The role of lysine, histidine and carboxyl residues in biological activity of equinatoxin II, a pore forming polypeptide from the sea anemone *Actinia equina* L.

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Equinatoxin II, a pore forming polypeptide from the sea anemone *Actinia equina* L. was subjected to chemical modifications with group specific reagents. Lysine residues were modified with pyridoxal-5'-phosphate, histidine residues with diethyl pyrocarbonate and carboxyl groups with the use of a water soluble carbodiimide. Modification of charged residues had no significant influence on the toxin interaction with serum lipoproteins. Lysine 5'-phosphopyridoxylated and histidine carbethoxylated derivatives of the toxin retained lethal and hemolytic activities, but the pH profile of hemolytic activity of 5'-phospho-pyridoxylequinatoxin II was markedly altered. Modification of the toxin carboxyl groups impaired both hemolytic and lethal activities, the latter, however, to the greater extent.

**Introduction**

Equinatoxin II (EqT II) from the sea anemone *Actinia equina* is a lethal, 20 kDa polypeptide belonging to a class of sphingomyelin inhibitable sea anemone cytolsins possessing a high degree of structural similarity [1-4]. According to present knowledge concerning the effects of the cytolytic sea anemones toxins either upon a variety of cells [5-11] or on artificial lipid membranes [11-16] a common mechanism of action is apparent insertion into the lipid bilayers with subsequent formation of pores. This primary event is probably responsible for a variety of pharmacological activities when the toxins are injected into experimental animals.

Several processes are pH dependant: the hemolytic activity of EqT II [17], pore formation in lipid bilayers induced by *Stichodactyla* (*Stoichactis*) *helianthus* cytolsin [14] as well as its development of surface pressure at the air:water interface [18]. This implies a possible role of charged groups in the mechanism of the sea anemone cytolytic toxins action. In this respect we modified lysine (Lys), histidine (His) and carboxyl groups in EqT II molecule by means of group-specific chemical reagents to obtain an insight into the role of these residues in determining toxin biological activity.

**Material and Methods**

**Isolation of equinatoxin II**

EqT II was isolated according to the method of Maček and Lebez [2] and its lethal and hemolytic activity was assayed as described previously [17].

**Modification of lysine residues with pyridoxal-5'-phosphate (PLP)**

Modification of Lys residues with PLP (Sigma, St Louis, U.S.A.) was performed as described by Nishigori and Toft [19]. To 1 ml of 5·10⁻⁵ M EqT II in 0.1 M phosphate buffer (pH 7.1) 1 ml of 10, 20 or 100 mM PLP was added, respectively. On a few occasions PLP concentrations lower than 10 mM were used (e.g., isoelectric focusing). The reaction mixture was incubated in the dark for 60 min at 4°C. In order to reduce the imine bond between Lys amino groups and PLP 0.5 ml of 60, 100 or 200 mM NaBH₄ was introduced into the reaction mixture. A drop of octanol was added to reduce foaming due to NaBH₄. The reaction mixture was then incubated in the dark at 4°C for 50 min and an additional 10 min at room temperature. After the reduction was complete, side products and excess reagents were removed on a Sephadex G-15...
column (1 × 7 cm), equilibrated and eluted with 0.1 M phosphate buffer (pH 7.1). During modification of the Lys residues of EqT II aliquots were removed from the reaction mixtures at different times and tested for the residual hemolytic activity.

Modification of histidine residues with diethyl pyrocarbonate (DEPC)

Histidine residues in EqT II were modified with DEPC (Sigma, St. Louis, U.S.A.) as described by Miles [20]. DEPC was dissolved in absolute ethanol to achieve a 2.23 M stock solution. An aliquot of 25 µl was then added to 3 ml of 10 mM imidazole-HCl buffer (pH 7.5). Absorbance of the resulting product, N-carbethoxyimidazole, was measured at a wave length of 330 nm. The concentration of the product was calculated from the absorbance data and extinction coefficient for N-carbethoxyimidazole at 230 nm (3.0 · 10³ cm⁻¹ M⁻¹). To 1 mg of EqT II dissolved in 1 ml of 0.1 M phosphate buffer (pH 6.0), 10 or 25 µl of DEPC stock solution were added. The achieved final concentration of DEPC was 4 · 10⁻⁴ M and 1 mM, respectively. The reaction was allowed to occur for 60 min. After 40 min hydroxylamine was added to reverse nonspecific side reaction of DEPC with tyrosine residues. Aliquots were removed from the reaction mixtures and tested for residual hemolytic activity.

Modification of carboxyl groups with a water soluble carbodiimide

Modification of carboxyl groups in the EqT II molecule was made according to the method of Fleer et al. [21]. Semicarbazide (Sigma, St. Louis, U.S.A.) was dissolved in 0.5 ml of 0.25 M cacodylate buffer (pH 5.5), to achieve a 1 M concentration. After addition of semicarbazide the pH value was adjusted to 5.5 with 5 M NaOH. The buffer system was diluted to 1 ml by the addition of 0.25 M cacodylate buffer, pH 5.5. EqT II (5 mg) was dissolved in the above described medium. 19 mg of solid water soluble 1-ethyl-3(3-dimethylaminopropyl) carbodiimide · HCl (EDC; Serva, Heidelberg, Germany) was then added and the suspension incubated for 30 min. After that an additional 5 mg of EDC was introduced into the reaction mixture. This procedure was repeated at every 30 min until there was no substantial decrease of hemolytic activity observed or alternatively the reaction was stopped after 150 min of incubation by the addition of solid ammonium acetate (75 mg). Side products, salts and excess reagent were removed from the modified toxin on a Sephadex G-25 column (1 × 7 cm), equilibrated and eluted with 0.2 M ammonium bicarbonate buffer (pH 8.0). Possible side reaction of Tyr residues with EDC was reversed by adding an equal volume of 1 M hydroxylamine (pH 7.5) to the modified toxin and incubating for 6 h at room temperature. Aliquots of the reaction mixture were removed at different time intervals and tested for residual hemolytic activity.

Spectrophotometrical evaluation of the reaction extent

The number of Lys residues modified with PLP was estimated using the extinction coefficient for pyridoxyl-lysine at 325 nm (4800 cm⁻¹ M⁻¹) according to Peach and Tolbert [22]. The number of modified histidine residues was calculated according to Miles [20] from the absorbance value and extinction coefficient for carbethoxyhistidine at 242 nm (3200 cm⁻¹ M⁻¹).

Amino acid analyses of EqT II with modified carboxyl groups

Amino acid analysis were performed using a Beckman 118 CL analyzer after protein hydrolysis in 6 M HCl at 108 °C for 24 h.

Ultraviolet, visible and difference absorbance spectra

Spectra were obtained on a double beam spectrophotometer Uvikon 860 (Kontron, Basel, Switzerland). In the case of PLP and carbodiimide modified EqT II ultraviolet and visible spectra were obtained in a wavelength range from 220 to 500 nm after desalting the protein. Difference spectra of EqT II modified with DEPC were directly recorded during the modification procedure in the wavelength range of 220 to 300 nm.

Isoelectric focusing

Isolelectric focusing of native, DEPC and PLP modified toxins was carried out on polyacrylamide gel plates with the pH gradient ranging from pH 5–11, stabilized with Pharmalyte (Pharmacia, Uppsala, Sweden). Protein standards were from Pharmacia and Serva (Heidelberg, Germany).

Precipitation of serum lipoproteins with equinatoxin II

Interaction of native and modified toxins with serum lipoproteins was tested according to Turk and Maček [23]. Non-immune human sera were electrophoretically separated on 1% agarose slides (2.5 × 7.5 cm) in diethylbarbital buffer (pH 8.2) for 3 h (17 mA, 200 V). Serum components were subsequently challenged with 80 µg of native EqT II, PLP modified toxin dissolved in 100 µl of 0.1 phosphate buffer (pH 7.1), DEPC modified EqT II dissolved in 0.1 M phosphate buffer (pH 6.0) or carbodiimide modified EqT II dissolved in 0.2 M ammonium bicarbonate (pH 8.0). After 24 h the agarose slides were washed with 0.9% NaCl and left overnight at room temperature and the washing procedure was then repeated. The agarose slides were stained with Amido black 10 B (Merck, Darmstadt, Germany) or Sudan black B (Serva, Heidelberg, Germany) and dried at room temperature.
**Hemolytic assays**

Hemolytic activity of native and modified toxins was measured by the turbidimetric method as described previously [17] using bovine erythrocytes. The pH of the erythrocyte suspensions was 7.4. Hemolytic activity of native, EqT II with modified Lys residues and EqT II with modified carboxyl groups was also determined in erythrocyte suspensions with different pH values ranging from 7.4 to 10.0.

**Lethality assays**

Intravenous lethality of native and modified toxins was estimated in male NIH mice (approx. 20 g each). The individual mouse LD$_{50}$ was calculated taking into account the weight of a single animal and an intravenous LD$_{50}$ of 35 μg/kg [2]. The amount of toxin injected into experimental animals was expressed as the number of mouse LD$_{50}$ which ranged from one LD$_{50}$ for the native EqT II to one to ten LD$_{50}$ for toxin modified with PLP or DEPC and from one to 80 LD$_{50}$ for EqT II with modified carboxyl groups. The experimental animals were observed for at least 24 h. Post mortem examination of treated mice was performed. Three mice were used for each applied dose.

**Results**

Modification of the EqT II 6–7 lysine residues with PLP resulted in an approximate 65% loss of native hemolytic activity. After the reduction of the Schiff base with NaBH$_4$, about 15% of hemolytic activity was restored so that the final loss was 50% irrespectively of the PLP concentration used (Fig. 1). During the extensive reaction of EqT II with PLP the solubility properties of the toxin slightly changed and partial precipitation of the modified toxin occurred. The solubility was particularly decreased at low ionic strength (as in isoelectric focusing). Ultraviolet and visible absorbance spectra of PLP modified EqT II revealed a new peak at 325 nm which is characteristic of pyridoxyl-lysine absorbance. The reaction of PLP with lysine residues was nearly quantitative since 8.9–9.2 of the ten present in the native EqT II were modified. In some experiments PLP concentrations lower than 10 mM were used. In this case the hemolytic activity was only slightly diminished and solubility was not decreased. Only 3–4 Lys residues were modified as estimated spectrophotometrically.

Modification of EqT II with DEPC did not have a significant influence on EqT II hemolytic activity. However, an oscillation in hemolytic activity appeared during the course of the reaction. After the first 15 min the loss of hemolytic activity was 15%, in the next 20 min it increased to nearly 50% of its initial value. Nevertheless, after addition of hydroxylamine the hemolytic activity of modified EqT II was almost completely restored. EqT II precipitated during the course of the reaction if a DEPC concentration higher than 5 · 10$^{-4}$ M was used. In this case addition of hydroxylamine only slightly restored activity which then remained steady even after 24 h of the preincubation of toxin with 1 mM DEPC. 35% of native toxin hemolytic activity still remained in spite of the decreased toxin solubility (Fig. 2). Difference absorbance spectral analyses during the course of the reaction of EqT II with DEPC revealed an increase of absorbance at 242 nm and a slight decrease at 280 nm, indicating nonspecific modification of tyrosyl residues. The extent of the reaction of histidine residues with DEPC estimated spectrophotometrically revealed modification of 2.4–2.8 histidyl residues out of four present in native EqT II.

Modification of EqT II with EDC and semicarbazide resulted in 60% loss of toxin hemolytic activity which was further slightly decreased when additional EDC was added. The final loss of hemolytic activity after 150 min incubation was 70% of the native activity. Treatment of modified toxin with hydroxylamine restored only 10% of its hemolytic activity (Fig. 3). About 7 to 10 of total carboxyl groups present in EqT II molecule were modified considering amino acid analysis.

While an increase of toxin pH was observed after modification of the carboxyl groups, pH values of EqT II modified with PLP and DEPC were shifted to the
Fig. 2. Chemical modification of equinatoxin II with diethyl pyrocarbonate (DEPC). Equinatoxin II was modified with 1 mM DEPC (○) or 4·10⁻⁴ M DEPC in 0.1 M phosphate buffer (pH 6.0) and hemolytic activity, 1/τ₅₀, was determined (●). Hemolytic activity of native EqT II as a control (Δ). The arrow indicates the addition of hydroxylamine.

Fig. 3. Hemolytic activity of equinatoxin II modified with 1-ethyl-3(3-dimethylaminopropyl) carbodiimide-HCl (EDC). EqT II was modified with the water soluble carbodiimide (EDC) and semicarbazide as a nucleophile in 0.25 M cacodylate buffer (pH 5.5). Light arrows represent additional introduction of EDC into the reaction mixture while bold arrow means the addition of 1 M hydroxylamine (pH 7.5) to an aliquot of modified EqT II (1:1 by volume). Hemolytic activity of native EqT II (●).

lower pH range. There were at least three bands observed in the case of the EqT II with modified histidine residues, and even more in the case of EqT II with modified lysine residues. The solubility of the PLP modified toxin was significantly reduced at the low ionic strength conditions which were required during isoelectric focusing, therefore less extensively modified toxin was applied to the plate (Fig. 4).

All modified toxins retained their full ability to precipitate with beta lipoproteins, and concomitantly, to interact with membrane lipids. It has previously been shown that EqT II causes precipitation of serum lipoproteins probably due to apoprotein delipidation, and this activity correlated with cytolytic and lethal activity [23]. Such an interference with serum lipoproteins was also shown and discussed for the Staphylococcus alpha-toxin [24].

Fig. 4. Isoelectric focusing of native and modified equinatoxin II. Native equinatoxin II (1 and 2), histidine modified (3), lysine modified (4), equinatoxin II with modified carboxyl groups (5), pH standards (6).

Fig. 5. pH dependence of hemolysis induced by native, carboxyl and lysine modified equinatoxin II. Concentrations of the native and both modified toxins were adjusted to achieve similar hemolysis rates, 1/τ₅₀, at pH 7.4. Native EqT II (190 ng/ml final concentration) (○), EqT II with four modified lysine residues (190 ng/ml final concentration) (●), EqT II with modified carboxyl groups (955 ng/ml final concentration) (△).
The increase in pH values of erythrocyte suspensions increased the rate of hemolysis induced with native or COOH modified EqT II, with a maximum rate achieved at pH 9.5. On the contrary, the pH-profile of hemolysis obtained with Lys modified toxin was flatter with an apparent pH optimum around 8.5. Note also the five times higher concentration of the carboxyls modified toxin used in Fig. 5.

Lethal properties of EqT II were not affected by modifications with either PLP or DEPC. Experimental animals died within a few minutes after injection of 1 to 2 intravenous LD₅₀ of the toxin. On the contrary, lethal activity of EqT II with modified carboxyl groups was reduced. Mice survived 40 intravenous LD₅₀ units and only died when 80 intravenous LD₅₀ units were injected. Even then the survival time was significantly prolonged up to 12 h. Post mortem examination revealed no macroscopically evident pathological changes.

Discussion

At present, only a few results about the structure-function relationship of sea anemone cytolytic toxins are available. Our previous experiments indicated the importance of Tyr residues for both hemolytic and lethal activities of EqT II and its interaction with serum lipoproteins, while arginine could be modified without impairment of the activities tested [23]. Intact tyrosine was also reported to be essential for hemolytic activity of the Stichodactyla helianthus cytolytin III while reductive methylation of certain lysine residues did not affect the binding and hemolytic activity [25,26].

In the present study, near quantitative modification of the EqT II molecule with pyridoxal-5'-phosphate, resulted in the formation of negatively charged 5'-phosphopyridoxyl-lysines and the corresponding decrease of toxin pI values. This modification clearly altered the pH profile of the hemolytic activity, but neither activity was substantially impaired. Modification of histidine residues in the EqT II molecule with DEPC did not alter either toxin activity. The decrease of hemolytic activity observed during the course of a reaction should be attributed to a nonspecific and reversible alteration of tyrosine residues with DEPC [20]. Such an accompanying modification could be deduced from the decrease of absorption at a wavelength of 280 nm and from the restored activity after the addition of hydroxylamine. Contrary to positively charged amino acid radicals, elimination of carboxylate negative charges by the water soluble carbodiimide did not definitely decrease either the hemolytic or the lethal activity, but the decrease of lethal activity was more pronounced. At the same time, lipid binding properties were preserved.

Considering sea anemone cytolytic toxins as polypeptides, which form transmembrane pores in lipid membranes [11–16], a model of their damage to cell membranes might be assumed for the purpose of the present discussion, which would be also relevant for their pathological activities [11]. According to this model, toxin molecule acting upon cell membrane undergoes several functionally discernible steps: association of toxin molecules with the surface of polar lipids as suggested by Doyle et al. [18]; their insertion into a hydrophobic moiety of lipids, lateral diffusion; and formation of stable or transient cation selective pores by oligomerization of toxin monomers. This latter characteristic has also been found for Stichodactyla helianthus cytolsin III [12,14] which is structurally related to EqT II. After all, newly formed pores in cell membranes mediate the ion fluxes and increase cytosolic calcium, a reason leading to cell specific cytopathological responses [11]. This view to the sea anemone cytolytic toxins is in a good agreement with the barrel stave model depicting the action of cytolytic pore-forming proteins and peptides upon lipid membranes [27].

Both electrostatic and hydrophobic interactions were proposed for the binding mechanism of Stichodactyla helianthus cytolsin III [18]. However, we found no correlation between pI of the modified toxin variants and alteration of the activities, neither equinatoxin II net charge was essential in the protein-lipoproteins interaction. Our results imply the prevalence of hydrophobic interactions in the insertion of the sea anemone toxin. This is also in accordance with the presence of distinctive hydrophobic amino acid sequences at the N-terminus and in the mid portion of sea anemone cytolytic toxins [28,29,30], as indicated by the hydrophatic index [29]. Unlike equinatoxin II, modification of histidine in Staphylococcus alpha-toxin, a bacterial pore forming toxin, affected the interaction with model lipid membranes and the cytolytic process [31].

Hence, it seems that variations in the charge of EqT II lysines and carboxyl residues, induced both by chemical modification and pH changes (see Fig. 5), play a remarkable role in the steps which follow the toxin insertion into a lipid membrane. Assuming that toxin charges are negligible for the rate of lateral diffusion of monomers in cell membranes, then lysine residues and particularly carboxylates should participate in the formation and/or permeability properties of a pore. This is consistent with cation selectivity of pores reported for Stichodactyla helianthus cytolsin III [12,14] and equinatoxin II [11]. Varanda and Finkelstein [14] who investigated conductance properties of ionic channels formed in lipid bilayers by cytolsin III suggested the presence of a titratable carboxyl group controlling cation permeability within or near the mouth of a channel. In addition, our recent results corroborate the importance of equinatoxin II carboxylates for both permeability of the toxin induced pores and binding of
divalent cations since Ni\textsuperscript{2+} completely blocked the hemolysis induced by native or lysine-modified toxin, while hemolysis induced by carboxyl groups modified toxin was only slightly affected by divalent cations [32]. Furthermore, differences in the pH profiles of hemolytic activity observed in the present study clearly demonstrate that besides carboxyl groups lysine residues are involved in modulation of hemolytic activity. For example, lysine residues of \textit{Staphylococcus} alpha-toxin have been shown to be important in determining the electrical properties of pores but not for formation of pores or for hemolytic activity [31]. Very recently, single channel experiments with carboxylate and lysine modified equinatoxin II provided the direct proof for participation of both carboxylate and lysine residues in controlling pore conductance and ion selectivity (G. Belmonte, Tesi di laurea, University of Trento, Italy, 1990).

We can conclude that the charged amino acid radicals are not essential for binding of equinatoxin II to lipid membranes. Lysine amino groups and carboxyl groups, very likely play a role in the formation and/or modulation of pore permeability. At present it is not clear whether toxin carboxylates have some additional functions since the correlation between lethal and cytolytic activity of \textit{EqT} II is not straightforward.

References