Role of the Amino Latch of Staphylococcal α-Hemolysin in Pore Formation

A CO-OPERATIVE INTERACTION BETWEEN THE N TERMINUS AND POSITION 217*

Received for publication, October 4, 2005 Published, JBC Papers in Press, October 14, 2005, DOI 10.1074/jbc.M510841200

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Staphylococcal α-hemolysin (αHL) is a β barrel pore-forming toxin that is secreted by the bacterium as a water-soluble monomeric protein. Upon binding to susceptible cells, αHL assembles via an inactive prepore to form a water-filled homoheptameric transmembrane pore. The N terminus of αHL, which in the crystal structure of the fully assembled pore forms a latch between adjacent subunits, has been thought to play a vital role in the prepore to pore conversion. For example, the deletion of two N-terminal residues produced a completely inactive protein that was arrested in assembly at the prepore stage. In the present study, we have re-examined assembly with a comprehensive set of truncation mutants. Surprisingly, we found that after truncation of up to 17 amino acids, the ability of αHL to form functional pores was diminished, but still substantial. We then discovered that the mutation Ser217 → Asn, which was present in our original set of truncations but not in the new ones, promotes complete inactivation upon truncation of the N terminus. Therefore, the N terminus of αHL cannot be critical for the prepore to pore transformation as previously thought. Residue 217 is involved in the assembly process and must interact indirectly with the distant N terminus during the last step in pore formation. In addition, we provide evidence that an intact N terminus prevents the premature oligomerization of αHL monomers in solution.

α-Hemolysin (αHL) 3 and leukocidin (Luk) are β barrel pore-forming toxins (βPFT) that are secreted by Staphylococcus aureus as water-soluble monomeric polyopeptides (1–5). On susceptible cells, the αHL and Luk polypeptides oligomerize to form water-filled transmembrane pores that cause cell permeation and in some cases lysis (1, 2, 6). The αHL monomer, a polypeptide of 293 residues, forms homoheptameric dimers, trimers, etc. (23, 24). The protease-resistant pre-stem regions of individual protomers in solution. Starting point in the assembly of this class of βPFTs (Fig. 1B).

The folds of all three monomers resemble that of the HL monomer, LukS-PV, have been determined (Fig. 2, A–C). The Luk structures serve as prototypes for the starting point in the assembly of this class of βPFTs (Fig. 1B).

A mechanistic framework with four distinguishable stages has been proposed for the assembly of αHL and related βPFTs (20, 22). In the water-soluble monomer (stage 1, Fig. 1B), the N terminus and the pre-stem are packed against the β sandwich core of the protein (20). At this stage, both the N terminus and the pre-stem are susceptible to proteolysis (22). When the monomer binds to membranes (stage 2, Fig. 1B), the pre-stem becomes resistant to proteolysis. However, the N terminus is still protease-sensitive. It is not clear whether any structural changes take place in stage 2, which is the most poorly defined of the four stages; the reduced susceptibility of the pre-stem to proteases could arise from a lack of accessibility to the enzymes at the bilayer surface. Lateral diffusion then gives rise to prepore formation (stage 3, Fig. 1B). The details of the pathway for oligomerization are not well understood (23, 24). Monomers could add onto a growing chain of subunits until it reaches the proper length to circularize or assembly could occur through random encounters between intermediates (e.g. dimers, trimers, etc.) (23, 24). The protease-resistant pre-stem regions of individual protomers reside inside the prepore (9, 22, 23, 25, 26), which is by definition not

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* This work was supported by the MRC/EPSRC and the ONR. Work at Texas A&M was supported by DARPA, the U.S. Department of Defense Tri-Service Technology Program, the U.S. Department of Energy, NASA, the National Institutes of Health, and the ONR. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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3 The abbreviations used are: αHL, α-hemolysin; βPFT, β barrel pore-forming toxin; BSA, bovine serum albumin; IVTT, in vitro transcription and translation; LukF, leukocidin F protein; LukS, leukocidin S protein; MBSA, MOPS-BSA; MOPS, 3-[(N-morpholino)propane sulfonic acid; RBC, rabbit erythrocytes; WT, wild type.
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FIGURE 1. Proposed assembly pathway for α-hemolysin. A, structure of the staphylococcal α-hemolysin (αHL) heptamer (15). Each subunit is shown in a different color. The position of the membrane is shown in red; B, proposed model for the assembly of αHL (15, 20, 22, 25). The N terminus is shown in magenta, and the pre-stem domain is shown in green (stage 1). Water-soluble monomer: both the N terminus and the pre-stem region are protease-sensitive (stage 2). Membrane-bound monomer: the N terminus remains protease-sensitive at this stage, but the pre-stem region becomes protease-resistant (indicated by the dashed line) (stage 3). Pre-pore: the N terminus remains protease-sensitive. One possibility is that it is exposed to the aqueous phase, as depicted (stage 4). Functional pore: latch formation makes the entire pore protease-resistant. Assembly of the heptameric αHL pore is shown, but a similar pathway is proposed for leukocidin, which forms an octameric pore containing four copies each of two subunits LukF and LukS.

Lytic (stage 3, Fig. 1B). The N terminus of the prepore remains protease-sensitive (22, 25). In our hands, no intermediates have been observed in the prepore to pore conversion, which may therefore be a cooperative process (stage 4, Fig. 1B) (25). In the case of wild-type αHL, formation of the transmembrane β barrel is accompanied by re-organization of the N terminus, which latches onto the neighboring subunit (15, 27). In the fully formed pore, the amino latch is hidden within the cavity of the cap domain and resists proteolysis (Fig. 2A) (22, 25).

The αHL pore has been extensively engineered for applications in biotechnology (28). For example, in stochastic sensing a wide variety of analytes can be detected at the single molecule level through the modulation of the current flowing through a single pore (29–31). Further, the αHL pore has recently been used as a nanoreactor to examine chemical reactions at the single molecular level (32–35). Accordingly, the αHL pore is emerging as a useful tool in both basic science and biotechnology (28, 31). Therefore, it is especially important to understand the assembly and structure of αHL and related βPFTs in detail.

In the present report, we shed additional light on the role of the N terminus in the assembly of αHL. In an early study, the analysis of truncation mutants suggested that the N terminus plays a crucial role in the transformation of the prepore to an active pore (22). Truncating the polypeptide by just two residues at the N terminus rendered αHL completely inactive and arrested assembly at the prepore stage (stage 3, Fig. 1B). However, a later study suggested that truncated polypeptides have limited pore-forming activity (36). In the present work, we resolve this issue through an examination of the original truncation mutants and a new series in which up to 22 residues are deleted. We find that: 1) up to 17 residues can be deleted from the N terminus of αHL without complete loss of activity, 2) latch formation is not necessary to form functional pores, 3) residue 217 cooperates with the N terminus during pore formation, and 4) the N terminus prevents the premature oligomerization of monomers in solution.

EXPERIMENTAL PROCEDURES

Construction of Mutants—All constructs were assembled in the pT7-SC1 expression vector (17, 37) and verified by DNA sequencing of the entire αHL inserts. Genes encoding the truncation mutants were generated by PCR mutagenesis and ligation-free in vivo recombination as described elsewhere (38, 39). Except for αHL-NΔ1, each forward mutagenic primer for N-terminal truncation contained an Ndel site at the initiation codon followed by an alanine codon (see Table 1). The WT αHL, αHL-NΔ3, and αHL-NΔ12 genes were then used to generate the point mutants: αHL-S217N, αHL-S217P, (αHL-NΔ3)-S217N, (αHL-NΔ3)-S217P, and (αHL-NΔ12)-S217N.

Synthetic LukS (hlgC) and LukF (hlgB) genes were used to produce point mutants of the leukocidin proteins: LukF-P215N, LukS-P201N, LukS-P202N, and LukS-P201N/P202N. The codon usage in these constructs differs from the natural genes, but the encoded amino acid sequences are unchanged. The PCR mutagenesis/in vivo recombina-

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tion technique was again used to generate these mutants (38, 39). In addition, codons 1–18 of the synthetic LukS gene were connected by PCR mutagenesis in vivo recombination to the gene segment encoding residues 22–293 of WT αHL to generate the mutant LukS(1–18)HL. Similarly, codons 1–21 of the synthetic LukF gene were connected to codons 22–293 of WT αHL to generate the mutant LukF(1–21)HL.

**Coupled in Vitro Transcription and Translation**—Proteins were generated by coupled in vitro transcription and translation (IVTT) by using an *Escherichia coli* T7-S30 extract system for circular DNA (Promega, no. L1130). The complete amino acid mixture (1 mM) minus cysteine and the complete amino acid mixture (1 mM) minus methionine, supplied in the kit, were mixed in equal volumes to obtain the working amino acid solution required to generate high concentrations of the proteins. The amino acids (2.5 μl) were mixed with premix solution (10 μl), 1-[35S]methionine (1 μl, MP Biomedicals, no. 51001H, 1175 Ci/mmol, 10 mCi/ml), plasmid DNA (4 μl, 400 ng/ml), and T7-S30 extract (7.5 μl), supplemented with rifampicin (20 μg/ml final) (37). Synthesis was carried out for 1 h at 37 °C (for αHL mutants) or 30 °C (for leukocidin mutants) to produce 25 μl of radiolabeled IVTT protein.

**Membrane Binding and Oligomerization**—WT αHL or mutant proteins (IVTT, 1.5 μl) were incubated with washed rRBC membranes (3 μl, 4.2 mg of protein/ml) in MBSA (30 μl, 10 mM MOPS titrated with NaOH, 150 mM NaCl, pH 7.4, containing 1 mg/ml BSA) for 1 h at 37 °C.

After centrifugation, the resulting membrane pellets were washed with MBSA and subjected to electrophoresis in 8% SDS-polyacrylamide gels. After drying and autoradiography.

**Limited Proteolysis Assay**—WT αHL and Luk proteins (WT or mutants) were obtained by IVTT. For the hemolytic assay, in the case of rRBC membranes, the samples were centrifuged (25,000 × g, 10 min), and the supernatants were separated. Supernatant (3 μl) was mixed with MBSA (22 μl) and incubated at room temperature for 1 h. Sample buffer (25 μl, 2×) (42) was then added to each tube. The unheated samples were subjected to electrophoresis in a 10% SDS-polyacrylamide gel (Fig. 6A). The gel was fixed for 30 min prior to drying and autoradiography.

**RESULTS**

**Construction of αHL Mutants to Analyze the Effect of the Residue at Position 217 on the Conversion of the Preparo to the Active Pore**—The role of the N terminus of αHL in pore formation was originally investigated before the availability of structural data. In the first study, Walker and coworkers in our laboratory generated four truncation mutants of αHL by deleting 2, 11, 22, and 38 residues from the N terminus (22). All formed SDS-stable oligomeric complexes on rRBC but were incapable of lysis. Therefore, these mutants were proposed to be arrested at a “prepro” stage, and it was inferred that the N terminus of αHL plays a crucial role in the conversion of the prepro to the active pore. However, as reported (22), all the mutants used in that study carried a point mutation at codon 217 (AGT → AAT) that had occurred during the construction of the expression vector. The altered codon encodes an Asn residue instead of a Ser residue. The mutation has no effect on the activity of the full-length protein as discussed in the report (22). Nevertheless, a later study from another laboratory suggested that truncated polypeptides might have limited activity (36). Further, Panchal and coworkers (43) from our laboratory had discovered that a mutation at position 217 could partially reactivate the non-lytic mutant αHL-H35N. Therefore, we decided to investigate whether the S217N mutation affects the activity of truncated proteins. In addition, in the Luk polypeptides, the corresponding position is occupied by a Pro residue, which is well conserved in all Luk variants: Pro215 in LukF and Pro201 in LukS (Fig. 2, B–D) (19). In consideration of these issues, we made seven mutants of αHL by changing Ser217 in the wide-type protein and selected truncation mutants (αHL-N23 and αHL-N12; Table 1) to either Asn or Pro: αHL-S217N, αHL-S217P, αHL-N23, (αHL-N23)-S217N, (αHL-N23)-S217P, αHL-N12, and (αHL-N12)-S217N. The mutant (αHL-N23)-S217N of the present study has the same polypeptide sequence as the mutant αHL(3–293) of the previous study (22). Similarly, (αHL-N12)-S217N is the same as αHL(A12–293) (22).
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Figure 2. Structural homology in β barrel pore-forming toxins. A, a single protomer of αHL within the heptameric pore. Each region of the polypeptide is shown in a different color: amino latch, magenta; β-sandwich domain, blue; rim domain, red; stem domain, green; triangle region, yellow. Ser217 is shown in stick form in orange. B, LukF water-soluble monomer (20). Each region is colored as in A. Pro215 is in stick form in orange. C, LukS-PV water-soluble monomer (19). Each region is colored as in A. Pro199 (Pro201 in the corresponding position in LukS) is shown in stick form in cyan. D, His35 is shown in stick form in cyan. All images were generated using PyMOL version 0.97. D, sequence comparison between αHL, LukF, and LukS in the region around Ser217 (αHL).

TABLE 1
Nomenclature of N terminal truncation mutants of αHL

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| WT αHL | A | D | S | D | I | N | I | K | T | G | T | T | D | I | G | S | N | T | T | V | K | T | G | D |
| αHL-NΔ1 | A | D | S | D | I | N | I | K | T | G | T | T | D | I | G | S | N | T | T | V | K | T | G | D |
| αHL-NΔ2 | A | D | S | D | I | N | I | K | T | G | T | T | D | I | G | S | N | T | T | V | K | T | G | D |
| αHL-NΔ3 | A | D | S | D | I | N | I | K | T | G | T | T | D | I | G | S | N | T | T | V | K | T | G | D |
| αHL-NΔ4 | A | D | S | D | I | N | I | K | T | G | T | T | D | I | G | S | N | T | T | V | K | T | G | D |
| αHL-NΔ5 | A | D | S | D | I | N | I | K | T | G | T | T | D | I | G | S | N | T | T | V | K | T | G | D |
| αHL-NΔ6 | A | D | S | D | I | N | I | K | T | G | T | T | D | I | G | S | N | T | T | V | K | T | G | D |
| αHL-NΔ7 | A | D | S | D | I | N | I | K | T | G | T | T | D | I | G | S | N | T | T | V | K | T | G | D |
| αHL-NΔ8 | A | D | S | D | I | N | I | K | T | G | T | T | D | I | G | S | N | T | T | V | K | T | G | D |
| αHL-NΔ9 | A | D | S | D | I | N | I | K | T | G | T | T | D | I | G | S | N | T | T | V | K | T | G | D |
| αHL-NΔ10 | A | D | S | D | I | N | I | K | T | G | T | T | D | I | G | S | N | T | T | V | K | T | G | D |
| αHL-NΔ11 | A | D | S | D | I | N | I | K | T | G | T | T | D | I | G | S | N | T | T | V | K | T | G | D |
| αHL-NΔ12 | A | D | S | D | I | N | I | K | T | G | T | T | D | I | G | S | N | T | T | V | K | T | G | D |
| αHL-NΔ13 | A | D | S | D | I | N | I | K | T | G | T | T | D | I | G | S | N | T | T | V | K | T | G | D |
| αHL-NΔ14 | A | D | S | D | I | N | I | K | T | G | T | T | D | I | G | S | N | T | T | V | K | T | G | D |
| αHL-NΔ15 | A | D | S | D | I | N | I | K | T | G | T | T | D | I | G | S | N | T | T | V | K | T | G | D |
| αHL-NΔ16 | A | D | S | D | I | N | I | K | T | G | T | T | D | I | G | S | N | T | T | V | K | T | G | D |
| αHL-NΔ17 | A | D | S | D | I | N | I | K | T | G | T | T | D | I | G | S | N | T | T | V | K | T | G | D |
| αHL-NΔ18 | A | D | S | D | I | N | I | K | T | G | T | T | D | I | G | S | N | T | T | V | K | T | G | D |
| αHL-NΔ19 | A | D | S | D | I | N | I | K | T | G | T | T | D | I | G | S | N | T | T | V | K | T | G | D |
| αHL-NΔ20 | A | D | S | D | I | N | I | K | T | G | T | T | D | I | G | S | N | T | T | V | K | T | G | D |
| αHL-NΔ21 | A | D | S | D | I | N | I | K | T | G | T | T | D | I | G | S | N | T | T | V | K | T | G | D |
| αHL-NΔ22 | A | D | S | D | I | N | I | K | T | G | T | T | D | I | G | S | N | T | T | V | K | T | G | D |

Membrane Binding, Oligomerization, and Activity of Ser217 Mutants of αHL—The mutant and WT polypeptides were tested for the ability to bind to rRBC membranes, to make SDS-stable oligomeric complexes, and to lyse rRBC. All seven mutants were able to bind to rRBC membranes as monomers (Fig. 3A). However, the S217P mutation affected the electrophoretic mobility of the monomers, both in the full-length αHL-S217P and truncated (αHL-NΔ3)-S217P proteins. Of the three full-length proteins, αHL-S217P migrated more quickly in an 8% SDS-polyacrylamide gel compared with WT αHL and αHL-S217N. In contrast, (αHL-NΔ3)-S217P showed a decreased mobility relative to the other two proteins with the same deletion. Therefore, the introduction of Pro at position 217 may have affected the folding of the monomers, which may not be fully denatured in SDS-polyacrylamide gels. All the mutants, except αHL-S217P, assembled on rRBC membranes as SDS-stable oligomeric complexes.

The ability of αHL polypeptides to lyse rRBC is characteristic of whether they are able to convert from non-lytic pre pores to functional pores. The hemolytic activity of αHL-S217N was only ~2-fold less than the WT protein (Fig. 3B). Therefore, as stated by Walker and coworkers (22), the S217N mutation does not have a significant effect on the full-length protein. However, in contrast to the previous studies, the deletion of two residues (αHL-NΔ3) and eleven residues (αHL-NΔ12) from the true WT αHL did not inactivate the protein. The αHL-NΔ3 mutant is only ~2–4-fold less active than WT, whereas the activity of the αHL-NΔ12 mutant is ~30-fold lower (Fig. 3B). Nonetheless, in complete agreement with the previous study, deleting two or eleven residues from the N terminus of the αHL-S217N mutant (αHL-NΔ3)-S217N and (αHL-NΔ12)-S217N, respectively) did abolish activity completely (Fig. 3B) (Walker et al. (22)). Therefore, the S217N mutation affects the activity of truncated mutants but not the full-length protein. Remarkably, the
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S217P mutation produced a different effect. Although the full-length αHL-S217P did not make SDS-stable oligomers, the mutant was active on rRBC (Fig. 3, A and B). The activity was at least 100-fold lower when compared with WT protein and, therefore, we cannot exclude the possibility that the observed activity is the result of an undetectable amount of SDS-stable oligomer. By contrast, deleting two residues from the N terminus of αHL-S217P ((αHL-ΔN3)-S217P) increased its activity by ~16-fold (Fig. 3B). This observation is paralleled by an increase in the amount of SDS-stable (αHL-ΔN3)-S217P oligomer.

Limited Proteolysis of αHL S217 Mutants—According to the present model for αHL assembly, the N terminus takes up different conformations in the prepore and the fully assembled pore (15, 22). As a result, the prepore and the pore show different sensitivities toward attack by proteinase K (22). Therefore, a limited proteolysis assay was carried out to evaluate the conformations of the N termini of the various truncation mutants. Gel-purified oligomers of αHL, αHL-S217N, αHL-ΔN3, (αHL-ΔN3)-S217N, and (αHL-ΔN3)-S217P were digested with proteinase K and heated to disrupt subunit-subunit interactions before analysis by electrophoresis (Fig. 3C). αHL-S217P was not tested, because it did not make SDS-stable oligomers. Similarly, the eleven-residue truncation mutants were omitted, because the protease digestion site is removed with the deletion of eleven residues (22). WT αHL heptamer was completely resistant to proteinase K digestion (Fig. 3C), confirming previous studies (22). Similar resistance was observed with αHL-S217N, suggesting that latch formation has been completed in this mutant and the N terminus is buried inside the cavity. However, all three truncation mutants, αHL-ΔN3, (αHL-ΔN3)-S217N, and (αHL-ΔN3)-S217P, were digested with proteinase K (Fig. 3C).

Effect of Amino Acid Substitution at the Position Corresponding to 217 in αHL on the Assembly of the Leukocidin Pore—According to sequence alignment and a structural comparison between αHL, LukS, and LukF, the position equivalent to 217 in αHL is occupied by a Pro residue in the Luk proteins (215 in LukF and 201 in LukS; Fig. 2D). Position 202 in LukS is also a Pro residue. To determine whether these positions in the Luk proteins affect the assembly of the Luk pore, four Luk mutants were generated: LukF-P215N, LukS-P201N, LukS-P202N, and LukS-P201N/ P202N. The N termini of LukF and LukS, respectively, are naturally one and seven residues shorter than that of αHL (19). The mutant proteins were tested for their ability to lyse rRBC (Fig. 3D). All the combinations of Luk mutants tested were hemolytic. No significant difference was observed in any of the mutant combinations with respect to the WT proteins (Fig. 3D). It should be noted that the hemolytic activity of the WT Luk subunits on rRBC is normally at least 500-fold lower than WT αHL (1).

Analysis of the N-terminal Truncation Mutants of Wild-type αHL—From the experiments described above, it was clear that the N terminus of the WT αHL is in fact not critically important for the formation of the active pore. Partial activity was preserved after two- and eleven-residue deletions. To further analyze the extent of deletion tolerated by WT αHL, a complete series of truncated mutants missing up to 22 residues was generated (Table 1). All 22 mutants were able to bind to rRBC membranes as monomers (Fig. 4A). All 22 mutants were also able to make SDS-stable oligomers on rRBC membranes (Fig. 4A). A hemolytic assay with rRBC was carried out to test for function (Fig. 4B). Within the 2-h assay period, mutants with up to 17 missing residues (αHL-ΔN1 to αHL-ΔN17) showed detectable activity. Across the series from WT αHL to αHL-ΔN17, the activity decreased by at least 1000-fold and was accompanied by an increase in the initial lag time. Within the assay period, we did not observe any lysis for the mutants αHL-ΔN18 to αHL-ΔN20. However, αHL-ΔN21 had very weak activity and αHL-
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This mutant has both a different length and a different sequence at the N terminus compared with WT αHL. The ability to oligomerize and the lytic activity of the mutants were compared with WT αHL. Both produced SDS-stable oligomers on rRBC membranes. However, the extent of oligomerization of LukS(1–18)HL is lower than that of WT αHL and that of LukF(1–21)HL lower still (Fig. 5A). Both oligomers also migrated faster on SDS-PAGE compared with the WT oligomer (Fig. 5A). A limited proteolytic assay was carried out to explore the conformations of the N termini in the LukS(1–18)HL and LukF(1–21)HL oligomers. Proteins were oligomerized on rRBC membranes, digested with proteinase K and heated to disrupt subunit-subunit interactions (Fig. 5B). Under these conditions, monomers that do not oligomerize are digested into small fragments. WT αHL was not digested, even with 0.5 mg/ml proteinase K (Fig. 5B). However, despite its similar length to WT αHL, LukF(1–21)HL was completely digested at the N terminus at 0.05 mg/ml proteinase K. At this concentration, LukS(1–18)HL was partly resistant to proteolysis. When the lytic activities of these proteins were measured, the full-length chimeric protein, LukF(1–21)HL, showed ~60-fold lower activity compared with WT αHL (Fig. 5C). Interestingly, the shorter chimeric protein, LukS(1–18)HL, was only 4-fold less active (Fig. 5C). For both mutants, the initial lag time was decreased, and the lytic activity increased compared with the truncated protein, αHL-NΔ21 (Figs. 4B and 5C).

Preoligomerization of Truncated Mutants—In the crystal structure of the αHL heptamer, the N terminus of each protomer extends away from the β-sandwich domain to form a protease-resistant latch with a neighboring subunit inside the cap domain. However, the protease sensitivity of the water-soluble monomer suggests that the N terminus is in an exposed conformation (22). Therefore, it is likely that the N terminus relocates during pore formation. Consistent with these structural considerations, fluorescent probes attached to the N terminus of αHL undergo a change in environment during pore formation (27). Further, in the structures of the LukF and LukS-PV monomers, the N termini adopt a β-strand conformation and thereby extend the inner β sheet of the β-sandwich domains by one strand (Fig. 2B) (19, 20). Because of the overall similarities between the folds in the αHL, LukF, and LukS-PV structures, it seems likely that the N terminus of the αHL monomer also folds against the β-sandwich domain. It is possible that this conformation of the N terminus prevents the oligomerization of αHL in solution by masking the surfaces of individual subunits (20). Therefore, we analyzed the ability of αHL to oligomerize in solution when the N terminus is truncated (Fig. 6A). The truncation mutants (Table 1) were expressed by IVTT and incubated at room temperature without the addition of red cell membranes (see “Experimental Procedures”). Although the IVTT reaction mixture itself contains ~1–2 μg/μl of E. coli lipids (including phosphatidylethanolamine and cardiolipin), the extent of premature oligomerization of WT αHL was found to be very low. However, as residues were removed from the N terminus, a gradual increase in the extent of oligomerization was observed up to αHL-NΔ18 as analyzed by SDS-PAGE. From αHL-NΔ19 to αHL-NΔ21, premature oligomerization decreased and only a very weak band of oligomers was seen for αHL-NΔ22. No oligomers were observed for truncation mutants between αHL-NΔ23 and αHL-NΔ29 (Fig. 6A). Monomers of αHL in solution are susceptible to proteolysis at two sites: at the N terminus and in the pre-stem domain. Oligomerization occludes the pre-stem inside the prepore, protecting it from proteolysis. Because the N terminus is already removed in the truncation mutants, an increased resistance to proteolysis in the stem domain would be

NΔ22 was as active as αHL-NΔ13 (Fig. 4B). To investigate these observations further, we made the αHL-NΔ21 and αHL-NΔ22 mutants again, together with seven additional mutants with deletions up to residue 29 (αHL-NΔ23 to αHL-NΔ29). The activities of αHL-NΔ21 and αHL-NΔ22 were confirmed (data not shown). The activity of αHL-NΔ23 was ~8-fold lower than αHL-NΔ22, and αHL-NΔ24 to αHL-NΔ29 did not show any activity during the 2-h assay period (data not shown). In accord with the observed activity, αHL-NΔ23 oligomerized on rRBC membranes. Although αHL-NΔ24 also showed very weak oligomerization, αHL-NΔ25 to αHL-NΔ29 did not (data not shown).

Sequence Specificity of the N Terminus of αHL in Its Role in Pore Formation—The previous experiments showed that, although the N terminus of αHL is not required for pore formation, truncations reduced activity and increased the initial lag time (Fig. 4B). To determine whether these findings are sequence specific, we generated two additional mutants of αHL by exchanging its N terminus with that of LukS or LukF. αHL showed a significant level of activity when 22 residues were removed from the N terminus (αHL-NΔ22, Fig. 4B). However, the activity of αHL-NΔ21 was very low. Therefore, we chose to replace missing residues in this mutant with the corresponding residues of LukF and LukS to check whether the activity could be increased. In the first mutant, the first 21 residues of αHL were replaced by the first 21 residues of LukF to generate LukF(1–21)HL. Therefore, this mutant has an identical length to WT αHL, but it has a different sequence at the N terminus. In the second mutant, the first 21 residues of αHL were replaced with the first 18 residues of LukS to generate LukS(1–18)HL.
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Knowledge about how αHL converts from its water-soluble monomeric form into the functional heptameric pore has contributed greatly to an understanding of the assembly and function of both small and large proteins in the βPFT family. For example, truncation mutagenesis of αHL produced the first evidence for a prepore intermediate in the assembly of the βPFT (22). The existence of prepores in small βPFT, including αHL, was later verified in other studies (25, 26, 44–46). Similar prepores intermediates were more recently reported for members of the family of cholesterol-dependent cytolysins, such as perfringolysin (47–50) and pneumolysin (51). This suggests that all βPFT share a common mechanism of pore formation (24).

Even before the crystal structure of the αHL pore was solved, the N terminus of αHL was thought to be vital for pore formation. Walker and co-workers (22) reported that αHL is unable to form lytic pores and arrests at the prepore stage when a deletion of two residues is made at the N terminus. Although Vandana and co-workers (36) later reported that αHL is active after the removal of four residues, the hemolytic activity of their truncation mutant was low. Nevertheless, we have re-examined this question, paying particular attention to the fact that our original mutants (22) contained the mutation S217N, which was introduced during the construction of the expression plasmid. Remarkably, as shown in the present work, this mutation, which is distant from the N terminus of αHL in both sequence and space, plays an important role in pore formation only when the N terminus of polypeptide is truncated.

In the present study, we have demonstrated that the true WT αHL is able to make SDS-stable oligomers even after the removal of 22 residues from the N terminus (Fig. 4A). This indicates that these mutants reach at least the prepore stage in assembly. To our surprise, mutants with up to 17 residues deleted were also able to lyse rRBC by forming functional pores, albeit with diminished activity compared with WT αHL (Fig. 4B). Therefore, we have demonstrated that the N terminus of αHL is not in fact necessary for pore formation. Our results also show that the N terminus of αHL can exist in different conformations in the fully functional pore (Table 2): 1) If the full-length N terminus is intact, it forms an amino latch, as in the cases of the WT polypeptide, corroborated by the crystal structure (Fig. 2A), and αHL-S217N. In this conformation, the N terminus is inaccessible to proteases (Fig. 3C); 2) If the N terminus is partly deleted, it does not form the amino latch. What is left of the N terminus, or more strictly the site of proteolytic cleavage, remains accessible, as in the cases of αHL-ΔN3 and (αHL-ΔN3)-S217P (Fig. 3C); 3) If the N terminus is completely removed, certain mutants still form active pores, as in the cases of αHL-ΔN21 and αHL-ΔN22 (Fig. 4B).

The prepores of βPFT are defined by two properties: 1) They are fully formed oligomers with subunits arranged around a central axis; 2) The lipid bilayer is not breached by them (22, 24). The central axis of the prepore lies perpendicular to the membrane surface (9, 50, 51), and the residues that will become the transmembrane β barrel have not yet undergone the required conformational change (25, 26, 46, 48). In the case of αHL, the prepore was believed to feature a protease-sensitive N terminus (Table 2) that was needed to form a protease-resistant latch upon transformation to the fully assembled pore (22). Therefore, protease sensitivity in the oligomeric state was regarded by us as a diagnostic test for prepore formation and to be absent in the fully assembled functional pore. Although this remains the case for WT αHL, the findings reported here show that the N termini of certain active αHL mutants remain accessible to proteases in the fully assembled pore (Fig. 3, B and C). Therefore, a protease-sensitive N terminus is no longer solely diagnostic of the prepore structure. For example, αHL-ΔN3 forms SDS-resistant oligomers with about the same efficiency as WT-αHL (Fig. 3A) and has ~25–50% of the activity (Fig. 3B). However, the oligomerized protein is completely (at least 95%) digested near the N terminus with proteinase K (Fig. 3C).

As documented above, when the N terminus of αHL is completely removed, the polypeptide can still form active pores (Fig. 4B). Mutants in which the N terminus was replaced also showed pore-forming activity (Fig. 5C). Latch formation was inhibited in a full-length protein made expected if these mutants prematurely oligomerize in solution. To analyze this possibility, a limited proteolysis assay was carried out on αHL-ΔN17 to αHL-ΔN21 after the described treatment (Fig. 6B). The WT protein was completely digested at 50 μg/ml of proteinase K showing the distinctive pattern of digestion at both the N terminus and the stem domain (22). By contrast, the truncated mutants were less susceptible to proteolysis and undigested polypeptides were apparent even at 500 μg/ml of protease in accord with the notion of premature oligomerization (Fig. 6B).
by the substitution of the first 21 residues of αHL with the corresponding residues of LukF, LukF(1–21)HL (Fig. 5B). The ability of this mutant to oligomerize was poor (Fig. 5A) and, accordingly, the activity was low (Fig. 5C), and we cannot exclude the possibility that it derives from an undetected fraction of protease-resistant oligomer. In LukS(1–18)HL, the first 21 residues of αHL are replaced by the first 18 residues of LukS. Latch formation was also absent (Fig. 5B), and this mutant showed increased oligomerization (Fig. 5A) and higher lytic activity compared with LukF(1–21)HL (Fig. 5C, ~25% of WT αHL). Therefore, LukS(1–18)HL is another example of an αHL mutant that exhibits lytic activity without latch formation.

Although the N terminus of αHL is not necessary for pore formation, deletions at the N terminus of the WT protein both increased the initial lag time and decreased the overall extent of hemolysis (Fig. 4B). The reduced activity could arise for one or more reasons: 1) Binding of the truncated mutants to red cell membranes is affected; 2) Assembly of monomers to form the prepore is inhibited; 3) The prepore to pore transformation is retarded; 4) The protein oligomerizes prematurely after synthesis, so that less is available for pore formation. Possibilities 1 and 2 cannot have a significant effect as truncation mutants up to αHL-N23 bind to red cells and oligomerize efficiently (Fig. 4A). Possibility 4 may contribute to the reduced activity of some of the mutants. αHL-N10 to αHL-N23 do show significant premature oligomerization (Fig. 6A), but the extent of premature oligomerization is not sufficient to account for the observed reduction in activity. Therefore, it is reasonable to infer that extended truncations at the N terminus of αHL retard the unfolding of the pre-stem domain during the prepore to pore transformation, possibility 3. This argument is further supported by experiments with LukS(1–18)HL and LukF(1–21)HL, in which 21 missing residues at the N terminus of αHL are replaced with residues from the N termini of LukS and LukF (Fig. 5C). In both cases, pore-forming activity is regained, and in the case of LukS(1–18)HL it is ~25% of the value for

TABLE 2
Table 2: Properties of the prepore and the pore of αHL

|             | Prepupe | Pore |
|-------------|---------|------|
| Oligomerization state | Fully oligomerized about a central axis | Fully oligomerized about a central axis |
| Membrane | Unbroken beneath the oligomer, therefore, non-lytic | Breached by the transmembrane β-barrel, therefore, lytic |
| Stem domain | Presumably folded inside the cavity | Folded into a transmembrane β-barrel |
| Is the N terminus necessary? | No, proteins truncated at the N terminus can form pores | No, proteins truncated at the N terminus can form pores |
| Conformation of the N terminus | Neither full length nor truncated N termini make latches inside the cavity, they are in a protease-accessible conformation | Full length N terminus makes a latch inside the cavity and is protease-resistant, N terminus can be completely deleted. If partly truncated, the N terminus adopts a conformation accessible to proteases |

FIGURE 6. Premature oligomerization of αHL truncation mutants. A, oligomerization of mutant proteins in solution. IVTT proteins were incubated with MBSA for 1 h at room temperature without the addition of membranes (see “Experimental Procedures”). Samples were then subjected to electrophoresis in a 10% SDS-polyacrylamide gel. B, limited proteolysis of the prematurely oligomerized truncation mutants. IVTT proteins were incubated with MBSA without additional membranes as in A and subjected to proteinase K digestion. Final concentrations of the enzyme in mg/ml: lane 1, 0; lane 2, 0.005; lane 3, 0.05; lane 4, 0.5. The samples were heated and subjected to electrophoresis in a 12% SDS-polyacrylamide gel.
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WT αHL. In the latter case, by contrast with WT αHL, the N-terminal latch is not formed. Therefore, the stimulation of the prepore to pore transition by the N terminus of αHL must be independent of the final conformation of the N terminus and its sequence. In an earlier study, Valeva and coworkers (52) provided evidence that, during heterohetero-amer formation by certain pairs of mutant αHL subunits, the N terminus in one class of subunits can activate its own pre-stem intramolecularly and that this activation is transmitted intermolecularly within the prepore to the pre-stem of a normally defective subunit. Further, when the part of the pre-stem that becomes the lower half of the β barrel is deleted, the resulting mutant assembles spontaneously in the absence of membranes to form heptameric structures in which the N termini have formed latches (37), again indicating a cooperative interaction between the N terminus and the pre-stem.

We have shown here that activity is completely lost in N-terminal truncation mutants of αHL only when the truncations are coupled with the S217N mutation (for example, see Fig. 3B). This observation suggests that the nature of the side chain at position 217 controls the prepore to pore transition together with the N terminus. Ser217 lies in a short loop in the rim domain that connects short stretches of 3_10 and α-helix (stretches C and D, Fig. 2A) (15). Both in the monomer and in the oligomer, the loop lies distant from the N terminus (Fig. 2A, Ala1→Ca to Ser217→Ca: monomer 30 Å, heptamer 36 Å (same subunit), 26 Å and 45 Å (adjacent subunits)). Therefore, it is likely that indirect, long distance interactions operate between the N terminus and position 217 during assembly. This effect is further illustrated by the properties of αHL-S217P, which has weak hemolytic activity that is strongly enhanced after truncation mutants of αHL. Interestingly, the WT Luk proteins, which already have Pro at the position corresponding to Ser217 in the truncations, have been shown to re-activate H35N (43). These mutations and Protein Toxins

ACKNOWLEDGMENT — We thank Frances Arnold for a helpful discussion.

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