Partial C-terminal Unfolding Is Required for Channel Formation by Staphylococcal \( \alpha \)-toxin*

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The pore-forming \( \alpha \)-toxin from Staphylococcus aureus is secreted as a soluble monomeric protein. In order to form a transmembrane channel, the protein has to undergo oligomerization and membrane insertion. Previous studies have shown that channel formation is favored by acidic pH. We have analyzed the effect of pH on the kinetics of channel formation as well as on the conformation of the toxin. Using a variety of spectroscopic probes for protein structure, we have shown that \( \alpha \)-toxin unfolded upon acidification and that the unfolding process occurred in at least three steps. The various steps could be selectively affected by modifying the salt concentration or the temperature. This unfolding was, however, only partial as the secondary structure remained native-like as witnessed by far UV CD measurements. The first unfolding step, corresponding to a region of the C-terminal half of the toxin, is of particular importance as it coincided with the exposure of hydrophobic patches on the surface of the protein as well as with the onset of channel formation. Our observations strongly suggest that transition of the C-terminal half of \( \alpha \)-toxin to a molten globule-like state is required for channel formation.

\( \alpha \)-Toxin is a 33-kDa protein secreted by most Staphylococcus aureus strains and is thought to be largely responsible for the pathogenesis of this bacterium in mammals (for review, see Refs. 1 and 2). The toxin has been shown to irreversibly damage the membranes of a great variety of cells including erythrocytes from different species (3), human platelets, endothelial cells, and mouse adrenocortical Y1 cells.

\( \alpha \)-Toxin is secreted as a soluble monomeric polypeptide that can bind to yet unidentified receptors on the surface of sensitive cells (2, 4). Lateral diffusion of membrane-bound monomers leads to the formation of non-lytic heptameric pore precursors that are thought to undergo a second conformational change in order to form the functional transmembrane channel (5, 6).

Since no crystal structure of \( \alpha \)-toxin has yet been obtained, the available structural information is derived from biochemical and biophysical studies. The protein is mainly composed of \( \beta \)-sheet as shown by circular dichroism (5, 7) and is in agreement with secondary structure predictions (8). Conformational analysis by limited proteolysis has suggested that the \( \alpha \)-toxin monomer is composed of two domains, corresponding roughly to the two halves of the protein, separated by a protease-sensitive, glycine-rich loop (5, 9–11). This hypothesis was reinforced by Bayley and co-workers (12) who have shown, by in vitro fragment complementation experiments, that cotranslation of the N- and C-terminal halves of the toxin leads to a product with significant hemolytic activity.

Very little is known about the mechanism by which \( \alpha \)-toxin inserts into a membrane. It has been shown that insertion requires proper oligomerization (13), that the glycine-rich loop penetrates into the bilayer (14) and lines the lumen of the final channel (15–18), and that channel formation is favored at acidic pH (19, 20). In this paper we have analyzed in detail the kinetics of channel formation by \( \alpha \)-toxin in artificial liposomes upon acidification. We show that the toxin is very sensitive to the local pH at the surface of the membrane rather than to the bulk pH. Finally, we have made use of these pH effects to study the structure of \( \alpha \)-toxin. Our data provide new insights into the multidomain organization of \( \alpha \)-toxin and suggest that the protein must partially unfold for channel formation to occur.

EXPERIMENTAL PROCEDURES

Protein Purification—\( \alpha \)-Toxin was produced by strain Wood 46 and purified as described previously (21). The protein concentration was determined by measuring the absorption at 280 nm, based on an optical density of 1.8 for a 1 mg/ml solution.

Preparation of Liposomes—Large unilamellar liposomes were prepared by reverse phase evaporation as described by Szoka and Papahadjopoulos (22, 23). Liposomes were prepared of either 95% egg phosphatidylcholine (EPC)† and 5% egg phosphatidic acid (EPA) or 100% egg phosphatidylglycerol (EPG) or of a mixture of EPC and EPG (1:1, w/w) in a buffer containing 100 mM KCl, 20 mM HEPES, pH 7.4, and 1.5 mg/ml of 6-methoxy-N-(3-sulfopropyl)quinolinium (SPQ).

Chloride Efflux Measurements—All fluorescence experiments were carried out using a PTI spectrophotometer operating in ratio mode with a spectral bandwidth of 5 nm for excitation and emission. The dye was excited at 350 nm and emission was recorded at 422 nm. Experiments were performed at 25 °C under continuous stirring. Liposomes were diluted to a final concentration of 50 μg/ml in a solution containing 100 mM KNO3 and 20 mM buffer at the desired pH. A phosphatecitric acid buffer was used between pH 3 and 3.5, MES between pH 4 and 6, and HEPES between pH 6.5 and 7. \( \alpha \)-Toxin was added to a final concentration of 80 ng/ml.

Estimation of the pH Near the Surface of Liposomes—The measurements of the surface potential and the calculation of the corresponding pH value were performed using the fluorescent probe 2-(p-toluidyl)-naphthalene-6-sulfonic acid (TNS) as described previously (24, 25).

Circular Dichroism—Circular dichroic experiments were carried out at room temperature on a Jasco 610 spectrometer. Quartz cells of 0.01- and 1-cm path length were used for measurements in the far (190–240 nm) and near (250–350 nm) UV spectra, respectively. The protein was diluted to a concentration of 0.66 and 0.44 mg/ml for measurements in

† The abbreviations used are: EPC, egg phosphatidylcholine; EPA, egg phosphatidic acid; EPG, egg phosphatidylglycerol; MES, 2-(N-morpholino)ethanesulfonic acid; SPQ, 6-methoxy-N-(3-sulfopropyl)quinolinium; TNS, 1-anilinonaphthalene-8-sulfonate.

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the far and near UV, respectively. The buffer contained 50 or 150 mM NaCl and 50 mM citric acid/Na2HPO4 adjusted to pH values ranging from 2.5 to 7.4. Spectra were measured after 10 min of incubation at the desired pH. Unless specified otherwise, this set of buffers was used for all experiments aimed at elucidating the effect of pH on the structure of α-toxin.

Tryptophan Fluorescence Measurements—Tryptophan fluorescence experiments were performed as described previously (26). The samples were allowed to incubate for 10 min at the desired pH before measuring the emission spectrum. The α-toxin concentration was 35 μg/ml.

ANS Binding—Binding of 1-anilinonaphthalene-8-sulfonic acid (ANS) was measured as described previously (23). The excitation and emission wavelengths were 380 and 480 nm, respectively, and the spectral bandwidths were of 5 nm. The final ANS concentration was 50 μM. The α-toxin concentration was 35 μg/ml.

RESULTS

Kinetics of Channel Formation as a Function of pH—Our goal was to study the channel-forming ability of α-toxin as a function of pH. Egg lipids were used in order to have a mixture of saturated and unsaturated lipids because it has been suggested that unsaturated fatty acids are required for channel formation by α-toxin (27, 28). Since different types of lipids and a wide range of pH values (3–7.4) were to be used in these experiments, we first determined whether α-toxin was able to cause membrane fusion under our experimental conditions. We used a technique previously described to measure membrane fusion events (29). This method consists of mixing liposomes containing pyrene-labeled phosphatidylcholine with unlabeled liposomes and measuring the ratio between the fluorescence intensities of the excimer to monomer peaks. If fusion occurs, pyrene-labeled phosphatidylcholine is diluted and the fluorescence ratio decreases. At all pH values and for the various types of liposomes we have studied (see "Experimental Procedures" and below), we could not detect any decrease in excimer fluorescence indicating that fusion did not occur (data not shown). In contrast, lysozyme, which is known to induce fusion, did lead to a decrease in the ratio of excimer to monomer fluorescence (data not shown).

Having established that liposome fusion did not occur, we studied the kinetics of channel formation by monitoring the kineticsof release of chloride from vesicles containing the chloride-sensitive dye SPQ. We first checked that chloride efflux occurred in a dose-dependent manner and that it could be inhibited by zinc, which has been shown to block the α-toxin channel (30) (data not shown). Kinetics were then measured at various pH values with liposomes having different lipid compositions. Fig. 1, A and B, shows that the kinetics obtained using an EPC/EPA (95:5%) mixture and pure EPG were very sensitive to pH. When plotting the maximal rate of chloride efflux as a function of pH, it clearly appears that chloride efflux occurred at far lower pH values for EPC than for EPG vesicles; the EPC/EPG (50:50) vesicles adopted an intermediate behavior (Fig. 1C). Chloride efflux could only be observed from EPC vesicles at pH values below 4, whereas for EPG vesicles, efflux readily occurred at pH 5.5. A decrease in the rate of chloride efflux was observed when the pH was further lowered (data not shown). This inhibition occurred at pH values below 4.5, 4.2, and 3 for pure EPG, EPC/EPG (50:50), and pure EPC vesicles, respectively.

We then determined whether the differences in kinetics observed for the various types of liposomes at the different pH values may reflect the amount of oligomers bound to the vesicles. Therefore, at the end of each chloride efflux measurement, the vesicles were recovered by ultracentrifugation and analyzed by SDS-polyacrylamide gel electrophoresis under non-boiling conditions. Remarkably similar amounts of oligomers could be seen at all pH values, whether efflux had occurred or not, confirming the existence of non-lytic oligomers (10, 31) (Fig. 2). The amounts of oligomer were also very similar for all the lipid compositions studied (data not shown). There seemed to be no correlation between the amount of oligomers and the chloride efflux nor did pH seem to have a significant effect on the oligomerization process.
The apparent preference at a given pH of α-toxin for EPG could thus be explained only by a high affinity of the toxin for the phosphatidylglycerol head group or for negatively charged head groups in general. Alternatively, α-toxin may be affected by the pH at the surface of EPG vesicles. A reduced local pH is expected since the negatively charged head groups give rise to an electrical surface potential that in turn decreases the pH at the membrane surface (32, 33). The ΔpH between the bulk and the vesicle surface depends on the surface charge density of lipids, on the local anion concentration, as well as on the size of the vesicles. The Gouy-Chapman theory predicts a ΔpH of 2.7 between a low ionic strength solution and a planar lipid bilayer composed of 100% negatively charged lipids. We have measured the pH near the surface of the EPG vesicles under our experimental conditions. For example, for a bulk pH of 5, the pH at the membrane surface was 1.47 pH units lower than the bulk pH, which is in close agreement with our previous measurements (25). When the maximal velocities of chloride efflux are expressed as a function of interfacial pH (Fig. 1C), it appears that the onset of efflux occurs at a similar pH for EPG and EPC vesicles, demonstrating that the toxin is sensitive to local pH rather than to the chemical structure of the lipid head group.

Effect of pH on the Structure of α-Toxin—Since acidic pH promotes membrane insertion, we have investigated whether acidification of the medium could induce a conformational change of the protein. We have analyzed the effect of pH on the structure of α-toxin using a variety of spectroscopic techniques. Changes in tertiary interactions were analyzed by near UV CD, in secondary structure by far UV CD, in environment of tryptophans by fluorescence spectroscopy, and in hydrophobicity of the protein by binding of the hydrophobic dye ANS. It is important to note that, if α-toxin is indeed composed of two domains corresponding to the N- and C-terminal halves of the protein as has been proposed, the use of near UV CD or tryptophan fluorescence as probes to follow unfolding of α-toxin introduces a bias. Indeed, one then preferentially analyzes the C-terminal half of the protein as it contains 7 out of the 8 tryptophans (Fig. 3). Even though the N-terminal half also contains a fair amount of aromatic residues, it will moderately contribute to the near UV CD spectrum.

Effect of pH on the Near and Far UV CD Spectra of α-Toxin—In order to investigate whether the tertiary structure of α-toxin is sensitive to pH, we have measured the near UV CD spectrum of the toxin at various pH values. The spectrum of the native toxin has three main peaks: two negative peaks at 266 and 295 nm and a positive peak at 281 nm (Fig. 4A). Down to pH 4.0, this spectrum remained unchanged. Upon a slight further acidification of only 0.5 pH unit, the negative ellipticity at 295 nm dropped by about 80% while the positive ellipticity at 281 nm became negative. The loss of signal at 295 nm, corresponding to the Lb transition of tryptophans (34), suggests that part of the tryptophan-rich C-terminal half of the protein underwent partial unfolding. The spectrum at pH 3.3 still retained a very broad and intense peak around 280 nm indicating that certain regions in the toxin still possessed a rigid tertiary structure. At lower pH values, the effects became dependent on the ionic strength of the medium. In the presence of 50 mM NaCl, a second, more gradual, unfolding step occurred leading to a full collapse of the spectrum (Fig. 4, A and B). In contrast, at physiological salt concentrations, the spectrum remained similar to the one observed at pH 3.3, and even at pH values as low as 2.3, indicating that a domain of the toxin was stabilized by salts (Fig. 4B).

This change in near UV CD signal was partially reversible. Indeed, 73% of the signal at 281 nm could be recovered when α-toxin was first incubated at pH 3.2 and then diluted into a buffer (50 mM NaCl, 50 mM citric acid/Na2HPO4) at pH 7. However, at the high concentration required for near UV CD, an incubation of α-toxin at pH 3.2 for several minutes led to aggregation upon neutralization.

To establish whether this acid-induced flexibility of the protein was accompanied by a loss of secondary structure, the far UV CD spectrum of α-toxin was measured at various pH values. As shown in Fig. 5, the spectrum is rather insensitive to pH. In particular, the ellipticity at 210 nm did not gradually decrease upon acidification indicating that there was no significant loss in secondary structure. Only for pH values below 2.5 and at 50 mM NaCl could we detect a significant contribution by random coil elements. In Fig. 5, a slight increase in the ellipticity can be observed upon acidification, but as the far UV CD spectra of β-sheet proteins are very sensitive to the environment (35), the appearance of the small changes observed is beyond interpretation. The observed variations are probably due to changes in the conformation of aromatic residues that are known to contribute significantly to the far UV CD spectrum and especially to that of β-sheet proteins (34, 36).

Effect of pH on the Intrinsic Fluorescence of Tryptophans—The effect of pH on the structure of α-toxin was further analyzed by measuring the intrinsic fluorescence of tryptophans. The maximal emission wavelength (λmax) of tryptophans, an indicator of solvent exposure, was measured at different pH values. For native α-toxin, λmax was found close to 322 nm, indicating that tryptophan residues are buried in a hydrophobic environment (Fig. 6). Down to pH 4, λmax remained unchanged. Upon further acidification, a red shift of λmax was observed suggesting that the environment of some tryptophan residues became hydrophilic. The shape of the unfolding curve.

![Fig. 2. Effect of pH on the amount of α-toxin oligomers bound to EPC/EPA vesicles.](image)

![Fig. 3. Distribution of aromatic residues along the α-toxin sequence.](image)
depended on the ionic strength as previously observed in the circular dichroism experiments (Fig. 4). At very low salt concentrations (3 mM NaCl), unfolding occurred essentially between pH 4 and 3.5 and seemed to follow an all or none process. However, at 150 mM NaCl, unfolding appeared to occur in two steps indicating again the stabilization of a folding intermediate around pH 3.5. As shown in the inset of Fig. 6, this intermediate showed a much higher fluorescence intensity than the native or the more unfolded toxin. Over a much narrower range of pH, a similar transient increase in fluorescence intensity was observed at 3 mM NaCl indicating that unfolding also occurred in two steps even though this was not apparent when analyzing $\lambda_{\text{max}}$ (data not shown). It is noteworthy that in the presence of 100 mM NaCl, full exposure of all tryptophans no longer occurred since $\lambda_{\text{max}}$ never exceeded 345 nm. As already indicated by the circular dichroism experiments, a domain of the protein seemed to be fully stabilized by salts (Fig. 4B). The effect of pH seemed reversible under all conditions used as $\lambda_{\text{max}}$ shifted back to 332 nm when the pH was raised back to 7.4.

We have also investigated the effect of temperature on the acid denaturation of α-toxin. When raising the temperature to 37 °C, the first unfolding step observed in the presence of 150 mM NaCl occurred at 0.5 pH unit higher than at 25 °C. Indeed, at 25 °C, $\lambda_{\text{max}}$ shifted to 333.5 ± 0.4 nm at pH 3.7; however, at 37 °C, a similar $\lambda_{\text{max}}$ ($\lambda_{\text{max}} = 333.2 ± 0.1$ nm) was already observed at pH 4.3. The second unfolding step was not significantly affected by this temperature shift.

Effect of pH on the Hydrophobicity of α-Toxin in Solution—Following the hydrophobicity of α-toxin as a function of pH is important for understanding the increased ability of the toxin to form channels at low pH as well as for the characterization of the acid unfolding process. Harshman et al. (20) had previously analyzed the effect of pH on the partitioning of α-toxin into the detergent phase of Triton X-114. They showed that at pH values below 4.5, α-toxin partially partitions into Triton X-114 and that all the toxin is found in the detergent phase at pH 3.75, indicating an increase in hydrophobicity upon acidification. We have investigated the effect of pH on the hydrophobicity by measuring the binding of the hydrophobic dye ANS (23, 37). We believe this technique is more suitable as it does not require the use of detergents that are likely to affect the structure of the protein. As shown in Fig. 7, a drastic increase of ANS binding was observed between pH 4 and 3.5 suggesting that a hydrophobic region of the protein became exposed. Upon further acidification, the binding decreased again suggesting a more pronounced unfolding of the toxin or aggregation. Indeed, it is known that ANS binds very little to fully unfolded or aggregated proteins.

The increase in hydrophobicity observed upon acidification was observed at all salt concentrations studied. The hydropho-
bic intermediate was, however, stabilized over a wider pH range in the presence of 150 mM NaCl. The effect of temperature on ANS binding was also analyzed. The appearance of the hydrophobic intermediate occurred at higher pH at 37 °C than at 25 °C (data not shown) in a manner similar to that observed when measuring \( \lambda_{\text{max}} \). These observations indicate that the increase in hydrophobicity is concomitant with the first unfolding step.

Similar experiments were performed using an excitation wavelength (\( \lambda_{\text{ex}} \)) of 295 nm instead of 380 nm. Under these conditions, tryptophan residues are excited and ANS can only be excited through energy transfer. The results were very similar to those obtained for \( \lambda_{\text{ex}} = 380 \) nm suggesting that ANS binds in close proximity to the tryptophan residues, most of which are clustered in the C-terminal half of the toxin.

**DISCUSSION**

The present study on the effect of pH on the conformation of \( \alpha \)-toxin, as well as its ability to form channels in artificial membranes, has led to new information on the three-dimensional structure of the toxin and on the mechanism of membrane insertion.

**Three-dimensional Structure of \( \alpha \)-Toxin**—Both CD and tryptophan fluorescence analyses of the effect of pH on the structure of \( \alpha \)-toxin clearly show, for the first time, that the toxin is composed of several structurally independent domains. Near UV CD spectroscopy, performed in the presence of 50 mM NaCl, shows a two-step unfolding suggesting the presence of two domains. At physiological salt concentration, however, unfolding seemed to occur in a single step leading to a stable species at acidic pH that still had regions with rigid tertiary structure (Fig. 4B). Under the same conditions (150 mM NaCl), unfolding as indicated by tryptophan fluorescence, revealed a two-step process (Fig. 6). Interestingly, the second increase of \( \lambda_{\text{max}} \) (Fig. 6) was not concomitant with a loss of near UV CD signal (Fig. 4B) suggesting that a region of the protein that had already lost its rigid tertiary interaction was undergoing further unfolding.

Our data would be consistent with the following multidomain organization of \( \alpha \)-toxin. The N-terminal half of the protein would form a single domain (domain 1) separated from the C-terminal region by the so-called glycine-rich loop. This domain 1 would be the less pH-sensitive region of \( \alpha \)-toxin, and the presence of salts would completely protect it toward acidic unfolding. In contrast, the C-terminal region would be very sensitive to pH. Upon acidification, it would unfold in a two-step process (Fig. 6). The fact that the two-step process could be selectively affected by varying the salt concentration or the temperature suggests that the C-terminal region might be formed by two domains. This, however, remains to be confirmed.

One must keep in mind that all mentioned unfolding steps are only partial as the secondary structure is essentially maintained under the different conditions. It seems that upon acidification, the various domains sequentially undergo a transition to a molten globule-like state. The most interesting folding intermediate was obtained, at pH 3.5, at physiological salt concentrations. In this folding intermediate, domain 1 is probably still fully folded, but the rest of the protein shows typical characteristics of the molten globule-like state: native-like secondary structure, increased flexibility of the tertiary structure, and binding of ANS. However, this folding intermediate does not seem as compact as most molten globule-like states since some tryptophans appear to be accessible to the solvent.

**Membrane Insertion**—Our study on the kinetics of chloride efflux induced by \( \alpha \)-toxin revealed that the protein is sensitive to the local pH at the membrane surface, rather than to the bulk pH, and that this pH had to be lower than 4 for channel formation to occur (Fig. 1C). The rate of channel formation was optimal at a local pH of 3.5 (Fig. 1C). Subsequent studies on the effect of pH on the structure of the toxin showed that below pH 4, the protein unfolds in a multistep process that could be explained by the sequential partial unfolding of domains. The first unfolding step, corresponding to the partial unfolding of a C-terminal region, correlates well with the increase in the rate of channel formation (when expressed as a function of local pH, Fig. 1C) suggesting that it is required for channel formation. In contrast, additional unfolding of the N-terminal region of the protein seemed to have an inactivating effect. Clearly, the pH values at which \( \alpha \)-toxin activation was observed are below the physiological range. Whether similarly low pH values may be encountered in the close vicinity of the plasma membrane in certain tissue is not clear. The present observations may also suggest that \( \alpha \)-toxin could act from within acidic endosomes or lysosomes. In any case, our goal in this study was to make use of the pH effects to understand unfolding and insertion.

**Conclusion**—Taken together, the above described observations suggest that the C-terminal half of \( \alpha \)-toxin has to undergo partial unfolding for channel formation to occur. It has been previously observed by us and by others that a variety of toxins including diphtheria toxin, colicin A, or Pseudomonas aeruginosa exotoxin A (for review, see Refs. 38 and 39) must undergo partial unfolding in order to insert into a membrane. The membrane-interacting domains of these toxins have a common \( \alpha \)-helical folding motif as described by Parker and Pattus (40). \( \alpha \)-Toxin, in contrast, belongs to a very different family of pore-forming toxins that are essentially formed of \( \beta \)-sheet, must oligomerize in order to insert into the membrane, and are thought to form porin-like channels. This family includes aerolysin, Clostridium septicum \( \alpha \)-toxin, protective antigen of anthrax toxin and some of the oxygen-labile toxins (41–44). The requirement of acidic pH for channel formation by toxins of this family has only been shown for anthrax toxin protective antigen (45) and \( \alpha \)-toxin, but the effects of pH have not been further characterized. This study is the first evidence that some \( \beta \)-sheet pore-forming toxins also require a transition to a molten globule-like state in order to form transmembrane channels. In this work, we have used pH to trigger unfolding. It is, however, tempting to speculate that in vivo other factors, such as interactions with a specific receptor on the target membrane, could induce a similar transition.

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