Cytolytic Activity and Molecular Feature of Cardiotoxin and Cardiotoxin-like Basic Protein: The Electrostatic Potential Field Is an Important Factor for Cell Lytic Activity

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Abstract. *Background/Aim: Cardiotoxin (CT) is a well-known cell lytic protein and has been purified from cobra venom. Cardiotoxin-like basic protein (CLBP) has two amino acid insertions and does not exhibit cell lytic activity. The molecular features of these CT family proteins were examined in the present study using molecular modeling and molecular simulation techniques. Materials and Methods: Molecular models of CT and CLBP were constructed based on the X-ray data of Naja mossambica mossambica CT VII4 (Protein Data Bank ID: 1CDT). The structural features of these models were examined using molecular orbital and electrostatic potential parameters. Results: The stereo-hydrophobicities and molecular torsions of CT and CLBP, which are indexes of structural features, were similar. Electrostatic potential fields (ESP) differed between CT and CLBP and this was considered one of the critical factors in molecular titer. Conclusion: The distribution of ESP fields may affect the cytolytic activity of the CT family.*

The electrostatic potential (ESP) field is one of the factors that affect the interactive property of proteins (1). The ESP field can produce an attractive or repulsive force between protein and target molecules. The ESP distribution pattern in insulin molecules has been applied to control the medicinal effects of insulin preparations (*e.g.* rapid-acting, intermediate-acting and long-acting insulin) (2, 3). In the present study, we examined the role of ESP fields in the cytolytic activities of cardiotoxin (CT) and cardiotoxin-like basic protein (CLBP). CT is a well-known cytolytic protein with a molecular weight of 7 kDa and has been obtained from snake (*e.g. Naja naja siamensis*) venom. CT consists of 60 amino acids and various types (CT-I, -II, -III, -IV) have been identified (4). These CTs exhibit cytolytic activities in various cells (*e.g.* human amnion Fogh-Lund cell). CLBP has also been purified from snake venom and consists of 62 amino acids (5, 6). The amino acid sequence of CLBP is very homologous to CT, with two amino acid residues being inserted in CT. However, CLBP does not exhibit cytolytic activity in cultured cells. Molecular modeling of four CTs (CT-I, -II, -III, -IV) from *Naja naja siamensis* and three CLBPs from *Naja naja siamensis* (CLBP-si), *Naja naja* atra (CLBP-at) and *Naja naja* (CLBP-na) were performed based on *Naja mossambica mossambica* cardiotoxin VII4 X-ray data (Protein data bank ID: 1CDT). The molecular features, including ESP fields, of these CTs and CLBPs were analyzed and their relationships with cytolytic activity were discussed.

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Materials and Methods

Molecular analysis of cardiotoxins. Molecular models of CTs and CLBPs were constructed based on the X-ray data of *Naja mossambica mossambica* CT VII4 (Protein Data Bank ID: 1CDT) using insightII-discover with homology module (Accelrys Inc., San Diego, CA, USA) (7, 8). The energy minimization of models was performed using a consistent valence force-field (CVFF). Electrostatic potential fields of CTs and CLBPs were calculated and the +1.0 kT/e (gray) and –1.0 kT/e (dark gray) contour was displayed (9). Molecular dynamics (MD) simulation (500 ps) of these proteins were performed using discover module. Molecular orbital (MO) parameters (*e.g.* solvation free energy, which is an index of stereo-hydrophobicity) were determined for every 15 amino acid fragments, which were derived from N-terminal of simulated protein (10). Dihedral angles of peptide-bond (-CONH-) at insertion sites (CTs: Cys^3-Asn^4, Lys^{23}-Met^{24}, CLBPs: Cys^3-His^4, His^4-Asn^5, Lys^{24}-Ala^{25}, Ala^{25}-Thr^{26}) were determined from MD trajectory.

Results

Cytotoxicity. The protein concentration of CTs and CLBPs that were required to lyse 50% (ED_{50}) of Fogh-Lund (FL) cells was summarized from references 4 and 5 (Table I). The ED_{50} values of CT-I, -II, -III and -IV were 5.4, 10.6, 16.1 and 12.6 μg/ml, respectively. CLBP-si, CLBP-at and CLBP-na had no cytolytic activity (>100 μg/ml) toward FL cells.

Molecular feature of CT and CLBP. Cardiotoxins consist of 60 amino acid residues and 2 amino acid insertions (His^4 and Ala^{25}) were detected in CLBP molecules (Figure 1). CTs and CLBPs were previously shown to possess four S-S bridges (CT: C^3-C^{21}, C^{14}-C^{38}, C^{42}-C^{53}, C^{54}-C^{59}, CLBP: C^3-C^{22}, C^{15}-C^{40}, C^{44}-C^{55}, C^{56}-C^{61}) (11). The molecular configuration of these proteins collapsed when a MD simulation was performed without these S-S bridges. The structural collapse was absent in the MD analysis that was tempered with four S-S bridges. The total energies of CT-I, -II, -III and -IV during the MD simulation (500 ps) converged within the range of 2,518.5 to 3,081.7 kcal/mol. The range of total energies in CLBP simulations converged from 3,106.3 to 3,166.4 kcal/mol and the total energies of CLBPs were higher than those of CTs (data not shown). The stable structures of CTs and CLBPs were extracted from the MD trajectory data and divided from the N-terminal into 10 peptides every 15 amino acids. The solvation free energies (dGWs) of these divided peptides were determined using a MO analysis and their stereo-hydrophobicities were estimated. The dGW values of CT-I peptides ranged from –1686.2 to –799.7 kJ/mol with an average of –1141.7 kJ/mol (Figure 2). In the CT-II, -III and -IV-derived peptide analysis, the dGW profiles were the same as that of CT-I and their averages were –1,111.7, –1,088.5 and –1,013.1 kJ/mol, respectively. The CLBP-derived peptides had similar dGW profiles with averages of -1,243.9 (CLBP-si), –1,115.7 (CLBP-na), –1,179.4 (CLBP-at) kJ/mol. No significant difference was observed in the stereo-hydrophobicities of these CTs and CLBPs.

To analyze the molecular distortion at two insertion sites (*e.g.* His^4 and Ala^{25} in CLBP), the dihedral angle changes in the insertion regions (CTs: 3/4, 23/24, CLBPs: 3/4, 4/5, 24/25, 25/26; Figure 1) were monitored during the MD simulation. The biases of dihedral angles were not observed in the CT or CLBP MD simulations and the angles were approximately –180 or +180 degrees. The average of the dihedral angles of CT and CLBP did not exhibit bias to either plus or minus values (Figure 3). No significant differences were observed in the structural features of the insertion regions in CT and CLBP. The dipole moment of His^4-inserted region in CLBP (CLBP-si: Leu^1-Cys^{15}) was 20.236 debye and its direction was the same as that of the CT-I dipole moment (18.327 debye, CT-I: Leu^1-Pro^{15}). In the Ala^{25} inserted region in CLBP (CLBP-si: Pro^{16}-Phe^{30}), the dipole moment was 66.277 debye and was in the opposite direction to that of CT-I (60.725 debye, Ala^{16}-Leu^{30}) (data not shown).

Table I. *50% Cytotoxicity (ED_{50}) of CTs and CLBPs.*

Protein	ED_{50} (μg/ml)
CT-I	5.4
CT-II	10.6
CT-III	16.1
CT-IV	12.6
CLBP-si	>100
CLBP-at	>100
CLBP-na	>100

Fogh-Lund (FL) cells were suspended in phosphate-buffered saline (PBS) at a concentration of 2.5×10^6 cells/ml. Various concentrations of each protein fraction were added separately to the cell suspensions and the mixtures were incubated at 37˚C for 30 min. The cytotoxic activity was measured by the trypan-blue exclusion test (4, 5). Cytotoxicity was expressed as ED_{50}: the protein concentration required to cause lysis of 50% of the cells. CT-I, -II, -III and -IV were prepared from *Naja naja siamensis*. CLBPs were prepared from the venom of *Naja naja siamensis* (CLBP-si), *Naja naja atra* (CLBP-at), *Naja naja* (CLBP-na) and their cytotoxicity was the same.

ESP fields of CT and CLBP. CT and CLBP molecules consist of one core region and three loops (Figure 4). A minus ESP field developed with the entire CT-I molecule. A minus ESP field was observed in CT-II, -III and -IV, similar to CT-I. On the other hand, a plus ESP field existed in the core region of the CLBP-si molecule, while a minus ESP field existed in the loop 1 and loop 2 region. A similar ESP distribution was confirmed in the CLBP-at and -na molecules. A significant difference was observed in the distribution of ESP between CTs and CLBPs. When His^4 and Ala^{25} of CLBP were deleted, the distribution of ESP changed to a CT-like pattern (data not shown). In the His^4-deleted CLBP model, a change was observed in the distribution of ESP and the ESP pattern was altered to a CT-like pattern. No changes were noted in the ESP pattern in the Ala^{25}-deleted CLBP model. Furthermore, the ESP pattern did not change to a CT-like pattern in the CLBP model in which Leu^1 and Lys^2 were removed to more simply arrange the N-terminal length.

Discussion

The total MD energies of CLBPs (3,106.3 to 3,166.4 kcal/mol) were higher than those of CTs (2,518.5 to 3,081.7 kcal/mol). These results suggest that the mobility and reactivity of CLBPs are higher than those of CTs. However, CLBPs did not exhibit any cytolytic activity that reflected

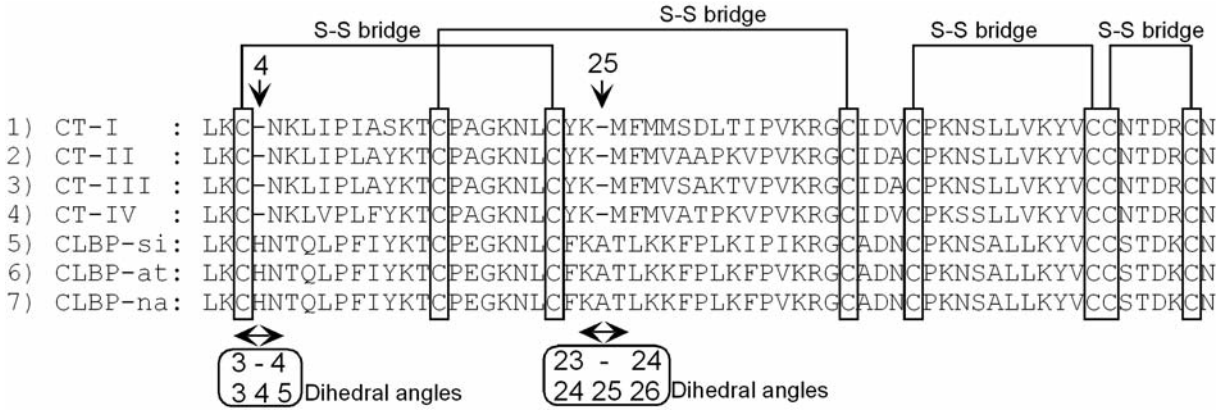

Figure 1. *Amino acid sequence of cardiotoxin (CT) and cardiotoxin-like basic protein (CLBP). Amino acid sequences of CTs and CLBPs were compared and their S-S bridges were indicated. CT-I, -II, -III, -IV: cardiotoxins from Naja naja siamensis. CLBP-si, -at, -na: CLBPs from Naja naja siamensis, Naja naja atra, Naja naja, respectively. Amino acid insertion was observed at* His^4 *and* Ala^{25} *in CLBP. Dihedral angles at insertion regions (CTs: 3/4, 23/24, CLBPs: 3/4, 4/5, 24/25, 25/26) were monitored during MD simulation.*

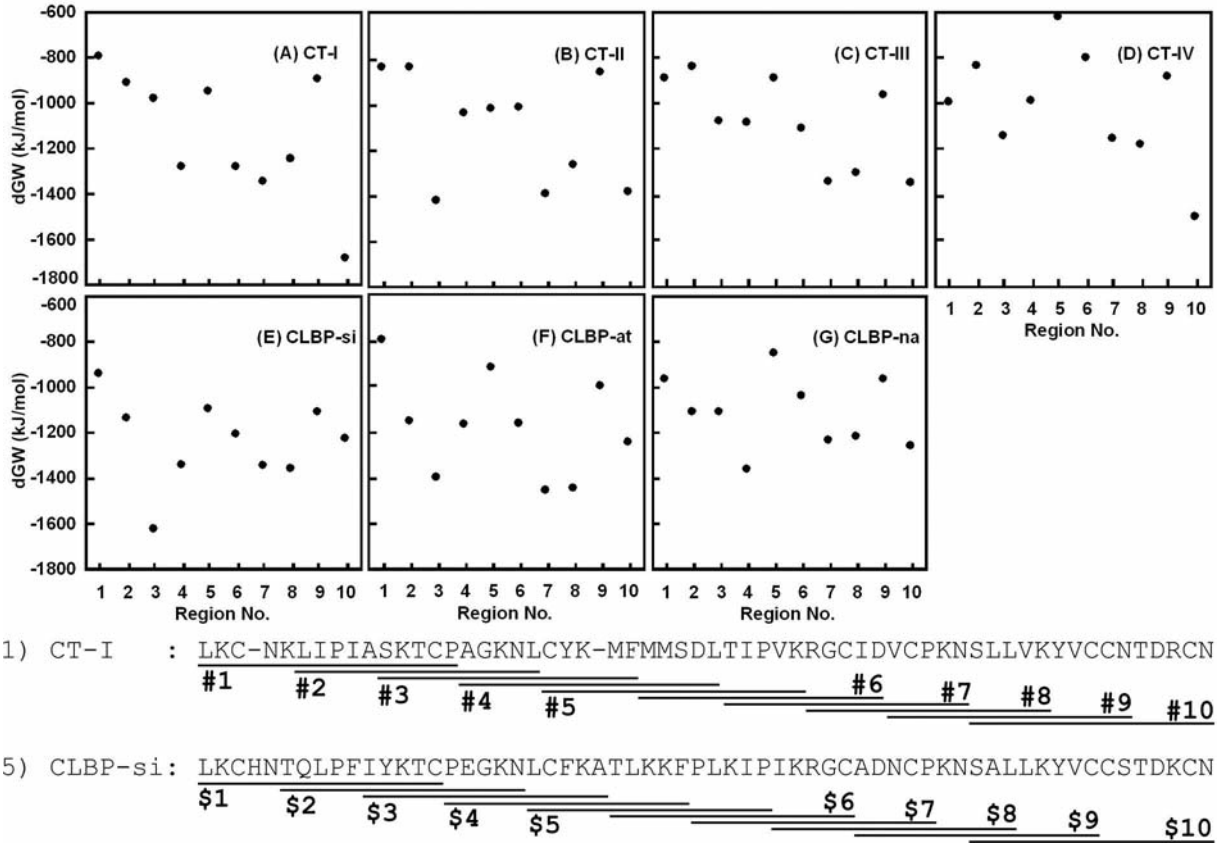

Figure 2. *Solvation free energy profile of CT and CLBP. Cardiotoxin-I (A: CT-I), -II (B: CT-II), -III (C: CT-III), -IV (D: CT-IV) from Naja naja siamensis. Cardiotoxin-like basic protein from Naja naja siamensis (E: CLBP-si), Naja naja atra (F: CLBP-at), Naja naja (G: CLBP-na), respectively. Stable structures of CTs and CLBPs were extracted from MD trajectory data and divided from N-terminal into 10 peptides every 15 amino acids. The region number of CT-I (#1 – #10) and CLBP-si ($1 – $10) peptides were shown according to the amino acid sequence of Figure 1. Solvation free energies (dGWs) of these divided peptides were determined using a molecular orbital (MO) analysis.*

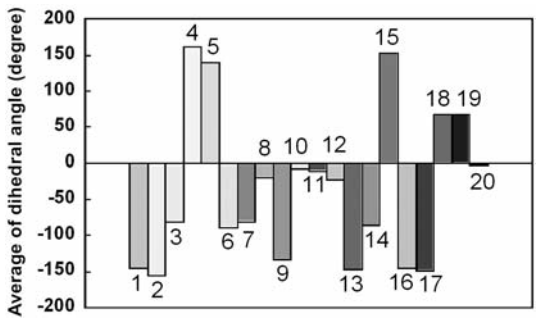

Figure 3. *Average of MD-simulated dihedral angles at insertion regions. Column numbers indicate the dihedral angle monitored insertion sites of CTs and CLBPs. Columns 1, 2: site 3/4, 23/24 of CT-I; columns 3, 4: site 3/4, 23/24 of CT-II; columns 5, 6: 3/4, 23/24 of CT-III; columns 7, 8: 3/4, 23/24 of CT-IV; columns 9–12: 3/4, 4/5, 24/25, 25/26 of CLBP-si; columns 13–16: 3/4, 4/5, 24/25, 25/26 of CLBP-at; columns 17–20: 3/4, 4/5, 24/25, 25/26 of CLBP-na, respectively.*

reactivity with the cell membrane. An appropriate balance between molecular mobility and MD energy may exist to show reactivity with the cell membrane. No significant difference was observed in the hydrophobicities of CTs and CLBPs (*e.g.* dGW profiles) (Figure 2). In the molecular torsion analysis at insertion regions of CT and CLBP, no significant difference was noted in the dihedral angles (one of the index of molecular structure) (Figure 3). These results indicate that other factors, besides molecular hydrophobicity and structural torsion, regulate cytolytic activity.

A significant difference was observed in the ESP field distribution of CLBPs and CTs, while CLBPs had plus and minus charged ESP fields. These ESP field features may be related to the loss of the cell lytic property of CLBPs. An analysis of protein function previously revealed that the ESP field pattern is an important index (1). The distribution of ESP on insulin molecules regulates its efficiency and various types of insulin molecules have been prepared, including rapid-acting, short-acting, intermediate-acting and long-acting ones (2). These stable structures with hexameric, dimeric and monomeric forms have been identified (12, 13). The insulin monomer molecule has the ability to react with receptors and is the main bioactive form. The insulin hexamer is converted into a monomer through the dimer leading to the re-appearance of activity. The amount of time required to express medicinal effect is dependent on the conversion time to the monomer form. The ESP field of rapid-acting insulin is designed so that it cannot easily interact with itself and exists in monomer forms. In long-acting insulin, the ESP field has been modified to easily form a hexameric structure. A time lag in the alteration from the hexamer to monomer form is expected due to this ESP modification resulting in a continuous medicinal effect. The modes of the inter-molecular interactions of CTs and CLBPs are considered to differ because their ESP field patterns were found to be significantly different. CLBP molecules had both plus and minus charged ESP field and could easily interact with other CLBP molecules (Figure 4). This inter-molecular interaction of CLBP appears to affect the titer of cytolytic activity. The amino acid sequence of CLBP was homologous with the

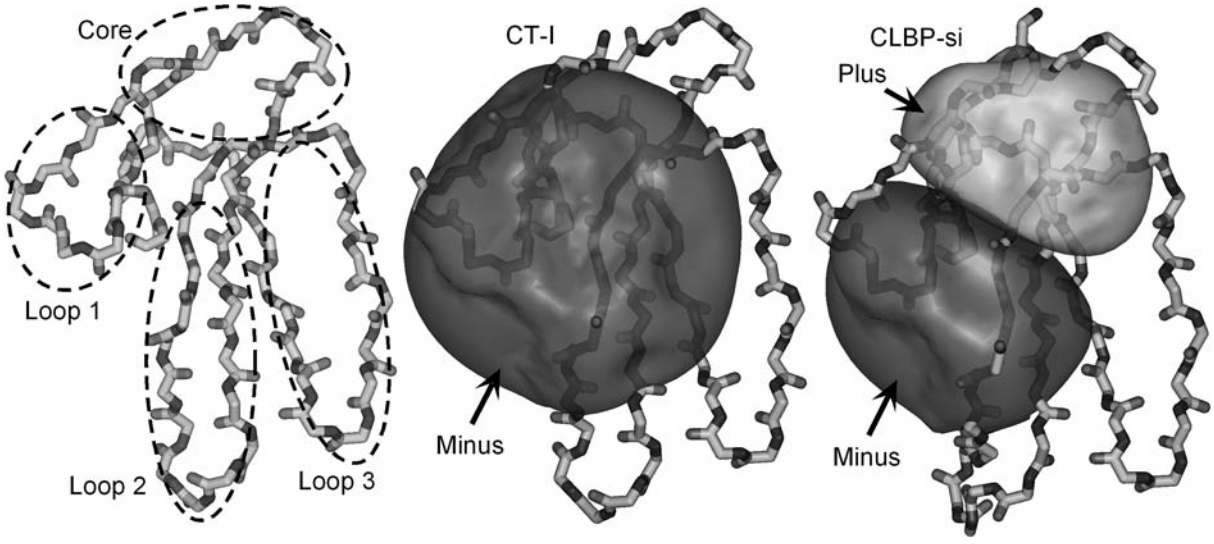

Figure 4. *Electrostatic potential field of CT and CLBP. CT-I molecule was covered with minus (dark gray) ESP field. CLBP-si was covered with pulse (gray) and minus (dark gray) ESP field.*

blood coagulation factor X-binding protein (X-bp) (14). X-bp is a component of cobra venom and the X-bp dimer has been shown to interact with the platelet membrane-binding Gla domain of coagulation factor X, thereby inhibiting the blood coagulation system. The CLBP dimer model had an appropriate ESP field and a good interactive profile with the factor X Gla domain was observed during the molecular simulation analysis (data not shown). These results indicate that the ESP profile of protein drugs (*e.g.* insulin) is one of the important factors determining function (titer) control. In order to examine the ESP-dependent function control of protein drugs, we designed a neo-CLBP molecule, which has a CT-type ESP field.

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