Site-Directed Mutagenesis of the Hole-Forming Toxin Aerolysin: Studies on the Roles of Histidines in Receptor Binding and Oligomerization of the Monomer†

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ABSTRACT: The six histidines of the channel-forming protein aerolysin have been replaced one at a time with asparagine by site-directed mutagenesis, and each of the modified proteins has been purified. Three proteins had the same hemolytic activity as native toxin, but the others, those changed at His107, His132, or His332, were less able to disrupt both human and rat erythrocytes. The largest reduction in activity, more than 100-fold, was observed with the His132 mutant protein. Studies with radioiodinated samples showed that it had approximately the same affinity as native aerolysin for the rat erythrocyte receptor. However, once bound to either rat or human erythrocytes, it was much less able to carry out the next essential step in hole formation, aggregation to form a stable oligomer. Aggregation was also reduced by replacing His107, but the contrast with native aerolysin and the effect on hemolytic activity were less pronounced. The protein modified at His332 behaved in a different way from those substituted at positions 107 and 132. Its affinity for the rat erythrocyte receptor was considerably lower than the affinity of the wild-type protein, but when bound it appeared to aggregate normally. The results suggest that His132 and perhaps His107 are involved in the aggregation of aerolysin whereas His332 may be at or near the receptor binding site.

Aerolysin is a hole-forming toxin which is largely responsible for the pathogenicity of *Aeromonas hydrophila* (Chakraborty et al., 1987). The bacteria export the protein as a protoxin which is activated by proteolytic removal of 20–30 amino acids from the C-terminus (Howard & Buckley, 1985). Any of a variety of proteases are capable of accomplishing activation, including those released by the bacteria itself (Garland & Buckley, 1986). Aerolysin binds with high affinity to a receptor on eucaryotic membranes which has tentatively been identified as glycophorin in rat erythrocytes (Howard & Buckley, 1982). Once bound, the toxin aggregates to form a pentameric or hexameric structure which is stable even to boiling in sodium dodecyl sulfate under reducing conditions. Oligomerization is followed by the formation of 3-nm channels in the membrane, causing cell disruption (Howard & Buckley, 1982). There is some evidence that aggregation occurs on the cell surface. Presumably it results in the conversion of the water-soluble monomer to a complex capable of inserting into the lipid bilayer. Proaerolysin can also bind to the membrane receptor, but it is completely unable to form a stable aggregate, which accounts for its complete lack of activity (Garland & Buckley, 1986).

We have cloned and sequenced the structural gene for proaerolysin (Howard & Buckley, 1986; Howard et al., 1987), and we have developed a simple procedure for the purification of the protoxin in very high yield (Buckley, 1989). The protein is quite hydrophilic, with no obvious sequences which could form transmembrane helices. Spectroscopic analysis and preliminary X-ray crystallographic data suggest that it consists almost entirely of β structure, analogous to the porin proteins from which aerolysin may have evolved (unpublished observations). The porins also form stable aggregates and transmembrane pores. There are six histidines and four cysteines in the primary structure of aerolysin. It is likely that none of the cysteines participates in oligomerization or channel formation, because activity is unaffected by alkylating agents and by reducing conditions. However, aggregation and hole formation are blocked by diethyl pyrocarbonate, suggesting that one or more histidines are required (Garland & Buckley, 1986). In this paper, we use site-directed mutagenesis to identify the histidines which influence the activity of the toxin.

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Table I: Primers Used in the Mutagenesis of Aerolysin

codon	oligonucleotides[a]	DNA codon change
107	GACTGGTAA*ATGACAGT	CAT to AAT
121	ATCTGGCCA*ATTACCTC	CAT to AAT
132	GCGGCAATA*ACAGCCAA	CAC to AAC
186	GCTTCAAGA*ATGGCGAT	CAT to AAT
332	GGTATACCA*ACCCGGAC	CAC to AAC
341	CTGGAACA*ACACCTTCG	CAC to AAC

[a] Sequences of mutagenic primers are written 5′ to 3′. The altered codons are underlined. The asterisk immediately follows the specific base changed.

Experimental Procedures

Materials

The heptadecanucleotides used for oligonucleotide-directed site-specific mutagenesis were synthesized by using a Sam One DNA synthesizer (Biosearch Inc., San Rafael, CA). Their sequences are given in Table I. Purification of mutagenic oligonucleotides was carried out by means of the C-18 Sep-pak chromatographic method (Zoller & Smith, 1984). All other reagents and materials were molecular biological grade.

Methods

Bacterial Strains and Vectors. The following strains of *Escherichia coli* K-12 were used: JM101, *supE*, *thi*, Δ(*lac-proAB*), [*F′*, *traD36*, *proAB*, *lacI*^{q}*ZΔM15*] (Pharmacia); JM105, *thi*, *rpsL*, *endA1*, *sbcB15*, *hsdR4*, Δ(*lacproAB*), [*F′traD36*, *proAB*, *lacI*^{q}*ZΔM15*] (Pharmacia). The other bacteria and vectors used have been previously described (Wong et al., 1989).

Cell Growth. E. coli JM101 and JM105 were grown as described (Zoller & Smith, 1984). HB101 and KUR1296 were grown at 37 °C in LB medium (Maniatis, 1972).

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Aeromonas salmonicida Rif-1 was grown at 26 °C in LB medium buffered with Davis minimal media (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), tetracycline (20 μg/mL).

Site-Directed Mutagenesis. All mutagenesis reactions were performed using the two-primer oligonucleotide-directed mutagenesis method (Zoller & Smith, 1984). Positive clones were screened initially by plaque hybridization followed by dot blot hybridization. Final confirmation of the nucleotide changes in the clones was achieved by chain-terminating DNA sequencing (Sanger et al., 1979). Restriction analysis was also performed on positive RF DNA to ensure that the clones contained the same restriction sites as the wild-type DNA. Once a clone was confidently identified by all of the above methods, the DNA was inserted into the vector pMMB66 (Furste et al., 1986) as we have described previously (Wong et al., 1989).

Plasmid Manipulations and Bacterial Matings. Plasmid preparations and cloning procedures were carried out by using methods reported previously (Wong et al., 1989). Bacterial matings between HB101 bearing mutagenized aerolysin/pMMB66 constructs and *A. salmonicida* Rif-1 were performed as described previously (Wong et al., 1989).

Protein Purification and Radioiodination. Each of the proteins was purified from culture supernatants of *A. salmonicida* Rif-1 as the form corresponding to unprocessed protoxin (Buckley, 1989). When required, conversion to mature protein was accomplished by treatment with trypsin. Iodination was carried out by the iodogen procedure (Fraker & Speck, 1978; Markwell & Fox, 1978) as we have described earlier (Howard & Buckley, 1982). Specific activities ranged from 2.6×10^7 to 4.6×10^7 cpm/nmol of protein. The hemolytic activities of all of the proteins were unaffected by iodination.

Hemolytic Titers. Wild-type and mutant proteins were titered as previously outlined (Howard & Buckley, 1982) using both rat and human erythrocytes. Titers are expressed as the $\log_2$ of the highest dilution resulting in complete hemolysis. Titers were repeated at least 3 times. There was no variation.

Binding of the Radioiodinated Proteins to Rat and Human Erythrocytes. All of the radioiodinated proteins were adjusted to the same specific activity (2.6×10^7 cpm/nmol) by the addition of appropriate amounts of unlabeled protein. They were then converted from the protoxin forms by treatment with 50 μg/mL trypsin for 15 min at room temperature, and then trypsin inhibitor (0.1 mg/mL) was added. Samples of 100 ng of each of the trypsin-treated proteins were incubated at 37 °C for 15 min in a total volume of 200 μL of 10 mM phosphate/0.15 M NaCl (PBS) containing 0.3 M sucrose and approximately 8×10^7 rat erythrocytes. After centrifugation, the cells were washed in 1 mL of cold PBS–sucrose and suspended in the sample buffer used for sodium dodecyl sulfate electrophoresis (SDS–PAGE; Neville, 1971). After separation, bands corresponding to aggregated and monomeric proteins were located by autoradiography. Binding to human erythrocytes was measured in a similar way except that 4 times as much of each of the proteins was used.

Binding Constants of Wild-Type Aerolysin and the His132 and His332 Mutants. Binding was measured in 1.5-mL Eppendorf tubes with 1–30 nM native or mutant radioiodinated protoxin and approximately 5×10^7 rat erythrocytes in PBS containing 0.1% (w/v) bovine serum albumin. After 30 min at 0 °C, the tubes were centrifuged, and supernatants and cells were counted separately. Nonspecific binding, which accounted for up to 10% of the total, was measured by adding a large excess of unlabeled protein. Binding constants and their standard errors were obtained from Scatchard plots (Scatchard, 1949) by regression analysis.

Table II: Hemolytic Activity of the Modified Proteins

histidine changed	hemolytic titer[a] human	rat
none (wild type)	9	12
107	7	10
121	9	12
132	2	7
186	9	12
332	7	11
341	9	12

[a] Titers were measured using 2 μg of each protein as described under Methods. Results are means of three identical experiments.

Results

Isolation of the Mutant Proteins. All of the modified proteins were exported by *A. salmonicida* in amounts comparable to the wild-type protein. They behaved in exactly the same way as native proaerolysin during purification (data not shown). This suggests that the structures of the proteins were not changed significantly by modification of any of the histidines. We have found that other changes in the primary structure of aerolysin lead to pronounced instability of the products. The main reason for this appears to be that the secondary structures of the proteins are altered enough by the mutations to make them much more sensitive to periplasmic proteases (unpublished observations).

Effects of the His Mutations on Hemolytic Activity. The hemolytic titers of the modified proteins are compared to wild-type aerolysin in Table II. Data obtained using both rat and human erythrocytes are presented. As we have shown earlier (Howard & Buckley, 1982), rat cells were more sensitive than human cells to native toxin. They were also more susceptible to all of the mutant proteins. Three of the new aerolysins were less active than native toxin. The largest reduction in activity was observed with the His132-modified protein. Its titer, measured with human erythrocytes, was seven wells lower than wild type under the conditions used, corresponding to a difference in activity of approximately 128-fold (titers are expressed in $\log_2$ units). Activity against rat erythrocytes was not as dramatically affected, but nonetheless was 32-fold lower than wild type. Replacing His107 or His322 also decreased hemolytic activity, although the reductions were much lower than those observed with the change at His132.

Binding to Human and Rat Erythrocytes. There are at least three ways that a change in the primary structure of aerolysin could lead to lower cytolytic activity. Replacing a histidine could result in lower affinity for the membrane receptor, decreased ability to aggregate, or failure to form an open channel. In order to distinguish between these possibilities, wild-type proaerolysin and each of the mutants were labeled with ^{125}I. The extent of binding and aggregation was estimated by using human or rat erythrocytes. Following incubation and SDS–PAGE, the proteins were detected by autoradiography, and the bands were cut from the gels and counted. The results are shown in Figure 1 and Table III. In every case, all or nearly all the radioactivity was confined to two bands, corresponding to the mature and aggregated forms of the proteins. Little or no protoxin was observed in any lane, and there were no low molecular weight breakdown products. Thus, each of

Table III: Binding of Radioiodinated Proteins to Rat Erythrocytes[a]

histidine changed	% of wild-type binding	monomer/aggregate ratio
none (wild type)	100	0.26
107	112	0.55
121	100	0.20
132	119	2.29
186	95	0.17
332	46	0.29
341	98	0.26

[a] Bands from the gels shown in Figure 1 were excised and counted. The results are the means of two separate determinations which did not vary by more than 10%.

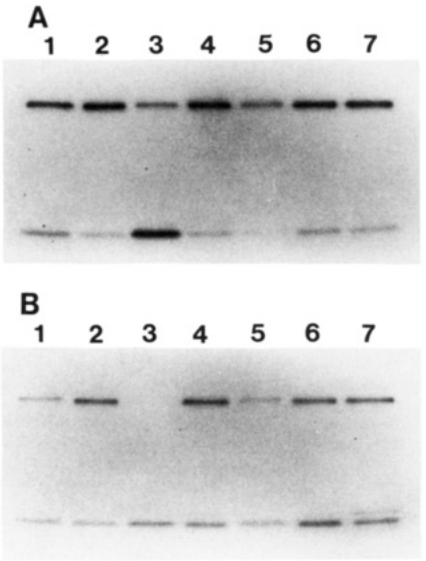

FIGURE 1: Autoradiograms of radioiodinated proteins bound to erythrocytes: (A) rat; (B) human. See text for experimental details. The samples were applied to the gels in order from the His107 mutant in lane 1 to the His341 mutant in lane 6. The mixture containing native aerolysin is in lane 7. The band near the top of the gels in all but one lane (3B) corresponds to aggregated aerolysin. A small amount of unprocessed proaerolysin can be seen just above monomeric mature aerolysin in lane 7B. This is one of three similar experiments.

the modified proteins shares essentially the same trypsin sensitivity as wild type. The three proteins shown in Table II to have normal titers each bound to rat and human erythrocytes in approximately the same amounts as native aerolysin. Furthermore, for all three, most of the radiolabel was recovered in the aggregates, and the proportions of monomer to aggregate were about the same as for aerolysin itself. The protein changed at His132 also appeared to bind to the rat cells as well as wild type, but it formed far less aggregate. In fact, no aggregate of this protein could be detected at all on the human erythrocytes at the concentration used (Figure 1). The proportion of monomer was also higher with the His107 mutant, although the contrast with wild type was not nearly as striking. Replacing the histidine at 332 resulted in quite different behavior. The protein appeared less able than wild type to bind to cells of both species; however, once bound, it formed aggregate in nearly the same proportion as native aerolysin.

Rat Erythrocyte Binding Constants. The results in Figure 1 and Table III indicated that binding of the 132 mutant to rat erythrocytes was similar to wild type, whereas binding of the 332 mutant was lower. In order to estimate the affinity of the proteins for the receptor, binding of the protoxin forms was measured over a range of concentrations (Figure 2). Scatchard (1949) analysis of the data showed that the number of binding sites was about 10^5/cell for each of the proteins,

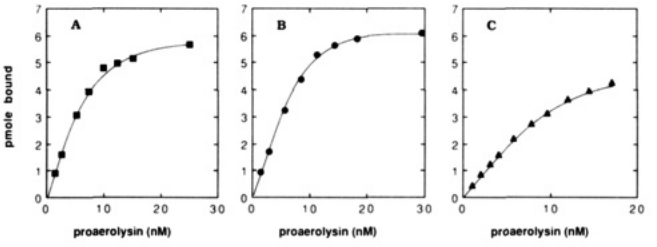

FIGURE 2: Binding to rat erythrocytes. Binding of native proaerolysin and the 132 and 332 mutants was measured as described in the text. Results shown are the means of three separate experiments. (A) Native proaerolysin; (B) 132 mutant; (C) 332 mutant.

indicating that they bound to the same receptor on the cell surface. The affinity constants of native proaerolysin and the His132 mutant for the receptor were about the same, $(2.93 \pm 0.30) \times 10^8$ and $(2.45 \pm 0.26) \times 10^8$ L/mol, respectively, whereas the affinity constant for the His332 mutant was lower, $(1.08 \pm 0.03) \times 10^8$ L/mol, consistent with the results in Figure 1 and Table III.

Discussion

There have been few attempts to quantitate the binding of hole-forming toxins to eucaryotic cells. Although several groups have measured the binding of *Staphylococcus aureus* α-toxin, widely different results have been reported (Bhakdi & Tranum-Jensen, 1988). Because they all aggregate not only once bound to the cell but also spontaneously in solution, accurate measurements of the binding of any cytolytic toxin may be impossible. The aerolysin system offers the very great advantage that both protoxin and toxin bind to cells, but the protoxin cannot aggregate and thus may be used in accurate binding studies. The results presented here show that proaerolysin has a high affinity for rat erythrocytes with an affinity constant of 2.9×10^8 L/mol. The number of binding sites approaches 10^5/cell, which approximates the number of glycophorin molecules in the membrane, assuming that the number of copies of glycophorin is about the same in rat and human erythrocytes (Bennett, 1985). This supports our contention that glycophorin is the aerolysin receptor (Howard & Buckley, 1982; Garland & Buckley, 1988). The number of sites is lower than we have previously estimated. In our earlier studies, however, we used mature aerolysin rather than the protoxin, and we did not achieve saturation of receptor sites. This could be taken as evidence that the toxin no longer binds to the receptor after aggregation, or after insertion into the lipid bilayer.

As many as three of the histidines in aerolysin may somehow be involved in the reaction mechanism which results in hole formation. It is therefore not surprising that we have previously observed that aggregation and hemolysis are inhibited by diethyl pyrocarbonate (Garland & Buckley, 1988). Changing His132 had the most profound effect on activity. The results show that this is due to a reduction in the ability of the protein to form oligomers, rather than a change in its affinity for the erythrocyte receptor. This mutant was proportionally more active against rat than human erythrocytes, presumably because the concentration of monomers was far higher on the surface of the rat cells, driving formation of oligomers. The much more modest effect of replacing His107 also seemed to be the result of decreased ability to aggregate. These observations suggest that His132 and perhaps His107 participate in the oligomerization process, or that they are structurally important in oligomer formation.

Changes in aggregation rate could not account for the decreased activity of the protein modified at position 332. In-

stead, the results clearly show that this protein has a lower affinity for the receptor on the rat erythrocyte, leading to the conclusion that His332 is at or near the binding site for the membrane receptor in aerolysin.

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