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Chemical Modification of *Staphylococcus aureus* α-Toxin by Diethylpyrocarbonate: Role of Histidines in Its Membrane-Damaging Properties

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Summary. *Staphylococcus aureus* α-toxin causes cell damage by forming an amphiphilic hexamer that inserts into the cell membrane and generates a hydrophilic pore. To investigate the role of the three histidine residues of this toxin we modified them with diethylpyrocarbonate, obtaining N-carbethoxy-histidine whose appearance may be followed spectrophotometrically. Despite the statistical nature of random chemical modification, it was possible to establish that modification of any one of the three histidines was enough to impair α-toxin activity on red blood cells and platelets. Two out of three histidines were essential for the interaction of the toxin with model membranes such as lipid vesicles and planar bilayers. Loss of lytic activity in both natural and model membranes was due both to defective binding and to defective oligomerization. When α-toxin hexamers inserted into lipid vesicles were assayed for chemical modifiability two histidines per monomer were found to be protected from diethylpyrocarbonate modification, whereas only one was protected after delipidation of the oligomer with a detergent. A possible model for the role of each histidine in the monomer is presented.

Key Words α-toxin · membrane damage · hemolysis · histidine modification · diethylpyrocarbonate · (*Staphylococcus aureus*)

Introduction

Clinical isolates of *Staphylococcus aureus* secrete a cytolytic exotoxin termed α-toxin, a water soluble 33-kDa basic polypeptide [10, 11]. This protein has been implicated in animals as a major virulence factor in staphylococcal infections [19]. It causes erythrocyte lysis in distinct steps: binding to the cell membrane, ion leakage and finally hemoglobin release [7]. Current evidence indicates that α-toxin oligomerizes on the cell surface formin an amphiphilic hexamer that inserts into the lipid bilayer, generating a transmembrane channel responsible for ion leakage [12, 13, 29]. Colloid-osmotic shock follows, leading to lysis of the membrane and eventually to cell death. For recent reviews on this toxin *see* refs. [5, 11, 28].

Consistent with the pore hypothesis, we observed that α-toxin makes channels in model membranes such as unilamellar vesicles and planar lipid membranes and that the aggregation of several toxin monomers is required for the formation of one pore [2, 9]. In an attempt to correlate the structure of this toxin with its function we have now studied the role of histidyl residues on toxin activity by chemically modifying them with the specific reagent diethylpyrocarbonate (DEPC). Interestingly, there are only three histidines per monomer of toxin and they are located in distinct positions: one near the C-terminus, one near the N-terminus and one in the middle of the chain [14].

The identification of single amino acid residues essential for α-toxin activity might facilitate the design of cross-reacting mutant proteins which could be used in the development of vaccines.

Materials and Methods

Chemicals

DEPC[1] (99%) and hydroxylamine-HCl (97%) were supplied from Janssen Chimica; calcein and Sephadex-G50 were obtained through Sigma; SDS and Brij-35 were from Pierce. Triton X-100, hystidine-HCl and imidazole were from Merck; egg PtdCho was supplied by Calbiochem and cholesterol by Fluka; POPtdCho and DPhPtdCho were purchased from Avanti Polar Lipids.

[1] *Abbreviations:* DEPC: diethylpyrocarbonate; PtdCho: phosphatidylcholine; POPtdCho: palmitoyl-oleoyl-phosphatidylcholine; DPhPtdCho: diphytanoyl-phosphatidylcholine; SUV: small unilamellar vesicles; RBC: red blood cells; TLC: thin-layer chromatography; SDS: sodium dodecyl sulfate; Triton X-100: octylphenoxy polyethoxy ethanol; Brij-35: polyoxyethylene lauryl ether

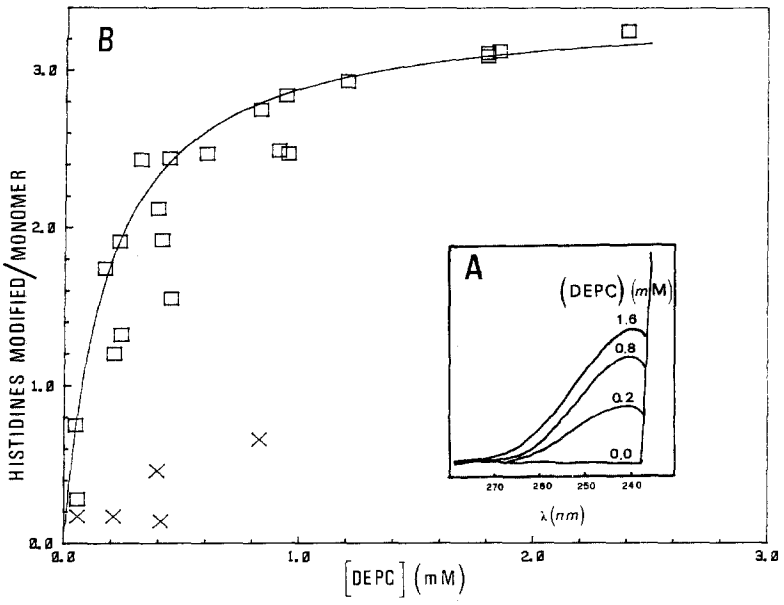

Fig. 1. Chemical modification of α-toxin histidines by DEPC. (*A*) The extent of histidine modification on α-toxin can be followed spectrophotometrically as an increased absorption in the region 230–260 nm, proportional to the amount of DEPC added. No change occurs in the 280 nm region. (*B*) The number of histidines modified per α-toxin molecule (derived from the absorbance at 242 nm using $\varepsilon = 3200\ M^{-1}\ cm^{-1}$ [25]) is reported as a function of DEPC concentration (open squares). The residual number of histidines modified after NH_2OH reconstitution is also given (crosses). The solid line is a simple titration curve assuming the presence of n histidines with identical affinity. Best fit yields an apparent dissociation constant $K_d = 0.18$ mM and $n = 3.4$

α-Toxin Modification

Samples of liophilyzed α-toxin from *Staphylococcus aureus* (kindly supplied by Dr. K. Hungerer, Behring, Marburg, FRG) were used without further purification.

DEPC Modification

Carbethoxylation was performed essentially as described by Miles [25]. DEPC was freshly diluted in anhydrous ethanol prior to each experiment to a nominal concentration of 20–30 mM. Because of hydrolysis of commercial DEPC the actual concentration of each diluted sample was determined by its reaction with free histidine or imidazole.

Carbethoxylation of α-toxin was initiated by adding aliquots of DEPC to a solution of protein in 10 mM phosphate buffer (pH 6.5) plus 125 mM NaCl and 0.2 mM EDTA (Buffer A). Protein concentrations used ranged 0.8 to 1.8 mg/ml (24–54 μM). The concentration of N-carbethoxyl-histidine residues was continuously monitored by difference spectrophotometry, in the range 300–235 nm, against a reference cuvette containing the same amount of toxin and ethanol as the sample; an absorption coefficient of $3.2 \times 10^3\ M^{-1}$ at 240 nm was used [25]. After each DEPC addition the reaction was allowed to proceed to completion (about 5 min at 25°C); thereafter a sample of modified toxin was collected and new DEPC was added. Spectrophotometric measurements were carried out in a Perkin-Elmer 551 spectrophotometer equipped with a temperature-controlled cell holder.

Reversal of DEPC Modification by Hydroxylamine

DEPC-modified toxin samples were incubated with hydroxylamine (at concentration of 0.33 M, pH 7.0) in Buffer A for 10–12 hr at room temperature and in the dark. These samples were then dialyzed against four changes of cold Buffer A plus 90 mM potassium phosphate, and the extent of residual modification was measured as above.

Assay of Toxin Activity on Red Blood Cells

Rabbit red blood cells were prepared as described by Bhakdi et al. [3]: venous blood was mixed with 1% (in volume) Na citrate and washed three times in isotonic buffer (0.85% NaCl, pH 6.0, hereafter called Buffer B).

Hemolytic Activity at Equilibrium

Hemolytic activity was titrated as described by Bhakdi et al. [3]. Samples of α-toxin were serially diluted in a 96-well microtiter plate (each well contained 50 μl Buffer A, and the toxin was twofold diluted at each step). An equal volume of rabbit red blood cells (2.5% (vol/vol) in Buffer B) was added to the wells and titers were read visually after 1 hr incubation at 37°C and expressed as the reciprocal of the last dilution giving >60% hemolysis. Hemolytic activity of native α-toxin was usually about 32,000 HU/mg.

Kinetic of Hemolysis

The time course of red blood cell lysis was followed spectrophotometrically as described by Ikigai and Nakae [16]. Turbidity was monitored at 700 nm in a semimicro quartz cuvette containing 2.5×10^6 cell/ml at 25°C. Initially $A_{700\ nm}$ was about 0.8.

Binding experiments

Toxin-treated erythrocyte membranes were prepared as follows: 500 μl of rabbit erythrocytes in Buffer B (at a cell count of 5×10^9 cell/ml) were mixed with 150 μl Buffer A plus or minus 1

mg/ml of α-toxin. After 1 hr incubation at 37°C the cells were hypotonically lysed in 5 mM Na phosphate and washed four times at 4°C (22,000 × g for 10 min) in this buffer. The final pellet was suspended in 150 μl of a physiological buffer (145 mM NaCl, 5 mM KCl, 1 mM MgCl2, 0.5 mM KH_2PO_4, 0.1 g/ml dextrose, 5 mM HEPES at pH 7.4, hereafter called Buffer C) and analyzed by SDS gel electrophoresis as described below.

Assay of Toxin Activity on Platelets

Bovine or porcine platelets were prepared from venous blood mixed with 1% (in volume) Na citrate, essentially as described by Radomski and Moncada [26]. Platelet-rich plasma was obtained by 20 min centrifugation at 250 × g (21°C). Platelets were then washed once by precipitating the cells at 900 × g for 10 min (21°C) and resuspending the pellet in 1.5 ml of buffer C (prewarmed at 37°C). Disposable plasticware was used throughout.

Typically, we obtained cell concentrations of 3 × 10^8 cell/ml in this way, as counted in a Bürker chamber placed under the objective of a phase-contrast optical microscope (Zeiss).

Platelet Aggregation

α-toxin induced aggregation of platelets was monitored by continuously measuring the absorbance at 450 nm of a platelet sample (concentration 1.5 × 10^7 cell/ml) mixed with 20 μg/ml toxin in a thoroughly stirred, 1-cm semimicro quartz cuvette held at 25°C.

Blood Coagulation

Clotting time was measured as follows: to a sample of whole citrated blood (collected as described above) 10 mM Ca^{2+} plus or minus α-toxin was added; the sample was then incubated at 37°C with stirring, and the time necessary for complete coagulation was determined.

Assay of Toxin Activity on Small Unilamellar Vesicles

Small unilamellar vesicles (SUV) consisting of egg PtdCho: cholesterol (molar ratio 1 : 1) were prepared by sonication of multilamellar liposomes (6 mg lipid/ml suspension) as descrilbed by Forti and Menestrina [9] in a buffer containing 80 mM calcein, 50 mM NaCl and enough NaOh to give pH 7.0. Both lipids were more than 99% pure, giving one spot by TLC.

After sonication SUV were washed in a Sephadex G50 column using an eluting buffer composed of 160 mM NaCl 10 mM HEPES adjusted to pH 7.0 by NaOH (Buffer D). External calcein was removed in this way and SUV appeared in the void volume at a lipid concentration of about 2 mg/ml.

Kinetic of Permeabilization

The assay for α-toxin induced permeabilization of the vesicles was performed as described previously [9, 23], briefly, as follows: aliquots of washed vesicles were introduced into a 1-cm quartz cuvette (continuously stirred at 21°C) containing 2.5 ml of buffer D; the final lipid concentration was typically 4 μg/ml. After mixing with toxin, the release of calcein from the vesicles produced an increase in fluorescence (excitation at 494 nm, emission at 520 nm) due to dequenching of the dye in the external medium. 100% release was determined by adding 0.8

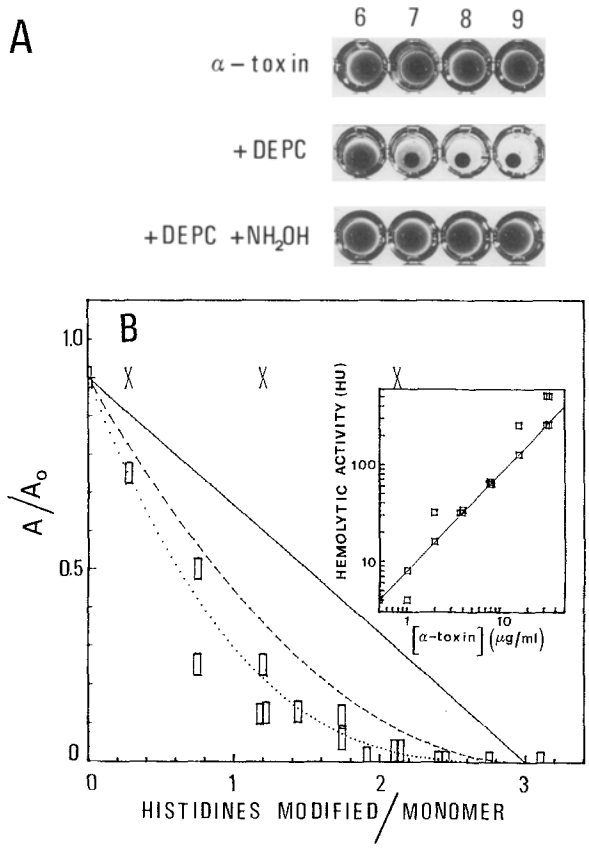

Fig. 2. Hemolytic activity of native and DEPC-modified α-toxin. (*A*) Microplate wells exemplifying the determination of the hemolytic activity. Numbers on top indicate the number of twofold dilutions to which the 1 mg/ml toxin sample was subjected. A diffuse color indicates wells in which hemolysis of rabbit red blood cells was complete. A central black spot indicates wells in which intact red blood cells deposited. *a*: native toxin, *b:* DEPC-modified toxin (2.8 histidines carbethoxylated), *c:* same as *b* but after NH_2OH reconstitution. (*B*) Plot of the residual activity (A/Ao) of α-toxin *vs.* the number of histidines modified by DEPC per toxin monomer (open symbols). A is calculated as 2^n where n is the position of the last well in which hemolysis occurred; Ao was 32,000 HU/mg. The relative activity after treatment with NH_2OH is given by crosses as a function of the number of histidines modified before reconstitution. Smooth lines are the theoretical predictions of Eq. (1) in the text using $n = 3$ and $i = 1$ (solid line), $i = 2$ (dashed line), $i = 3$ (dotted line). *Inset:* Linear dependence of the hemolytic activity on the concentration of native α-toxin administered, in the range 0.5 to 50 μg/ml. The solid line traced has a slope of 1.

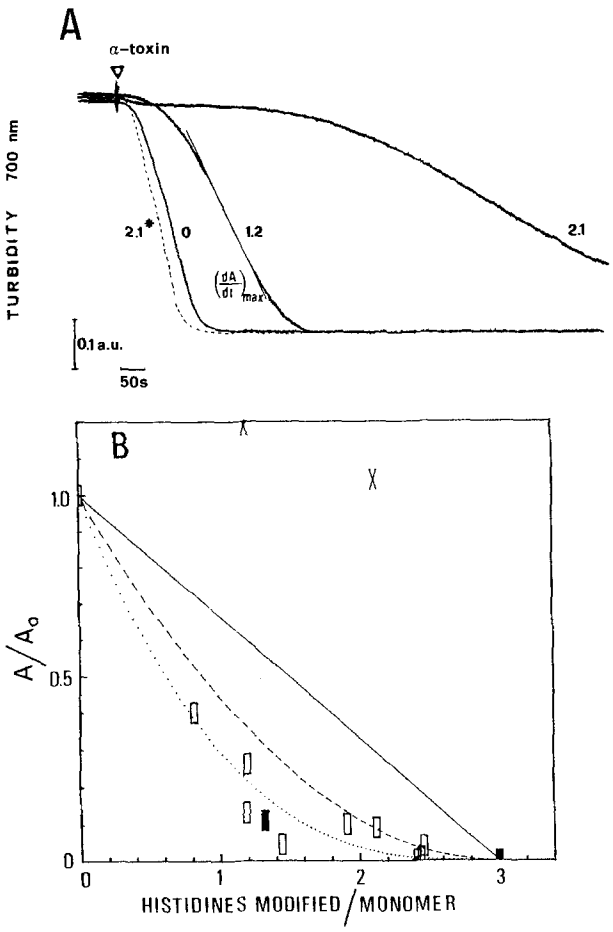

Fig. 3. Kinetic of hemolysis by native and modified α-toxin. (*A*) Cell lysis was measured as a decrease in the absorption at 700 nm; the concentration of rabbit erythrocytes was 2.5×10^6 cell/ml; toxin concentration was 20 μg/ml. Native or DEPC-modified toxin was used (the number of histidines modified per toxin monomer is indicated next to each trace). The dashed trace is for the most modified toxin sample after treatment with NH_2OH (the number of histidines modified before treatment with hydroxylamine is indicated with the asterisk). The maximal slope of the absorbance decrease (taken as indicated) was divided by the initial absorbance to calculate the activity *A* of α-toxin. (*B*) Plot of the residual activity (*A*/*Ao*) of α-toxin *vs*. the number of histidines modified per toxin monomer. RBC were from rabbit (open symbols) or pig (filled symbols); activity of samples reconstituted by NH_2OH on rabbit erythrocytes is indicated by crosses. *Ao* was 18×10^{-3} sec^{-1} for rabbit and 5.7×10^{-3} sec^{-1} for pig red blood cells. Theoretical lines have the same meaning as in Fig. 2*B*

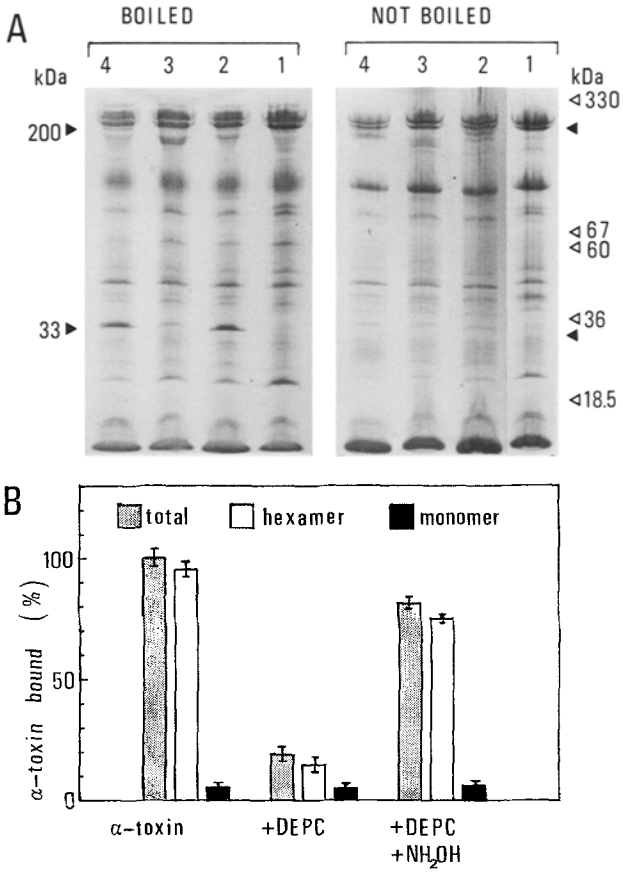

Fig. 4. Binding of native and modified α-toxin to erythrocyte membranes. (*A*) SDS gel electrophoresis of Triton X-100-solubilized hypotonically-lysed rabbit red blood cells exposed to: buffer A, lanes *1*; native α-toxin, lanes *2*; DEPC-modified α-toxin (1.9 histidines modified per toxin monomer), lanes *3*; same toxin as in lanes *3* but after hydroxylamine treatment, lanes *4*. Samples in the left panel were boiled in SDS before being applied to the gel. Open arrowheads mark the position of standard proteins of different molecular weight, filled arrowheads are extrapolated positions of mol wt 33 and 200 kDa corresponding to toxin monomers and hexamers, respectively. (*B*) Histograms showing the amount of α-toxin bound to the erythrocyte membrane obtained by densitometry of the gels in *A* for native, DEPC-modified and NH_2OH-reconstituted α-toxin, respectively. Monomeric toxin, filled bins; hexameric toxin, open bins; total, shaded bins. The total amount of native α-toxin bound is used as 100%

mM Triton X-100. Spontaneous release of calcein was negligible under these conditions.

Binding Experiments

Toxin binding to lipid vesicles was assayed as follows: 100 μl of washed vesicles were mixed with 150 μl of toxin (1 mg/ml in Buffer A) and incubated for 1 hr at 37°C. Unbound toxin was removed by twofold filtration over Amicon filters YMT100 (cut off at mol wt 100 kDa) mounted in an Amicon MPS-1 micropartition system (20 min centrifugation at 1000 × *g*). The final semi-dry retentate was resuspended in buffer D to a final volume of 70 μl. The filtrate was collected and concentrated to the same volume by about 30 min centrifugation at 5000 × *g* in Centricon 10 microconcentrators (equipped with Amicon PM10 membranes with cut-off 10 kDa).

Retentate and filtrate were then subjected to SDS gel electrophoresis as described below. In control experiments to assess the selectivity of the filters, we estimated by densitometry of the gels that more than 95% of a sample of monomeric α-toxin (in the

absence of lipid) crossed the YMT100 membrane whereas more than 95% was retained by the PM10 membrane.

Preparation of Planar Lipid Bilayers and Assay of Toxin Activity

Planar lipid bilayers were prepared by the apposition of two monolayers on a 0.2-nm hole in a thin Teflon septum separating two buffered salt solutions as described earlier [2]. The monolayers were spread using either palmitoyl-oleoylphosphatidylcholine (POPtdCho) or di-phytanoyl-phosphatidylcholine (DPhPtdCho) dissolved at 10 mg/ml in *n*-hexane.

S. aureus α-toxin was added to the *cis* compartment of a preformed stable bilayer, and the current flowing through the membrane, under voltage-clamp conditions, was sent to an *I-V* converter (built around a virtual grounded AD515K operational amplifier). The *cis* compartment was connected to the virtual ground, and voltage signs refer to it; current is defined positive when cations flow into this compartment. Baseline conductance of the membranes did not exceed 50 pS. Ag-AgCl electrodes were used. Bathing solutions, 4 ml of Buffer A on each side, were kept at room temperature.

Polyacrylamide Gel Electrophoresis

SDS gel electrophoresis was performed according to Laemmli [20] on precast polyacrylamide minigels (Pharmacia). We used gradient gels with densities ranging from 10 to 15%. Electrophoresis was run at 10°C in buffer containing a 0.5% SDS. Gels were stained with Coomassie brilliant blue or with silver stain, and the amount of protein was quantitated by total densitometry with a Shimadzu CS-930 densitometer (at 600 nm). All protein samples were made 0.8% in SDS before running on the gel; in the case of toxin that had been incubated with lipid vesicles or erythrocytes, 1 mM Triton X-100 was also included to solubilize the lipid matrix.

Results

Characterization of the Chemical Modification of α-Toxin by DEPC

DEPC reacts primarily with the imidazole of histidines on proteins, but simultaneous modification of other residues cannot be excluded *a priori* [25] and has to be verified.

The extent of histidine modification can be followed spectrophotometrically as an increased absorption in the region 230–260 nm (Fig. 1*A*). The number of histidines modified per toxin molecule can thus be calculated (Fig. 1*B*). As expected, modification saturates (with large amounts of DEPC) around the value of three histidines per α-toxin monomer, which is the reported histidine content of α-toxin [14]. Though histidine carbethoxylation is

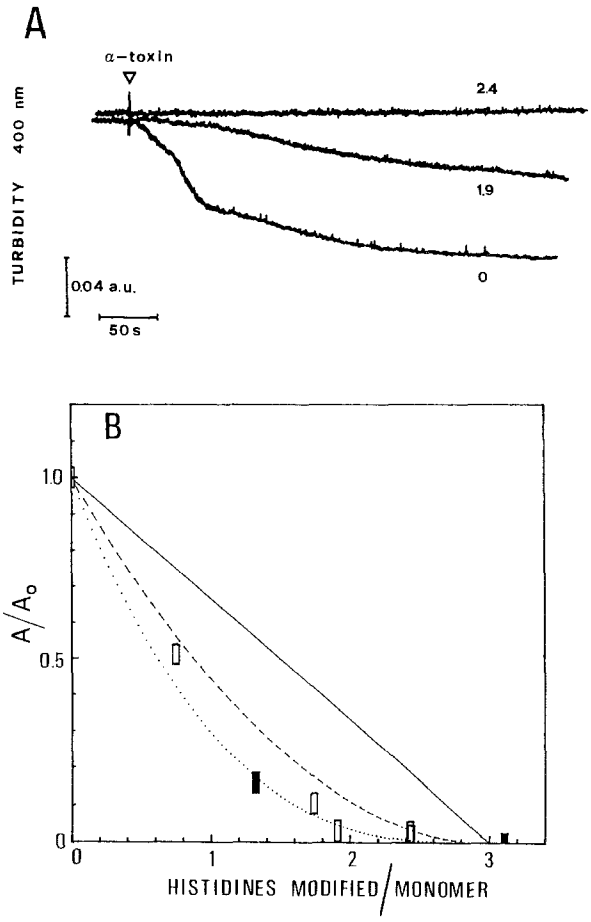

Fig. 5. Inhibition of α-toxin-induced platelet aggregation by DEPC treatment. (*A*) Cell aggregation was measured as a decrease in the turbidity at 400 nm; bovine platelet concentration was 1.5×10^7 cell/ml; toxin concentration was 20 μg/ml. Native or DEPC-modified toxin was used, and the number of histidines modified per toxin monomer is indicated next to each trace as in Fig. 3*A*. (*B*) Plot of the residual activity (A/Ao) of α-toxin *vs.* the number of DEPC-modified histidines per toxin monomer obtained as in Fig. 3. Platelets used were bovine (open symbols) or porcine (filled symbols). Ao was 7×10^{-3} sec^{-1} for bovine and 2.5×10^{-3} sec^{-1} for porcine platelets. Theoretical lines have the same meaning as in Fig. 2*B*

practically irreversible in this buffer, the reaction reaches an apparent equilibrium because of competing hydrolysis. Accordingly, we can introduce an apparent dissociation constant K_d. Data fitted adequately well to a single titration curve with $K_d = 1.8$ mM (which corresponds in these experiments to a DEPC/α-toxin molar ratio of about 6), indicating that the three histidines have similar reactivity and accessibility. The apparent K_d is introduced for the sake of comparing modifications run under different experimental conditions. In agreement with that reported previously [25], we found that histidine car-

Table. Effect of native and DEPC-modified α-toxin on blood clotting time[a]

Source of blood	Toxin	No. modified histidines	t_0 (sec)	t/t_0	No. experiments
Bovine	−		310 ± 80	1.00 ± 0.25	4
	+	0		0.47 ± 0.04	7
	+	0.75		0.59 ± 0.06	3
	+	1.74		0.77 ± 0.25	3
	+	2.43		0.83 ± 0.12	5
Human	−		460 ± 40	1.00 ± 0.09	3
	+	0		0.67 ± 0.09	5
	+	2.75		0.96 ± 0.12	3
	+	3.00		1.09 ± 0.07	3
	+	2.75[b]		0.60 ± 0.05	3

[a] Citrated human or bovine blood was recalcified with 15 or 10 mM Ca^{2+}, respectively, and clotting time in the absence of α-toxin, indicated as t_0, was measured (mean ± SEM). Clotting time in the presence of 20 μg/ml α-toxin at various stages of modification, called t, was used to calculate the ratio t/t_0 reported (mean ± SEM). The number of histidines modified per toxin monomer was calculated as shown in Fig. 1 for each sample.

[b] Sample treated with hydroxylamine; the number of modified histidines per toxin monomer prior to treatment with NH_2OH is given. After treatment this was reduced to 0.65.

bethoxylation was largely reversed by hydroxylamine (Fig. 1*B*).

Other amino acids (tyrosine, tryptophan, cystein and lysine [18, 24, 25]) can also be modified by DEPC though less specifically. We have found that only lysine residues are modified in parallel to histidines in the case of α-toxin. In fact, while cystein residues are absent in this toxin [14], modification of tyrosine is ruled out because there is no decrease in the absorbance at 280 nm (Fig. 1*A*) and tryptophan modification is excluded because no change [24] in the emission fluorescence spectrum between 300 and 400 nm is observed (*data not shown*).

Modification of lysyl residues was evidenced by the appearance in native-PAGE at pH 8.8 of additional bands, besides that of native toxin, indicating the presence of toxin with lower net charge (companion paper [8]). This was what we expected because carbethoxylation removes the lysine positive charge. Histidyl residues do not contribute because they are uncharged anyway at this pH. Accordingly, we found that the pattern of these bands was unaffected by extensive treatment with hydroxylamine [8], in agreement with the notion that DEPC modification of lysyl residues is irreversible [25].

Thus, treatment with hydroxylamine provided a unique way of unambiguously distinguishing between the effects due to histidine modification (which were fully reversed by hydroxylamine) and those due to lysine modification (which were completely irreversible). Based on this distinction we separated the effects of histidine modification on toxin activity, which will be described in the following, and those of lysine modification which will be described elsewhere [8].

Histidine Modification with DEPC Reduces the Activity of α-Toxin on Blood Cells

Red Blood Cells

DEPC modification of α-toxin results in a loss of hemolytic activity measured in a microtiter plate assay (Fig. 2*A*), which is a simple and effective test of the concentration of active toxin present (*see* insert in Fig. 2*B*).

The residual hemolytic activity of α-toxin, (i.e., the ratio between the activity of the modified toxin, A, and that of the native toxin, Ao) is a function of the number of histidines modified per monomer (Fig. 2*B*). As discussed later, this plot gives information on the number of residues necessary for activity. Hemoliticity is completely restored by reversing DEPC modification with hydroxylamine.

As a complementary approach, we measured spectrophotometrically the kinetics of hemolysis (Fig. 3*A*). DEPC treatment considerable increases the lag time before the onset of hemolysis and in addition reduces its rate. Both these effects are re-

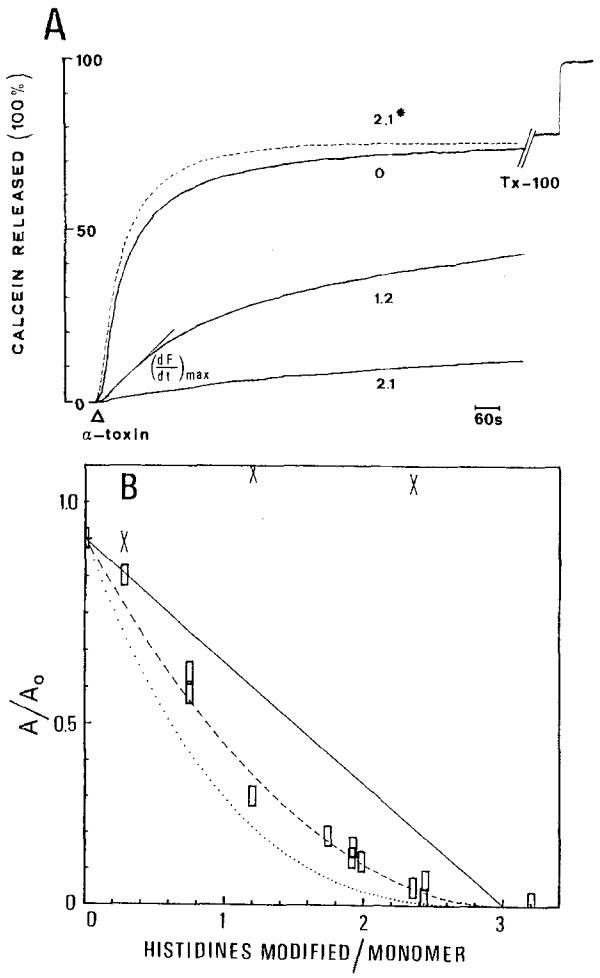

Fig. 6. Inhibition by DEPC of α-toxin-induced permeabilization of PtdCho/cholesterol (1 : 1) small unilamellar vesicles. (*A*) Permeabilization of calcein-loaded SUV was measured as an increase in the fluorescence at 520 nm (excitation was at 490 nm) due to calcein release. Toxin concentration was 20 μg/ml. Native or DEPC-modified toxin was used (the number of histidines modified is indicated as in Fig. 3*A*). The dashed trace is for the most modified toxin sample after reconstitution with NH_2OH. The maximal slope of the fluorescence increase (as indicated) divided by the fluorescence value for 100% release (obtained by addition of the detergent) is used to calculate the activity *A* of α-toxin. (*B*) Plot of the residual activity (*A*/*Ao*) of α-toxin on lipid vesicles *vs.* the number of histidines modified by DEPC per toxin monomer. Activity of samples reconstituted by NH_2OH is indicated by crosses. *Ao* ranged from 2 to 9 $\times$ 10^3 sec^{-1} for different vesicle preparations. Theoretical lines are the same as in Fig. 2*B*

versible upon treatment of the modified samples with hydroxylamine.

As demonstrated by Ikigai and Nakae [16], the rate of hemolysis is a sensitive test of toxin activity, and accordingly we used it to plot the residual α-toxin activity *vs.* the number of modified histidines per monomer (Fig. 3*B*).

It is well documented that α-toxin-induced lysis of red blood cells is due to the assembly of an hexameric toxin aggregate onto their membrane [7, 12, 13, 17, 29]. The binding step occurs in monomeric form at an earlier stage [6, 7, 27]. In order to distinquish whether the reduced hemolytic activity of histidine-modified α-toxin is due to a defective binding of the toxin to the cells or to a defective hexamerization once it is bound, we have investigated quantitatively both binding and oligomerization (Fig.4).

Rabbit erythrocrytes were incubated plus or minus α-toxin for 1 hr at 37°C and then hypotonically lysed and washed free of unbound toxin by centrifugation. Packed cell membranes were solubilized by Triton X-100 and divided in two aliquots, one boiled in SDS the other kept on ice. Membrane proteins of these samples were analyzed by SDS-PAGE (Fig. 4*A*).

As already shown by Füssle et al. [13], the presence of monomeric α-toxin among the other intrinsic membrane proteins of red blood cells is readily recognized. It is less easy to detect toxin hexamers because they are masked by endogenous spectrin. Anyhow, the presence of the hexamer may be indirectly determined by the increased density of the monomeric α-toxin band when the samples are boiled in SDS, a procedure which quantitatively reconverts hexamers into monomers [13]. Densitometry of each monomeric toxin band then allows us to derive the histogram in Fig. 4*B*, which shows that the total amount of toxin bound to erythrocytes is strongly reduced by histidine modification, suggesting that binding is inhibited. On the other hand, the amount of hexamer bound decreases much more than the amount of monomer bound (which is in fact relatively constant), suggesting that also the oligomerization step is inhibited. It is not likely that inhibition of the aggregation step alone could be enough to block overall binding because it has been shown that at a temperature around 0°C α-toxin firmly and quantitatively binds to red blood cells in monomeric form [27] even if hexamers are not assembled. Thus our data indicate that both the binding step and oligomerization are actually inhibited. It is noteworthy that this inhibition is almost completely reversed upon hydroxylamine treatment.

Platelets

Human platelets are much more sensitive than human red blood cells to α-toxin [4] (they approach the sensitivity of rabbit erythrocytes which is 100 to 1000 times that of human erythrocytes [21]). α-toxin elicits platelet aggregation, thus promoting blood coagulation [4]. We were thus prompted to investigate

the effects of native and DEPC-modified α-toxin also on platelets. Figure 5*A* shows that toxin-induced aggregation of bovine platelets is inhibited by DEPC treatment: the lag time is increased and the time course is slowed. As for hemolysis, the rate of this reaction can be used to calculate the residual activity of the toxin plotted in Fig. 5*B*.

The effect of histidine modification also becomes clear when the ability of α-toxin to accelerate the coagulation (induced by re-calcification) of whole citrated blood is considered (Table). Native α-toxin markedly reduces the clotting-time, whereas modified α-toxin loses this property. Treatment with hydroxylamine restores the aggregating properties of modified α-toxin.

Histidine Modification with DEPC Reduces the Activity of α-Toxin on Model Membranes

To try to discriminate the effects of α-toxin modification on its interaction with the protein component and with the lipid component of the cell, we applied it to simpler, purely lipidic systems such as vesicles and planar bilayers.

Lipid Vesicles

Small unilamellar vesicles composed of a 1 : 1 mixture of cholesterol and PtdCho, release their internal content very quickly when exposed to native α-toxin [9, 31]. This effect is considerably inhibited by DEPC modification, which reduces the rate of permeabilization (Fig. 6*A*). As for hemolysis, the permeabilizing effect is completely recovered when the histidine groups are reconverted to the native state by NH_2OH. As a matter of fact, both in the case of vesicle permeabilization and of erythrocyte lysis the activity of NH_2OH reconstituted samples is actually somewhat larger than controls (Figs. 3 and 6). We have checked that NH_2OH-treatment of unmodified toxin (under otherwise the same conditions) does not increase toxin activity both on red blood cells and on lipid vesicles, but rather reduces it slightly. The increased activity observed is thus probably due to the change in electrical charge of the reconstituted toxin due to the effects on lysyl residues [8].

By analogy with blood cell, we used the maximal rate of permeabilization as a measure of the residual activity of α-toxin after DEPC treatment and we plotted it *vs.* the number of modified histidines per toxin monomer (Fig. 6*B*).

To distinguish whether the reduced permeabilization of lipid vesicles is due to defective binding, to defective hexamerization of the toxin once it is bound, or to both, we have quantitatively investigated binding and oligomerization also on this model system (Fig. 7).

PtdCho/cholesterol (1 : 1) lipid vesicles were incubated with α-toxin at different stages of histidine modification and then separated from unbound toxin by a microseparation system. The presence of toxin in the vesicle phase (bound toxin) and in the water phase (unbound toxin) was then analyzed by SDS gel electrophoresis (Fig. 7*A*). Histidine modification reduces the amount of toxin bound to the vesicles both in the form of monomers and in the form of hexamers. The amount of unbound toxin, which is present solely in the form of monomers, is increased correspondingly. All these effects are reversible upon treatment with hydroxylamine.

Quantitation by densitometry of such gels is shown in Fig. 7*B*. As for red blood cells, bound toxin is reduced by histidine modification, suggesting that binding is inhibited; however, bound hexamer is reduced much more rapidly than bound monomer, indicating once again that oligomerization is also inhibited.

This result was confirmed by investigating the effect of DEPC treatment on the spontaneous oligomerization that α-toxin undergoes in a water solution when subjected to mild heating [1]. We observed that indeed spontaneous hexamerization is also reduced by DEPC treatment in a hydroxylamine-reversible way (Fig. 7*C*).

Planar Bilayers

α-toxin forms ionic channels in planar lipid bilayers composed of pure phosphatidylcholine when administered through the water phase [2, 22]. This effect, revealed by the appearance of discrete increments of the ionic current flowing through the bilayer clamped at +40 mV, is also inhibited by DEPC treatment (Fig. 8*A*). Chemical modification decreases the rate of formation of pores and diminishes their conductance (i.e., the amplitude of the current jumps). The first effect is reversible with NH_2OH treatment but the latter is not. This suggests that reduction of the pore conductance is due to modification of lysyl residues and thus will be discussed in a separate paper [8].

In this case, too, we used the maximal rate of pore opening across the bilayer as an indication of the residual activity of α-toxin, and we plotted it as a function of the number of histidines modified per monomer of toxin (Fig. 8*B*).

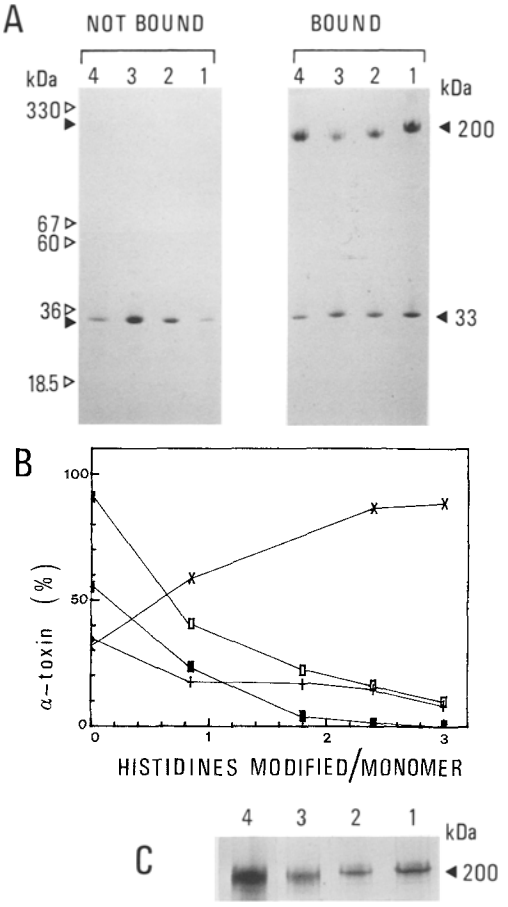

Fig. 7. Effects of DEPC modification on the binding of α-toxin to PtdCho/cholesterol vesicles. (*A*) SUV composed of PtdCho/cholesterol in a 1 : 1 molar ratio were exposed to the action of a fixed amount of native, DEPC-modified or NH_2OH reconstituted α-toxin. After 1 hr incubation at 37°C the SUV were separated from unbound toxin by filtration over Amicon filters (cut off at 100 kDa), and the content of α-toxin in the retentate (bound toxin panel) and the filtrate (unbound toxin panel) was assayed by SDS gel electrophoresis. Lanes *1*, native α-toxin; lanes *2* and *3*, DEPC-treated α-toxin, with 1.2 and 2.3 histidines modified per toxin monomer, respectively; lanes *4*, same toxin samples as lanes *2* but after hydroxylamine treatment. Molecular weights indicated by arrows were obtained as in Fig. 4*A*. (*B*) Densitometry of a gel as in *A* allowed independent calculation of the amount of toxin bound to the lipid vesicles in the form of hexamers (■), toxin bound in the form of monomers (+), and toxin unbound (×). These three quantities are shown in the figure together with total amount of toxin bound (□), as a function of the extent of DEPC modification. The average amount of toxin recovered (bound + unbound) is used as 100%. (*C*) Heat-induced hexamerization of native, DEPC-modified or NH_2OH reconstituted α-toxin in the water phase. After 1 hr incubation at 60°C toxin samples were separated on a gel which was then silver-stained. Only the bands corresponding to the hexamer are shown; they constitue a minor part (at most 4%) of the toxin applied. Lane *1*, native α-toxin; lanes *2* and *3*, DEPC-treated α-toxin, modified on 0.3 and 2.3 histidines per toxin monomer, respectively; lane *4*, same toxin sample as in lane *3* but after hydroxylamine treatment. Molecular weight indicated by the arrow was obtained as in Fig. 4*A*

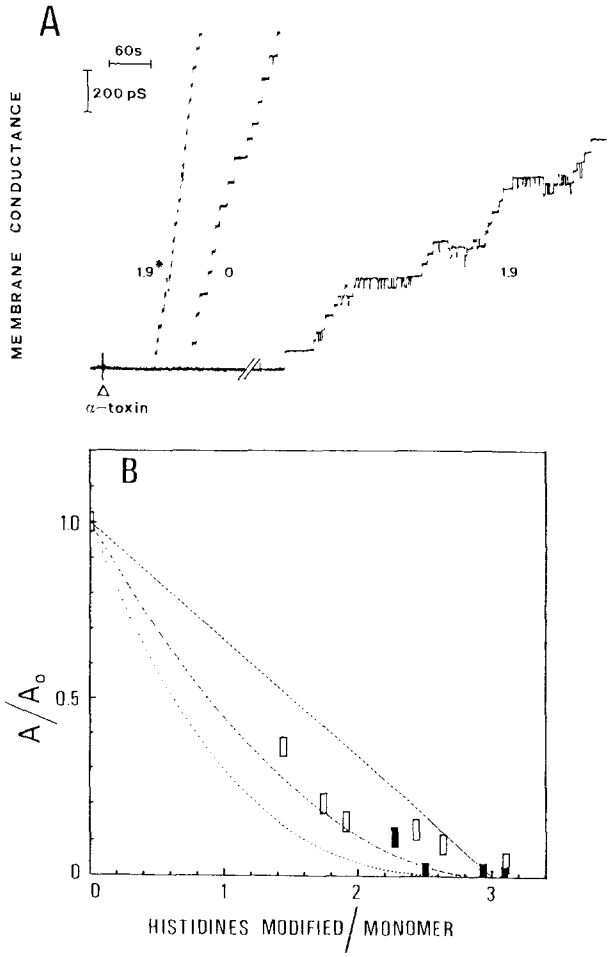

Fig. 8. Channel formation in planar lipid membranes by native and DEPC-modified α-toxin. (*A*) POPtdCho membranes clamped at +40 mV were exposed to α-toxin either native, or DEPC-treated, or the same but reconstituted with NH_2OH (trace with the asterisk). The number of histidines modified per toxin monomer is indicated next to each trace as in Fig. 3*A* (the asterisk indicates the number of histidines modified before hydroxylamine treatment). 1.25 μg/ml of toxin were given where indicated. Discrete current jumps indicate opening and occasionally closing of single ion channels. The single channel conductance G can be evaluated from these steps. We get $G = 123 \pm 18$ pS (mean ± SD) for native toxin, and $G = 70 \pm 13$ pS and 82 ± 13 pS for modified toxin before and after NH_2OH treatment, respectively (the number of steps used are 35, 58 and 48 in the same order). The rate of pore formation can also be evaluated directly from these traces. We have used it to calculate the specific toxin activity, A, by dividing it by the concentration of toxin administered. Calibration bars are the same for all traces. A double bar in the experiment with DEPC-modified toxin indicates an interruption of 3 min during which the trace was flat. (*B*) Plot of the residual activity (A/Ao) of α-toxin on planar lipid membranes *vs.* the number of histidines modified by DEPC per toxin monomer. Each symbol represents the average of one to three independent determinations on different membranes. Ao was 0.8×10^{3} pores $sec^{-1}/\mu g\ ml^{-1}$ for DPhPtdCho (open symbols) and 0.06×10^{3} pores $sec^{-1}/\mu g\ ml^{-1}$ for POPtdCho (filled symbols). Theoretical lines are the same as in Fig. 2*B*

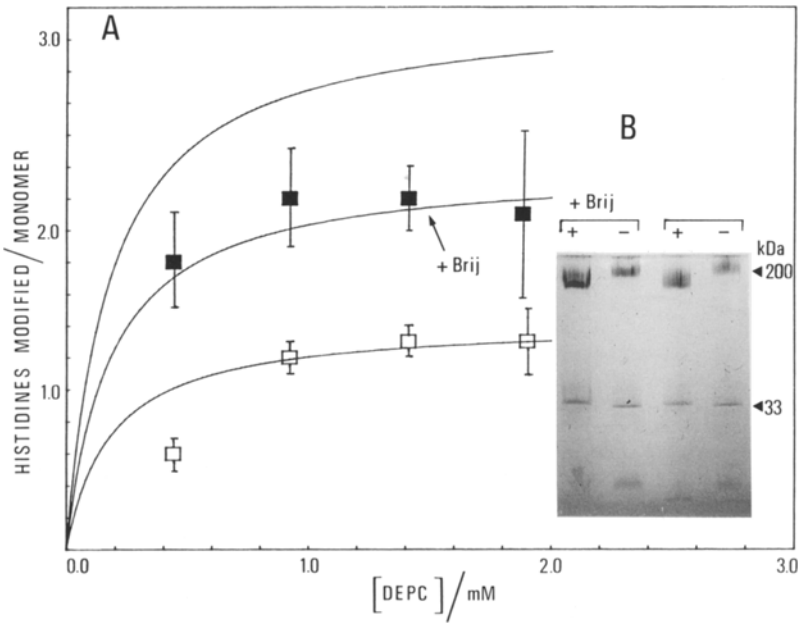

Fig. 9. Chemical modification of preformed α-toxin hexamers by DEPC. (*A*) The extent of DEPC modification of α-toxin preincubated with lipid vesicles was followed as in Fig. 1*A*, and the number of histidines modified per α-toxin molecule was derived. Filled symbols were obtained adding 1 mM Brij-35 before DEPC. Upper solid line is the same titration curve as in Fig. 1*B*, whereas lower and middle curves were obtained using the same apparent K_d but a maximum number of 1.4 and 2.4 histidines modifiable per toxin monomer, respectively. (*B*) SDS gel electrophoresis of α-toxin which was preincubated (1 hr at 37°C) with lipid vesicles, and then either modified by addition of 1.85 mM DEPC (+) or not (−). In the two left lanes vesicles were solubilized by 1 mM Brij-35 after incubation with the toxin but before DEPC addition. Molecular weights indicated by arrows were obtained as in Fig. 4*A*

Chemical Modification by DEPC of Preformed α-Toxin Hexamers

The finding that DEPC modification of α-toxin histidines may prevent its binding to lipid vesicles and its later oligomerization suggests that histidines may have a role in both these steps. As a consequence, one might expect that toxin aggregates preassembled on lipid vesicles are less prone to histidine modification by DEPC than free monomers, because the histidyl residues involved in binding and aggregation are hidden into the lipid matrix and/or into the oligomer.

We investigated this possibility by carbethoxylating (as in Fig. 1) α-toxin which had been preincubated 1 hr at 37°C with PtdCho : cholesterol vesicles (Fig. 9). Under these conditions most of the toxin is in the hexameric form (*see* Fig. 9*B*). The number of modified histidines per monomer in this case saturated at around 1.4 (Fig. 9*A*), which is consistent, after correcting for the amount of unbound monomeric toxin still present (less than 10%), with only one histidine being modified per monomer. When the oligomers were delipidated with a detergent before DEPC addition about one more histidine per monomer was modified, indicating that it became accessible to the reagent.

SDS gel electrophoresis of lipid vesicles incubated with α-toxin plus or minus DEPC in excess (Fig. 9*B*), indicates that preformed hexamers are resistant to this chemical treatment, i.e., are not converted to monomers, even when delipidated with a detergent. DEPC-treated hexamers, however, run a bit faster than controls, and this may be attributed to the modification of a few accessible lysine residues as we discuss in a separate paper [8].

Discussion

α-toxin is a 293-residue polypeptide chain lacking disulphide bridges [14] with only three histidines located in position 35, 144, and 259, i.e., near the N-terminus, in the middle of the chain and near the C-terminus, respectively.

Reaction of α-toxin with the specific histidyl modifier DEPC decreases its lytic activity both on cells (Figs. 2–5) and on model membranes (Figs. 6–8). Because hydroxylamine reactivates the toxin, the loss of activity may be correlated with the modification of histidyl residues [25].

The average number of modified histidine residues per monomer of α-toxin can be determined from the absorbance change at 240 nm [25] (Fig. 1). When the residual activity of the toxin is plotted *vs.* the number of residues modified per monomer, it is evident that carbethoxylation of all three histidines is necessary for complete inactivation of the toxin (Figs. 2, 3, 5, 6, 8).

However, it is known that this method does not, in general, give the number of residues which are really essential for activity [15, 30]. Therefore, we used the statistical method of Tsou [30] to derive that number. In its simplest form this method as-

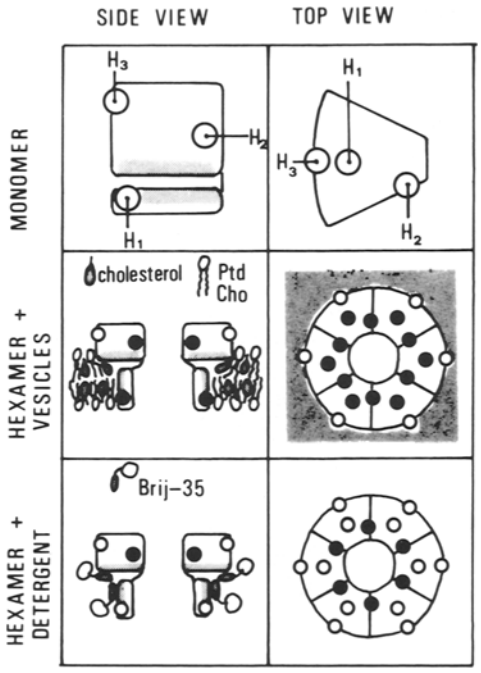

Fig. 10. Tentative model for the interaction of α-toxin with amphiphilic molecules. *Upper panels:* top and side views of the water-soluble α-toxin monomer. As proposed in [29] a hydrophobic surface (shaded area) is cryptic and becomes exposed only when the protein interacts with amphiphilic molecules (*see* lower panels). The three histidine residues are indicated. *Middle panels:* top and side views of the hexameric aggregate assembled in a PtdCho/cholesterol (1 : 1) bilayer membrane. The hydrophobic surfaces are exposed to the interior of the lipid film. A hydrophilic pore is thus formed through the membrane. As indicated in [13] the pore protrudes into the water phase and has a ring shape when seen from the top (the plane of the membrane has been shaded in the top view). Two histidines per monomer (filled circles) become inaccessible to DEPC added in the water phase. One of these (the one in the lipid binding site) is shown to interact with a PtdCho head group (as suggested in [31]). The other is involved in the hexamerization. *Lower panels:* top and side views of the hexameric aggregate delipidated by the detergent Brij-35. The hydrophobic region of this amphiphile is a 12-carbon chain which could leave the lipid binding site exposed to the solution. Only one histidine per monomer (filled circle) is protected from DEPC modification in this case, i.e., the one involved in hexamerization

sumes that all histidine residues are equally reactive, which is consistent with the results in Fig. 1 in our case. Accordingly, one can write

$$m = n\,(1-(A/Ao)^{1/i}) \qquad (1)$$

where n is the number of modifiable residues, m the number of residues modified at a given stage, i the number of essential residues, A and Ao the residual and the original activity, respectively.

Comparison of the experimental results in Figs. 2*B*, 3*B*, and 5*B* with the predictions of Eq. (1) indicates that modification of even one of the three histidines of α-toxin is enough to impair its activity on blood cells, i.e., $i = 3$.

However, when model membranes comprised of pure lipids are considered (Figs. 6*B* and 8*B*) a best fit is obtained with $i = 2$. This indicates that only two histidines are essential in such simpler systems. Since loss of activity on model membranes is due to defective binding but also to a defective oligomerization (*see* Fig. 7), it is tempting to suggest that one of the two essential histidines is located in, or near, a toxin site necessary for binding to lipids, whereas the other is located in, or near, a site involved in intermolecular bonding which becomes active during oligomerization.

This idea is consistent with the results in Fig. 9 which show that in toxin hexamers inserted into a lipid bilayer, two histidines per monomer become inaccessible to DEPC modification. In our model these two histidines (which we will call H1 and H2) would be those involved in lipid binding and in oligomerization. These residues would become protected by lipid molecules and by toxin molecules, respectively. In the mean time, one residue, in this model the nonessential one (hereafter called H3), is still fully accessible. When the same hexamers are delipidated by a detergent, one more residue (conceivably that involved in lipid binding) becomes modifiable. A cartoon depicting this tentative model is shown in Fig. 10.

We have no experimental indication of which of the three α-toxin histidines are actually H1, H2 and H3. However, since His_{259} is located in a highly hydrophilic region of the molecule [14] we suggest that this is the residue not involved in the formation of the lipid-bound hexamer, i.e., it is H3, the residue which remains always accessible to DEPC.

The fact that all three histidine residues appear to be essential for the activity of α-toxin on blood cells further suggests that H3 may still have a role in the interaction of the toxin with a component of such cells which is absent from model membranes. This component could be a protein receptor which facilitates, at an early stage, the interaction of the toxin with the surface of the cellular membrane. The inhibition of such an early step is probably reflected in the huge increase in the lag time before the onset of hemolysis which is introduced by DEPC treatment (Fig. 4) and is not observed in the permeabilization of lipid vesicles (Fig. 7).

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