Increased Stability upon Heptamerization of the Pore-forming Toxin Aerolysin*

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Aerolysin is a bacterial pore-forming toxin that is secreted as an inactive precursor, which is then processed at its COOH terminus and finally forms a circular heptameric ring which inserts into membranes to form a pore. We have analyzed the stability of the precursor proaerolysin and the heptameric complex. Equilibrium unfolding induced by urea and guanidinium hydrochloride was monitored by measuring the intrinsic tryptophan fluorescence of the protein. Proaerolysin was found to unfold in two steps corresponding to the unfolding of the large COOH-terminal lobe followed by the unfolding of the small NH2-terminal domain. We show that proaerolysin contains two disulfide bridges which strongly contribute to the stability of the toxin and protect it from proteolytic attack. The stability of aerolysin was greatly enhanced by polymerization into a heptamer. Two regions of the protein, corresponding to amino acids 180–307 and 401–427, were identified, by limited proteolysis, NH2-terminal sequencing and matrix-assisted laser desorption ionization-time of flight, as being responsible for stability and maintenance of the heptamer. These regions are presumably involved in monomer/monomer interactions in the heptameric protein and are exclusively composed of β structure. The stability of the aerolysin heptamer is reminiscent of that of pathogenic, fibrillar protein aggregates found in a variety of neurodegenerative diseases.

The pore-forming toxin aerolysin is secreted by the human pathogen Aeromonas hydrophila as an inactive precursor, called proaerolysin (for review, see Refs. 1 and 2). According to the crystal structure of proaerolysin, each monomer is formed of two domains, a small globular NH2-terminal region (domain 1, amino acids 1–82) and a long elongated lobe that can be divided into 3 domains that are not continuous in the linear sequence (Fig. 1) (3). In the crystal structure proaerolysin is organized as a dimer in which the two monomers are arranged in an antiparallel fashion with the two NH2-terminal domains clasping each other in a crossover fashion (3). Dimeric aerolysin was also observed in solution at high toxin concentrations (4, 5). However, at concentrations below 1 mg/ml, proaerolysin starts separating into monomers as recently suggested by gel filtration experiments.1

In order to become active, the precursor proaerolysin must be proteolytically processed at its COOH terminus (7–10). The mature aerolysin is then able to oligomerize by circular assembly of seven monomers (11, 12) which leads to the formation of a central channel in a manner similar to that which has been observed for other toxins as well as for GroEL, Trp RNA-binding attenuation protein, and the proteasome (for review, see Ref. 13). The aerolysin heptamer exposes hydrophobic surfaces (14) which enable the complex to spontaneously insert into a lipid bilayer and form a channel (15).

Here we have analyzed the stability of both proaerolysin and the aerolysin heptamer. We show that the unfolding of proaerolysin occurs in at least two steps and is reversible under non-reducing conditions. The two disulfide bridges in the proaerolysin molecule greatly contribute to the overall stability of the protein. These studies moreover show that the NH2-terminal domain 1 of the toxin constitutes a separate folding domain and is the most stable part of the toxin. The second part of this work concerns the analysis of the effects of heptamerization on the stability of aerolysin. Urea (8 m) had no effect on the structure of the complex. Partial unfolding, without disassembly of the complex, was, however, observed in GdnHCl2 (6 m). Again the disulfide bonds were found to significantly contribute to the overall stability. In order to identify regions of the protein involved in maintaining the oligomer assembled, limited proteolysis studies were performed. Results from NH2-terminal sequencing of the fragments indicated that domains 1 and 2 could be entirely removed and that domains 3 and 4 are crucial for the maintenance and the stability of the heptameric complex.

EXPERIMENTAL PROCEDURES

Protein Purification—Wild type and mutant proaerolysins were purified according to a published procedure (16). Generation of C159S was described previously (17, 18). Concentrations were determined by measuring the optical density at 280 nm, considering that a 1 mg/ml sample has an OD of 2.5 (9). Heptameric aerolysin was prepared as described previously (10, 12). Hemolytic activities were measured as described previously (19). When oligomerization had to be prevented activation was performed in 150 mM NaCl, 50 HEPPS, pH 8.5 (20).

Cyanogen Bromide Fragmentation—Cyanogen bromide fragments

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1 Fivaz, M., Velluz, M.-C., and van der Goot, F. G., J. Biol. Chem., in press.
2 The abbreviations used are: GdnHCl, guanidine hydrochloride; HEPPS, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid, Tricine, N-[2-hydroxy-1-(2-hydroxyethyl)ethyl]glycine; C159S, mutant in which cysteine 159 was replaced by serine; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; PVDF, polyvinylidene difluoride.
were obtained by treating proaerolysin for 24 h with a 500-fold molar excess of cyanogen bromide in 75% formic acid at room temperature. The sample was then frozen and evaporated by vacuum centrifugation. The sample was then rinsed with water, frozen, and evaporated again. This procedure was performed twice.

**Unfolding with Chaotropic Agents—Equilibrium unfolding experiments were performed as described by Pace et al. (21). Urea and GuHCl stock solution were prepared as described by Pace et al. (21) in either 150 mM NaCl, 20 mM HEPES, pH 7.4, or 150 mM NaCl, 20 mM HEPES, pH 8.4. The denaturant concentrations of each stock solution were determined by weight and refractive index (21). These solutions were used if the difference between the two values was less than 1%. Buffer, denaturant, and protein (20 μg/ml) from concentrated stocks were mixed and samples were incubated in a water bath at 25 °C for at least 24 h, unless specified otherwise. All experiments were performed at least 3 times. To test for reversibility, a solution of proaerolysin in the post-transitional region (4–6 μM urea) of the denaturation curve was submitted to a 2-fold serial dilution with buffer in a 96-well plate. The samples were then incubated for 2 h at room temperature to allow refolding of the protein. Trypsin (1/50 protease/protein ratio, w:w, 10 min incubation) was then added to each well to convert proaerolysin into aerolysin and the hemolytic activities were then determined as described previously (19).

**Tryptophan Fluorescence—**Fluorescence measurements were made with a PTI spectrofluorimeter equipped with a thermostated cell holder. The excitation wavelength was either 278 or 295 nm and slit widths were 5 and 2.5 nm for excitation and emission, respectively. For each record spectrum, the Raman scatter contribution was removed by subtraction of a buffer blank. Very similar unfolding curves were obtained whether the samples were excited at 278 or 295 nm. In order to measure the effect of denaturants on the fluorescence intensity, samples were excited at 278 nm (allowing excitation of both tryptophans and tyrosines). The ratio between the intensity at 345 nm (which varied significantly) and the intensity at 315 nm (which varied little) was calculated for each condition. Experiments were performed at least three times and the average ratio calculated. The protein concentration was 20 μg/ml.

**Limited Proteolysis with Trypsin and NH2-terminal Sequencing—**Heptameric aerolysin was prepared as described previously (10, 12). Limited proteolysis of the heptamer with trypsin was either performed in solution or in a SDS gel. For cleavage in solution, the heptamer was first incubated with 0.1% SDS for 3 h. Tryptsin was then added in a 1:1 (w:w) ratio and the sample was incubated for 24 h at 37 °C. The same amount of trypsin was added every 24 h. After 72 h incubation, 10-fold excess of trypsin was added and the sample was analyzed by SDS-PAGE. For in-gel proteolysis, the heptamer was run on a 7.5% SDS gel. The gel was subsequently incubated for 24 h at 37 °C. The trypsin to protein ratio between 0.1:1 and 12:1 were tested and led to the same results. The samples were concentrated 2-fold using a Speed-Vac. The sample was then rehydrated with 7.5% gels and transferred onto a PVDF Porablot membrane (0.2 μm, Macherey-Nagel, Düren, Germany) according to a procedure adapted from Laurière (22). After electrophoretic migration, the gel was sealed in a plastic pocket containing water and frozen at −80 °C for 15 min between two glass plates. The gel was then incubated in a buffer containing 0.1% (w/v) SDS, 15 mM lactic acid, 25 mM Tris, pH 8.4, for 15 min. The membrane was activated in methanol for 1 min. Proteins were transferred under semi-dry conditions on the Bio-Rad semi-dry element transfer cell (Trans-blot SD). The following buffers were used: Buffer I contained 1.2% (w/v) SDS, 60 mM lactic acid, 100 mM Tris, pH 8.4; buffer II, 0.1% (w/v) SDS, 15 mM lactic acid, 25 mM Tris, pH 8.4; buffer III, 20% methanol, 60 mM lactic acid, 20 mM Tris, pH 3.8; and buffer IV, 25% methanol, 100 mM Tris, pH 10.4. The gel and blotting membrane were assembled with Whatman No. 3 paper and sponges in a blotting sandwich as follows, going from the cathode to the anode: 1) sponge in buffer I; 2) Whatman papers in buffer II; 3) gel; 4) plastic frame; 5) PVDF membrane; 6) 3 Whatman papers in buffer III; and 7) sponge in buffer IV. Samples were transferred for 120 min at 150 mA. The membrane was then washed three times for 10 min in water, dried between two Whatman papers, and stained twice for 5 min with Coomassie Blue. The membrane was then dehydrated in methanol, dried under vacuum for 30 min, and kept at 4 °C.

**Limited Proteolysis with Boilysin—**Heptameric aerolysin (0.4 mg/ml, in 30 mM NaCl, 20 mM HEPES, pH 7.4) was incubated in the presence of 5 mM CaCl₂ and 0.1% SDS for 10 min at 70 °C. Then boilysin, which was kindly provided by Dr. van den Burg (23) was added to a protein to protease ratio of 200 and the sample was incubated for an additional 10 min at 70 °C. Proteolysis was performed in the presence of 0.1% SDS in order to disperse aggregated heptamers. Similar results were obtained in the absence of SDS but lead to bands that were not as sharp (not shown). The boilysin-treated sample was then precipitated with chloroform/methanol. A fraction of the sample was analyzed by SDS-PAGE. The remainder of the sample was treated with 70% formic acid for 30 min at room temperature to disassemble the heptameric complexes. The obtained peptide sample was split in two. Part was analyzed by matrix-assisted laser desorption ionization-time of flight mass spectrometry and the rest was analyzed on Tricine-SDS gels (24). The peptide gel was transferred onto a PVDF Porablot membrane overnight at 40 V using a buffer transfer containing 12.5 mM Tris, 92 mM glycine, and 10% methanol.

**Other Methods—**In order to disrupt the aerolysin heptamer, the complex was treated with 70% formic acid for 30 min at room temperature. The sample was then frozen and evaporated using a Speed-Vac. The sample was resuspended in water, frozen, and evaporated again. Unless specified, protein transfer onto PVDF membranes was performed using classical procedures. Amino-terminal amino acid sequencing was performed using an ABI 477A or 473A protein sequencer and the standard Edman chemistry provided by the manufacturer (Applied Biosystems and ABI, Weiterstadt, Germany).

**RESULTS**

**Identification of Disulfide Bridges—**Proaerolysin contains 4 cysteine residues (Fig. 1), which according to the crystal structure are involved in two disulfide bridges: Cys¹⁵⁻⁹⁻Cys⁷⁵ and Cys¹⁵⁹⁻⁴⁻Cys¹⁶⁴ (3). For our studies, it was important to confirm the existence of these disulfide bridges experimentally.

To reveal the existence of Cys¹⁵⁻⁹⁻Cys⁷⁵ disulfide bridge, we made use of the fact that methionine residues, which constitute cyanogen bromide (CNBr) cleavage sites, separate these two cysteines. CNBr fragmentation of proaerolysin is predicted to give rise to a variety of fragments among which two fragments of 9 and 4.5 kDa, respectively, containing Cys75 and Cys19. If these two cysteines are bridged, a 13.5-kDa fragment should be observed under nonreducing conditions but not under reducing conditions. As shown in Fig. 2A, this is indeed the case.

To confirm the existence of a bridge between Cys¹⁵⁹⁻⁴⁻Cys¹⁶⁴ we used another approach. It has been previously shown that treatment of proaerolysin with trypsin only leads to the cleavage of a COOH-terminal peptide and that the mature aerolysin thus obtained is very resistant to further proteolysis (7). However, as shown Fig. 2B, when wild type proaerolysin was treated with trypsin in the presence of 10 mM DTT, two additional bands appeared with apparent molecular masses of 16 and 30 kDa indicating that an additional cleavage site became accessible upon reduction of disulfide bridges. The...
same cleavage pattern was observed when cleaving a proaerolysin mutant in which Cys159 was mutated to serine thereby preventing the formation of a disulfide bond. Amino-terminal sequencing revealed that fragment 1 in Fig. 2B contained two fragments starting at Arg163 and Cys164, respectively, indicating that cleavage had occurred between the two cysteines, and band 2 had as expected the same NH2 terminus as the full-length toxin. When the wild type toxin is treated with trypsin in the absence of DTT, NH2-terminal sequencing revealed that fragment 1 in Fig. 2B contained two fragments of 9.0 and 4.5 kDa. The difference in migration pattern indicate that Cys19 and Cys 75, that are separated by 3 methionine residues, formed a disulfide bridge in the native protein. B, wild type proaerolysin was incubated in the presence of 10 mM DTT for 15 min at room temperature and then treated with trypsin (1/50, mol:mol) for 20 min. SDS-PAGE analysis of the sample revealed the presence of two bands in addition to the aerolysin band. The same migration pattern was observed after treatment of the C159S proaerolysin mutant with trypsin in the absence of DTT. NH2-terminal sequencing revealed that the 30-kDa band contained two fragments with the following NH2 termini, XGDKTAI and XXXXDKTAIKV, and the 16-kDa band contained one fragment with the same NH2 terminus as the full-length toxin. When the wild type toxin is treated with trypsin in the absence of DTT, the precursor is fully converted to aerolysin without any evidence for further breakdown.

**FIG. 2.** Proaerolysin contains two disulfide bridges. A, proaerolysin was subjected to CNBr fragmentation and analyzed on a Tricine-SDS gel under reducing (with β-mercaptoethanol, + ) and nonreducing (without β-mercaptoethanol, − ) conditions. As predicted, in the absence of β-mercaptoethanol, the three largest peptides had molecular masses of 27.8, 13.6, and 8.8 kDa. Upon reduction the 13.6-kDa fragment led to two fragments of 9.0 and 4.5 kDa. The difference in migration pattern indicate that Cys19 and Cys75, that are separated by 3 methionine residues, formed a disulfide bridge in the native protein. B, wild type proaerolysin was incubated in the presence of 10 mM DTT for 15 min at room temperature and then treated with trypsin (1/50, mol:mol) for 20 min. SDS-PAGE analysis of the sample revealed the presence of two bands in addition to the aerolysin band. The same migration pattern was observed after treatment of the C159S proaerolysin mutant with trypsin in the absence of DTT. NH2-terminal sequencing revealed that the 30-kDa band contained two fragments with the following NH2 termini, XGDKTAI and XXXXDKTAIKV, and the 16-kDa band contained one fragment with the same NH2 terminus as the full-length toxin. When the wild type toxin is treated with trypsin in the absence of DTT, the precursor is fully converted to aerolysin without any evidence for further breakdown.

The biphasic nature of the urea unfolding curve (Fig. 3B) suggests that domains of the protein unfold sequentially. The possibility that the two steps correspond to the unfolding of two independent folding units is further strengthened by the observation that both temperature and pH selectively affected only the first step (not shown).

**Effects of Disulfide Bonds on the Stability of Proaerolysin in**
Thermodynamic parameters of proaerolysin unfolding

Thermodynamic parameters of wild type and mutant proaerolysin and of the aerolysin heptamer were calculated as described by Pace et al. (21). $\Delta G(H_2O)$ represents the free energy in the absence of denaturant, $m$ is a measure of the dependence of $\Delta G$ on denaturant concentration, i.e. of the ability of the denaturant to unfold a protein, and $[D]_{1/2}$ the urea concentration for which the midpoint of the transition is reached. Calculations were performed considering the contribution of proaerolysin or aerolysin monomers, even in the case of heptamer where the complex never dissociates.

| Protein                  | Denaturant | $\Delta G(H_2O)$ kcal/mol | $m$ | $[D]_{1/2}$ M | pH | °C |
|--------------------------|------------|----------------------------|-----|--------------|----|----|
| Wild type Proaerolysin   | Urea       | 7.5                        | 2.9 | 7.4          | 25 |    |
| Wild type Proaerolysin   | Urea       | 2.0                        | 1.6 | 7.4          | 37 |    |
| Wild type Proaerolysin   | Urea       | 7.6                        | 2.4 | 8.4          | 25 |    |
| Wild type Proaerolysin   | GdnHCl     | 10.0                       | 0.9 | 7.4          | 25 |    |
| Wild type Proaerolysin   | GdnHCl     | 4.6                        | 2.9 | 7.4          | 25 |    |
| Wild type Proaerolysin + DTT | Urea     | 2.4                        | 7.4 | 25          |    |    |
| Heptamer                 | GdnHCl     | 20.0                       | 3.8 | 7.4          | 25 |    |
| C159S Proaerolysin       | Urea       | 5.1                        | 2.0 | 7.4          | 25 |    |
| C159S Proaerolysin       | Urea       | 6.9                        | 2.7 | 7.4          | 25 |    |

**Urea**—We next studied the contribution of the two disulfide bridges to the stability of proaerolysin. Urea denaturation experiments were first performed using the wild type toxin in the presence of DTT. Under these conditions unfolding was irreversible and therefore thermodynamic parameters could not be calculated except for the midpoint transition (Table I). As shown in Fig. 4, DTT had a dramatic effect on both unfolding steps leading to an apparently monophasic curve. These observations indicate that at least one of the two bridges strongly contributes to the stability of the protein.

To specifically investigate the role of the Cys159-Cys164 bridge, we analyzed the urea denaturation of the C159S proaerolysin mutant. Interestingly breaking of the Cys159-Cys164 bridge by this mutation only affected the first unfolding transition, whereas the second was similar to the one observed for the wild type protein (Fig. 4). Thus the Cys159-Cys164 bridge appears to be important for stability despite the fact the two cysteines are only 5 amino acids apart. The fact that DTT did have an effect on the stability of the C159S mutant indicates that breaking the Cys159-Cys164 bridge had a strong destabilizing effect. Therefore both disulfide bridges contribute to the stability of proaerolysin.

These results also indicate that the biphasic unfolding of proaerolysin corresponds to the unfolding of the large lobe of the protein (containing the Cys159-Cys164 bridge), followed at higher urea concentrations by the unfolding of domain 1 (containing the Cys138-Cys275 bridge). The two disulfide bridges strongly contribute to the stability of the protein domain in which they are located.

**Unfolding of the Aerolysin Heptamer**—We next studied the stability of the aerolysin heptamer, which is its channel conforming configuration by analyzing the effect of the urea on the tryptophan fluorescence of the heptamer. Quite remarkably, no change in fluorescence intensity or in maximal emission wavelength could be observed after 24 h incubation of the complex with 8 M urea (Fig. 5A). Unfolding of the heptamer required high concentrations of a more potent chaotropic agent, guanidinium hydrochloride (GdnHCl). Unfolding appeared very cooperative and occurred in a single step with a midpoint transition at 3.8 M (Fig. 5B, Table I). For comparison, unfolding of proaerolysin in GdnHCl was also analyzed. As observed in urea, unfolding followed a two-step process (Fig. 5B). This first midpoint transition was at 0.9 M GdnHCl and the second at 2.9 M. We found that DTT affected unfolding of the heptamer indicating that the disulfide bridges contributed to its stability.

The above experiments illustrate the uncommon stability of the noncovalent aerolysin heptamer. In order to identify the regions of the protein that are involved in maintaining the heptamer, we have performed limited proteolysis under conditions that would not lead to full heptamer disassembly.

**Partial Proteolysis of the Aerolysin Heptamer**—We were able to generate a partially degraded aerolysin heptamer after prolonged incubation (7 days) in the presence of 0.1% SDS and of residual trypsin remaining after activation (Fig. 6). This truncated complex ran as a double band slightly below the 200-kDa molecular mass marker on acrylamide gradient gels. To determine where cleavage had occurred, the truncated complex was dissociated into monomers by formic acid treatment (25) (Fig. 6A, lane 2). In addition to the aerolysin band (presumably resulting from the nondegraded full-length heptamer), a major polypeptide with an apparent molecular mass of 37 kDa and a less abundant band (corresponding to a doublet) with an apparent molecular mass of 22 kDa were detected. Amino-terminal sequencing revealed that the 37-kDa fragment and one of the fragments running at 22 kDa had the same NH$_2$ terminus, i.e. Asp 139, whereas the second 22-kDa fragment started at Trp 149. The calculated molecular mass of a fragment going from Asp 139 to the trypsin activation site at Lys 267 is 32.4 kDa. A heptamer formed by such fragments would have a mass of 227 kDa, which is in close agreement with the apparent molecular mass of the digested complex (Fig. 6A, lane 2). It is therefore likely that all monomers in the truncated heptamer lacked the first 138 amino acids and that some also underwent COOH-terminal cleavage.

We next performed limited proteolysis of the heptamer with trypsin at an enzyme to protein ratio of two to one for 3 days. Shorter incubation times could be used when the heptamers were treated with trypsin within the SDS gel itself (see "Experimental Procedures"). As shown Fig. 6B (lane 2), trypsin treatment led to the accumulation of three oligomeric species of approximately 200, 160, and 75 kDa apparent molecular mass. The fact that all species ran higher than the aerolysin monomer indicates that the heptameric organization was at least
partially maintained. This was confirmed by the fact that these bands could no longer be seen when the trypsin-treated sample was incubated with formic acid prior to SDS-PAGE (not shown).

Truncated oligomers were transferred to PVDF membranes and NH$_2$-terminal sequencing was carried out to identify the fragments. Transfer of the 200-kDa complex was so inefficient that we were unable to obtained sequence information. The 160-kDa complex was found to contain a single NH$_2$ terminus, starting at Gln195 (Table II, Fig. 6). However, considering that the limit of sensitivity is about 1 pmol, we cannot exclude that other less abundant NH$_2$ termini were present. The 75-kDa complex contained 5 detectable NH$_2$ termini. The two most abundant NH$_2$ termini, each corresponding to ~27% of the total, were His186 and Gly402 (Table II, Fig. 6B). The three less abundant NH$_2$ termini found in this complex were Gln195 (~18% of total), Ser192 (~14% of total), and Ala398 (~14% of total). The detectable NH$_2$ termini present the 75-kDa complex indicate that cleavage occurred in the 185–194 region (in domain 3) and in the 397–401 region (at the domain 2-domain 3 boundary). The simplest interpretation of these results is that trypsin had released at least 185 residues for the NH$_2$ terminus in all seven monomers and that all contained the COOH-terminal region (amino acids 398 to the end, i.e. Lys$^{427}$).

**Fig. 5.** Effect of heptamerization on the stability of the toxin in GdnHCl. Proaerolysin and heptameric aerolysin (20 μg/ml) in 150 NaCl, 30 mM HEPES, pH 7.4, were denatured in urea (A) or GdnHCl (B) for 24 h at 25 °C and the ratio between the fluorescence intensities at 345 and 315 nm was calculated as a function of the urea concentration. Denaturation of the heptamer in GdnHCl was also performed in the presence of 10 mM DTT 24 h at 25 °C.

| Band | Apparent $M_r$ | NH$_2$-terminal sequence | Starting amino acid | Calculated full-length $M_r$ |
|------|----------------|--------------------------|--------------------|-----------------------------|
| Fragment 1 | 37 | DMDVTRDG | 139 | 32.4 |
| Fragment 2a | 22 | DMDV | 139 | 32.4 |
| Fragment 2b | 22 | WVIRG | 149 | 31.7 |
| Trypsin | | | |
| Band 2 | 160 | XXXXTVGGXAN | 195 | 26.3 |
| Band 3a | 75 | HGDTVQSDRQ*** | 186 | 27.3 |
| Band 3b | 75 | GDFSAESQFA*** | 402 | 2.6 |
| Band 3c | 75 | QVLKTVVXAXA** | 195 | 26.3 |
| Band 3d | 75 | SDRQVKTVV* | 398 | 2.9 |
| Band 3e | 75 | AGITG | |
| Boilysin | | | |
| Fragment 3a (major band) | 14.4 | LDPDSFK | 179 |
| Fragment 3b (minor band) | 13.8 | FKHGDVT | 184 |
| Fragment 3c (minor band) | 13.2 | VTQS | 189 |

**Table II**

NH$_2$-terminal sequencing of the aerolysin heptamer proteolytic fragments

The NH$_2$-terminal sequence of the proteolytic fragments shown in Figs. 6 and 7 are summarized. The calculated full-length molecular weight ($M_r$) corresponds to the calculated weight of the peptide started at the determined N terminus and ending at Lys$^{427}$, the trypsin activation site. The NH$_2$-terminal labeled with three asterisks (***), each accounted for 27% of all NH$_2$-terminal in the 75-kDa complex, the NH$_2$-terminal labeled with two asterisks (**) for 18%, and the NH$_2$-terminal labeled with one asterisk (*) for 14% each.
Boilysin cleavage led to a similar cleavage pattern as trypsin with two major bands with apparent masses of 160 and 75 kDa (Fig. 7A, lane 3). Upon dissociation of the complex by formic acid treatment, three bands of similar molecular weights could be detected on Tris glycine peptide gels (Fig. 7B, indicated by lines in lane 3). The three bands were sequenced separately by cutting the blot in very thin strips and not sequencing as a mixture. The top major peptide started at Leu^{179} as determined by NH\textsubscript{2}-terminal sequencing (Table II). The second minor peptide started at Phe^{184}, and finally the smallest minor peptide started at Val^{189} (Table II). The cross-contamination levels between the three bands were low. Matrix-assisted laser desorption ionization-time of flight mass spectrometry analysis indicated that these three peptides had a mass of 14.38, 13.82, and 13.24 kDa, respectively. When taking into account the NH\textsubscript{2} termini and the masses, it appears that all three ended at Glu\textsuperscript{307}.

The above proteolysis studies show that amino acids 1–178 can be removed without triggering full disassembly of the heptamer. Since the boilysin fragments ended at Glu\textsuperscript{307} and no other peptides could be detected, it appears that domains 1 and 2 had been completely removed from the heptamer complex. Although peptides containing residues 401 to 427 (corresponding to the trypsin activation site) were not detected in the boilysin-treated samples it seems unlikely that they had been removed by the enzyme. They indeed are part of a 4-stranded \(\beta\)-sheet in domain 3 (Fig. 1). More likely this small peptide of 2.7 kDa was not detected on our peptide gels. This hypothesis is supported by the presence of NH\textsubscript{2} termini starting in the 397–401 regions in the truncated complexes obtained by trypsin treatment (Fig. 6B, Table II).

**DISCUSSION**

In the present work we have analyzed the stability of the pore-forming toxin aerolysin both in its precursor form and in its heptameric complex. Unfolding of the proaerolysin in urea was biphasic. The first phase could be attributed to the unfolding of the COOH-terminal elongated lobe (domains 2 to 4) of the proaerolysin molecule and the second to the unfolding of domain 1 (NH\textsubscript{2}-terminal domain) which was by far the most stable part of the protein. Both the large COOH-terminal domain and the NH\textsubscript{2}-terminal domain 1 were found to owe their stability in part to the presence of a disulfide bridge (Cys\textsuperscript{159}, Cys\textsuperscript{164} and Cys\textsuperscript{199-Cys\textsuperscript{174}}, respectively). It was somewhat surprising to find that the Cys\textsuperscript{159-Cys\textsuperscript{164}} (Fig. 1) had a role in stability since this bridge links two cysteines that are only 5 amino acids apart in the primary sequence. This bridge was also found to protect a surface exposed loop at the top of domain 2 from proteolysis.

The stability of the toxin was dramatically enhanced upon heptamerization of aerolysin. Incubation for 24 h with 8 \(\times\) urea failed to disassemble the subunits and did not even affect the fluorescence of tryptophan residues indicating that they were still in a rigid environment. Also, incubation of the heptamer with 1\% SDS for 3 h did not affect the tertiary structure as witnessed by near ultraviolet circular dichroism (not shown). Loosening of the structure, without separating the subunits, however, required a 24-h incubation with 4 \(\times\) of the more potent chaotropic reagent GdnHCl. Such incredible stability is reminiscent of fibrillar protein aggregates which form upon self-assembly of prion protein or Alzheimer \(\beta\)-amyloid peptide (26–28). Extremely high stability is also found in enzymes produced by extremophilic bacteria (29). In many of these proteins, increased stability is reached by association of subunits (30).

To identify the regions of the protein that are responsible for maintaining the heptamer so tightly assembled, we established proteolysis conditions that would cleave the protein but not fully disassemble the complex. Not surprisingly, considering our observations on the very high stability of the complex, the heptamer was very insensitive to proteolysis. Nicking required either very high trypsin concentrations and several days of incubation or treatment at 70 °C with the engineered thermostable enzyme boilysin (23). It is attractive to speculate that the resistance of the heptamer to proteolysis under physiological conditions would be an explanation for the inability of mammalian cells to recover after aerolysin treatment. We have

**FIG. 7. Limited proteolysis of the aerolysin heptamer using boilysin.** The aerolysin heptamer was incubated with a 1/200 molar ratio of boilysin for 10 min at 70 °C in the presence of 0.1% SDS. The truncation products were analyzed on a SDS 10% gel (A). In parallel the boilysin-treated sample was treated with 70% formic acid in order to disassemble the truncated complexes. The obtained fragments were analyzed on Tricine-SDS gels (B). In A: lane 1, proaerolysin; lane 2, marker for full-length heptameric aerolysin; lane 3, boilysin-treated sample. In B: lane 1, proaerolysin; lane 2, boilysin-treated sample; lane 3, boilysin-treated sample after formic acid treatment.

**FIG. 8. Region in aerolysin involved in maintaining the heptamer assembled.** Limited proteolysis studies suggest that two amino acid stretches, 180–307 and 401–427, which composed domains 3 and 4, are involved in the maintenance of the aerolysin heptamer. These amino acids have been **underlined** with a **black bar** on the figure. The two disulfide bonds are also indicated on the primary sequence of aerolysin. Domain 1, residues 1 to 82, are **unboxed**; domain 2, residues 83–178, 311–389 are **boxed in gray**; domain 3, residues 179–194, 224–274, 299–409 and 399–409 are **boxed in black**; domain 4, 185–223, 275–298, 410–427 are **boxed in white**.
indeed never seen recovery of aerolysin-treated cells in contrast to
the situation reported for Staphylococcal α-toxin (31) and



Vibrio cholerae



El Tor cytolytic (32), two toxins that have
modes of action very similar to that of aerolysin. In contrast to
the aerolysin heptamer, the Staphylococcal α-toxin heptamer is
heat labile and sensitive to proteases (33, 34) and might there-
fore be degraded by cellular proteases after internalization of
plasma membrane patches that contain the pore. This type of
repair mechanism has indeed been proposed but remains to be
demonstrated.

The analysis of the fragments obtained after harsh proteo-
lytic treatment showed that the truncated complexes contained
residues ~180 to 307 and 401 to 427 of aerolysin, which
constitute domains 3 and 4 (Fig. 8, in dark gray). These results
suggest that domain 1 and domain 2 could be excised from the
heptamer without provoking complete disassembly of the com-
xplex. This indicates that domain 1 is not involved in main-
taining the heptamer assembled, even though it is the most stable
part of proaerolysin and appears to be crucial for dimerization
at high toxin concentrations. Heptamerization, in contrast to
dimerization, might therefore not occur through a domain-
swapping interaction. Alternatively it could occur by a swap-
ning mechanism involving different domains. The fact that
domain 2, which is the largest domain in the protein (Figs. 1
and 8), could also be excised from the complex in addition to
domain 1 is in agreement with the model of the aerolysin
channel proposed by Parker et al. (3). According to this model,
domain 1 of one monomer interacts with domain 2 of the next
monomer in the circular assembly. Therefore, upon removal of
domain 1, domain 2 would no longer be involved in monomer-
monomer contacts and could become accessible to proteases.

Thus domains 3 and 4, and more specifically residues 180 to
307 and 401–427, are sufficient to maintain the complex as-
sembled and to confer SDS resistance since the truncated hept-
amers could be detected after SDS-PAGE. Domains 3 and 4
are exclusively composed of β-strands and random structure
indicating that, as for amyloids, stable self-assembly of aerolysin
involves β-sheet domains (26, 30, 35). Self-association of
β-sheet domains resulting in the formation of pathogenic pro-
tein aggregates is a characteristic feature of various medical
disorders including Huntington’s, Alzheimer’s, and Creutzfeld-
Jacob’s diseases (36). Further studies on the dissociation and
reassociation mechanisms of aerolysin, which forms a smaller
complex that is more amenable to biochemical and spectroscop-
ical analyz. es, are likely to contribute to understanding the
aggregation processes of these pathogenic protein complexes.

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