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Avidin–FITC Topological Studies with Three Cysteine Mutants of Equinatoxin II, a Sea Anemone Pore-Forming Protein

Gregor Anderluh,*[,1] Ariana Barlič,* Igor Križaj,† Gianfranco Menestrina,‡ Franc Gubenšek,† and Peter Maček*

**Department of Biology, Biotechnical Faculty, University of Ljubljana, Večna pot 111, 1000 Ljubljana, Slovenia; †CNR-ITC Centro Fisica Stati Agrregati, 38050 Povo, Italy; and ‡Department of Biochemistry and Molecular Biology, Jožef Stefan Institute, 1000 Ljubljana, Slovenia*

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Equinatoxin II (EqtII) is a cysteinless pore-forming protein from sea anemone *Actinia equina*. Three cysteine mutants were produced in an *E. coli* expression system in order to study the topology of lysine 77, arginine 126, and alanine 179. Accessibility of an introduced thiol group in the water soluble mutants was studied by using the thiol specific reagent fluorescein maleimide. In aqueous solution all three mutants were readily modified with the probe, indicating their accessibility to the solvent. Mutants were also biotinylated with biotin maleimide, enabling coupling with avidin–fluorescein isothiocyanate (avidin-FITC). After binding and insertion of biotinylated toxins into liposomes, avidin-FITC, which is unable to enter intravesicular compartment through toxin-created pores, was used to discriminate intra- or extravesicularly located thiols. All the mutated residues are found to be located on the outside of the lipid vesicles. The results proved the biotin-avidin system as suitable for topological studies of proteins creating pores in membranes.

Pore-forming toxins are the most widely spread type of toxins in living organisms (1). Mostly, they are water-soluble (poly)peptides which are able to insert into biological or artificial lipid bilayers and permeabilize them. Several bacterial pore-forming toxins have been well characterized (2,3).

More than 30 cytolytic polypeptide toxins have been isolated from sea anemones and classified into three groups according to their molecular mass, isoelectric point and mechanism of action (4). The largest group consists of basic and sphingomyelin-inhibitable toxins with a molecular mass of 20 kDa. Equinatoxin II (EqtII), a 179 amino acid residues representative from *Actinia equina* L., exhibits lethality (5), cardiotoxicity (6), platelet aggregation (7), cytotoxicity (8) and hemolytic activity towards a variety of red blood cells (9). In lipid membranes, EqtII forms cation-selective pores with diameter of 2 nm, permeable to the fluorescein derivative, calcein (10,11). Based on cross-linking experiments and kinetic data (11) it was suggested that the pores are composed of 3 or 4 monomers. Pore formation appears to be a multi-step process including conformational changes, binding and insertion of toxin monomers into the membrane, and oligomerization finally resulting in a functional pore (12–14).

In the present study, we took advantage of the fact that sea anemone pore-forming toxins lack cysteine or cystine (15-18). In order to investigate the topology of the molecule, EqtII cysteine mutants K77C, R126C, and A179C were produced and purified to homogeneity. They were labelled with either fluorescein maleimide or biotin maleimide. The latter modification allows binding of avidin-FITC, a complex large enough to be unable to enter vesicles through toxin-created pores. Labelling with fluorescein maleimide indicated the exposed location of the mutated residues on the water soluble molecules. These residues were neither transferred to the lipid phase nor to the intravesicular side of the membrane after binding and insertion of the biotinylated toxin molecules, since they were accessible to avidin-FITC.

MATERIALS AND METHODS

Mutagenesis, expression and purification of EqtII cysteine mutants. Mutations were introduced by PCR. For mutation Ala179→Cys,

[1] To whom correspondence should be addressed. Fax: +386 61 273 390; e-mail: gregor.anderluh@uni-lj.si.

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E2(+) (15) was used as a sense and E2.3 (−) (5′- CGA ATT CTA TCA GCA TTT GCT CAC GTG AAT TT- 3′) as an antisense oligonucleotide primer. For changing Lys77 and Arg126 to cysteine, T7 (5′- TAA TAC GAC TCA CTA TAG-3′) was used as a sense and T7-40 (5′- GTT TAC TCA TAT ATA CTT TAG-3′) as antisense oligonucleotide primer. They bind 61 bases upstream and 71 bases downstream, respectively, of in frame cloned EqtII cDNA in pAG2.1 (18). Additional mutagenic oligonucleotide primers, K77C (5′- TAC AAC GGT CAG TGC GAT CGT GGT CC- 3′) and R126C (5′- TAT AAG GGA AAG TGC CGA GCA GAC C- 3′), were added into a reaction mixture and amplification was performed as described (19). Codons for cysteines are underlined. After amplification, fragments were restricted with *Nde*I and *Eco*RI and cloned into pT7-7. The vectors were used to transform *E. coli* BL21(DE3) strain. Mutants were expressed and purified from the lysed bacteria by using ion-exchange chromatography on SP-Sepharose and gel filtration on Sephacryl S-200 (18).

Characterization of mutant toxins. SDS-PAGE was performed on a Phast System (Pharmacia). The N-terminal sequence was determined by Edman degradation using a Procise Sequencing system 492A (Perkin Elmer). Hemolytic activity was measured as described (20). One hemolytic unit (HU) was defined as a quantity of the toxin which reduced an apparent absorbance of the bovine erythrocyte suspension at 700 nm from 0.5 to 0.25 in 2 min. The concentration of mutant toxins was determined spectrophotometrically by the method of Perkins (21) or by using an ϵ_{280} of 3.61×10^4 $M^{-1}cm^{-1}$ (5).

Labelling sulfhydryl groups. Prior to labelling with fluorescein maleimide, the mutants were reduced with DTT at a final concentration of 5 mM and the excess of DTT removed by gel filtration on Sephadex G-15 (Sigma) with 50 mM Na-phosphate buffer, pH 7.5. Active fractions were eluted with the void volume and concentrated by Centricon-10 (Amicon). A 20-fold molar excess of fluorescein maleimide (Pierce), dissolved in the phosphate buffer, was then added and the mixture incubated at room temperature for 1 h. Labelled mutants were separated by HPLC (Waters) on SigmaChrom IEX-S column (Supelco) and detected with a diode array UV and fluorescence detector. Wild-type EqtII, treated in the same way, was not labelled. A value of 78×10^4 M^{-1} cm^{-1}, declared by the supplier, was used for ϵ_{490} of fluorescein maleimide. Experimentally, we found it decreased by 34% at 280 nm. This value was used in determining the extent of fluorescein labelling by assuming an equimolar fluorescein : protein ratio for isolated mutants.

For labelling with biotin maleimide, toxins (few μg) were incubated in 0.05 M Tris-HCl, pH 7.2, with biotin maleimide (probe/toxin ratio of 10) for 30 min at room temperature. DTT in excess was added to stop the reaction. In order to determine the yield of labelling, mixture was analysed by an analytical HPLC using HIC column (Brownlee). The amount of labelling was determined from absorption peak areas at 280 nm.

Binding avidin-FITC. For observing the binding of avidin-FITC to biotinylated mutants in a vesicle- bound form the following protocol was used. Toxins (3 μg) were biotinylated as described above and added to vesicles (large, unilamellar vesicles produced by extrusion technique (LUVETs) (22), 100 nm in diameter, composed of sphingomyelin/1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine 3/1; lipid/toxin molar ratio 400). After 30 min the mixture was centrifuged at 4°C and 54 000 rpm for 90 min (Eppendorf ultracentrifuge). Supernatant containing unbound toxins was removed and titered for residual hemolytic activity in order to determine the amount of binding. To the pellet, 4.5 μg of avidin-FITC (Sigma) were added and incubated for 30 min at room temperature. The mixture was centrifuged again under the same conditions, in order to remove the unbound avidin-FITC. The pellet was resuspended in 20 μl of the vesicle buffer, 140 mM NaCl, 20 mM Tris-HCl, 1 mM EDTA, pH 7.0. Ten μl of resuspended pellet was added to 960 μl of the vesicle buffer and fluorescence was measured on a FluoroMax (SPEX) photon-counting spectrofluorimeter. Excitation wavelength was set at 495 nm and emission spectra were recorded from 500-540 nm. The fluorescence of vesicle-biotinylated-mutant-avidin-FITC complex was corrected by a control value obtained for an avidin-FITC-vesicles mixture only. The amount of reacted avidin-FITC was determined from the calibration curve and the molar ratio of avidin-FITC/mutant was calculated. Avidin-FITC itself did not precipitate under this conditions.

RESULTS AND DISCUSSION

Cysteine mutagenesis has proved to be useful in structure and function studies of various membrane proteins (23-25). Replacement of a protein amino acid residue by cysteine enables the use of numerous thiol-specific probes. We employed the bacterial expression system for recombinant wild-type EqtII (18) to produce and isolate its K77C, R126C, and A179C mutants. The residues in the wild-type protein have been predicted to be solvent exposed (17), however, their exact position in a lipid bound form of the toxin has not been clear.

Characteristics of the purified mutants are presented in Table I. All mutants were hemolytically active. The activity of the R126C mutant did not differ significantly from that of wild-type, while A179C was 50% more active. Mutant K77C retained less than 1% of activity. The differences in hemolytic activity could be caused by an alteration of the protein structure after the introduction of cysteine. The following arguments suggest that the overall conformation of the mutants is not altered: i) intrinsic tryptophan fluorescence was mea-

TABLE I

Characteristics of EqtII Cysteine Mutants

Mutant	Relative hemolytic activity[a]	Yield (mg/l of bacterial broth)	N-terminal serine (mol %)	Extent of labelling with fluorescein (mol %)	Dimer formation[b]
Wild-type	1.00	—	n.d.	0	—
K77C	0.0077	1.5	100	91	+
R126C	1.14	3.2	100	71	+
A179C	1.51	10	65	90	+

[a] Specific hemolytic activity was determined for each mutant and normalized to the activity of native wild-type EqtII.
[b] Dimer formation was observed on SDS-PAGE. n. d. not determined.

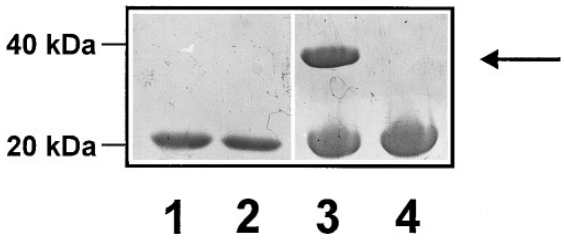

FIG. 1. Formation of dimers by cysteine mutants of EqtII. The appearance of dimers for mutants and wild-type was checked by SDS-PAGE. Lane 1, wild-type reduced and boiled; lane 2, wild-type without reduction and boiling; lane 3, A179C without reduction and boiling; lane 4, A179C mutant reduced and boiled. An arrow denotes dimers present in lane 3.

sured for all mutants and was the same as for the wild-type protein (data not shown); ii) the yield of mutants isolated from the bacterial supernatant has proved to be a good indication of the proper folding (26). In our case, the final yield was in the same range (Table I) as was obtained for the wild-type (18); and iii) for mutant K77C, exhibiting the lowest activity, the recorded far-UV CD spectra did not differ from that of the wild-type (data not shown). The decreased activity of K77C mutant will be discussed elsewhere.

N-terminal amino acid sequencing revealed that none of the mutants contained the initiator methionine. As shown in Table I, mutants K77C and R126C were correctly processed, on the other hand, about 35% of mutant A179C did not contain Ser-1. The reason for the partly improper processing might be in the changed local conformation of the N-terminal part of EqtII, which promote cleavage after Ser-1 by bacterial aminopeptidases.

The thiol-specific reagent, fluorescein maleimide, was used to determine the accessibility of the cysteine residues of the water soluble mutants. The yield of labelling ranged from 71-91% (Table I), indicating that all mutated residues are exposed to the solvent and readily modified. Wild-type toxin did not react with fluorescein maleimide (data not shown). Furthermore, reactivity of exposed thiols was proved by observing spontaneous formation of the mutant covalent dimers in non-reducing medium, as compared to monomeric wild-type EqtII (Figure 1).

Avidin-FITC has two to four molecules of fluorescein attached. It is widely used in fluorescence *in situ* hy-

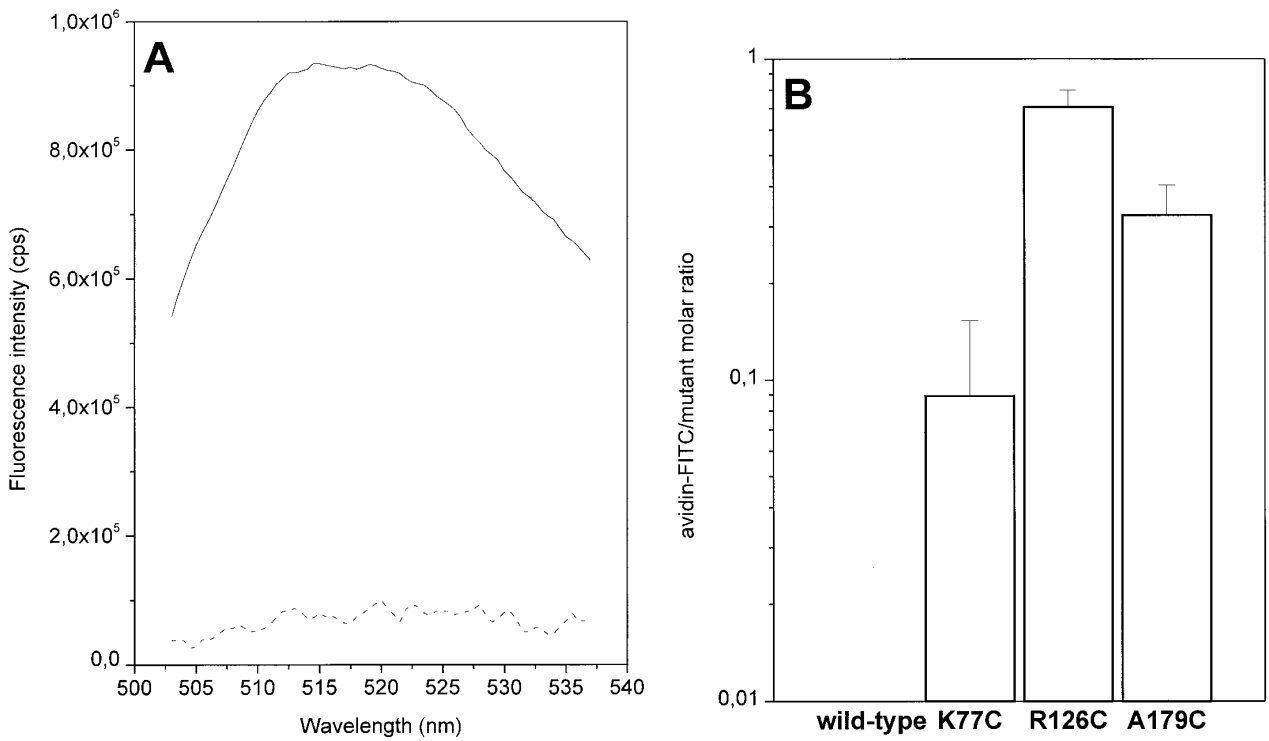

FIG. 2. Binding of avidin-FITC to biotinylated membrane bound forms of EqtII cysteine mutants. **(A)** The fluorescence of pellet, in which avidin-FITC was bound to the membrane bound form of mutants, was measured as an increase of fluorescence at 515 nm (a representative experiment with the A179C mutant is shown, solid line). Wild-type toxin did not precipitate avidin-FITC (broken line). **(B)** The molar ratio of avidin-FITC bound per molecule of mutant protein. From fluorescence intensity the amount of bound avidin-FITC was estimated. The ratio was calculated taking into consideration extent of binding and biotinylation. Vertical bars indicate s.d. of mean (N= 2).

bridization (27,28) and cell staining (29,30). In our study, this water soluble probe has been employed as it is too large to enter toxin pores, and as such it could be used to label only biotinylated residues on the *cis* (outer) side of the vesicular membrane. The yield of labelling with biotin maleimide, as determined from peak areas, was similar to that of labelling with fluorescence maleimide, except for R126C mutant which was labelled only up to 21%. Results of avidin-FITC coupling with membrane-bound wild-type or biotinylated toxins are presented in Fig. 2. Avidin-FITC bound in the membrane pellet was detected by an increase of fluorescence with an emission maximum at 518 nm, characteristic for fluorescein. With the exception of wild-type EqtII, avidin-FITC combined with all mutant variants. In Fig. 2A, an experiment with A179C mutant is shown. Figure 2B shows extents of binding of avidin-FITC to the lipid-bound mutants. The estimated probe/mutant molar ratio of about 0.7 for R126C suggests that Arg-126 is more exposed to the extravesicular solvent than Ala-179, and in particular Lys-77. This contrasted the lower extent of labelling of R126C when protein was free in solution. This apparent contradiction is probably due to the different conformation of the mutant in water and in the lipid-bound form.

In conclusion, we found that cysteine residues, introduced by mutagenesis at positions 77, 126 and at the C-terminus of EqtII, are exposed to the solvent as predicted for the wild-type (17). After insertion of the toxin into vesicle lipid bilayer, they all face its *cis* side as they could be accessed by the large, water soluble probe, avidin. Cysteine 126, which is less accessible for fluorescein and biotin maleimides, moves to a more exposed position after the protein-lipid interaction. The study demonstrates that the combination of cysteine mutagenesis and a high affinity biotin-avidin system may be useful approach in topological studies of protein pores in membranes.

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