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Modification of Lysine Residues of *Staphylococcus aureus* α-Toxin: Effects on Its Channel-Forming Properties

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Summary. *Staphylococcus aureus* α-toxin opens an ion channel in planar phospholipid bilayers which is selective for anions over cations, supposedly because of the presence of positively charged groups along the ion pathway. To remove some positive charges of this protein toxin, we chemically modified part of its lysine residues either with diethylpyrocarbonate, followed by histidine regeneration with hydroxylamine, or with trinitrobenzenesulfonic acid. The extent of chemical modification can be followed accurately by native polyacrylamide gel electrophoresis and isoelectric focusing. Ethoxyformilation of two to three lysine residues per toxin monomer does not impair hemolysis of rabbit red blood cells nor formation of pores in model membranes. It reduces the conductance and the anion selectivity of the channel and changes the shape of its current-voltage characteristic. This indicates that positively charged lysine residues are actually important in determining the electrical properties of the pore. Ethoxyformilation of channels preassembled in planar bilayers produces the same changes as modification of toxin monomers before channel formation. Furthermore, it can be performed by adding diethylpyrocarbonate on either side of the bilayer. This suggests that the lysine residues relevant for the electrical properties of the pore are located inside its lumen where they can be reached by diethylpyrocarbonate diffusing from either entrance of the channel.

Key Words α-toxin · pore formation · ion selectivity · lysine modification · diethylpyrocarbonate · trinitrobenzenesulfonic acid · *Staphylococcus aureus*

Introduction

α-toxin is a cytolytic exotoxin secreted by virulent strains of *Staphylococcus aureus* as a single water-soluble 33 kDa polypeptide with pI around 8.4 [7, 10, 31]. It causes hemolysis of red blood cells and damages a variety of other cells by forming an amphiphilic hexamer that inserts into the cell membrane and generates a hydrophilic pore [7, 10, 11, 30–32].

Nucleated cells can survive the action of the toxin under favorable conditions [3]. For this reason and because the cutoff of the α-toxin pore is relatively small (at a molecular weight between 2000 and 4000, corresponding to a diameter of about 2 nm [6, 11]), this protein toxin has been widely used in the last few years as a means to make cells permeable to ions and small molecules but not to cytosolic proteins and enzymes [1, 2, 17, 21].

From this point of view and, more in general, to understanding the molecular basis of the damage caused by this toxin, a detailed knowledge of the structure and permeability properties of the toxin-pore is desirable. Though several models of the channel structure have been presented [18, 31, 32] up to now, they are still entirely speculative and await for more experimental data.

We have shown recently that the ionic transport properties of the pore formed by α-toxin can be conveniently studied in a very simple system such as the planar lipid membrane [3, 23, 24]. In these model membranes we demonstrated that the aggregation of several toxin monomers is required for the formation of one pore [4]. We found that the pore has an average diameter of 1.1 ± 0.1 nm, is water filled and slightly anion selective [4, 23]. The selectivity increases at low pH, suggesting that it is due to the presence of charged amino acid residues on the pore, conferring an overall positive charge to its interior.

Natural candidates to provide this charge are lysine and arginine residues because they are positively charged at neutral pH and very hydrophilic. Futhermore, they are generally located on the outer surface of globular proteins [19] and hence are likely to line also the hydrophilic interior of the pore.

To test this hypothesis we have modified some of the α-toxin lysine residues with two agents,

DEPC[1] and TNBS, which both remove the positive charge of the amino group, and we have investigated the effect on the permeability properties of the channel. Effects of arginine modification could not be studied because this modification inactivates the toxin [14].

Materials and Methods

Toxin and Other Reagents

Liophilyzed α-toxin from *Staphylococcus aureus* (kindly provided by Dr. K. Hungerer, Behring, Marburg, FRG) was reconstituted in 10 mM KH_2PO_4, 125 mM NaCl, 0.2 mM EDTA at pH 6.5 (called Buffer A) shortly before use. TNBS was obtained through Sigma; sources of all the other reagents are as in [27].

Ethoxyformilation of α-Toxin

DEPC modification of histidines and primary amines as well as reconstitution of imidazoles with hydroxylamine [25] were performed exactly as described in [27]. The average number of histidine residues modified per toxin monomer, before and after hydroxilamine treatment, was determined spectrophotometrically as described in [27].

Trinitrophenylation of α-Toxin

TNBS was used to modify α-toxin primary amines according to a standard procedure [22]. Briefly, 61 or 228 μM TNBS was mixed with 10 μM α-toxin in a solution (hereafter called Buffer B) containing one part of $NaHCO_3$ 4% (wt : vol) and two parts of Buffer A, final pH 8.1. After one hour stirring at 20°C (in the dark) the reaction was stopped by adding 20 mM NH_2OH, and the sample was immediately dialyzed against Buffer B plus 20 mM NH_2OH for 6 hr at 4°C and for an additional 12 hr against Buffer B alone. Thereafter the number of modified lysine residues was determined at 345 nm using the known molar extinction coefficient of 1.45×10^4 M^{-1} cm^{-1} [22].

Assay of Toxin Hexamerization on Lipid Vesicles

Small unilamellar vesicles (SUV) composed of egg PtdCho and cholesterol (molar ratio 1 : 1) were prepared as described [9, 27]. Toxin oligomerization onto lipid vesicles was assayed as follows: 100 μl of vesicles (2 mg/ml of lipid) were mixed with 150 μl of α-toxin (1 mg/ml in Buffer A) and incubated 1 hr at 37°C. Unbound toxin (mol wt 33 kDa) was removed by washing the vesicles over Amicon filters YMT100 (cut off at mol wt 100 kDa) as described [27]. The final retentate was analyzed by SDS gel electrophoresis as specified below.

Preparation of Planar Lipid Bilayers

Planar lipid bilayer membranes composed of either POPtdCho or DPhPtdCho and separating two aqueous solutions (4 ml on each side) containing buffer A if not otherwise specified were prepared exactly as in [27].

The ionic current flowing through the membrane, under voltage-clamp conditions, was converted to voltage by a virtual grounded operational amplifier (Analog Devices AD515K) with a feed-back parallel of 100 MΩ and 10 pF. Ag-AgCl electrodes were used either directly immersed into the electrolyte solution or (for the selectivity experiments) connected to it via agarose bridges saturated with 2 M NaCl.

S. aureus α-toxin was added on one side only (termed *cis*) of a preformed stable bilayer of low conductance. Voltage and current directions are defined as in [27]. Experiments were performed at room temperature.

Modification of Pores Preformed on Planar Bilayers

Preformed pores were modified by DEPC directly on the planar bilayer. To do this the membrane was first exposed to native α-toxin (at a concentration of 5–10 μg/ml for the case of POPtdCho membranes or 0.25 μg/ml for the case of DPhPtdCho membranes) and the pores were allowed to insert. When a steady state was reached with a very small pore-formation rate (usually after 30 to 50 min) DEPC was added on one side of the membrane (either *cis* or *trans*) at the concentration specified in the text. Thereafter the electrical properties of the population of pores did change with time and got stabilized after about 12 to 15 min. Selectivity and rectification given in the text were measured under these final conditions.

The pH of the compartment where DEPC was added was determined at the end of the experiment and was usually found to be 0.2–0.3 pH units more acidic than the starting value, pH 6.5 (in no case was the final value less than 6.0). Control experiments did show that similar changes in pH alone can by no means account for the observed effects on the channel properties.

Electrophoresis

All the electrophoresis were performed with a semi-automatic unit (PhastSystem by Pharmacia) according to the procedures suggested by the purchaser. For SDS-PAGE we used precast minigels (Pharmacia) with polyacrylamide density gradients 10–15% or 8–25% as specified; other conditions were as in [27].

In native-PAGE, proteins were separated in an SDS-free buffer at pH 8.8 and hence migrated according to their intrinsic net charge [16]. Minigels with linear gradients 8–25% were used and the run was performed at 10°C.

For isoelectric focusing (IEF) homogeneus 5% polyacrylamide gels containing ampholytes (Pharmacia) were used. A pH gradient from 3 to 9 was established across the gel by a pre-run. Samples were then applied in the middle of the gel. The pH gradient on the gel was mapped by running standard proteins with different pI (Pharmacia Broad pI Calibration Kit).

[1] *Abbreviations:* DEPC, diethylpyrocarbonate; TNBS, trinitrobenzenesulfonic acid; IEF, isoelectric focusing; PtdCho, phosphatidylcholine; POPtdCho, palmitoyl-oleoyl-phosphatidyl-choline; DPhPtdCho, diphytanoyl-phosphatidylcholine; SUV, small unilamellar vesicles; TLC, thin-layer chromatography; SDS, sodium dodecyl sulfate; Triton X-100, octylphenoxy polyethoxy ethanol.

Gel staining and densitometric evaluation were performed as in [27].

Results and Discussion

Characterization of Lysyl Modification of α-Toxin

DEPC mainly reacts with the amino and imidazole groups of proteins [22]. We have shown elsewhere [27] that all three histidines of α-toxin can be modified by DEPC and that this modification is reversed by hydroxylamine. We found that in the case of α-toxin modification of primary amines on lysine residues occurs together with histidine modification. This is not surprising because lysine residues, in spite of being less reactive, are present in a much larger proportion than histidines (28 Lys against 3 His) [12].

Modification of lysyl residues becomes apparent when the native and the modified toxin are electrophoresed under nondenaturing conditions at pH 8.8 (Fig. 1). In fact, ethoxyformilation changes the net electrical charge of the protein at any pH below the pK of the lysine ε-amino group (which is around 10) by removing its positive charge [22, 25]. Accordingly, though SDS-PAGE shows no change in the molecular weight of the modified toxin monomer (Fig. 1*A*), native-PAGE at pH 8.8 reveals the splitting of the main toxin band into an increasingly large number of bands with increasing DEPC modification (Fig. 1*B*). All these new bands are displaced towards the anodic side of the gel.

Since in native-PAGE proteins with the same molecular weight migrate at different rates depending on their intrinsic electrical charge, the presence of faster bands in the modified toxin indicates that these molecules became more negatively charged, as expected from the neutralization of the positive charges on the lysine residues. As shown by Creighton [16] this technique provides an effective way of counting the number of modified residues per toxin molecule. In fact (as we show in Fig. 2), the spacing of the bands is such to suggest that they each differ from the preceding one by addition of the same discrete charge to the molecule, i.e., by modification of one single amino acid residue.

A contribution of histidine modification to this pattern can be ruled out because it does not change the electrical charge of the protein at pH 8.8, since at this pH the histidine is uncharged. Consistently we found that the pattern of bands is unaffected by exposure of the treated toxin to hydroxylamine (Fig. 1), which removes DEPC from imidazole [27] but not from the lysyl ε-amino group [22, 25]. Further confirming this interpretation, we found that modification with low concentrations of the specific lysine modifier TNBS [22] produces the same pattern in native-PAGE (Figs. 1 and 2).

Complementary information can be obtained by isoelectric-focusing the toxin (Fig. 3). Unmodified toxin gives a major band of pI around 8.3, as expected [10], whereas both DEPC- and TNBS-modified samples present a number of additional bands shifted towards lower values of pI. We assume (by comparison with Fig. 2) that each band differs from the preceding one by the modification of one additional amino acid residue. Careful observation shows that major bands are often degenerated into multiplets, indicating that modification does not always simultaneously affect the same residues on all the toxin molecules [16]. Also in this case hydroxylamine treatment of the DEPC-modified samples does not change the pattern of the major bands, but it produces a rearrangement within the minor bands forming a multiplet if the multiplet is located in a region of pI ≤ 6.5. This is most probably due to the regeneration of histidine residues, which are charged at this pH unless they are ethoxyformilated. The finding suggests that histidine modification may (to some extent) be detected by IEF.

Both DEPC modification followed by NH_2OH reconstitution, and TNBS modification, produce toxin monomers with modified lysine residues. The relative concentration of unmodified, single-modified, double-modified, etc., monomers can be estimated by scanning densitometry of the pertinent native-PAGE or IEF gels. The average number of lysine residues modified per toxin monomer (called m) can be calculated from these concentrations according to

$$m = \Sigma_j j\, c_j / \Sigma_j c_j \qquad (1)$$

where j is the number of lysine residues modified in each band (i.e., zero in the first, one in the second, etc.), and c_j is the concentration (or density) of that band. In the case of TNBS-modified toxin the value of m determined in this way agreed within ± 20% with the average number of lysine residues modified, measured spectrophotometrically.

In principle, samples obtained with both modification methods may be used to investigate the role of positively charged residues on the interaction of the toxin with model membranes. However, when the hemolytic activity of these samples is tested on rabbit erythrocytes (as described in [27]) we found that DEPC-modified NH_2OH-reconstituted α-toxin is fully active [27], whereas TNBS-modified toxin has a decreased hemolytic activity (fourfold and eightfold decrease with TNBS concentrations 61 and 228 μM, corresponding to $m = 1.5$ and $m = 2.5$,

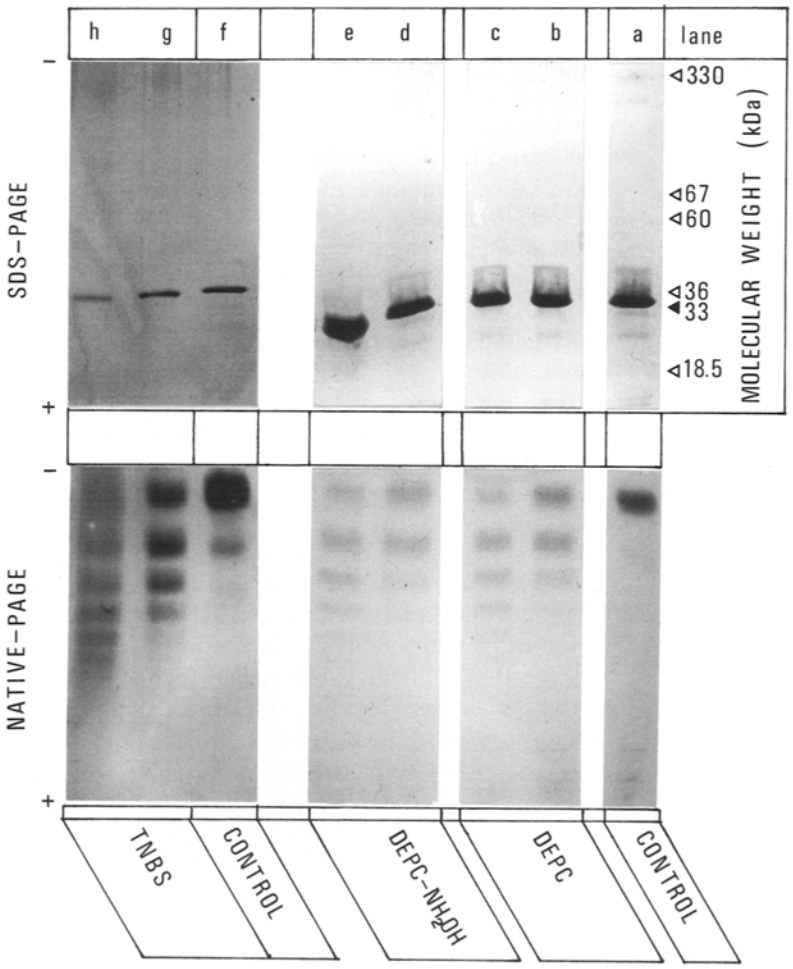

Fig. 1. Lysine modification of α-toxin with DEPC and TNBS. *Upper panel:* SDS-PAGE of α-toxin samples. Lanes *a* and *f*, native toxin; lanes *b* and *c*, toxin modified with 0.06 mM or 0.04 mM DEPC, respectively; lanes *d* and *e*, same samples as lanes *b* and *c* but after NH_2OH treatment; lanes *g* and *h*, toxin modified with 0.06 mM or 0.2 mM TNBS, respectively. Lanes *a* to *e* were stained with Coomassie blue; lanes *f* to *h* were silver stained. Protein concentration was 0.74 mg/ml in lanes *a* to *e* and 0.25 mg/ml in lanes *f* to *h*. Open arrowheads point at the position of standard proteins of different mol wt, full arrowheads extrapolate the positions of mol wt 33 kDa corresponding to toxin monomers. *Lower panel:* Native-PAGE of the same samples as in the upper panel and in the same order. Lanes *a* to *e* were stained with Coomassie blue (protein concentration was 1 mg/ml); lanes *f* to *h* were silver stained (protein concentration was 0.33 mg/ml). The direction of the electric field applied is indicated in both panels

respectively). Thus it appears that the DEPC-NH_2OH procedure is to be preferred.

Similarly, the effects of arginine modification (which we performed by hydroxyphenylglioxal or succinic anhydride) on the channel properties could not be studied because this modification inactivated the toxin, as reported also by others [14].

Effect of Lysyl-Modification on the Electrophoretic Behavior of α-Toxin Hexamers

When α-toxin is incubated with PtdCho/cholesterol (1 : 1) lipid vesicles, a characteristic hexameric aggregate appears on their surface which is responsible for an increased membrane permeability [9, 11]. The presence of the aggregate is evidenced by SDS-PAGE of these vesicles (incubated 1 hr at 37°C and washed free of unbound toxin by a microseparation system as described by Pederzolli et al. [27]). It appears that most of the toxin runs in the gel as a 200-kDa aggregate, i.e., a hexamer (Fig. 4).

Lysyl modification performed either with mild TNBS-treatment or with the DEPC-NH_2OH treatment does not impair the ability of the toxin to form the hexamer as it does not reduce its permeabilizing effect revealed by the release of internally trapped markers (*data not shown*). However, lysyl-modified hexamers run slightly faster than controls (an effect probably related to the altered electrical charge of the molecule) and in a broader band, possibly because of an heterogeneous number of modified lysine residues (Fig. 4).

Interestingly, when toxin hexamers preformed on lipid vesicles are treated similarly with DEPC (a procedure which we have found to modify at most one histidine per monomer [27]) an aggregate is produced with the same electrophoretic properties as the one assembled starting from lysyl-modified monomers (Fig. 4). This demonstrates

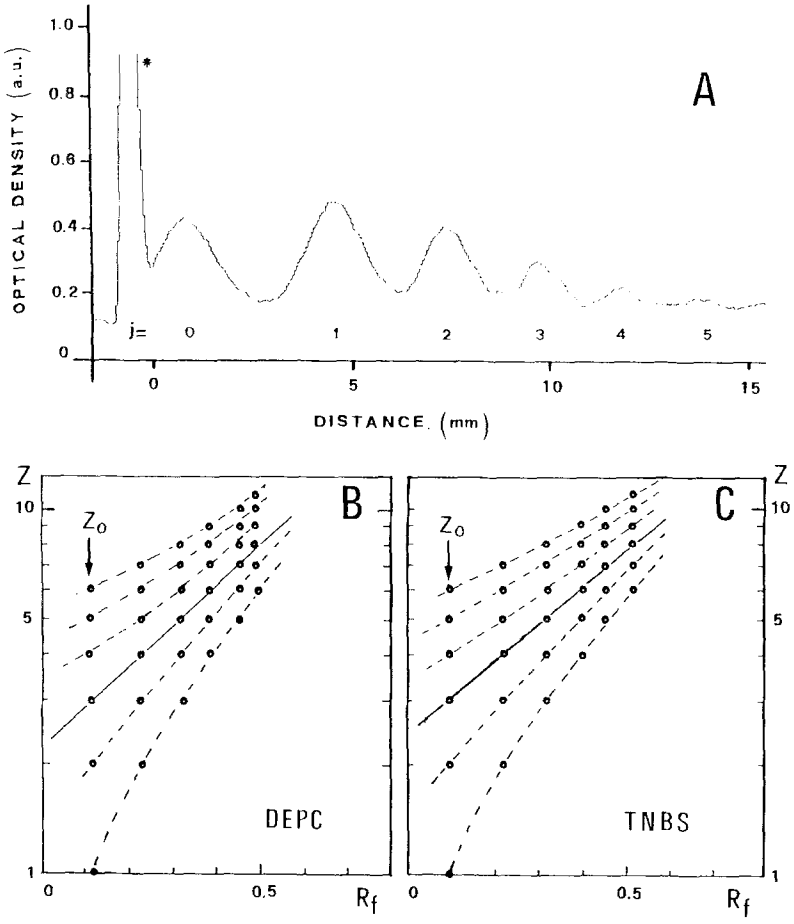

Fig. 2. Effect of lysyl modification on the electrical charge of α-toxin. (*A*) Densitometry of native-PAGE (the example shows lane *e* of the lower panel in Fig. 1) allows independently calculating the position and the relative protein content of each band. Bands are labeled with the index *j* which gives the number of lysine residues modified per toxin monomer. Density is given in absorption units. The asterisk marks the interface with the stacking gel. (*B* and *C*) Half-logarithmic plots of the relative mobility R_f (i.e., distance of the band/distance of the front end) of α-toxin in native-PAGE *vs.* its negative charge *Z* in modulus. (*B*) DEPC-modified-NH_2OH-reconstituted α-toxin; (*C*) TNBS-modified α-toxin. The charge (expressed in arbitrary units) was attributed assuming that each band differs from the preceding one by the addition of one discrete unit of charge. Different values of Z_0 (the charge of the native toxin at pH 8.8) were used. Points with the same Z_0 are connected by lines. Both plots are linear if one assumes that Z_0 is three arbitrary units (solid lines)

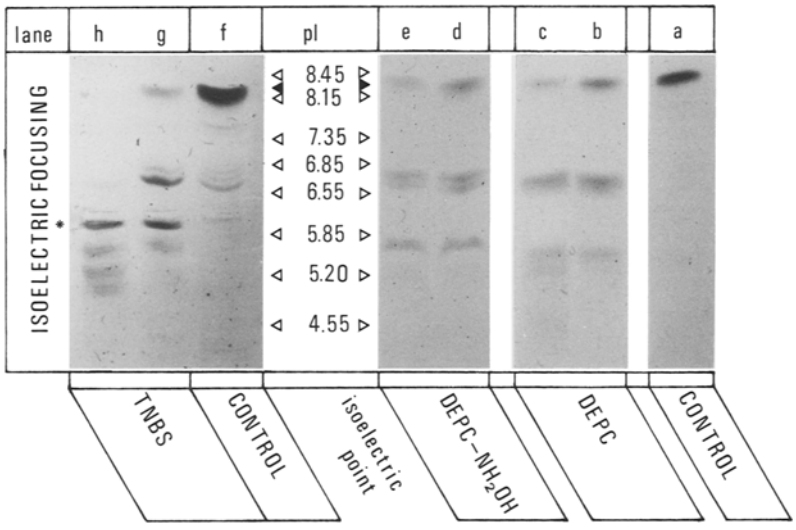

Fig. 3. Effect of lysine modification on the isoelectric point of α-toxin. IEF of α-toxin samples on 5% polyacrylamide gels: lanes *a* and *f* native toxin; lanes *b* and *c*, toxin modified with 0.06 mM or 0.4 mM DEPC, respectively; lanes *d* and *e*, same samples as lanes *b* and *c* but after NH_2OH treatment; lanes *g* and *h*, toxin modified with 0.06 or 0.2 mM TNBS, respectively. Lanes *a* and *e* were stained with Coomassie blue (protein concentration was 0.66 mg/ml); lanes *f* to *h* were silver stained (protein concentration was 0.33 mg/ml). Open arrowheads point at the position of standard proteins with known pI, filled arrowheads extrapolate the pI of native α-toxin. An asterisk marks the position where the samples were applied to the gel; in lanes *g* and *h* part of the protein failed to migrate, probably because of partial denaturation due to TNBS treatment

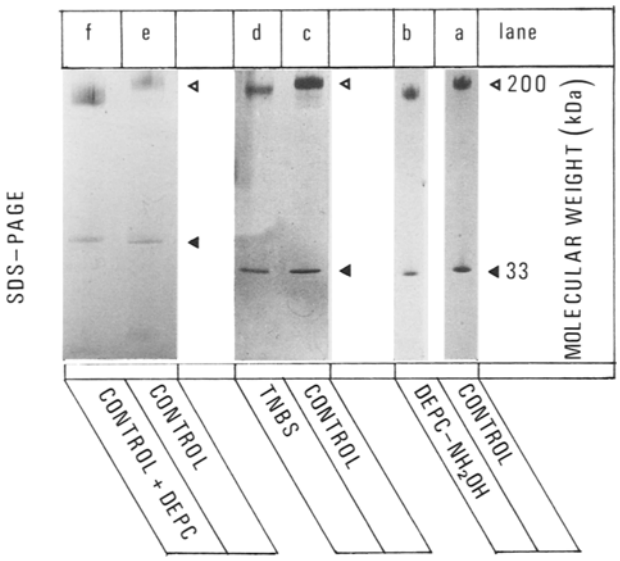

Fig. 4. Effects of lysyl modification on the electrophoretic behavior of α-toxin hexamers formed on lipid vesicles. SUV composed of PtdCho/cholesterol in a 1 : 1 molar ratio were exposed to the action of a fixed amount of either native α-toxin (lanes *a*, *c* and *e*), or DEPC-modified NH_2OH-reconstituted α-toxin (lane *b*) or TNBS-modified α-toxin (lane *d*). After 1 hr incubation at 37°C the vesicles were separated from unbound toxin by filtration over Amicon filters (cut-off at mol wt 100 kDa) and the content in bound α-toxin of the retentate was assayed by SDS gel electrophoresis. In lane *f* vesicles incubated with native α-toxin were subsequently treated with 1.85 mM DEPC. Toxin concentration during incubation was 0.60 mg/ml in lanes *a* and *b*, 0.14 mg/ml in lanes *c* and *d* and 0.43 mg/ml in lanes *e* and *f*. The concentration of α-toxin applied to the gel was 0.41 mg/ml in lanes *a* and *b*, 0.09 mg/ml in lanes *c* and *d* and 0.29 mg/ml in lanes *e* and *f*. Gels used were linear polyacrylamide gradients ranging 10–15% (lanes *a* to *d*) or 8–25% (lanes *e* and *f*). Lanes *c* and *d* were silver stained; all the other were stained by Coomassie blue. The position of mol wt 33 kDa (corresponding to toxin monomers) and 200 kDa (corresponding to toxin hexamers) are indicated by filled and open arrowheads, respectively. They were extrapolated by comparison with the position of standard proteins as in Fig. 1

that a number of lysine residues are still accessible to DEPC modification even after the hexamer is assembled in the lipid membrane.

Effect of Lysyl-Modification on the Electrical Properties of α-Toxin Channels

α-toxin forms ionic channels in PtdCho planar lipid bilayers when introduced into the bathing solution. Opening of one pore is revealed by a sudden increase of the ionic current flowing through the bilayer when the voltage is clamped at +40 mV (Fig. 5*A*). A number of such current jumps may be used to build up a histogram relating the number of events observed with their conductance (Fig. 5*A*). From this histogram the average conductance of the pore can be calculated. Ethoxyformilation of the toxin decreases such conductance (trace *b*). This effect is not reversible by NH_2OH, suggesting that it is due to lysine modification (trace *c*).

The dependence of the pore conductance on the mean number of lysine residues modified per toxin monomer (determined by means of Eq. (1)) is shown in Fig. 5*B* together with the theoretical predictions of a model which will be later described in detail. This model is based on a few simple considerations: (i) a change in the pore conductance after lysine neutralization was expected because the conductance of an ion channel is largely determined by the charged amino acid residues lining its inner surface; (ii) being anion selective at pH 7.0 (*see* Fig. 6) the α-toxin pore is conceivably lined by positive charges which attract anions and repel cations; (iii) lysine residues most probably contribute to this charge because they are usually located at the hydrophilic surface of proteins; (iv) ethoxyformilation of a few such residues per toxin monomer would be enough to decrease the attraction for anions (chloride in this case) and hence also the conductance of the pore.

According to these considerations the selectivity of the channel should also change when the positive charge is removed from the lysine residues. To test this, we measured the reversal voltage (i.e., the voltage at which the net current flowing through the channel is zero) when membranes containing a large number of toxin pores separate a tenfold concentration gradient of NaCl (Fig. 6*A*). From the reversal voltage, V_{rev}, it is possible to derive the ratio between the permeability of chloride and that of sodium ($P(Cl)/P(Na)$) using the Nernst-Planck equation [29]. We found that indeed ethoxyformilation of α-toxin decreases the magnitude of V_{rev} and hence the anion selectivity of the pore (Fig. 6*A*). This effect is resistant to NH_2OH treatment, implying that it is independent from histidine modification. The dependence of the permeability ratio $P(Cl)/P(Na)$ on the mean number of lysine residues modified per toxin monomer is shown in Fig. 6*B* and is correctly predicted by our model.

It is worth noting that also in the case of some porins it was shown that lysine residues are essential in determining the selectivity and the conductance of the channel [5, 13].

The peculiar role of lysine groups in the pore properties is confirmed by the analysis of its current-voltage (I–V) characteristic (Fig. 7). As originally shown by Menestrina [23] the I–V curve of native

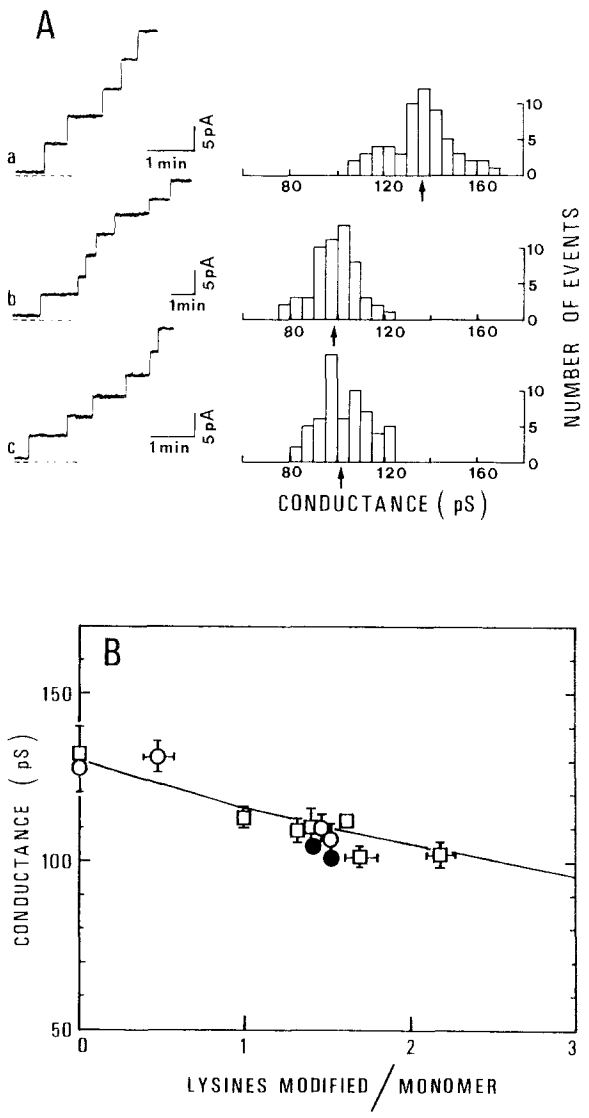

Fig. 5. Conductance of the channel formed by native and lysyl-modified α-toxin in planar lipid membranes. (*A*) POPtdCho membranes were exposed to α-toxin either native (trace *a*), or DEPC treated (trace *b*), or the same but reconstituted with NH_2OH (trace *c*). The number of lysine residues modified per toxin monomer (derived as shown in Fig. 2) was 1.5 in traces *b* and *c*. Histidines modified (per toxin monomer) were 2.3 before and 0.4 after NH_2OH treatment. 5 μg/ml toxin was administered in all cases. Discrete current jumps revealed the opening of single ion channels. A dashed line indicates zero current. Applied potential was +40 mV. Next to each trace a histogram shows the number of events with a given conductance observed in different (2 to 4) experiments run with the same toxin sample. Mean conductance values obtained from those histograms are indicated by arrows. (*B*) Dependence of the single channel conductance at +40 mV on the number of DEPC-modified lysine residues per toxin monomer (derived as shown in Fig. 2). Full points are for NH_2OH-reconstituted toxin samples. Squares are for DPhPtdCho membranes, circles for POPtdCho membranes. Error bars are SEM of 20 to 60 events. The solid line is the prediction of the theoretical model discussed in the text

α-toxin pores is not linear since the current flowing at positive voltages is much larger than that flowing at negative voltages of the same value. In terms of the most general electrodiffusional models this implies that the potential profile of the pore (generated by its own fixed charges) is asymmetrical along the ion pathway [20, 29]. Lysyl-ethoxy-formilation decreases (to a large extent) the nonlinearity of the *I–V* curve and once again this effect is not reversed by NH_2OH treatment (Fig. 7*A*), ruling out a role for histidine. This finding suggests that neutralization of some lysine residues at well-defined positions along the ion pathway changes the potential profile of the pore.

As a measure of the nonlinearity of the *I–V* curve we calculated the ratio between the current flowing at +120 mV and that at −120 mV (called $I(+V)/I(-V)$). The dependence of this ratio on the mean number of lysine residues modified per toxin monomer is shown in Fig. 7*B*, and again it is well predicted by our model.

Preformed α-toxin channels can also be modified with DEPC, which produces a strong reduction of their anionic selectivity and a linearization of their *I–V* curve (Table). This is consistent with what we have shown in Fig. 4, i.e., that lysine residues of preformed hexamers can still be modified by DEPC. Further, it indicates that at least some of these accessible residues are actually located near the ion pathway where they can influence the ion flux.

In principle it is possible, by adding DEPC only on one side of the membrane, to probe the disposition of the relevant lysines on the two sides of the channel. Interestingly, addition of DEPC only to the *cis* side or only to the *trans* side of the bilayer has exactly the same final effect both on the selectivity and on the *I–V* curve of the pore (Table). This seems to mean that the lysyl groups responsible for the electrical properties of the pore are modified in both cases, implying that they are actually located inside the lumen of the pore where they may be approached by DEPC diffusing from either side of the channel (DEPC has mol wt 162 and thus it is expected to move freely into the pore which has a cut-off at a mol wt between 2,000 and 4,000 [6, 11]). It is not likely that these results are affected by diffusion of DEPC (which is actually a rather lipophilic molecule) through the membrane. In fact, in a planar lipid bilayer experiment the concentration on the side opposite of addition is always buffered to zero because of the exceedingly large volume of the compartment compared to the small area of the membrane. Even for free diffusion through the open hole (when the membrane is broken) the concentration of the diffusant on the other side would not appreciably increase over a period of several hours.

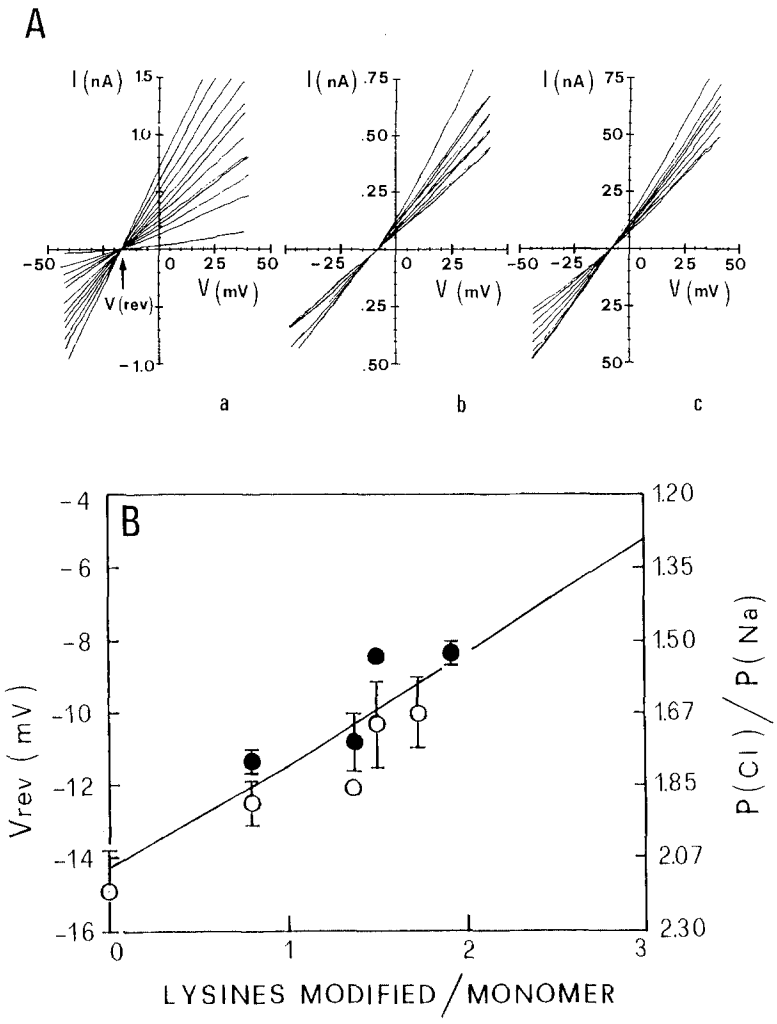

Fig. 6. Selectivity of the native and lysyl-modified α-toxin pore. (*A*) Current-voltage curves shown were obtained by applying a slowly changing triangular wave of voltage to membranes containing a population of many α-toxin channels as observed several minutes after the addition of the protein. The membranes separated two asymmetrical solutions containing 500 mM NaCl on the *cis* side and 50 mM NaCl on the *trans* side (pH 6.5). Curves obtained at successive intervals of time are superimposed; their slope becomes steeper with time because the number of channels inserted into the bilayer increases. All the curves intercept the voltage axis at one point. Such voltage, at which no net current flows through the channel, is called *reversal voltage* (V_{rev}). The negative value of V_{rev} indicates that the pore is anion selective; ideal anion selectivity under these conditions would give $V_{rev} = -52$ mV. POPtdCho membranes were exposed either to native α-toxin (panel *a*), or DEPC-treated toxin (panel *b*), or to the same but reconstituted with NH_2OH (panel *c*). The number of lysine residues modified per toxin monomer (derived as shown in Fig. 2) was 1.5 in panels *b* and *c*. Histidines modified (per toxin monomer) were 2.3 before and 0.4 after NH_2OH treatment. Toxin was 5 μg/ml. (*B*) Dependence of V_{rev} upon the number of lysine residues modified by DEPC per α-toxin monomer (derived as shown in Fig.2) either before (open symbols) or after (closed symbols) NH_2OH treatment. Error bars are standard deviations of 2 to 8 independent experiments, similar to those in *A*, run on different membranes. Membranes were composed of POPtdCho. Other conditions were as in Fig. 5. The chloride to sodium permeability ratio ($P(Cl)/P(Na)$) given on the right scale was calculated from V_{rev} according to the Nernst-Planck equation [29]. The solid line is the prediction of the theoretical model discussed in the text

Finally, we have found that chemical modification of α-toxin before or after channel formation are not additive in the sense that the addition of DEPC on one side of a membrane containing preformed channels attains the same final result whether we start from unmodified toxin or from a heavily modified toxin sample (2.9 lysines and 1.9 histidines modified per monomer, *see* the Table). This proved to be true both for the *I–V* nonlinearity and for the ionic selectivity.

A Model for the Role of Lysine Residues on the Channel Properties

To design a possible model for the structure of the α-toxin channel we made a few considerations: (i) its lysine-dependent anion selectivity (Fig. 6) indicates that a few of these residues are located along the ion pathway; (ii) the accessibility of such residues in preformed pores from either side of the pore (Table) implies that they are actually located inside the lumen of the pore; (iii) the nonlinearity of the *I–V* curve in the native channel (chloride currents going from the *cis* to the *trans* side are larger than chloride currents going the other way round) suggests that an excess of positive charge is located near the *cis* entrance of the pore; (iv) the fact that DEPC added to preformed pores can reverse the nonlinearity of their *I–V* curve (Table) indicates that negative fixed charges are also present at the *cis* entrance and may prevail when all lysine residues are neutralized.

A tentative picture of the channel is given in Fig. 8. We envisaged it as a hollow cylinder resulting from the assembly of six toxin monomers. To fit the experimental data we had to assume the presence of at least three positively charged lysine residues and one negatively charged acidic (glutamic or aspartic) residue per monomer. These charges are located at

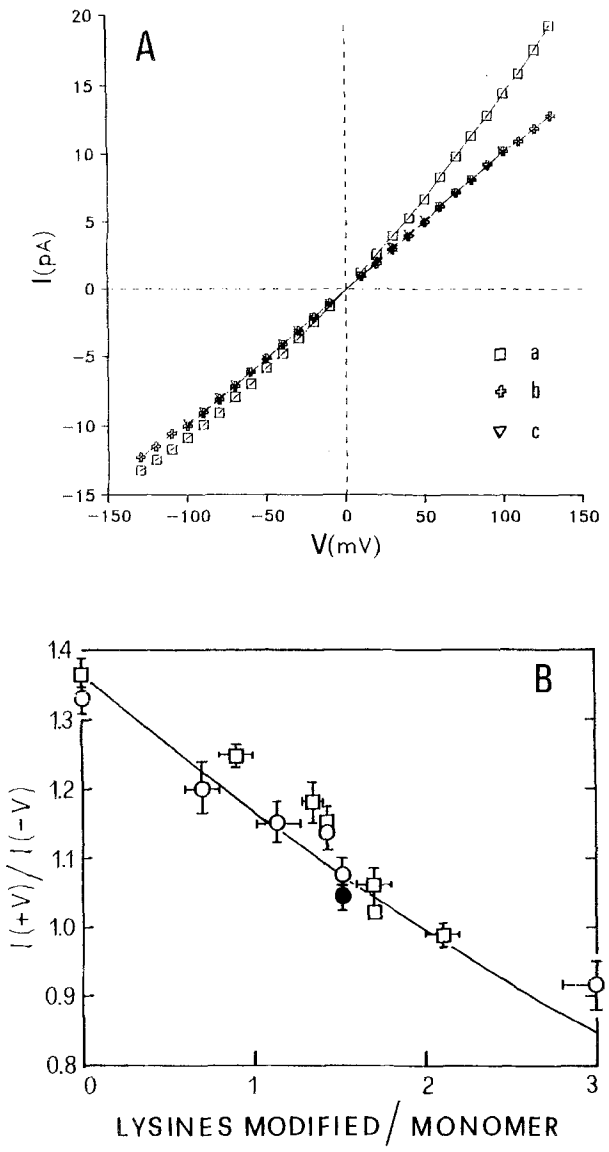

Fig. 7. Current-voltage characteristic of the native and lysyl-modified α-toxin pore. (*A*) Current-voltage curves obtained applying short square-shaped voltage pulses [23] to a membrane containing many α-toxin channels are shown. The membrane separated two symmetrical solutions containing 125 mM NaCl on both sides (pH 6.5). Curves were scaled to the current flowing through a single ion channel using the discrete current jumps observed at the beginning of the experiment (as shown in Fig. 5*A*). POPtdCho membranes were exposed either to native α-toxin (curve *a*), or DEPC-treated toxin (cruve *b*), or to the same but reconstituted with NH_2OH (curve *c*). The number of lysine residues modified per toxin monomer (derived as shown in Fig. 2) was 1.5 in curves *b* and *c*. Histidines modified (per toxin monomer) were 2.3 before and 0.4 after NH_2OH treatment. Toxin was 5 μg/ml. (*B*) dependence of the ratio $I(+V)/I(-V)$ (in absolute value) on the number of lysine residues modified by DEPC per α-toxin monomer (derived as shown in Fig. 2) either before (open symbols) or after (filled symbol) NH_2OH treatment. Squares are for DPhPtdCho membranes, circles for POPtdCho membranes. Other conditions were as in Fig. 5. The solid line is the prediction of the theoretical model discussed in the text

the *cis* entrance of the pore and, for the sake of simplicity, we placed them symmetrically around its center. They contribute to create an entrance potential at the *cis* side which we call Φ_c.

The most general expression for the current I flowing through a channel is given by the Goldman-Hodgkin-Katz (GHK) equation [20, 28, 29], which (in the case of a symmetrical NaCl solution of concentration C on both sides of the pore) reads

$$I = \Sigma_i I_i \tag{2}$$

$$I_i = (a/l)\, u_i\, e_0\, z_i\, (V + \Phi_t - \Phi_c)\, C \frac{\exp(-z_i \Phi_t')(1 - \exp(-z_i V'))}{1 - \exp(z_i(\Phi_c' - \Phi_t' - V'))} \tag{3}$$

where the index i refers to the ion species present (Na and Cl); a and l are the cross sectional area and length of the pore; u and z are the absolute mobility and valence of the ion species; e_0 is the electronic charge; V is the applied potential and Φ_c, Φ_t are the entrance potentials (due to the fixed charges on the pore) at the *cis* and *trans* side, respectively. All the primed potentials are reduced potentials obtained by dividing the corresponding unprimed potentials by the factor kT/e_0 (k: Boltzmann constant; T: absolute temperature) which is 25 mV at room temperature.

In our model only Φ_c is not nil and hence Eq. (3) simplifies to

$$I_i = A\, u_i z_i (V - \Phi_c) \frac{1 - \exp(-z_i V')}{1 - \exp(z_i(\Phi_c' - V'))} \tag{4}$$

where we have introduced the constant $A = e_0 C (a/l)$.

Combining Eqs. (2) and (4) it is easy to calculate the ratio $I(+V)/I(-V)$. In the limit of large V, we get

$$|I(+V)/I(-V)| = \exp \Phi_c'. \tag{5}$$

Fitting Eq. (5) to the results in Fig. 7 we get $\Phi_c = +8$ mV for the native channel.

When the entrance potential Φ of a pore with a symmetric distribution of point charges located around its mouth is calculated using the Debye-Hückel theory [8] (or even the more refined calculation of Nelson and McQuarrie [26, 28]), one simply gets that Φ is directly proportional to the total amount of charge present. Accordingly,

$$\Phi_c = k\, Q_c. \tag{6}$$

Where Q_c is the algebraic sum of the charges present at the *cis* entrance and K is a proportionality constant.

In our model we have two positive charges in excess (per toxin monomer) in the native channel.

Table. Effects of DEPC modification on the electrical properties of preformed α-toxin pores[a]

Lipid	$[DEPC]_{cis}$ (mM)	$[DEPC]_{trans}$ (mM)	$I(+V)/I(-V)$	V_{rev} (mV)	$P(Cl)/P(Na)$
DPhPtdCho	—	—	1.34 ± 0.03 (4)[b]		
	4.0	—	0.88 ± 0.03 (1)		
	7.8	—	0.83 ± 0.03 (2)		
	3	—	1.00 ± 0.02[c]	−7.5 ± 0.5[c]	1.46 ± 0.04[c]
	—	3	0.93 ± 0.02[c]		
POPtdCho	—	—	1.28 ± 0.03 (6)[b]	−14.8 ± 0.7 (6)[b]	2.16 ± 0.08[b]
	4.9	—	0.73 ± 0.05 (2)		
	—	3	0.89 ± 0.05 (3)		
	4	—		−6.6 ± 2.1 (2)	1.38 ± 0.14
	—	4		−8.0 ± 0.5 (4)	1.50 ± 0.05
DPhPtdCho			1.33[d]	−14.3[d]	2.10[d]
or POPtdCho			0.85[e]	−5.5[e]	1.30[e]

[a] The ratio $I(+V)/I(-V)$, in absolute value, has been calculated at $|V| = 110$ mV from the I–V characteristic of membranes containing many channels (as shown in Fig. 7). The reversal voltage V_{rev} was measured as shown in Fig. 6. Values are average ± SEM of different experiments (the number of experiments is given in parenthesis). The permeability ratio $P(Cl)/P(Na)$ has been calculated from V_{rev} according to the Nernst equation [29]. DEPC was added (at the given concentration) either to the *cis* or to the *trans* side of a bilayer containing preformed channels. Mean values before addition of DEPC and theoretical predictions of the model presented in the text are also indicated.

[b] Before DEPC addition.

[c] Values obtained starting from a modified α-toxin sample (2.9 lysines modified); before DEPC addition $I(+V)/I(-V)$ was 1.02 ± 0.03 and V_{rev} was −7.5 ± 0.5 mV.

[d] Theoretical expectation for native toxin (no lysine residue modified).

[e] Theoretical expectation for most modified toxin (three lysine redisues modified per monomer).

From this it is easy to calculate Φ_c (and $I(+V)/I(-V)$ by Eq. (5)) also for the case of one, two, or three lysine residues modified per toxin monomer. This is shown in Fig. 7 as a solid line which fits rather well to the experimental data.

Using Eqs. (2) and (4) it is also easy to calculate the conductance of the pore ($G = I/V$). In the limit of V large and positive anions will move from the *cis* to the *trans* compartment, experiencing an entrance potential Φ_c at the pore mouth, whereas cations will move the other way round, experiencing no entrance potential. Accordingly, we get

$$G = A\,(u_{Na} + u_{Cl} \exp \Phi'_c). \tag{7}$$

A least-squares fitting of Eq. (7) to the experimental points of Fig. 5*B*, using a length of the pore of 12 nm as derived from electron microscopy data [11], allows us to estimate its cross-sectional area which is 1.14 nm^2 (corresponding to a pore diameter of 1.2 nm). This section is about 12 times that of the rectifying potassium channel and three times that of the acetylcholine-receptor channel [15]. Such large section (about 450 water molecules find place in a pore of this size) prevents the flowing ions from interacting strongly with the walls of the pore and thus justifies the use of a simple diffusional model.

Finally, Eq. (4) may be used to calculate the ratio between the anion and the cation permeability through the pore. In fact, we have that [29]

$$P(Cl)/P(Na) = I_{Cl}/I_{Na} \tag{8}$$

and this ratio does not depend on the applied potential. In fact, when Eq. (4) is used to calculate I_{Cl}/I_{Na} both in the limit of V large and positive and in the limit of V large and negative one gets

$$I_{Cl}/I_{Na} = u_{Cl} \exp \Phi'_c/u_{Na}. \tag{9}$$

It is evident from Eq. (9) that in this model the anion selectivity of the channel is a direct consequence of the fact that Φ_c is positive. Thus we expect that changing the fixed charge (e.g. by modifying lysine residues) would also change the selectivity of the pore. This is exactly what was found experimentally (Fig. 6). The predictions of Eq. (9) are shown in Fig. 6*B* as a solid line. We feel that the fit between experimental points and theoretical expectation is quite good, particularly considering that there is no adjustable parameter in this plot.

In conclusion, although admittedly simplistic, the model well describes the dependence of the electrical properties of the channel on the modification of a few of its lysine residues.

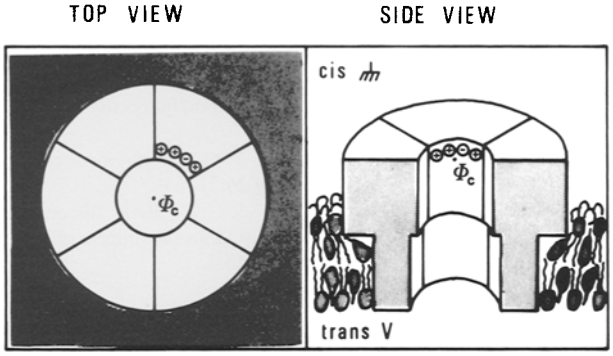

Fig. 8. Proposed model for the structure of the channel formed by α-toxin in lipid membranes. Top and side (dissected) view of the α-toxin pore in a PtdCho/cholesterol (1 : 1) bilayer membrane. The channel is shown as a hollow cylinder resulting from the assembly of six equal toxin monomers. Protein hydrophobic surfaces are exposed to the lipid phase whereas hydrophilic groups are exposed to the water phase. A hydrophilic pore is thus formed through which ions can cross the membrane. As indicated by electron microscopy [11], the pore protrudes into the water phase on the *cis* side (i.e., the side of addition of the toxin) and is ring-shaped when seen from the top (the plane of the membrane has been shaded in the top view). In conductance experiments the voltage V is applied to the *trans* side whereas the *cis* side is grounded. We assume the presence of three positively charged lysine residues and one negatively charged acidic residue per monomer (indicated by circles on one monomer only). These charges are located at the *cis* entrance of the pore and are symmetrically distributed around its center. They create an entrance potential Φ_c at the *cis* mouth which influences the flux of ions through the pore.

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References

1. Ahnert-Hilger, G., Brautigam, M., Gratzl, M. 1987. Ca^{++}-stimulated catecholamine release from α-toxin-permeabilized PC12 cells: Biochemical evidence for exocytosis and its modulation by protein kinase C and G proteins. *Biochemistry* **26:**7842–7848
2. Bader, M.-F., Thiersè, D., Aunis, D., Ahnert-Hilger, G., Gratzl, M. 1986. Characterization of hormone and protein release from α-toxin-permeabilized chromaffin cells in primary culture. *J. Biol. Chem.* **261:**5777–5783
3. Bashford, C.L., Alder, G.M., Menestrina, G., Micklem, K.J., Murphy, J., Pasternak, C.A. 1986. Membrane damage by haemolytic viruses, toxins, complement and other agents: A common mechanism blocked by divalent cations. *J. Biol. Chem.* **261:**9300–9308
4. Belmonte, G., Cescatti, L., Ferrari, B., Nicolussi, T., Ropele, M., Menestrina, G. 1987. Pore formation by *Staphylococcus aureus* alpha-toxin in lipid bilayers: Dependence upon temperature and toxin concentration. *Eur. Biophys. J.* **14:**349–358
5. Benz, R., Schmid, A., Van der Ley, P., Tommassen, J. 1989. Molecular basis of porin selectivity: Membrane experiments with OmpC-PhoE and OmpF-PhoE hybrid proteins of *Escherichia coli* K-12. *Biochim. Biophys. Acta* **981:**8–14
6. Bhakdi, S., Muhly, M., Füssle, R. 1984. Correlation between toxin binding and hemolytic activity in membrane damage by Staphylococcal α-toxin. *Infect. Immun.* **46:**318–323
7. Bhakdi, S., Tranum-Jensen, J. 1987. Damage to mammalian cells by proteins that form transmembrane pores. *Rev. Physiol. Biochem. Pharmacol.* **107:**147–223
8. Bockris, J.O.M., Reddy, A.K.N. 1970. Modern Electro-Chemistry. Vol. 1. Plenum, New York
9. Forti, S., Menestrina, G. 1989. Staphylococcal α-toxin increases the permeability of lipid vesicles by a cholesterol and pH dependent assembly of oligomeric channels. *Eur. J. Biochem.* **181:**767–773
10. Freer, J.H., Arbuthnott, J.P. 1983. Toxins of *Staphylococcus aureus*. *Pharmacol. Ther.* **19:**55–106
11. Füssle, R., Bhakdi, S., Sziegoleit, A., Tranum-Jensen, J., Kranz, T., Wellensiek, H.J. 1981. On the mechanism of membrane damage by *Staphylococcus aureus* α-toxin. *J. Cell Biol.* **91:**83–94
12. Gray, G.S., Kehoe, M. 1984. Primary sequence of the α-toxin gene from *Staphylococcus aureus* Wood 46. *Infect. Immun.* **46:**615–618
13. Hancock, R.E.W., Schmid, A., Bauer, K., Benz, R. 1986. Role of lysines in ion selectivity of bacterial outer membrane porins. *Biochim. Biophys. Acta* **860:**263–267
14. Hebert, T.E., Fackrell, H.B. 1987. Inhibition of staphylococcal α-toxin by covalent modification of an arginine residue. *Biochim. Biophys. Acta* **916:**419–427
15. Hille, B. 1984. Ionic channels of excitable membranes. Sinauer Associates, Sunderland (MA)
16. Hollecker, M., Creighton, T.E. 1980. Counting integral numbers of amino groups per polypeptide chain. *FEBS Lett.* **119:**187–189
17. Homan, R.J. 1988. Aggregation of IgE receptors induces degranulation in rat basophilic leukemia cells permeabilized with α-toxin from *Staphylococcus aureus*. *Proc. Natl. Acad. Sci. USA* **85:**1624–1628
18. Ikigai, H., Nakae, T. 1987. Assembly of the α-toxin-hexamer of *Staphylococcus aureus* in the liposome membrane. *J. Biol. Chem.* **262:**2156–2160
19. Kendrew, J.C. 1963. Myoglobin and the structure of proteins. *Science* **139:**1259–1266
20. Lindemann, B. 1982. Dependence of ion flow through channels on the density of fixed charges at the channel opening. Voltage control of inverse titration curve. *Biophys. J.* **39:**15–22
21. McEwen, B. F., Arion, W. J. 1985. Permeabilization of rat epthocytes with *Staphylococcus aureus* α-toxin. *J. Cell Biol.* **100:**1922–1929
22. Means, G.E., Feeney, R.E. 1971. Chemical modification of proteins. Holden-Day, San Francisco
23. Menestrina, G. 1986. Ionic channels formed by *Staphylococcus aureus* alpha-toxin: Voltage-dependent inhibition by divalent and trivalent cations. *J. Membrane Biol.***90:**177–190
24. Menestrina, G. 1988. Pore formation by *Staphylococcus aureus* alpha-toxin: A study using planar bilayers. *In*: Bacterial Protein Toxins. F.J. Fehrenbach et al., editors. *Zentralbl. Bakteriol.* Suppl. 17, pp. 295–302. Gustav-Fischer Verlag, Stüttgart

25. Miles, E.W. 1977. Modification of histidyl residues in proteins by diethylpyrocarbonate. *Methods Enzymol.* **47:**431–442
26. Nelson, A.P., MacQuarrie, D.A. 1975. The effects of discrete charge on the electrical properties of a membrane. I. *J. Theor. Biol.* **55:**13–27
27. Pederzolli, C., Cescatti, L., Menestrina, G. 1991. Chemical modification of *Staphylococcus aureus* α-toxin by diethylpyrocarbonate: Role of histidines in its membrane-damaging properties. *J. Membrane Biol.* **119:**41–52
28. Ropele, M., Menestrina, G. 1989. Electrical properties and molecular architecture of the channel formed by *E. coli* hemolysin in planar lipid membranes. *Biochim. Biophys. Acta* **985:**9–18
29. Schultz, S.G. 1980. Basic Principles of Membrane Transport. Cambridge University Press, New York
30. Thelestam, M. 1983. Membrane damage by staphylococcal α-toxin to different types of cultured mammalian cells. *Biochim. Biophys. Acta* **762:**481–488
31. Thelestam, M., Blomqvist, L. 1988. Staphylococcal α-toxin—recent advances. *Toxicon* **26:**51–65
32. Tobkes, N., Wallace, B.A., Bayley, H. 1985. Secondary structure and assembly mechanism of an oligomeric channel protein. *Biochemistry* **24:**1915–1920

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