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Relationship between the structure and the enzymatic activity of crotoxin complex and its phospholipase A_2 subunit: An *in silico* approach

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ABSTRACT

Crotoxin, one of the major toxins of South American rattlesnake *Crotalus durissus* subspecies, is an heterodimeric complex composed of two distinct subunits: a basic phospholipase A_2 (PLA_2, CB) and an acidic nontoxic catalytically inactive protein, crotapotin (CA). It's well known that CB has a high enzymatic activity; however the molecular aspects that determine this fact remain unknown. In this study, an *in silico* approach was used to predict the CA structure by homology modeling, and the crotoxin structure by means of molecular docking. CA structure was built using the software Modeller taking *Crotalus atrox* PLA_2 (1PP2:R) as a template. Different criteria measured by Procheck, Verify 3D and ProSA were indicative of the reliability and the proper fold for the predicted structural model of CA. Then, a combination of this model and CB crystal structure was used to build the structure of crotoxin complex through rigid-body protein–protein docking. The crotoxin-3D model suggested that by means of hydrophobic and π–π stacking interactions, CA-Y24 and CA-F119 interact with CB-F24 and CB-F119, respectively. Those interactions could prevent the interfacial adsorption of the CB onto the lipid/water interface by blocking part of the interfacial binding surface of the PLA_2. This fact could explain the differences regarding to enzymatic activity between the crotoxin complex and CB. In addition, the crotoxin-3D model showed solvent-exposed regions of CA that could bind the receptor expressed in target cells.

1. Introduction

Crotoxin, the major toxin of South American rattlesnakes *Crotalus durissus* subspecies, is an heterodimeric complex composed of two distinct subunits: a basic phospholipase A_2 (PLA_2, CB) and an acidic nontoxic catalytically inactive protein, crotapotin (CA) [1]. This toxin is responsible for neurotoxicity and myotoxicity of these venoms [2–6]. The enzymatic activity of CB subunit alone is higher than the crotoxin complex but weakly toxic; however CA potentiates its toxicity enhancing its pharmacological activity and lethal potency [1,7,8]. CA consists of three polypeptide chains (α, β and γ) linked together by disulfide bridges and acts as an inhibitory 'chaperone' subunit. CA prevents non-specific interactions between PLA_2 and phospholipids on membrane surfaces other than its target (neuromuscular junction). The currently accepted model suggests that when the crotoxin complex reaches the membrane target, a transient ternary complex between the receptor and the crotoxin's subunits is formed, then CA is released in solution [9]. However, the identity of this receptor/acceptor has not been established yet.

Interspecies and intraspecies variation has been described for crotoxin complex. Intraspecies variations arises from the presence of diferent isoforms of CA or CB as it was demonstrated in the venom of *C. d. terrificus* [10], *C. d. cascavella* [2], *C. d. collilineatus* [4] and *C. d. ruruima* [5]. Those isoforms differ in potency and biological activity [10]. Such multiplicity and diversity could be explained because CA isoforms are apparently the result of posttranslational modifications of a unique PLA_2-type precursor of crotoxin or also due to the expression of different mRNAs [11]. Moreover the observed synergy of those subunits can be consequence of different affinity associations of those. Evidence from Choumet et al. [12] and Faure and Bon [13] suggest that the instability of this complex favors the dissociation of the subunits before the target is reached. Interestingly, when CB associates CA at higher affinity, the CTXs present greater toxicity and minor enzymatic activity [14].

On the other hand, interspecies variation derives from the geographic location, genetic differences, among others. Thus, identification of crotoxin isoforms and homologs have been reported in several *C. durissus* subspecies [2,4–6,10–18]. Nevertheless, the enzymatic and pharmacological activities of these proteins are

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generally similar to those described for crotoxin from *C. d. terrificus* venom.

Data about crystallization of crotoxin proteins has been obtained during the last years [19–24]. In the earliest studies was impossible to obtain the structure of crotoxin complex due to the fact it seems that the highly diffuse diffraction pattern of CA crystals does not allow that the adquisition of the three-dimensional structure for this subunit [20]. However, the CB structure from *C. d. terrificus* and *C. d. collilineatus* are known [21–23]. After this, recently the structure of a crotoxin isoform from *C. d. terrificus* has been described [24].

Even after all the recent advances on crystallographic (X-ray) and nuclear magnetic resonance (NMR) for structure determination only a very small proportion of the determined structures correspond to protein–protein complexes. X-ray and NMR techniques have practical difficulties that make impossible to obtain the structure of all known proteins. That is because X-ray needs large amounts of protein and does not give information about the protein dynamics and his hydrogen composition. In contrast, the biggest problem of NMR is its powerlessness to resolve the structure of the big proteins or protein complexes [25]. A viable solution when we know the atomic structures of the individual proteins involved in an interaction, either by experiment or by modeling, several computational methods are available that suggest the structure of the interaction [26]. These methods are each time more accurate to predict native or native-like complexes [27]. The generated model could give important structural information that could guide new experiments and also give additional information for the complexes whose structures were experimentally determine (movements of the domains, alternative complexes, among others) [26,27].

In this study an *in silico* approach was performed to obtain the three-dimensional structure of CA. After the evaluation of this model, it was used to generate a structure of the crotoxin complex. In addition this information was employed to perform a structure–activity relationship study and suggest a hypothesis regarding the differences in enzymatic activity between CB and crotoxin complex.

2. Materials and methods

2.1. Sequence alignment and homology modeling of CA

The model for the three-chain molecule of CA was derived from a previous one for the one-chain CA precursor, a seven disulfide-bonded PLA_2. The amino acid sequence of the CA precursor from *C. d. terrificus* was retrieved from the Swiss-Prot database (Primary Accession No. P08878). The NCBI Basic Local Alignment Search Tool (BLAST), for the sequence similarities was used for searching the crystal structures of the closest homologues available in the Brookhaven Protein Data Bank (PDB). The results yielded by NCBI BLAST revealed chain R from *Crotalus atrox* PLA_2 (PDB ID: 1PP2:R) with a resolution of 2.5 Å as a suitable template and with an identity score of 69% and E value 9×10^{-47}. For the alignment between sequences of CA precursor, CB1 (an isoform of CB, Primary Accession No. P24027) and PLA_2 from *C. atrox* (Primary Accession No. P00624), CLUSTALW was used from its web site at http://www.ebi.ac.uk/Tools/msa/clustalw2/ [28]. Renetseder et al. [29] method of numbering of amino-acid residues was used. The three-dimensional model of CA from *C. d. terrificus* was built using the crystal structure coordinates of chain R from *C. atrox* PLA_2. This step was performed using EasyModeller [30] a graphical user interface for the program Modeller (9v8) [31]. This program is completely automated and is capable of generating energy minimized protein models by satisfying spatial restraints on bond distances and dihedral angles extracted from the template PDB file. Modeller performs an automatic loop modeling and model optimization. The input for the program Modeller consisted of the sequence of CA precursor and PDB file from chain R from *C. atrox* PLA_2. Many runs of Modeller were carried out in order to obtain the most plausible model. After the model generation, the three-chain molecule of CA was obtained by removing the proteolyzed peptides.

2.2. Model evaluation

The stereochemical excellence of the protein structure and overall structural geometry were confirmed by Procheck program [32]. The Verify 3D program was used to determine the compatibility of an atomic model (3D) with its own amino acid sequence (1D) by assigning a structural class on the basis of its location and environment (alpha, beta, loop, polar, non-polar, *etc.*) as well as comparing the results with good database structures [33]. The energy of residues was checked by ProSA, using the web service ProSA-web [34,35].

2.3. Molecular docking

The evaluated three-chain molecule of CA and CB (chain B from PDB ID: 2QOG) were used to build a model for crotoxin complex. The structures were prepared by using the Protein Preparation module implemented in the Maestro program. Firstly, hydrogen atoms are automatically added to each protein according to the chemical nature of each amino acid, on the basis of the ionized form expected in physiological condition. This module also controls the atomic charges assignment. Secondly, each 3D structure of the protein was relaxed through constrained local minimization using the OPLS force fields in order to remove possible structural mismatches due to the automatic procedure employed to add the hydrogen atoms. The rigid-body molecular docking procedure was performed using the program PatchDock [36]. For this process CB and CA were chosen as receptor and ligand, respectively. CB was used as receptor because in other neurotoxic complexes present in snake venoms, the PLA_2 subunit is taken as receptor for the other subunits [37]. Then, the PatchDock solutions were submitted to a refinement process, using the tool FiberDock [38,39]. PatchDock is a geometry-based molecular docking algorithm. It is aimed at finding docking transformations that yield good molecular shape complementarity. Such transformations, when applied, induce both wide interface areas and small amounts of steric clashes. A wide interface is ensured to include several matched local features of the docked molecules that have complementary characteristics. The PatchDock algorithm divides the surface representation of the molecules into concave, convex and flat patches. Then, complementary patches are matched in order to generate candidate transformations. Each candidate transformation is further evaluated by a scoring function that considers both geometric fit and atomic desolvation [36]. On the other hand, FiberDock models both side-chain and backbone flexibility and performs rigid-body optimization on the ligand orientation. The backbone and side-chain movements are modeled according to the Van der Waals forces between the receptor and ligand. Finally, FiberDock ranks the refined solutions by a binding energy scoring function. This score includes Atomic Contact Energy, softened van der Waals interactions, partial electrostatics and additional estimations of the binding free energy [38,39]. After Fiberdock refinement the solution with the highest global energy (binding energy) was chosen. Table with ranked solutions is provided as Supplementary material.

2.4. Analysis of protein interfaces

The interface between CA and CB in crotoxin complex was characterized with the Protein interfaces, surfaces and assemblies

service PISA used from its web site http://www.ebi.ac.uk/msd-srv/prot_int/pistart.html [40]. The interface contacts between two polypeptide chains were obtained through a contact map analysis and characterized with the SPACE bioinformatics tool CMA (contact map analysis) [41]. Only interface contacts for which the contact area is equal to or greater than 10 Å^2 are shown.

2.5. Phospholipase A_2 activity

For enzymatic analysis, isoforms of crotoxin and CB were isolated from Colombian *C. d. cumanensis* venom, as described by Pereañez et al. [18].

2.6. Activity on monodisperse substrate

The monodisperse and chromogenic substrate 4-nitro-3-octanoyloxy-benzoic acid was used to determine the kinetic behavior of CB and crotoxin. The assay described by Cho and Kézdy [42] and Holzer and Mackessy [43], modified for 96-well plates was used. The standard assay mixture contained 200 μL of buffer (10 mM Tris–HCl, 10 mM $CaCl_2$, 100 mM NaCl, pH 8.0), 20 μL of substrate at different concentrations in acetonitrile, 20 μL of water and 20 μL of sample (70 μM of each protein). After the addition of the sample, the mixture was incubated 40 min at 37 °C, and the absorbance was read at 425 nm at 10 min intervals. The enzymatic activity, expressed as the initial velocity of the reaction (V_o), was calculated based on the increase in absorbance after 20 min. The V_{max} and K_m parameters were calculated for CB and crotoxin. The assay was performed by triplicate.

2.7. Activity on phosphatidylcholine

This activity was assayed according to the method reported by Dole [44], with titration of free fatty acids (FA) released from phosphatidylcholine (from dried egg yolk, Sigma) suspended in 1% Triton X-100, 0.1 M Tris–HCl, 0.01 M $CaCl_2$, pH 8.5 buffer, using 70 μM of crotoxin and CB, respectively. The time of reaction was 15 min at 37 °C. The enzymatic activity was expressed as μEq-g FA/min of six independent determinations.

2.8. Statistical analysis

In order to obtain the V_{max} and K_m values, a Michaelis–Menten analysis using the least squares fit was applied. The two-tailed Student's *t*-test was performed, with the aim to determine significant differences between hydrolytic activities of crotoxin and CB on phosphatidylcholine. This same test was applied in order to determine significant differences between the K_{cat}/K_m values of crotoxin and CB. A difference with a $p < 0.05$ was considered significant. Results are shown as mean ± SEM of n indicated in each case.

3. Results

3.1. Sequence alignment, homology modeling of CA and model evaluation

The sequence alignment showed a high number of conserved residues in CA, CB1 and *C. atrox* PLA_2 (Fig. 1A). CA precursor displayed identities of 68% and 45% with CB and *C. atrox* PLA_2,

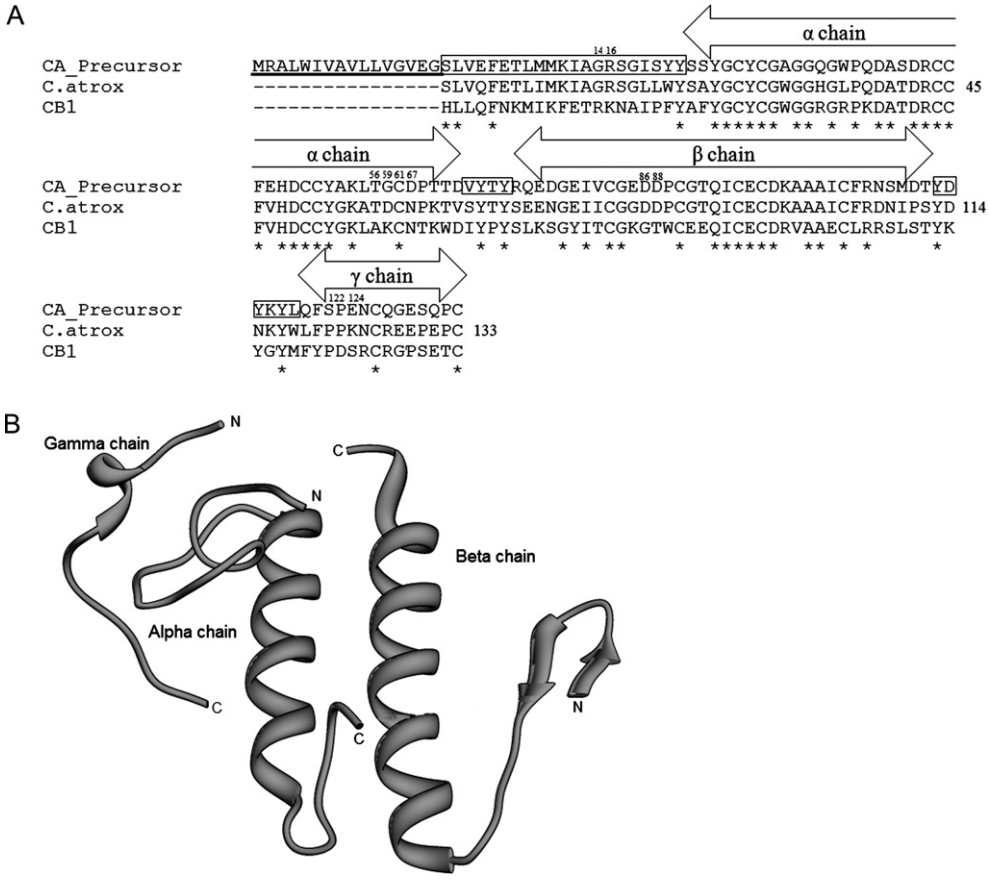

Fig. 1. (A) Sequence alignment of target (CA precursor), CB and template (*C. atrox* PLA_2). Conserved residues are marked with an asterisk (*). The signal peptide of CA precursor are underlined. The α, β and γ chains of matured CA are shown. Renetseder et al. [29] method of numbering of amino-acid residues was used. (B) Model of three-dimensional structure of CA. The α, β and γ chains of matured CA are shown. The N and C terminals of each chain are indicated.

respectively. When the proteolyzed peptides of CA were removed, great changes in identities were not observed (68% and 51% with CB and *C. atrox* PLA_2, respectively). The chain R of *C. atrox* PLA_2 was used as template for modeling the CA three-dimensional structure. This automated process resulted in the CA structure with α, β and γ chains (Fig. 1B). The quality of the homology model was evaluated by Procheck, the detailed residue-by-residue stereochemical quality of the CA model was found to be good (91.3% in most favored regions, 7.2% in additional allowed regions and 1.4% in disallowed regions, Asp79) (Fig. 2A). The Verify 3D program was used to determine the compatibility of an atomic model (3D) with its own amino acid sequence (1D). The scores were between 0.06 and 0.50 (Fig. 2B). The energetic architecture of protein folds was determined by using the program ProSA. This analysis of the model revealed a *Z*-score value of −5.35 and it is in the range of native conformations of the template (−5.12) (data not shown). The energy profile of the CA predicted model was found to be good (Fig. 2C). This model showed that most of the residues are in the negative region.

3.2. Molecular docking and protein interface analysis

A model for the structure of crotoxin complex was generated by PatchDock and further refinement with FiberDock. An interaction of the C-terminal region of CB and the α-helix of α chain of CA was shown (Fig. 3A). No hydrogen bonds were displayed; however, important Van der Waals and π–π stacking interactions were exhibited between amino acids of diverse nature. This was confirmed with the contact map analysis of the complex. In this analysis it was observed that most of the favorable contacts are aromatic–aromatic or hydrophobic–hydrophobic residues (Fig. 3B and C). Nevertheless, a destabilizing contact between Arg125 from CB and His48 from CA was detected. Finally, an interface area of 765.2 $Å^2$ was shown in the contact analysis.

3.3. Phospholipase A_2 activity of crotoxin complex and its catalytic subunit

A monodisperse and chromogenic substrate was used to perform a kinetic analysis of crotoxin and CB. Crotoxin showed K_m and V_m values of 9.59 mM and 18.22 μM/min. Whereas, CB displayed K_m and V_m values of 15.85 mM and 15.85 μM/min. In addition, the K_{cat} values for these toxins were determined (15.6 seg^{-1} and 13.8 seg^{-1} for crotoxin and CB, respectively). Furthermore, the catalytic efficiency was measured by calculating the K_{cat}/K_m parameter [1.63×10^3 M^{-1} s^{-1} and 2.10×10^3 M^{-1} s^{-1} for crotoxin and CB ($p > 0.05$), respectively]. These results indicate that crotoxin and CB have similar catalytic efficiency to hydrolyze the monodisperse and chromogenic substrate. When the aggregated substrate was used, CB showed higher activity than crotoxin [0.21 ± 0.01 μEq-FA/min and 0.14 ± 0.01 μEq-FA/min, respectively ($p < 0.05$)].

4. Discussion

In this study, an *in silico* approach was used to obtain the three-dimensional structure of CA subunit of crotoxin complex. The stereochemical quality of the model was acceptable as shown in the Ramachandran plot being only Asp79 in disallowed regions. Then, to analyze the compatibility of an atomic model (3D) with its own amino acid sequence (1D) the CA model was evaluated with Verify 3D. For each residue an structural class is assigned based on its location and environment (alpha, beta, loop, polar, nonpolar, *etc.*). The scores for the residues from CA model always were above zero. This is in accordance with the reliable models, which should have positive scores [33,45]. Finally the CA model was analyzed using ProSA software. The *Z*-score value of −5.35 showed

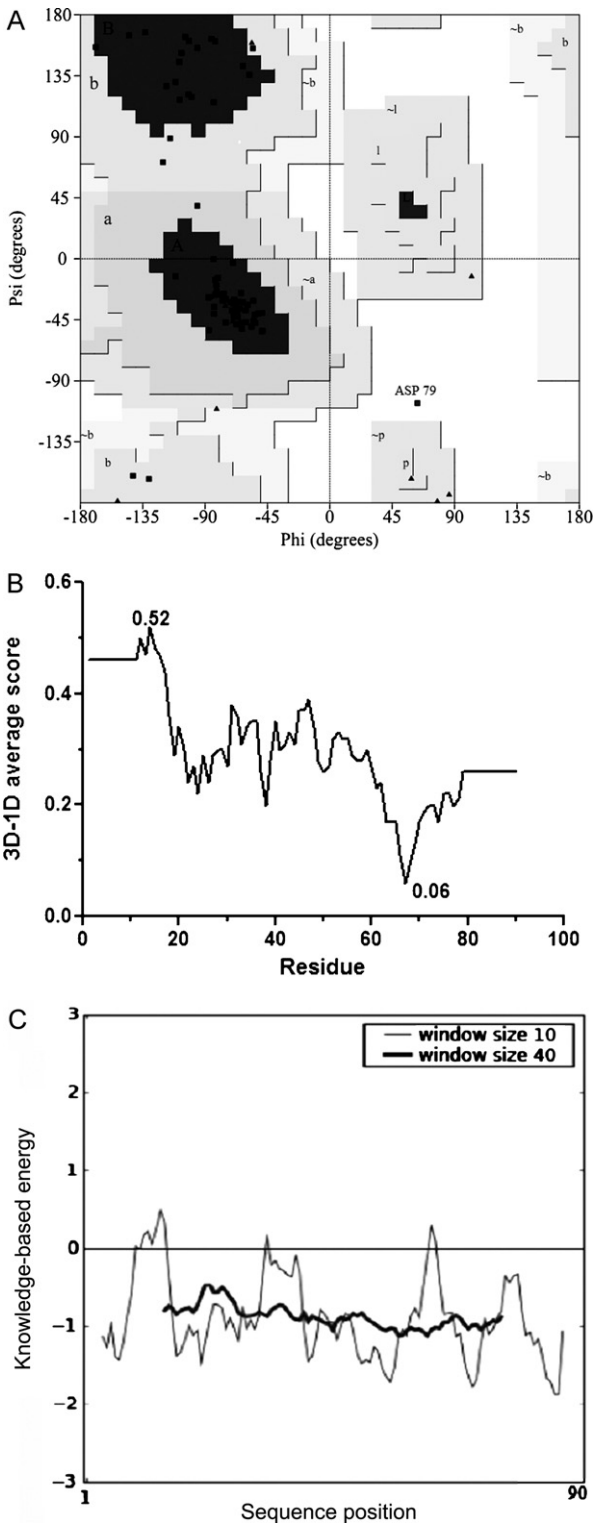

Fig. 2. (A) The Ramachandran plot of modeled CA. The favored and most favored region is dark gray and gray respectively, pale gray is the generally allowed and disallowed regions is white. (B) Verifyed-3D analysis. Positive scores suggest that the residues are compatible with their environments in the model build for CA. The lowest and the highest values are shown. (C) ProSA energy plot calculated for the CA homology model.

by the CA model is in between values for native proteins of similar size (including the *C. atrox* PLA_2). The energy plot displayed by ProSA shows the local model quality by plotting energies in function of the amino acid sequence position. In brief, positive values correspond to problematic or erroneous parts of a model. When the

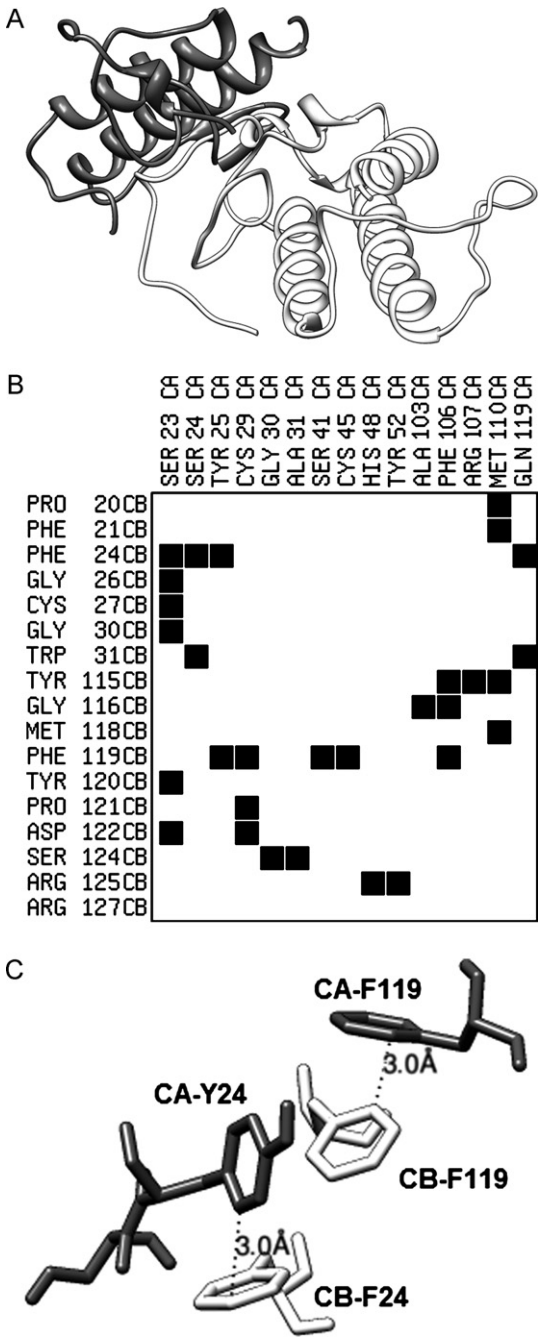

Fig. 3. (A) Model of the crotoxin complex obtained by rigid-body docking between CB and CA. CB and CA are white and gray, respectively. (B) Contact map analysis of the crotoxin complex. Only residues for which the contact area is equal or greater than 10 Å^2 are shown. (C) $\pi-\pi$ stacking interactions between CA and CB in crotoxin complex. CA residues are shown in gray and CB amino acids are displayed in white.

fragment of 10 residues was evaluated, most of them were in the negative region. However, when a fragment of 40 residues was evaluated none of the residues is outside of the negative region. To sum up, the stereochemical quality, the compatibility of the 3D structure with its amino acid sequence and the energy profile of the CA three-dimensional model confirm that this is a reliable model. The modeled structure of CA was similar to those previously described [46,47]; however, in these studies the quality and the reliability of the model were not demonstrated.

The modeled structure of CA and the crystal structure of CB were used to build a model of crotoxin complex. The rigid-body protein–protein docking is one of the most used approaches to build protein complexes [25,26,48]. This methodology was used to generate a model of crotoxin complex. However, due to the natural flexibility of proteins to predict the structure of a protein is a great challenge. Nevertheless, this problem was confronted in part by using a flexible induced-fit backbone refinement method (FibeDock) [38,39]. This method optimizes the side chain conformations in the interface, models backbone movements and minimizes the relative rigid body orientations of the molecules. The resulting solution of rigid-body protein–protein docking and further refinement showed an important interaction between C-terminal region of CB and α-helix of α chain of CA.

Crotoxin and CB showed similar catalytic efficiency to hydrolyze a monodisperse substrate. This is in accordance with Canziani et al. [49,50] who reported that crotoxin and CB have similar rates of hydrolysis of dihexanoyllecithin monomers. On the other hand, crotoxin displayed lower enzymatic activity than CB on aggregated substrate (phosphatidylcholine). Similar results were described by Breithaupt [51], Canziani et al. [49] and Radvanyi and Bon [52]. The molecular aspects that determine the minor enzymatic activity of crotoxin in comparison with CB remain unknown. In the earliest studies, Jeng and Fraenkel-Conrat [53] examined the effects of p-bromophenacyl bromide (p-BPB) on the crotoxin complex and its subunits. They observed that it was not possible to alkylate His48, the catalytic residue of CB, with p-BPB when this subunit was part of the crotoxin complex. However, that alkylation occurred when the complex was dissociated, resulting in catalytically inactive CB. These results suggested that CA occludes the CB active site. In addition, these observations agree with the three-dimensional model of crotoxin complex proposed by Mascarenhas et al. [46], in which CB active site was shielded by CA. However, the results described above do not explain the intrinsic PLA_2 activity of crotoxin complex. In contrast, Radvanyi and Bon [52] reported that p-BPB inactivates the CB subunit, indicating that CA did not mask the catalytic site of the PLA_2. Equivalent conclusions were described by Canziani et al. [49], who demonstrated that crotoxin complex is able to bind phosphatidylcholine monomers, which supports the free accessibility of the active site of CB in the crotoxin complex.

PLA_2s require interfacial activation for their activity. This activation is performed by mean of the "interfacial binding surface" (i-face), which mediates the adsorption of the enzyme onto a lipid/water interface from the cell membrane [54]. In this interaction are involved positively charged, hydrophobic and aromatic residues [55]. The model of crotoxin described in this study showed a cluster of hydrophobic and $\pi-\pi$ stacking interactions as it is shown in Fig. 3C. In such interactions are involved CB F24 and F119, and CA Y24 and F119. In addition, as shown in Fig. 4A, the Phe-mentioned CB amino acids are implicated in the i-face of the PLA_2. Therefore, the CA–CB interactions in crotoxin complex impede the interfacial activation of CB in the crotoxin by blocking some residues of the i-face of the PLA_2 (F24 and F119). This suggestion is in agree with Canzani et al. [50] who proposed the mechanism of inhibition of phospholipase A_2 activity of CB by CA. Based on a large series of kinetics and binding studies using the crotoxin complex and its subunits, these authors concluded that in the crotoxin complex, the binding of the subunit B to the phosphatidylcholine–water interface is prevented by CA, thus, the interfacial activation of the enzyme is not allowed. Consequently, this could provoke that crotoxin leaves the vesicle and hydrolyzes the substrate in the aqueous solution. In addition, this could explain why the rate of hydrolysis with the crotoxin complex is slower than with CB, as it was observed in this and other studies [49,51,52]. Furthermore, this fact could also clarify the intrinsic PLA_2 activity of crotoxin *in vitro*. Canzani et al. [49] also suggested that the interaction of CB with phosphatidylcholine–water interfaces may require the exposure of a specific area on the enzyme surface (known today as i-face) different from the active site and shielded in the crotoxin

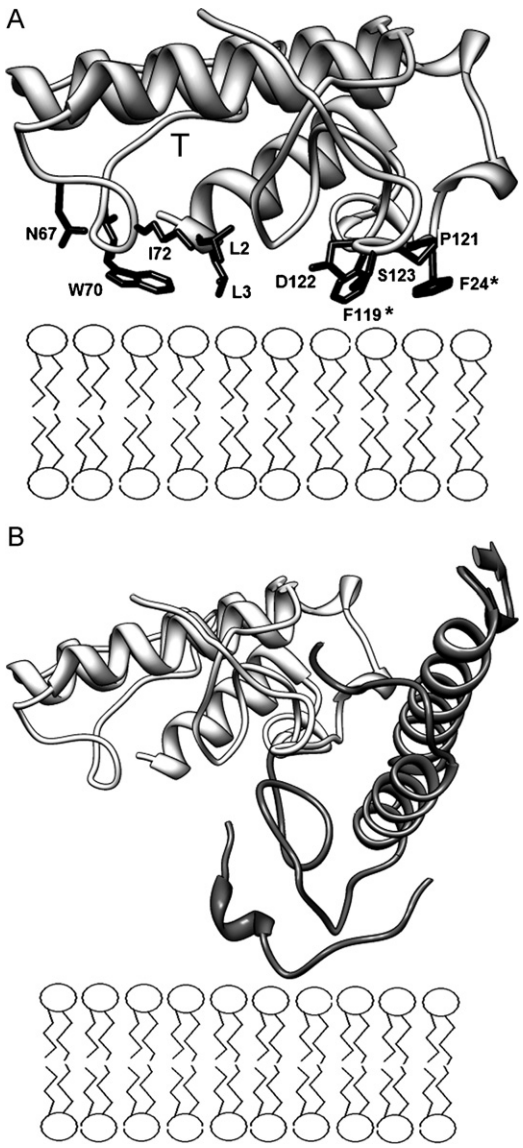

Fig. 4. (A) Amino acids of CB involved in the interfacial binding surface. The i-face of CB was inferred after structural and sequence alignment between CB and human group IIA phospholipase A_2, whose i-face was described by Winget et al. [55]. An asterisk (*) indicates the CB residues that are blocked by CA in the crotoxin complex. (B) Model of crotoxin complex in the lipid/water interface. CB and CA are white and gray, respectively. Note that CA impedes the interfacial activation of CB.

complex hence this area would probably be located within the region of interaction of CA and CB. This assumption is consistent with three-dimensional model of crotoxin exposed in this study since –as mentioned above– there are residues of the i-face that are also involved in the region of the interaction between CA and CB.

In a recent work about the crotoxin the 3D structure of the complex was revealed by X-ray [24]. The 3D arrangement of the complex showed that CA subunit not only sterically occludes substrate access to the catalytic site but also inhibits the binding of the most i-face residues to phospholipid aggregates. This condition does not explain the intrinsic PLA_2 activity of the crotoxin complex because the hydrolytic activity requires interfacial activation of the enzyme and free access to active site. Even though in our model some residues of the i-face of PLA_2 are blocked by the interaction with CA, the active site of CB has a free access. This fact could explain the intrinsic PLA_2 activity of crotoxin complex, since the prevention of the interfacial activation could provoke that crotoxin leaves the vesicle and hydrolyzes the substrate in the aqueous solution. Nevertheless, the crystallographic structure of crotoxin and the theoretical model described in this study share some residues on the interface between CA and CB, in these two structures CB-F24 and CB-F119 are interacting with different residues of CA [24].

In conclusion, a reliable three-dimensional model for CA was built using homology modeling. After, a combination of this model and CB-crystal structure was used to generate the structure of crotoxin complex. The crotoxin-3D model suggests that by mean of hydrophobic and $\pi-\pi$ stacking interactions, CA-Y24 and CA-F119 interact with CB-F24 and CB-F119, respectively. These interactions would prevent the interfacial adsorption the CB onto lipid/water interface by blocking part of the i-face of the PLA_2 (Fig. 4A and B). However, this would provoke that crotoxin leaves the vesicle and hydrolyzes the substrate in the aqueous solution. This fact could explain the differences respect to enzymatic activity between different crotoxin complexes in comparison with CB. In addition, the crotoxin-3D model showed solvent-exposed regions of CA that could bind the receptor expressed in target cells.

Conflict of interest statement

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmgm.2012.01.004.

References

[1] R.A. Hendon, H. Fraenkel-Conrat, Biological roles of the two components of crotoxin, Proc. Natl. Acad. Sci. U. S. A 68 (1971) 1560–1563.
[2] D.G. Beghini, M.H. Toyama, S. Hyslop, L. Sodek, J.C. Novello, S. Marangoni, Enzymatic characterization of a novel phospholipase A_2 from *Crotalus durissus cascavella* rattlesnake (Maracambóia), venom, J. Protein Chem. 19 (2000) 603–607.
[3] D.G. Oliveira, M.H. Toyama, J.C. Novello, L.O.S. Beriam, S. Marangoni, Structural and functional characterization of basic PLA_2 isolated from *Crotalus durissus terrificus* venom, J. Protein Chem. 21 (2002) 161–168.
[4] L.A. Ponce-Soto, M.H. Toyama, S. Hyslop, J.C. Novello, S. Marangoni, Isolation and preliminary enzymatic characterization of a novel PLA_2 from *Crotalus durissus collilineatus* venom, J. Protein Chem. 21 (2002) 131–136.
[5] L.A. Ponce-Soto, P.A. Baldasso, F.F. Romero-Vargas, F.V. Winck, J.C. Novello, S. Marangoni, Biochemical, pharmacological and structural characterization of two PLA_2 isoforms Cdr-12 and Cdr-13 from *Crotalus durissus ruruima* snake, Protein J. 26 (2007) 39–49.
[6] M.H. Toyama, D.G. Oliveira, L.O.S. Beriam, J.C. Novello, L. Rodrigues-Simioni, S. Marangoni, Structural, enzymatic and biological properties of new PLA_2 isoform from *Crotalus durissus terrificus* venom, Toxicon 41 (2003) 1033–1038.
[7] E. Habermann, H. Breithaupt, The crotoxin complex: an example of biochemical and pharmacological protein complementation, Toxicon 16 (1978) 19–30.
[8] C. Bon, in: R.M. Kini (Ed.), Venom Phospholipase A2 Enzymes: Structure, Function and Mechanism, Wiley, Chichester, 1997, pp. 269–285.
[9] E. Délot, C. Bon, Model for the interaction of crotoxin, a phospholipase A_2 neurotoxin, with presynaptic membranes, Biochemistry 32 (1993) 10708–10713.
[10] G. Faure, C. Bon, Several isoforms of crotoxin are present in individual venoms from the South American rattlesnake *Crotalus durissus terrificus*, Toxicon 25 (1987) 229–234.
[11] G. Faure, J.L. Guillaume, L. Camoin, B. Saliou, C. Bon, Multiplicity of acidic subunit isoforms of crotoxin, the phospholipase A_2 neurotoxin from *Crotalus durissus terrificus* venom, results from posttranslational modifications, Biochemistry 30 (1991) 8074–8083.
[12] V. Choumet, C. Bouchier, E. Delot, G. Faure, B. Saliou, C. Bon, Structure and function relationship of crotoxin, a heterodimeric neurotoxic phospholipase A2 from the venom of a South-American rattlesnake, Adv. Exp. Med. Biol. 391 (1996) 197–202.
[13] G. Faure, A.L. Harvey, E. Thomson, B. Saliou, F. Radvanyi, C. Bon, Comparison of crotoxin isoforms reveals that stability of the complex plays a major role in its pharmacological action, Eur. J. Biochem. 214 (1993) 491–496.

[14] G. Faure, V. Choumet, C. Bouchier, L. Camoin, J.L. Guillaume, B. Monegier, M. Vuilhorgne, C. Bon, The origin of the diversity of crotoxin isoforms in the venom of *Crotalus durissus terrificus*, Eur. J. Biochem. 223 (1994) 161–164.
[15] S. Hernandez-Oliveira, M.H. Toyama, D.O. Toyama, S. Marangoni, S. Hyslop, L. Rodrigues-Simioni, Biochemical, pharmacological and structural characterization of a new PLA_2 from *Crotalus durissus terrificus* (South American rattlesnake) venom, Protein J. 24 (2005) 233–242.
[16] M.H. Toyama, D.O. Toyama, P.P. Joazeiro, E.M. Carneiro, L.O. Beriam, L.S. Marangoni, A.C. Boschero, Biological and structural characterization of a new PLA_2 from the *Crotalus durissus collilineatus venom*, Protein J. 24 (2005) 103–112.
[17] E.B.S. Diz Filho, S. Marangoni, D.O. Toyama, F.H.R. Fagundes, S.C.B. Oliveira, F.V. Fonseca, A.K. Calgarotto, P.P. Joazeiro, M.H. Toyama, Enzymatic and structural characterization of new PLA_2 isoform isolated from white venom of *Crotalus durissus ruruima*, Toxicon 53 (2009) 104–114.
[18] J.A. Pereañez, V. Núñez, S. Huancahuire-Vega, S. Marangoni, L.A. Ponce-Soto, Biochemical and biological characterization of a PLA_2 from crotoxin complex of *Crotalus durissus cumanensis*, Toxicon 53 (2009) 534–542.
[19] A. Achari, F.R. Radvanyi, D. Scott, C. Bon, P.B. Sigler, Crystals of crotoxin suitable for high resolution X-ray diffraction analysis, J. Biol. Chem. 260 (1985) 9385–9587.
[20] K.F. Santos, M.T. Murakami, A.C. Cintra, M.H. Toyama, S. Marangoni, V.P. Forrer, J.R. Brandão Neto, I. Polikarpov, R.K. Arni, Crystallization and preliminary X-ray crystallographic analysis of the heterodimeric crotoxin complex and the isolated subunits crotapotin and phospholipase A_2, Acta Crystallogr. Sect. F: Struct. Biol. Cryst. Commun. 63 (2007) 287–290.
[21] D.P. Marchi-Salvador, L.C. Corrêa, A.J. Magro, C.Z. Oliveira, A.M. Soares, M.R. Fontes, Insights into the role of oligomeric state on the biological activities of crotoxin: crystal structure of a tetrameric phospholipase A_2 formed by two isoforms of crotoxin B from *Crotalus durissus terrificus* venom, Proteins 72 (2008) 883–891.
[22] D.P. Marchi-Salvador, L.C. Corrêa, G.H. Salvador, A.J. Magro, C.Z. Oliveira, J. Iulek, A.M. Soares, M.R. Fontes, Preliminary X-ray crystallographic studies of a tetrameric phospholipase A_2 formed by two isoforms of crotoxin B from *Crotalus durissus terrificus* venom, Acta Crystallogr. Sect. F: Struct. Biol. Cryst. Commun. 63 (2007) 1067–1069.
[23] G.H. Salvador, C.A. Fernandes, L.C. Correâ, N.A. Santos-Filho, A.M. Soares, M.R. Fontes, Crystallization and preliminary X-ray diffraction analysis of crotoxin B from *Crotalus durissus collilineatus* venom, Acta Crystallogr. F65 (2009) 1011–1013.
[24] G. Faure, H. Xu, F.S. Saul, Crystal structure of crotoxin reveals key residues involved in the stability and toxicity of this potent heterodimeric β-neurotoxin, J. Mol. Biol. 412 (2011) 176–191.
[25] M.J. Forster, Molecular modelling in structural biology, Micron 33 (2002) 365–384.
[26] D.W. Ritchie, Recent progress and future directions in protein–protein docking, Curr. Protein Pept. Sci. 9 (2008) 1–15.
[27] I.S. Moreira, P.A. Fernandes, M.J. Ramos, Protein–protein docking dealing with the unknown, J. Comput. Chem. 31 (2010) 317–342.
[28] J.D. Thompson, D.G. Higgins, T.J. Gibson, CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weightmatrix choice, Nucl. Acids Res. 22 (1994) 4673–4680.
[29] R. Renetseder, S. Brunie, B.W. Dijkstra, J. Drenth, P.B. Sigler, A comparison of the crystal structures of phospholipase A_2 from bovine pancreas and *Crotalus atrox* venom, J. Biol. Chem. 260 (1985) 11627–11634.
[30] B.K. Kuntal, P. Aparoy, P. Reddanna, EasyModeller: a graphical interface to MODELLER, BMC Res. Notes 3 (2010) 226.
[31] A. Sali, T.L. Blundell, Comparative protein modelling by satisfaction of spatial restraints, J. Mol. Biol. 234 (1993) 779–815.
[32] R.A. Laskowski, M.W. MacArthur, D.S. Moss, D.S. Hornton, PROCHECK: a program to check the stereochemical quality of protein structures, J. Appl. Crystallogr. 26 (1993) 283–291.
[33] J.U. Bowie, R. Lüthy, D. Eisenberg, A method to identify protein sequences that fold into a known three-dimensional structure, Science 253 (1991) 164–170.
[34] M.J. Sippl, Recognition of errors in three-dimensional structures of proteins, Proteins 17 (1993) 355–362.
[35] M. Wiederstein, M.J. Sippl, ProSA-web: interactive web service for the recognition of errors in three-dimensional structures of proteins, Nucleic Acids Res. 35 (2007) W407–W410.
[36] Schneidman-Duhovny, Y. Inbar, R. Nussinov, H.J. Wolfson, PatchDock and SymmDock: servers for rigid and symmetric docking, Nucleic Acids Res. 33 (2005) W363–W367.
[37] R. Doley, R.M. Kini, Protein complexes in snake venom, Cell Mol. Life Sci. 66 (2006) 2851–2871.
[38] E. Mashiach, R. Nussinov, H.J. Wolfson, FiberDock: flexible induced-fit backbone refinement in molecular docking, Proteins 78 (2010) 1503–1519.
[39] E. Mashiach, R. Nussinov, H.J. Wolfson, FiberDock: a web server for flexible induced-fit backbone refinement in molecular docking, Nucleic Acids Res. 38 (2010) W457–W461.
[40] E. Krissinel, K. Henrick, Inference of macromolecular assemblies from crystalline state, J. Mol. Biol. 372 (2007) 774–797.
[41] V. Sobolev, E. Eyal, S. Gerzon, V. Potapov, M. Babor, J. Prilusky, M. Edelman, SPACE: a suite of tools for protein structure prediction and analysis based on complementarity and environment, Nucleic Acids Res. 33 (2005) W39–W43.
[42] W. Cho, F.J. Kézdy, Chromogenic phospholipase A_2 substrates and assays, Methods Enzymol. 197 (1991) 75–79.
[43] M. Holzer, S.P. Mackessy, An aqueous endpoint assay of snake venom phospholipase A_2, Toxicon 34 (1996) 1149–1155.
[44] V.P. Dole, A relation between non esterified-fatty acids in plasma and the metabolism of glucose, J. Clin. Invest. 35 (1956) 150–154.
[45] D. Eisenberg, R. Lüthy, J.U. Bowie, Verify 3D: assessment of protein models with three-dimensional profiles, Methods Enzymol. 277 (1997) 396–404.
[46] Y.P. Mascarenhas, P.F. Stouten, J.R. Beltran, C.J. Laure, G. Vriend, Structure–function relationship for the highly toxic crotoxin *from Crotalus durissus terrificus*, Eur. Biophys. J. 21 (1992) 199–205.
[47] C. Demangel, R.C. Maroun, S. Rouyre, C. Bon, J.C. Mazié, V. Choumet, Combining phage display and molecular modeling to map the epitope of a neutralizing antitoxin antibody, Eur. J. Biochem. 267 (2000) 2345–2353.
[48] S. Vajda, D. Kozakov, Convergence and combination of methods in protein–protein docking, Curr. Opin. Struct. Biol. 19 (2009) 164–170.
[49] G. Canziani, C. Seki, J.C. Vidal, Accessibility of the active site of crotoxin B in the crotoxin complex, Toxicon 20 (1982) 809–822.
[50] G. Canziani, C. Seki, J.C. Vidal, The mechanism of inhibition of phospholipase activity of crotoxin B by crotoxin A, Toxicon 21 (1983) 663–674.
[51] H. Breithaupt, Enzymatic characteristics of crotalus phospholipase A_2 and the crotoxin complex, Toxicon 14 (1976) 221–233.
[52] F.R. Radvanyi, C. Bon, Catalytic activity and reactivity with p-bromophenacyl bromide of the phospholipase subunit of crotoxin. Influence of dimerization and association with the noncatalytic subunit, J. Biol. Chem. 257 (1982) 12616–12623.
[53] T.W. Jeng, H. Fraenkel-Conrat, Chemical modification of histidine and lysine residues of crotoxin, FEBS Lett. 87 (1978) 291–296.
[54] O.G. Berg, M.H. Gelb, M.D. Tsai, M.K. Jain, Interfacial enzymology: the secreted phospholipase A_2-paradigm, Chem. Rev. 101 (2001) 2613–2654.
[55] J.M. Winget, Y.H. Pan, B.J. Bahnson, The interfacial binding surface of phospholipase A_2s, Biochim. Biophys. Acta 1761 (2006) 1260–1269.