation that substitutions in a short amino-acid sequence in the C-terminal region influence the myotoxic activity of BthTx-I suggests that this region determines a specific interaction with the target membrane. Recent models for the myotoxic activity of Lys⁴⁹- PLA_2 s assume that a myofibril membrane protein acts as an acceptor for myotoxic PLA_2 s [22], and that binding to this protein provokes pathological cellular responses which ultimately lead to cell death [8]; however, the putative myotoxic acceptor for the Lys⁴⁹- PLA_2 remains to be identified.

Recent mutagenesis studies have indicated that the C-terminal loop regions of other snake venom PLA_2 s act as structural determinants in diverse pharmacological activities. Charge-reversal mutagenesis of cationic residues in the C-terminal loop region of ammodytoxin A from *Vipera ammodytes ammodytes* resulted in a decreased neurotoxic activity [23]. Furthermore, a decreased effect on the inhibition of platelet-aggregation was observed by mutagenesis of negatively charged (Asp¹¹⁵ → Lys) and aromatic (Trp¹¹⁹ → Thr) residues in the C-terminal region of the acidic PLA_2 from *Agkistrodon halys Pallas* [24]. It is worthy of note that in both of these Asp⁴⁹- PLA_2 s, mutation of the C-terminal region resulted in the unambiguous separation of catalytic and pharmacological activities, and that in the case of BthTx-I, myotoxic activity is present in the absence of measurable catalytic activity. Furthermore, in all three venom PLA_2 s investigated, the pharmacological effects were reduced, although not abolished, by mutagenesis in the C-terminal region, which suggests that additional regions in the respective PLA_2 s contribute to the structural determinants of the pharmacological activities. Such observations lead us to suggest that these venom PLA_2 s which demonstrate diverse pharmacological effects have overlapping structural determinants in the C-terminal region. Indeed, this concept provides a possible explanation for the combined pharmacological activities observed in many snake venom PLA_2 s.

A key question with respect to the biological function of BthTx-I is whether there is a possible correlation between the myotoxic and Ca^{2+} -independent membrane-damaging activities. Although the C-terminal region is clearly important for both functions, individual amino acid substitutions result in differing effects on myotoxic and membrane-damaging activities, which demonstrates that no correlation exists between these two effects. However, further insights into the Ca^{2+} -independent membrane-damaging activity were revealed from calcein-release experiments, which suggested that the C-terminal loop is divided into two functional regions. Alanine-substitution of positively charged amino acids between positions 115–117 and at position 122 resulted in a significant reduction in the Ca^{2+} -independent

membrane-damaging activity, whereas the substitutions of Arg¹¹⁸ → Ala and of cationic residues between positions 127 and 133 had no significant effect. This suggests that the cationic residues flanking positions 118–119 serve a role in the membrane-damaging mechanism, and is consistent with the effects of cationic residue charge-reversal mutagenesis in the C-terminal region of Asp⁴⁹-PLA₂s, in which a reduced affinity for zwitterionic lipid membranes was observed [25]. The result also provides supporting evidence for the electrostatic interaction of the C-terminal region of Asp⁴⁹-PLA₂s with the lipid membrane. Although the distribution of the charges influencing the binding effect is different in Asp⁴⁹-PLA₂s, our data indicates that electrostatic forces may also play a significant role in the association and membrane-damaging activity of the Lys⁴⁹-PLA₂s.

In Class I/II PLA₂s, the interfacial binding face (i-face) is defined by hydrophobic residues at the entrance to the active-site cleft and substrate binding pocket, which are encircled by a ring of positively charged residues, including residues 116–119 of the C-terminal loop region [26,27]. Substitution of hydrophobic and aromatic residues with tryptophan in the i-face of bovine [28] and human type-II secreted PLA₂ [29] results in an increased affinity for model membranes, whereas elimination of tryptophan residues in the i-face of *Naja atra atra* decreases this affinity [30]. Furthermore, insertion of a tryptophan residue at position 117 of the C-terminal region of the porcine pancreatic PLA₂ increases the affinity for model membranes [31]. Therefore, the effects of substituting aromatic residues with tryptophan on BthTx-I Ca²⁺-independent membrane-damage may be interpreted in the light of the current understanding of the role of aromatic residues in the interaction of membrane proteins with both model and synthetic membranes [32]. The polar atoms in the aromatic rings of both tyrosine and tryptophan results in a preferential location between the polar head groups and the acyl chains in phospholipid membranes, and the double ring system of the indole side chain of tryptophan results in an enhanced effect for this interfacial localization [33]. In contrast, the unsubstituted aromatic ring of phenylalanine leads to a preference for a deeper and more hydrophobic location in the bilayer. The Phe¹²⁵ → Trp mutant in BthTx-I shows a decreased Ca²⁺-independent membrane-damaging activity; a possible explanation is that upon interaction of the native protein with membrane, Phe¹²⁵ is inserted into the depth of the acyl chains, but that substitution with tryptophan results in a more superficial location, with the resultant loss of activity. In contrast, the increased activity observed in the Tyr¹¹⁷ → Trp indicates that an enhanced interfacial localization of this position results in an increased activity. These conclusions are consistent with recent studies in which a synthetic peptide from the C-terminal region of myotoxin II from *B. asper*, in which all aromatic residues were substituted by tryptophan residues, demonstrated an enhanced affinity for membranes [34]. Taken together, the results from the cationic charge-elimination and aromatic-substitution experiments suggest that the C-terminal region of BthTx-I may be functionally divided into two regions: a region around residue 125 (corresponding to the kink in the C-terminal loop), which partially inserts into the outer leaflet of the lipid bilayer, and a region including residues 115–119, which interacts in a more superficial manner, immobilizing the distal region of the C-terminal loop, and thereby favouring the partial insertion of the kink region. This idea is supported by the comparison of the *in vivo* effects of synthetic peptides from the C-terminal regions of Lys⁴⁹-PLA₂s from *B. asper* and *Agkistrodon piscivorus piscivorus*, which suggests that a large hydrophobic residue at position 121 is important in the membrane destabilization process of Lys⁴⁹-PLA₂s [35].

In conclusion, site-directed mutagenesis has identified specific cationic and aromatic residues in the C-terminal region of BthTx-I that are involved in both myotoxic and Ca²⁺-independent membrane-damaging activities. Furthermore, the differing effects of mutants in the C-terminal loop demonstrate that these two activities are independent with discrete, yet partially overlapping, structural determinants on the surface of the molecule. The results lend support to a model of Ca²⁺-independent membrane-damage by BthTx-I [7] in which the C-terminal region interacts with and penetrates the outer leaflet of the membrane bilayer. Further site-directed mutagenesis of the protein surface is in progress in order to map in greater detail the structural determinants for the myotoxic and Ca²⁺-independent membrane-damaging activities of BthTx-I.

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