SHORT COMMUNICATION

N-Terminal truncation mutagenesis of equinatoxin II, a pore-forming protein from the sea anemone Actinia equina

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The role of the N-terminal segment 1–33 of equinatoxin II, a 20 kDa pore-forming protein from the sea anemone Actinia equina, was studied by N-truncation mutagenesis. A part of this segment was classified as being amphiphilic and membrane seeking. Wild-type equinatoxin II and its mutants lacking 5, 10 and 33 amino acid residues, respectively, were produced in Escherichia coli using T7 RNA polymerase-based expression vector. Soluble recombinant proteins were isolated from bacterial lysates and assayed for their inhibition by sphingomyelin, binding to red blood cells and hemolytic activity. The N-terminal deletion of 33 amino acids resulted in an insoluble protein, while mutants lacking 5 and 10 residues expressed increased relative avidity for sphingomyelin and red blood cell membranes. Their specific hemolytic activity was decreased, however, with increasing truncation. The results suggest that the N-terminus, which has been found to be conserved in sea anemone pore-forming toxins, contributes to the solubility of the equinatoxin II, but it is not essential for binding to lipid membranes. It is very likely that the N-terminus play a role in the formation of functional pores.

Keywords: cytolysin/deletion mutagenesis/pore-forming toxin/recombinant protein/sea anemone

Introduction

Pore-forming toxins (Harvey, 1990) are water-soluble polypeptides which insert into cell or model lipid membranes and permeabilize them. They have been found, for example, in bacteria (Menestrina et al., 1994), sea anemones (Maček, 1992) and insect venoms (Dempsey, 1990). The tertiary structure and precise mechanism of pore assembling have been determined for only a few of them (Dempsey, 1990; Parker et al., 1994; Stroud, 1995).

From sea anemones, more than 30 pore-forming cytolsins have been isolated. They are highly basic proteins with a molecular mass of ~20 kDa (Kem, 1988; Turk, 1991; Maček, 1992). So far, the primary structure has been completed for only three of them (Blumenthal and Kem, 1983; Simpson et al., 1990; Belmonte et al., 1994). Analysis of the complete and partial N-terminal amino acid sequences of several similar sea anemone cytolsins revealed no similarity with other proteins and they probably represent a novel protein superfamilies. Typical of the sea anemone cytolsins is their inhibition by sphingomyelin. Experiments with model lipid membranes showed that sphingomyelin in the membrane acts as a lipid acceptor (Linder et al., 1977; Belmonte et al., 1993; Maček et al., 1995; Meinardi et al., 1995). Equinatoxin II (EqtII), a lysin from the sea anemone Actinia equina L., is one of the more extensively studied cytolsins (Maček and Lebez, 1988). Its binding constant for lipid ranges from 9.4×102 to 142×103 M⁻¹, being dependent on the lipid composition (Maček et al., 1995). EqtII creates pores in both cell and model lipid membranes (Zorec et al., 1990; Maček et al., 1994). A cation-selective channel with a hydrodynamic inner diameter of 1–2 nm is composed of three or four monomers as deduced from kinetic measurements and cross-linking experiments (Belmonte et al., 1993).

Circular dichroism experiments with EqtII indicated a significant increase of α-helical structure at the expense of β-structure upon interaction with lipid vesicles (Belmonte et al., 1993). In this respect, particular attention was paid to a highly conserved N-terminal segment which was predicted to be in an amphiphilic α-helix conformation. Furthermore, considerable structural analogy of the EqtII segment 11–33 with mellitin and virus fusion peptides exists. It has been suggested that this part of the molecule may act as an anchor which facilitates insertion of the toxin in lipid bilayer (Belmonte et al., 1993).

Here, we report the production of N-terminal truncated mutants of this sea anemone pore-forming toxin and the significance of this particular part of the molecule for cytolytic activity. Gradual deletion of 5 or 10 N-terminal amino acid residues results in mutant proteins with an increased susceptibility for sphingomyelin binding, although the truncated proteins are less or completely inactive in producing hemolysis.

Materials and methods

Materials

Restriction and DNA-modifying enzymes were purchased from Boehringer-Mannheim or New England Biolabs. All other chemicals of analytical grade were obtained from Sigma or Serva, unless stated otherwise. Oligonucleotides were purchased from Ransom Hill Bioscience or synthesized on an Applied Biosystems 381A DNA synthesizer and then purified by a double precipitation with sodium acetate and ethanol. The bacterial strains Escherichia coli DH5α and E.coli BLR(DE3) were obtained from Gibco BRL and Novagen, respectively.

Cloning of EqtII mutants

The wild-type EqtII coding region was cloned as described previously (Anderluh et al., 1996). The coding regions for N-terminal truncated mutants were amplified by PCR using the EqtII cDNA. The 3′-primer was in all cases E2.2(–) (5′-CGAATTCCATATGGGGGC-CGTTATTTGATGGTGTGC-3′). It is complementary to the last 14 nucleotides of the EqtII cDNA coding region and contains two termination codons and EcoRI and BamHI sites. The 3′-primer was used in combination with the 5′-primers: EDI(+) (5′-GGAAATTCATATGGGGGCGTTATTTGATGGTGTGC-3′) for construction of EqtIIA–5, ED2(+) (5′-GGAAATTCATATGGGGGC-GCGTTATTTGATGGTGTGC-3′) for construction of EqtIIA–5
for EqtIIΔ1–10 and ED3(+) (5'-GGAACTCATATGACT- TGTGCGGTTGTCAC-3') for EqtIIΔ1–33. They all contain EcoRI and NdeI sites followed by a sequence corresponding to the desired 5' ends of the particular EqtII mutant. The NdeI site includes the initiator codon and is followed by a codon for an amino acid with a short side chain (Gly or Ala) which ensures efficient co-translational removal of the N-terminal methionine in vivo (Macák et al., 1989). PCR was performed using a Perkin-Elmer Cetus kit for 30 cycles with the following conditions: denaturation, 94°C for 1 min; annealing, 48°C for 1 min; and extension, 72°C for 1 min. The PCR products were digested at the ends with EcoRI and cloned into pUC19, where the nucleotide sequence was verified using a T7 sequencing kit (Pharmacia) and 35S-dATPoxS (Amersham). The coding regions were excised from pUC19 with NdeI and EcoRI and cloned into the pT7-7 expression vector (Tabor, 1994). The expression vectors named pAG4.1, pAG4.2 and pAG4.3 allow the production of EqtIIΔ1–5, EqtIIΔ1–10 and EqtIIΔ1–33, N-terminal deletion mutants of EqtII, respectively.

Expression and isolation of EqtII mutants

The constructed vectors were used to transform the E. coli BLR(DE3) strain and expression of EqtII mutants was induced as described (Anderluh et al., 1996). The same amount of bacterial cells was taken for the determination of the hemolytic activity of supernatants. Cells were spun down, then 50 µl of STE buffer (8% sucrose, 0.1% Triton X-100, 50 mM EDTA and 50 mM Tris–HCl, pH 8.0) and 1 µl of 50 mg/ml lysozyme were added to the bacteria and incubated at room temperature for 10 min. Bacterial cells were briefly sonicated with a microtip at 70 W (Ultrasonics W185 sonic cell disruptor) and centrifuged for 10 min. The same amount of supernatant, usually 10 µl, was then used to determine the half time of hemolysis as described (Maček and Lebez, 1988) (see also characterization of proteins). Native and recombinant wild-type and mutant Eqts were isolated as described previously (Maček and Lebez, 1988; Anderluh et al., 1996).

Characterization of recombinant proteins

A molar extinction coefficient of 3.61 × 10^4 M^-1 cm^-1 (Maček and Lebez, 1988) were used to determine the concentration of native EqtII. The concentration of recombinant proteins was determined spectrophotometrically by the method of Perkins (1986) or by Bio-Rad Protein assay using known concentrations of native EqtII as a standard.

Proteins were analyzed using an SDS–PAGE Phast system from Pharmacia. For the native electrophoresis 8–25% gradient gels with reversed electrodes were used following the instructions of the manufacturer.

Polyclonal antibodies against EqtII were raised in a rabbit and serum was used for immunoblotting without further purification. Proteins were blotted for 2 h to a polyvinylidene difluoride membrane (Millipore). The membrane was blocked in buffer B, i.e. 1 M NaCl, 20 mM EDTA and 0.2 M Tris–HCl, pH 7.4 (buffer A) containing 4% BSA for 2 h. Incubation with 30 µl of antisemur in 5 ml of buffer B was followed by incubation with peroxidase conjugated goat anti-rabbit antibodies (Jackson Immunoresearch), 1:1000 in buffer B. The membrane was extensively washed with buffer A between the two steps. After the last incubation, the membrane was washed again and developed using 3-aminomethylcarbazole as a substrate.

Analytical RP-HPLC (Milton Roy) was performed on a C18 column (Applied Biosystems) equilibrated in 0.1% trifluoroacetic acid in water. Elution was performed with a 30 min linear gradient to 80% (v/v) acetonitrile with 0.1% (v/v) trifluoroacetic acid at a flow rate of 1 ml/min and absorbance was monitored at 215 nm. The N-terminal sequence was determined by Edman degradation using an Applied Biosystems 470A protein sequencer.

Hemolytic activity was measured turbidimetrically on bovine red blood cells at 25°C as described previously (Maček and Lebez, 1988). Toxins were added to 2 ml of a red blood cell suspension in 0.13 M NaCl, 20 mM Tris–HCl, pH 7.4 (erythrocyte buffer) with an apparent absorbance of 0.5 at 700 nm. The measured half time of hemolysis, t50, was the time in which an apparent absorbance reduced to 0.25. One hemolytic unit (1 HU) was defined as a quantity of the toxin which reduced an apparent absorbance of the erythrocyte suspension at 700 nm from 0.5 to 0.25 in 2 min.

Inhibition with sphingomyelin

A stock 4 mg/ml sphingomyelin dispersion was prepared in distilled water using sonication with a microtip three times for 20 s. The suspension was always vortex mixed before use. A 1 HU amount of the toxin was preincubated with sphingomyelin at a chosen concentration in 10 µl of erythrocyte buffer for 2 min at room temperature and the hemolytic activity of the mixture was then measured as described.

Binding to red blood cells

A 6.2 µg amount of the toxin variants was added to 500 µl of the red blood cell suspension in erythrocyte buffer, supplemented with 30 mM PEG 4000, with an apparent absorbance of 1.0 at 700 nm at 4°C. The mixture was incubated on ice for 5 min and then briefly centrifuged at the same temperature. A 100 µl volume of the supernatant was then used to measure hemolytic activity on an erythrocyte suspension as described. For the control, toxins were added to 500 µl of the PEG buffer and hemolysis was measured as before. By using equivalents of hemolytic units for each particular toxin variant, the binding of toxins was quantified. For SDS–PAGE and immunoblot analysis, toxins (0.7 µg in 5 µl) were added to 20 µl of red blood cell suspension containing PEG. After incubation for 2 min on ice, the erythrocytes were centrifuged briefly and the supernatant was removed. An electrophoresis loading buffer containing SDS was then added separately to erythrocytes and supernatant and both samples were analyzed on a Phast system with subsequent immunoblotting.

Results

Cloning and expression of N-truncated mutants of EqtII

The PCR amplified products coded for N-deletion mutants lacking 5, 10 or 33 amino acids from the N-terminal end. In order to exclude errors during PCR amplification using Taq polymerase, the fragments were cloned into pUC19 where nucleotide sequences was checked. In all cases they matched perfectly the corresponding regions of the EqtII cDNA. In Figure 1A immunoblot analysis of the expressed proteins in bacterial lysate is shown. Recombinant Eqts are all of the expected size. The immunoblot shows that the level of expression was approximately the same for all three mutants and wild-type EqtII, which was about 5% of the total bacterial proteins.

Isolation and characterization of N-truncated mutants

Certain amounts of Eqts were expressed in the inactive form of insoluble inclusion bodies and part in the form of soluble
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Fig. 1. Expression and characterization of recombinant Eqts. (A) Immunoblot analysis of Eqts production. Lane 1, wild-type EqtII; lane 2, induced cells with pT7–7; lane 3, EqtIIΔ1–5; lane 4, EqtIIΔ1–10; lane 5, EqtIIΔ1–33; lane 6, native EqtII as positive control. The lower band represents an unspecific reaction of serum with one of the bacterial proteins. (B) Analysis of purified recombinant Eqts. Lane 1, SDS–PAGE of EqtIIΔ1–10; lane 2, EqtIIΔ1–5; lane 3, wild-type EqtII; M, molecular mass standards. (C) Non-denaturing PAGE of native EqtII purified from the sea anemone (lane 1) compared with recombinant wild-type EqtII (lane 2) and EqtIIΔ1–5 (lane 3).

Fig. 2. Hemolytic activity of recombinant Eqts in bacterial supernatants. Left, designations and N-terminal sequences of the truncated forms compared with wild-type EqtII are shown. Right, the same volume of supernatants was used for hemolysis assay as described in Materials and methods. Rates of hemolysis, 1/t50, were compared by taking wild-type EqtII activity as 100%. Means ± s.d. were obtained from three independent transformation and expression experiments using plasmids as denoted.

Table I. Some of the characteristics of recombinant Eqts

<table>
<thead>
<tr>
<th>Protein</th>
<th>Yield (mg/l)</th>
<th>N-Terminal methionine (mol%)</th>
<th>Specific hemolytic activity (HU/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native EqtII</td>
<td>–</td>
<td>–</td>
<td>3610</td>
</tr>
<tr>
<td>Recombinant EqtII</td>
<td>1.00</td>
<td>5</td>
<td>3860</td>
</tr>
<tr>
<td>Recombinant EqtIIΔ1–5</td>
<td>1.08</td>
<td>0</td>
<td>3200</td>
</tr>
<tr>
<td>Recombinant EqtIIΔ1–10</td>
<td>0.06</td>
<td>10</td>
<td>1130</td>
</tr>
</tbody>
</table>

hemolytically active proteins. The bacterial supernatant of EqtIIΔ1–33 showed no hemolytic activity, whereas the activity of the EqtIIΔ1–5 and EqtIIΔ1–10 supernatant was reduced to 44 and 4%, respectively, compared with wild-type EqtII (Figure 2). Homogeneous EqtIIΔ1–5 and EqtIIΔ1–10 were isolated from the bacterial supernatant as shown in Figure 1B. Some of the properties of recombinant Eqts are presented in Table I. While recombinant EqtII has the same biochemical properties as the native (Figure 1C and Table I), the mutants differ in size and overall net charge. As deduced from native PAGE, EqtIIΔ1–5 mutant was more positive (Figure 1C). Approximately 1 mg of active wild-type or the EqtIIΔ1–5 mutant was produced from 1 l of bacterial broth. The recovery of EqtIIΔ1–10 was 17 times lower (Table I). It is produced mainly in the form of insoluble inclusion bodies. We tried to renature the recombinant EqtIIΔ1–10 by first extracting inclusion bodies in 0.1 M Tris–HCl, pH 6.8, containing 7 M Gn–HCl followed by dialysis against 0.1 M Tris–HCl, pH 7.6. We failed to obtain any hemolytically active protein using this procedure. The initiator methionine was cleaved off efficiently in the case of recombinant EqtII and EqtIIΔ1–5 (Table I). EqtIIΔ1–10 still possessed ~10% of the methionine form, but it was not possible to separate the isoforms by RP-HPLC (data not shown).

Biological activity of recombinant proteins
The specific hemolytic activity of EqtIIΔ1–5 and EqtIIΔ1–10 mutants was reduced to 89 and 31%, respectively, compared with native and wild-type EqtII (Table I).

Progressive removal of 5 and 10 amino acids results in increased inhibition by sphingomyelin. Preincubation of the
results of both chemical modification and EqtII intrinsic tryptophan fluorescence. Toxin activity is completely impaired if a single residue of either tyrosine (Turk et al., 1989) or tryptophan (Turk et al., 1992) is modified. Furthermore, tryptophan residue(s), which are not present in the N-terminal moiety, were shown to interact with lipids (Maček et al., 1995). Our results are also in agreement with a proposed multi-step model of pore formation by EqtII, based on the kinetic changes in intrinsic tryptophan fluorescence and of vesicle permeabilization (Maček et al., 1995). The existing data suggest a model of EqtII binding to lipid membrane and pore formation could be as follows. Initial interaction with sphingomyelin at the membrane surface, the nature of which is unknown at present, triggers a conformational change in the toxin, including that of the amphiphilic N-terminus. This may reveal a larger hydrophobic surface of the protein which promotes an insertional step leading to toxin oligomerization.

In structure–function studies of non-bacterial pore-forming toxins, molecular biological approaches have rarely been reported, unlike other types of toxins. A short cardiotoxic neurotoxin from the sea anemone Anthopleura xanthogrammica and mutant forms, for example, were expressed in E. coli by using synthetic genes (Gallagher and Blumenthal, 1992). Our study has proved the use of a bacterial expression system suitable for producing N-truncated mutants of the sea anemone pore-forming protein, and further mutagenesis of EqtII combined with biochemical and biophysical studies is under way to obtain more details on the protein–lipid interactions.

**Acknowledgements**

The authors are grateful to Dr Vladimir Cotič for the preparation of the antibodies and to Professor Roger Fain for critical revision of the manuscript. This work was supported by the Ministry of Science and Technology of Slovenia.

**References**


Received September 18, 1996; revised January 20, 1997; accepted March 13, 1997

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