A Non-classical Assembly Pathway of Escherichia coli Pore-forming Toxin Cytolysin A

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Background: Cytolysin A (ClyA) is an α-pore-forming toxin secreted from pathogenic E. coli.

Results: ClyA monomer assembles to an oligomeric pre-pore structure independently of lipid membrane and detergent.

Conclusion: Our results support a model that ClyA proteins may oligomerize to a prepore within outer membrane vesicles before arriving on the target cell membrane.

Significance: The proposed model for ClyA represents a non-classical pathway to attack eukaryotic host cells.

Pore-forming toxins (PFTs)² represent the largest family of bacterial protein toxins and constitute important bacterial virulence factors (1, 2). Their cytolytic function operates by introducing a large, water-filled pore into target cell membranes. These pores either deliver toxic effector proteins to the target cell or lead to cell lysis through leakage (3). Most bacterial PFTs are secreted into the extracellular environment in a water-soluble form, where they subsequently diffuse and assemble on host cell membranes. Pore-forming toxins are classified as α-PFTs or β-PFTs depending on the structure of the transmembrane pore, i.e. α-PFTs contain α-helical transmembrane domains, and β-PFTs form a β-barrel (1, 2).

Cytolysin A (ClyA), also known as silent hemolysin A (SheA) or hemolysin E (HlyE), is a cytolytic α-PFT that causes the hemolytic phenotype of several Escherichia coli (E. coli) strains (4–9). Its homologs are also found in other pathogenic organisms, including Salmonella typhi and Shigella flexneri (10, 11). The E. coli ClyA monomer is a 34-kDa soluble protein that has a rod shape formed by a core bundle of four long α-helices (~90 Å long) (10). At the end of the bundle that contains the N-terminal region, an additional shorter (~30 Å long) helix from the C-terminal region packs against the core bundle, forming a five-helix bundle for about one-third the length of the molecule. The structure of the transmembrane pore shows a hollow funnel consisting of 12 subunits (protomers) (12, 13). Each protomer contributes one amphipathic α-helix that packs in an iris-like structure to form the transmembrane barrel. The narrowest opening of the channel at the transmembrane site has a diameter of 35 Å while the top of the funnel is 70 Å (12).

The general model for multimeric PFT attack action involves three steps (1): (i) docking of the soluble monomer to the target cell membrane; (ii) assembly of the monomer into a ring-like prepore structure that lacks the transmembrane domain structure at the membrane surface; (iii) penetration of the transmembrane domain across the target cell membrane. In the first step, cholesterol, carbohydrates, or membrane proteins on the host cell surface may serve as receptors for the association of the toxins with the membrane (14–18). ClyA toxin is also believed to follow a similar strategy when attacking host cells (1). However, unlike the well-studied β-toxins α-hemolysin and protective antigen from anthrax toxin, which are secreted by Gram-positive bacteria into the extracellular environment as a soluble monomer (19, 20), ClyA is secreted from E. coli via a vesicle-mediated pathway (21–23). Similar to the budding of yeast cells, the outer membrane of E. coli bubbles out and pinches off to form the outer membrane vesicles (OMVs) (24). During the formation of the OMV, many outer membrane proteins as well as periplasmic proteins are incorporated into the OMV. The vesicle-mediated pathway has been found to deliver several toxins, including heat-labile enterotoxin and Shiga toxin (25–27). So far, it remains unclear how these toxins are released from the OMVs to carry out their cytolytic function (25).

Of note, Wai et al. discovered that ClyA in the OMV adopted a ring-like oligomeric structure when viewed under an electron-microscope (21). This observation contradicts the typical
PFT strategy of assembling pores from water-soluble monomers at the surface of the target cell. These ClyA oligomers were speculated to represent transmembrane pores, thus raising the question about how transmembrane pores already embedded in E. coli lipid membranes could then transfer to the host cellular membrane. Nevertheless, its secretion pathway is distinct from the well-studied β-toxins, which suggests that ClyA may have an unexpected mechanism to carry out its cytolytic function.

In this study, we report that ClyA assembles into hemolytically active oligomers in the absence of lipid bilayers or detergents. The water-soluble oligomer may represent a prepore intermediate undergoing the transition from the monomer to the transmembrane pore. These data provide important evidence to elucidate the structural organization of the oligomeric ClyA proteins in the OMV, which is a step further toward understanding the attack mechanism for the ClyA toxin.

**EXPERIMENTAL PROCEDURES**

**Materials**—All the chemicals were purchased from Fisher Scientific unless specified. Luria Broth (LB) medium was from Boston BioProducts.

**Cloning and Mutagenesis of ClyA**—The E. coli ClyA gene was amplified from the E. coli K-12 genome (ATCC) and cloned into a pT7 vector with a C-terminal hexa-histidine tag. The cysteine-less ClyA (C87S, C285S) was obtained by the overlap PCR method. The PCR was performed using Phusion polymerase (New England Biolabs) with pT7-ClyAw as a template. The primers for C87S were 5’-ATGAATGTCTGTGTTGGAACAGCAAATG (forward) and 5’-ACTTTCAATGAGTATCAGAAAAGAC (reverse). The primers for C285S were 5’-GAGCCAAATG (forward) and 5’-CTCAAAG (reverse). All primers were obtained from Fisher Scientific unless specified. Luria Broth (LB) medium was from Boston BioProducts.

**Expression and Purification of ClyA**—The resulted plasmids were transformed into E. coli BL21 (pLys). Cells were grown in LB medium at 37 °C to 

**Liposome Preparations**—All lipids were purchased from Avanti Polar Lipids (Alabaster, AL). E. coli total extract or brain total extract lipids in chloroform was dried at 20–23 °C under nitrogen and then kept under vacuum for at least 3 h. Lipids were re-hydrated in buffer 50 mM Tris-HCl, pH 8.0, 150 mM NaCl to a 25 mg/ml final concentration of total lipids, and incubated for 30 min at 20–23 °C with vortexing at 5 min intervals. The suspended lipid mixture was frozen in liquid nitrogen and thawed at 37 °C for a total of three cycles to reduce the number of multi-lamellar liposomes. Hydrated lipids were extruded 21 times through a 0.4 μm pore size polycarbonate filter (Whatman) using an Avanti Mini-Extruder (Alabaster, AL). The resultant liposomes were stored at 4 °C and used within 2 weeks of production.

**Gel Filtration Analysis of Oligomerization**—Gel filtration was performed at room temperature using a Superdex 200 10/300 gel filtration column (GE Healthcare). For detergent-triggered oligomerization, samples were analyzed in buffer 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.01% (w/v) n-dodecyl β-d-maltoside (DDM) at room temperature. For detergent-independent oligomerization, samples were incubated with buffer containing no DDM at the indicated temperature prior to GFC analysis. The buffer used for GFC was 50 mM Tris-HCl, pH 8.0, 150 mM NaCl.

**Intrinsic Fluorescence Studies**—ClyA proteins were analyzed at a concentration of ~3 μM at 25 °C. For intrinsic fluorescence measurements the excitation wavelength was 280 nm and emission spectra were collected at 290–410 nm using a Fluorolog-3 spectrophotometer. The fluorescence emission spectra of corresponding buffers were subtracted from the emission spectrum of each ClyA sample. For DDM-induced oligomerization studies, 15 μl of DDM stock solution (10% w/v) was added to 1.5 ml of ClyA monomer at a final concentration of 0.1% (w/v). Subsequently, the emission fluorescence spectra were recorded at 3-min intervals.

**Tb(DPA)₃³⁻-loaded liposomes were prepared as above, except that HBS buffer (50 mM HEPES, 100 mM NaCl, pH 7.5) including 3 mM TBCl₂, 9 mM 2,6-pyridinedicarboxylic acid (DPA) was added to 6.4 mg of dried lipids to yield a final total lipid concentration of about 30 mM (final volume, 0.30 ml). The resulting liposomes were separated from non-encapsulated Tb(DPA)₃³⁻ by gel filtration (Sephacore CL-6B-200, 0.7 cm inner diameter ×50 cm) in HBS buffer, and the resulting concentration (~2 mM) was measured by absorbance at 400 nm.
Analysis of Hemolytic Activity—The liquid hemolysis assay with sheep blood cells was used to measure the hemolytic activity of the ClyA proteins (28). Briefly, Remel sheep defibrinated blood (Thermo Scientific) was washed with HyClone Dulbecco’s phosphate-buffered saline (DPBS) buffer (Thermo Scientific) and diluted 4-fold in DPBS buffer. ClyA proteins (7 μg) were added to 250 μl of blood cells and incubated at 37 °C for 15 min. Samples were then centrifuged at 22,000 × g at 4 °C for 8 min. The absorbance of the supernatant at 540 nm was measured to determine the released hemoglobin. Total hemolysis (100%) was defined by incubation of red blood cells in MilliQ water, in place of buffer.

Analysis of Pore-forming Activity by Fluorescence Quenching—The pore-forming activity of ClyA proteins was also assessed by using a Tb(DPA) fluorescence quenching assay. Tb(DPA)3−-loaded liposomes (6 μl of ~2 mM giving a final concentration of 12.5 μM total lipids) were added to 994 μl of quenching buffer (100 mM NaCl, 50 mM HEPES, 5 mM EDTA, pH 7.5) containing 250 nM protein just before the measurement. Samples were excited at 278 nm and the net initial emission intensity (F0) at 544 nm was determined after equilibration of the sample at 25 °C for 2 min. The samples were then incubated for 1 h at 37 °C. After re-equilibration at 25 °C, the final net emission intensity (Ff) of the sample was determined and the fraction of quenched fluorophore was calculated using 1 - Ff/F0. Measurements were repeated 4 times for each condition.

Oxidation of ClyA Proteins—The formation of intramolecular disulfide bonds were catalyzed using the oxidizing reagent Cu(phenanthroline)2 following a previous protocol (29). ClyA proteins were incubated with 1.5 mM Cu(phenanthroline)2 at room temperature for 30 min. EDTA (5 mM, final concentration) was then added to quench the reaction. All the chemicals were then removed by buffer exchange using a Centricon (Millipore) with a 3 kDa cut-off. To reduce the sample, oxidized proteins were incubated with 20 mM DTT for 60 min at room temperature. DTT was then removed by buffer exchange before the analysis of hemolytic activity.

Single Channel Study of ClyA—Planar lipid bilayer experiments were performed in an apparatus partitioned into two chambers with a 25 μm-thick Teflon film. An aperture of ~100-μm diameter had been made near the center of the film with an electric arc. Each chamber was filled with 25 mM Tris-HCl, pH 8.0, 1 M KCl. A Ag/AgCl electrode was immersed in each chamber with the cis chamber grounded. A positive potential indicates a higher potential in the trans chamber. The ClyAO8 (12, 33) was incubated with 0.1% (w/v) DDM overnight at 4 °C and then analyzed by GFC with a running buffer 50 mM Tris-HCl, pH 8.5, 150 mM NaCl, and 0.01% DDM. ClyA monomers were incubated at 37 °C for 2 h. The running buffer of GFC was 50 mM Tris-HCl, pH 8.5, 150 mM NaCl. c and d, conversion of ClyA08 and ClyA09 to transmembrane pore. The ClyA08 (c) and ClyA09 (d) were incubated with 0.1% (w/v) DDM overnight at 4 °C and then analyzed by GFC with a running buffer 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.01% DDM.

RESULTS

Assembly of ClyA in the Absence of Detergent—It is often observed that PFT monomers assemble to form the omycogenic transmembrane pores upon contacting with detergents or lipid vesicles (30–32). Similar to previous findings, the retention volume of ClyA monomers shifted from 15 ml to 10 ml by gel filtration chromatography (GFC) after incubation with 0.1% (w/v) DDM overnight at 4 °C (Fig. 1a) (12, 33). This high molecular weight oligomer was previously shown to represent the transmembrane pore structure and is termed ClyA_TM here (12,
During the purification, we noticed that the ClyA protein showed a tendency to form high molecular weight oligomers even in the absence of detergent or lipids. We therefore explored the oligomerization of ClyA in the absence of detergent or membranes using GFC. Two peaks with retention volumes of 8.5 ml and 9.5 ml appeared in chromatograms after incubation of the ClyA monomer at 37 °C in buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl for 2 h (Fig. 1b). Fractions corresponding to two peaks were named oligomer 8 (ClyA8O8) and oligomer 9 (ClyA9O9) based on their respective retention volumes. Notably, these two fractions migrated faster in the GFC column than ClyATm (Fig. 1, a and b). Because the ClyA monomer contains two cysteines, it is possible that ClyA formed oligomers through disulfide bond formation during the incubation at 37 °C. To assess this, the same experiment was repeated in buffers containing freshly prepared 10 mM DTT at 37 °C. The presence of DTT did not modify the elution profile of ClyA oligomers, excluding the involvement of disulfide bridges (data not shown). The ClyA8O8 and ClyA9O9 were pooled separately and re-analyzed by GFC. The two proteins were still eluted at their original retention volumes of 8.5 ml and 9.5 ml, respectively (supplemental Fig. S2). No monomer was observed in the chromatogram, indicating that ClyA8O8 and ClyA9O9 did not dissociate. Thus, we conclude that ClyA was able to oligomerize in the absence of detergents/membranes and that the isolated oligomers were stable in solution.

Conversion of Oligomer 8 and Oligomer 9 to the Transmembrane Pore—To investigate whether ClyA8O8 and ClyA9O9 can convert to the transmembrane pore, we incubated them with 0.1% DDM overnight at 4 °C. The ClyA8O8 shifted to a peak eluted at 15 ml, corresponding to the monomer, and a peak at 10 ml, corresponding to the fully-assembled transmembrane pore (Fig. 1c). The ClyA9O9 also shifted to peak at 10 ml but little monomer is observed during this process (Fig. 1d). These results indicate that ClyA8O8 may contain loosely packed monomers that can be solubilized by DDM. By contrast, ClyA9O9 had a stable structure that did not disassemble in detergent solution. Rather, DDM triggered a conformational change in ClyA9O9 that transformed it into the transmembrane pore. Because a portion of ClyA8O8 also appeared to have converted to ClyATm, it may contain a mixture of ClyA8O8-like oligomers with a stable structure and loosely packed monomers.

Many assembled β-toxin oligomers have shown tolerance to sodium dodecyl sulfate (SDS) treatment and remain as oligomers on SDS-PAGE (31, 34–36). We were interested to see if ClyA oligomers would have different stabilities in SDS and migrate differently in the SDS-PAGE. We found that all three oligomeric forms of ClyA dissociated to monomer on SDS-PAGE (31, 34–36). We were interested to see if the ion conductance of a membrane channel is proportional to the pore size, which, in turn, is determined by the number of protomers in the transmembrane pore (38), we could address this question by comparing the conductance of ClyA channels formed in two different pathways. Here, single channel insertion of ClyA proteins into planar lipid bilayers was monitored by bilayer current recording experiments (Fig. 3a). Similar to the results of the electrophysiological studies of E. coli ClyA and its homologs from Salmonella typhi (6, 39, 40), the ClyA channels formed by monomer show a broad distribution from 5–15 nS with a major peak at around 11 nS (Fig. 3b), suggesting that ClyA might create pores of variable size. The broad distribution of conductance observed by electrophysiology agrees with the cryo-EM data showing ClyA pores of variable size in detergent solution (12). The ClyA channels formed by ClyA9O9 and ClyA8O8 exhibited similar conductance distribution patterns with the majority of ClyA8O8 pores exhibiting a conductance between 6–10 nS and ClyA9O9 pores a conductance between 9–16 nS (Fig. 3, c and d).
The population of ClyA\textsubscript{O8} channels shifted to slightly larger pores than those formed directly from monomer while ClyA\textsubscript{O9} pores appeared smaller. So far, it is unclear why the conductance of the ClyA\textsubscript{O8}, ClyA\textsubscript{O9}, and ClyA\textsubscript{TM} pores are different considering that they migrate at the same retention volume by GFC (Fig. 1). Further detailed structural studies will be carried out to address this issue in future.

Interestingly, when we measured the conductance of the ClyA monomer sample after 7 days incubation at 4 °C in DDM, the histogram of conductance showed almost a single population of 11 nS pores (Fig. 3\textit{e}). This suggests that the 11 nS pore is the most stable form, since it survives a long-time incubation in detergent. By contrast, many other forms of ClyA lose the ability to insert into the lipid bilayer, probably due to aggregation or degradation. The dodecameric structure of the transmembrane pore determined by x-ray crystallography was also obtained from a DDM sample which contained a large variety of ClyA pores of different sizes (12). We suggest that the ClyA transmembrane pore with dodecameric structure might correspond to the 11 nS pores observed in the electrophysiology recordings.

**Temperature- and Concentration-dependent Oligomerization**—We were interested in how the temperature and the protein concentration affect ClyA assembly in solution. ClyA monomer was incubated at temperatures ranging from 4°C to 42°C for 2 h. At a temperature lower than 37°C, little ClyA oligomerization was observed (Fig. 4). Unlike the assembly in the detergent micelles which proceeded to completion at 4°C for 30 min, the detergent-independent assembly was strictly temperature-dependent and was a much slower process relative to the former. To see if oligomerization was also concentration dependent, we incubated 0.1 mg ClyA protein at concentrations ranging from 1 μg/ml to 0.1 mg/ml at 42°C for 2 h. Samples were then analyzed by GFC. In order to load enough protein sample to the GFC column for detection, the two lower concentration samples, 10 μg/ml and 1 μg/ml, were concentrated to 1 ml using an Amicon centrifuge concentrator at 4°C. To eliminate the possibility that oligomerization might also be induced during the centrifugation concentrating procedure, we incubated 100 ml 1 μg/ml at 4°C for 2 h and concentrated the sample to 1 ml and loaded it to the GFC column as a control. We found ClyA remained as a monomer and no protein was eluted at either 8 or 9 ml retention volume demonstrating the concentration step does not induce oligomer formation (data not shown). The percentage of each population (ClyA\textsubscript{O8}, ClyA\textsubscript{O9} and monomer) was calculated from the area of the peaks eluted at 8.5 ml, 9.5 ml, and 15 ml in the chromatogram that were analyzed by the Unicorn software. The data are summarized in Fig. 5.

The population of ClyA\textsubscript{O9} channels shifted to slightly larger pores than those formed directly from monomer while ClyA\textsubscript{O8} pores appeared smaller. So far, it is unclear why the conductance of the ClyA\textsubscript{O8}, ClyA\textsubscript{O9}, and ClyA\textsubscript{TM} pores are different considering that they migrate at the same retention volume by GFC (Fig. 1). Further detailed structural studies will be carried out to address this issue in future.

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The population of ClyA\textsubscript{O9} channels shifted to slightly larger pores than those formed directly from monomer while ClyA\textsubscript{O8} pores appeared smaller. So far, it is unclear why the conductance of the ClyA\textsubscript{O8}, ClyA\textsubscript{O9}, and ClyA\textsubscript{TM} pores are different considering that they migrate at the same retention volume by GFC (Fig. 1). Further detailed structural studies will be carried out to address this issue in future.

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lowest concentration (1 μg/ml, ~30 nm), the ratio of the oligomer to monomer was not reduced compared those of the higher concentrations, suggesting the oligomerization was independent of the protein concentration. Because of the detection limit of GFC, we were not able to investigate lower concentrations. This result demonstrates that ClyA has a strong tendency to oligomerize even at a low concentration at physiological temperature.

Probing the Structural Arrangement of Oligomers by Fluorescence—To understand the structural arrangement of ClyA proteins, we measured their intrinsic fluorescence spectra. The ClyA monomer contains two tryptophan and 13 tyrosine residues. As shown in Fig. 6a, the monomer had a fluorescence maximum at 315 nm while the fully assembled pore ClyA_Tm had a maximum at 340 nm. The fluorescence emission spectra of the ClyA微量元素 and ClyA_O9 were very similar with the fluorescence maxima also at 340 nm, close to that of the transmembrane pore. The spectra from 345–400 nm overlapped well with that of the transmembrane pore while there is a blue shift of 2 nm in the spectra from 300–340 nm (Fig. 6a). Because the spectra of ClyA微量元素 and ClyA_O9 appeared to be in between the monomer and transmembrane pore, we suspected they might be an intermediate state. To test this, we studied a series of intermediate states of ClyA proteins during the transition to transmembrane pores. Here, DDM was added to ClyA monomer to a final concentration of 0.1% (w/v) of DDM at 23 °C to trigger the pore formation. The spectrum was taken every 3 min until the spectra stabilized. Fig. 6b shows that the fluorescence intensity rose sharply upon the addition of detergent and the wavelength of the peak maximum shifted from 315 to 340 within the first 3 min. After the initial fluorescence jump, the fluorescence intensity gradually decreased and became stable after 30 min. The normalized spectra of these intermediates revealed the peak maxima undergo subtle blue-shift during the transition (Fig. 6c). Interestingly, the spectrum taken 9 min after the addition of the DDM overlaid well with the spectrum of ClyA微量元素 and ClyA_O9 (Fig. 6d), indicating that the structure of ClyA微量元素 and ClyA_O9 might mimic an intermediate state of the oligomerization process.

We also investigated how the composition of the lipid membrane affects the assembly of ClyA. To avoid the occurrence of oligomerization in solution that would contribute to fluorescence spectrum, the ClyA monomers were incubated with liposomes prepared from porcine brain lipids or E. coli lipids extract at room temperature in a 1:50 protein to lipid molar ratio. The spectrum of the brain lipid liposomes sample overlapped very well with the spectrum of ClyA_Tm (Fig. 6e), which demonstrated that ClyA proteins have transformed into transmembrane pores on the brain lipid liposomes. In contrast, the spectrum of the ClyA incubated with E. coli liposomes did not resemble the DDM treated sample (Fig. 6f). Instead, it was located in between the monomer and the spectrum of the ClyA微量元素 suggesting that ClyA does not form the transmembrane pore on E. coli lipid membranes.

Effect of Lipid Composition on Pore-forming Activity—To further examine the pore-forming activity of ClyA proteins on liposomes, we used a fluorescence quenching assay. The liposomes with Tb(DPA)_3 encapsulated inside were added to buffers containing the fluorescence quencher EDTA. EDTA can diffuse into the liposome through ClyA pores on the liposome membrane and quench the fluorescence. Fig. 7 shows that ClyA monomer caused a significant fluorescence decrease in the brain lipid liposomes. Consistent with our hemolytic assay, ClyA微量元素 and ClyA_O9 also induced fluorescence quenching but with reduced activity. On the contrary, no fluorescence quenching was observed with any of the ClyA protein samples incubated with E. coli liposomes. This experiment confirms that ClyA proteins do not form transmembrane pores on E. coli lipid membranes.

Direct Conversion of ClyA微量元素 and ClyA_O9 to Transmembrane Pores—The data above revealed that ClyA微量元素 and ClyA_O9 can convert to the transmembrane pore when in contact with detergent micelles or lipid membranes. This could be achieved through two possible pathways: (i) ClyA微量元素 and ClyA_O9 dissociate into monomers then re-assemble in the micelles/lipid membrane or, (ii) ClyA微量元素 and ClyA_O9 directly convert to the transmembrane pore without undergoing the dissociation step. To distinguish between these pathways, we monitored the conversion of ClyA微量元素 and ClyA_O9 in the presence of brain lipid membranes by intrinsic fluorescence. Liposomes were added to monomer and the fluorescence was recorded (Fig. 8a). After 5 min, the spectrum showed two peak maxima at 314 nm and 340 nm indicating there was still monomer remaining in the sample. This result suggests the association process of the ClyA monomers to oligomers on the lipid membrane occurs on the time scale of minutes, which is much slower than the αH toxin (<5ms) (41). If ClyA微量元素 and ClyA_O9 dissociate into monomers, we expect to observe the spectrum of the monomeric species due to the relatively slow association process of ClyA monomers. Adding liposomes to ClyA微量元素 and ClyA_O9 induced a slight change at the 300–340 nm range (Fig. 8, b and c), however we did not observe a significant shift of the spectrum toward the monomer peaks. Thus, this experiment suggests that ClyA微量元素 and ClyA_O9 directly convert to the transmembrane pore on the lipid membrane without first dissociating to monomers.
Effect of Redox Environment on the Activity of ClyA

ClyA monomer contains two cysteines (C87, C285). The structure of ClyA monomer revealed that these cysteines were located within 5.2 Å (C87 to C285), a distance that would allow a disulfide bond to form. In the transmembrane pore structure, the cysteines move away from each other to be 6.8 Å apart, which is the longest possible distance for a disulfide bond to form between to cysteine residues (42). This raised a possibility that the disulfide formed between the cysteines might act as a switch to control ClyA activity. To test this, we induced the disulfide bond formation by incubating the protein with Cu(phenanthroline)2. GFC analysis of the sample showed ClyA proteins remained monomeric, indicating the protein did not form any inter-molecular disulfide bonds (supplemental Fig. S4). The oxidized ClyA was subjected to the hemolytic assay. Fig. 9 shows that the oxidized ClyA lost 90% of hemolytic activity. Incubation in 20 mM DTT for an hour could recover 60% the activity. The incomplete recovery of the activity is likely due to the close packing of the helix bundles that occludes the disulfide bond from the reducing chemicals. As a control, the double cysteine knock out mutant ClyA/ΔCys exhibited no response to the oxidation/reduction procedure. These results indicated that formation of the intra-molecular disulfide bond could inhibit the pore-forming activity presumably by preventing the occurrence of conformational change necessary for the formation of oligomeric transmembrane pore.

DISCUSSION

Previous work on ClyA has also shown that ClyA oligomerizes in solution without detergent at 37 °C (43). In this example, monomer, dimers and high molecule-weight oligomers (8–10mers as speculated by the authors) were observed by GFC (43). The 8–10mer fraction lacked hemolytic activity. However, our data clearly demonstrated that both ClyA08 and ClyA09 lysed blood cells while the thermally denatured ClyA did not.
Because ClyA₀₈ and ClyA₀₉ retains both a stable structure and hemolytic activity, we expect that they are not a disordered aggregate but rather an active oligomeric form of ClyA proteins. In addition, the intrinsic fluorescence of these soluble oligomers matched that of an intermediate state. This suggests the detergent-independent oligomer resembles an intermediate state between the monomer and fully assembled transmembrane pore. Since ClyA₀₈ and ClyA₀₉ were formed in solution, it is unlikely that the α-helical transmembrane barrel has formed, as this would expose the hydrophobic outer surface of the barrel to aqueous solution. Therefore, we believe that ClyA₀₈ and ClyA₀₉ could be oligomeric forms without the hydrophobic transmembrane barrel domain, i.e., the prepore structure. Also, we have shown that ClyA₀₈ and ClyA₀₉ can convert directly to transmembrane pore. In summary, we propose that ClyA₀₈ and ClyA₀₉ are intermediate states between the monomer and transmembrane ClyA channel. Since these two populations were eluted earlier than transmembrane pores in GFC, it might have a less compact structure than the transmembrane pore.

Although many PFTs require detergents or lipid bilayers for assembly to oligomeric prepore, the protective antigen of anthrax toxin also oligomerizes to a prepore structure in the absence of detergents or lipid membranes (35, 44). In fact, the water-soluble prepore was formed by the oligomerization of protective antigen 63 which derives from the proteolytic removal of a 20 kDa fragment from the full length anthrax monomer. Similarly, Monalysin, a PFT from the pathogen Pseudomonas entomophila, forms a multimeric structure in solution after proteoactivation (45). Therefore, not all PFTs require lipid membranes or similar environments to form prepore structures. Although in vitro the PA63 and Monalysin undergo oligomerization in solution after protease treatment, in nature the oligomerization of the PA63 and Monalysin is triggered by the proteases expressed on the host plasma membrane, where they subsequently assemble. The activation of ClyA may be regulated by the change of its redox status during the secretion of ClyA into OMVs (10, 21, 43). ClyA in the periplasm contains a C87-C285 disulfide bond (6, 21, 46), whereas ClyA within OMV is reduced (21). The disulfide bond may prevent assembly of functional pore complexes, as oxidized ClyA showed a decreased hemolytic activity compared with reduced ClyA, which is consistent with previous studies (10, 21, 43). This notion is further supported by the observation that ClyA expressed in an E. coli (dsbA⁻ and dsbB⁻) strain, which is deficient in periplasmic disulfide bond formation, displays a significant hemolytic activity on blood agar plates, an effect that is not detectable for ClyA.

FIGURE 8. Determine the pathway of ClyA₀₈ and ClyA₀₉ conversion. Brain lipid liposomes (0.025 mg/ml, final concentration) were added to ClyA proteins (0.025 mg/ml): monomer (a), ClyA₀₈ (b), and ClyA₀₉ (c) solution at 25 °C. The fluorescence spectra were recorded at the indicated time intervals.

FIGURE 9. Regulation of the ClyA pore-forming activity by redox change. The pore-forming activity of various proteins was examined by hemolytic assay. The oxidized sample contained wild type ClyA (ClyAwt) and ClyA double cysteine mutant C87S, C285S (ClyA ΔCys) incubated with Cu(phenanthroline)₂. The reduced samples were obtained by DTT treatment of oxidized proteins. Blank samples were phosphate-buffered saline.
expressed in wild-type strain (21). Therefore, the secretion of ClyA to OMVs where DsbA and DsbB are absent may reduce the intracellular disulfide bonds and activate the toxin for oligomerization (21).

Some speculate that ClyA forms transmembrane pores upon interaction with the membrane of the OMV (21). However, this contradicts two observations: (i) the pre-formed ClyA transmembrane pore lacks hemolytic activity as demonstrated by our study and previous data (37). A transmembrane ClyA pore embedded in the OMV membrane loses functionality for further attack. (ii) the pore-forming activity of ClyA is strongly dependent on cholesterol in the membrane, which suggests that cholesterol may facilitate the transmembrane domain insertion (46). Since bacterial outer membranes contain no cholesterol, the transformation of ClyA into the transmembrane pore inside of the OMV should be very slow. Our intrinsic fluorescence analysis supports this notion: the spectra of ClyA with liposomes reveal transmembrane pores in brain lipids but ClyA cannot transform in the presence of E. coli liposomes. The Tb fluorescence quenching assay further confirmed that ClyA cannot form transmembrane pores on E. coli lipid membranes. Thus, it is reasonable to believe the oligomeric structure of ClyA in the OMV may not be the transmembrane pore.

The average expression level of a protein in E. coli is ~1000 copies, roughly 1 µmol in the cytosol (47). Our data demonstrated that ClyA forms oligomers at 37 °C at concentrations as low as 30 nM. Under physiological conditions ClyA can associate into ClyA_{prep} like prepore structures once activated in the OMV and this process can occur independently of lipid membrane. Taken together, these data strongly suggest that the secreted ClyA proteins oligomerize to a prepore structure instead of the transmembrane pore inside the OMV before they reach the target cell membrane.

We propose that ClyA adopts a non-classical assembly pathway including the following steps (Fig. 10): (i) ClyA is expressed as a monomer in the cytosol and exported to the periplasmic space where it remains inactive in an oxidized form; (ii) ClyA is secreted in outer membrane vesicles in monomer form; (iii) Cleavage of the disulfide bond in the OMV activates ClyA monomers which subsequently oligomerize to the prepore structure. How the OMV then delivers the active ClyA prepore to the host cell remains unknown. One plausible mechanism involves internalization of the vesicle and subsequent release of the cargo proteins to the cytoplasm of the target cells (25). Further studies are underway to identify how ClyA is released.

In conclusion, we have shown that ClyA protein assembles into a functional oligomer in the absence of detergents and membranes. The oligomeric form represents a prepore intermediate state that may resemble the ClyA oligomer structure in the OMVs. These data provide insight regarding the oligomeric state of ClyA proteins in the OMV, which represents an important step toward resolving the overall mechanism of ClyA. High-resolution crystal structures of the prepore might be obtained in the near future, and they will be essential for determining how the prepore becomes the hemolytic pore.

![Schematic model showing the proposed assembly pathway for the E. coli ClyA](image_url)

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