Journal of Biomolecular NMR, **18:** 281–282, 2000.
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Letter to the Editor: Sequence-specific resonance assignments of the potent cytolysin equinatoxin II

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Received 3 August 2000; Accepted 7 September 2000

Key words: assignments, cytolysin, sea anemone, toxin

Biological context

Sea anemone cytolysins constitute a novel class of toxins that functions by forming channels in cell membranes. In contrast to the bacterial pore-forming toxins, there is little detailed information on the mechanism of action of these toxins and as yet no 3-D structures have been determined. These highly basic toxins of mass ~20 kDa generate pores in membranes containing sphingomyelin by forming assemblies of three or more monomers (Belmonte et al., 1993) in the membrane. The channels formed by this oligomerisation are permeable to small molecules and solutes and the resulting osmotic imbalance promotes cell lysis. The cytolysins share no significant sequence identity with the bacterial pore-forming proteins such as α-hemolysin, where a heptameric complex forms the transmembrane pore (Gouaux, 1997), making it likely that the cytolysins have a unique structure.

Equinatoxin II (EqtII) is a 179-residue, 19.8-kDa cytolysin isolated from the Mediterranean anemone *Actinia equina* L. (Maček and Lebez, 1988). It is essentially identical with tenebrosin-C, a cytolysin isolated from the Australian red waratah anemone, *Actinia tenebrosa* and the first of this class of toxins to be sequenced in full (Simpson et al., 1990); tenebrosin-C has a variant S177T and EqtII a variant P81D (Belmonte et al., 1994). EqtII and tenebrosin-C show a high degree of sequence similarity (>60%) to cytolysins of *Stichodactyla helianthus, Heteractis magnifica* and other sea anemones (Wang et al., 2000). The transmembrane pore formed by EqtII consists of 3 to 4 non-covalently linked subunits (Belmonte et al., 1993) and there is some evidence of a conformational change upon binding to the membrane (Belmonte et al., 1994; Menestrina et al., 1999), with a more α-helical structure present in the membrane bound form, but no details at atomic resolution are available.

Our goal is to determine a high-resolution structure for EqtII both in solution and in a membrane environment. We are also interested in elucidating the structural basis for its interaction with sphingomyelin, which appears to be a necessary constituent of membranes susceptible to lysis by EqtII and other anemone cytolysins, and in understanding how EqtII oligomerises to form pores. In this paper we report essentially complete, sequence-specific, backbone and side-chain assignments for the ^{1}H, ^{13}C and ^{15}N resonances of double-labelled EqtII. Based on chemical shift data and sequential NOEs, the secondary structure of EqtII consists of eight strands of β-sheet and two short stretches of α-helix.

Methods and results

Labelled EqtII was prepared in an *Escherichia coli* expression system (Anderluh et al., 1996). Plasmid pAG2.1 was used to transform *E. coli* BL21(DE3) strain. Bacterial cells were grown in 400 ml of M9 medium in 2 l flasks at 37 °C and 250 rpm. The expression of EqtII was induced in the early log phase by addition of IPTG to a final concentration of 0.1 mM. Medium was supplemented with 1 g/l of $^{15}NH_4Cl$ (Isotec), and 0.3% (w/v) of ^{13}C-glucose (Cambridge Isotopes), in order to produce ^{15}N-labelled or ^{13}C,

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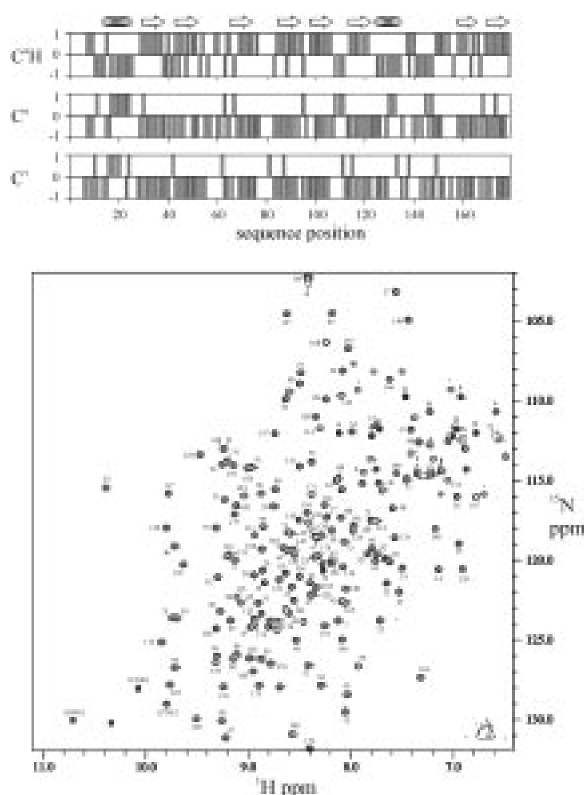

Figure 1. Chemical shift index (CSI) plot of EqtII with the secondary structure elements indicated (above), and ^{1}H-^{15}N 2D HSQC spectrum at 600 MHz of ^{15}N-labelled EqtII in 90% H_2O/10% $^{2}H_2O$ at pH 3.9 and 30 °C (below).

^{15}N-labelled protein. Soluble EqtII from the bacterial cytoplasm was isolated as described previously (Anderluh et al., 1996).

All NMR data were recorded at 30 °C on VARIAN Unity *Inova* 600 and 750 MHz spectrometers equipped with triple-resonance gradient probes. Sample concentrations were ∼1 mM and the pH was 3.9. DQF-COSY, TOCSY and NOESY spectra were acquired on an unlabelled protein sample, both in H_2O and $^{2}H_2O$. ^{15}N HSQC, HNHA and ^{15}N-edited NOESY and TOCSY spectra were recorded on ^{15}N-labelled protein. HNCA, HN(CO)CA, HNCACB, HN(CO)CACB, HNCO, H(CC)-TOCSY-(CO)NH, CC-TOCSY-(CO)NH, HCCH-TOCSY experiments from the ProteinPack (Varian Associates) were recorded with ^{13}C, ^{15}N-labelled protein (Sattler et al., 1999). NMR data were processed with the VNMR software and analysed with XEASY (Bartels et al., 1995).

The sequential assignment was accomplished by identifying peptide backbone connectivities using HNCA, HN(CO)CA and H(CC)-TOCSY-(CO)NH spectra, together with sequential NOEs in the ^{15}N-edited NOESY spectrum. Side-chain chemical shift assignments were made using HNCACB, HN(CO)CACB, H(CC)-TOCSY-(CO)NH, CC-TOCSY-(CO)NH, HCCH-TOCSY and ^{15}N -edited TOCSY spectra. $^{3}J_{HNH\alpha}$ coupling constants were determined from the HNHA spectrum.

Extent of assignments and data deposition

Sequential assignments have been determined for 173 of the 179 residues. Figure 1 shows an annotated ^{1}H-^{15}N HSQC spectrum of EqtII. For the assigned residues, 95% ^{1}H, 95% backbone ^{15}N, 97% ^{13}Cα and 90% side-chain ^{13}C resonances have been assigned.

A chemical shift index (CSI) plot is also shown in Figure 1. Based on these shifts, as well as backbone coupling constants and sequential NOEs, the secondary structure of EqtII consists of 8 strands of β-sheet and 2 short stretches of α-helix, the locations of which are indicated in Figure 1. The ^{1}H, ^{15}N and ^{13}C assignments have been deposited in the BioMagResBank database (http://www.bmrb.wisc.edu) with accession number 4797.

Acknowledgements

The authors would like to thank the Carlsberg Foundation for travel support for M.G.H. and the Danish Natural Science Research Council for support for W.Z. The use of the facilities of the Danish Instrument Center for NMR Spectroscopy of Biological Macromolecules at the Carlsberg Laboratory is acknowledged.

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