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Lysine 77 is a Key Residue in Aggregation of Equinatoxin II, a Pore-forming Toxin from Sea Anemone *Actinia equina*

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Abstract. Among eighteen point mutants of equinatoxin II produced in *E. coli,* containing a single cystein substitution at variable position, EqtIIK77C was chosen for its peculiar properties. It was almost 100 times less hemolytic than the wild-type, but its hemolytic activity could be restored by chemical modification of the thiol group, provided that a positive charge was reintroduced. This indicates that a positive charge at this position is necessary for toxin activity. The mutant formed larger pores as compared to the wild type, but displayed the same cation selectivity. The pores reverted to normal size upon reintroduction of the positive charge. The conformation of EqtIIK77C and its binding to lipid membranes, either vesicles or red blood cells, was almost normal. However the kinetics of calcein release from lipid vesicles was substantially slower than that of the wild-type. Taken together with the different size of the pore formed, this is an indication that mutation of Lys77 → Cys influences the normal development of the aggregate which is required for assembling the functional pore.

Key words: Pore-forming toxin — Sea anemone — Secondary structure — Chemical modification — Sulfhydryl reagents — Pore size — Ion selectivity — *Actinia equina*

Introduction

Equinatoxin II (EqtII) is a potent hemolytic protein of 179 amino acids found in the venom of the sea anemone *Actinia equina* L. It belongs to a larger group of actiniaria proteins, called actinoporins, which share very similar characteristics: size of around 20 kDa, isoelectric point higher than 9, inhibition by sphingomyelin and common ability to lyse red blood cells by colloid-osmotic shock [8, 13, 28]. Little is known about their structural organization. Salient features are a high content in β-structure, nearly 65% on average [17], and the presence of an amphiphilic α-helix at the N-terminus [4]. Their mechanism of action is also not fully understood at the molecular level, however a common tract is the opening of toxin-induced channels in the membrane of the attacked cell. Pore-formation requires at least two steps, as shown by kinetic experiments [5, 15, 27]: binding of the protein to the membrane in monomeric form and subsequent oligomerization into the functional channel. The membrane-bound EqtII monomer interacts with the lipid with at least two extended regions: the N-terminal helix and a hydrophobic core comprising residues 105–120, and makes some additional contacts near the C-terminus [1, 2]. The final pore aggregate is not stable and the existence of oligomeric structures was envisaged from the analysis of permeabilization kinetics and from chemical cross-linking experiments [5, 27]. Although possessing a wide spectrum of action against different cell types [13], we could show that EqtII can be usefully targeted against human cancer cells [20] or human parasites [26].

To make some progress in these applications or, more in general, to improve our understanding of protein-membrane interactions, a better knowledge of toxin structure/function relation is clearly needed. In this paper we investigate the properties of an EqtII mutant, EqtIIK77C, which was produced in *E. coli.* This mutant was almost 100 times less active than the wild-type, however by chemical modification we were able to re-

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store its hemolytic activity just by reintroducing a positive charge in its side chain. This mutant also formed larger pores as compared to the wild type. Finally, while the binding was preserved, the kinetics of calcein release was substantially slower, indicating that mutation of Lys77 → Cys had an influence only on the second part of the pore-formation mechanism, i.e., the aggregation into a functional pore. We also proved that chemical modifications on an introduced thiol group can be used in alternative to extensive site-directed mutagenesis to provide the same kind of information.

Materials and Methods

Production and Isolation of Toxins

Native EqtII was isolated as described [14]. EqtIIK77C was produced in an *E. coli* expression system as described previously [3]. The expression and purification of all other mutants has been reported [2]. Native and SDS-PAGE were performed on PHAST system from Pharmacia. Circular dichroism spectra of the wild-type and mutant in water were recorded by a Jasco J-710 spectropolarimeter in a 1 cm cell at 25° as in [4]. Tryptophan fluorescence measurements were made at 25°C using a FP-750 fluorimeter (Jasco). Excitation wavelength was 295 nm (in order to minimize tyrosine contribution [11]) and emission spectra were recorded from 300 to 400 nm. Excitation and emission slits were set at 10 nm. The concentration of proteins was 2.5 μM. Background fluorescence of the buffer was subtracted.

Preparation of Vesicles

Multilamellar liposomes were prepared either in the presence of 80 mM calcein (neutralized to pH 7.0 with NaOH) or in 140 mM NaCl, 20 mM Tris-HCl, 1 mM EDTA, pH 7.0 (vesicle buffer) and subjected to 6 cycles of freezing and thawing. Large unilamellar vesicles (LUV) were prepared by extruding a solution of multilamellar liposomes using a two-syringe extruder (LiposoFast Basic unit, Avestin, Canada) through polycarbonate filters (Nucleopore) with pores of an average diameter of 100 nm [12]. Small unilamellar vesicles (SUV) were prepared by sonication of multilamellar vesicles for 40 min at room temperature using pulsed sonicator (Vibracell VC 500 Sonics & Materials). Lipids used were sphingomyelin (Avanti Polar Lipids) and 1-palmitoyl-2-oleyl-sn-glycero 3-phosphocholine (POPC) (Lipoid) at ratio 3:1. To remove untrapped calcein, the vesicles were spun through minicolumns (Pierce) loaded with Sephadex G50 preequilibrated with vesicle buffer. The final concentration of lipid was determined using Menagent phospholipid assay (Menarini Diagnostics, Florence, Italy) as described earlier [27].

Hemolytic Activity

Hemolytic activity was measured by an automated microplate reader (UVmax, Molecular Devices, Sunnyvale, CA). Toxins were subjected to serial twofold dilution in 140 mM NaCl, 10 mM Tris-HCl, pH 7.4, with 0.1 mg/mL Prionex (porcine hydrolysed collagene of average mass 20 kD purchased from Pentapharm, Basel, Switzerland) in 96 well microtiter plates. Human red blood cells (HRBC), collected intravenously and washed in the same buffer, were then added. The time course of hemolysis was followed turbidimetrically at 650 nm for 30 min at room temperature. Percentage of hemolysis was determined as follows:

$$\text{Hemolysis (\%)} = (A_{max} - A_{fin})/(A_{max} - A_{min}) \times 100$$

where A_{max} and A_{min} represent the absorbance values for intact and completely hemolysed RBC respectively and A_{fin} is the value in each well at the end of the assay. For osmotic protection experiments glycerol, and a series of polyethylenglycols (PEGs) i.e., PEG200, PEG400, PEG600, PEG1000, PEG1500 or PEG2000, where the numbers denote average molecular mass (all purchased from Fluka) were added to erythrocyte buffer in a final 30 mM concentration. Erythrocytes were incubated in such buffer at least half an hour before starting the experiment. The radii of these molecules, in hydrated form, were taken from [10]. Control experiments were performed in the presence of 2 mM dithiothreitol (DTT).

FTIR Studies of Lipid-Free and Membrane-Bound Toxins

Secondary structure of the toxins, with or without lipid, was investigated by FTIR spectroscopy essentially as described earlier [16, 17]. Spectra were collected in the attenuated total reflection (ATR) configuration [6], on a Bio-Rad FTS 185 spectrometer, equipped with DTGS detector and KBr beamsplitter, and purged with 10 l/min dry air. Absorption spectra were obtained in the region between 4,000 and 600 cm^{-1}, at a resolution of one data point every 0.25 cm^{-1}, 40 to 120 interferograms were collected, Fourier transformed and averaged. 25 μg of toxins in water (concentration between 0.6 and 0.8 mg/ml) were deposited on one side of a 10-reflections Ge crystal and gently dried under nitrogen in a thin layer. The crystal was housed in a liquid cell and, before collecting a spectrum, flushed with D_2O-saturated nitrogen for 20–30 min to allow deuteration of the sample, in particular replacement of the fast-exchangeable amide hydrogen ions [25]. The spectrum of the sample-free crystal was used as the background. If present, residual H_2O vapor bands were subtracted. The relative content of secondary structural elements was estimated by the method of Susi and Byler [24] exactly as we have already described [17]. To estimate the number, position, amplitude and width of the Lorentzian components which were finally used to curve fit the amide I′ band between 1700 and 1600 cm^{-1}, was first deconvoluted with a resolution enhancement factor $k = 2$ [16].

For membrane-bound samples, aliquots of SM:POPC (3:1) SUV, prepared as described above, were diluted to 125 μM in 1 ml of Hepes-NaOH 10 mM pH 7.0 and incubated with 1.25 μM of one of the two toxins (final lipid:toxin molar ratio 100:1). After incubation at room temperature for ½ hr, the SUVs were separated from the residual unbound toxin by precipitation in an ultracentrifuge (Optima TL 100 by Beckman), using a fixed angle rotor (TLA 100.2) run at 100 krpm (≈450,000 × *g*) for 3 hr at 4°C. The supernatants were collected and tested for the presence of free toxin by measuring the hemolytic activity. At these conditions, more than 90% of the protein coprecipitated with the SUV. The pellet, consisting of vesicles and bound toxin, was recollected in an aliquot of 20 μl, diluted with the same volume of H_2O and applied to the Ge crystal for FTIR analysis. Controls in which the same amount of toxin was centrifuged in the absence of the vesicles indicated that, without lipid, not more than 5% of the protein could be collected in the bottom 20 μl of the tube. The contribution of the lipid to the lipid plus toxin spectra was removed by subtracting the spectrum of the lipid alone (collected following the same centrifugation procedure, except that the protein was omitted) weighted by a coefficient that eliminated the residual band at 1740 cm^{-1}, due to the stretching of the carboxyl groups of the phospholipid. This was necessary also in view

of the fact that sphingomyelin, comprising the ceramide moiety, contributed a signal in the amide I region which was between 23 and 28% of the total. After removal of the lipid contribution, differential spectra were also obtained by subtracting the spectrum of the toxin in soluble form, with a weight that minimizes the remaining integral in the amide I′ region.

Permeabilizing Activity

Permeabilization activity on unilamellar vesicles loaded with calcein was measured using a fluorescence microplate reader (Fluostar, SLT, Austria). Samples were excited at 485 nm and the fluorescence emitted at 538 was measured. A 96 well microtiter plate was filled with 200 μL of vesicles buffer and the desired amount of toxin and vesicles. The concentrations used are reported in the text. The experiments were run at room temperature. The percentage of release, R%, was calculated as follows:

$$R(\%) = (F_{fin} - F_{in})/(F_{max} - F_{in}) \times 100$$

where F_{in} and F_{fin} represent the initial and the final (steady state) value of fluorescence before and after addition of toxin, respectively.

Toxin Binding

To determine the binding of toxins to HRBC at steady state, 2 μg of proteins were added to 20 μL of HRBC ($A_{650} = 1.0$) in erythrocyte buffer supplemented with 30 mM PEG4000 in a final volume of 25 μL. They were incubated for 10 min and then centrifuged for 2 min at 14,000 × *g*. Supernatant was removed and pellet resuspended in 5 μL of electrophoresis buffer (0.2 M Tricine, 0.2 M Tris, 0.55% SDS, pH 7.5), subjected to SDS-PAGE electrophoresis using PhastSystem, transferred to PVDF (polyvinylidene difluoride) membranes and stained with anti-EqtII serum as described [2]. To estimate the velocity of binding to model lipid membranes, toxins at a concentration of 2.5 μg/mL were added to a solution of SUV at a final lipid/toxin ratio 500. After a variable delay, HRBC were added and residual hemolytic activity was determined by measuring turbidimetrically the rate of hemolysis. By comparison with a calibration curve of toxin alone, the amount of free toxin remaining in solution at each moment was then determined.

Chemical Modifications

Toxins, at a final concentration of 8.6 μM, were incubated in 0.05 M Tris-HCl, pH 8.2, with DTT (at a DTT/toxin ratio 80) for 10 min at room temperature. Thereafter, either iodoacetic acid or iodoacetamide (Sigma) in 0.5 M Tris-HCl pH 8.2, were added to toxins in a final ratio of probe/DTT = 30 (meanwhile the ratio probe/toxin was 2400). The reaction was stopped with excess of DTT after 30 min of labeling at room temperature and in the dark. For labeling with bromoethylamine (Sigma) the same conditions were used, except that the concentration of bromoethylamine was higher (usually probe/toxin was 12,000) and toxin was incubated at room temperature overnight. Labeled proteins were used for hemolytic assays, release of calcein or native-PAGE without further purification.

Black Lipid Membranes (BLM)

Bilayer lipid membranes were prepared at room temperature by the painting technique [19]. The lipid used was a mixture in a molar ratio 20:1 of diphytanoyl-phosphatidylcholine (DPhPC) and egg sphingomyelin (SM) which were both provided from Avanti Polar Lipids. A droplet from a 10 mg/mL solution of these lipids in n-decane was spread over a 1 mm hole in a Teflon septum separating two stirred buffer solutions (4 ml of KCl 100 mM, Tris 10 mM, pH 8). The hole was pretreated with a few μL of the same lipid solution allowed to dry in air. The membrane spontaneously thinned to a bilayer with an average capacitance of 1 nF and a conductance of 100 pS. Toxin was added on one side only (called the *cis* side) to stable preformed bilayers. However, in the case of EqtIIK77C, we sometimes reformed the membrane in the presence of the toxin in order to improve the detection of pores. The current flowing through the membrane, under voltage clamp conditions, was collected through Ag-AgCl and converted to voltage by a virtual grounded operational amplifier (Burr Brown OPA 104C). *Trans* compartment was the reference and the current was defined positive when cations flew into the *cis* compartment. For the selectivity experiments the solutions contained 10 mM Hepes (pH 8.0) plus either 20 mM KCl in the *cis* compartment or a variable concentration of KCl (ranging from 20 to 600 mM as specified) in the *trans* compartment. The electrodes were connected through agar bridges saturated with 3 M KCl.

Results

In an attempt to identify domains of EqtII that are important for its function, a collection of point mutants was produced in which a single cysteine was introduced at different positions [2]. We then screened the importance of having a charge at that position, by chemically modifying the single cysteine with either iodoacetic acid (introducing a negative charge) or bromoethylamine (introducing a positive charge). The hemolytic activity of each mutant, before and after chemical modification, is shown in Fig. 1 together with that of the wild-type. The introduction of the cystein was well tolerated by all mutants except those in the interval between R31C and K77C and by S167C (Fig. 1*A*). Chemical modification gave additional information (Fig. 1*B*). It appears that a polar residue is required at position 20 and 43 (normally occupied by lysines) because the introduction of a charge of either sign increased the specific activity. Conversely, a nonpolar residue seems to be necessary at position 54 (normally occupied by a serine), since the introduction of a charge of either sign decreased the specific activity of the mutant. Most interesting was mutant EqtIIK77C, because in this case the presence of a positive charge at position 77 (like in the native protein) was able to restore its hemolytic activity nearly to the level of the wild-type, whereas the presence of a negative charge reduced it even more. Therefore, we decided to study the effects of the mutation at position 77 in more detail.

Structural Properties of EqtIIK77C

EqtIIK77C is produced in a bacterial expression system as a homogeneous protein with similar yield as the wild-

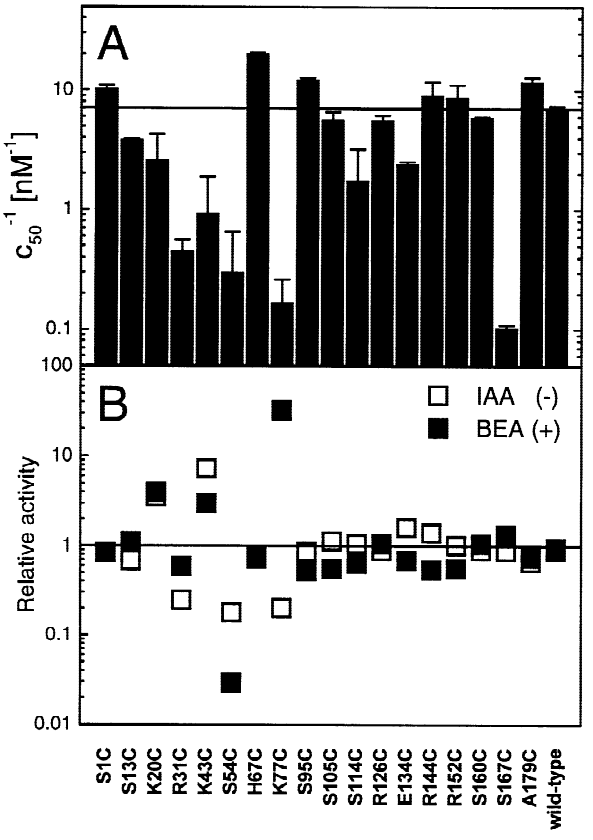

Fig. 1. Dependence of the hemolytic activity of EqtII mutants containing a single cystein at variable position upon introduction of a charged group at the thiol. (*A*) Hemolytic activity of each mutant was measured in a 96-well microtiter plate and compared to the wild-type. It is expressed as the reciprocal of the toxin concentration required for 50% of lysis of HRBC. Reported values are mean of triplicates ± SD. (*B*) Relative change of activity (measured as in part *A*) for each mutant after modification with either iodoacetic acid, which introducing a negative charge at the thiol group (open squares) or bromoethylamine, introducing a positive charge (closed squares).

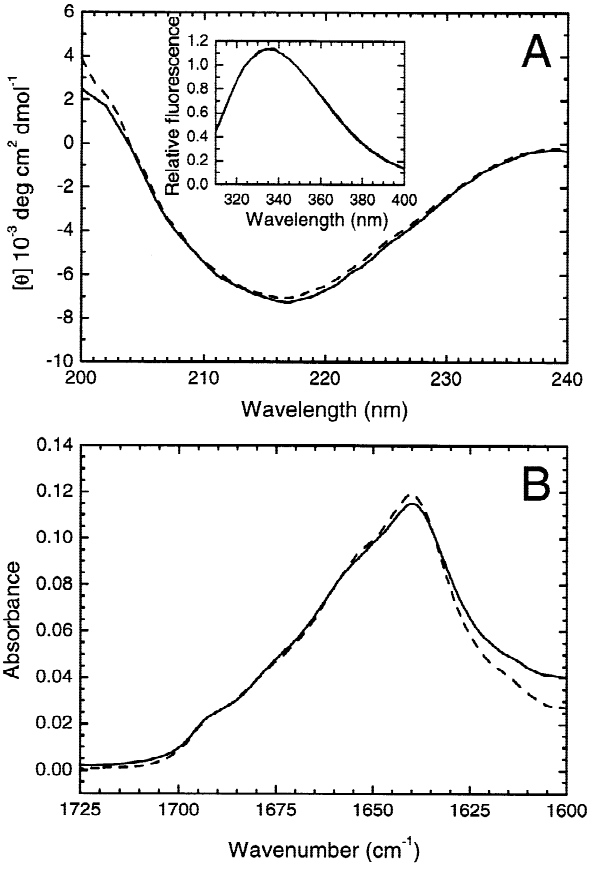

Fig. 2. Spectroscopic properties of the wild-type and EqtIIK77C (*A*) CD spectra measured in water at 25°C in the presence of 0.23 mg/mL for the wild-type and 0.45 mg/mL for EqtIIK77C. Inset: tryptophan emission fluorescence spectra measured in water at 25°C, with excitation wavelength at 295 nm and both slits at 10 nm. The concentration of both proteins here was 0.05 mg/mL. In both panels, solid lines are for the wild-type, dashed lines for EqtIIK77C. (*B*) FTIR-ATR spectra of deuterated films of the wild-type and EqtIIK77C in the amide I′ region. 25 μg were applied for both toxins. As in part *A*, solid lines are for the wild-type, dashed lines for EqtIIK77C. For a proper superimposition, the spectrum of the mutant was multiplied by a constant factor (1.3) calculated dividing the area of the amide I′ band of the wild-type by that of the mutant.

type, nevertheless it is about 80 times less hemolytic (Fig. 1*A*). To clarify the reasons for such a low activity, we first checked if its structure was preserved. In SDS-PAGE it was running normally, whereas in native-PAGE, it appeared as a single band running towards the cathode at a slightly slower rate than the wild-type because of its less positive charge (*not shown*). A small portion of covalently bound dimers was present (as indicated by the difference in the SDS-PAGE pattern with and without DTT). They amounted to less than 10% and did not influence the hemolytic properties of the mutant since hemolysis in the presence or the absence of DTT was the same. The global protein fold was checked by measuring the far-UV CD spectrum (Fig. 2*A*), which had a pronounced minimum at 217 nm and was superimposable to that of the wild-type, and tryptophan fluorescence emission which was also practically identical to that of the wild-type (inset of Fig. 2*A*). An evaluation of the secondary structure composition of both proteins was obtained from analysis of their amide I′ vibrational band in FTIR spectra (Fig. 2*B*). The two spectra were indeed very similar, suggesting the presence of a large content of β-structure. According to [24], we calculated that the percent composition of β-sheet, β-turn, α-helix and random coil was 56%, 10%, 17% and 17% for the wild-type and 58%, 15%, 15% and 12% for EqtIIK77C, i.e., the mutant had only slightly less random coil and more β-turn than the wild-type. These values are consistent with those already reported for two sticholysins, similar actinoporins produced by the anemone *Stichodactyla he-*

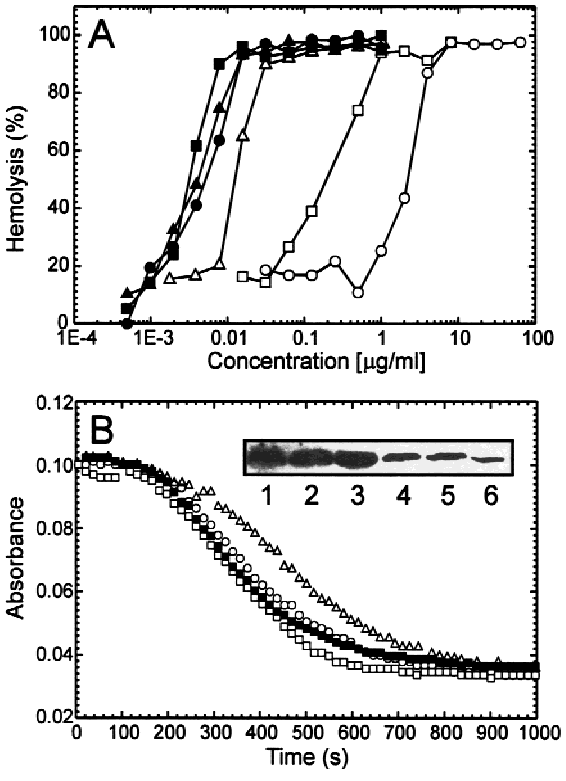

Fig. 3. Hemolytic activity of EqtIIK77C and its derivatives. Hemolysis of HRBC (initial A_{650} = 0.1) was measured with an automated microplate reader at room temperature in 140 mM NaCl, 10 mM Tris-HCl, 0.1 mg/mL Prionex, pH 7.0. (*A*) Percentage of hemolysis calculated after 30 min. Black symbols, the wild-type; open symbols, EqtIIK77C. Squares, unmodified protein; circles, protein modified with iodoacetic acid; triangles, protein modified with bromoethylamine. Representative results are shown in all cases, relative variation was within 15%. (*B*) Time course of hemolysis, symbols are the same as in part *A*. Toxin concentrations were: the wild-type 30 ng/mL; EqtIIK77C 1 μg/mL; *S*-carboxymethylcysteine EqtIIK77C 8 μg/ml and *S*-aminoethylcysteine EqtIIK77C 63 ng/mL. Inset: western-blot of protein monomers bound to HRBC membranes (toxin concentration during incubation was always 80 μg/mL). Lanes 1 to 3, EqtIIK77C and its derivatives; lanes 4 to 6, the wild-type; proteins in lanes 2 and 5 were treated with iodoacetic acid; proteins in lanes 3 and 6 were treated with bromoethylamine; lanes 1 and 3 were unmodified.

lianthus [17]. All experiments thus lead us to the conclusion that the overall 3D structure of EqtIIK77C was preserved.

Pore Forming Activity of EqtIIK77C and Its Derivatives

The effects of introducing different functional groups at the single cysteine residue of EqtIIK77C, by covalent modification of the sulfhydryl group, was further investigated (Fig. 3). Indeed we observed that the hemolytic activity of EqtIIK77C was dependant on the kind of side chain attached on Cys77 (Fig. 3*A*, Table). While the activity of EqtIIK77C alone was reduced approximately 80 times, as compared to the wild-type, that of variants having either *S*-carboxymethylcysteine, *S*-aminoethylcysteine or *S*-carboxyamidomethylcysteine at position 77 was reduced approximately 570, 2.7 or 37 times, respectively. Controls indicated that hemolysis by the wild-type was not affected by the same treatment with iodoacetamides or bromoethylamine, indicating that, in the absence of the sulfhydryl group, side reactions at other amino acids did not occur. The 30-fold rise in activity of EqtIIK77C, upon modification with bromoethylamine, clearly indicates that a positive charge at position 77 is required for the wild-type activity. The reason that the restoration of hemolysis was still incomplete is that while modifications with iodoacetic acid and iodoacetamide were extensive (according to native PAGE and ion-exchange chromatography), that with bromoethylamine was not (Table). Due to the different chemistry involved, the yield was in that case around 35% (in agreement also with the results of other investigators that used the same reagent [21]), practically matching the recover in activity and suggesting that the portion of protein that was modified was indeed fully active.

Despite the differences in activity, the mechanism of binding and lysis of EqtII, EqtIIK77C and the derivatives, was apparently the same. In fact, the binding to HRBC was even slightly better for the mutant (inset in Fig. 3*B*), and independent of the charge at position 77, since both variants, with positive or negative charge,

Table 1. Effects of point mutations and chemical modifications at residue 77 on the hemolytic activity of EqtII

Protein[a]	Lateral chain[b]	$1/c_{50}$ [nM^{-1}][c]	Modification yield (%)[d]
Wild-type	NH_3^+	5.7 ± 0.9	—
EqtIIK77C	SH	0.10 ± 0.03	—
EqtIIK77C-BEA	S NH_3^+	3.7 ± 2.3	35
EqtIIK77C-IAA	S COO^-	0.01 ± 0.02	100
EqtIIK77C-IAM	S $CONH_2$	0.14 ± 0.01	100

[a] EqtIIK77C was derivatized with either bromoethylamine (EqtIIK77C-BEA) or iodoacetic acid (EqtIIK77C-IAA) or iodoacetamide (EqtIIK77C-IAM).
[b] Structural formulae of the side chains resulting from point mutation or chemical modification are indicated.
[c] Hemolytic activity, expressed as the reciprocal of the toxin concentration required to obtain 50% of lysis of HRBC, was obtained as shown in Fig. 3.
[d] The yield of modification was determined by ion-exchange chromatography.

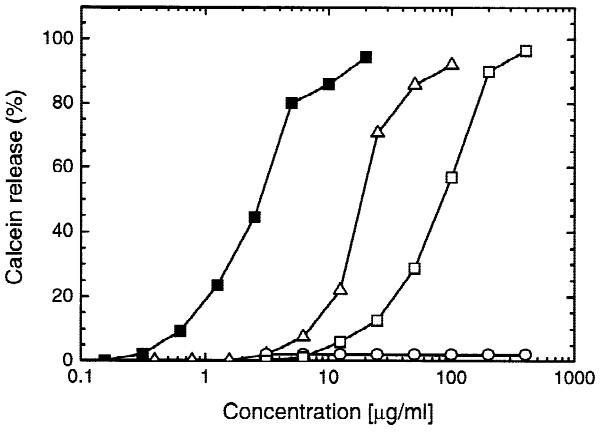

Fig. 4. Permeabilizing activity of EqtIIK77C and its derivatives on lipid vesicles. LUV were composed of SM/POPC (3/1) and loaded with calcein. The release was measured with SLT microplate reader at room temperature in 140 mM NaCl, 20 mM Tris-HCl, 1 mM EDTA, pH 7.0. The percentage of release was calculated after 45 min with reference to the total release induced by 1 mM TritonX-100. Black squares, the wild-type; open symbols, EqtIIK77C: squares, unmodified protein; circles, protein modified with iodoacetic acid; triangles, protein modified with bromoethylamine. Representative results are shown in all cases, relative variation was within 15%.

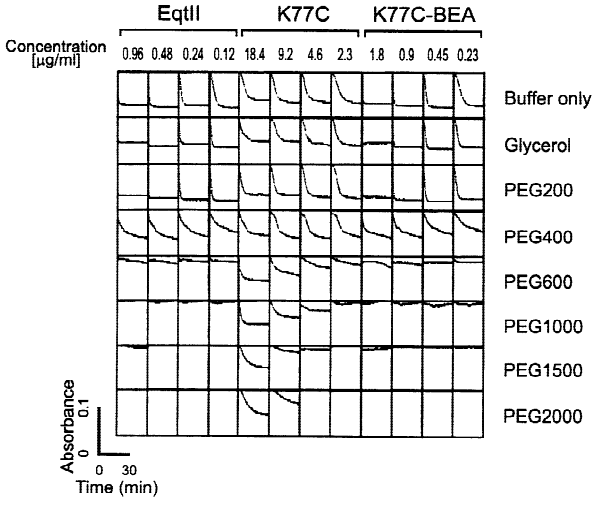

Fig. 5. Osmotic protection of hemolytic activity by PEGs. The hemolytic activity was titrated by measuring the change in turbidity. PEGs of different size, as indicated on the right, were present in the erythrocyte buffer at a final 30 mM concentration. Toxins were twofold serially diluted and then HRBC added and the time course of hemolysis was measured for 30 min. Toxin concentrations are reported; EqtIIK77C-BEA indicates the mutant modified with bromoethylamine.

bound equally well to the cells. Furthermore, also the time course of hemolysis was very similar, albeit occurring at different toxin concentrations (Fig. 3*B*).

Similar results were obtained when one simple system, the release of calcein from liposomes, was used to investigate the interaction of EqtIIK77C and its derivatives with pure lipid membranes (Fig. 4). As compared to the wild type, the activity of EqtIIK77C alone was reduced about 30 times, while that of *S*-aminoethylcysteine EqtIIK77C was reduced approximately 7 times (representing almost a fourfold recovery upon reintroduction of the positive charge) and that of *S*-carboxymethylcysteine EqtIIK77C was nil, at least up to a concentration of 0.4 mg/mL.

The Pore Formed by EqtIIK77C is Larger Than That of the Wild Type

The addition to the erythrocyte buffer of osmotic protectants like PEGs, can considerably prolong the lag time and reduce the rate of hemolysis by the wild-type [5]. In the case of the wild-type, addition of PEG600 (radius 0.7 nm) already strongly diminished the hemolytic activity, whereas PEG1000 (radius 0.9 nm) completely abolished it, at all toxin concentrations (Fig. 5). The hemolytic activity of EqtIIK77C was also reduced, but in this case PEGs of larger size were required. For example, PEG1000 was active only at lower toxin concentrations, whereas at higher, only PEG2000 (radius 1.4 nm) was inhibitory. If a positively charged side chain was attached to Cys77 (by bromoethylamine) the protective effect of smaller PEGs was again observed.

Electrical Properties of the Pore Formed by EqtIIK77C

EqtIIK77C formed ionic pores in BLM composed of a PC/SM mixture (Fig. 6*A*). While the wild-type readily formed pores at 5 nM concentrations, the concentration of EqtIIK77C required was much higher, usually around 60 nM. The number of channels formed was also always smaller with the mutant, which is in accordance with its reduced hemolytic activity. The conductance of the wild-type single channel was approximately 400 pS (Fig. 6*B*). On the contrary, EqtIIK77C formed channels with larger conductance, of approximately 500 pS, in agreement with the increase in pore size observed in the hemolysis experiments with PEGs.

Despite the increase in conductance, the selectivity of the pores remained practically the same, i.e., the permeability of K^+ was roughly 10 times that of Cl^- with both toxins (inset of Fig. 6*B*). Furthermore, the addition of methane-thio-sulphonate (MTS) reagents, which are commonly used for thiol modification of preformed ion channels or toxin pores [9, 18], did not show any effect on the ion currents flowing through preformed EqtIIK77C pores, at least up to a concentration of 2 mM (*data not shown*).

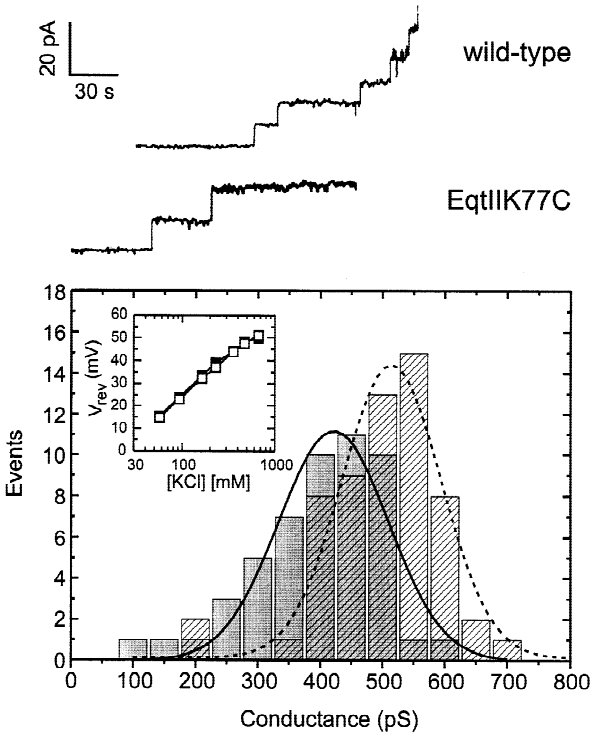

Fig. 6. Channel formation in BLM. Upper traces: ionic currents flowing through membranes exposed either to the wild-type (upper trace) or to EqtIIK77C (lower trace). Upward steps indicate the formation of single pores into the BLM. Applied voltage was +20 mV. Concentration of protein (added only to the *cis* side of the membrane) was 20 nM for the wild-type and 25 nM for EqtIIK77C. BLM were formed of DPhPC with 5% SM. Buffer on both sides was 4 mL of KCl 100 mM, Tris 10 mM, pH 8. (*B*) Histograms showing the number of events with a given conductance observed in experiments as in part *A*. Average conductance (±SD) was 397 ± 101 pS for the wild-type (51 events from 3 different membranes, shaded bins) and 501 ± 94 pS for EqtIIK77C (59 events from 5 different membranes, hatched bins). Superimposed are Gaussian distributions. Inset: Voltage reversal (i.e., the voltage V_{rev} at which no net ion current flows) measured under different concentration gradients. KCl was 20 mM on the *cis* side and the reported amount on the *trans* side. Wild-type (open squares) was 5 nM whereas EqtIIK77C (closed squares) was 35 nM. Adaptation of the Goldman-Hodgkin-Katz expression [23], to the observed V_{rev} yields a cation to anion permeability ratio P_{K^+}/P_{Cl^-} of 9.9 for the wild-type (closed squares) and 9.7 for EqtIIK77C (open squares), which are both very similar to the values reported for Na^+ and Cl^- with the wild-type [5].

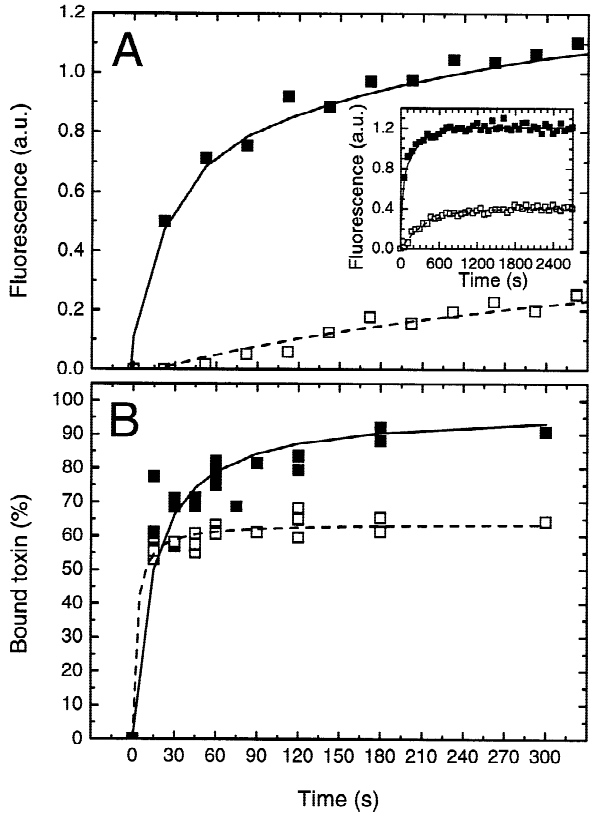

Fig. 7. Comparison between toxin binding and calcein release with EqtIIK77C and the wild-type. (*A*) Calcein release, in the first 5 min, from calcein-loaded SUV of the same composition as in the lower panel and at the same toxin concentration (L/T ratio 1.25). The inset shows the time course of release on a longer time scale. Other conditions are as in Fig. 4. Open squares and filled squares are as in the lower panel. At a concentration of 10 μg/mL, the wild-type reached 50% of calcein release in 30 sec, while EqtIIK77C took 270 sec to get to the same level. The final release of EqtIIK77C was only one-third of that of the wild-type. (*B*) binding of toxins to liposomes was estimated by incubation with POPC/SM (1/3) SUV at L/T ratio 50 for desired amount of time (toxin concentration was 10 μg/ml) Thereafter HRBC were added and the rate of hemolysis was determined turbidimentrically. Using calibration curves obtained with known concentrations of toxins, the amount of toxin remaining in solution was calculated at each time. Open squares are for EqtIIK77C whereas filled squares are for the wild-type.

Aggregation Rather than Binding is Impaired in EqtIIK77C

To determine at which step mutation Lys77 → Cys affects the permeabilizing activity of EqtIIK77C, the binding to SUV and their permeabilization was simultaneously measured in comparison with the wild type (Fig. 7). Although both the rate and the extent of calcein release were much lower for the mutant (Fig. 7*A*), we observed that the initial binding was virtually the same, and the final value was only slightly lower with EqtIIK77C (Fig. 7*B*).

FTIR spectroscopy, performed under similar conditions, confirmed that the extent of binding to SUV was similar (approximately 90% for the wild-type and 84% for EqtIIK77C, Fig. 8). Furthermore, both the wild-type and the mutant underwent a small conformational change with lipid binding, that can be summarized as a decrease of random coil and an increase of α-helical and β-structure (inset in Fig. 8). In the lipid-bound form, the percent composition of β-sheet, β-turn, α-helix and random coil was 55%, 16%, 18% and 10% for the wild-type and 56%,

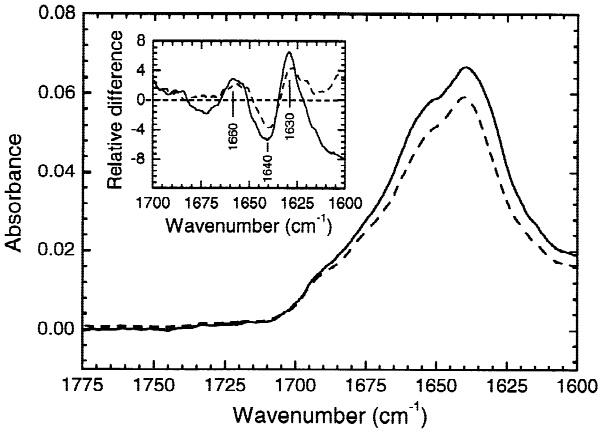

Fig. 8. FTIR-ATR spectra, in the amide I′ region, of the wild-type and EqtIIK77C adsorbed to lipid vesicles. Toxin incubated at a lipid/toxin ratio of 100 with SUV comprised of SM/PC (3:1) were separated from unbound toxin by centrifugation and applied to the ATR crystal as in Fig. 2. The spectra were corrected by removing the contribution of the lipid as explained in Materials and Methods. By comparison with spectra generated by known amounts of the toxin it was evaluated that around 90% of the wild-type (solid line) and 84% of EqtIIK77C (dashed line) coprecipitated with the SUV. Inset: Differential spectra, obtained by subtracting the soluble form from the normalized lipid-bound form, are shown in the inset, for either the wild-type (solid line) or EqtIIK77C (dashed line). They are reported as a percent of the amplitude of the lipid-bound form. With both toxins an excess of signal around 1660 cm^{-1} and 1634 cm^{-1} was present, indicating an increase in α-helix, β-turn and β-sheet structure, which was compensated by a decrease at 1640 cm^{-1}, suggesting a reduction of the random coil.

14%, 18% and 11% for EqtIIK77C, i.e., almost identical. Such a conformational change was already described for sticholysins [17] and appears to be typical of actinoporins.

Discussion

Cysteine mutants of EqtII have been recently used to determine the regions of this molecule that interact with the membrane [1, 2]. During this investigation we observed that three neighboring mutants, S54C, H67C and K77C showed the largest changes in hemolytic activity, suggesting that this region is important for proper functioning of EqtII. While mutant H67C was three times more active than the wild-type, mutants S54C and K77C were approximately 100 times less active. Here we have investigated the reasons for the decreased activity of EqtIIK77C further.

We have observed that the overall structure of the monomer of EqtIIK77C is preserved and that its binding to the membrane matrix is practically normal (Figs. 2, 3, 7 and 8). This is consistent with the fact that, after membrane binding, residue 77 remains readily accessible to thiol reactive reagents added on the *cis* side [2]. In fact, from a low resolution topological study, we determined that EqtII binds to the membrane using other parts of the protein molecule, i.e., an 18 residues long stretch near the N-terminus, predicted to be organised as an amphipathic α-helix, and a hydrophobic core extending from residue 105 to 120 [2].

It is therefore conceivable that residue 77 is either involved in the final steps of the formation of the pore (i.e., the aggregation of pre-inserted monomers) or that it drastically diminishes the flow of ions through the channel, for example being located in the pore lumen. Experiments with BLM clearly demonstrated that the conductance of EqtIIK77C pore is not reduced, but, rather increased with respect to that of the wild-type. This was confirmed by the osmotic protection experiments which indicated that larger molecules could pass through the EqtIIK77C pore, suggesting it has a radius larger than 1.4 nm (the hydrodinamic radius of PEG2000) as compared to a radius of about 0.9 nm for the wild-type (Fig. 5 and [5]). Furthermore, in BLM we did not observe any effect either on the cation selectivity of the pore or on the current flowing through preformed EqtIIK77C pores exposed to the action of MTS reagents, indicating that the side chain of residue 77 was not facing the pore lumen. Conversely, both the hemolytic activity and the size of the pore in RBC were restored by pretreatment with thiol-modifying agents that reintroduced the positive charge at position 77 (Fig. 5).

Altogether these data clearly suggest that residue 77 is involved in the aggregation of EqtII rather than having effects on the flow of ions through the pore. This remembers the situation found with another pore-forming toxin, i.e., staphylococcal α-toxin, for which it was observed that modifications of His35 lead to severe loss of activity, despite normal binding, because of a faulty oligomerization [7, 30]. In that case, it was demonstrated that His35, albeit not completely embedded in the contact surface between monomers [22], controls a conformational step (the formation of the 'stem') which renders the α-toxin oligomer stable and competent to make the pore [29, 31]. In the case of EqtII it is possible that Lys77 actually lies on the contact surface between neighboring monomers or that it is involved in some other crucial step occurring during oligomerization.

The use of selective covalent modifications of the single introduced thiol group with various reagents, has proven very useful. In fact, by introducing of *S*-carboxymethylcysteine and *S*-aminoethylcysteine we could mimic the side chains of Glu and Lys at position 77 without the need of time-consuming production of other point mutants. In this way we could prove that a positive charge is required in that position. This might imply that Lys77 is involved in the formation of an important salt bridge for example between adjacent mono-

mers as in the case of Lys110 and Lys147 of staphylococcal α-toxin.

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