Assembly of the Oligomeric Membrane Pore Formed by Staphylococcal α-Hemolysin Examined by Truncation Mutagenesis*

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The α-hemolysin (αHL) from Staphylococcus aureus causes the lysis of susceptible cells such as rabbit erythrocytes (rRBCs). Lysis is associated with the formation of a hexameric pore in the plasma membrane. Here we show that truncation mutants of αHL missing 2 to 22 N-terminal amino acids form oligomers on the surfaces of rRBCs but fail to lyse the cells. By contrast, mutants missing 3 or 5 amino acids at the C terminus are very inefficient at oligomerization but do lyse rRBCs, albeit extremely slowly. The C-terminal truncation mutants, retarded as monomers on the cell surface, undergo a conformational change in which the protease-sensitive loop located near the midpoint of the polypeptide chain becomes occluded. Judged by this criterion, polypeptides truncated at the N terminus, frozen as nonlytic oligomers, are in a similar conformation. A second proteolytic site near the N terminus of the polypeptide becomes inaccessible in the lytic pore formed by the wild-type polypeptide, supporting the idea that a second conformational change occurs upon pore formation. These findings suggest a pathway for assembly of the lytic pore in which αHL first binds to target cells as a monomer, which is converted to a nonlytic oligomeric intermediate before formation of the pore. In keeping with this model, an N-terminal truncation mutant blocks the slow lysis induced by a C-terminal truncation mutant, presumably by diverting the weakly lytic subunits into inactive oligomers.

Information about the assembly of oligomeric membrane proteins is important for understanding the biosynthesis of integral membrane proteins, the actions of cytolytic toxins and immune proteins, and how receptors aggregate with each other or with regulatory proteins. While considerable knowledge is accumulating about the integration into membranes of individual protein subunits (1-4), including large polypeptides with multiple domains (5), far less is known about how membrane protein subunits come together. Recent findings concerning Na/K-ATPase (6), acetylcholine receptor (7), complement C9, and perforin (8) have demonstrated intermediates in assembly in complex cellular systems. A more complete picture would be available if such work were combined with in vitro biochemical and biophysical studies of assembly intermediates (4).

Because of its simplicity, α-hemolysin (αHL) is a model system for studying the assembly of an oligomeric membrane pore (9). The αHL polypeptide of 33,200 Da (293 amino acids) is secreted by Staphylococcus aureus as a water-soluble monomer, which assembles into cylindrical pores in susceptible cell membranes (10, 11). The secondary structure of both the monomer and hexamer is predominantly β-sheet (12, 13). It has been proposed that αHL undergoes a conformational change during assembly, involving the separation of two rigid domains connected by a loop near the midpoint of the polypeptide chain, converting the molecule into an amphipathic rod, six of which form a pore (12). A glycine-rich sequence (residues 119-143) (14), thought to be the loop, is susceptible to proteases in the monomer but not in the hexamer (12). Here, we use deletion mutagenesis to demonstrate two potential intermediates in assembly: a membrane-bound monomer in which the loop is occluded and a nonlytic oligomeric pore precursor of variable stoichiometry.

EXPERIMENTAL PROCEDURES

Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis was carried out as described by Tobeke et al. (12). Gels showing oligomers were run according to Fairbanks (15) and contained 1% SDS. After limited proteolysis (see below) gels were run according to Laemmli (16). Radiolabeled markers (Amersham) were 14C-methylated proteins: myosin (M, 200,000); phosphorylase b (M, 92,500); bovine serum albumin (M, 69,000); ovalbumin (M, 46,000); carbonic anhydrase (M, 30,000); lysozyme (M, 14,300).

Deletion Mutagenesis—Fragments of the αHL gene were amplified by the polymerase chain reaction from the plasmid pT7NPH-8 in which the internal NdeI site in the gene has been removed by mutagenesis (17). This copy of the αHL gene contains a G → A substitution at position 1058 resulting in the replacement of Ser-217 by a Gin residue, which has no effect on the activity of the polypeptide. For N-terminal truncations, the 5' primer was 5' AAACATTTTCTGAACCTTGGCCTAAAG, which is complementary to a sequence in the proximal 3'-untranslated region, except for two mismatches introduced to form a HindIII site. The 3’-primer was used in combination with the 5’-primers: 5’ GCCGGAAATCTGCGAGGAGGTACATATGCGAGATTTATTTAAAACGC, αHL (A39-293); 5’ GCCGGAAATCTGCGAGGAGGTACATATGCGAGATTTATTTAAAACGC, αHL (A39-293); 5’ GCCGGAAATCTGCGAGGAGGTACATATGCGAGATTTATTTAAAACGC, αHL (A39-293). The EcoRI, PstI, and ribosome binding sites at the 5'-ends of these primers were not used in the work described here. Each primer contained an NdeI site at the initiation codon followed by an alanine codon. The latter ensures efficient cotranslational removal of the N-terminal methionine (17) and limits proteolytic degradation (18). Residue 1 in staphylococcal αHL (s-αHL) is also alanine. The 5'-primer for the C-
terminal truncations was 5’ CGGGATCTTAACTGACTCTACATTAGGG, which hybridizes to the T7 promoter region upstream from the Ndel site in pT7NH-8. The 3’-primers were 5’ CGCGAACTTCTTCATTTCTCCTTTCTTCTTCCTCTTCCTGC, aHL(1-290); 5’ CGCGAACTTCTCATTATTTTTCCCAATCG, aHL(1-288); 5’ GGGCAATGTTACTTATTTCTTCTTATATC, aHL(1-285). These primers encode a HindIII site followed by two terminal anacodons and the desired anacodons of the αHL sequence. The amplification products were cut with NdeI and HindIII.

CAAGCTTTCATTATTTTTCCCAATCGATTTTATATC, aHL(l-290), GCGCAATGTTACTTATTTTTCCCAATCG, aHL(l-288), which hybridizes to the T7 promoter region upstream from the Revichia coli S30 extract (Promega L464) in the presence of T7 RNA polymerase, rifampicin, and [35S]methionine (17). For hemolysis as- tion of methionine, ensuring the synthesis of concentrations of mu-

Three times with each of three independent clones. The following polymerase chain reaction errors the number of cycles was minimized.

The stoichiometries of the oligomers, as recorded in Table I, were assigned by assuming that the major species formed by [35S]r-αHL is a hexamer (12, 17) and a linear relationship between the mass of an oligomer and the negative log of the migration distance. For entries A and B in the Table, it was assumed that homo-oligomers were formed. For entry C and the experiments with DOC (see below), it was assumed that hetero-oligomers containing a single mutant sub-unit were formed.

Oligomer Formation in DOC—To investigate oligomer formation in detergent, mutant [35S]-labeled polypeptides in S30 mix (7.5 µl) were mixed with 10 mM methionine, 100 mM Tris-Cl, pH 8.0, containing 1 mM β-mercaptoethanol, followed by the addition of 1 mM leupeptin (1 µl) and s-αHL (1.8 mg/ml; 30 µl). Sodium DOC (82 mM, pH 8.2, containing 10 mM Tris-Cl) was then added over 30 min to 0.4 mM (4 X 2.5 µl), followed by a 20-min incubation, all at room temperature. After each addition the mixture was stirred for a few seconds. Finally, one volume of 2 X Fairbanks loading buffer was added at room temperature, followed by analysis in a 4.5% SDS-polyacrylamide gel gel electrophoresis and autoradiogra-

Hemolysis Assay—S30 extracts were serially diluted in microtiter wells with 20 mM KH$_2$PO$_4$, 150 mM NaCl, pH 7.4, containing 1 mg/ml bovine serum albumin (K-PBSA) before the addition of an equal volume of 1% rabbit red blood cells (rRBCs). The extent of hemolysis, based on protein chemistry, the ability to bind to erythrocytes and the properties of single channels formed in lipid bilayers (17). In the present work, truncation mutants of αHL

Membranes or cells were recovered by centrifugation, dissolved in 2 X Fairbanks loading buffer, warmed at 45 °C for 5 min, and subjected to electrophoresis in a 4.5% SDS-polyacrylamide gel (17). The stoichiometries of the oligomers, as recorded in Table I, were assigned by assuming that the major species formed by [35S]r-αHL is a hexamer (12, 17) and a linear relationship between the mass of an oligomer and the negative log of the migration distance. For entries A and B in the Table, it was assumed that homo-oligomers were formed. For entry C and the experiments with DOC (see below), it was assumed that hetero-oligomers containing a single mutant sub-unit were formed.

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Limited Proteolysis—Mutant [35S]-labeled polypeptides in S30 mix (1 µl) were allowed to bind to 1% rRBCs in K-PBSA (90 µl) for 1 h at 20 °C. In the experiment displayed in Fig. 2, the cells were washed twice with K-PBSA, resuspended in the same buffer (20 µl), and treated with proteinase K (0.1 µg) for 0, 2, 15, and 60 min at 20 °C. [35S]-Labeled polypeptides, diluted 80-fold in K-PBSA without the prior addition of rRBCs, were also treated with proteinase K under the same conditions. After the addition of phenylmethylsulfonyl fluoride (0.4 µl, 50 mM) to each sample, it was heated in Laemmli loading buffer for 5 min at 95 °C and subjected to electrophoresis in a 12% SDS-polyacrylamide gel (17). In the experiment displayed in Fig. 3, proteolysis was with 1 µg of proteinase K for 5 min at 20 °C.

RESULTS AND DISCUSSION

Hemolytic Activity of αHL Truncation Mutants—α-Hemolysin expressed in E. coli cells or lysates (r-αHL) is essentially identical to staphylococcal αHL (s-αHL) as judged by criteria based on protein chemistry, the ability to bind to erythrocytes and to form oligomers on cells or in detergent, hemolytic potency, and the properties of single channels formed in lipid bilayers (17). In the present work, truncation mutants of αHL were made by ligating segments of the hemolysin gene, generated by polymerase chain reaction amplification, into a T7 transcription vector followed by coupled in vitro transcrip-

![Fig. 1. Oligomer formation on rRBCs and in DOC examined by SDS-polyacrylamide gel electrophoresis and autoradiography.](image-url)

A: left panel, 35S-labeled truncation mutants were incubated with rRBCs followed by a chase with excess s-αHL; right panel, duplicate experiment with C-terminal truncations, film overexposed. B, 35S-labeled truncation mutants were mixed with excess s-αHL and treated with DOC at around the critical micelle concentration. Related data is given in Table I.
Assembly of α-Hemolysin

**TABLE I**

Properties of αHL deletion mutants

Hemolytic activity is recorded as the well in a 2-fold serial dilution in which 50% hemolysis of RBCs occurred. The final dilution in well 1 was 1:4. In parentheses are the relative concentrations at which hemolysis took place (r-αHL = 1). The ability of a mutant polypeptide to bind to RBCs was determined by experiments such as that shown in Fig. 1A. Oligomer formation on RBCs was determined using three separate procedures: A, no chase with unlabeled s-αHL; B, chase with unlabeled s-αHL (for an example see Fig. 1A); C, mutant polypeptide and unlabeled s-αHL premixed. Oligomer formation in DOC was determined as described in Fig. 1B. Key: s, more than 50% conversion to oligomer; w, weak oligomer band; vw, very weak oligomer band; ?, extremely weak oligomer band of uncertain origin in DOC experiment. Protease resistance was determined as shown in Fig. 2: +, protease-resistant loop region in >10% of the polypeptide bound to RBCs; −, protease-sensitive loop region.

| αHL mutant | Hemolytic activity | Binding to RBC | Oligomer formation | Protease resistance |
|------------|-------------------|---------------|-------------------|--------------------|
|            | 1 h | 24 h | rRBC | A | B | C | DOC | |
| 1-285      | 0   | 0   | +   | 0 | 0 | 0 | ?  | −  |
| 1-288      | 0   | 0   | +   | 0 | α5 vw | α6 vw | α6 vw | +  |
| 1-290      | 0   | 0   | +   | 0 | α6 vw | α6 vw | α6 vw | +  |
| r-αHL (1-293) | 9 | 11-12 (1) | + | α5 s | α6 s | α6 s | α6 s | +  |
| A3-293     | 0   | 0   | +   | α5 s | α5 s | α6 s | α6 s | +  |
| A12-293    | 0   | 0   | +   | α5 s | α6 w | α6 w | α6 s | +  |
| A23-293    | 0   | 0   | +   | α6 vw | α6 w | α6 w | α6 s | +  |
| A39-293    | 0   | 0   | +   | 0 | 0 | 0 | ?  | −  |

**Fig. 2.** Time courses of limited proteolysis of αHL truncation mutants in solution and on the RBC surface with proteinase K. A, r-αHL; B, αHL(A3-293); C, αHL(1-290). The filled arrows indicate the position to which peptides from αHL cleaved in the loop region migrate (molecular mass of ~17 kDa based on 125I-labeled markers; the initially formed N- and C-terminal fragments comigrate (12)). The open arrows indicate fragments generated after cleavage at sites near the N terminus (molecular mass of ~32 kDa based on 125I-labeled markers).

**Fig. 3.** Limited proteolysis of αHL truncation mutants on the RBC surface. Key: −, untreated cells; +, cells treated with proteinase K for 5 min after αHL had bound. The proteolytic fragments of the C-terminal truncation mutants are of increased electrophoretic mobility, suggesting that cleavage occurs at the N terminus. Except for αHL(A3-293), the C-terminal truncation mutants bound to RBCs are unaffected by proteinase K. This suggests that a proteinase K cleavage site lies between residues 3 and 11.
of hexamer, and a lower mass species, probably a tetramer. An N-terminal truncation mutant lacking 22 amino acids (αHL(A23–293)) formed a hexamer, albeit inefficiently. Removal of 38 amino acids or more (αHL(A39–293), and data not shown) resulted in complete loss of the ability to form stable oligomers on rRBCs. Closely similar results were obtained whether or not treatments of rRBCs with mutant polypeptides were followed with a chase with unlabeled s-αHL. Oligomers on rRBCs were very similar to those in DOC (Table I, entry C). The size distributions of these structures differed from those formed by the mutant subunits on rRBCs (Fig. 1A) suggesting that these N-terminal mutants, defective in lysis but capable of self-assembly, can form hetero-oligomers containing s-αHL subunits. This finding was confirmed by experiments with rRBCs using mutant subunits premixed with s-αHL in which the size distributions of the oligomers were very similar to those in DOC (Table I, entry C).

Oligomerization of C-terminal Truncation Mutants—Deletions of 3 or 5 amino acids at the C terminus (αHL(1–290), αHL(1–288)) greatly inhibited the ability of αHL to form oligomers on rRBCs. Traces of hexamer were observed, which were no longer detectable after the deletion of eight or more C-terminal amino acids (αHL(1–285)) (Fig. 1A). Hexamer formation by αHL(1–290) and αHL(1–288) was even less efficient when the s-αHL chase was omitted, suggesting that these mutants are better incorporated into oligomers that include the full-length polypeptide. Traces of hexamer were also observed after treatment of αHL(1–290) and αHL(1–288) with DOC in the presence of s-αHL. It is presumably hexamer that causes the slow lysis of rRBCs by αHL(1–290) (Table I).

Conformations of Mutant αHL Polypeptides Probed by Limited Proteolysis—The conformations of αHL(A3–293) and αHL(1–290), the mutant polypeptides with the smallest N- and C-terminal truncations, were probed in solution and on the surface of the red cell by limited proteolysis with proteinase K (12). After binding to rRBCs, both resisted cleavage in the loop region (Fig. 2). Proteinase K has previously been shown to cleave αHL in solution before Val-140 and Ile-136 (major sites) and before Asn-139 and Gly-134 (minor sites) (25). Even though αHL(1–290) is almost entirely monomeric under these conditions (Fig. 1A), quantitative autoradiography indicated that the glycine-rich loops of 30 ± 15% of the bound polypeptide were resistant to proteolysis. A plausible interpretation is that this fraction of αHL(1–290) undergoes a conformational change on the rRBC surface (see below). The resistant fraction of bound αHL(1–290) was nonetheless cleaved at additional sites that were deduced to lie within 11 amino acids of the N terminus by comparison of the proteolytic patterns generated from several truncation mutants bound to rRBCs (Fig. 3). The N-terminal site of cleavage was further confirmed by using the labeled mutants [35S-Cys]αHL-S3C and [35S-Cys]αHL-T292C. Wild-type αHL contains no cysteine residues so the radiolabel acts as a marker for an intact N or C terminus. Radiolabel was present in membrane-bound, ~32-kDa fragments of αHL-T292C, but not in fragments of αHL-S3C.

The N-terminal truncation mutant (αHL(A3–293)) was also cleaved near the N terminus (Figs. 2B and 3). The cleavage proceeded to completion but more slowly than with αHL(1–290), perhaps because of steric hindrance to proteolysis in the nonlytic oligomeric state. By contrast, a large fraction of the full-length polypeptide, r-αHL, was completely resistant to cleavage (Fig. 2A). While this may indicate a second conformational change upon formation of the αHL pore, it must be recognized that the N-terminal truncation could itself affect the rate of proteolysis. The small fraction of full-length r-αHL bound to rRBCs that is cleaved at the N-terminal site is likely to be residual monomer (Fig. 2A).

Interpretation of the Mutagenesis Experiments—One drawback of any study using mutagenesis is the possibility that the mutant polypeptides are incorrectly folded (26). Several arguments suggest that the present study is less vulnerable than many to this criticism. First, while several of the mutants

2 B. J. Walker and H. Bayley, unpublished results.
were more sensitive than r-αHL to proteinase K in solution, they did exhibit initial cleavage in the loop region. When bound to rRBCs the loop was protected, but a site near the N terminus was cleaved. This conservation of the proteolytic sites found in wild-type αHL suggests that the folding of the mutant polypeptides approximates the native structure. Second, the mutant polypeptides that have been described in most detail here are not inactive but defective in specific and varied aspects of assembly. Third, although modifications that alter the phenotype of αHL need not be drastic (e.g. in αHL(A3-283) the N-terminal Ala-Asp-Ser... becomes Ala... and in αHL(1-290) just 3 residues...Met-Thr-Asn are removed from the C terminus), we are not advocating the involvement of deleted amino acids in specific functions. Rather, we are suggesting that selective modification of the N- or C-terminal domains leads to the accumulation of interesting assembly intermediates. This reserved interpretation remains valid if the mutations produce regional disturbances in folding rather than simple voids in the protein structure. The recent finding that the N- and C-terminal halves of αHL can be separately synthesized and reconstituted into a functional hemolysins strengthens the assumptions that the polypeptide can be considered as a two-domain structure (12) and that at least the unaltered half of each truncation mutant is properly folded. In summary the main conclusions of the study seem incontrovertible: 1) N-terminal lesions in αHL yield polypeptides that form oligomers without hemolytic activity; 2) C-terminal lesions yield polypeptides that bind to rRBCs as monomers in which the loop connecting the N- and C-terminal domains is occluded.

Model for Assembly of the αHL Pore—The data are consistent with a working model for the assembly of αHL (Fig. 4), which provides a succinct summary of the biochemical findings. At this point, other possibilities cannot be strictly excluded, including those in which the monomer is lytic (for discussions see Refs. 11, 27-29). The N-terminal truncation mutants, αHL(A3-293), αHL(A12-293), and αHL(A23-293), are trapped as zymolytic oligomers (3), suggesting that the step 3 → 4 is blocked. By contrast, the C-terminal truncation mutants, αHL(1-290) and αHL(1-288), appear to be retarded at 2, membrane-bound monomers with an occluded loop region. These mutant polypeptides are very slowly converted to hexamers with concomitant cell lysis (4). In keeping with the suggested sequence of events, αHL(A3-293) blocks the slow hemolysis induced by αHL(1-290) (Fig. 5), without affecting the binding of the latter to rRBCs (data not shown). The model is consistent with earlier findings (12, 27, 30, 31). For example, a conformationally altered monomer (2) has been suggested based on photolabeling studies with hydrophobic reagents (27), while a nonlytic oligomeric pore precursor (3) has been proposed, because proteolytically cleaved s-αHL forms oligomers but does not lyse cells (30). Preliminary structural studies also suggest that oligomeric forms of s-αHL that do not fully penetrate the membrane can be trapped in two-dimensional crystals (31). Besides unifying previous mechanistic data and proposals, the present findings suggest that the C terminus of the polypeptide is involved in the initial aggregation of the αHL monomers, while an intact N terminus is required to complete pore formation (Fig. 4). As r-αHL can be obtained in milligram amounts (17), it should now be possible to isolate the putative intermediates for biophysical studies after treatment of αHL mutants with DOC (24) or by fractionation of αHL-treated rRBCs (32).

Acknowledgments—We thank Stephen Cheley, Rob Jackson, John Kasianowicz, and Rekha Panchal for help and comments, and John Goodchild and Vipin Kohli for oligonucleotides from the Worcester Foundation for Experimental Biology Cancer Center Core Facility.

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