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Primary and secondary structure of a pore-forming toxin from the sea anemone, *Actinia equina* L., and its association with lipid vesicles

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Abstract

The complete amino acid sequence of equinatoxin II, a potent pore-forming toxin with hemolytic, cytotoxic and cardiotoxic activity from the venom of the sea anemone, *Actinia equina* L., is reported. In addition, circular dicroism was used to estimate the secondary structure of this toxin either in the water-soluble or in the membrane-anchored form. Equinatoxin II when in water was found to contain about 29–33% of α-helical structure, 53–58% of β-strand + β-turn and 10–16% of random structure. Upon association with phospholipids, in particular with sphingomyelin, a rearrangement of the secondary structure occurs resulting in an increase of the α-helix content. An amphiphilic α-helical segment is predicted at the N-terminus, which shares structural homology with membrane active peptides like melittin and viral fusion peptides. In analogy to the behaviour of these peptides we propose that at least part of the α-helix content increase of equinatoxin II is due to the insertion of its N-terminus into the lipid bilayer. As in the case of melittin, association of 3–4 equinatoxin molecules is necessary to induce membrane permeabilisation.

Key words: Pore-forming toxin; Primary structure; Secondary structure; Protein–lipid interaction

1. Introduction

Equinatoxin II (molecular mass 19.5 kDa) from *Actinia equina* [1] belongs to a class of about 30 polypeptide cytolytic toxins, with a molecular mass ranging from 10–20 kDa, which have been isolated from sea anemones [2–4]. Lethality of equinatoxin II in mammals has been ascribed to severe vasospasm of coronary vessels [5,6]. In addition, cardiac arrhythmia, negative and positive inotropic effects [7] (very similar to those induced by tenebrosin C from *Actinia tenebrosa* [8,9]) have also been observed. The cytolytic effects of sea anemones toxins reported so far include red blood cells hemolysis, platelet aggregation and lysis, cytotoxic and cytostatic effects on fibroblasts [10–13]. These effects are, at least in part, due to the ability of these molecules to bind to the cell membrane [14] and to open pores [15]. At an early stage in fact, pore formation results in a rise of intracellular calcium which, as a second messenger, can trigger a number of secondary effects, for instance platelet aggregation. As a later step an osmotic imbalance is created which leads to cell swelling and lysis [15]. This mechanism has been strongly supported by experiments with several sea anemone toxins using lipid vesicles [16,17], lipid monolayers [18] and bilayers [17,19,20]. Formation of rectifying cation-selective pores have been reported for *Stichodactyla helianthus* cytolysin III [19,20] and equinatoxin II [17]. Their diameter was sized as "larger than 1.0 nm", and "around 2.3 nm", respectively. In both cases, an oligomerisation of 3–4 toxin molecules in the lipid membrane was suggested to be necessary for rising pores.

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Abbreviations: BNPS-skatole, 2-(2-nitrophenylsulfenyl)-3-methyl-3-bromoindolenine; CNBr, cyanogen bromide; HU, hemolytic unit; PC, phosphatidylcholine; RBC, red blood cells; SM, sphingomyelin; TFA, trifluoroacetic acid.

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The particular role in the activity of equinatoxin II of specific amino acid residues (i.e., charged groups, tyrosine and tryptophan residues) has been studied by chemical modification [17,21–23]. However, the elucidation of the protein primary and secondary structure is a prerequisite for further detailed and comparative structure-function studies. Despite the considerable number of cytolytic toxins purified from sea anemones, only the primary structure of *S. helianthus* cytolysin III [24] and *A. tenebrosa* tenebrosin C [25] were completed, whereas a partial amino acid sequence of equinatoxin, now named equinatoxin II [26], and the N-terminus 49 amino acids of a hemolytic toxin from *Heteractis magnifica* were reported [27].

Here we report the complete amino acid sequence of equinatoxin II and CD spectral estimates of its secondary structure. It is shown that the interaction of this sea anemone cytolytic toxin with lipids induces a significant increase of its α-helical content implying a rearrangement of the polypeptide backbone. A putative role of the N-terminus of this toxin is suggested in accordance to experimental data and structural predictions.

2. Materials and methods

2.1. Materials

Equinatoxin II was isolated and assayed as described elsewhere [1]. A molar extinction coefficient of $3.61 \cdot 10^4$ $M^{-1} cm^{-1}$ was used for spectrophotometric determination of its concentration [8]. Bovine serum albumin (BSA) was from Calbiochem, melittin and transferrin were from Sigma. β-Trypsin (EC 3.4.21.4) for tryptic degradation of equinatoxin II was prepared by the method of [28]. Sequential grade CNBr, maleic anhydride and BNPS-skatole were obtained from Pierce, hydroxylamine hydrochloride from Fluka, methanoic acid from Merck. Sphingomyelin (SM) was from Fluka and phosphatidylcholine (PC) from Calbiochem. All other chemicals were commercial products of sequential or analytical reagent grade.

2.2. Amino acid sequence determination

Cyanogen bromide cleavage of equinatoxin II (2 mg) was performed in 85% TFA containing 50-fold molar excess of CNBr over the protein and 0.3 mM tryptophan in order to protect the protein tryptophan residues from oxidation. The cleavage reaction proceeded in the dark at room temperature for 21.5 h. The reaction mixture was diluted 10-fold with distilled water and freeze-dried. BNPS-skatole fragmentation of 1.1 mg of protein was done according to Ref. [29]. After the maleylation of the native toxin [30] and dialysis against 0.1 *N*-methylmorpholine acetate (pH 7.95), tryptic cleavage was carried out at 37°C for 4 h by the twofold addition of β-trypsin to a final concentration of 2% (w/w). Hydroxylamine hydrolysis of Asn-Gly bonds in the toxin was performed according to the method of [31].

Peptide purification was performed by HPLC (Milton Roy) on Chrompack reverse-phase C8 and C18 columns (100×3 mm) equilibrated with 0.1% TFA in water (solvent A) and eluted, at a flow rate of 1 ml/min, by various gradients to 80% acetonitrile in solvent A. Peptides were separated also on a Sephacryl S-200 (150×0.5 cm) column at a flow rate of 2.4 ml/h with 50% methanoic acid. The absorbance was monitored either at 215 and 280 nm.

Amino acid analyses of peptide hydrolysates, obtained with 6 M HCl at 110°C for 24 h, were performed by HPLC (LKB) using a SuperPac ODS 2 column and pre-column *o*-phthalaldehyde derivatization. Detection was fluorimetric, with excitation at 330 nm and emission at 450 nm. The amino acid sequence of intact equinatoxin II and derivative peptides was determined by automated Edman degradation on an Applied Biosystems liquid-pulsed sequencer (model 475A) connected on-line to a model 120A phenylthiohydantoin-amino acid analyser.

2.3. Preparation of vesicles

Small unilamellar vesicles (SUV) were prepared from SM, PC or mixtures of PC and SM (1:1 molar ratio) by sonication as described previously [17]. Briefly, dry lipid or lipid mixture was dissolved in chloroform and solvent was evaporated under reduced pressure. Lipids at a concentration of 5 mg/ml were suspended in buffer 140 mM NaCl, 10 mM Tris-HCl (pH 7.4) and vortexed. Lipid dispersions were sonicated for 30 min in a pulsed sonicator (Vibracell VC 500 Sonics & Materials) at room temperature. Cleared lipid suspensions were centrifuged at 12000 rpm, stored at 4°C and used within three days.

2.4. Spectroscopy

Circular dichroism (CD) spectra were recorded on a Jasco J-710 spectropolarimeter in the range from 190 nm to 240 nm. The instrument was calibrated with d-10-camphorsulfonic acid. Protein secondary structure was evaluated with the SSE-338 program using the reference CD spectrum method [32]. A best fit procedure provides the calculated contents in helix, β-form, β-turn and unordered form. Applying this procedure to a set of 7 reference spectra of proteins with widely different secondary structure we found that the best agreement with crystal data was obtained when the evaluation range was restricted to the region 200 nm–

240 nm. The CD spectrum of transferrin was measured and used to assess the constrained fitting procedure. Seven circular dichroism scans were collected and averaged for each sample. This procedure resulted in fairly good estimates, in fact the predicted secondary structure was: α-helix 30%, β-strand 29%, β-turn + coil 41% as compared to the X-ray determined structure: α-helix 37%, β-strand 25%, β-turn + coil 38% [33,34].

The same procedure was used for analysing the secondary structure of both melittin and equinatoxin II either in buffer or after preincubation with SUVs. All CD spectra were recorded with a 0.2 cm pathlength cell. Apparent CD spectra of SUV suspension, without protein, were recorded at different times to control their contribution to the CD signals. These blanks were subtracted from the appropriate sample CD spectra. Because equinatoxin II has been reported to precipitate serum lipoproteins [22], an aggregation of lipid vesicles in certain toxin-lipid suspensions, cannot be 'a priori' excluded and this could be the source of erroneous interpretation of the CD spectra. However, no turbidity increase, which should ensue from vesicle aggregation, was noted in these experiments, and this is consistent with observations we have done previously on calcein loaded vesicles that are permeabilised but not aggregated by equinatoxin II [35]. We also performed a direct experiment using quasi elastic light scattering (QELS) to determine vesicle size before and after addition of 100 μg/ml toxin. Vesicles extruded through 50 nm holes in polycarbonate filters were used, which are slightly larger than sonicated vesicles and give more scattering. Furthermore, the lipid concentration was larger than in the CD experiments (lipid to toxin ratio was 600); however, this should only promote vesicle aggregation. We did not observe any change in the size of the vesicles, which remained 60 ± 15 nm for up to one hour, thus excluding that the toxin induces the formation of lipid aggregates. These results, which were obtained using the Zetasizer 3 particle sizer of Malvern, are not shown for the sake of conciseness.

2.5. Hemolytic assays

The hemolytic activity of native equinatoxin II and the residual hemolytic activity following a preincubation with SUV (i.e., the activity of the unbound toxin), were determined turbidimetrically at a wavelength of 650 nm with a microplate reader (UVmax from Molecular Devices) supported by the computer program SOFTmax. Human RBC were prepared from fresh heparinized blood by washing three times with isotonic saline, and resuspending in isotonic buffer: 160 mM NaCl, 10 mM Tris-HCl (pH 7.5). Finally, the concentration of RBC was adjusted with the buffer to an apparent absorbance of 1.0 at 700 nm in a 1 cm pathlength cuvette.

Binding of equinatoxin II to SUV was studied as follows. Equinatoxin II (7.5 μg/ml) was incubated with SUVs, of different composition and concentration, in isotonic buffer for 10 and 60 min at 37°C. Unbound toxin was recovered in a filtrate obtained by passing the mixtures through a polysulfone filter with a molecular mass cut-off of 300 kDa which retained only vesicles and bound toxin. Before use, to saturate unspecific protein binding sites, the filters (Ultrafree-MC from Millipore) were washed with a 0.2 mg/ml solution of BSA in isotonic buffer. Hemolytic activity of filtrates was assayed by serial 2-fold dilutions in 96-well microplates. One hemolytic unit was arbitrarily defined as the reciprocal of the dilution of equinatoxin II changing the optical density with a maximal rate of 0.01/min, which in this assay corresponds to a lysis of around 50% RBC after 30 min. Results were expressed as percentages of the control, which was a filtrate of toxin without SUV preincubation.

2.6. Prediction of secondary structure

The amino acid sequences were analysed for occurrence of amphiphatic α-helixes and β-strands as described by [36]. Alignments were done using the program CLUSTAL. Estimation of the mean hydrophobic

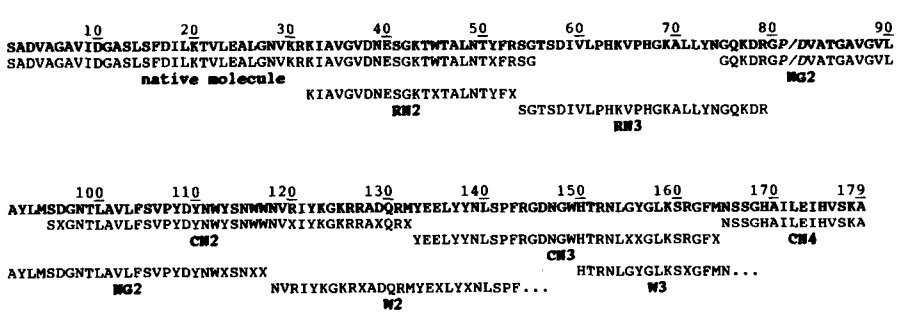

Fig. 1. Amino acid sequence of equinatoxin II (bold). The sequences of the toxin N-terminal (native molecule), CNBr (CN2, CN3, CN4), tryptic (RN2, RN3), hydroxylamine hydrolytic (NG2), and BNPS-skatole derived peptides (W2, W3) were determined by automated Edman degradation. In position 81, approximately equimolar Pro and Asp (italic) were found. × stands for poorly identified amino acid residues.

moment and Eisenberg plot of α-helixes were done as in Ref. [37].

3. Results

3.1. Primary structure

The N-terminal sequence determination of native equinatoxin II, the most abundant isotoxin of the three purified from *A. equina* [1], provided its primary structure up to position 55. Cleavage of the toxin with CNBr resulted in four fragments with identical amino acid contents as it was already reported [8,26]. Three of them, comprising the C-terminal part of the molecule, were isolated and sequenced (peptides CN2, CN3 and CN4 in Fig. 1). The CN4 peptide represented the C-terminus as it ended with Ala and not homoserine which is the usual product of CNBr cleavage. A second set of peptides was obtained by BNPS-skatole cleavage after Trp residues. Three fractions were isolated and the shorter two, providing overlaps to the CN peptides, were analysed (peptides W2 and W3 in Fig. 1). The Trp^{45}–Thr^{46} bond was not cleaved, probably due to the intramolecular participation of the Thr hydroxyl group, which prevented the hydrolysis. In W2 peptide, Arg^{120} appeared in low yield, suggesting its partial modification.

The β-tryptic hydrolysis of the maleylated toxin resulted in RN peptides. The amino acid sequences of RN2 and RN3 are shown in Fig. 1. The last overlaps were made by fragmenting equinatoxin II at Asn-Gly bonds with hydroxylamine. Out of three resulting peptides, only the NG2 peptide was sequenced. In the 7th cycle of this run (position 81 in the equinatoxin II molecule) Pro and Asp were detected at an about equimolar concentration. The Pro/Asp micro heterogeneity at position 81 indicates that the equinatoxin II under study consists of two nearly equimolar isoforms, both of 179 amino acid residues and lacking cysteine or cystine. A similar micro heterogeneity, Ser^{177}/Thr^{177}, was found with tenebrosin C from *A. tenebrosa* [25] despite all preparative and analytical methods had apparently proven, as in the case of equinatoxin II, its homogeneity [8]. Furthermore, an additional equinatoxin isoform (less than 2%) was detected unless equinatoxin II was subjected to rechromatography. In this contaminant isoform the following substitutions were observed: Phe^{107}-Ile^{108}-Val^{109} for Pro^{107}-Tyr^{108}-Asp^{109} (Fig. 1). Overall, the primary structures of equinatoxin II variant Pro^{81} and tenebrosin C variant Ser^{177} [25] are identical, as it was previously suggested [8]. Furthermore, both toxins are closely related to *S. helianthus* cytolysin III, a 54% similarity score was reported [25].

3.2. Changes of secondary structures as assessed by CD

The far UV CD spectrum of native equinatoxin II shows a broad negative minimum peaked at 218 nm, and becomes positive below 205 nm (Fig. 2). When fitted to the given reference spectra, as explained in Materials and methods, only a general agreement could be achieved. This was not unexpected in view of the relatively high Trp content in this protein [38] and due to some limitations of the method (i.e., the solving equation and the use a preselected reference protein set [39]). Altogether, native equinatoxin II was estimated to contain about 29–33% of α-helical, 53–58% of β-strand + β-turn and 10–16% of random structure. These values, which did not depend on the concentration of toxin used, have to be regarded as somehow tentative in view of the limitations explained above.

Incubation of equinatoxin II with various SUV preparations resulted in a significant increase in its α-helical content as indicated by the decrease in the ellipticity value at 222 nm (Fig. 2). Rearrangement of the toxin secondary structure was dependent both on the type of lipid and on the lipid/toxin ratio (Fig. 3A). SM, the most potent acceptor molecule for sea anemone toxins [10,17,40], was the most efficient as

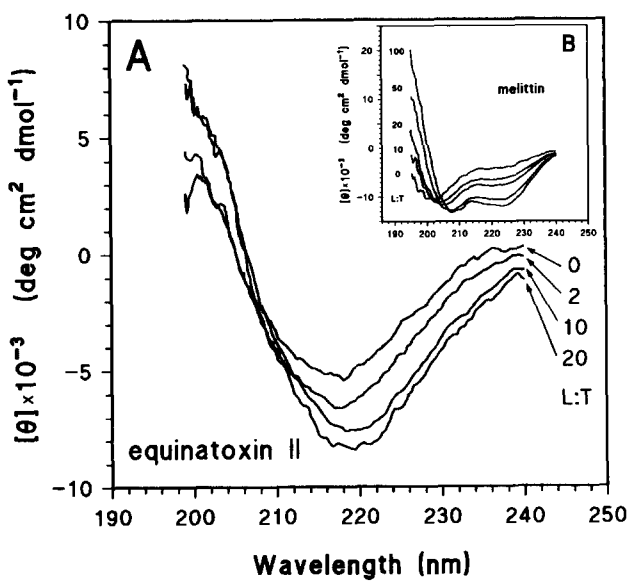

Fig. 2. CD spectra of equinatoxin II with and without added lipid. The toxin (100 μg/ml), was incubated for 10 min with SUV of sphingomyelin (SM) in buffer 140 mM NaCl 10 mM Tris, pH 7.4 at 37°C at the indicated lipid/toxin ratios (L:T). These and all other spectra were averages of 7 consecutive runs. Fractions of α-helix, β-strand, β-turn, and random coil structure were calculated as described in Material and methods with a best fit procedure and used for Fig. 3. (Inset) similar experiments with melittin (also 100 μg/ml), and DMPC vesicles. In this case, fractions of calculated α-helix, β-strand, β-turn, and random coil changed from 1.8, 47.0, 5.0, 46.1% (in the absence of lipid), respectively, to 41.4, 19.5, 3.8, 35.4% in the presence of DMPC vesicles at a lipid/toxin ratio of 100.

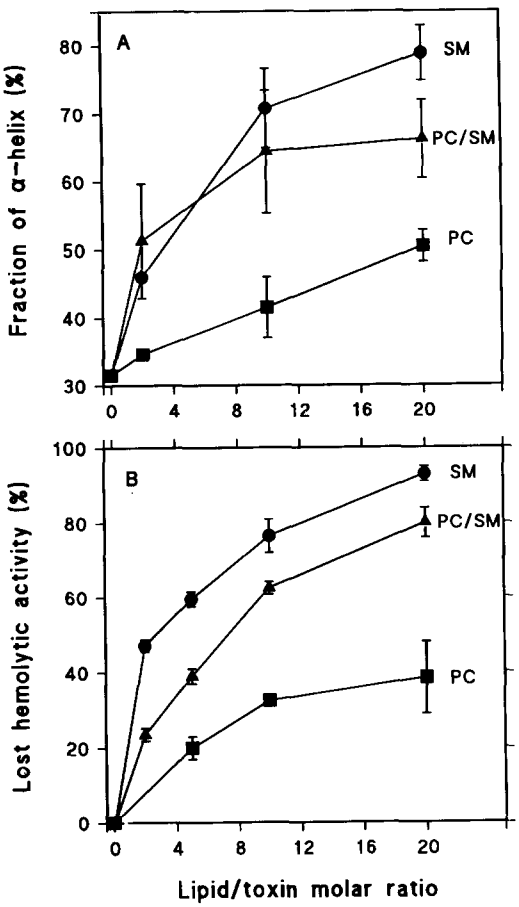

Fig. 3. Effect of lipid on secondary structure and activity of equinatoxin II. (A) SUV were mixed with toxin at the indicated lipid/toxin ratios and incubated in buffer 140 mM NaCl 10 mM Tris, pH 7.4 at 37°C (toxin concentration was 100 μg/ml). After 10 min seven CD spectra were scanned and averaged and fractional content of α-helix (plotted), β-strand, β-turn, and random coil (not shown) were calculated. Values are means ± S.D. of four different experiments (error bars within the dimension of the symbol were omitted). Results after 12 h incubation were similar. SUV used were comprised of SM (circles), PC (squares), and PC/SM (triangles) (B) Effect of lipid vesicles on the hemolytic activity of equinatoxin II. Residual hemolytic activity, i.e., that of the toxin remaining unbound in solution, was determined as described in Methods following an incubation of 10 min at 37°C of the toxin (7.5 μg/ml) with SUV in 160 mM NaCl 10 mM Tris-HCl (pH 7.5). The reported loss of hemolytic activity was calculated as the difference between the activity of the control (set to 100%) and the activity of the sample. SUV used were comprised of SM (circles), PC (squares), and PC/SM (triangles), at the indicated lipid/toxin ratio. Control was the toxin incubated without lipids. Using a toxin concentration of 100 μg/ml (as in part A) or an incubation for 60 min gave essentially the same results.

compared to the mixture PC/SM or to pure PC. The increase in α-helical content occurred at the expenses of both β and random structure. At maximal change, with SM, the toxin was estimated to contain about 75% α-helical, 25% β-strand + β-turn structure, and no random coil. Although these quantitative values must be considered with some caution for the reasons explained above, they clearly suggest an increase of α-helical structure at the expense of both β-structure and unordered coils. The transition caused by pure PC was similar albeit much less prominent.

Such a lipid-induced increase in α-helical content has been observed with many amphipathic membrane-active polypeptides. One example is melittin [41,42], which we have used as a control (Fig. 2).

3.3. Hemolytic activity

In parallel to this conformational changes, incubation of equinatoxin II with SUV resulted in a decrease of its hemolytic activity, due to the binding and the inactivation of lipid-bound form [40]. There is a strong correlation between the extent of structural change at a given lipid to toxin ratio (Fig. 3A), and the binding of equinatoxin II to SUV (Fig. 3B), as determined from the loss in hemolytic activity. Altogether these facts reveal that binding of the toxin to lipid membranes, consequent conformational rearrangements, and loss of activity of bound molecules are interrelated processes.

4. Discussion

The completed primary structure of one isoform of equinatoxin II (variant Pro^{81}) reported herein is identical to that of one isoform of tenebrosin-C (variant Ser^{177}) from the Australian sea anemone *A. tenebrosa* [25]. The biological significance of nearly identical primary structure in toxins from geographically and genetically remote species of the sea anemones, like *A. tenebrosa* and *A. equina*, and of the abundance of isotoxins, were already discussed [8]. Our main interest here is to correlate some features emerging from the analysis of the primary structure of equinatoxin II, and from the comparison with completed and partial sequences of other sea anemone cytolysins, with the mechanism of its association with lipid membranes and pore-formation.

Pore formation experiments in planar membranes and permeabilisation of lipid vesicles by sea anemone toxins [15,17,19,20], all strongly imply a structural rearrangement when these water soluble toxins insert into the lipid bilayer. In general such a rearrangement can involve changes of both the secondary and the tertiary structure of the peptide. However, in the case of bacterial pore-forming protein toxins it appears that the rearrangement is only at the level of the tertiary structure, with little or no change in secondary structure. This is what was found for instance for colicin [43], staphylococcal α-toxin [44] and aerolysin from *Aeromonas hydrophila* [45]. Our CD results (Figs. 2 and 3), actually support the occurrence of a conformational change also of the secondary structure in the case of

equinatoxin II, implying the formation of new α-helical structure at the expense of both β-structure and unordered coil. This is not an odd case, in fact equivocal stretches, which have propensity to adopt both α and β structure, have been found in many proteins and it was shown that they can take on one or the other of these structures depending on the polarity of the environment [46–49].

Particular attention goes to the N-terminus of the toxin, which appears to be extremely well conserved among the five sea anemone cytolysins for which it was sequenced (Fig. 4A) and to posses a stretch which can be arranged as an amphiphilic α-helix (Fig. 5A). Previous authors [24] reported that a search for homology in the protein data base unravelled only that a 221–346 segment of *E. coli* porin was partially analogous (20.3%) to *S. helianthus* cytolysin III. However, we noticed that the N-terminal sequences of sea anemone cytolytic toxins can be aligned and inspected for structural analogy with some amphipathic polypeptides which are known to interact with lipid membranes i.e., the honey bee peptide melittin and SV5, the fusion peptide of simian virus 5 fusion glycoprotein (Fig. 4B).

Alignment with SV5 shows 37% identity and further 19% similarity without introducing any gap in the two peptides. Such analogy also extends, to a slightly smaller degree, to some other related viral fusion peptides which operate at neutral pH, i.e., those from simian, Sendai and human immunodeficiency virus [50]. Alignment with melittin evidences 35% identity and further 26% similarity, with the introduction of just a unit gap.

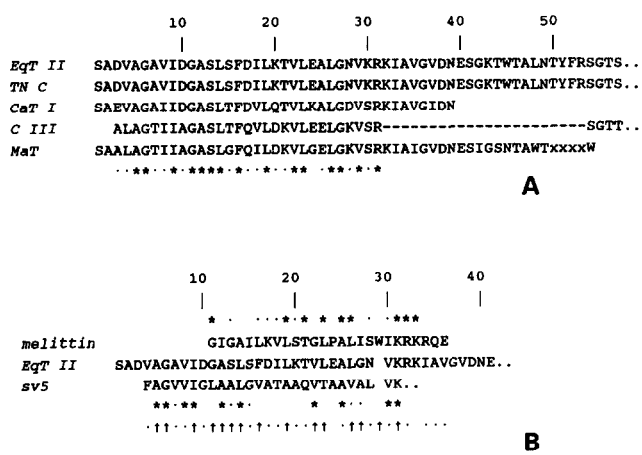

Fig. 4. (A) N-terminal amino acid sequences of the sea anemone toxins, cytolysin III from *S. helianthus* (C III, [24]), caritoxin I from *Actinia cari* (CaT I), tenebrosin-C from *Actinia tenebrosa* (TN C, [25]), equinatoxin II (EqT II) and cytolysin from *Heteractis magnifica* (MaT, [27]), are aligned. Asterisks denote identical amino acid residues, dots those similar. Caritoxin I was purified as reported in Ref. [57] and its N-terminus sequence was determined essentially as described in Materials and methods for equinatoxin. Alignment was done using the program CLUSTAL. (B) The N-terminal amino acid sequence of equinatoxin II is aligned with two amphipathic peptide: melittin and SV5, the fusion peptide of simian virus glycoprotein. As in part A, asterisks denote identical amino acid residues and dots those which are similar. In the lower line the consensus of the N-terminal sequences of the different sea anemone toxins is reported. Vertical arrows denote strictly conserved residues, dots those similar.

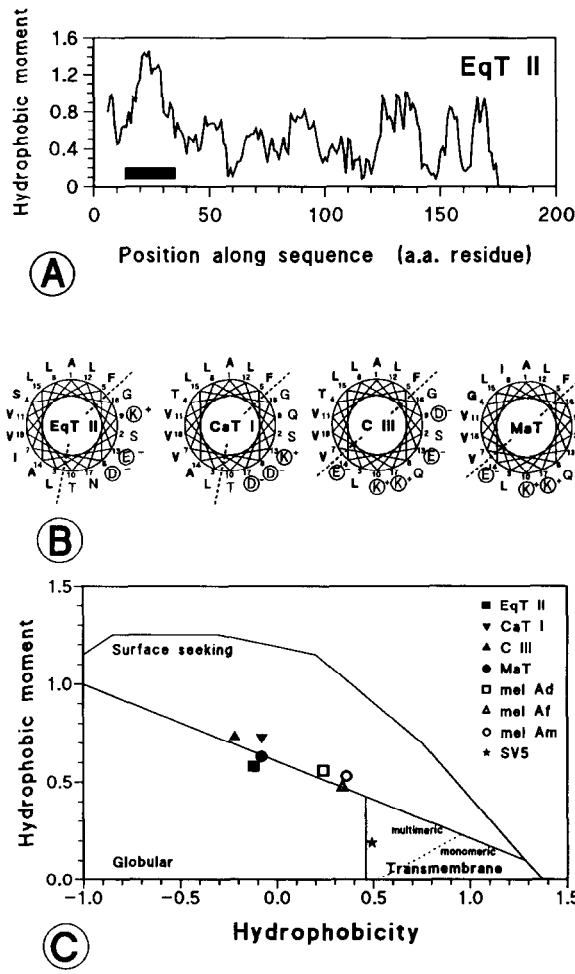

Fig. 5. Amphipathic properties of the N-terminus peptide of sea anemone cytolysins. (A) Profile of mean hydrophobic moment (calculated as in Ref. [37], using a window of eleven residues) along the sequence of EqT II. The bar highlights the occurrence of one major peak of hydrophobic moment (i.e., of tendency to form an amphiphilic α-helix) which is about 20 residues long and is located near the N-terminus. (B) α-helical wheel projections [58] of segments 12–29 of EqT II (but TnC is identical), CaT I, MaT and of segment 10–27 of C III. Residues in bold are those hydrophobic, the other are hydrophilic, those circled are charged. Dotted lines suggest the border between the hydrophobic and the hydrophilic face of each helix. (C) Eisenberg plot of the predicted N-terminal α-helixes of sea anemone cytolysins (closed symbols) of three different isoforms of melittin (open symbols) and of SV5 (asterisk). Mean hydrophobicity and mean hydrophobic moment were calculated as in Ref. [37], using a window of eleven residues. Both sea anemone and bee peptides locate at the borderline of the surface seeking peptides, suggesting they have the tendency to bind to lipid membranes lying flat at their surface. SV5 is overall more hydrophobic and would probably insert perpendicular to the plane of the membrane.

Also in this case, the analogy extends to some melittin-related peptides like bombolitin and bombinin. Furthermore, the analogy between equinatoxin N-terminus helix and melittin becomes even more striking when one considers that amino acid residues at positions 10–13 of melittin, a so called 'hinge region' including a proline which is absent in the sea anemone toxin, may be omitted from the bee peptide without loss of hemolytic and antimicrobial activity [51]. Instead, 5 out of the 8 melittin residues which cannot be omitted without severe impairment of its activity, are conserved in equinatoxin II (including 3 regularly spaced leucin residues appearing on one side of the helix). This suggests they may have a similar role in membrane penetration, which is evidenced also by a similar position in the surface seeking region of the Eisenberg plot (Fig. 5B).

Interestingly, both these families of peptides, from bee venom and from viruses, are known to increase their α-helical structure content upon binding to lipid bilayers [41,42,52,53], by forming an ordered helix. Furthermore, recent cross-linking experiments revealed that 3–4 equinatoxin molecules transiently assemble in the lipid bilayer to form a channel [17], as it is also the case with both melittin [54] and viral fusion peptides [50,52]. The same number of monomers was estimated to be required also for pore formation by *S. helianthus* cytolysin III, based on kinetic experiments [19,20]. No aggregate was observed in solution, i.e., in the absence of lipid [17]. Although we do not want to claim here that there is a genetic link between these peptides, it appears quite reasonable that through evolution they have converged to analogous structural properties. In particular, a part of the increase in helical structure of lipid bound equinatoxin II probably stems from the insertion of its N-terminus peptide into the membrane. Oligomerisation on the membrane could also contribute to stabilize this newly formed α-helical structure.

From the CD experiments and the loss in hemolytic activity (Fig. 3) it appears that approximately half of the toxin is already bound to the lipid vesicles at a lipid to toxin ratio of around 2–3. Although somehow unexpected, this result is consistent with micro calorimetry experiments recently reported for a very similar toxin from the sea anemone *Radianthus macrodactilus*. In that case it was found that, at saturation, one toxin molecule binds to six lipid molecules [55]. This seems to suggest that only a small portion of the toxin molecule is inserted in the membrane, perhaps just one helix, or one helical hairpin. It is possible that the anchored N-terminus could facilitate the insertion into the lipid phase of another region of the molecule, adopting the helical hairpin structure, this in fact appears to be a very common pathway for membrane-penetrating protein toxins [56]. Preliminary experiments that we have performed on the transfer of equinatoxin tryptophan residues into the lipid membrane would strongly reinforce this idea.

In conclusion, equinatoxin II seems to share some properties both with short membrane-damaging peptides, i.e., a change in secondary structure favouring a higher α-helical content in the membrane bound form, and with larger protein toxins, i.e., insertion of only a few helixes into the lipid phase.

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