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Minireview

Crossing three membranes

Channel formation by aerolysin

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Aerolysin is a channel-forming toxin responsible for the pathogenicity of *Aeromonas hydrophila*. It crosses the inner and outer membranes of the bacteria in separate steps and is released as a 52-kDa inactive protoxin which is activated by proteolytic removal of approximately 40 amino acids from the C terminus. The toxin binds to the erythrocyte transmembrane protein glycophorin and oligomerizes before inserting into the membrane, producing a voltage gated, anion selective channel about 1 nm in diameter. Remarkably, proaerolysin appears to be dimeric, whereas the oligomer is a heptamer. Using chemical modification and site-directed mutagenesis, we have identified some of the regions of the molecule which appear to be involved in secretion and in channel formation.

Aerolysin; Hemolysin; Proteolytic activation; Channel formation

1. INTRODUCTION

Many species produce peptides or proteins that can make holes in cell membranes. Some of these cytolysins are not much more than simple detergents, molecules like mellitin that disrupt the lipid bilayer directly. Others, such as aerolysin, the hemolysin released by *Aeromonas hydrophila*, produce channels in far more complex ways. They typically bind to specific cells and puncture their plasma membranes by forming organized channels with characteristic, well-defined properties. Aerolysin is one of the few examples of such a toxin that is secreted by a Gram-negative bacteria [1]. This makes it especially intriguing to those interested in the interaction of proteins with lipid bilayers, as the protein has to navigate no fewer than three membranes, the inner and outer membranes of the secreting organism, and the membrane of the target cell, which it must span in order to form a channel. An outline of the steps believed to be involved in this process is presented in Fig. 1.

2. GETTING OUT OF THE BACTERIA

One of the most remarkable features of aerolysin is the route it takes out of the cell. Like other Gram-negative species, *A. hydrophila* is surrounded by two membranes that are separated by the periplasmic space. The protein crosses the inner membrane cotranslationally as a prepropeptide, directed by a typical signal sequence that is removed during transit [2]. Proaerolysin is released into the periplasm and it then traverses the outer membrane in a separate step. How this occurs is a puzzle of great interest. It is not yet clear what provides the energy for the protein to cross the outer membrane, or why it does not go back across the inner membrane and enter the cytoplasm. It seems certain that the molecule is not released by rupture of the outer membrane, because other proteins remain periplasmic. Nor is it modified by post-translational modification, since the molecular weight of proaerolysin determined by mass spectrometry matches the predicted molecular weight almost exactly (unpublished). Presumably the set of proteins first shown to be required for secretion by *Klebsiella pneumoniae* [3] and more recently by *A. hydrophila* [4] is somehow involved, but there is as yet no evidence for their direct participation.

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3. PROPERTIES OF THE PROTEIN

The structural genes from both *A. hydrophila* and *A. sobria* have been cloned and sequenced [5–7]. The translated products are very similar hydrophilic proteins of 52 kDa with little or no predicted α-helical structure, much like the porins that make channels in the bacterial outer membrane. Spectroscopic analysis of the purified *A. hydrophila* protoxin indicates that the molecule consists almost entirely of β structure, supporting the prediction. Proaerolysin forms tetragonal crystals that

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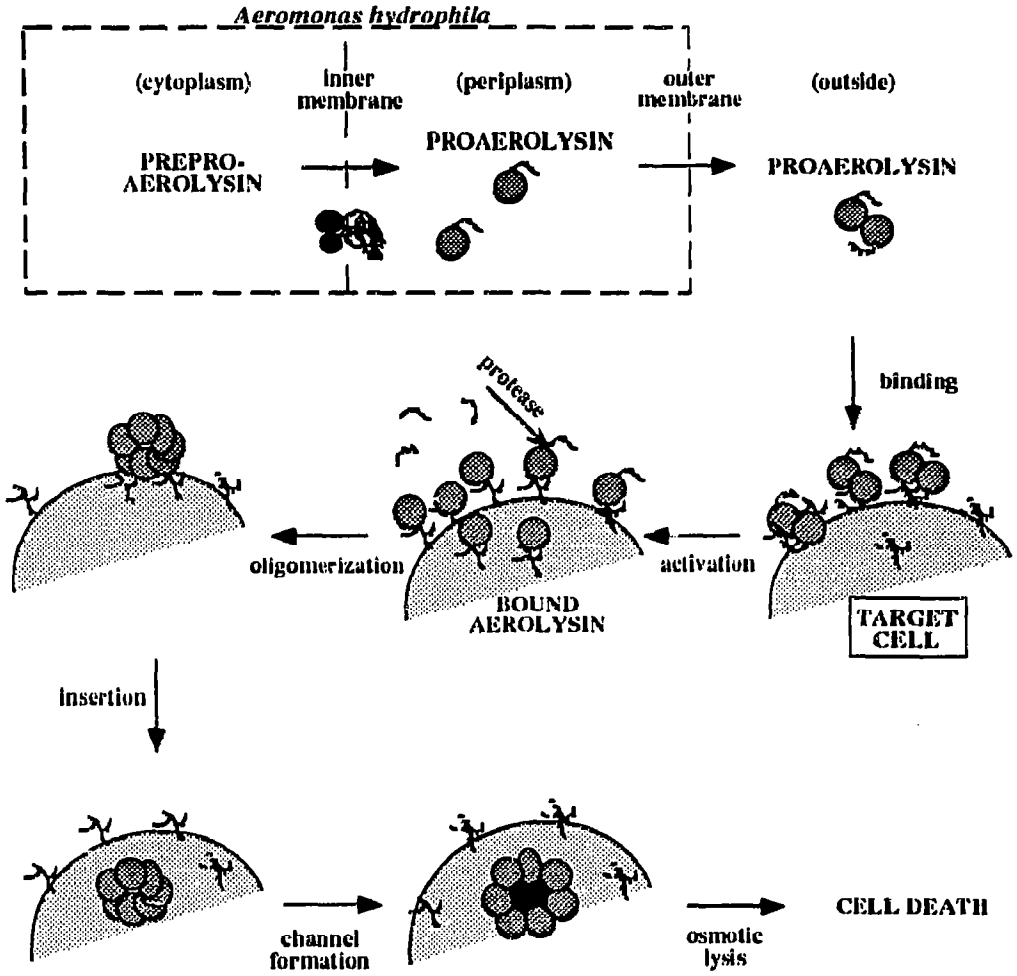

Fig. 1. Secretion and activation of aerolysin and the pathway of channel formation.

contain a dimer in the asymmetric unit and a partial analysis of the electron density map obtained with four derivatives suggests that the molecule is organized into three domains, each consisting of remarkably long β strands [8]. Using a combination of chemical modification and site-directed mutagenesis, we have identified several important regions of the molecule. They are depicted in Fig. 2.

4. ACTIVATION

Since at high concentrations the protein can insert directly into any lipid bilayer [11], the bacteria must protect itself from damage while aerolysin is in transit. It accomplishes this by producing the protein in an inactive proform [13]. The protoxin is only converted to active aerolysin after it is released, by proteolytic removal of a C-terminal peptide. In laboratory cultures, proteases secreted by the bacteria are responsible for processing, whereas in vivo the protoxin may be activated by several common proteases [14]. Recently using mass spectrometry (unpublished) we have identified the sites at which trypsin and chymotrypsin cut the polypeptide chain and activate proaerolysin. Trypsin cleaves proaerolysin after the lysine at position 427, removing a 43 amino acid C-terminal fragment, whereas chymotrypsin cuts after the arginine at 429.

5. BINDING

As with other toxins, cell sensitivity is largely determined by the presence or absence of a receptor, and by the affinity with which aerolysin binds [11,12]. In erythrocytes, we have shown that the receptor is the transmembrane protein glycophorin [11]. This glycoprotein is present in large copy number, and by binding aerolysin with high affinity it effectively concentrates the toxin on the surface of the cell, thus facilitating the oligomerization step that follows [14]. Both proaerolysin and aerolysin have the same affinity for the receptor, and during an *A. hydrophila* infection it seems likely that the protoxin is activated directly on the cell surface after it binds. This arrangement minimizes the risk that the toxin could be formed and oligomerize in solution,

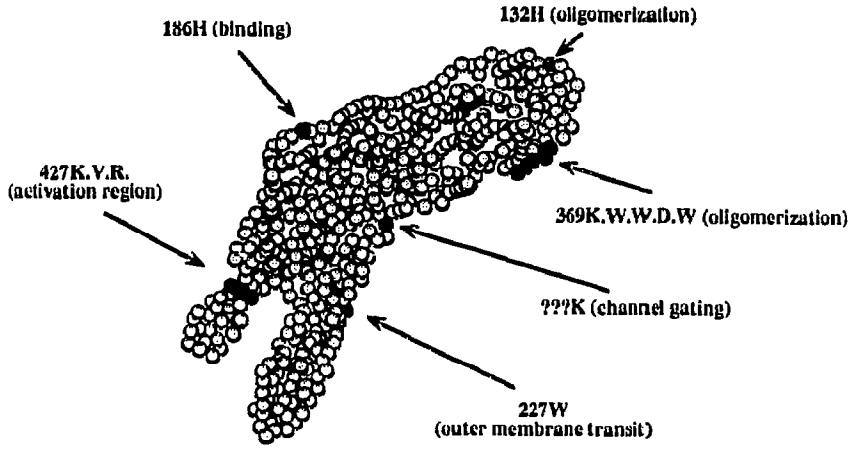

Fig. 2. A representation of the structure of proaerolysin, portraying the importance of some regions of the polypeptide chain. The structure is loosely based on a preliminary analysis of the the electron density map. **However, the regions identified in the figure are placed arbitrarily, for illustration only, as there is no structural information yet available.** With the exception of the sequence K.W.W.D.W, which we have recently found from unpublished results to be involved in oligomerization, all of the regions are described in publications cited in the text.

which could be hazardous to the bacteria, or could lead to aggregation of the oligomers and irreversible precipitation.

6. OLIGOMERIZATION IS REQUIRED FOR ACTIVITY

Although oligomerization must normally occur on the surface of the target cell, it will also take place in solution if the protein concentration rises above about 100 μg/ml. However this must happen only very rarely in vivo. We have shown that oligomerization is an essential step in channel formation and that proaerolysin is inactive because it cannot oligomerize [14], and we have identified several amino acids that appear to have important roles in the oligomerization process (ref. [9] and Fig. 2). Although there is no evidence that covalent bonds are formed during oligomerization, the oligomers are extraordinarily stable, withstanding treatment with high concentrations of chaotropic agents, detergents, weak acids and bases, heat, and reducing conditions. Recently, using electron microscopy to examine two dimensional crystals of the oligomers, we have concluded that they are heptameric. Additional evidence for this very uncommon symmetry has been obtained by stem measurements of the mass of the oligomer, as well as by molecular weight estimations by sodium dodecyl sulfate polyacrylamide electrophoresis [15]. Preliminary results acquired with the analytical ultracentrifuge (unpublished) indicate that proaerolysin is a dimer in solution and since we have found that the active oligomer is heptamer, it follows that the protein must first dissociate after activation. Perhaps this is the effect of removing the C-terminus, leading to the exposure of a surface that promotes oligomerization. This would account for our failure to observe any major structural changes in the protein by circular dichroism or tryptophan fluorescence. Unfortunately we have so far been unable to find conditions that will allow us to measure the oligomerization state of active aerolysin by analytical ultracentrifugation.

7. WHICH COMES FIRST, INSERTION OR OLIGOMERIZATION?

How does a water soluble, hydrophilic protein penetrate a membrane and end up spanning the lipid bilayer? We only understand fragments of the process. Neither aerolysin nor proaerolysin is surface active, and there is no evidence that either will spontaneously insert into a membrane at neutral pH. The protein contains no hydrophobic sequences longer than a few amino acids, and circular dichroic measurements indicate that there is virtually no alpha helical structure. Thus we were not surprised to find that aerolysin oligomerizes before it enters the membrane. We also observed that oligomerization is accompanied by the appearance of a hydrophobic surface and this presumably plays a role in breaching the lipid bilayer although we have no idea how this occurs or why the bilayer is not measurably damaged during the penetration step (unpublished). Nor do we know the orientation of the oligomer in the membrane or to what extent it is exposed on the trans side. Because aerolysin contains no hydrophobic stretches, and because it has a very high proportion of β structure, it seems likely that the membrane-associated form will share some features with porin proteins.

8. THE AEROLYSIN CHANNEL. IS IT THE SAME IN REAL AND ARTIFICIAL MEMBRANES?

Aerolysin forms voltage-gated channels in planar lipid bilayers that are slightly anion selective [16–17]. Zinc ions not only prevent channel formation by inhib-

iting oligomerization, but they also induce closure of preformed channels. In this and several other ways aerolysin is similar to alpha toxin of *Staphylococcus aureus*. Recently we have found that amino-reactive reagents also cause preformed channels to shut and we are in the process of trying to identify which amino acids are modified. Every observation we have made on the properties of channels in the artificial system has been consistent with our results with human erythrocytes, so we can conclude that they correspond to the channels formed in vivo. Based on the permeability of molecular weight markers, we have estimated that the pores formed in erythrocyte membranes are about 1 nm in diameter [12], and we have obtained a very similar value from our analysis of the aerolysin 2-D crystalline array by electron microscopy [15].

9. CONCLUSIONS

Although we now know many details of several of the steps in the formation of channels by aerolysin, we do not seem to have made much progress towards understanding how the protein crosses the outer membrane of the bacteria, or how it penetrates the eucaryotic plasma membrane. Once the crystal structure of the protein has been solved, we hope to be able to identify regions of the protein that are involved in these steps.

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