The Channel-forming Protein Proaerolysin Remains a Dimer at Low Concentrations in Solution*

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Proaerolysin, the proform of the channel-forming protein aerolysin, is secreted as a dimer by Aeromonas sp. The protein also exists as a dimer in the crystal, as well as in solution, at least at concentrations in the region of 500 μg/ml. Recently it has been argued that proaerolysin becomes monomeric at concentrations below 100 μg/ml and that only the monomeric form of the protein can bind to cell surface receptors (Fivaz, M., Velluz, M.-C., and van der Goot, F. G. (1999) J. Biol. Chem. 274, 37705–37708). Here we show, using non-denaturing polyacrylamide electrophoresis, chemical cross-linking, and analytical ultracentrifugation, that proaerolysin remains dimeric at the lowest concentrations of the protein that we measured (less than 5 μg/ml) and that the dimeric protoxin is quite capable of receptor binding.

The channel-forming toxin aerolysin is secreted as an inactive precursor called proaerolysin by bacteria in the Aeromonas family. Proaerolysin binds to glycosylphosphatidylinositol-anchored receptors on sensitive cells and is converted to aerolysin by proteases such as furin. Aerolysin then oligomerizes, and the heptamers that are formed insert into the bilayer, producing channels (1). Proaerolysin exists as a dimer in the crystal, and the structure of the dimer has been solved (2). In previous studies we have shown that proaerolysin is secreted as a dimer by Aeromonas salmonicida (3), and we have also found, using analytical ultracentrifugation and chemical cross-linking, that it is a dimer in solution (4), at least at concentrations in the range of 500 μg/ml (10 μM). The dimeric form of the protein may have several advantages over the monomer. First of all, it appears that only the dimer is recognized by the Aeromonas secretion machinery, which is responsible for transit of proaerolysin across the outer membrane of the bacteria. Thus dimerization in the periplasm is necessary for secretion by the bacteria (3). Second, a large area of the surface of the protein is protected in the dimer, and this could contribute to the unusual protease stability of aerolysin (2, 5). Third, two regions of the molecule appear to be involved in receptor binding, one at the top of the large lobe and another in the small lobe (6). In the dimer the large lobe of one monomer is much closer to the small lobe of the other monomer than it is to its own small lobe (2). Finally, dimerization may help control the oligomerization step in channel formation. The monomers presumably must separate for oligomerization to occur because the oligomer is heptameric (7, 8). The stability of the dimer may reduce the risk that the protein will oligomerize in the absence of a target membrane.

Recently, it has been argued that proaerolysin is a monomer in solution at concentrations below 100 μg/ml (2–2 μM) and that it is the monomer that binds to cell surface receptors (9). These conclusions were based on the results of binding and cross-linking studies with radioiodinated proaerolysin, on concentration-dependent changes in the elution profile of proaerolysin using gel permeation chromatography with an agarose matrix, and on the apparent inability of a variant of proaerolysin (M41C) that is a covalent dimer to bind to surface proteins. In this paper we present contradictory evidence and alternative explanations of the data of the earlier study. We show that proaerolysin remains dimeric in solution at the lowest concentrations that we measured (less than 5 μg/ml) and that covalent proaerolysin dimers can indeed bind to cell surfaces.

EXPERIMENTAL PROCEDURES

Materials—Trypsin and trypsin inhibitor were obtained from Sigma. Native proaerolysin and the proaerolysin variants were purified according to our published procedure (10). All of the samples migrated with an apparent mass of 52 kDa on reducing SDS-PAGE (data not shown).

Production of Proaerolysin Variants That Form Covalent Dimers—The production of M41C proaerolysin and the properties of this variant have been described previously (3). Two other covalent variants were made in the same way. Briefly, the structure of the native proaerolysin dimer was examined to locate single residues that lie within a few Ångstroms of each other in the dimer (as Met-41 does). Two residues, Ser-13 and Lys-185, were identified, and two proaerolysin variants, S13C and K185C, were created by site-directed mutagenesis and purified. Some of the purified S13C migrated in nonreducing SDS-PAGE with the same apparent mass as M41C (100 kDa), indicating that an intermolecular bridge had formed between the cysteines at position 13 in the two monomers (see Fig. 1A). The rest of the protein migrated as a monomer (non-covalent dimers are dissociated by the SDS in SDS-PAGE), indicating that intermolecular bridge formation was not complete. Consistent with this result, the S13C variant retained appreciable channel-forming activity, although it was rendered more active by reduction with dithiothreitol (data not shown). The K185C variant migrated entirely as a single band, more slowly than the S13C and M41C dimers (see Fig. 1A), presumably because the new intermolecular bridge prevented complete unfolding in SDS. It migrated with the same apparent mass as native proaerolysin under reducing conditions (data not shown). Like the M41C variant, K185C was virtually inactive unless reduced.

Chemical Cross-linking—Samples of proaerolysin in 150 mM NaCl, 20 mM HEPES, pH 7.4 were treated with 0.25 mM diethylditosylsuccinimidyl propionate (DSP, Pierce) at room temperature. After 30 min, 1 μM Tris, pH 8, was added to a final concentration of 90 mM, and the incubation was continued for 5 min before adding sample buffer for SDS-PAGE.

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1 The abbreviations used are: SDS-PAGE, SDS-polyacrylamide gel electrophoresis; DSP, diethylditosylsuccinimidyl propionate.
molecular mass markers are indicated. (w/v) bovine serum albumin, pH 7.4, containing either 5

The cells were resuspended to 4% (v/v) in 150 mM NaCl, 10 mM poly-

untreated proaerolysin is in the

0.745 cm³/g was used for the calculations (4).

employed an An-60 Ti (titanium) rotor. Temperature and speed condi-

using an An-55 Al (aluminum) rotor. Sedimentation equilibrium runs

aluminum Kel-F 12-mm double sector cells. Sedimentation velocity

ice-cold 5 mM sodium phosphate, pH 8.0. The membranes were pelleted

after 30 min of incubation on ice, the cells were pelleted and lysed by suspending them in

proaerolysin or one of the proaerolysin variants. After 30 min of incubation on ice, the cells were pelleted and lysed by suspending them in ice-cold 5 mM sodium phosphate, pH 8.0. The membranes were pelleted and washed in the phosphate buffer. They were then dissolved in sample buffer, separated by SDS-PAGE, and blotted to detect proaero-

lysin that had bound.

Analytical Ultracentrifugation—Proaerolysin was dialyzed exten-
sively against 150 mM NaCl, 20 mM HEPES, pH 7.4 and loaded into

Human erythrocytes from outdated blood were washed with 150 mM NaCl, 20 mM phosphate, pH 7.4.

RESULTS

Covalent Proaerolysin Can Bind to Cell Surfaces—Fivaz et al. (9) reported that the M41C variant of proaerolysin was apparently unable to bind to proteins from baby hamster kidney cells and drew the conclusion that the proaerolysin dimer must dissociate for binding to occur. We compared the binding of native proaerolysin to human erythrocytes with the binding of the three covalent variants, including M41C. A blot of the membrane-associated proteins separated by nonreducing SDS-PAGE is shown in Fig. 1B. For comparison, a Coomassie-stained nonreducing SDS-PAGE gel of the proteins used is shown in Fig. 1A. Dimers of both S13C and K185C were readily detected in amounts comparable with the amount of bound native monomer, indicating that both covalent dimers could bind to the erythrocyte membranes. Much less M41C was detected, suggesting that this variant binds more poorly than wild type and the S13C and K185C dimers, a result that is consistent with the previous finding (9).

Proaerolysin Dimers Can Be Cross-linked at Low Protoxin Concentrations—Previously we have found that treating native proaerolysin with dimethylsuberimidate produces cross-linked dimers; however, we did not try protein concentrations lower than ~500 µg/ml or 10 µM (4). Fivaz et al. (9) used glutaralde-

hyde and proaerolysin concentrations as low as 50 µg/ml to study dimer cross-linking. They concluded that cross-linking was concentration-dependent and that this was an indication that the dimer was dissociating as the protein concentration was lowered (9). They drew this conclusion despite the fact that their results appear to show that proportionally as much cross-linked dimer was formed at 50 µg/ml proaerolysin as at 100 µg/ml. In preliminary experiments we found that DSP cross-links proaerolysin much more efficiently than either dimethyl-

suberimidate or glutaraldehyde, and we used this reagent to look for evidence of the presence of dimers at proaerolysin concentrations as low as 4 µg/ml. The results in Fig. 2 show that even at the lowest concentration, nearly all of the protoxin migrated with an apparent mass of ~100 kDa after treatment

FIG. 1. Mobilities and binding of covalent proaerolysin dimers. A, Coomassie-stained gel illustrating the mobilities of the proaerolysin variants compared with native proaerolysin under nonreducing conditions. Under reducing conditions all of the proteins had the same mobility. B, binding of proaerolysin variants to human erythrocytes. After exposing erythrocytes to the different proaerolysins, membranes were re-

covered and separated by nonreducing SDS-

PAGE. Native proaerolysin and the variants were detected by Western blotting. The positions of 116- and 80-kDa molecular mass markers are identified: wt, wild type.

FIG. 2. Cross-linking of proaerolysin with DSP. Proaerolysin at 20 µg/ml (lane 1) or 4 µg/ml (lane 2) was treated with DSP, electrophoresed, and blotted as described under “Experimental Procedures.” Untreated proaerolysin is in the third lane. The positions of three molecular mass markers are indicated.

Binding to Human Erythrocytes—Human erythrocytes from outdated blood were washed with 150 mM NaCl, 20 mM phosphate, pH 7.4. The cells were resuspended to 4% (v/v) in 150 mM NaCl, 10 mM poly-

ethylene glycol (average molecular weight, 8000), 17.5 mM HEPES, 1%

(w/v) bovine serum albumin, pH 7.4, containing either 5 × 10⁻⁹ M native proaerolysin or one of the proaerolysin variants. After 30 min of incubation on ice, the cells were pelleted and lysed by suspending them in ice-cold 5 mM sodium phosphate, pH 8.0. The membranes were pelleted and washed in the phosphate buffer. They were then dissolved in sample buffer, separated by SDS-PAGE, and blotted to detect proaero-

lysin that had bound.

Analytical Ultracentrifugation—Proaerolysin was dialyzed exten-
sively against 150 mM NaCl, 20 mM HEPES, pH 7.4 and loaded into

Aluminum Kel-F 12-mm double sector cells. Sedimentation velocity runs were carried out in a Beckman Analytical XL-A ultracentrifuge using an An-55 Al (aluminum) rotor. Sedimentation equilibrium runs employed an An-60 Ti (titanium) rotor. Temperature and speed condi-

tions are indicated in the figure legends. A partial specific volume of 0.745 cm³/g was used for the calculations (4).

Sedimentation velocity scans were analyzed using XL-A Ultra Scan version 4.1 sedimentation data analysis software (Borries Demeler, Missoula, MT), which employs a published method of boundary analysis (11). Sedimentation equilibrium scans were analyzed using a nonlinear, least squares, curve-fitting algorithm (12), contained in the XL-A data analysis software.

Electrophoresis and Blotting—SDS-PAGE was carried out under re-

ducing or nonreducing conditions (13). Non-denaturing gel electro-

phoresis was performed in 10% gels in the absence of reducing agents (14). When necessary, proteins were blotted onto nitrocellulose and detected using a mouse anti-proaerolysin monoclonal antibody and anti-mouse horseradish peroxidase (15). Blots were developed by en-

hanced chemiluminescence (PerkinElmer Life Sciences).

FIG. 3. Proaerolysin migrates as a dimer in non-denaturing gels regardless of concentration. A, Coomassie-stained gel comparing the mobilities of native proaerolysin and the covalent proaerolysin dimers M41C and K185C. B, Western blot comparing the mobilities of several concentrations of native proaerolysin (in µg/ml) with M41C proaerolysin: wt, wild type.
Proaerolysin Migrates as a Dimer in Non-denaturing Gels at All Concentrations—Our ability to cross-link proaerolysin dimers at low protoxin concentrations led us to believe that the protein might be stable enough to remain dimeric during non-denaturing gel electrophoresis. The results in Fig. 3 show that this is the case. Native proaerolysin migrated with the same mobility as M41C and K185C, both of which are covalent dimers under these conditions. Diluting the protein to concentrations as low as 0.1 mg/ml had no effect on its mobility (Fig. 3B).

Sedimentation Velocity Centrifugation Indicates That Proaerolysin Is a Dimer—The sedimentation velocity analysis of proaerolysin at 16 and 80 μg/ml is illustrated in Fig. 4. It is clear that the protein was very homogeneous at both concentrations, as indicated by the convergence of the lines in the “fan plots” (11). The sedimentation coefficient was $5.2 \pm 0.1$ S at both protein concentrations, in agreement with the previous result obtained with 500 μg/ml proaerolysin (4). Thus there is no significant change in the sedimentation coefficient or the homogeneity of proaerolysin, and hence no evidence for dimer dissociation, in the concentration range between 16 and 500 μg/ml.

Proaerolysin Has the Mass of a Dimer by Sedimentation Equilibrium Centrifugation—Sedimentation equilibrium analysis confirmed that proaerolysin is entirely dimeric at 16 μg/ml. At this concentration the data could be fitted to a single ideal component model of molecular mass 94.9 kDa (Fig. 5A), very similar to the mass of 95.6 kDa we have obtained previously at higher protein concentrations (4). At the higher concentration of 91 μg/ml, the best fitting line for a monodisperse sample gave a molecular mass of 95.2 kDa (Fig. 5B); however the fit to a single ideal component model was not as good as it was at the lower concentration. This is indicated by the non-random trend exhibited by the residuals. The systematic pattern observed at the higher concentration is indicative of the presence of some aggregation (16).

**FIG. 4.** Sedimentation velocity analysis of proaerolysin. In A the $A_{230}$ was 0.2 and in B the $A_{280}$ was 0.2. The run was performed at 20 °C and 42,000 rpm in 150 mM NaCl, 20 mM HEPES, pH 7.4. The analysis was carried out according to the method of van Holde and Weischet (11) in which the lines converging toward a common $s_{20,w}$ (Svedbergs) value and the number of lines are proportional to the fraction of sample represented. Time is shown in seconds.

**FIG. 5.** Sedimentation equilibrium analysis of proaerolysin. The $A_{230}$ was 0.24 in A, and the $A_{280}$ was 0.22 in B. The protein concentration in A was 16 μg/ml, and in B the protein concentration was 96 μg/ml. The continuous lines indicate the fitting to a thermodynamically single ideal species of mass 94.9 kDa (A) and 95.2 (B). The top plots show the $\chi^2$ residuals (18) as a function of the radial distance. The run was performed at 6 °C and 15,000 rpm using 150 mM NaCl, 20 mM HEPES, pH 7.4.

with DSP, indicating that little or none of it was dissociated into monomers and contradicting the conclusion of the glutaraldehyde study (9).

**DISCUSSION**

The results of the present study of the state of proaerolysin in solution lead to different conclusions than those reached by Fivaz et al. (9). Our data indicate that the protoxin is a dimer, even at very low concentrations, and that dimer dissociation is not required for binding to surface receptors.

We believe that several factors may account for differences between our results and conclusions and those of Fivaz et al. (9). These authors observed that the M41C variant did not bind to surface proteins and they interpreted this to mean that
dimer separation is necessary for binding. A more likely explanation is that the M41C substitution reduces the ability of the protein to interact with the receptor. The mutation is in the carbohydrate binding fold or aerolysin pertussis toxin domain of proaerolysin, which we have identified previously (17). We have shown that changing other amino acids in this region can have pronounced effects on receptor binding (6).

Fivaz et al. (9) also found that they were unable to cross-link proaerolysin dimers with 3,3′-dithiobis[sulfosuccinimidyl propionate] after exposing baby hamster kidney cells to native proaerolysin that had been radioiodinated using IODO-GEN. They concluded that this was because dimers had dissociated and that only monomers were bound to surface receptors (9). However, even in control experiments using protoxin concentrations at which they readily observed dimer with glutaraldehyde, very little cross-linked dimer was produced with 3,3′-dithiobis[sulfosuccinimidylpropionate]. This may indicate that this reagent is a poor cross-linking agent for proaerolysin. The use of radioiodinated proaerolysin may also have affected the outcome of their attempt to cross-link dimers to surface proteins. We have found that the use of IODO-GEN results in a product that is less active than normal proaerolysin,2 perhaps because one or more tyrosines important for binding are affected. Two candidates are Tyr-61 and Tyr-162, because variants in which these residues are replaced bind more poorly to glycosylphosphatidylinositol-anchored proteins than does native proaerolysin (6).

Fivaz et al. (9) noted an increase in the elution volume from a Sephadex gel filtration column with decreasing concentration of the proaerolysin applied and concluded that this was evidence that the dimer dissociated at low concentrations. We have found that proaerolysin will bind to agarose-based matrices such as Sephadex under a variety of conditions and that this can lead to increased retention with decreased concentration (data not shown here). It is possible that this kind of interaction may account for their observations.

The dimeric state of proaerolysin in solution, which we confirm here, is consistent with several things we know about the protein, including its secretion, its stability to proteases, and the extensive interaction between monomers in the crystal (2). Nevertheless, some time after activation by proteolytic nicking, the monomers must separate, not only because there are an odd number of monomers in the oligomer, but because all of the monomers are thought to be in the same orientation in the oligomer, whereas they are in opposite orientations in the dimer (2). How and when dimer separation occurs remains to be established.

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