Oligomerization of the Channel-Forming Toxin Aerolysin Precedes Insertion into Lipid Bilayers†

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ABSTRACT: Oligomerization is a necessary step in channel formation by the bacterial toxin aerolysin. We have identified a region of aerolysin containing two tryptophans which influence the ability of the protein to oligomerize. Changing the tryptophan at position 371 or 373 to leucine resulted in mutant proteins that oligomerized at much lower concentrations than the wild-type toxin. Near-ultraviolet circular dichroism measurements showed that the tertiary structures of the L-371 and L-373 mutant toxins may be slightly different from the structure of wild type. Other single amino acid replacements in the same region of the protein as the two tryptophans appeared to have little or no effect on any properties of the protein. None of the changes we made had any measured effect on secretion of the protein by the bacteria. The L-373 and L-371 proteins induced chloride release from liposomes at lower concentrations than native toxin. Wild-type aerolysin solutions were completely unable to cause release when oligomeric toxin was absent or when it was removed by centrifugation. Aerolysin changed at H-132, which cannot form oligomers, was also inactive against liposomes. We conclude that aerolysin channels are produced by direct insertion of oligomers formed in solution, or assembled on the surface of the cell after binding to the receptor, and not by lateral diffusion of the monomer after it enters the lipid bilayer.

Aerolysin is a channel-forming toxin released by the Gram-negative bacterium *Aeromonas hydrophila* that is responsible for the pathogenicity of this organism in mammals [for a review see Buckley (1992)]. We have shown that the protein is secreted as a protoxin that is activated by proteolytic removal of a C-terminal fragment (Howard & Buckley, 1985). The activated toxin binds with high affinity to the erythrocyte transmembrane protein glycophorin. This effectively concentrates aerolysin on the surface of the cells and leads first to the production of extremely stable oligomeric structures and then to channel formation and cell lysis. Channel formation by means of "barrel–stave" assembly of monomers is common to many cytolytic proteins (Ehrenstein & Lecar, 1977; Ojcius & Young, 1991), but it has not been clearly established for any toxin whether oligomers are formed prior to insertion into the membrane or by lateral movement of monomers in the membrane after they insert.

Aerolysin shares many properties with α toxin of *Staphylococcus aureus*, in spite of the fact that the primary structures of the two proteins seem to be nearly completely unrelated. They are water-soluble hydrophilic proteins that must oligomerize in order to form channels, and oligomerization of both toxins depends on at least one histidine residue and is blocked by zinc (Garland & Buckley, 1988; Wilmsen et al., 1990; Pederzolli et al., 1991). Bhakdi and Tranum-Jensem (1991) have proposed that α toxin oligomerizes after it inserts into the cell membrane. In contrast, we have circumstantial evidence, based on the kinetics of channel formation in erythrocytes (Garland & Buckley, 1988), that oligomerization of aerolysin occurs before insertion. This is consistent with the hydrophilic nature of the monomer and with our recent observation that oligomerization, which can occur spontaneously in solution at high protein concentrations, is accompanied by the exposure of a hydrophobic surface (van der Goot et al., 1992).

In this communication we describe several aerolysin mutants obtained by site-directed mutagenesis of the sequence KWWDW in aerolysin. A similar sequence is conserved in the oxygen-labile toxins, and de Kruijff (1990) has proposed that the tryptophans it contains may play a role in membrane penetration. We show that two of the aerolysin mutants oligomerize more readily than the native protein and we exploit their properties to examine the role of the aerolysin oligomer in channel formation.

EXPERIMENTAL PROCEDURES

Materials. Dilauroylphosphatidylglycerol (DLPG)[1] was obtained from Avanti Lipids. Phosphatidylcholine (EPC) and phosphatidic acid (EPA) from egg yolk were purchased from Lipid Products. L-α-Dioleoylphosphatidylglycerol (DOPG), soybean trypsin inhibitor, and trypsin were from Sigma. The chloride-sensitive dye 6-methoxy-*N*-(3-sulfopropyl)quinolinium (SPQ) and 8-anilino-1-naphthalenesulfonate (ANS) were obtained from Molecular Probes (Eugene, OR).

Bacterial Strains and Plasmids. Escherichia coli strain TG1 was supplied by Amersham Corp. and used in site-directed mutagenesis experiments. Mutant plasmids were constructed by digesting replicative form preparations with restriction enzymes *Eco*RI and *Hin*dIII and ligating into plasmid pMMB66HE (Furste et al., 1986). Plasmid constructs were transformed into *E. coli* strain HB101 (*recA13 hsdS20 ara-14 proA2 LacY1 galK2 leuB6 rpsL20 xyl-5 mtl-1 supE44*). Plasmids were moved into *Aeromonas salmonicida* strain CB3

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[1] Abbreviations: ANS, 8-anilino-1-naphthalenesulfonate; DLPG, dilauroylphosphatidylglycerol; DOPG, L-α-dioleoylphosphatidylglycerol; EPA, phosphatidic acid from egg yolk; EPC, phosphatidylcholine from egg yolk; SPQ, 6-methoxy-*N*-(3-sulfopropyl)quinolinium.

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(Buckley, 1990) using the conjugative helper plasmid pRK2013 (Figurski & Helinski, 1979).

Cell Growth. *E. coli* HB101 was grown at 37 °C in LB medium (Maniatis et al., 1982) and TG1 was grown as recommended by the supplier. *A. salmonicida* Rif-1 and CB3 were grown at 27 °C in LB medium buffered according to Davis (Miller, 1972) and supplemented with 0.2% glucose. When appropriate, antibiotics were added to the following concentrations: ampicillin (100 μg/mL), rifampicin (40 μg/mL), and kanamycin (40 μg/mL).

Site-Directed Mutagenesis. Tryptophan 371 was replaced with leucine by changing the codon TGG to TTG in *aerA*. In the same way W373 (TGG) was changed to L373 (TTG), K369 (AAG) to Q (CAG), and D372 (GAC) to N (AAC). All mutagenesis reactions were performed using the methods of Eckstein et al. (1985, 1986, 1988) with materials supplied by the Amersham Corp. Positive clones were screened initially by plaque hybridization or by dot blot hybridization. Final confirmation of the nucleotide changes in each of the clones was accomplished by chain-terminating DNA sequencing of the entire gene using Sequenase version 2.0, with materials and procedures provided by U.S. Biochemical Corp. Once a clone was identified by these methods, the DNA was inserted into the vector pMMB66HE as we have described previously (Wong et al., 1989, 1990).

Plasmid Manipulations and Bacterial Matings. Plasmid preparations and cloning procedures, as well as bacterial matings between HB101 bearing mutagenized *aerA*/pMMB66 constructs and *A. salmonicida* Rif-1 or CB3, were carried out as before (Wong et al., 1990).

Protein Purification. Proaerolysin and the mutant proteins were purified according to our published procedure (Buckley, 1990). Each protein migrated as a single band upon sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Neville, 1971).

Hemolytic Titers. Titers were obtained with human erythrocytes as previously described (Howard & Buckley, 1982). Values are expressed as the $\log_2$ of the highest dilutions that resulted in complete cell lysis.

Activation of Aerolysin. Unless otherwise specified, proaerolysin was activated by adding 5 μL of 100 μg/mL trypsin to 100 μL of protoxin at a concentration of 1–2 mg/mL in 0.3 M NaCl and 20 mM Tris, pH 7.4. After 10 min at room temperature, 5 μL of 1 mg/mL trypsin inhibitor was added and the mixture was stored on ice. This procedure results in the quantitative conversion of the protoxin to aerolysin but does not cause degradation of the toxin (Garland & Buckley, 1988).

Circular Dichroism. Circular dichroic experiments in the near ultraviolet were carried out at 25 °C with a Jobin Yvon Mark V spectrometer. Quartz cells with a 1-cm path length were used. The protein concentration was typically 0.5–0.6 mg/mL in 100 mM NaF and 10 mM Hepes, pH 6.8.

Tryptophan Fluorescence. Fluorescence measurements were made with an SLM 8000 spectrofluorometer (Urbana, IL) operating in ratio mode. The excitation wavelength was 295 nm and slit widths were 8 nm for both excitation and emission. Samples were continuously stirred in thermostated 3-mL quartz cuvettes at 25 °C. Unless otherwise specified, the buffer was 150 mM NaCl and 20 mM Tris–acetate, pH 7.4. For each recorded spectrum, the Raman scatter contribution was removed by subtraction of a buffer blank.

ANS Binding. Binding of 8-anilino-1-naphthalenesulfonate (ANS) was measured in 0.5-mL quartz cuvettes containing 20 mM Tris–acetate, pH 6.8, at 37 °C. The excitation and emission wavelengths were 380 and 480 nm, respectively, with spectral bandwidths of 4 nm. The final ANS concentration was 50 μM.

Light Scattering. Light scattering experiments were performed in 0.5-mL quartz cuvettes containing 150 mM NaCl and 20 mM Tris–acetate, pH 7.4, at 36 °C. The SLM 8000 spectrofluorometer was used in ratio mode, with a spectral bandwidth of 8 nm for excitation (λ_{ex} = 450 nm).

Vesicle Preparation. Large unilamellar liposomes were prepared by reverse-phase evaporation according to Szoka and Papahadjopoulos (1978) using a mixture of EPC, EPA, and DOPG (6:2:2 w/w/w) suspended by brief sonication in 100 mM KCl and 10 mM Hepes, containing 1.5 mg/mL SPQ, pH 7.4. The suspension was extruded through 0.2-μm polycarbonate filters (Nucleopore, Pleasanton, CA) and passed down a gel-filtration column (PD10, Pharmacia, Uppsala, Sweden) equilibrated with the same buffer in order to remove the extravesicular dye.

Chloride Efflux Measurements. These experiments were also carried out with the SLM 8000 spectrofluorometer, operating in ratio mode with spectral bandwidths of 8 nm for both excitation and emission. The dye was excited at 350 nm and emission was measured at 422 nm. Disposable polystyrene fluorometer cuvettes (Sigma) were used to minimize the risk of cross-contamination. Liposomes were diluted to a final lipid concentration of 48 μM in a buffer containing 100 mM KNO_3 and 10 mM Hepes, pH 7.4. Samples were stirred continuously in a thermostated cell holder at 25 °C. Spontaneous leakage of chloride from liposomes prepared in this way was negligible within the time frame of our experiments.

Other Methods. Sodium dodecyl sulfate–polyacrylamide electrophoresis (SDS–PAGE) was performed using the Laemmli (1973) buffer system for the acrylamide gradient gels (3–15% acrylamide, 2.6% cross-linker) and the Neville (1971) system for regular gels. Proteins were stained with Coomassie blue. Aerolysin concentrations were calculated using an absorbance of 1.5 at 280 nm for pure 1 mg/mL solutions of toxin and protoxin.

RESULTS

Secretion of Mutant Proteins by Aeromonas salmonicida and Hemolytic Activity. All of the four mutant proaerolysins, including those with leucine replacing tryptophan at 371 or 373, were secreted by *A. salmonicida* containing the cloned structural genes in amounts comparable to wild-type protein (not shown here). These results should be contrasted with our earlier observation that changing W-227 of proaerolysin to leucine causes a nearly complete block in the secretion of the resulting mutant protein (Wong & Buckley, 1991). In addition, all four of the mutant aerolysins had hemolytic titers that were not very different from aerolysin itself (also not shown), although L-371 consistently had slightly lower titers than native toxin.

CD Spectroscopy in the Near-Ultraviolet of Wild-Type and Mutant Proaerolysin. We have previously shown that the near-ultraviolet CD spectrum of proaerolysin contains a very broad peak around 270 nm and two distinct peaks at 289 and 296 nm (van der Goot et al., 1992). The spectra of Q-369 and N-372 proaerolysins were very similar to the spectrum of native protein (not shown), whereas the spectra of the L-371 and L-373 proaerolysins, compared to that of native protein in Figure 1, appeared to be different. The changes in the spectrum of the L-373 mutant were rather small, suggesting that the tryptophan which was replaced was not a major contributor (there are 18 tryptophans, as well as 21 tyrosines

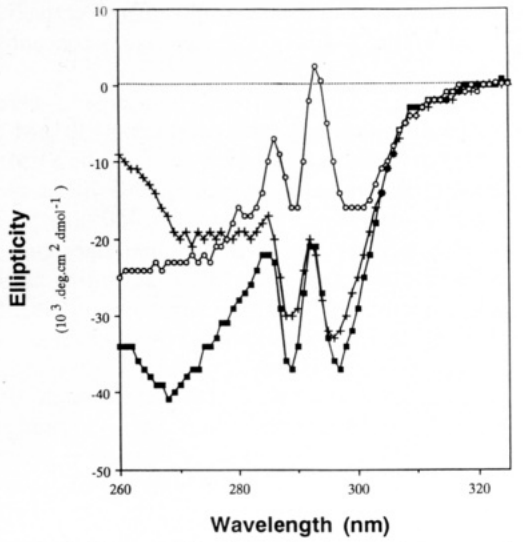

FIGURE 1: Influence of single tryptophan mutations on the near-ultraviolet CD spectrum of proaerolysin. Circular dichroism spectra of wild-type proaerolysin (+) as well as mutant L-371 (○) and L-373 (■) measured in the near ultraviolet. Ellipticities are expressed per mole of protein.

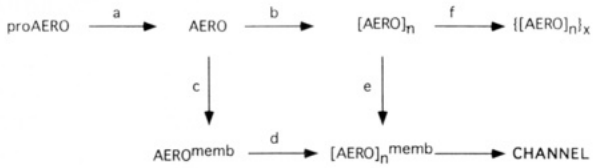

FIGURE 2: Illustration of possible pathways to channel formation by aerolysin. Monomeric aerolysin is formed by protease activation in step a. The monomer may insert in the membrane (c) and oligomerize (d), or it may oligomerize (b) and then insert (e). Small aggregates $\{[AERO]_n\}_x$ may be insertion-competent, but larger aggregates ($y > x$) are inactive.

and 11 phenylalanines, in the native protein) and secondly that the structure of the protein was only slightly altered. In contrast, the difference observed with the L-371 protein was much larger, indicating either that the tryptophan we removed contributed disproportionately to the spectrum or that the structure of the protein was changed. It is worth noting that there was no wavelength shift in the peaks, but rather the spectrum was less intense, suggesting a similar but looser tertiary structure than that of native protein.

Tryptophan Fluorescence upon Activation. We have previously found (van der Goot et al., 1992) that activation of proaerolysin by trypsin induces an increase in tryptophan fluorescence as well as a slight red shift. The kinetics of activation can be followed by measuring tryptophan fluorescence at 350 nm as a function of time. Since this method gives quite reproducible kinetics, it was used to compare the proaerolysin mutants to wild type. At the protein concentration used (0.3 mg/mL), none of the point mutations appeared to cause a change in the activation kinetics (data not shown).

Effect of Mutation on the Ability of Aerolysin to Oligomerize. Native proaerolysin is activated by treatment with trypsin, which removes 43 amino acids from the C-terminus of the protein (van der Goot et al., 1992). The aerolysin that is formed is resistant to further proteolysis (Garland & Buckley, 1988). At the concentrations used here, a typical sample of aerolysin is composed of at least three populations (Figure 2). Immediately after activation, most of the toxin is monomeric; however the monomer gradually oligomerizes in a time- and concentration-dependent manner, producing

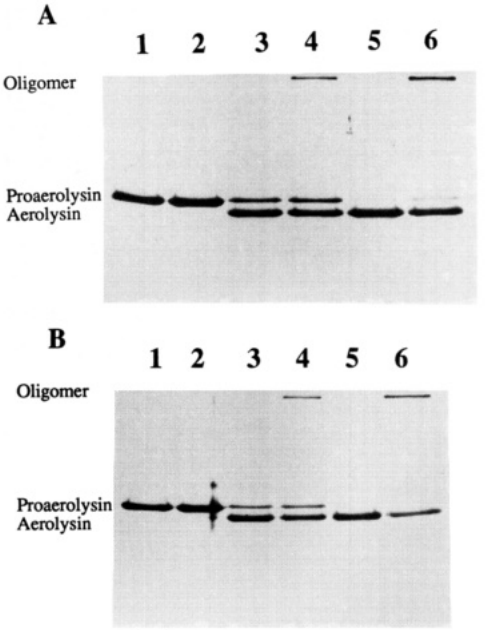

FIGURE 3: Abnormal oligomerization of L-371 and L-373 mutant aerolysins. Wild-type proaerolysin and the proteins modified at W-371 and 373 were activated with trypsin as described in the text. At the indicated times, aliquots were taken, mixed with SDS–PAGE sample buffer, and boiled. (A) Lanes 1, 3, and 5, wild type, 0, 15, and 30 min; lanes 2, 4, and 6, L-371, 0, 15, and 30 min. (B) Same as (A) except that L-373 replaced L-371.

oligomers that can be observed by SDS–PAGE (Figure 3; Garland & Buckley, 1988) as well as by electron microscopy (Wilmsen et al., 1992). Once formed, the oligomers rapidly aggregate, causing concentrated aerolysin preparations to become cloudy. Aggregates of the oligomer are less stable. They can be dissociated to monodisperse oligomers by treatment with SDS or urea (not shown here) and consequently are not seen by SDS–PAGE. The SDS–PAGE results in Figure 3 compare the effects of trypsin on native proaerolysin and the L-371 and L-373 protoxins. It may be seen that both mutant proteins were reduced to the molecular weight of wild-type aerolysin by exposure to trypsin, and there were no signs of further breakdown, indicating that they were correctly processed and that the amino acid replacements had not caused major structural changes in the proteins. However, it is clear from the figure that they had a greater tendency to oligomerize than wild type at comparable concentrations, as evidenced by the appearance of oligomers at the top of the gel. In contrast, the Q-369 and N-372 proaerolysins behaved just like wild type when treated with trypsin (not shown here).

Rayleigh light scattering was used to study the oligomerization of the mutant proteins in a more quantitative way. The results are shown in Figure 4. It may be seen that replacing W-371 or W-373 with leucine had a pronounced effect on the ability of the protein to oligomerize, corroborating the results shown in Figure 3. Whereas we have previously shown that, under the same experimental conditions, activation of wild-type aerolysin does not induce any increase in scattered light at concentrations as high as 0.2 mg/mL (van der Goot et al., 1992), oligomerization of L-373 aerolysin could be detected at protein concentrations as low as 0.05 mg/mL (Figure 4A), and L-371 aerolysin could still oligomerize at concentrations as low as 0.02 mg/mL (Figure 4B).

ANS Binding during Activation. ANS binds to solvent-accessible clusters of nonpolar residues (Stryer, 1965), which are absent in the unfolded state of proteins and relatively rare in the native state. We have shown that wild-type aerolysin

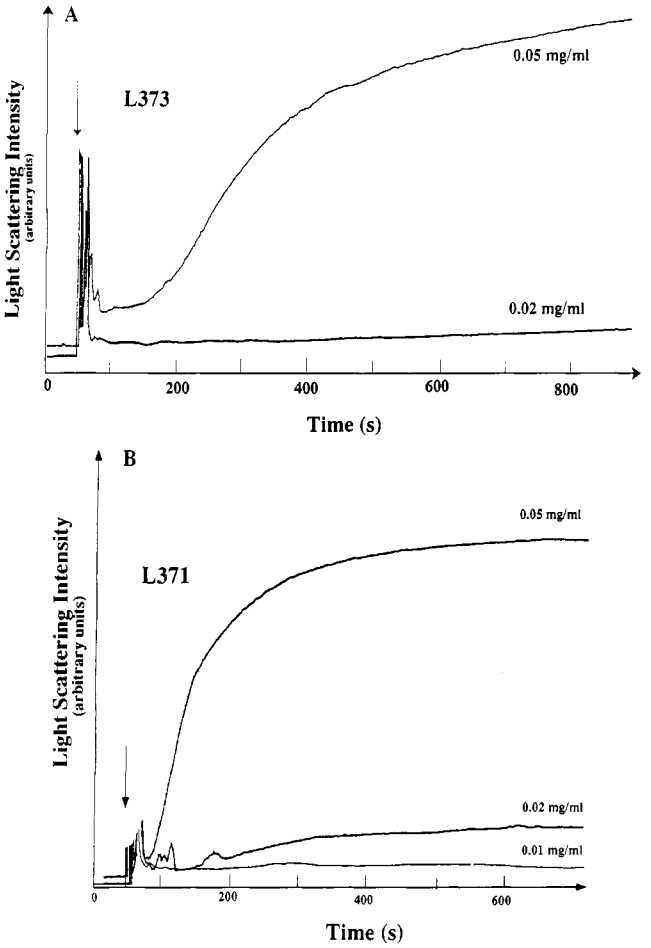

FIGURE 4: Effect of concentration on the ability of L-371 and L-373 aerolysin to oligomerize. Oligomerization of aerolysin after activation was followed by measuring the scattered light (λ_{ex} = 450 nm) as a function of time (T = 36 °C). In all experiments, the protoxin was diluted into 150 mM NaCl and 10 mM Tris–acetate, pH 6.8, and after 50 s of acquisition, trypsin (1 μg/mL) was added (indicated by an arrow). Measurements were performed on L-373 (A) and L-371 (B) at the indicated concentrations.

at a concentration of 0.5 mg/mL (where it oligomerizes spontaneously in solution) binds the hydrophobic probe ANS, indicating that a nonpolar surface is exposed. The N-132 mutant aerolysin, which is unable to oligomerize, does not bind the dye (van der Goot et al., 1992). If ANS binding is a consequence of oligomerization, the L-371 and L-373 mutants should bind the dye at much lower concentrations than wild type. The results in Figure 5 show that this is the case. The experiments were performed at a protein concentration of 0.03 mg/mL. At this concentration, wild-type aerolysin and the Q-369 mutant do not oligomerize at all, whereas both the L-371 and L-373 mutant toxins form oligomers (see below). It may be seen that only the latter two proteins bound ANS after activation, indicating that oligomerization must occur in order for the dye to bind. It may be seen from the results in Figure 6 that the kinetics of ANS binding induced by activation of native proaerolysin with trypsin are very similar to the kinetics observed with light scattering, further demonstrating that both these techniques are measuring the appearance of oligomers.

Effect of Trypsin Activation of Wild-Type and Mutant Proaerolysins on Chloride Release from Liposomes. Aerolysin will also form channels in artificial lipid vesicles (Howard & Buckley, 1982). The release of the chloride-sensitive dye SPQ from large unilamellar liposomes by aerolysin is shown in Figure 7A. The SPQ is initially quenched by the anion

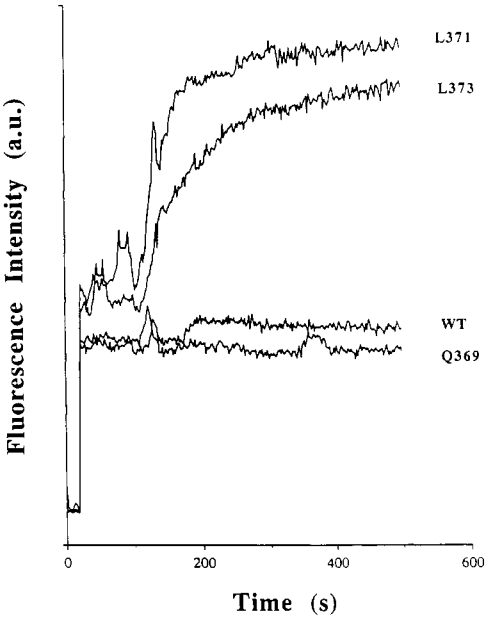

FIGURE 5: ANS binding to wild-type and mutant aerolysins. ANS was diluted into 20 mM Tris–acetate, pH 6.8, to a final concentration of 50 μM (T = 37 °C). After 40 s, mutant or wild-type (WT) proaerolysin was added to the solution (0.03 mg/mL final concentration). At t = 100 s, trypsin was added (1 μg/mL).

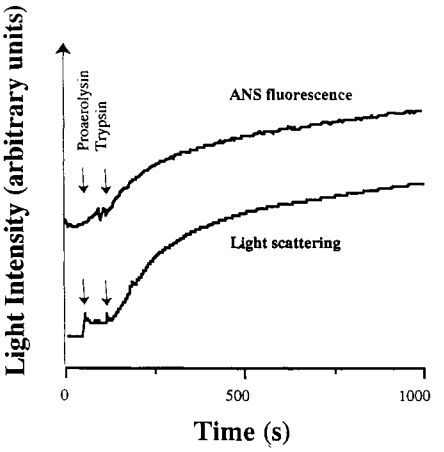

FIGURE 6: Comparison of the kinetics of ANS binding and light scattering upon activation of wild-type aerolysin. ANS was diluted into 70 mM NaCl and 20 mM Tris–acetate, pH 7.4, to a final concentration of 50 μM (T = 37 °C). After 40 s, wild-type proaerolysin was added to a final concentration of 0.12 mg/mL. At t = 100 s, trypsin was added (1 μg/mL). Either ANS fluorescence or light scattering was monitored as a function of time.

inside the vesicles, but when the liposome is punctured, the chloride leaks out and an increase in fluorescence can be observed. Under these conditions, proaerolysin was completely unable to induce chloride leakage at any concentration, an indication that chloride release by activated toxin was not due to some artifactual interaction of the protein with the liposomes. The results show that efflux depended on the concentration of aerolysin, indicating that we could use this procedure to compare the activity of different preparations. The experiment in Figure 7A was performed by taking different aliquots from the same 2.2 mg/mL stock solution of aerolysin, so that the relative amounts of aggregate, oligomer, and monomer remained nearly constant. Two observations led us to conclude that oligomerization was necessary for chloride release. First, when proaerolysin was treated with trypsin in the presence of 5 mM zinc, which prevents oligomerization, the aerolysin produced had no effect on the liposomes (Figure

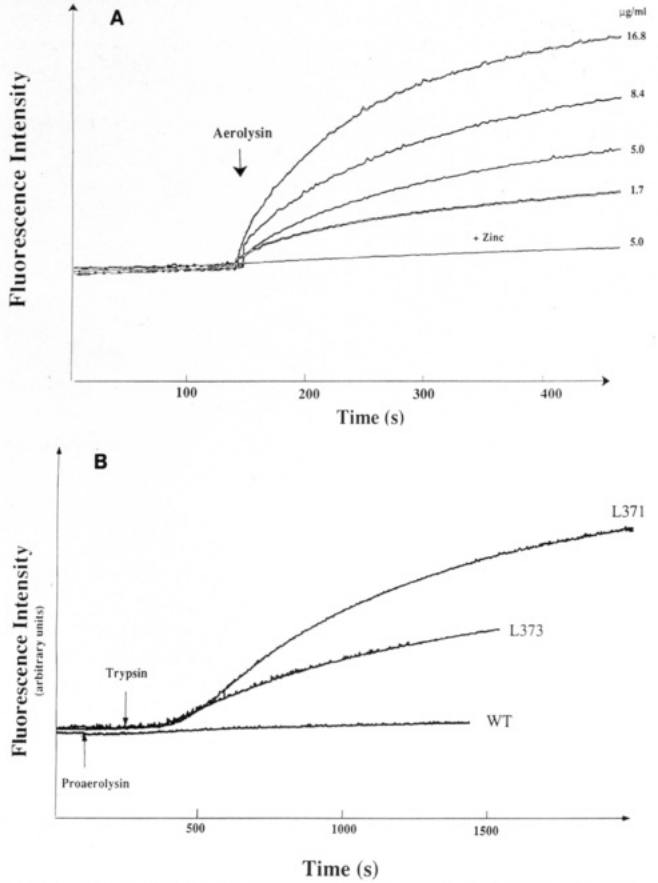

FIGURE 7: Chloride efflux from liposomes containing a chloride-sensitive dye induced by aerolysin. (A) Concentration dependence of efflux with wild-type toxin. Liposomes containing 100 mM KCl, 10 mM Hepes, and 1.5 mg/mL SPQ, pH 7.4, were diluted into a chloride-free buffer (100 mM KNO_3 and 10 mM Hepes, pH 7.4). At 150 s, various amounts of aerolysin were added to the sample. The lower curve was obtained using aerolysin that had been activated by trypsin in the presence of 5 mM zinc acetate. The final Zn^{2+} concentration in the liposome suspension was 60 μM. (B) Efflux with tryptophan mutants. The protoxin (14 μg/mL) was added to liposomes (48 μM lipids) containing the chloride-sensitive dye SPQ. After about 150 s, trypsin (0.2 μg/mL) was added to activate the toxin. The experiment was performed with wild-type proaerolysin as well as with the mutants L-371 and L-373.

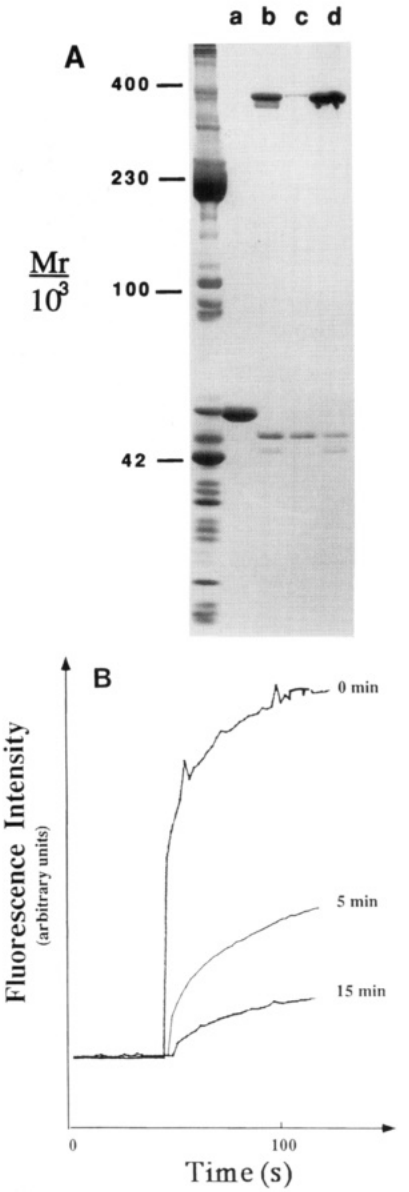

FIGURE 8: Effect of sample centrifugation on the composition of aerolysin samples and on their channel-forming ability. (A) Proaerolysin (1.93 mg/mL, lane a) was activated by trypsin as described in Experimental Procedures and left at 37 °C for 1 h, and the sample containing oligomeric and monomeric aerolysin (lane b) was centrifuged. The pellet contained all the oligomer as well as some monomer (lane d), whereas the supernatant was nearly devoid of oligomer (lane c). (B) Aerolysin was added to liposomes containing a chloride-sensitive dye (final protein concentration 2 μg/mL). Aliquots of the same sample were centrifuged at 10000*g* for 5 or 15 min and the supernatant was added to the liposomes suspension (same final protein concentration).

7A). Second, the aerolysin mutant modified at H-132, which lacks the ability to oligomerize (Green & Buckley, 1990), was also unable to cause chloride release (data not shown).

When native proaerolysin was added to liposome suspensions to a final concentration of 14 μg/mL (too low for oligomerization to occur) and then converted to aerolysin in situ by the addition of trypsin, no chloride efflux from liposomes could be seen (Figure 7B). On the other hand, when trypsin was added to the same concentrations of L-371 or L-373 proaerolysin (high enough for them to oligomerize after activation), fluorescence increases could be observed after a lag time of a few minutes, and L-371 aerolysin induced chloride efflux more rapidly than the L-373 toxin (Figure 7B).

Effect of Removing Oligomeric Aerolysin on Channel-Forming Ability. Additional evidence showing that it is the oligomer that is insertion-competent came from experiments in which the relative proportions of monomer, oligomer, and aggregate were changed. The SDS–polyacrylamide gel in Figure 8A shows that when an aerolysin sample was centrifuged, only monomers remained in the supernatant. Oligomers must aggregate very quickly before or during centrifugation and are therefore pelleted. The effect of centrifugation on the ability of the supernatant to induce chloride efflux is illustrated in Figure 8B. The results show that anion release decreased as the concentration of oligomers diminished. The monomer concentration in the supernatant was apparently unaffected by centrifugation, and the fact that the supernatant was nearly inactive further supports the conclusion that the monomer cannot insert into the membrane and form channels. It should be noted that no zinc was present during this experiment and the small residual activity evident in the figure was probably due to continued oligomerization after centrifugation.

Increased conversion of oligomers to aggregates (as evidenced by increasing cloudiness of the samples) results from increasing the incubation temperature or allowing the samples to stand after activation. Figure 9 shows that the ability of

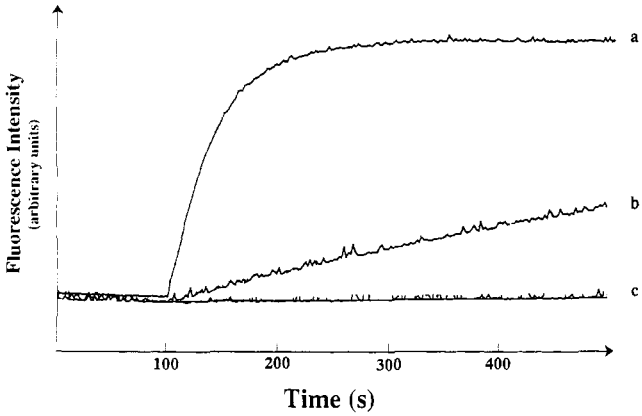

FIGURE 9: Influence of incubation time and temperature after activation on channel formation by L-371 aerolysin. The proaerolysin L-371 stock solution (0.89 mg/mL) was activated by trypsin as described in Experimental Procedures. The active toxin was added to liposomes containing a chloride-sensitive dye to reach a final concentration of 17 μg/mL after 4 min of activation at 20 °C (a), 40 min of activation at 20 °C (b), and 40 min of activation at 20 °C plus 10 min at 37 °C (c).

L-371 aerolysin to release chloride from liposomes was greatly reduced by simply incubating the sample for 40 min (at 20 °C) instead of the 4 min used in previous experiments, and the activity was almost abolished when the same sample was incubated an additional 10 min at 37 °C. Thus we can conclude that aggregation results in inactivation of aerolysin oligomers. We have observed that ANS binding to native aerolysin oligomers is progressively reduced as they aggregate (data not shown).

DISCUSSION

We chose to modify the sequence KWWDW of proaerolysin because of its similarity to a conserved sequence in the oxygen-labile toxins and because of the possibility that the clustered tryptophans might be involved in membrane penetration (de Kruijff, 1990). All of the changes we made were rather substantial, but none of them affected secretion of the protein by the bacteria, and there were only modest changes in cytolytic activity. In contrast, we found in a previous study that even the relatively conservative change of W-227 to phenylalanine had a significant effect on the secretion of proaerolysin across the outer membrane of the bacteria, as well as on its activity (Howard et al., 1987; Wong & Buckley, 1991). Even substituting the bulky W-371 or W-373 of proaerolysin with leucine, which has a more linear side chain, did not seem to affect the overall structure of the protoxin in any major way. The environments of the remaining tryptophans were unchanged, since the tryptophan fluorescence of the two mutant proteins was identical to that of wild type, and perturbations in the arrangement of the side chains were modest, as the near CD spectra of the proteins were only slightly altered. Nevertheless, the leucine replacements had a dramatic effect on oligomerization of the toxin. This is shown qualitatively in Figure 3 and in a more quantitative way in Figure 4. The fact that aerolysin's ability to oligomerize was changed by replacing either of these tryptophans does not necessarily mean that they are located at the monomer/monomer interface within the oligomer. The leucine substitutions may destabilize the three-dimensional structure of aerolysin as suggested by the lower CD signal of the L-371 mutant, thereby reducing the energy barrier associated with a conformational change that leads to oligomerization.

Solutions of mature aerolysin contain a mixture of monomers, oligomers, and aggregated oligomers, and different pathways can be imagined leading to channel formation (Figure 2). By changing the relative proportions of monomeric, oligomeric, and aggregated aerolysin and by comparing native aerolysin with mutant proteins, we were able to determine that only the oligomer can produce channels in vitro (via step e in Figure 2). Pore-forming ability was evaluated by measuring chloride efflux from SPQ-containing liposomes. We first showed that chloride release increased proportionally with increasing amounts of a stock solution of preactivated toxin, which contained a fixed proportion of monomer, oligomer, and aggregate. The relative amounts of the three components were then modified by varying the protein concentration or the temperature, by centrifuging, or by using mutant proteins which oligomerize more readily than native aerolysin (L-371 and L-373) or not at all (N-132). The results in Figures 7–9 show that any change that increased the amount of oligomer increased chloride release, and any change which diminished the amount of oligomer lowered release. We cannot exclude the possibility that monomeric aerolysin can also insert into the membrane (step c in Figure 2), but since our evidence shows clearly that this does not lead to channel formation, if there is a step c, it is not followed by oligomerization and formation of a functional channel (step d).

The ANS binding experiments support the conclusion that it is the aerolysin oligomer alone which is insertion-competent. The results in Figures 5 and 6 show that only the oligomer was capable of binding the hydrophobic dye ANS. Proaerolysin, monomeric aerolysin, and aggregated oligomers do not appear to have hydrophobic patches exposed to the solvent. Nor does N-132 aerolysin, which is unable to oligomerize (Green & Buckley, 1990). On the other hand, L-371 and L-373 aerolysin, which oligomerize at unusually low concentrations, also bind ANS at much lower concentrations than wild-type aerolysin.

In light of these and previous results, the following model can be proposed for the mechanism of action of aerolysin in vivo. At the surface of the target cell, proaerolysin binds to glycophorin and is processed by host proteases. The role of glycophorin might be to enable local concentrations of aerolysin to rise above the threshold needed for oligomers to form (Garland & Buckley, 1988). Oligomerization somehow exposes hydrophobic patches which may provide a driving force for the initial penetration of the oligomer into the lipid membrane. The energetic advantage of this mechanism is that the hydrophilic face of the large water-filled lumen (17 Å; Wilmsen et al., 1992) is never in contact with the hydrophobic core of the membrane. The high threshold concentration required for oligomerization minimizes the likelihood that oligomers will form in the absence of the receptor, increasing the chances of proper targeting and reducing any risk to the bacterium producing the toxin.

A very similar mechanism of channel formation has been proposed for complement protein C9, which polymerizes spontaneously in solution (Podack & Tschopp, 1982) and then inserts into membranes where it forms large channels (Tschopp et al., 1982). In vivo, C9 first binds to membrane-bound C5b–9 complex and then inserts into the membrane. This complex may serve the same purpose as glycophorin does for aerolysin, concentrating C9 on the cell surface (Peitsch & Tschopp, 1991).

It thus seems that the general scheme for channel formation proposed by Ojcius and Young (1991) does not apply to aerolysin, nor to C9. Bhakdi and Tranum-Jensem (1991) have suggested that the scheme is followed by α toxin, but it

is worth noting that, in spite of early evidence that the α toxin hexamer formed in the absence of membranes cannot cause cell lysis (Arbuthnott et al., 1967, 1973; Füsse et al., 1981), there are inconsistencies in the α toxin literature. It is not clear whether oligomerization occurs when the α toxin monomer is bound to the membrane (Tobkes et al., 1985), partially inserted (Ikigai & Nakae, 1987), or completely inserted into the bilayer (Reichewein et al., 1987; Harsman et al., 1989; Forti & Menestrina, 1989). Perhaps site-directed mutagenesis will produce oligomerization mutants of this toxin which will allow the insertion question to be resolved directly.

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