Unfolding of *Vibrio cholerae* Hemolysin Induces Oligomerization of the Toxin Monomer*

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*Vibrio cholerae* hemolysin (HlyA) is a pore-forming toxin that exists in two stable forms: a hemolytically active water-soluble monomer with a native molecular weight of 65,000 and a hemolytically inactive SDS-stable heptamer with the configuration of a transmembrane diffusion channel. Transformation of the monomer into the oligomer is spontaneous but very slow in the absence of interaction with specific membrane components like cholesterol and sphingolipids. In this report, we show that mild disruption of the native tertiary structure of HlyA by 1.75 M urea triggered rapid and quantitative conversion of the monomer to an oligomer. Furthermore, the HlyA monomer when unfolded in 8 M urea refolded and reconstituted on renaturation into the oligomer biochemically and functionally similar to the heptamer formed in target lipid bilayer, suggesting that the HlyA polypeptide had a strong propensity to adopt the oligomer as the stable native state in preference to the monomer. On the basis of our results, we propose that (a) the hemolytically active HlyA monomer represents a quasi-stable conformation corresponding to a local free energy minimum and the transmembrane heptameric pore represents a stable conformation corresponding to an absolute free energy minimum and (b) any perturbation of the native tertiary structure of the HlyA monomer causing relaxation of conformational constraints tends to promote self-assembly to the oligomer with membrane components playing at most an accessory role.

*Vibrio cholerae* hemolysin (HlyA), an extracellular membrane-damaging protein with a native monomeric molecular weight of 65,000 (1–4), belongs to a unique class of dimorphic proteins that can exist in two stable states, a water-soluble monomer and an oligomeric integral membrane protein (5). These proteins are commonly known as pore-forming toxins (PFTs) (6) for their ability to lyse target eucaryotic cells by punching holes in the plasma membrane and are released as water-soluble monomeric proteins by a wide variety of pathogenic bacteria. PFTs interact with specific target membrane components and self-assemble by noncovalent interactions involving oligomerization domains into exceptionally stable and rigid β-barrel oligomers that eventually insert into the membrane by using amphipathic β-hairpins as anchors to the nonpolar core of the lipid bilayer (5, 7). High resolution structural data are available for only two of the PFTs, viz. *Staphylococcus aureus* α-toxin (8) and aerolysin of *Aeromonas hydrophila* (9). Our understanding of the mode of action of PFTs is inadequate in at least two aspects: the driving force for self-assembly of the toxin monomer and the precise role of membrane components in triggering this transition.

HlyA is synthesized as an 82-kDa preprohemolysin by *V. cholerae* El Tor O1 and non-O1 strains and exported to the culture medium as the 79-kDa prohemolysin (proHlyA) (10). Proteolytic removal of the 132-residue N-terminal stretch (Pro-region) generates the mature 65-kDa HlyA with a specific hemolytic activity of approximate 100 pM toward rabbit erythrocytes (11). HlyA transforms itself in contact with target biomembranes and synthetic lipid vesicles containing cholesterol into water-filled transmembrane heptameric channels of internal diameter of 1.5 nm (3, 4). Recently, Harris et al. (4) have shown by transmission electron micrographic study that HlyA interacts with cholesterol and forms oligomers at the interface of cholesterol microcrystals and water. Despite its apparent solubility in water, the HlyA monomer is strongly amphipathic and partitions nonspecifically to lipid-water interface (12).

There is considerable disruption in the tertiary structure of the HlyA monomer in the lipid-water interface (12), caused apparently by low solvent polarity (13) and moderately by acid pH (14). However, it is not clear whether the unfolding of HlyA plays any role in the reconstitution of the monomer into an oligomer. Significantly, we observed slow conversion of the HlyA monomer to the oligomer in water even in complete absence of membrane components, suggesting that self-assembly is basically a spontaneous event guided by the greater thermodynamic stability of the oligomer (12). In addition to its pore-forming activity, HlyA binds specifically to β1-galactosylterminated complex glycoconjugates (15) through a C-terminal region related to the sugar-binding domain of the plant lectin ricin (16).

In this communication, we show that perturbation of the tertiary structure of HlyA was sufficient to induce complete and rapid conversion to the oligomer. Our data suggest that the oligomer might be the unique final structure to which the HlyA polypeptide is committed with the apparently stable monomer representing a conformation corresponding to a local free energy minimum or trap. To strengthen our hypothesis that self-assembly of HlyA is driven by a search for stability, we needed to demonstrate that a protein closely similar to HlyA in amino acid sequence and tertiary structure but lacking oligimerization activity tends to switch over to an oligomeric state in suitable conditions. We show that proHlyA is such a protein, and by including it in the present study, we evaluate the contributions of the oligimerization domain and global conformational characteristics of the monomer toward self-assembly.
RESULTS

Physicochemical Analysis of proHlyA—In primary structure, proHlyA differs from the mature toxin in having a 15-kDa polypeptide appendage at the amino terminus and it is reasonable to expect some degree of resemblance in solution and biochemical properties between the two. During phase partitioning with Triton X-114 (21), proHlyA was localized exclusively in the detergent-rich phase (data not shown), indicating that it was similar to HlyA in possessing a strongly amphipathic surface (12). The intrinsic tryptophan fluorescence emission spectra of proHlyA and HlyA overlapped completely (data not shown), indicating similar microenvironments for indolyl residues scattered throughout the length of the polypeptide chains (10). Such a coincidence is strongly suggestive of a close similarity in global conformations for the two proteins. Incubation of proHlyA with PC and PC-cholesterol vesicles induced a massive change in the tryptophan fluorescence spectrum of the protein that included a red shift in the emission wavelength maximum from 320 to 340 nm (Fig. 1). This indicates a strong interaction of the protoxin with lipid vesicles as well as its partial unfolding in the lipid-water interface, similar to that reported for HlyA (12). In contrast to HlyA, however, proHlyA failed to form SDS-stable oligomer in PC-cholesterol vesicles (Fig. 2, lane 2) and was not capable of inserting in the lipid bilayer as indicated by the absence of fluorescence resonance energy transfer from the protein to the dansyl moiety embedded in the core of the lipid bilayer (Fig. 3). Following trypsinization, proHlyA regained the ability to form SDS-stable oligomer (Fig. 2, lane 4) with an insertion-competent configuration (Fig. 3). ProHlyA did not bind to immobilized asialofetuin, indicating that the precursor toxin had no lectin-like affinity for specific sugars (data not shown). Although proHlyA failed to oligomerize in synthetic lipid vesicles, it was hemolytically active toward rabbit erythrocytes with a specific activity of 1.6 nM. This could be attributed to a partial conversion of proHlyA to HlyA by rabbit erythrocyte membrane proteases as seen in Western immunoblot (22) analysis of the protoxin incubated with rabbit erythrocytes (data not shown).

Unfolding and Oligomerization of HlyA—To extend our previous observations on the spontaneous self-assembly of HlyA in water (12), we studied whether reconstitution of the toxin into a more stable structure could be promoted by releasing the polypeptide from the configurational constraints maintaining the native conformation by incubation in urea. The tryptophan fluorescence emission spectra of the HlyA monomer in 0, 2, 3, 3.5, and 8 M urea are shown in Fig. 4A. Appreciable perturbation of the native tertiary structure was observed at urea concentrations of 3 M and above. However, in place of the expected shift in wavelength from 330 to 345 nm on complete unfolding (12), fluorescence intensity remained virtually constant in this wavelength region, suggesting that considerable heterogeneity in tryptophan environments persisted in 8 M urea. To have a better indication of unfolding, fluorescence emission data were plotted in terms of the ratio of the emission intensity at 350 nm to that at 290 nm (I350/I290), the increase of which correlates with exposure of tryptophan residues to water (Fig. 4B). There was a slow, almost linear increase in the ratio I350/I290 from 0.9 to 1.8 in the urea concentration range of 3–8 M with no indication of an all-or-none type of transition to a specific thermodynamic state like the molten globule or unfolded polypeptide (23). Since the I350/I290 ratio approached a value of 1.8 in comparison to a value of 2.6 for free tryptophan in water (24), it seems that a significant fraction of indolyl residues remained shielded in apolar environment even in 8 M urea. Notably, the I350/I290 for the HlyA oligomer remained essentially unchanged at approx-
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**Fig. 2.** SDS-PAGE analysis of proHlyA to detect self-assembly in lipid vesicles. Samples were treated with 1% SDS at 50 °C for 15 min, a temperature at which the oligomer does not dissociate into monomer. Lane 1, proHlyA; lane 2, proHlyA incubated with PC-cholesterol liposome at a protein:lipid weight ratio of 2:1 (w/w), a ratio at which the sensitivity of the assay was found to be maximal. The incubation mixture was excited at 280 nm, and fluorescence emission was recorded at 512 nm ±10 s after mixing the protein with liposome. Curves 1 and 2 refer to proHlyA and trypsinized protoxin, respectively. Slit-widths were 5 nm. Dansylated liposome suspension without protein served as the control.

**Fig. 3.** Fluorescence energy transfer from proHlyA to dansyl-phosphatidylethanolamine incorporated in PC-cholesterol liposomes. Samples were incubated at a protein:lipid ratio of 2:1 (w/w), a ratio at which the sensitivity of the assay was found to be maximal. The variation of I350/I320 with GdmCl concentration (Fig. 5A) was calculated for proHlyA (○), mature HlyA (○), and HlyA oligomer (○) as a function of urea concentrations. Experiments were performed at least three times, and the average ratios were calculated. Inset, the fraction of HlyA (○) and proHlyA (●) present as oligomers in the urea concentration range from 2.5 M to 8 M was calculated by combining the spectrofluorimetric data in Fig. 4B and the transverse urea gradient gel electrophoresis profiles in Fig. 5. The I350/I320 values in 2.5–8 M urea concentration range were assumed to be weight-averages of fluorescence emission contributions of the oligomer (I350/I320 = 0.9) and those of the fully unfolded polypeptide (I350/I320 = 2.6).

To explain the persistence of ordered structure even in 8 M urea, we subjected the toxin monomer to transverse urea gradient gel electrophoresis, a technique that allowed us to visualize species with altered conformations and constitutions (25). Surprisingly, the HlyA monomer self-assembled at approximately 1.75 M urea and the conversion was essentially complete at approximately 2.5 M urea (Fig. 5A), a condition that normally promotes dissociation of multimeric protein complexes. The HlyA monomer and oligomer had the same I350/I320 value (Fig. 4B), and oligomerization could be detected by its characteristic slow mobility in urea gel electrophoresis. Concomitant with the change in tryptophan fluorescence at approximately 3.5 M urea (Fig. 4B), an additional component with intermediate electrophoretic mobility appeared and increased in relative intensity with increasing urea concentration (Fig. 5A). By combining the electrophoretic and spectrofluorimetric data, we identified the new component as the unfolded polypeptide. Because the increase in I350/I320 ratio was contributed solely by the unfolded polypeptide, the values were weight averages of fluorescence emission contributions of tryptophan buried in the nonpolar core of the oligomer and those exposed to water in the fully unfolded polypeptide, allowing us to calculate the relative amount of the HlyA oligomer in the urea concentration range from 2.5 to 8 M (Fig. 4B, inset). It seems that limited unfolding of the HlyA monomer in the urea concentration range of 1.75 to 3 M triggered quantitative conversion to the oligomer. However, at higher urea concentrations, self-assembly of the toxin had to compete with complete unfolding but remained significant even in strongly dissociating condition at 8 M urea. The HlyA monomer, equilibrated with urea in the concentration range from 1.75 to 8 M, renatured upon removal of urea by dialysis to an SDS-stable (Fig. 6, lane 2) hemolytically inactive oligomer with strong carbohydrate-dependent hemagglutinating activity toward rabbit erythrocytes. The HlyA oligomers generated by prolonged exposure of the monomer to water, by limited unfolding in urea, and by contact with target lipid vesicles were indistinguishable in stoichiometry, stability in SDS (Fig. 6), affinity and specificity for sugars, action on target cells, and intrinsic tryptophan fluorescence emission spectrum (data not shown).

If reconstitution of the HlyA monomer into the SDS-stable oligomer was motivated primarily by the gain in thermodynamic stability, the process should in principle be independent of the method used to perturb the native conformation of the toxin. To test this hypothesis, we monitored conformational and constitutional alteration in the HlyA monomer equilibrated with various concentrations of GdmCl, LiCl, and ethanol. The variation of I350/I320 with GdmCl concentration (Fig. 7A) resembled closely the corresponding curve (Fig. 4B) in urea. Because it was not possible to visualize oligomerization of HlyA in GdmCl by electrophoresis, we quantified self-assembly of the toxin to the heptamer (Fig. 7B) by disappearance of hemolytic activity and also by SDS-PAGE of the mixture following removal of the denaturant by dialysis. It is seen that 0.75 M GdmCl triggered quantitative conversion of the monomer to the oligomer with increasing denaturant concentrations favoring complete unfolding. However, 5 M LiCl and 20% ethanol unfolded HlyA without inducing significant oligomerization at these or lower concentrations of the denaturants (data not shown). These data suggest that although HlyA with a disrupted tertiary structure never renatured to the hemolytically active monomer, the conditions for reconstitution into the heptamer were somewhat stringent.

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next how far self-assembly of PFTs was motivated by interactions involving oligomerization domains and global conformational characteristics of the toxin monomer. To do this, we repeated the urea-unfolding experiment described above for HlyA with proHlyA, the HlyA precursor that was incapable of self-assembly but resembled the mature toxin in amphipathicity and conformation. Perturbation of the native tertiary structure by urea at a concentration as low as 1 M induced complete conversion of the monomer to the oligomer (Fig. 5B), suggesting that proHlyA was intrinsically as prone to undergo self-assembly as the mature toxin. As evident from the transverse urea gradient gel electrophoresis (Fig. 5B) and implied in the rapid increase of I350/I320 with increasing urea concentration (Fig. 4B), the efficiency of protoxin self-assembly decreased rapidly with increasing urea concentration and was effectively zero at 8 M urea, suggesting that the oligomerization domain of proHlyA existed in an active conformation in a relatively narrow range of urea concentration, reverting to the dormant or unfolded configuration at lower or higher urea concentrations, respectively. Collectively, these data suggest that the tendency to self-assemble was intrinsic and dictated primarily by global conformational characteristics of the protein with the oligomerization domain playing a key role in attainment of the stable quaternary structure.

**DISCUSSION**

In this communication, we show that a relatively mild disruption of the native HlyA tertiary structure by urea at a concentration as low as 1.75 M triggered conversion of the toxin monomer to a species that was biochemically and functionally similar to the oligomer generated in lipid vesicles. Self-assembly of the HlyA monomer was quantitative and irreversible and faster by several orders of magnitude than spontaneous oligomerization of the toxin observed in water. To our knowledge, this is the first report of the reconstitution of a PFT monomer into an oligomer without assistance of any of the biophysical or biochemical attributes of a membrane lipid bilayer. Delinking of oligomerization of the HlyA monomer from interaction of the toxin with cell surface molecules might provide us with new insights into the mechanism of transformation of this intriguing class of proteins from one native state to another.

Exceptional stability and rigidity of oligomeric pores created by PFTs in target membrane (26), together with our observation that the purified HlyA monomer converted irreversibly to the oligomer when kept in water for days (12), led us to speculate whether the oligomer was the most stable configuration for the HlyA polypeptide with the monomer representing an alternative arrangement that corresponded to a local free energy minimum or trap and was stable enough to survive for a sufficient length of time. If this is so, a mild perturbation of the native HlyA structure might enable the protein to scale the energy barrier separating the two minima by relaxing the conformational constraints that contribute to the height of the barrier and thereby trigger its reconstitution into the energetically more favorable heptameric state. To test this hypothesis, we incubated HlyA with urea and observed a dramatic transi-
tion to the oligomer at approximately 1.75M urea (Fig. 5A). A relatively mild disruption in tertiary structure caused by urea in the concentration range from 1.75 to 3M was enough and indeed most effective in inducing oligomerization of HlyA. The tendency of HlyA to refold and reconstitute into the oligomer was so great that the monomer converted to roughly a 50:50 mixture of the oligomer and the unfolded polypeptide even in 8M urea, a condition that strongly promotes unfolding of proteins to random coil configuration and dissociation of protein complexes. Because GdmCl unfolds protein more efficiently than urea, it is no surprise that the above observations were reproduced at substantially lower concentrations of the denaturant (Fig. 7, A and B). These data, together with the fact that renaturation of the unfolded polypeptide led to its recovery exclusively as the SDS-stable oligomer, established that HlyA could fold and reconstitute autonomously into the oligomer without assistance of any protein, lipid, or carbohydrate component of the target membrane.

The irreversible denaturation of HlyA induced by ethanol and LiCl with absence of oligomerization might be attributed to several factors. Although it did not contradict the view that the native monomer was not the energetically favored conformation, it suggested that self-assembly of the toxin as distinct from nonstoichiometric association of partially or completely unfolded polypeptides depended on persistence of critical elements of tertiary structure of the native HlyA in the denaturant-induced disrupted state of the toxin and therefore on the mode of denaturation. This is plausible because the HlyA monomer and oligomer had similar global conformations (12) and also is supported by our data that the efficiency of self-assembly relative to complete unfolding was reduced in high concentrations of urea and GdmCl. It is also possible that polarity and ionic strength of the medium affected efficiency of oligomerization for integrity of the heptamer assembly depended ultimately on the strength of electrostatic and hydrophobic interactions between protomers.

We next evaluate how far the preceding observations were pertinent to the self-assembly of HlyA in water or at the target cell surface. Although renaturation of urea-unfolded HlyA to SDS-stable β1-galactosyl-specific hemagglutinating oligomer did not reflect nonspecific aggregation of random coil polypeptides, it is not clear whether refolding and self-assembly in urea followed a pathway essentially similar to that involved in oligomerization of the toxin in water or lipid-water interface. There are no data in this work that provide a categorical answer to this question. However, the striking difference in stability of the urea-induced HlyA and proHlyA oligomer in high concentrations of urea as well as water suggested that the oligomerization domain played a key role in self-assembly of HlyA in urea. Because the oligomerization domain plays a critical role in functional interaction of a PFT with the target membrane (5), the pathways of urea and lipid-induced oligomerization might differ in details but converge at key points. In connection with the differential behavior of urea-unfolded HlyA and proHlyA in renaturation experiments, it is worth recalling the previous observations of Nagamune et al. (27). They reported that GdmCl-unfolded proHlyA renatured to the native state but HlyA failed to regain hemolytic activity in a similar experiment. We note that there is no contradiction between these early findings and the present data and interpret the failure of unfolded HlyA to renature as a reflection of the fact that the hemolytically active conformation did not represent the minimum energy configuration. On the other hand, proHlyA renatured successfully because the minimum energy arrangement was inaccessible by blockage of oligomerization domain in the native state.

Previous work on the mechanism of membrane permeabilization by HlyA established a critical role for cholesterol and related sterols in triggering oligomerization of the toxin at the target membrane surface (4). Is the role of cholesterol compatible with the present data, suggesting that a relatively mild disruption in tertiary structure might be instrumental in inducing irreversible self-assembly? Because the precise nature and magnitude of conformational changes in HlyA in response to various perturbants were not defined, it is difficult to speculate a straightforward answer. A corollary of the proposition that self-assembly of HlyA was driven by the tendency of the system to attain a minimum energy configuration is that the process was intrinsically spontaneous and would be promoted by any factor that reduced the height of the barrier separating the relative and absolute energy minima. Therefore, it is not unlikely that there might exist several pathways of promoting oligomerization of HlyA, all of which might not be relevant to the process taking place at the membrane surface. There is indeed considerable disruption in the native HlyA structure in the lipid-water interface, although the conformational change seems to be inadequate to trigger by itself its rapid reconstitution into the oligomer as indicated by slow oligomerization observed in phospholipid vesicles devoid of cholesterol (12). Specific affinity of the toxin for cholesterol would cause sequestration of the protein at the lipid-water interface, and the massive increase in local concentration would augment the kinetics and equilibrium of oligomerization of the partially unfolded HlyA by several orders of magnitude. Extension of the present study to other PFTs, preferably those with known three-dimensional structures, would enable us to explore whether transition of the hydrophilic monomer to a transmembrane oligomeric pore is basically guided by the propensity of the protein to adopt the most stable configuration with the membrane components playing at most a secondary role.

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