Structural Analysis of Human Specific Cytolysin Intermedilysin Aiming Application to Cancer Immunotherapy

KAZUTO OHKURA1,2, HIDEAKI NAGAMUNE1 and HIROKI KOURAI1

1Department of Biological Science and Technology, Faculty of Engineering, University of Tokushima, 2-1 Minamijosanjima-cho, Tokushima 770-8506; 2Graduate School of Bioagricultural Science, Nagoya University, Furo-cho, Chikusa-ku, Aichi 464-8601, Japan

Abstract. Background: Intermedilysin (ILY) is a human specific cytolysin secreted by Streptococcus intermedius. In the present study, we performed molecular modeling of ILY and cholesterol-dependent cytolysins (CDCs) (pneumolysin, PLY; listeriolysin O, LLO; streptolysin O, SLO; alveolysin, ALV; suliysin, SLY; pyohyn, PLO) to compare the membrane binding domains including the undecapeptide (11mer) region which is thought to be necessary for the cytolytic activity of CDCs. Materials and Methods: The molecular models of cytolysins were constructed using InsightII with Homology module with X-ray data of perfringolysin O (PFO). Results: The ILY molecule was long and rod shaped, and comprised four domains. ILY was shown to possess stereocomplementary surfaces within the molecule and the potential to stack with 8 degrees of curvature leading to a ring cluster of 45 molecules or so in the human erythrocyte cell membranes. Conclusion: From the molecular orbital calculations and isostatic potential analysis, we considered that the ILY 11mer region has different features from those of traditional CDCs, and the ILY domain 4 should be very useful to apply the human cell-specific targeting module.

The genus Streptococcus includes several pathogenic species, among them S. pneumoniae, an etiological agent of pneumonia, and S. pyogenes (group A hemolytic streptococcus). Cytolytic toxins from streptococci, such as pneumolysin (PLY) from S. pneumoniae and streptolysin O (SLO) from S. pyogenes, are amongst the virulence factors of these pathogens and actually cause cell lysis and cardiac dysfunction. These streptococcal cytolysins are known to be members of the cholesterol-dependent cytolysins (CDCs), that include pyolysin (PLO), tetanolysin, perfringolysin O (PFO) and listeriolysin O (LLO), which are produced by many Gram-positive pathogens, Arcanobacterium pyogenes, Clostridium tetani, Clostridium perfringens and Listeria monocytogenes, respectively (1). The secreted forms of CDCs are water-soluble monomers (47–60 kDa), forming homo-oligomeric complexes which become embedded in the cell membranes. These oligomers contain about 50 individual monomers (2). Subsequently CDCs disrupt the cell membranes by the formation of pores, with the CDCs requiring cholesterol (CHL) molecules in cell membranes as receptors. It is well-known that CDCs contain an almost invariant undecapeptide sequence (PFO: E458CTGLAWEWWR468) located near the C-terminus, and that the region is important for activity and CHL binding as determined in experiments using point mutants and chemical modifications (3-5).

Streptococcus intermedius is one of the species classified in the Anginosus group streptococci and a significant opportunistic pathogen (2). It is of clinical interest as it tends to be associated with abscesses in deep-seated organs including the brain and liver (6). It has been reported that S. intermedius produces many putative virulence factors such as glycosidases and proteases (7-10). Recently, we revealed that a human-specific cytolysin, intermedilysin (ILY), is secreted from a strain of S. intermedius originally isolated from a human liver abscess (11,12). ILY is a cytolysin genetically related to CDCs, showing different characteristics such as a low affinity to cholesterol (CHL) and a human specificity. ILY has a unique sequence (G485ATGLAWEPWR495) corresponding to the undecapeptide (11mer) region of CDCs (13,14). Similarly PLO, has a unique 11mer region (E491ATGLAWDPWW501). Although a discrepancy was found in the affinity of PLO to CHL in the literature (15,16), these substitutions in PLO are similar to those found in ILY and strongly suggest a lower affinity for PLO to CHL than other CDCs. Thus, among these toxins, analyses of the relationship between the structure of the 11mer region and affinity to CHL should provide useful information for understanding the cytolytic mechanism of ILY.
and other CDCs, and for the design of toxin molecules more suitable as a cell permeabilizer and other membrane-associated cell technological tools. In the present study, we initially performed molecular modeling of ILY and CDCs using X-ray data of PFO (17), and compared the structures and molecular orbital features in attempt to apply the ILY membrane-binding region (domain 4) to the targeting modules.

Materials and Methods

Molecular modeling. Sequences of ILY, PLO and CDCs were aligned using clustalX (Figures 1 and 2) (18). The homology model of ILY was based on the crystal structure of PFO (1PFO) using the InsightII Homology module (Accelrys Inc., USA) on a Silicon Graphics Octane Workstation (17). The initial model was built in two stages: [1] identification of significant regions of sequence identity between PFO and ILY, and assigning coordinates to ILY within these regions; [2] designation of ILY coordinates in the regions that showed less convincing regions of sequence identity to PFO using a database of peptide fragments. Since PFO included no hydrogen data in its X-ray crystallographic structure, the hydrogens were added using the Biopolymer module. The model was visually checked to remove any obvious steric clashes, followed by energy minimization using a Consistence Valence Forcefield (CVFF) with Discover3. The RMS deviation for all C· positions on superposition between PFO and ILY was 0.165 Angstrom. The quality of the final model was assessed using PROCHECK and X-PLOR (19,20). Homology models of PLO, PLY, LLO, SLO, alveolysin (ALV), and suilysin (SLY) were designed as well as ILY.

Molecular orbital and electrostatic potential analysis. The coordinate data of the 11mer region were extracted from the minimized molecular models of ILY, PLO, PLY, LLO, SLO, ALV and SLY, and hydrogen atoms were added to each N- and C-terminus of the peptides. Energy calculations were performed with PM3 Hamiltonian using MOPAC (Fujitsu Limited, Japan), and the stable and transient structures were initially built with general parameters of bond length, bond angle and dihedral angle, and refined with the eigen-vector following (EF) optimization method. The electrostatic potential fields were calculated using MM geometry with PM3 parameter by CAChe (Fujitsu Limited). Each electrostatic potential isosurface that showed both the polarity of the potential and where in space the potential equals ±0.030 a.u. (±18 kcal/mol, ±75 kJ/mol) was generated (red: positive, blue: negative potential field).

Electron microscopic observation of ILY cluster. Human erythrocytes were washed twice with physiological saline and finally suspended at a concentration of 50% (v/v). Eighty ìl of the 50% suspension of erythrocytes was treated with 20 ìl of ILY fraction. The mixture was incubated at 37ÆC for 10 min. The 20 ìl of suspension of lysed erythrocytes was dropped onto the surface of distilled water. After 20 min, floating ghost membrane were mounted on supporting films on grids. Membrane samples were fixed with 2.5% glutaraldehyde for 1 min at room temperature and washed with PBS. The varying oligomeric forms in specimens negatively-stained with 2% ammonium molybdate (pH 7.0) were investigated by transmission electron microscopy using a transmission electron microscope (H-800; Hitachi Co., Japan) running at 100KeV.

Crystallization. All crystallization experiments were performed at 4ÆC using the hanging drop vapor diffusion methods, in which 2 ìl of 7.5 mg/ml protein solution was mixed with an equal volume of mother liquor, which included 14 to 26% polyethylene glycol 10000, 0.1M citrate buffer (pH 5.5). The crystals grew within 2 to 4 weeks, and obtaining large crystals up to about 0.7mm in size. A single crystal was sealed into a standard glass capillary and intensity data were collected at room temperature using Dip2030 (Mac Science Co., Ltd., Japan). The X-ray generator produced 3.6kW (40kV, 90mA). The data were processed using the programs DENZO and SCALEPACK (22,23).
Ohkura et al.: Molecular Modeling of ILY

Figure 2. Alignment of primary structure of cytolysins. The sequences of cytolysins were aligned using clustalX. Gray arrows indicate the starting position for construction of homology models, which were constructed based on the crystal structure of PFO (1PFO: X-ray crystal data initiated at Asp30). An assignment of each domain (domain 1 ~ domain 4) was indicated.
Results

Molecular modeling of ILY and CDCs. The sequences of ILY, PLO and CDCs (PFO, PLY, LLO, SLO, ALV, SLY) were aligned using clustalX (Figure 2)(18). The identity of the amino acid sequence between PFO and ILY, PLO, PLY, LLO, SLO, ALV and SLY is 40.7, 39.0, 46.5, 42.7, 65.7, 71.0 and 40.7%, respectively. The ILY model exhibits a good stereochemistry with greater than 80% of residues in the most favored region and no residues in the disallowed region of the Ramachandran plot (Table I). The root mean square (RMS) deviation of C· positions between PFO and ILY was 0.165 Angstrom. RMS deviation of bond length, bond angle, dihedral angle and improper angle of each model was 0.010 Angstrom, 2.187 degrees and 27.159 degrees, respectively. The ILY molecular model was long and rod-shaped, and it comprised four domains: domain 1 (residues 55-79, 116-205, 255-301, 377-400), domain 2 (residues 80-115, 401-417), domain 3 (residues 206-254, 302-376) and domain 4 (residues 418-532) (Figures 2 and 3). The model consists of 48.7% sheet and 22.8% helix. ILY was shown to possess stereocomplementary surfaces within the molecule. The molecule size was 120 (H) x 40 (W) x 70 (D) Angstroms, and the electrostatic potential is indicated as red (negative), blue (positive) and white (neutral) (Figure 4). The resulting models of PLO and other CDCs were the same overall long rod-shapes as ILY, and they had four domains annotated as domain 1 ~ 4 (Figure 2). In the Ramachandran plots, over 90.0% of the amino acid residues of these were located in the most favored and additional allowed regions. RMS deviation in bond length, bond angle, dihedral angle and improper angle of each model was 0.008 ~ 0.305 Angstrom, 1.668 ~ 2.633 degree and 21.102 ~ 27.465 degree, respectively (Table I). The rate of sheet and helix content in these models was strikingly similar to that of PFO molecule.

Table I. Statistics of modeled cytolysins.

<table>
<thead>
<tr>
<th></th>
<th>Most favoured regions (%)</th>
<th>Additional allowed regions (%)</th>
<th>∆Cα PFO-toxin (Å)</th>
<th>Bond Angle (deg)</th>
<th>Dihedral Angle (deg)</th>
<th>Improper Angle (deg)</th>
<th>Sheet (%)</th>
<th>Helix (%)</th>
<th>Homology to PFO (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PFO</td>
<td>87.0</td>
<td>12.8</td>
<td>0.000</td>
<td>1.230</td>
<td>27.716</td>
<td>4.576</td>
<td>49.5</td>
<td>23.1</td>
<td>100.0</td>
</tr>
<tr>
<td>PLY</td>
<td>83.5</td>
<td>16.5</td>
<td>0.155</td>
<td>2.278</td>
<td>26.944</td>
<td>5.143</td>
<td>49.6</td>
<td>23.2</td>
<td>46.5</td>
</tr>
<tr>
<td>LLO</td>
<td>83.7</td>
<td>14.3</td>
<td>0.158</td>
<td>1.904</td>
<td>26.978</td>
<td>4.682</td>
<td>47.4</td>
<td>22.9</td>
<td>42.7</td>
</tr>
<tr>
<td>SLO</td>
<td>76.0</td>
<td>12.0</td>
<td>0.109</td>
<td>1.668</td>
<td>27.217</td>
<td>3.294</td>
<td>49.5</td>
<td>23.1</td>
<td>65.7</td>
</tr>
<tr>
<td>ALV</td>
<td>74.5</td>
<td>16.4</td>
<td>0.001</td>
<td>2.106</td>
<td>27.465</td>
<td>2.817</td>
<td>47.1</td>
<td>23.2</td>
<td>71.0</td>
</tr>
<tr>
<td>SLY</td>
<td>85.7</td>
<td>11.2</td>
<td>0.305</td>
<td>2.633</td>
<td>27.241</td>
<td>6.604</td>
<td>43.8</td>
<td>17.8</td>
<td>40.7</td>
</tr>
<tr>
<td>PLO</td>
<td>89.4</td>
<td>8.7</td>
<td>0.183</td>
<td>2.595</td>
<td>21.102</td>
<td>5.607</td>
<td>48.7</td>
<td>22.8</td>
<td>39.0</td>
</tr>
<tr>
<td>ILY</td>
<td>82.7</td>
<td>16.3</td>
<td>0.165</td>
<td>2.187</td>
<td>27.159</td>
<td>5.791</td>
<td>48.7</td>
<td>22.8</td>
<td>40.7</td>
</tr>
</tbody>
</table>

Each model was evaluated using PROCHECK and X-PLOR, and the most favored and additional allowed region were determined.

1) The root mean square (RMS) deviation of Cα between PFO and each toxin was indicated.
2) RMS deviation in bond length, bond angle, dihedral angle and improper angle of each model.

Molecular orbital analysis of undecapeptide region. In the 11mer region of PFO, the highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) localized at W467 and W466, respectively, and the next HOMO and next LUMO both localized at W464 (Figure 6A). Dipole moment was calculated as 12.499 debye directed to the outer side of the molecule (Figure 7 Type C). The orbitals (HOMO, LUMO, next HOMO, next LUMO) localized at three Trp residues (data not shown) and their dipole moments (7.485 ~ 9.163 debye) directed to the outer space (Figure 7 Type C) as well as PFO. On the other hand, the molecular orbitals of ILY localized between two Trp residues (W494: HOMO, next LUMO, W491: LUMO, next HOMO) (Figure 6B), and the dipole moment (1.731 debye) was significantly smaller than that of PFO and the direction faced to the intramolecular site crowded with other amino acid residues (Figure 7 Type A). The orbitals in PLO located at three Trp residues (W500, HOMO; W497, LUMO; W501, next HOMO, next LUMO). Its dipole moment (2.522 debye) was smaller than that of PFO but directed to the outer side (Figure 7 Type B). PFO did not exhibit a significant potential
field at the 11mer region (Figure 8). However, ILY formed a negative potential field at the 11mer region, especially at Pro493. Although PLO had a Pro499 residue (instead of Trp466 in PFO) as well as ILY, it had no marked electrostatic field. The electrostatic isopotential fields in the 11mer region of other CDCs (PLY, LLO, SLO, ALV, SLY) were impartially distributed as with PFO (data not shown).

Cluster formation of ILY on human erythrocyte membrane. By electron microscopic observation, it was revealed that numerous ring-shaped structures, with a diameter of 400 Angstroms and rim thickness of 70 Angstroms, were formed in the human erythrocyte membranes treated with ILY (Figure 9). However, no ring structure was found in rabbit erythrocyte membranes reacted with ILY (data not shown). This ring structure seemed to be a ILY homo-oligomer judging from the structural homology between ILY and CDCs, which also form ring clusters. Indeed, this interpretation does not conflict with the deduced structure of monomeric ILY (Figure 4). Then we estimated that ILY seemed to stack with 8 degrees of curvature and then form a ring cluster of 45 molecules or so in the human cell membranes. In this case, the 11mer region should be faced towards the inner side of the ring cluster, because the molecular thickness of the 11mer region of ILY (i.e., N-terminal side) was less than that of the C-terminal side as shown in Figure 4 and Figure 9.
Figure 4. Surface model of ILY. Molecular surface model was visualized by solvent surface, then electrostatic potential was indicated as red (negative), blue (positive) and white (neutral). Dimensions of height (H=120), width (W=40), depth (D=70) Angstrom was decided from scale in InsightII.

Figure 5. Domain 4 solvent surface models of cytolysins. Coordinate data of domain 4 was extracted from toxin models, and visualized the molecular surface as Figure 4.
Crystal analysis. The best block-shaped single crystals of ILY were obtained using 14% PEG10000 as a precipitant, 0.1M citrate buffer (pH 5.5) at 4°C. They grew to approximate dimensions of 0.7 x 0.3 x 0.5 mm within 3 weeks (Figure 10). One crystal was used to collect native data set to 4.4 Ångstroms. The crystals belonged to the P2₁2₁2₁ space group (a = 91.6, b = 104.3, c = 174.2 Ångstrom, α = β = γ = 90°). Data were collected by three-degree oscillation photography, and were collected up to 4.4 Ångstroms (R=3.0%/completeness=70.4%). The Matthews coefficient of native crystal was 3.7, assuming 2 mol/asymmetric unit (24), and the solvent content was 66.87%.

Discussion

We used the crystal data of PFO (1PFO), which began from the 30th Asp residue, as a template for the molecular modeling. Since the N-terminal structure in the first domain of each mature form of the cytolsins varied in length, we first aligned the primary sequence of cytolsins to match the N-terminal amino acid sequences of each cytolsin to that of PFO (gray arrows in Figure 2). The ILY and CDC models exhibit a good stereochemistry judging from calculated RMS deviations and their Ramachandran plots and we therefore consider the present model structure of cytolsins to be realistic (Table I). The modeled ILY was long, rod-shaped, and comprised four domains (Figure 3). Although domains 1–3 were not formed as a sequentially continuous cluster, domain 4 (from the 418th Asp) was formed as a sequentially continuous structure (Figure 2). ILY was shown to possess stereocomplementary surfaces within the molecule, and the molecular size was determined to be 120(H) x 40(W) x 70(D) Ångstroms (Figure 4). This deduced ILY size was consistent with the rim thickness (70 Angstroms) in the ring cluster structure of ILY formed in human erythrocyte membranes, which was revealed by an electron microscopic observation (Figure 9). The diameter of this ring cluster was 400 Ångstrom, and it seemed to stack with 8 degrees of curvature towards the inside in an ILY 11mer region, then form a ring cluster of 45 molecules or so in the cell membranes (Figure 9). We compared the domain
4 of cytolysins, and observed the convex-shape at the 11mer region (arrow in Figure 5) in CDCs (i.e. Trp466 in PFO). In contrast, we observed the concave-shape in the ILY 11mer region (at Pro493). Because of this difference, we considered that ILY may show a different behavior in the interaction with cholesterol from those of ordinary CDCs. Although PLO is regarded as a member of CDCs at present, PLO indicated the concave-shaped 11mer region (Pro499) as well as ILY. This suggests that PLO shows unique binding characteristics to cholesterol compared to other CDCs. According to an electrostatic potential field analysis of the 11mer region, a potential field was localized close to each region of PFO (Figure 8) and other CDCs (data not shown). In contrast, the negative potential field was formed mainly at Pro493 in ILY. From these results, we consider that the 11mer region of ILY has a significantly different characteristic from those of other CDCs. In addition, as shown in Figure 5, PLO showed a concaved feature at Pro499 in surface modeling but, interestingly, PLO possessed a CDC-like form on measuring the electrostatic potential field in the 11mer region (Figure 8). Therefore, PLO seems to be intermediate between ILY and other typical CDCs in its interaction with cholesterol.

The importance of the Trp residue in the 11mer region of PFO has been reported previously in which mutants with either W464, W466 or W467 substituted by a Phe residue exhibited a markedly decreased hemolytic activity (4,5). We analyzed the 11mer region by molecular orbital calculation, and observed localization of the major molecular orbitals (HOMO, LUMO, next HOMO, next LUMO) at three Trp residues in PFO (W464, W466, W467 in Figure 6A) and other CDCs (PLY, LLO, SLO, ALV, SLY) (data not shown). Moreover, since the dipole moment of 7.485 – 12.499 debye occurred toward the outside of the CDC molecules (Figure 7 Type C), we considered that these CDCs seem to easily interact with cholesterol at the 11mer region. In the ILY 11mer region, the major orbitals were observed between W491 and W494. A dipole moment of ILY was smaller (1.731 debye) than those of other CDCs and its direction was towards the crowded side of the ILY molecule (Figure 6B, Figure 7 Type A). From these results,
we consider that the molecular characteristics of the ILY 11mer region differs from those of other CDCs, and it seems to be concerned with the low affinity of ILY to cholesterol. The major orbitals at three Trp residues (W497, W500, W501) were observed in PLO as PFO (Figure 6C). However the dipole moment was smaller (2.522 debye) than CDCs and the direction differs (Type B in Figure 7). This result suggests that the PLO 11mer region will show a characteristic intermediate to ILY and CDCs.

Thus the human-specific ILY molecule (especially domain 4) has different features from traditional CDCs, and it should be very useful to develop the human cell-targeting module. As shown in Figure 10, we obtained a single ILY crystal, and assigned it belonged to the P2$_1$2$_1$2$_1$ space group. Then we considered that the handling of the ILY molecule is easy, and a high purity protein sample can be obtained for various applications. Moreover, we observed a low immunogenicity of the ILY domain 4 (ILY4D) in rabbits, immunized with ILY4D fused with glutathione S-transferase. This immunological feature of ILY4D is preferable when considering certain applications in medicine: if effector cells (i.e. macrophages or cytotoxic T lymphocytes activated by cytokines) are coated with a chimeric protein (i.e. an anti-cancer antigen antibody) which linked with ILY4D, and injected into the veins or tissue of cancer patients, then these effector cells can effectively target the cancer cells through recognition by the chimeric protein and subsequently kill them. ILY4D or chimeric ILY4D easily bind to human cell membranes when mixed with human cells and this binding process is minimally reduced by serum cholesterol. Therefore, ILY4D should be a very convenient molecule for development of cell delivery systems, which will be applied to the cell-drug treatment of targets (e.g., cancer immunotherapy and gene therapy). We are currently proceeding with the development of various cell membrane attaching modules based on the ILY4D structure deduced in the present study.

Acknowledgements

We thank Dr. Hideaki Tsuge, Tokushima Bunri University, for technical advice in the X-ray crystallographic study. This work was supported, in part, by Grant-in-Aid for Scientific Research (15590098) from the Ministry of Education, Science, Sports and Culture, Japan.
Figure 9. Observation of ILY cluster in human erythrocyte membranes. Left: human erythrocyte membranes treated with ILY. Bar = 100 Angstroms. Right: modeled ILYs stacked with 8 degree of curvature then form a ring cluster (11mer region faced to inner side of the cluster) of 45 molecules or so in the human cell membranes.

<table>
<thead>
<tr>
<th>Data collection</th>
<th>Native</th>
</tr>
</thead>
<tbody>
<tr>
<td>Max resolution (Å)</td>
<td>4.4</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>15025</td>
</tr>
<tr>
<td>Redundancy</td>
<td>2.9</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>70.4</td>
</tr>
<tr>
<td>R symm (%)</td>
<td>0.162</td>
</tr>
<tr>
<td>I/σ (I)</td>
<td>3.0</td>
</tr>
<tr>
<td>Resolution range (Å)</td>
<td>5.25~5.00</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>70.2</td>
</tr>
<tr>
<td>R symm(%)</td>
<td>0.315</td>
</tr>
</tbody>
</table>

Crystal is $P2_12_12_1$

$a=91.6$ Å, $b=104.3$ Å, $c=174.2$ Å, $α=β=γ=90°$

Data were collected by DIP2030 using $λ=1.00$ Å

$R$ symm=$\Sigma h\Sigma i|h,i-<h>|\Sigma h,i|$

Figure 10. ILY Crystal screening. Left: Data collection statistics of native ILY crystal. Right: ILY crystal photography.
References


22 Minor W: "XDISPLAYF Program", Purdue Univ., West Lafayette, IN, USA 1993.
