# Spectroscopic Study of the Activation and Oligomerization of the Channel-Forming Toxin Aerolysin: Identification of the Site of Proteolytic Activation†

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ABSTRACT: The channel-forming protein aerolysin is secreted as a protoxin which can be activated by proteolytic removal of a C-terminal peptide. The activation and subsequent oligomerization of aerolysin were studied using a variety of spectroscopic techniques. Mass spectrometric determination of the molecular weights of proaerolysin and aerolysin permitted identification of the sites at which the protoxin is processed by trypsin and chymotrypsin. The results of far- and near-UV circular dichroism measurements indicated that processing with trypsin does not lead to major changes in secondary or tertiary structure of the protein. An increase in tryptophan fluorescence intensity and a small red shift in the maximum emission wavelength of tryptophans could be observed, suggesting that there is a change in the environment of some of the tryptophans. There was also a dramatic increase in the binding of the hydrophobic fluorescent probe 1-anilino-8-naphthalenesulfonate during activation, leading us to conclude that a hydrophobic region in the protein is exposed by trypsin treatment. Using measurements of light scattering, various parameters influencing oligomerisation of trypsin-activated aerolysin were determined. Oligomerization rates were found to increase with the concentration of aerolysin, whereas they decreased with increasing ionic strength.

The pathogenic Gram-negative bacteria *Aeromonas hydrophila* secretes the channel-forming toxin aerolysin into the culture supernatant. We have shown that the protein is released as a protoxin which can be activated by a variety of mammalian proteases, as well as by proteases released by the bacteria itself (Howard & Buckley, 1985). Although the exact site at which proaerolysin is processed has not been established, it is clear that trypsin removes a C-terminal peptide and that this results in a decrease of approximately 2500 Da in the molecular mass of the protein, based on the change in its mobility during sodium dodecyl sulfate–polyacrylamide electrophoresis (Howard & Buckley, 1985; Garland & Buckley, 1988).

Activated aerolysin binds to the transmembrane protein glycophorin on the surface of mammalian erythrocytes (Howard & Buckley, 1982). In this way, the toxin is effectively concentrated on the cell surface, and this leads to oligomerization. Once formed, the oligomers insert into the membrane, producing uniform channels approximately 1 nm in diameter which cause death of the cell (Howard & Buckley, 1982; Wilmsen et al., 1990). The oligomers have several remarkable features, including extraordinary stability and the fact that they appear to be heptamers (Garland & Buckley, 1988; Wilmsen et al., 1992), yet the mechanism of oligomerization has not been studied in any detail. Proaerolysin is completely unable to oligomerize, and as a result it is inactive (Garland & Buckley, 1988; Green & Buckley, 1990). It seems likely that the C-terminal fragment which is removed by proteolysis either prevents a structural change which must accompany oligomerization or shields a region of the protein which must be exposed for oligomers to form. In this paper we use several spectroscopic methods to examine the activation and oligomerization of the protein.

## EXPERIMENTAL PROCEDURES

*Protein Purification.* Wild-type proaerolysin and a mutant form of the protein in which H132 is replaced by an asparagine (Green & Buckley, 1990) were purified according to our published procedure (Buckley, 1990).

*Mass Spectrometry.* Monomeric trypsin-activated aerolysin was produced by treating proaerolysin (1 mg/mL) with trypsin attached to cross-linked beaded agarose (Sigma, 0.5 unit of insoluble trypsin/mL of solution) for 1 h at room temperature in a buffer containing 150 mM NaCl, 20 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES), and 1 mM zinc acetate, pH 6.8. The sample was then centrifuged in order to pellet the trypsin, and the supernatant, which contained only aerolysin monomer, was dialyzed overnight against 0.9% NaCl and 20 mM HEPES, pH 6.8. Chymotrypsin-activated aerolysin was obtained by incubating proaerolysin with the enzyme (50:1 w/w). After 30 min at 37 °C, phenylmethanesulfonyl fluoride (PMSF) was added to 1 mM, and the sample, which was very cloudy, was centrifuged for 10 min. The pellet, essentially free of proaerolysin, was resuspended in 20 mM Tris, 150 mM NaCl, and 1 mM PMSF, pH 7.4. Proaerolysin and aerolysin samples were dialyzed for 24 h against 5 mM morpholinoacetate, pH 7.4, aliquoted, and then lyophilized prior to use. Samples were dissolved in $H_2O/CH_3OH$ (50:50 v/v) containing 1% acetic acid to approximately 10 pmol/$\mu$L, and average mass measurements were made on a VG Bio-Q mass spectrometer

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(Bio Tech, Manchester, U.K.) as described by Van Dorsselaer et al. (1990). Results are expressed as the means ± standard deviations.

*Circular Dichroism (CD).* Circular dichroic experiments were carried out at 25 °C in a Jobin Yvon Mark V spectrometer calibrated using the ammonium salt of 10-(+)-camphorsulfonic acid. Quartz cells of 0.2- and 1-cm path length were used for measurements in the far-ultraviolet (195–250 nm) and near-ultraviolet (250–350 nm), respectively. The protein concentration was 0.1–0.2 mg/mL in 150 mM NaCl and 10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), pH 8, for far-ultraviolet measurements and 0.5–0.6 mg/mL in 100 mM NaF and 10 mM HEPES, pH 6.8, for near-ultraviolet measurements.

*Tryptophan Fluorescence.* Fluorescence measurements were made with an SLM 8000 spectrofluorimeter (Urbana, IL) operating in ratio mode. The excitation wavelength was 295 nm, and the emission wavelength was 350 nm. The spectral band width was 8 nm for both. 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. The barycentric mean wavelengths ($\lambda_m$) of the spectra were calculated using the equation $\lambda_m = \sum[F(\lambda) + \lambda]/\sum F(\lambda)$, where $F(\lambda)$ is the point fluorescence at wavelength $\lambda$. Measurements were taken every 1 nm.

*ANS Binding.* Binding of 1-anilino-8-naphthalenesulfonate (ANS) was measured in 0.5-mL quartz cuvettes containing 20 mM Tris–acetate, pH 6.8 at 37 °C. Once again the SLM 8000 spectrofluorimeter was used, with an excitation wavelength of 380 nm, an emission wavelength of 480 nm, and 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, using the SLM 8000 spectrofluorimeter operating in ratio mode, with a spectral bandwidth of 8 nm for excitation ($\lambda_{ex}$ = 450 nm).

*Other Methods.* Sodium dodecyl sulfate–polyacrylamide electrophoresis was carried out as described by Neville (1971). Protein concentrations were calculated using an absorbance of 1.5 at 280 nm for pure 1 mg/mL solutions of aerolysin and proaerolysin. This relationship was obtained from amino acid analysis data. Amino-terminal amino acid sequencing was performed using an ABi 477A protein sequencer and the standard Edman chemistry provided by the manufacturer (Abi, Weiterstadt, Germany).

## RESULTS

*Site of Proteolytic Activation.* Native proaerolysin is converted to the active toxin by treatment with trypsin, which removes a fragment from the C-terminus of the protein without affecting the N-terminus (Howard & Buckley, 1985). Chymotrypsin will also activate the protoxin, although proaerolysin is much less sensitive to this enzyme (Garland & Buckley, 1988). The cut site for chymotrypsin must be very close to the trypsin site, as the processed forms of the toxin are indistinguishable by SDS–PAGE (Garland & Buckley, 1988), and Edman sequencing demonstrated that, as with trypsin, the amino terminus of the protein was unchanged by treatment with chymotrypsin (data not shown). In order to determine the exact sites of processing, the masses of the unprocessed and processed proteins were measured by mass spectrometry before and after activation. A value of 51 945 ± 4 Da was obtained for proaerolysin, very close to the mass of 51 932 Da calculated from the translated DNA sequence, which is shown in Figure 1. Also noted in the figure are several changes from the originally published sequence, which we have uncovered during the course of repeated resequencing of the DNA. Aerolysin produced by trypsin treatment had a measured mass of 47 488 ± 4 Da, indicating that trypsin cuts after K427, as this would result in a protein with a predicted mass of 47 472 Da. Thus much more of the protein is removed by trypsin than we had estimated from the change in its mobility by SDS–PAGE (Howard & Buckley, 1985). The mass of aerolysin produced by chymotrypsin was 47 731 ± 7 Da, leading to the conclusion that the protein is cut between the two arginines at 429 and 430. Although this is an unusual site for chymotrypsin to act, it would produce a protein with a predicted mass of 47 727 Da. The proposed activation sites for both proteases are shown in Figure 1.

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AEPVYPDQLR LFSLGQGVCG DKYRPVNREE AQSVKSNIVG MMGQWQISGL  50
ANGWVIMGPG YNGEIKPGTA SNTWCYFTNP VTGEIPTLSA LDIPDGDEVD 100
VQWRLVHDSA NFIKPTSYLA HYLGYAWVGG NHSQYVGEDM DVTRDGDGWV 150
IRGNNDGGCD GYRCGDKTAI KVSNFAYNLD PDSFKHGDVT QSDRQLVKTV 200
VGWAVNDSDT PQSGYDVTLR YDTATNWSKT NTYGLSEKVT TKNKFKWPLV 250
GETELSIEIA ANQSWASQNG GSTTTSLSQS VRPTVPARSK IPVKIELYKA 300
DISYPYEFKA DVSYDLTLSG FLRWGGNAWY THPDNRPNWN HTFVIGPYKD 350
KASSIRYQWD KRYIPGEVKW WDWNWTIQQN GLSTMQNNLA RVLRPVRAGI 400
TGDFSAESQF AGNIEIGAPV PLAADSKVRR ARSVDGAGQG LRLEIPLDAQ 450
ELSGLGFNNV SLSVTPAANQ
                        ↑ ↑
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FIGURE 1: Proaerolysin sequence and protease activation sites. Changes from the sequence originally published are underlined. The proposed sites of processing by trypsin (T) and chymotrypsin (C) are marked with arrows.

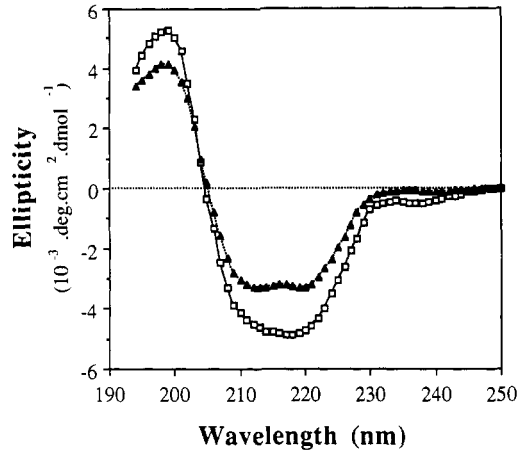



FIGURE 2: Far-ultraviolet circular dichroism of proaerolysin (□) and aerolysin (▲). CD spectra of the toxin were measured before and after activation by trypsin as described in the Experimental Procedures. Ellipticities are expressed per mole of peptide bonds.

*CD Spectroscopy of Aerolysin and Proaerolysin in the Far-Ultraviolet.* The far-UV CD spectra of proaerolysin and aerolysin are depicted in Figure 2. Estimates of the secondary structure of the two proteins were made using the computer program CONTIN (Provencher & Glöckner, 1981). Proaerolysin was calculated to contain 50% β-sheet, 20% β-turn, 11% α-helices, and 19% unassigned structure, whereas trypsin-activated aerolysin was estimated to have 54% β-sheet, 28% β-turn, 4% α-helices, and 15% unassigned structure.

*CD Spectroscopy in the Near-Ultraviolet.* The near-ultraviolet CD spectrum of proaerolysin at pH 6.8 in the

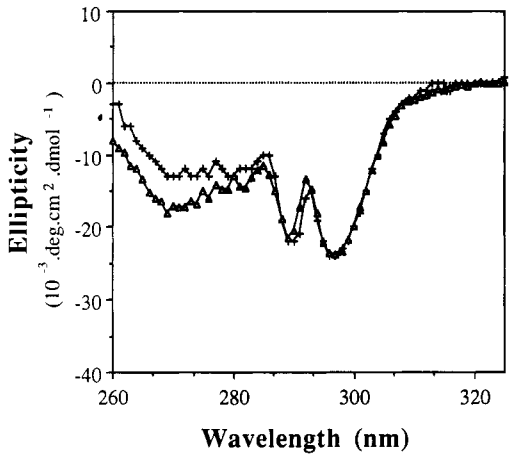



FIGURE 3: Effect of activation on the near-ultraviolet CD spectrum of proaerolysin. Ellipticities are expressed per mole of protein. (+) aerolysin + $Zn^{2+}$; (Δ) proaerolysin + $Zn^{2+}$.

presence of 2 mM $Zn^{2+}$ is represented in Figure 3. Three main peaks may be seen in the figure, one very broad one near 270 nm and two quite distinct peaks at 289 and 296 nm. The well-structured spectrum is the product of 50 aromatics, so the individual contribution of the various residues could not be established. Adding 2 μg/mL trypsin to the proaerolysin in order to convert it to the active toxin resulted in a small change in the spectrum which was probably due to a modest reorientation of the protein's side chains. It could not have been a consequence of aerolysin oligomerization, as this does not occur in the presence of $Zn^{2+}$ (Wilmsen et al., 1990).

*Tryptophan Fluorescence upon Activation.* Both proaerolysin and aerolysin contain 18 tryptophans. The fluorescence emission spectrum of proaerolysin displays a maximum at 335 nm (Figure 4A), suggesting that these residues are in a rather hydrophobic environment. Converting proaerolysin into aerolysin by activation with trypsin led to an increase in fluorescence as well as a slight red shift (1.5-nm shift of the maximum emission wavelength). Calculation of the barycentric emission wavelength also revealed a 2-nm red shift due to the broadening of the spectrum. The increase in fluorescence which accompanied trypsin treatment provided us with a way to follow the kinetics of activation (Figure 4B). Under the conditions used in the figure, the reaction was completed within 100 s of the addition of trypsin. The kinetics of activation measured in this way were extremely reproducible from one experiment to the next.

*ANS Binding.* In order to examine the possibility that a hydrophobic surface is exposed upon activation of proaerolysin, binding of the hydrophobic fluorescent probe ANS was followed (Turner & Brand, 1968). The results are presented in Figure 5. Adding proaerolysin to a solution of ANS caused only a small change in fluorescence. However, subsequent addition of trypsin led to a rapid and dramatic increase. In contrast, when the same experiment was performed in the presence of 2.5 mM zinc acetate, there was little or no increase in ANS binding. We know that, under these conditions, aerolysin is prevented from oligomerizing, although processing by trypsin is not affected (Wilmsen et al., 1990). Aerolysin in which H132 is replaced by asparagine is also unable to form oligomers (Green & Buckley, 1990). The results in the figure show that this mutant toxin behaved in the same way in the absence of $Zn^{2+}$ as wild type did with $Zn^{2+}$. Thus no increase in fluorescence was observed upon addition of trypsin.

*Light Scattering.* Rayleigh light scattering was used as a means to follow oligomerization of native trypsin-activated aerolysin. The results are shown in Figure 6. Solutions of

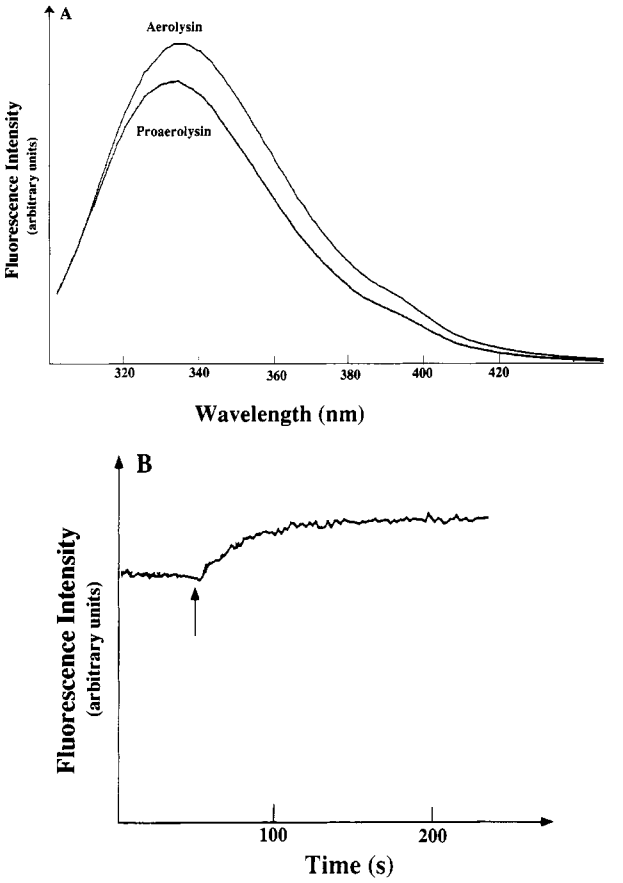



FIGURE 4: Changes in tryptophan emission spectra of proaerolysin upon activation by trypsin. (A) The emission spectrum of proaerolysin (40 μg/mL) was measured. Then trypsin (0.2 μg/mL) was added and the emission of the aerolysin which was produced was measured. The small peak at 395 nm is due to a Woods anomaly of the emission monochromator. (B) The kinetics of proaerolysin activation by trypsin were followed by measuring tryptophan fluorescence.

proaerolysin (first 50 s in the figure) scatter very little light, as the protoxin does not oligomerize. Addition of trypsin led to the production of aerolysin, and as the toxin began to oligomerize, an increase in light scattering was observed. The results suggest that this process was concentration dependent: at protein concentrations of 0.2 mg/mL and lower, no change could be detected upon trypsin addition. It should be noted that the difference between the curves obtained for proaerolysin at 0.5 and 1 mg/mL in Figure 6 is not necessarily due to a faster rate of oligomerization per se, but rather to the fact that the amount of oligomer formed (and thus the amount of light scattered) is quantitatively higher.

The results in Figure 7 show that the rate at which oligomerization occurs can be modified by changing the ionic strength of the buffer. As the ionic strength is increased, the initial velocity of increase in light scattering is considerably slowed down. This effect was confirmed by SDS–PAGE (Figure 7B). Oligomer can be seen in the gel within the first minute after activation in the low ionic strength medium, but the amount of oligomer is greatly diminished at higher ionic strengths.

The results in Figure 8 show that the additional two amino acids left at the C-terminus of aerolysin when chymotrypsin was used to activate instead of trypsin had a pronounced effect on the protein's ability to oligomerize. Aerolysin formed by treatment with chymotrypsin oligomerized at a lower concentration than the toxin produced with trypsin, an indication that the C-terminus may play a role in this process.

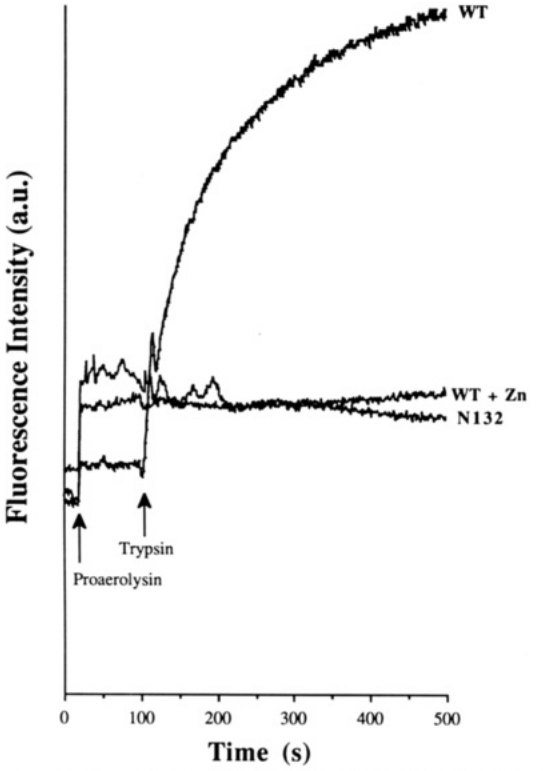



FIGURE 5: Activation of proaerolysin monitored by ANS binding. Proaerolysin (0.5 mg/mL final concentration) was added to the ANS solution after 40 s, and 20 s later trypsin was added (1 μg/mL). The same experiment was performed as follows: (1) with wild-type proaerolysin with and without 2.5 mM zinc acetate; (2) with proaerolysin mutant protein in which H132 is replaced by an asparagine (in the absence of zinc).

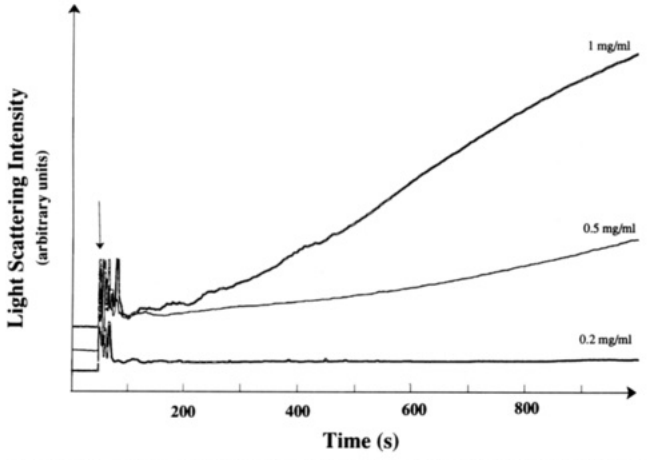
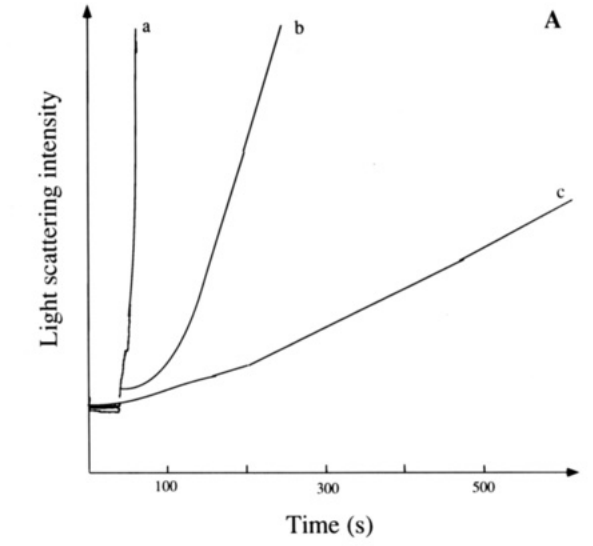



FIGURE 6: Effect of concentration on the ability of trypsin-activated aerolysin to oligomerize. After 50 s of data acquisition with the indicated concentrations of protoxin, trypsin (1 μg/mL) was added (shown with an arrow).

## DISCUSSION

Although the activation of proaerolysin by trypsin eventually results in the conversion of a water-soluble protein into an oligomeric structure capable of inserting into membranes, there appears to be remarkably little change in secondary or tertiary structure, at least as measured by far- and near-ultraviolet circular dichroism. It is worth noting here that far-UV CD may not be a reliable technique to study the secondary structure of this protein because of the high proportion of aromatic amino acids. Proaerolysin contains 18 tryptophans, 21 tyrosines, 11 phenylalanines, and 6 histidines, and they must contribute not only to the near-UV CD spectrum but also in some significant way to the far-UV CD spectrum (Manning & Woody, 1989). Furthermore, many of these residues may



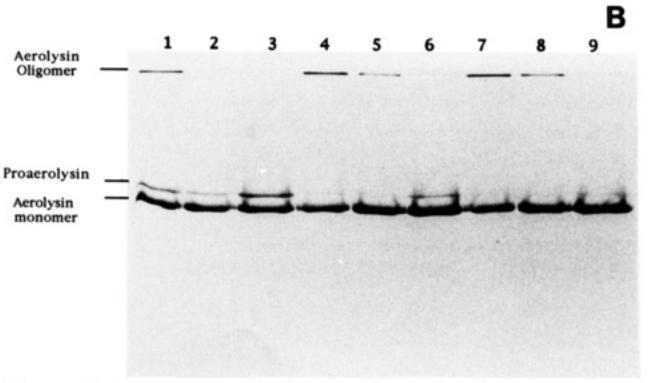




FIGURE 7: (A) Effect of ionic strength on the oligomerization of trypsin-activated aerolysin. Proaerolysin was diluted to a final concentration of 0.5 mg/mL in 20 mM Tris–acetate, pH 7.4, containing the following concentrations of NaCl: (a) 75 mM, (b) 190 mM, and (c) 825 mM. After 40 s, trypsin was added (1 μg/mL). (B) Oligomerization followed by SDS–PAGE analysis. Proaerolysin was activated as described in panel A. At the indicated times, aliquots were taken, mixed with SDS–PAGE sample buffer, and boiled. Lanes 1, 2, and 3, samples in 75, 190, and 825 mM NaCl, respectively, at 1 min; lanes 4–6, 2 min; lanes 7–9, 3 min.

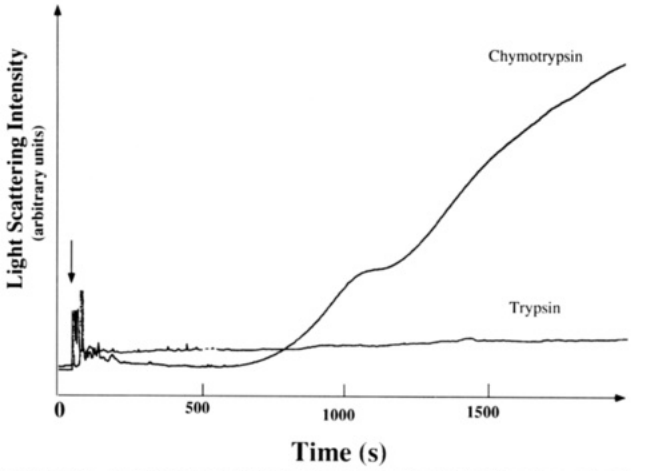



FIGURE 8: Comparison of the oligomerization kinetics of aerolysin after activation by trypsin or chymotrypsin. Proaerolysin was diluted into 150 mM NaCl and 20 mM Tris–acetate, pH 7.4, to a final concentration of 0.02 mg/mL.

be close enough to each other to allow electronic coupling between them. One can identify several possible aromatic clusters in the primary sequence of proaerolysin, including Y118-Y122-Y125-W127, Y304-Y306-F308, Y357-W359-Y363, and W370-W371-W373-W375. Even amino acid

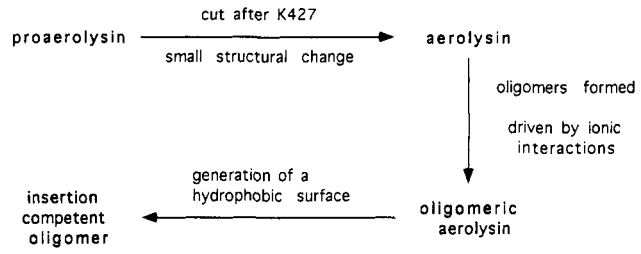



FIGURE 9: Steps in the activation and oligomerization of aerolysin.

couples such as W74-Y76, F175-Y177, or F245-W247 might be important if they are located in β-strands (not unlikely considering the very large proportion of β-structure in aerolysin), as the side chains could be very close to each other on the same side of a strand. Thus the polypeptide backbone cannot be the only contributor to the far-UV signal of this protein, and quantitative comparison of secondary structures between aerolysin and proaerolysin is not reliable.

Although the circular dichroic and tryptophan fluorescence measurements indicate that activation results in only small changes in the structure of aerolysin, the results of ANS binding experiments show that there is a pronounced increase in the ability of the protein to bind the hydrophobic dye after trypsin treatment. The simplest way to explain how this occurs is to imagine that the peptide masks a hydrophobic region until it is removed by proteolysis; however, the results with the N132 mutant protein and with $Zn^{2+}$ suggest that the sequence of events must be somewhat more complicated. There is no increase in ANS binding when the mutant toxin is treated with trypsin, or when native proaerolysin is activated in the presence of $Zn^{2+}$. Since N132 aerolysin cannot oligomerize, and since $Zn^{2+}$ prevents oligomerization of native trypsin-activated aerolysin, it seems reasonable to conclude that oligomerization is required for the appearance of the hydrophobic region to occur. Although the two events may happen simultaneously, the hydrophobic surface is apparently not the major determinant for oligomerization, as we have found that oligomers will form in the presence of rather high concentrations of nonionic detergents (not shown here).

On the basis of the results of the present study, channel formation by aerolysin may proceed according to the scheme depicted in Figure 9. From the molecular weights of proaerolysin and aerolysin obtained by mass spectrometry, we now know that trypsin cleaves the protein after K427. Chymotrypsin appears to cut at position 429, between two arginines. Although this is an unusual position for chymotrypsin to attack, the enzyme is known to hydrolyze many different peptide bonds at low rates, in addition to those involving aromatic amino acids (Smyth, 1967), and this may explain the need to use much higher levels of this enzyme than trypsin for activation (Garland & Buckley, 1988). Of course the peptide which is removed by either enzyme may be cut in many other places, and this may even be necessary to expose or free a region of the protein. Once activated, aerolysin can proceed to form oligomers, depending on the toxin concentration and other factors such as temperature (unpublished) and ionic strength. Since oligomerization is inhibited by increasing ionic strength, but not by nonionic detergents, it is likely driven by ionic interactions between activated aerolysin monomers. It is this step which is blocked by $Zn^{2+}$, and which the N132 mutant cannot carry out. It appears that the new C-terminus produced by proteolysis affects the rate of oligomerization since chymotrypsin-activated aerolysin, which has an extra two amino acids, Val-Arg, oligomerizes more easily than the toxin produced with trypsin. We have shown that proaerolysin can also be activated by *A. hydrophila*'s own proteases (Howard & Buckley, 1985). If they produce the same C-terminus as chymotrypsin, oligomers may be formed at very low toxin concentrations in situ.

Although we do not know the specificity of the *Aeromonas* proteases, the size of the aerolysin which they form is the same as the size of trypsin and chymotrypsin–aerolysin on SDS–PAGE, indicating that all these enzymes cut proaerolysin in the same region. This region falls in a string of 11 amino acids which is strongly conserved between the aerolysins of *A. hydrophila* (Howard et al., 1987) and *Aeromonas sobria* (Hussein et al., 1988) and which contains four positive charges bracketed by two negative charges. All of the structure prediction algorithms we tried (five secondary structure methods, hydrophobic moment, accessibility) indicated that there is a small α-helical stretch in the string, flanked by two regions rich in glycine and proline which are likely to be in turns or in coil conformation (not shown here). The fact that even chymotrypsin cuts proaerolysin here points to the unusual nature of this region.

The last step in the production of a competent channel-forming complex is the appearance of a hydrophobic surface. This occurs either during or as a consequence of oligomerization. It may be this surface which transforms aerolysin from a water-soluble protein to a form which can insert into the lipid bilayer. Evidence that it is the aerolysin oligomer, and not the monomer, which is surface active and which penetrates membranes will be presented in a separate communication.

## ACKNOWLEDGMENT

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**Registry No.** Aerolysin, 53126-24-2; trypsin, 9002-07-7; chymotrypsin, 9004-07-3.