The Role of the Amino Terminus in the Kinetics and Assembly of α-Hemolysin of Staphylococcus aureus*

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The nature of the involvement of an intact NH2 terminus in the assembly of α-hemolysin of Staphylococcus aureus was reinvestigated. For the first time, a deletion of the first four amino acids at the NH2 terminus of α-hemolysin yielded a novel mutant that undergoes all of the conformational changes to form a lytic pore. The experimental evidence shows unequivocally that the mutant toxin forms heat- and sodium dodecyl sulfate-stable heptameric oligomers. The concentration required to achieve 50% lysis of red blood cells is around 58–116 ng/ml, and the time taken to achieve lysis to the same extent as that of intact toxin is considerably longer. Transmission electron microscopic studies also suggest that the pores formed by this deletion mutant are similar to those by the full-length toxin. This is in contrast to the previously reported 2- and 11-amino acid deletions that failed to proceed further from a presumed prefinal nonlytic pore to a lytic pore. Studies on the kinetics of assembly indicate that this mutant can form heat- and sodium dodecyl sulfate-stable oligomers as fast as full-length α-hemolysin but that pore opening is slowed down. The data strongly suggest that these amino acids (Ala-Asp-Ser-Asp) are involved in the final stages of assembly of α-hemolysin in target membranes.

The assembly of proteins into membranes is an intricate phenomenon in which these proteins undergo a large degree of conformational changes in exerting their biological functions. Ion channels (1), integral membrane proteins (2), and other membrane-associated proteins (3) are a few well known examples. Pore-forming toxins such as α-hemolysin of Staphylococcus aureus, streptolysin O of Streptococcus pyogenes, aerolysin of Aeromonas hydrophila, and α-toxin of Clostridium septicum, secreted by several bacteria in water-soluble forms, also assemble spontaneously in target cell membranes to form transmembrane channels (4–6). Unlike ion channels, membrane-associated proteins, and cell surface receptors, these pore-forming toxins do not have well defined stretches of hydrophobic amino acids in their sequences. The localized environment experienced by stretches of amino acids that insert into membranes appears to control the assembly and function of some of these toxins. Hence, a detailed study of such toxins would provide valuable information regarding the spatial and structural organization of the polypeptide chain segments that span the membrane bilayer. This would eventually lead to a better understanding of their mode of action as well as pave the way for the rational design of novel lytic molecules (7). Among the molecules available for the study of such pore-forming toxins, α-hemolysin (α-HL) of S. aureus is a relatively small protein. It is therefore amenable for redesign of its functions (8). α-HL is a 293-amino acid polypeptide that binds to the target cells as a monomer, and the cell-bound monomers undergo extensive conformational changes to form a transmembrane pore. The pore formation takes place through a series of intermediates, and the activity of α-HL is critically dependent on intact NH2 and COOH termini. A deletion at the NH2 terminus of α-HL led to the formation of oligomers devoid of lytic property, whereas a deletion at the COOH terminus has resulted in a monomer that was deficient in its ability to oligomerize (9). In the recently reported crystal structure of α-HL heptamer, the NH2 terminus has been shown to form a latch that participates in interprotomer interactions (10). The NH2-terminal segment was shown earlier to be accessible to site-specific chemical modification agents in three states, namely, monomer in solution, membrane-bound monomer, and membrane-bound heptamer (11). This observation appears to be consistent with the crystal structure of the heptamer. Yet how a well exposed segment controls the assembly and the role it plays during the pore formation still remain unexplored.

To gain further insight into these questions, it is necessary to delineate the role of an intact NH2-terminal segment during pore formation. This can be accomplished by detailed biochemical and biophysical analyses of NH2-terminal deletion mutants of the toxin. Unfortunately, all of the NH2-terminal deletion mutants of α-HL known so far have not provided any significant information regarding the extent of the involvement of this segment in assembly and pore formation (9). Moreover, in the absence of electron microscopic evidence, the status of oligomers formed by these deletion mutants remains open to alternate interpretations. In this paper we describe an NH2-terminal deletion mutant, which, for the first time, is capable of undergoing all of the conformational changes that are necessary to form a functional pore. The biochemical and electron microscopic evidence of the mutant clearly shows that it assemblies as fast as the full-length α-HL but forms functional pores with altered kinetic behavior.

MATERIALS AND METHODS

S. aureus wood 46 strain was obtained from ATCC (10832); the other bacterial strains used were obtained from commercial sources. All restriction and modifying enzymes were purchased from New England Biolabs. The cloning, expression vectors, Taq DNA polymerase, and RNasin H were products of the Promega Corporation. Expression vector pET-23d(+) was obtained from Novagen. The expression vectors were

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FIG. 1. Cloning of the genes of α-HL and α-HL(5–293). Panel A, the gene of α-HL excluding the 26-amino acid 5′-leader sequence (5′ Is) was PCR amplified from genomic DNA by Tag DNA polymerase using an upstream primer containing an NcoI site and a downstream primer containing a HindIII site (present in the 3′-untranslated region of the gene). The ATG sequence present in the NcoI site (ccATGgG) acts as the initiation codon for the translation of the mature α-HL polypeptide. Temperature conditions used for PCR amplification were: 1 min at 95 °C for denaturation, 2 min at 60 °C for annealing, and 3 min at 72 °C for extension. At the end of 20 cycles an additional 10 min at 72 °C was given for extension. Panel B, the gene for the deletion mutant was obtained by shifting the ATG start codon down to the fourth amino acid of the mature polypeptide chain, and the fifth amino acid was changed to alanine. The upstream and downstream primers again contain an NcoI and a HindIII site, respectively. The downstream primer is the same as used in panel A. The α-HL full-length gene was used as a template for PCR amplification of the α-HL(5–293) gene, and the conditions for amplification are the same as in panel A. The numbers shown below are the start of the mature polypeptide chain with respect to the full-length α-HL. Panel C, the above PCR products (panels A and B) were first polished with Klenow and cloned at the SmaI site of the pGEM-3Zf(+) vector (P2271, Promega). Respective inserts were obtained by digesting the plasmids pGEM-3Zf(+)/α-HL and pGEM-3Zf(+)/α-HL(5–293) with NcoI and HindIII, which were subcloned into the pT7 vector of pGEMEX-1 (P2211, Promega) origin, which was constructed as follows. A 163-bp fragment from pET-23d(+) (+) between BglII and HindIII containing the T7 promoter and the ribosome binding site was taken and inserted into pGEMEX-1 to derive the expression vector with NcoI at the initiation site for translation instead of NdeI. A second NdeI site present in pGEMEX-1 (close to AaiI) was removed by digestion, filled in with Klenow, and religated. It should be noted that in the pGEMEX-1 vector, proteins can be expressed only as a fusion of the T7 gene 10 leader and the coding strand. By employing the above modifications, the T7 gene 10 protein could be expressed in the control vector under the control of the T7 promoter.

modified to suit the experiments as described here. Protein extractions were carried out with the Bradford reagent (Bio-Rad) using ultrapure lipid-free bovine serum albumin (Sigma) as the standard. All hemolysis assays were carried out with freshly drawn blood from New Zealand rabbits from the local animal facility. All other chemicals used were of analytical grade.

Cloning of α-HL and α-HL(5–293)—The genomic DNA of S. aureus wood 46 strain was made according to Lema et al. (12) with minor modifications. The strategy for cloning the genes of α-HL and its mutant is shown in Fig. 1. The α-HL gene was obtained by PCR amplification of the above genomic DNA using the 5′ primer 5′-TAATCTT-GTGCCTGcactcgAGAgTTTCTGAGT, which creates an NcoI site (shown in italics) just before the start of the polypeptide chain (shown in italics). The 3′ primer is same as reported earlier (13). The 26-amino acid hydrophobic leader sequence (5′ Is) as shown in Fig. 1 of the full-length α-HL was deleted for the present work. The α-HL gene was verified by screening several independent PCR isolates exploiting the unique internal restriction sites present in the gene. The deletion mutant α-HL(5–293) was constructed by shifting the ATG start codon down to the fourth amino acid (Asp) of the full-length α-HL (amino acid numbers are with respect to mature full-length α-HL as mentioned in Fig. 1), and the fifth amino acid is changed to alanine for efficient processing of fMet as in the NH2-end rule (14–16). The gene for the present deletion mutant was obtained by PCR amplification of the α-HL gene using the upper primer 5′-GcAgATCTGcactgCTAATATTGAAACCGG, which creates an NcoI site; the downstream primer is the same as that used for full-length α-HL as mentioned above. As a result of this approach, the deletion mutant carries a mutation at the fifth amino acid (i.e. Ile to Ala, 15A) so that the polypeptide has the sequence AaNIKTG ... (see Fig. 1 for numbering). The PCR-amplified fragments were first polished with Klenow polymerase followed by ligation at the SmaI site of pGEM-3Zf(+) vector. The insertions between NcoI and HindIII were removed from the pGEM-3Zf(+) for subcloning into an expression vector under the control of T7 promoter as described in Fig. 1. The pT7 plasmids containing the inserts were used for generation of [35S]Met-labeled proteins using coupled in vitro transcription and translation (IVTT) as well as for expression in JM109(DE3). At least four independent PCR isolates of the present deletion mutant were cloned into the expression vector to check the consistency of the data. The data presented here were obtained with PCR isolate α-HL(5–293)-4 (4 represents the isolate number used for all of the studies).

Dideoxy Sequencing—The sequence of the coding strand of the constructs in the expression vector was determined with an Applied Biosystems Prism model 377 automated DNA sequencer using T7 promoter and α-HL internal primers. The sequence was consistent with our cloning strategy and is in agreement with the changes reported earlier (13, 17). Only one mutation, R56K, was observed. Apart from being a conserved mutation it has been shown earlier that a replacement of Arg with Cys (R56C) at this position does not affect the binding or lytic properties of the full-length α-HL (18). Hence, this change is considered insignificant for the present studies.

Purification of α-HL and α-HL(5–293)—The soluble forms of the respective toxins from various JM109(DE3) expression cultures were purified by standard protocols. Briefly, an overnight inoculum (10 ml) was prepared by picking a single colony from a freshly transformed plate. The inoculum was washed with LB-ampicillin to remove any secreted β-lactamase present in the medium before its addition to 2 liters of LB medium containing 100 μg/ml ampicillin. The culture was grown at 30 °C for 10–12 h. The cells were harvested by centrifugation at 2,000 × g for 20 min and lysed once with a French press at 6,000 p.s.i. The resultant cell lysate was centrifuged at 40,000 × g for 1 h and the supernatant treated with polyethyleneimine to a final concentration of 0.25%. The suspension was then left on ice for 15 min and clarified by centrifugation. The supernatant was dialyzed extensively at 4 °C against 10 mM sodium acetate, pH 5.2, by changing the buffer at least six times over a period of 2 days. The dialyzed supernatant containing the deletion mutant was loaded after clarification by centrifugation and purified on an S-Sepharose column preequilibrated with 10 mM sodium acetate, pH 5.2. The mutant typically eluted in the range of 160–220 mM NaCl. The S-Sepharose-purified proteins were then subjected to further purification by fast protein liquid chromatography on a Mono S column using the above buffer. The yield for the mutant is 0.2 mg/liter. The purity and presence of the mutant were checked routinely by SDS-PAGE (19).

Protein Sequencing—The purified protein was blotted onto a Problot polyethylene dine difluoride membrane (Millipore Corp.) using 10 mM CAPS, pH 11.0, as reported earlier (20). The blot was stained briefly with Ponceau S and destained overnight before sequencing. Edman degradation was performed with an Applied Biosystems model 476A sequencer using Fastblot cycles. The first 10 amino acids of the mutant were determined with good repetitive yields.

In Vitro Transcription and Translation—[35S]Met-labeled proteins were generated by in vitro transcription and translation (13, 18), using the Escherichia coli T7 S30 extract system for circular DNA (L-1130, Promega). Briefly, supercoiled plasmid DNA (250 ng) was incubated with S30 premix (20 μl), T7 S30 extract (15 μl), amino acid mix minus methionine (5 μl), [35S]Met (1 μl of 15 μCi/μl) in the presence of RNAasin H (1/25 units), rifampicin (50 ng), and nuclease-free water to a final volume of 50 μl for 45 min at 37 °C. The samples were centrifuged immediately at 4 °C for 5 min at 16,000 × g, aliquoted, and stored at −70 °C for further use. Each aliquot was thawed and used immediately.

Limited Proteolysis—The purified mutant (30 μg) was treated with 0.1 μg of proteinase K in water for 0, 2, 15, and 60 min. At the end of each time point, an aliquot was taken directly into a microcentrifuge tube containing 5 × Laemmli sample buffer and boiled at 100 °C for 5 min. The samples were then loaded to 14% SDS-PAGE gels. The gel was stained with Coomassie Brilliant Blue for visualization of the bands.

Oligomerization with Deoxycholate and Heat Stability—The mutant protein (1 mg/ml) was treated with 7 × 1-μl aliquots of 25 mM sodium deoxycholate in 100 mM Tris-HCl, pH 8.2, at an interval of 10 min/μl with brief stirring to a final concentration of −6.5 mM. After the last addition the mixture was left for an additional 10 min at room temper-
ature. At the end of the incubation period, the samples were mixed with Laemmli sample buffer and subjected to 8 and 10% SDS-PAGE.

**Hemolysis Assays**—The quantitative lysis of rabbit red blood cells (rRBCs) was measured by taking 1.8 μg of purified deletion mutant or α-HL in 1 ml of K-PBSA (150 mM NaCl, 20 mM KH₂PO₄, pH 7.4, containing 1 mg/ml bovine serum albumin) in tube 1, out of which a 500-μl aliquot was 2-fold serially diluted in more than 15 tubes. An equal volume of 2% rRBCs was added to all of the tubes, which were left at 25 °C for 24 h in the case of mutant and for 30 min for the full-length toxin. At the end of the incubation period absorbance at 545 nm (hemoglobin release) was recorded against nonlytic supernatants of the last tubes.

**Kinetics of Lysis**—The time course was studied as reported earlier (18) by incubating a known amount of toxin in 1 ml of 0.032% rRBCs. The light scattering at 595 nm was measured at regular intervals with gentle mixing to resuspend the sedimented rRBCs.

**Competitive Hemolysis**—The competitive hemolysis assay was performed by mixing the full-length toxin and the mutant in appropriate ratios in a 500-μl volume of K-PBSA. An equal volume of 2% rRBCs was then added and left at 25 °C for 30 min. At the end of incubation period, the absorbance at 545 nm was recorded. The absorbance (at 545 nm) obtained after suspending the rRBCs (1%) in 5 mM sodium phosphate buffer, pH 8.0, was taken as the 100% lysis value. The percentage of lysis for a given toxin ratio was calculated with respect to the 100% lysis value.

**Kinetics of Assembly of Toxins**—In these experiments freshly translated [³⁵S]Met proteins (5 μl) were mixed with 5% rRBCs (25 μl), prepared as mentioned above, and at a given time point a 6-μl aliquot was withdrawn. The cells were then pelleted. The cell pellet was put directly in 20 μl of 1 × Laemmli sample buffer, incubated at 60 °C for 5 min, and subjected to 12% SDS-PAGE. After electrophoresis the gel was fixed for autoradiography without staining. The respective heptamer bands were analyzed further as reported earlier (11). Briefly, after obtaining a suitable autoradiogram, the respective heptamer bands were excised from the dried gel and allowed to reswell in 1× Laemmli sample buffer overnight. The samples were boiled at 95°C for 10 min and electrophoresed as mentioned earlier with appropriate controls.

Such an analysis confirmed that the heptamer bands are composed of constituent monomers of α-HL/mutants (data not shown).

**Electron Microscopy of α-HL and α-HL(5–293) Pores**—Purified full-length α-HL or α-HL(5–293), 30 μg in 50 μl, was incubated with an equal volume of 10% rRBCs and left for 24 h at 20 °C. Subsequently, the lysed rRBC membranes were collected by high-speed centrifugation (16,000 × g) and washed extensively with 5 mM sodium phosphate, pH 8.0, several times. The membrane pellet was then resuspended in 50 μl of the same buffer. The samples for electron microscopy were prepared by placing a few microliters of the sample on a carbon-coated grid for 5 min, and the excess sample was removed with blotting paper. The grid was then stained for 1 min with freshly prepared 2% sodium phosphotungstic acid, pH 7.3, and examined in a JEOL transmission microscope.

**RESULTS AND DISCUSSION**

Initial observations of the NH₂-terminal deletion mutant presented here, generated by the coupled in vitro transcription and translation system, exhibited slow lysis of the rRBCs which is in contrast to an earlier observation (9). To develop a conceptual framework about the assembly and function of pore-forming toxins, in particular α-HL, a detailed study of the NH₂-terminal deletion mutant of α-HL becomes imperative. Hence, we have revisited the NH₂-terminal part of α-HL with an aim to dissect further the mechanism of its assembly.

**Characterization of α-HL and α-HL(5–293) Toxins**

The cloning strategy depicted in Fig. 1 was different from the approach published earlier (13), and it readily yielded the desired construct. The DNA sequence of the construct is in agreement with earlier reports (13, 17). In addition, the cloning strategy used was proven to be valid as the NH₂-terminal sequence of the mutant was found to be ANIKTG as expected. All products of independent PCR isolates showed the same biological activities. The protein band corresponding to α-HL(5–293) was very prominent and migrated slightly ahead of α-HL on a 13-cm-long SDS-PAGE gel. To examine the overall folding of α-HL(5–293), both the mutant toxin and α-HL were digested with protease K, which cleaves α-HL at around the 130–135 position of the polypeptide yielding an approximate two halves of the toxin. Identical patterns were observed for both α-HL and α-HL(5–293). Hence, it can be concluded that the overall folding of α-HL(5–293) approximates that of α-HL.

**Properties of α-HL(5–293) Vis à Vis Native α-HL**

The assembly and function of α-HL have been elucidated by several groups during the recent years (9, 13, 21). Based on these studies, a working model has emerged to explain the activity of α-HL. According to this model, the water-soluble toxin (stage 1) first binds to the rRBC membrane as a monomer (stage 2). The membrane-bound monomer then recruits other monomers in the membrane to form a heptameric prefinal pore (stage 3). This prefinal pore, however, is devoid of lytic property. Further conformational changes are required to form a functional lytic pore (stage 4).

According to the current understanding of α-HL pore formation, intact NH₂ and COOH termini are absolutely critical for the formation of a functional lytic pore (stage 4). Deletion of three amino acids at the COOH terminus arrests this toxin at stage 2 (as a membrane-bound monomer), whereas deletion of two amino acids at the NH₂ terminus arrests the toxin at stage 3, which is unable to lyse the target cells (8). Based on these observations it was concluded that an intact NH₂ terminus is an absolute requirement for the formation of a functional pore. However, the exact nature of the involvement of the NH₂ terminus is not yet clear. Interestingly, the α-HL(5–293) presented here shows lytic properties similar to those of α-HL as judged by the criteria that follow.

**Oligomer Properties**—Although it is still debatable whether α-HL has specific receptors on rRBCs or not, it is well understood that α-HL homo-oligomers can be generated in vitro, by treatment of the toxin with sodium deoxycholate. The homo-oligomers formed by α-HL(5–293) after treatment with deoxycholate are depicted in Fig. 2 (panel B, lanes 1–4, and panel C, lanes 1–3). The oligomers of α-HL(5–293) exhibit properties of heat and SDS stability, similar to the oligomers of α-HL, which are characteristic features of the staphylococcal toxin. The
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The purified toxin (α-HL or α-HL(5–293)) in K-PBSA was 2-fold serially diluted to more than 15 tubes. An equal volume (500 μl) of 2% rRBCs was added to make up the volume to 1 ml. The concentration of the toxin present in the first tube was 900 ng/ml. The samples were incubated at room temperature for various times. At the end of the incubation period, the samples were centrifuged, and the absorbance at 545 nm was recorded against the supernatants of the last tubes. Open symbols (□ and △) represent α-HL at 30 min and 24 h, respectively. Filled symbols (● and ▽) represent α-HL(5–293) at 4 and 24 h, respectively. The quantity of α-HL(5–293) required to achieve 50% lysis of rRBC was recorded after a 4-h incubation. The data presented here are an average of three independent experiments.

Quantitative Lysis—The lysis pattern observed for α-HL is consistent with earlier reports (22). The quantity of α-HL(5–293) required for achieving 50% lysis was found to be about 2-fold higher than that reported earlier for α-HL (~30 ng/ml; Ref. 13), but it is within the range of experimental error. The lysis pattern for α-HL(5–293) is linear and gradual, whereas it is sigmoidal for α-HL at optimal concentrations (see Fig. 4). α-HL has a shorter lag time (the time difference between the addition of toxin to rRBCs and observation of lysis), the same for α-HL(5–293) is much longer. The slow lysis could be for two reasons: (i) the intact NH$_2$ terminus plays a major role in recruiting other subunits to form a prefinal pore, i.e. between stages 2 and 3, or (ii) it helps in conversion of the prefinal pore to a final functional pore, i.e. between stages 3 and 4. The data presented in the following paragraphs show that it plays a major role after the toxin assembles successfully into a prefinal oligomer. It is to mention here that the previously constructed mutants, i.e. α-HL(3–293) or α-HL(12–293), did not exhibit any lytic property (9). In addition, the deletion mutant, α-HL(3–293), was found to inhibit the slow lysis exhibited by a COOH-terminal deletion mutant (α-HL(1–290)). It is not yet known whether α-HL(3–293) can form at least ring-like structures or oligomers of α-HL (see Fig. 6).

Electron Microscopy of the Pores of α-HL and α-HL(5–293) The pores formed by α-HL(5–293) on rRBCs were visualized by transmission electron microscopy (Fig. 5). The average diameter of the pore appears to be 1.5–2.0 nm, and the pores formed by α-HL(5–293) appear to have all of the characteristics of α-HL pores as seen in panels A and B, which are consistent with earlier reports (24, 25). In addition, this mutant also forms tetragonal two-dimensional crystalline arrays like α-HL (Fig. 5, A and B). Each individual pore of α-HL is composed of seven monomeric units. If the number of subunits/pore is different, then one would expect a different packing arrangement such as hexagonal or other patterns because intersubunit interactions play a major role in two-dimensional crystalline arrays. It is clear from Fig. 5 that α-HL(5–293) forms identical two-dimensional crystalline arrays like α-HL. If the earlier observation about the formation of pentameric oligomer by α-HL(3–293) is correct, then α-HL(5–293) should have also formed a pentameric oligomer. Accordingly, it should have led to the formation of a different two-dimensional crystalline array. All of the properties of α-HL(5–293) described above strongly suggest that the mutant oligomer is also a heptamer.

Assembly of α-HL and α-HL(5–293)The NH$_2$ terminus of α-HL can play multiple roles during the process of assembly and pore formation. The earlier observation that the α-HL(3–293) deletion mutant was capable of inhibiting the slow lysis of a COOH-terminal deletion mutant suggests two distinct possibilities. First, the NH$_2$ terminus can play a major role in stabilizing interprotomer interactions during the process of assembly. Second, the NH$_2$-terminal segment can participate directly in opening up the pore. These two possibilities, although interrelated, can be examined by studying the kinetics of assembly and by competitive hemolysis studies. If the NH$_2$ terminus participates actively in stabilizing the interprotomer interactions during the assembly, one can anticipate formation of loosely associated oligomers that are unable to withstand heat and SDS during the early stages of assembly and more stable (heat- and SDS-resistant) oligomers at later stages of assembly. In view of the above, we have carried out kinetic studies on the assembly of α-HL(5–293) and...
α-HL. In these studies [35S]Met-labeled toxins were allowed to bind to rRBCs to monitor the formation of heat- and SDS-stable oligomers. Simultaneously, their lytic potencies were also measured. It is clear from Fig. 6 that α-HL(5–293) can form stable oligomeric structures as fast as α-HL (lanes C—H). It is very surprising that SDS- and heat-stable structures for α-HL(5–293) were seen within 1–2 min of its binding to rRBCs, and yet no cell lysis was observed until ~4 h. In contrast, complete cell lysis was observed for α-HL in 5–7 min (from the intensity of monomer and heptamer bands shown in Fig. 6, it can be seen that both α-HL and α-HL(5–293) have almost identical quantities). This observation is consistent with the kinetics of lysis shown in Fig. 4. The data from the kinetics of lysis and assembly taken together clearly dissect the pore formation mechanism further. The NH2-terminal segment has no role in binding nor in the recruitment of other subunits to form the prefinal nonlytic pore. In other words, the deleted amino acids, ADSD, appear to have no role at all until the toxin forms a successful prefinal nonlytic pore because the oligomers formed by α-HL(5–293) in 2–5 min have completely acquired the characteristics of the oligomer of α-HL. Hence, it can be surmised that the role of NH2 terminus is to accelerate the later events of conformational changes necessary for the formation of a functional pore.

The interaction of NH2-terminal region of one protomer with other protomers during pore formation was also examined using competitive hemolysis experiments. If the NH2 terminus of one protomer is to interact with other protomers during assembly, then one would expect that α-HL(5–293) should retard the cell lysis of α-HL. In these experiments, cell lysis was monitored after the α-HL(5–293) and α-HL were mixed in different ratios, typically ranging from 10:1 to 1:1 (where α-HL was kept constant) and incubated with rRBCs for 30 min. It is clear from Table I that α-HL(5–293) competitively retards the cell lysis by α-HL. It should be noted that the observed cell lysis is caused by three distinct types of pores present on the cell: α-HL pores, α-HL(5–293) pores, and α-HL and α-HL(5–293) hybrid pores in different ratios. It is clear from Figs. 3 and 4 that α-HL pores are very fast in lysing the cells, and α-HL(5–293) pores are much slower. Lysis is retarded dramatically as the number of α-HL(5–293) monomeric units increases per hybrid pore. We have not carried out the competitive hemolysis experiments with a COOH-terminal deletion mutant, as done earlier, because any deletion at the COOH terminus leads to destabilization of the toxin monomer itself. Hence, these competitive hemolysis studies clearly suggest that the deleted amino acids play a major role in organizing the polypeptide chains during the last stages of assembly and pore formation. The α-HL monomer undergoes extensive conformational changes when it converts itself from a prefinal pore to a functional pore. During this process, the NH2 terminus latch and the glycine-rich loop (stem) were said to cooperate with each other (26). A close examination of the crystal structure of the heptameric α-HL reveals that the distance between the NH2 terminus and the stem (glycine-rich loop) is approximately 75–80 Å (10). This is a substantial degree of long range interactions requiring pre-

N. Sangha and M. V. Krishnasastry, unpublished observations.

| Competitive inhibition of hemolysis by αHL(5–293) | |
|---|---|---|---|
| αHL | αHL(5–293) | Ratio | Hemolysis | % |
| ng/ml | ng/ml | αHL/αHL(5–293) | after 30 min |
| 50 | 50 | 1:1 | 100 |
| 50 | 100 | 1:2 | 80 |
| 50 | 150 | 1:3 | 32 |
| 50 | 200 | 1:4 | 5 |
| 50 | 250 | 1:5 | 4 |
| 50 | 300 | 1:6 | 3 |
| 50 | 350 | 1:7 | 0 |
| 50 | 400 | 1:8 | 0 |
| 50 | 450 | 1:9 | 0 |
| 50 | 500 | 1:10 | 0 |

FIG. 5. Electron microscopic detection of the pores formed by α-HL(5–293). Panel A, two-dimensional pattern observed for full-length α-HL. Panel B, two-dimensional pattern observed for α-HL(5–293). Panel C, magnified area of two-dimensional crystalline pattern shown for α-HL(5–293) in panel B. The magnification bars represent 50 nm in panels A and B and 25 nm in panel C.

FIG. 6. Kinetics of assembly of α-HL(5–293). [35S]Met-Labeled (5 μl) α-HL and α-HL(5–293) were incubated with 25 μl of 5% rRBCs. At a given time interval a 6-μl aliquot was withdrawn, and the cells were pelleted. The cell pellet was put directly into 20 μl of Laemmli sample buffer and incubated at 60 °C for 5 min. The samples were loaded on a 12% SDS gel. Lane A, unboiled IVTT generated α-HL; lane B, unboiled IVTT-translated α-HL(5–293); lane C, α-HL, 2 min; lane D, α-HL, 5 min; lane E, α-HL(5–293), 2 min; lane F, α-HL(5–293), 5 min; lane G, α-HL(5–293), 60 min; lane H, α-HL(5–293), 180 min; lane I, IVTT-translated α-HL protein kept at 60 °C for 5 min; lane J, IVTT-translated α-HL(5–293) protein kept at 60 °C for 5 min; lane K, lane D sample boiled at 95 °C for 10 min; lane L, sample boiled at 95 °C for 10 min. M1 and O represent monomer and heptameric oligomer, respectively. The heptamer bands from lanes C–H, respectively, were excised from the above gel and were analyzed further as reported earlier (11).
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