Importance of the Carboxyl Terminus in the Folding and Function of α-Hemolysin of Staphylococcus aureus*

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The physical state of two model mutants of α-hemolysin (αHL), αHL(1–289), a carboxyl-terminal deletion mutant (CDM), and αHL(1–331), a carboxyl-terminal extension mutant (CEM), were examined in detail to identify the role of the carboxyl terminus in the folding and function of native αHL. Denatured αHL can be refolded efficiently with nearly total recovery of its activity upon restoration of nondenaturing conditions. Various biochemical and biophysical studies on the three proteins have revealed the importance of an intact carboxyl terminus in the folding of αHL. The CDM exhibits a marked increase in susceptibility to proteases as compared with αHL. αHL and CEM exhibit similar fluorescence emission maxima, and that of the CDM is red-shifted by 9 nm, which indicates a greater solvent exposure of the tryptophan residues of the CDM. In addition, the CDM binds 8-anilino-1-naphthalene sulfonic acid (ANS) and increases its fluorescence intensity significantly unlike αHL and CEM, which show marginal binding. The circular dichroism studies point that the CDM possesses significant secondary structure, but its tertiary structure is greatly diminished as compared with αHL. These data show that the CDM has several of the features that characterize a molten globule state. Experiments with freshly translated mutants, using coupled in vitro transcription and translation, have further supported our observations that deletion at the carboxyl terminus leads to major structural perturbations in the water-soluble form of αHL. The studies demonstrate a critical role of the carboxyl terminus of αHL in attaining the native folded state.

α-Hemolysin (αHL)1 of Staphylococcus aureus has attracted lot of attention from structural biologists and biotechnologists for its potential applications in biotechnology and therapeutics (1). It is a 293-amino acid polypeptide that binds target cells as a monomer, and the cell-bound monomers undergo lateral diffusion to form a transmembrane heptameric pore. The heptamer is a rigid mushroom-like structure, resistant to SDS and temperatures up to 80 °C (2). The water-soluble monomer undergoes a series of conformational changes to form the heptameric pore on the membrane. The amino acid residues 110–148, termed the stem domain, penetrate the membrane bilayer to access the interior of a target cell for the functional pore formation.

The activity of αHL was earlier shown to be critically dependent on an intact amino and carboxyl termini (3). Recent studies from our laboratory have shown that deletion of four amino acids at the amino terminus of αHL leads to delayed pore opening. Although this mutant (αHL(5–293)) could undergo the oligomerization process as fast as native αHL, the conformational changes that lead to the opening of the pore were retarded (4). Fluorescence spectroscopic studies carried out by Valeva et al. (5) have also arrived at similar conclusions regarding the role of the amino terminus. Previous studies have shown that deletion of three, five, or eight amino acids at the carboxyl-terminal end of αHL impairs its oligomerization and pore formation abilities. The carboxyl-terminal deletion mutants, however, have been shown to bind to rabbit red blood cells (rRBCs), where they remain in a cell-bound monomer form (3). In addition, the carboxyl-terminal deletion mutants are very inefficient in forming heterooligomers with full-length αHL. All of the studies conducted so far have attributed the role of the amino terminus for pore opening and the carboxyl terminus for the initial oligomerization process of αHL. However, the reasons for the inefficient oligomerization of the carboxyl-terminal deletion mutants are not yet clear.

It has been observed that the carboxyl-terminal end of αHL becomes more exposed to the solvent in the oligomeric state than in the monomer form in solution, as revealed by IASD modification of single cysteine mutants (IASD is a membrane-impermeant reagent that covalently modifies surface-accessible cysteine residues in proteins (6, 7)). This observation was supported by the crystal structure of the fully assembled αHL pore, which shows that the carboxyl terminus is well exposed to solvent. Thus, the carboxyl terminus does not appear to be critically involved in interpore interactions (3). Hence, a deletion of as few as three carboxyl-terminal residues ought not to have any drastic effect on the oligomerization process. Another possibility is that the carboxyl-terminal deletion is hampering a process prior to the oligomerization step, which might occur in the water-soluble monomer or at the membrane-bound monomer stage. Therefore, in order to have a better understanding of the role of the carboxyl terminus in the structure and function of αHL, we have aimed to examine the properties of a carboxyl-terminal deletion and an extension mutant.

The extension mutant was constructed with an aim to see whether or not αHL can accept a polypeptide at the carboxyl terminus and still carry out its folding and function. For this
C Terminus in the Folding and Function of S. aureus αHL

MATERIALS AND METHODS

All bacterial strains employed in this study were obtained from commercial sources. Ultrapure bovine serum albumin, S-Sepharose, and 8-anilino-1-naphthalene-sulfonate (ANS) were obtained from Sigma. Protein estimations were carried out with the Bradford reagent (Bio-Rad) using ultrapure lipid-free bovine serum albumin as the standard. Hemolysis assays were carried out with freshly drawn blood from New Zealand White rabbits of the local animal facility. All other chemicals used were of analytical grade.

Cloning of αHL, CDM, and CEM—The cloning of αHL was described in detail by Vandana et al. (4). The PCR amplification of CDM was achieved by advancing the stop codon present in αHL at 294 to Gln296 by a downstream primer that contains a HindIII site. The upstream primer is the same as that used for αHL. The resultant PCR product was digested with NcoI and HindIII and ligated to the pT7 vector described earlier (4). The CEM was constructed in two stages by PCR amplification of the αHL gene using an upstream primer that contains an EcoRI site at Asn529 and the downstream primer (with HindIII site) of αHL reported earlier (4). The upstream primer eliminates the stop codon present in the αHL gene. The primers containing the EcoRI and HindIII sites were first joined by PCR using αHL template. The CEM in final form was obtained by re-PCR of αHL template using a T7 promoter primer and the EcoRI and HindIII joined product obtained in the first stage. The resultant PCR product was digested with NcoI and EcoRI to remove the 3’-untranslated region of αHL. This double-digested PCR product was ligated to the parent pT7 vector between NcoI and EcoRI, resulting in a result of removal of the stop codon of αHL. The translation proceeds beyond the EcoRI site and incorporates the following 38-amino acid stretch from the vector backbone: 5′-GATATGGATCAG——AGTTGGCTGG——GCCATTTGTTCCGTTTTG-3′.

Purification of αHL—αHL was purified from S. aureus wound 46 (ATCC 10832) as reported earlier (8). Briefly, a 2% mid-log phase inoculum of S. aureus was added to 1 liter of tryptic soy broth, and the culture was harvested for 18 h at 37 °C. The cells were resuspended in 0.5 M sodium acetate, pH 5.2. The supernatant was centrifuged and loaded on an S-Sepharose column pre-equilibrated with 10 mM sodium acetate, pH 5.2, 0.5% Triton X-100 in buffer A, rinsed with buffer A, and stored at 4 °C or by simple dilution as desired for specific experiments, and hereafter this protein is referred to as renatured. The renatured protein was found to be >95% pure as judged by SDS-PAGE (9).

Limited Proteolysis—The proteins (native and refolded as applicable) were subjected to digestion with Proteinase K at 25 °C by keeping the substrate-enzyme ratio at 50:1. At appropriate time points, the enzyme was inactivated by the addition of 5× Laemmli sample buffer and boiled at 100 °C for 5 min. The samples were analyzed by 14% SDS-PAGE.

RESULTS

Purification and Characterization of αHL, CDM, and CEM—All of the toxins employed for the present study were constructed under the control of the T7 promoter. The relative sizes of the polypeptides are in agreement with the cloning strategy (Fig. 1A). Unlike recombinant αHL and CEM, the soluble form of CDM could not be obtained due to extensive inclusion body formation (>95%). Since inclusion body formation is often due to temperature-sensitive denaturation of the protein and can be avoided at lower culture growth temperatures (10), the CDM culture was grown at 30 °C. However, this had no effect on the extent of inclusion body formation. Hence, we have purified CDM by solubilizing its inclusion bodies as described under “Materials and Methods.” The purity of all
three proteins was routinely assessed by SDS-PAGE and was found to be >98% as shown in Fig. 1A.

We have carried out limited proteolysis of the three proteins in solution with Proteinase K. Limited proteolysis is a sensitive probe for analyzing the conformation of proteins (11). Polypeptides that are devoid of tertiary structure have been observed to be very sensitive to proteolysis (12, 13). Proteinase K cleaves αHL monomer in solution between residues 131 and 136 of the polypeptide chain, yielding an approximate two halves of αHL. Such a cleavage does not occur for the membrane-bound forms of monomer and oligomer because these residues get occluded in the membrane (14). As seen in Fig. 1B, the refolded CDM was completely digested by Proteinase K in minutes, whereas αHL gave the expected two halves, which were resistant toward further protease attack. This enhanced susceptibility of CDM towards protease suggests that its structure is not as rigid as that of αHL.

Hemolytic Activity—The hemolytic activity of the three proteins was compared by a quantitative assay. As seen from Fig. 2, the CDM exhibited no lysis at all in the time course of the experiment, whereas the CEM showed efficient lysis. However, it is about 5-fold weaker than αHL and increasing the amount of CEM 5 times in the assay gave an identical curve like that of αHL.

Unfolding and Refolding of αHL—The denaturation and renaturation of αHL were carried out by employing a wide spectrum of denaturing conditions as described under “Materials and Methods.” It is clear from Table I that >95% of hemolytic activity of αHL can be recovered upon restoration of nondenaturing conditions. However, when αHL was incubated at pH 3.5 at room temperature, the recovery of hemolytic activity decreases with longer incubation times. At pH 3.5, the carboxyl-terminal portion of αHL is said to undergo a transition to a molten globule-like state with exposed hydrophobic regions (15). The apparent reason for the loss of activity at pH 3.5 could be due to aggregation of the partially unfolded intermediate. However, total recovery of activity was achieved when the incubation was carried out at 4 °C, which could be either due to slow denaturation or due to a significant decrease in hydrophobic interactions at the lower temperature.

Fluorescence Studies—The normalized fluorescence emission spectra of native and denatured states of αHL, CDM, and CEM are shown in Fig. 3, and the emission maxima obtained are 336, 345, and 336 nm, respectively. It is interesting to note that the emission maximum of CDM exhibited a 9-nm red shift with respect to native αHL, indicating a change in the polarity of environment of tryptophans due to solvent exposure (16). The emission maxima for all three toxins were further red-shifted in presence of 8 M urea to 352.5 nm. In contrast to CDM, the fluorescence spectrum of CEM was identical to that of αHL, indicating that the extra 38 residues at the C-terminal end of αHL did not have any influence on its folding and function. The residues that were deleted in the case of CDM are Glu290-Met291-Thr292-Asn293, and the residues extended in the case of CEM do not contain any tryptophans. Hence, αHL, CDM, and CEM contain an equal number of aromatic residues in their primary sequence, and the differences in their fluorescence spectra clearly reflect the degree of compactness of the individual toxins.

The fluorescence emission maximum as a function of pH for αHL and CDM is depicted in Fig. 4. The curve obtained for αHL
were observed in contrast to renatured CDM, and high molecular weight forms of CDM. This hysteresis could be due to formation of soluble aggregates resistant to acid denaturation compared with the case 1 CDM. The toxins were incubated in 50 mM citrate-phosphate buffer at various pH values, and their fluorescence emission maxima were recorded as described in the legend to Fig. 3. □, aHL; ▲ and ○ represent CDM refolded and denatured, respectively.

The increase in case of CDM was rather dramatic, as shown in Fig. 5. In addition, the emission maximum of ANS had blue-shifted from 513 nm in buffer to 483.5 nm upon binding to CDM. On the other hand, the denatured states of the three proteins did not bind any ANS (data not shown). This result indicates that CDM has hydrophobic regions exposed to the solvent, unlike aHL and CEM. It is interesting to note that the CEM did not show any concomitant increase in fluorescence intensity of ANS. These observations reflect the compactness of native aHL and the role of the carboxyl terminus in maintaining such a compact structure.

CD Studies—The secondary structure of aHL and CDM was examined by far UV-CD spectroscopy. The CD spectrum of CDM shows a significant content of secondary structure and is characteristic of a predominantly β-sheet protein, as is the case for aHL. However, comparison with the aHL spectrum suggests some minor conformational differences between the two species (Fig. 6A). The spectrum of aHL shows a minimum at 214.5 nm and is consistent with earlier reports (14, 15, 20). In case of CDM, the minimum was red-shifted to 218.5 nm. This might result from a change in the polarity of the environment of β-sheets, since it is well known that the β-sheet is very sensitive to a change in environment conditions (21).

The near UV-CD spectra of aHL and CDM are shown in Fig. 6B. The aHL spectrum is consistent with the previously published reports. Comparison of the two spectra shows a drastic difference in the tertiary structure of the two proteins. In case

**TABLE I**

| Denaturing agent          | Buffer used for renaturation | Time period of incubation in denaturant before renaturation |
|---------------------------|-----------------------------|----------------------------------------------------------|
| Native control (no denaturant) | A, B, C                      | 2 min 15 min 30 min 60 min |
| 8 M urea                  | A, B                         | 6 6 6 6                                                   |
| 6 M guanidine HCl         | A, B                         | 6 6 6 6                                                   |
| Low pH                    | C                            | 6 5 3 2                                                   |
| 50 mM citrate-phosphate, pH 3.5 | C                           | 6 6 6 5                                                   |
| 50 mM citrate-phosphate, pH 2.5 | C                           | 6 6 6 5                                                   |

* Buffers are as follows: A, 10 mM sodium acetate, pH 5.2; B, 20 mM potassium phosphate, 150 mM NaCl, pH 7.2, containing 1 mg/ml bovine serum albumin; and C, 50 mM citrate-phosphate, pH 7.0.

The numbers shown are the well number exhibiting 50% lysis of RBCs in a 96-well plate.

**Fig. 3.** Fluorescence spectra of aHL, CDM, and CEM in the presence and absence of 8 M urea. The fluorescence spectra of the respective toxins (30 μg/ml) in 10 mM MOPS, pH 7.0, were recorded in quartz cuvettes with excitation fixed at 295 nm and 5-nm slit widths for both excitation and emission. The spectra are corrected for buffer. Solid line, aHL; dotted line, CDM; short dashed line, CEM. Very short dashed line, dashed line and dot, and long dashed line are aHL, CDM, and CEM, respectively, in 8 M urea. Spectra for CDM were obtained by renaturation of urea-solubilized inclusion bodies by dilution to the appropriate concentration followed by clarification by high speed centrifugation. AU, arbitrary units.

**Fig. 4.** Effect of pH on the fluorescence emission aHL and CDM. The toxins were incubated in 50 mM citrate-phosphate buffer at various pH values, and their fluorescence emission maxima were recorded as described in the legend to Fig. 3. ■, aHL; ▲ and ○ represent CDM refolded and denatured, respectively.

is in agreement with previous results (15). The CDM in 8 M urea was either 1) diluted with an appropriate buffer to the desired pH (final urea concentration was kept at about 80 mM) or 2) renatured by dialysis, and the pH of the dialysate was adjusted to the desired value. The curves obtained by both of the approaches do not overlap (as one would expect) even after incubation for 48 h, in that the case 2 CDM appears to be more resistant to acid denaturation compared with the case 1 CDM. This hysteresis could be due to formation of soluble aggregates among the renatured CDM molecules, because it has been observed that aggregation commonly interferes with the correct equilibrium, giving rise to such hysteresis (17). This possibility was examined by glutaraldehyde cross-linking of the renatured CDM, and high molecular weight forms of CDM were observed in contrast to aHL (data not shown).

**Probing the Hydrophobic Regions of aHL, CDM, and CEM—**

ANS has been extensively used to characterize the hydrophobic pockets of proteins and enzymes for understanding their folding and function (18). The fluorescence emission of ANS is sensitive to a change in the polarity of the environment of proteins and enzymes for understanding their folding and function (18). The fluorescence emission of ANS is sensitive to a change in the polarity of the environment of hydrophobic regions and is marginally increased in the presence of aHL and CEM.
of CDM, the negative peaks at 265 and 295 nm, which correspond to the vibrionic regions of phenylalanine and tryptophan, respectively (22), are totally absent. This indicates that most of the phenylalanine and tryptophan are in a more mobile environment. However, CDM still possesses the positive peak at 280 nm, whose ellipticity is about 30% of the corresponding peak in the aHL spectrum. This reveals that some rigid tertiary contacts are present in CDM, particularly around a fraction of the tyrosine residues, but the overall spectrum reflects a significant disorder in the tertiary structure of CDM.

DISCUSSION

In the present study, we have designed a carboxyl-terminal deletion and a carboxyl-terminal extension mutant of aHL in order to investigate the contribution of the carboxyl-terminal residues of the protein to its structural organization and function. Our efforts to isolate the CDM have met with partial success, since it appears to be unstable. The protein is almost exclusively found in inclusion bodies, unlike recombinant aHL and CEM, which can be isolated in soluble, active form. A variety of attempts to purify the CDM by ion exchange and gel filtration techniques have led to precipitation of the protein in the column. Hence, a wide spectrum of conditions was employed in an attempt to stabilize the CDM, and the conditions suitable for spectroscopic studies are low ionic strength and pH 5.0–7.0. The CEM, on the other hand, can be purified like aHL. The hemolytic data presented in Table I show that aHL can be unfolded and refolded to its native form in a variety of conditions. Refolding is extremely efficient, with almost total recovery of activity. Hemolysis studies carried out with the mutants showed that the CDM is very weakly lytic, which is in agreement with prior studies with other C-terminal deletion mutants (3). On the other hand, the CEM, which has 38 extra amino acids, was able to lyse the rRBCs efficiently (Fig. 2).

The CDM has a tendency to aggregate both in vitro and in vivo (as seen by the extensive inclusion body formation). Studies have shown that inclusion bodies form due to aggregation of partially folded intermediates (23). Hence, the occurrence of CDM in inclusion bodies suggests that the mutant protein was unable to achieve the final folded conformation in vivo. Limited proteolysis experiments have revealed that the CDM gets completely digested unlike aHL, which exhibited its typical “two halves” pattern. This pronounced susceptibility of CDM to proteolytic digestion suggests a more relaxed structure in which many proteolytic sites that are otherwise hidden in aHL are getting exposed. All these observations suggest that the CDM possesses a non-aHL like structure.

The fluorescence emission of the CDM lies in between the native and denatured states of aHL. This red shift observed for the CDM indicates that its tryptophan residues are more exposed to the solvent as compared with aHL. This was further corroborated with binding studies with ANS, a dye widely used to probe the molten globule states of proteins. The fluorescence intensity of ANS increased by about 10-fold upon binding to CDM but showed negligible increase in the presence of aHL.
and the denatured states of the two proteins. The increase is accompanied by a blue shift of the emission maximum by 29.5 nm, which is an indication of specific binding. These results indicate the presence of exposed hydrophobic regions in CDM, which are sequestered in native αHL.

The far UV-CD spectrum of CDM shows that it possesses nearly native-like secondary structure. However, its tertiary structure as analyzed by near UV-CD is greatly diminished. The near UV-CD spectrum indicates that most of the phenylalanine and tryptophan of CDM are in a mobile, symmetric environment but some tertiary contacts are present in the environment of some of the tyrosines. A cursory glance at the distribution of the aromatic residues along the αHL polypeptide chain shows that seven of the eight tryptophan residues are in the carboxyl-terminal half of αHL, and eight of the 14 tyrosine residues are located on the N-terminal half. This gives rise to the possibility that the tertiary contacts may be localized on the first half of the polypeptide chain.

All of these observations strongly suggest that significant perturbations to the αHL structure had occurred in the absence of the carboxyl terminus. Interestingly, the additional amino acids at the carboxyl terminus (as in CEM) did not have any influence either on the structure or the function of native αHL. The CEM exhibited all of the characteristics of αHL. Proteolytic digestion revealed an αHL-like pattern, and the protein was able to oligomerize and cause the lysis of rRBCs. It has the same fluorescence emission maximum as αHL, which suggests that the tryptophans of both of the proteins are in a similar environment. Like αHL, it does not have any of its hydrophobic regions exposed to the solvent, as demonstrated by the negligible ANS binding. All of these points illustrate that it is possible to build residues at the carboxyl terminus of αHL without inhibiting its folding or function.

The non-αHL like structure of the CDM can be interpreted as its being unable to “attain/maintain” the native, αHL-like conformation, and for that the carboxyl terminus is very crucial. We have tested for such a possibility by in vitro transcription and translation, whereby the activity of the CDM was assessed as it emerged from the ribosome during in vitro translation. The hemolytic activity of αHL, CDM, and CEM was examined as a function of initiation of translation. It is clear from Fig. 7 that at constant rate of transcription and translation, αHL begins to show lysis within minutes after initiation of translation, whereas the CDM fails to show any lysis. On the other hand, the CEM lyses rRBCs efficiently but marginally slower when compared with αHL. This shows that freshly synthesized αHL folds rapidly to attain its native, active structure, but the CDM is unable to do so although it lacks just four amino acids. This suggests that the CDM is probably stuck in a partly folded inactive state.

The physical state of the carboxyl-terminal deletion mutants of αHL is intriguing, since these mutants cannot participate in interprotomer interactions to form oligomers on rRBCs. They can however, bind the rRBCs and remain in the form of a cell-bound monomer (3). This is not surprising, because partially folded states of proteins are shown to bind membranes with high affinity (24). The crystal structure of αHL reveals that the N-terminal latch makes extensive contacts with the inner β-sheet of an adjacent protomer. Yet, when as many as 22 residues are deleted from the N terminus, αHL is still able to form oligomers (although yet to be fully characterized). This is because in a heptamer, each protomer participates in about 120 salt bridges and hydrogen bonds and 850 van der Waals contacts (2). In the absence of a few of these interactions, as in the case of an N-terminal deletion mutant, the net force of the remaining interactions is enough to drive the process to the oligomeric state. However, this does not seem to happen in the case of carboxyl-terminal deletion mutants, and they are unable to carry out such fruitful interactions either among themselves (i.e., formation of homooligomer) or with αHL (i.e., formation of heterooligomer). Our data are clearly able to reveal the reason behind the inefficient oligomerization of carboxyl-terminal deletion mutants. The present data unequivocally prove that the CDM possess a partially folded nonnative conformation with greatly diminished tertiary structure. Such a species could still bind rRBCs through nonspecific hydrophobic interactions but does not possess the requisite motifs for carrying out interprotomer interactions. As a result, it cannot form either homooligomers with itself or heterooligomers with αHL. We have also examined the lysis efficiency of the heterooligomers of αHL and CEM by mixing a fixed amount of αHL with increasing amounts of CEM. As seen in Table II, the percentage of lysis increases with increasing amounts of the CEM, which shows that the extended carboxyl terminus does not interfere with the assembly of αHL (i.e., during formation of functional pore). This is in contrast to the amino-terminal deletion mutant αHL (5–293), which retards the lysis of αHL as reported by us earlier (4). This again supports our conclusion that the carboxyl terminus is not very critical for the oligomerization process but instead is involved in the folding of the monomer form.

**FIG. 7.** Monitoring the hemolytic activity of αHL, CDM, and CEM during translation. Coupled in vitro transcription and translation was carried out as mentioned earlier (3). Typically, an aliquot of translation mix was withdrawn and added to 0.5% rRBCs, and the decrease in light scattering of rRBCs at 595 nm due to lysis was monitored at regular time intervals. [●, □, and ▲] represent αHL, CDM, and CEM, respectively at 3.5 min after initiation of translation. No detectable lysis was observed before 3 min.

**TABLE II**

Hemolytic activity of heterooligomers of different ratios of αHL and CEM

| Ratio | Lysis |
|-------|-------|
|       | %     |
| 1:0   | 68    |
| 1:1   | 76    |
| 1:2   | 81    |
| 1:3   | 83    |
| 1:4   | 86    |
| 1:5   | 89    |
The molten globule states of proteins have been attributed with four distinct features, viz. compactness, a near native secondary structure, loss of tertiary structure, and exposure of hydrophobic regions (25). High affinity of CDM for ANS reveals that it has hydrophobic regions exposed to the solvent. In addition, the CDM exhibited the presence of substantial secondary structure accompanied by a significant loss of tertiary structure. We could not, however, determine the compactness of the CDM due to its tendency to interact with gel filtration matrices. All these observations suggest that in the absence of the carboxyl terminus, the physical state of a HL may lie close to a molten globule-like state. Furthermore, the observations presented here strongly suggest that a HL is able to fold to its native form only after its complete synthesis, and/or the CDM is not able to “latch on” to native aHL-like structure because of the absence of the carboxyl terminus.

In summary, for the first time, the role of the carboxyl terminus of aHL has been delineated in greater detail. While the amino terminus is important for the functional pore formation step, the carboxyl terminus is crucial for correct folding and maintenance of the water-soluble monomer form of aHL. Removal of just four residues from the carboxyl terminus makes the protein unable to proceed beyond a molten globule-like state. This information would be valuable to understand how membrane binding toxins like aHL carry out their folding and function. Since single amino acid substitutions at the carboxyl terminus do not affect the function, the length (i.e. backbone) of the protein appears to be vital instead of the actual sequence at the carboxyl terminus. In addition, we have shown that the addition of an extra sequence at the carboxyl terminus does not affect the folding or function of aHL. Such molecules could form the basis for construction of new molecules in the future.

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