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Dimer Dissociation of the Pore-forming Toxin Aerolysin Precedes Receptor Binding*

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Marc Fivaz, Marie-Claire Velluz, and F. Gisou van der Goot‡

From the Department of Biochemistry, University of Geneva, 30 quai E. Ansermet, 1211 Geneva, Switzerland

The pore-forming toxin aerolysin is secreted by *Aeromonas hydrophila* as an inactive precursor. Based on chemical cross-linking and gel filtration, we show here that proaerolysin exists as a monomer at low concentrations but is dimeric above 0.1 mg/ml. At intermediate concentrations, monomers and dimers appeared to be in rapid equilibrium. All together our data indicate that, at low concentrations, the toxin is a monomer and that this species is competent for receptor binding. In contrast, a mutant toxin that forms a covalent dimer was unable to bind to target cells.

The pore-forming toxin aerolysin is produced by the human pathogen *Aeromonas hydrophila* as an inactive precursor, proaerolysin (for reviews, see Refs. 1–3). Subsequent steps include binding to the target cells and activation of proaerolysin into mature aerolysin. Binding requires the presence of specific cell surface receptors. Although the identity of the receptors varies from one cell type to another (4–6), all receptors identified so far are proteins anchored to the membrane via a glycosylphosphatidyl inositol (GPI)[1] anchor. Activation of proaerolysin involves proteolytic removal of a C-terminal peptide by proteases such as trypsin (7, 8) or the mammalian endoprotease furin (9). The toxin must then oligomerize into a heptameric ring (10, 11) which inserts into the membrane to form a pore. Channel formation provokes the loss of small molecules triggering signaling cascades in a dose-dependent manner (12), selective vacuolation of the endoplasmic reticulum (6), and apoptosis in certain cell types (13).

We have previously shown by analytical centrifugation that proaerolysin and aerolysin can exist as a dimer (14) because it was found in the crystal structure of proaerolysin (15). A puzzling question, therefore, was how a dimer (even number of monomers) converts into a heptamer (odd number of monomers). The crystal structure shows that the monomers in the dimer are oriented in an anti-parallel fashion (15), whereas in the heptamer, monomers are parallel as deduced from low resolution micrographs of the aerolysin channel (10). Here we show, by cross-linking and gel filtration, that the dimer separates into monomers at low concentrations and that monomers, rather than dimers, are detected on target cells. The possible role of the proaerolysin receptor in preventing dimerization is also discussed.

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‡ To whom correspondence should be addressed. Tel./Fax: (41) 022 702-6414; E-mail: Gisou.vandergoot@biochem.unige.ch.

[1] The abbreviations used are: GPI, glycosylphosphatidyl inositol; M41C, mutant in which methionine 41 was replaced by cysteine; PNS, post-nuclear supernatant; BHK, baby hamster kidney cells; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; DTSSP, 3,3′-dithiobis[sulfosuccinimidylpropionate].

EXPERIMENTAL PROCEDURES

Protein Purification and Iodination—Wild type and M41C mutant proaerolysins were purified as previously published (16, 17). Iodination of proaerolysin was performed as previously published (6, 18).

Binding to Baby Hamster Kidney Cells and Preparation of Post-nuclear Supernatants—Monolayers of baby hamster kidney (BHK) cells were grown and maintained as described (6). Confluent monolayers of BHK cells were incubated at 4 °C with wild type or mutant proaerolysin (50 ng/ml) in Glasgow minimal essential medium buffered with HEPES, pH 7.4, 1 μg/ml trypsin/chymotrypsin inhibitor. Cells were then washed three times for 10 min with PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS^{2+}) at 4 °C and scraped from the dish. Post-nuclear supernatant (PNS) was prepared as described (6, 19) and analyzed for the presence of proaerolysin by SDS-PAGE followed by Western blotting using a monoclonal anti-proaerolysin antibody (4) and horseradish peroxidase-labeled secondary antibodies, which were revealed using enhanced chemiluminescence (Pierce). The sample buffer did not contain any reducing agent unless specified. For Western blot analysis, a 12.5% gel was transferred onto nitrocellulose membrane for 2.5 h at 86 V in a Tris-glycine-methanol buffer using a Bio-Rad wet transfer chamber.

DTSSP Cross-linking—BHK cells were treated with 50 ng/ml ^{125}I-proaerolysin as described above. After extensive washing, DTSSP at a final concentration of 0.2 mM in PBS^{2+} was added to the cells at 4 °C. After different incubation times at 4 °C, the medium was removed and replaced with PBS^{2+} containing 20 mM glycine, pH 7.4, to quench the reaction. As a control, one dish was treated with the cross-linker in the presence of 20 mM glycine, pH 7.4. After 10 min of incubation with the quencher, cells were scraped and PNSs were prepared. DTSSP cross-linking was also performed on proaerolysin in solution (0.47 mg/ml ^{125}I-proaerolysin). ^{125}I-proaerolysin was incubated with 0.2 mM DTSSP. After various times, aliquots were taken and quenched by 20 mM glycine, pH 7.4. All cross-linking products were analyzed by SDS-PAGE on 9–15% acrylamide gradient gels followed by autoradiography.

Glutaraldehyde Cross-linking—Wild type or mutant proaerolysin was prepared at various concentrations in 150 mM NaCl, 20 mM HEPES, pH 7.4. After 30 min of incubation at room temperature, 4 μg of each sample was loaded at the bottom of a Pyrex tube and 1 ml of 1% glutaraldehyde added. After 2 min, 50 μl of NBH_4 (2 M) in 0.1 M NaOH and 50 μl of lysine (2M) in 0.1 M NaOH were added to stop the reaction. After 20 min at room temperature, samples were precipitated with trichloroacetic acid and deoxycholate as a carrier. Cross-linking products were analyzed by SDS-PAGE (20) followed by silver staining. Reducing sample buffer was used in these experiments.

Gel Filtration—All gel filtration experiments were performed using the Amersham Pharmacia Biotech micro FPLC SMART system equipped with a precision Superdex 75 PC column. The column was equilibrated with 150 mM NaCl, 20 mM HEPES, pH 7, and the system was thermostatted at 20 °C. The flow was set to 40 μl/min, 10 μl of sample at the desired toxin concentration was injected, and the optical density at 280 nm was monitored as a function of the elution volume.

Analytical Techniques and Proaerolysin Overlays—Protein concentrations of cellular fractions were determined with bicinchoninic acid (BCA, Pierce). Proaerolysin overlays, to detect proaerolysin-binding proteins, were performed as described previously (6).

RESULTS AND DISCUSSION

The Dimeric M41C Proaerolysin Mutant Is Unable to Bind to the Toxin Receptors—We, and others, have previously established that proaerolysin can bind to its GPI-anchored receptors in a toxin overlay assay (4, 6, 9, 13). On BHK cells, this led to

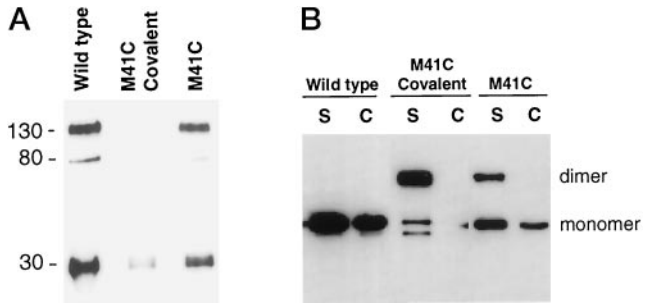

FIG. 1. **The M41C dimeric mutant is unable to bind to proaerolysin receptors.** *A*, post-nuclear supernatants were prepared from BHK cells that had not been treated with toxin and were centrifuged at 50,000 rpm for 20 min using a TL100.3 Beckman rotor to prepare a membrane fraction. 40 μg of membrane-associated proteins were separated on a 9–15% acrylamide gradient gel and blotted onto a nitrocellulose membrane. A toxin overlay assay was then performed using either the wild type toxin, the M41C covalent dimer, or the mixture of covalent and non-covalent M41C dimers (see "Results"). *B*, wild type and proaerolysin M41C mutants were added to BHK monolayers at 4 °C at a final toxin concentration of 0.4 nM. Cells were then washed and post-nuclear supernatants were prepared. Both the toxin sample prior to addition to cells (*S*, solution) and the post-nuclear supernatant (*C*, cell) were analyzed by SDS-PAGE followed by Western blotting using an anti-proaerolysin monoclonal antibody. Electrophoresis was performed under non-reducing conditions to detect the disulfide-linked M41C dimers. Dimeric toxin could not be detected on the cellular extracts.

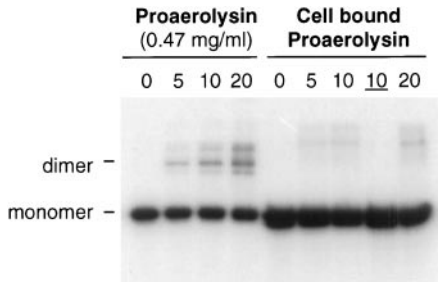

FIG. 2. **Proaerolysin dimers could not be detected on BHK cells after DTSSP cross-linking.** ^{125}I-proaerolysin was cross-linked for different times with the thiol-cleavable reagent DTSSP (0.2 mM) either in solution (0.47 mg/ml) or bound to BHK cells. The times of cross-linking are indicated in minutes at the *top* of each *lane*. As a control, 10-min cross-linking of ^{125}I-proaerolysin bound to BHK cells was also performed in the presence of glycine as a quencher (*lane 10*). Cross-linking products were analyzed by SDS-PAGE on a 9–15% acrylamide gradient gel under non-reducing conditions followed by autoradiography. When β-mercaptoethanol was added to the sample buffer, none of the cross-linking products could be detected (not shown). A cross-linked product corresponding to the proaerolysin dimer could be observed when experiments were performed at 0.47 mg/ml in solution. This product was absent when cross-linking the toxin at the cell surface. Higher molecular weight products were then observed presumably corresponding to proaerolysin bound to its 130-kDa receptors.

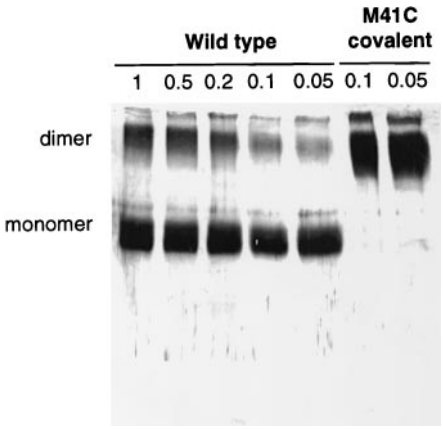

FIG. 3. **The efficiency of wild type proaerolysin dimer cross-linking is concentration-dependent.** Wild type proaerolysin was cross-linked with glutaraldehyde at different concentrations (indicated in mg/ml at the *top* of each *lane*). As a positive control, the M41C proaerolysin mutant was also cross-linked. The same amount of protein (4 μg) was loaded in each lane. β-Mercaptoethanol was present in the sample buffer to break the inter-monomer disulfide bound of M41C; the dimers observed are therefore because of cross-linking.

the identification of three receptors with apparent molecular masses of 30, 80, and 130 kDa (6, 19).

We performed here a proaerolysin overlay assay using the M41C mutant proaerolysin instead of the wild type toxin. Upon overexpression, this mutant, in which Met-41 was replaced by cysteine, forms covalent dimers in the bacterial periplasm and is then secreted as such (17) (the M41C dimer can be visualized by SDS-PAGE under non-reducing conditions, Fig. 1*B*). As shown in Fig. 1*A*, we could no longer detect the three GPI-anchored proaerolysin binding proteins when performing overlay assays with the M41C mutant, indicating that the covalent dimer was unable to bind to the receptors. This was confirmed by the fact that we could not detect any M41C covalent dimers on BHK cells that were treated with the mutant toxin in contrast to what is observed when using the wild type toxin (Fig. 1*B*).

The above experiments suggest that either the proaerolysin dimer cannot bind to cell surface receptors or that the M41C mutant has altered properties. We therefore analyzed the binding of the M41C mutant obtained from bacteria that had been grown in the presence of β-mercaptoethanol to prevent as much as possible the formation of the disulfide bond between the two Cys-41 residues (Fig. 1*B*, *M41C*, *lane S*). When the M41C mutant lacked the engineered disulfide bridge between two monomers, it recovered its ability to bind to cells (Fig. 1*B*) and more specifically to the receptors (Fig. 1*A*). These experiments show that the M41C mutation itself had no effect on receptor binding and suggest that the dimeric nature of the mutant might hinder binding.

Proaerolysin at the Surface of BHK Cells—We next addressed the issue of the dimer binding using a different approach. Cross-linking experiments were performed after binding wild type proaerolysin to target cells to analyze the presence of toxin dimers. For this, BHK cells were incubated with ^{125}I-proaerolysin at 4 °C to allow binding of the toxin but not processing (19). After extensive washing, cells were incubated with the thiol-cleavable cross-linker DTSSP, and the cross-linking products containing the toxin were revealed by autoradiography. As a positive control, we performed cross-linking, under the same conditions, of proaerolysin in solution at 0.47 mg/ml, a concentration where cross-linking of dimers has previously been observed (14). Dimers of proaerolysin could indeed be observed after DTSSP cross-linking of the toxin in solution at 0.47 mg/ml (Fig. 2). After cross-linking at the surface of BHK cells, however, no product with the molecular weight of the dimer could be observed (Fig. 2). Only a cross-linked complex with an apparent molecular mass of ≈200 kDa was detected, presumably corresponding to monomeric proaerolysin cross-linked with its 130-kDa GPI-anchored receptor. It seems unlikely that the cross-linked product corresponds to dimeric toxin cross-linked to the receptor. Indeed, if dimers were present at the cell surface, monomer-monomer, monomer-receptor, and dimer-receptor complexes would be expected after cross-linking, the later product being the less abundant because three polypeptides must be cross-linked together. We thus conclude that proaerolysin was a monomer when bound to the cells.

The Efficiency of Dimer Cross-linking Decreases with Protein Concentration—The absence of detectable dimers at the cell

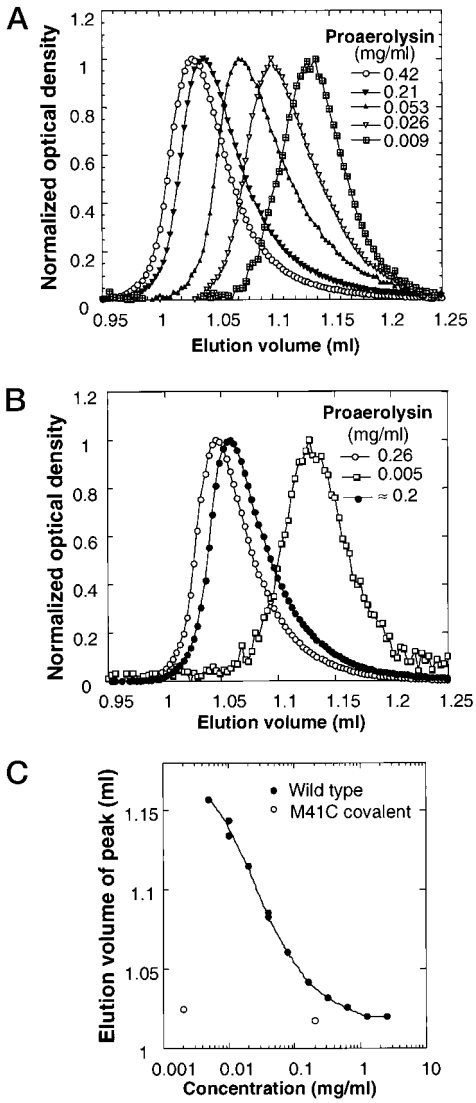

FIG. 4. **The elution profile of wild type proaerolysin on a gel filtration column is concentration-dependent.** *A*, 10 μl of wild type proaerolysin at different concentrations was injected onto a Sephadex 75 PC column using the Amersham Pharmacia Biotech SMART system, and the optical density at 280 nm was measured as a function of the elution volume. The retention time was strongly dependent on the initial toxin concentration. The base line was subtracted from each curve, and the maximum OD was normalized to 1 to enable comparison between the different profiles. *B*, the elution profile of wild type proaerolysin was measured at 0.26 mg/ml and after dilution to 0.005 mg/ml. A 0.005 mg/ml proaerolysin was also concentrated by centrifugation on an Amicon 30 filter to approximately 0.2 mg/ml (see Fig. 5*A*, *lane b*), and the elution profile was measured. *C*, the peaks of the elution profiles were plotted as a function of toxin concentration for the wild type toxin and the M41C covalent dimer (see Fig. 5).

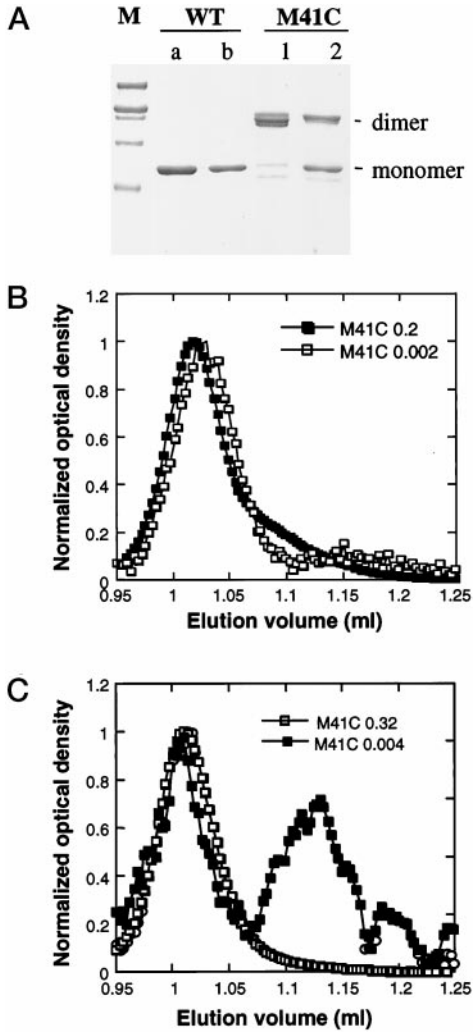

FIG. 5. **Elution profiles of the M41C covalent dimer.** *A*, SDS-PAGE analysis of wild type and mutant proaerolysins. Proaerolysin at 0.26 mg/ml (*lane a*) was diluted to 0.005 mg/ml and then concentrated again by centrifugation on an Amicon 30 filter to approximately 0.2 mg/ml and analyzed by 12.5% SDS-PAGE. The M41C mutant in its essential covalently dimeric form (*lane 1*) and in a partly covalent form (*lane 2*) is also shown. Electrophoresis was performed under non-reducing conditions. *B*, the M41C proaerolysin mutant (see *lane 1* in *panel A*) was injected onto the Sephadex column at two concentrations (0.2 and 0.002 mg/ml, 10 μl). Even a 100-fold dilution did not affect the elution profile. The base line was subtracted from all curves, and the maximums were normalized to 1 for comparison. *C*, the M41C mutant containing a mixture of covalent and non-covalent dimers (see *lane 2* in *panel A*) was analyzed at 0.32 and 0.004 mg/ml. At low concentration, two peaks can be observed corresponding to the covalent dimer and the proaerolysin monomer.

surface suggests that either receptor binding triggers dimer dissociation or that dissociation precedes interaction with the receptors. The dimeric nature of proaerolysin was established using analytical centrifugation (14) and small angle neutron scattering.[2] These techniques require toxin concentrations above 0.1 mg/ml. In contrast, studies addressing the interaction of proaerolysin with target cells require toxin concentrations neighboring 10 ng/ml because of the high sensitivity of mammalian cells (4, 6, 9, 13, 19). One possible explanation for the observed difference in stoichiometry between previous studies (14) and the present work is that the higher order assembly of proaerolysin is concentration-dependent.

To address this issue, we performed cross-linking experiments at decreasing toxin concentrations. The experiments were carried out using a brief 2-min glutaraldehyde cross-linking procedure to minimize complex formation because of stochastic encounters. As a positive control, we used the covalent dimeric M41C mutant. As can be seen in Fig. 3, the extent of cross-linking of the wild type dimer decreased as cross-linking was performed at lower toxin concentrations. In contrast, dimers were observed even at low concentrations for the M41C mutant. Previous observations have shown that proaerolysin can be dimeric, the present results suggest that at low concentrations, however, proaerolysin is monomeric.

Effect of Proaerolysin Concentration on the Gel Filtration Elution Profile—To further study the stoichiometry of proaero-

[2] C. Lesieur, G. G. van der Goot, and P. Timmins, unpublished data.

lysin as a function of concentration, we have analyzed the elution profile of proaerolysin on a Precision Superdex 75 gel filtration column mounted onto the Amersham Pharmacia Biotech micro FPLC SMART system. As can be seen in Fig. 4*A*, the retention time of proaerolysin was strongly concentration-dependent and increased as the concentration of the toxin injected onto the column decreased. This change in behavior was reversible because re-concentration of the sample by centrifugation on an Amicon filter followed by gel filtration analysis led to the predicted decrease in retention time (Figs. 4*B* and 5*A*). The fact that the elution time of proaerolysin varied with the toxin concentration indicates that either the conformation of the toxin changed upon dilution (for example, partial unfolding which would increase the Stocks radius) or that the dimer (104 kDa) separated into monomers (52 kDa). We could not detect any change in conformation by analyzing the trypsin sensitivity and the maximal emission wavelength of tryptophans of proaerolysin at different toxin concentrations (not shown). The elution profile of mature aerolysin was also found to change with toxin concentration, indicating that proaerolysin and aerolysin undergo similar changes as a function of concentration (not shown). As for proaerolysin, the change in elution profile of mature aerolysin upon dilution was reverted upon subsequent concentration of the protein (not shown).

Combined with the glutaraldehyde cross-linking experiments, these gel filtration data suggest that the proaerolysin and the aerolysin dimers separate into monomers. Because of the detection limits, we could not perform gel filtration experiments below 5 μg/ml and were therefore unable to estimate the elution volume for a proaerolysin population composed only of monomers. The relative abundance of monomers and dimers at a given toxin concentration, therefore, could not be determined. What clearly appears on the plot of the elution peak as a function of toxin concentration is that, at 0.1 mg/ml and above, corresponding to the concentrations used in the studies that established that proaerolysin was dimer (14), the protein is essentially dimeric (Fig. 4*C*). It is interesting to note that the elution profiles showed a single symmetrical peak at all concentrations, indicating that dimers and monomers were in rapid equilibrium.

To confirm that the change in elution profile with concentration was because of dimer dissociation rather than to a change in conformation only, we have analyzed the elution profile of the M41C mutant, both in its covalent dimer form and a partially covalent form (Fig. 5*A*). The covalent M41C mutant had exactly the same elution profile as the wild type toxin at high concentrations (Fig. 4*C* and 5*B*). In contrast to the wild type toxin, however, the elution profile was independent of toxin concentration. When analyzing the partially covalent M41C mutant, however, two peaks could be detected at low concentrations, presumably corresponding to the covalent dimer and monomeric M41C, respectively (Fig. 5*C*). The fact that the elution profile of the M41C covalent dimer was not sensitive to toxin concentration, whereas that of non-bridged M41C was, suggests that observations with the wild type toxin indeed reflected a dimer to monomer transition.

Conclusion—In the present work we have shown, by cross-linking and gel filtration experiments, that proaerolysin is a monomer at low, probably more physiologically relevant, concentrations and therefore encounters its target cell as a monomeric species. Moreover, our experiments using the M41C covalent dimer suggest that the dimer is in fact unable to bind to the GPI-anchored receptors. We can, however, not rule out that the lack of binding of the M41C mutant is because of an altered conformation or reduced flexibility of the covalent dimer (21). Therefore whether dissociation is obligatory remains to be established. It is not clear at present whether during *Aeromonas* infection, as opposed to overexpression of the toxin, proaerolysin ever exists as a dimer. Using the M41C covalent dimer, Hardie *et al.* (17) have previously shown that the dimer is a secretion-competent form, which does not exclude the possibility that the monomer can also be secreted and is prevalent during infection.

After binding to the cell surface, proaerolysin is converted into aerolysin, which then oligomerizes into a heptameric ring that forms the pore. We have shown that dimerization of proaerolysin and aerolysin is reversible. It is unlikely that dimers would re-form at the cell surface after receptor binding because they were not observed in our DTSSP cross-linking experiments. This lack of re-assembly of the dimer could be due to the fact that the local concentration at the cell surface is not high enough, despite clustering of the toxin in cholesterol-rich microdomains (19). It is, however, attractive to believe that binding to the receptors prevents dimer formation. Because aerolysin can form both dimers (14) and heptamers (11), these two reactions are likely to be in competition especially because the concentration threshold that allows oligomerization of aerolysin (14) is very similar to the concentration that allows dimerization. One possible role for the receptor could, therefore, be to prevent dimerization to drive the reaction toward oligomerization.

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