Cytotoxin ClyA from *Escherichia coli* assembles to a 13-meric pore independent of its redox-state

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ClyA is a pore-forming toxin from virulent *Escherichia coli* and *Salmonella enterica* strains. Here, we show that the intrinsic hemolytic activity of ClyA is independent of its redox state, and that the assembly of both reduced and oxidized ClyA to the ring-shaped oligomer is triggered by contact with lipid or detergent. A rate-limiting conformational transition in membrane-bound ClyA monomers precedes their assembly to the functional pore. We obtained a three-dimensional model of the detergent-induced oligomeric complex at 12 Å resolution by combining cryo- and negative stain electron microscopy with mass measurements by scanning transmission electron microscopy. The model reveals that 13 ClyA monomers assemble into a cylinder with a hydrophobic cap region, which may be critical for membrane insertion.

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Introduction

Pore-forming toxins (PFTs) kill target cells by inserting channels into their membranes. Strikingly, PFTs are soluble in aqueous solution before converting to a membrane-embedded state. This transition requires the exposure of hydrophobic surfaces that are buried in the water-soluble state, and frequently includes assembly of monomers to an annular, oligomeric pore complex (reviewed in Parker and Feil, 2005). PFTs are classified into α PFTs and β PFTs (Gouaux, 1997). α PFTs span the target membrane with α

helices. Well-known members of this class are the colicins (Zakharov and Cramer, 2002) and diphtheria toxin (Choe *et al.*, 1992). Conversely, β PFTs insert into membranes by forming transmembrane β barrels. This class includes *Staphylococcus aureus* α -hemolysin (Song *et al.*, 1996), *Aeromonas hydrophila* aerolysin (Parker *et al.*, 1994), and cholesterol-dependent cytolysins (Tweten *et al.*, 2001).

ClyA (also referred to as HlyE or SheA) is a recently discovered PFT (Ludwig *et al.*, 1995; Oscarsson *et al.*, 1996; del Castillo *et al.*, 1997), which could not be classified so far due to the lack of structural data. It is responsible for the hemolytic phenotype of several *Escherichia coli* and *Salmonella enterica* strains (del Castillo *et al.*, 1997; Oscarsson *et al.*, 2002; Ludwig *et al.*, 2004). ClyA lyses erythrocytes from different species, shows cytotoxicity towards cultured mammalian cells, and induces apoptosis of macrophages (Ludwig *et al.*, 1999; Oscarsson *et al.*, 1999; Lai *et al.*, 2000). In addition, sublytic amounts of ClyA induce slow intracellular Ca^{2+} oscillations in epithelial cells (Soderblom *et al.*, 2005). These observations suggest that ClyA contributes to the virulence of pathogenic bacterial strains (Ludwig *et al.*, 2004; Kerenyi *et al.*, 2005).

The crystal structure of monomeric, soluble ClyA (34 kDa) revealed a rod-shaped, mostly α helical molecule (Wallace *et al.*, 2000; Figure 1). It consists of a long four-helix bundle (helices A, B, C, and F) with a short C-terminal helix (helix G) alongside at one end. A subdomain consisting of a short β hairpin (the ‘ β tongue’) flanked by two short helices (helices D and E) is located on the other end of the bundle. The β tongue region forms the major hydrophobic patch on the ClyA surface, and mutational analysis indicated that this region interacts with target membranes (Wallace *et al.*, 2000). The two cysteine residues of ClyA, located in helix B (C87) and helix G (C285), are sufficiently close to form a disulfide bond. In the crystal structure of ClyA, the cysteines are reduced and bridged by a mercury atom of a Hg(II)-derivative compound.

Based on the crystal structure of the monomer and on electron microscopy (EM) data, Wallace *et al.* (2000) constructed a model of the ClyA pore, in which eight protomers are aligned perpendicular to the membrane surface with the β tongue interacting with lipid. In this octameric assembly, the conformation of the monomer is essentially retained, apart from a minor 20° reorientation of the subdomain containing the β tongue with respect to the rest of the molecule.

Many aspects of the biogenesis of functional ClyA are largely unknown. Translocation to the periplasm is neither mediated by a signal sequence nor requires cytolytic activity of ClyA (del Castillo *et al.*, 1997; Ludwig *et al.*, 1999; del Castillo *et al.*, 2001). Export from the bacterial cell occurs in outer membrane vesicles (OMVs) where ClyA forms oligomeric, membrane-embedded pores (Wai *et al.*, 2003a). Soluble ClyA in the periplasm contains the C87–C285 disulfide bond (Ludwig *et al.*, 1999; Oscarsson *et al.*, 1999; Wai *et al.*, 2003a), whereas OMV-embedded ClyA is reduced

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Figure 1 Structure of Monomeric ClyA. Atomic structure of soluble, monomeric ClyA_{red} (Wallace *et al*, 2000) with the four-helix bundle formed by helices (A, B, C and F (blue), the β hairpin (yellow) with its flanking helices D and E (orange), and the C-terminal helix G (cyan) that is linked to helix C by a disulfide bridge (C87-C285) in ClyA_{ox}.

(Wai *et al*, 2003a). Recent results indicated that oxidized ClyA (ClyA_{ox}) has a lower intrinsic hemolytic activity than reduced ClyA (ClyA_{red}) (Atkins *et al*, 2000; Wallace *et al*, 2000; Wai *et al*, 2003a). Therefore, it was postulated that the disulfide bond may prevent assembly of functional pore complexes by anchoring helix G to the four-helix bundle (Atkins *et al*, 2000). Since secretion and activation seem to occur hand in hand with a change in the redox state of ClyA, it was hypothesized that the assembly of active ClyA pores is redox-regulated (Atkins *et al*, 2000; Wallace *et al*, 2000; Wai *et al*, 2003a). This is supported by the observation that *E. coli dsbA*[−] and *dsbB*[−] strains, which are deficient in periplasmic disulfide bond formation, display a ClyA-dependent hemolytic phenotype on blood agar plates that is not detectable for the corresponding wild-type strain (Wai *et al*, 2003a).

Here, we provide firm evidence that, in contrast to previous results, ClyA is assembly-competent and active independent of its redox state. We show that detergent or lipid induces a slow structural change in monomeric ClyA to the conformation required for the assembly into the cylindrical pore complex. Using cryo-EM and single particle analysis we have established the three-dimensional (3D) structure of the oligomeric pore complex at 12 Å resolution. It comprises 13 subunits, consistent with the mass measured by scanning transmission electron microscopy (STEM). Our 3D model combined with spectroscopic data suggest a large structural change of the ClyA monomers during assembly of the pore complex. Projections from vitrified proteoliposomes bearing lipid-inserted pore complexes document that the architectures of the detergent-induced and the lipid-inserted ClyA pore complexes are highly similar.

Results

Redox state of ClyA cysteines does not modulate hemolytic activity

To date, controversy exists about the activity of ClyA_{ox} (Atkins *et al*, 2000; Wallace *et al*, 2000; Wai *et al*, 2003a, b).

In order to clarify the functional and structural role of the disulfide bond of ClyA, we prepared homogeneous samples of ClyA_{red} and of ClyA_{ox}. ClyA_{red} was overexpressed and isolated from the cytoplasm of *E. coli*. ClyA_{ox} was obtained by Cu²⁺-mediated air oxidation of purified ClyA_{red}, and subjected to gel filtration to remove high molecular weight aggregates that formed during oxidation. The redox state of ClyA_{ox} and ClyA_{red} was verified by Ellman's assay and by labeling of free cysteines under denaturing conditions with 5-(((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid (IAEDANS) (Supplementary Figure 1).

We used gel filtration to analyze the oligomerization state of freshly purified ClyA_{red} and ClyA_{ox}. At 15°C both ClyA_{red} and ClyA_{ox} are stable monomers even at very high protein concentrations (100 μ M, cf Figure 2A). The observed retention volumes correspond to that of a globular 43 kDa protein. Considering that the elongated shape of ClyA will lead to a lower retention volume, this clearly indicates the monomeric state of ClyA_{ox} and ClyA_{red} (34 kDa). Thus, our experiments contradict the hypothesis of redox-regulated oligomerization of soluble ClyA (Atkins *et al*, 2000; Wai *et al*, 2003a). The only difference between ClyA_{ox} and ClyA_{red} with regard to the oligomeric state was the much higher tendency of ClyA_{ox} to form inactive, high molecular weight aggregates upon prolonged incubation, especially at 37°C (Supplementary Figure 2).

We next studied the hemolytic activity of ClyA_{red} and ClyA_{ox}. As shown in Figure 2B, ClyA_{red} and ClyA_{ox} displayed the same hemolytic activity in a liquid hemolysis assay with horse erythrocytes. To test whether the difference in hemolytic activity observed by other authors (Atkins *et al*, 2000; Wai *et al*, 2003a) reflects different kinetics of hemolysis induced by ClyA_{red} and ClyA_{ox}, we followed the time course of hemolysis (Figure 2C). Yet again, ClyA_{red} and ClyA_{ox} were identical in activity. In addition, we tested whether ClyA changes its redox state upon contact with erythrocytes. Exposure of ClyA_{red} and ClyA_{ox} to IAEDANS under denaturing conditions before and after incubation with erythrocytes revealed that neither ClyA_{red} nor ClyA_{ox} changes the redox state upon membrane insertion (Figure 2D). Taken together, our data show that the intrinsic hemolytic activity of ClyA is independent of its redox state.

Oligomerization of ClyA is triggered by detergent

Since no functional differences were detectable between ClyA_{red} and ClyA_{ox}, and since biologically active ClyA is exported from the bacterial cell in its reduced state (Wai *et al*, 2003a), we focused our structural analysis on the pore complex formed by ClyA_{red}. In search of conditions that would allow the pore structure to be investigated by single particle analysis, we screened a variety of detergents for their ability to trigger assembly of ClyA. Many different detergents induced the formation of oligomeric complexes (data not shown) with dimensions similar to those of lipid-embedded complexes reported by Wallace *et al* (2000). The most homogeneous samples were obtained with *n*-dodecyl- β -D-maltopyranoside (DDM). The kinetics of DDM-induced oligomerization of ClyA_{red} were analyzed by gel filtration (Figure 3A). We selected a temperature of 15°C, at which the assembly process is slow enough to allow for identification of assembly intermediates (see below). A shift of the retention volume of ClyA_{red} from 15.1 to 12.3 ml was observed after only 2 min of exposure to DDM. This peak

Figure 2 Activity of reduced and oxidized ClyA. **(A)** Oligomerization state of ClyA_{red} and ClyA_{ox} in PBS at 15°C. ClyA_{red} (100 μM) and ClyA_{ox} (5 μM) were subjected to analytical gel filtration. The protein is monomeric, even at high concentrations. Arrows indicate retention volumes of soluble mass standard proteins. v_0 , void volume. **(B)** Hemolytic activity of ClyA_{red} and ClyA_{ox} at 37°C. Hemolysis of a 1% suspension of horse erythrocytes by ClyA_{red} and ClyA_{ox} (concentration range between 0.01 and 1000 nM) was examined using a liquid hemolysis assay according to Rowe and Welch (1994). **(C)** Kinetics of hemolysis induced by ClyA_{red} and ClyA_{ox} at 37°C. The decrease in turbidity upon hemolysis of a 0.1% suspension of horse erythrocytes exposed to 250 nM ClyA_{red} or ClyA_{ox} was monitored at 650 nm. **(D)** Redox state of ClyA after insertion into erythrocyte membranes. 250 nM ClyA_{red} (lanes 1 and 3) or ClyA_{ox} (lanes 2 and 4) were added to a 1% suspension of horse erythrocytes (lanes 3 and 4) or PBS (lanes 1 and 2) and incubated for 30 min at 37°C. The samples were thereupon exposed to IAEDANS under denaturing conditions and analyzed by SDS-PAGE (there are no prominent bands of erythrocyte proteins in the mass range of ClyA).

decreased with increasing incubation time while another peak at a retention volume of 9.6 ml appeared. As retention volumes of soluble proteins are not affected by DDM (soluble mass standard proteins showed the same retention volumes with and without DDM, cf Figures 2A and 3A), we conclude that association of monomeric ClyA_{red} with detergent causes the lower retention volume of ClyA_{red} observed after short exposure to DDM. The dominant peak at 9.6 ml contained oligomers, as documented by EM (see below). Quantitative analysis of the peak areas at 9.6 and 12.3 ml showed that DDM-associated, monomeric ClyA_{red} (intermediate I₁) disappeared with virtually the same rate as oligomeric ClyA_{red}

was formed, with half-lives of 1000 ± 300 and 1100 ± 200 s, respectively (Figure 3A, right panels). To test whether intermediate I₁ indeed represents monomeric ClyA_{red} in complex with detergent, rather than an oligomeric form, ClyA_{red} was exposed to DDM for different times, crosslinked with dithio-bis(succinimidylpropionate) (DSP), and the products were examined by SDS-PAGE (Figure 3B, left panel). The protein was predominantly monomeric after 2 min exposure to DDM (Figure 3B, left panel, lane 2). With longer exposure to DDM (lanes 3–6), the band corresponding to monomeric ClyA_{red} gradually disappeared and the band corresponding to the covalently linked pore complex appeared (Supplementary Figure 3). Quantitative densitometric analysis showed that the band of monomeric ClyA_{red} exhibits the same half-life (1100 ± 200 s) as intermediate I₁ identified by gel filtration (Figure 3A and B, right panels). Together, our data suggest a model in which ClyA_{red} inserts into the detergent micelle as a monomer and then assembles via faintly populated intermediates to a defined oligomeric complex.

Electron micrographs of negatively stained protein eluting at 9.6 ml revealed homogeneous, ring-shaped and rectangular projections corresponding to top- and side views of the ClyA_{red} oligomer (Figure 3C), top views representing the majority of particle projections. The outer diameter of the top view averages varied between 90 and 125 Å (Supplementary Figure 4). This is consistent with the diameter of ClyA oligomers reconstituted into lipid vesicles (Wallace *et al*, 2000), and with that of ClyA pores found in isolated OMVs (data not shown). The negative-stain side view average revealed a cylindrical structure that converges to a cap at one end and has a total length of about 160 Å (Figure 3E), in contrast to monomeric ClyA, which exhibits a length of only 110 Å (Wallace *et al*, 2000). Single particle analysis of negatively stained pore complexes was carried out to obtain the fine structural features of the pore complex. A total of 2876 top- and 826 side views were extracted from the micrographs, aligned, and classified by multivariate statistical analysis to calculate class averages. The negative-stain top view class average of Figure 3D shows a cylindrical pore complex with an inner structure. Angular periodicity analysis indicated a dominant 13-fold symmetry, which was present for all top view class averages (Supplementary Figure 4). To compare the structures of pores formed by either reduced or oxidized ClyA, pores formed by ClyA_{ox} were also examined by negative stain electron microscopy. Class averages of these ClyA_{ox} pores revealed a cylindrical structure with 13-fold symmetry and the same dimensions as ClyA_{red} (Supplementary Figure 5).

STEM mass measurements of ClyA indicate a 13-mer

Freeze-dried detergent solubilized ClyA complexes revealed three distinct populations: single complexes, as well as double and triple forms, where two or three single complexes were associated (Figure 4A, galleries). According to sequence the mass of the ClyA_{red} monomer is 34.45 kDa. In comparison, the mass of the complex determined by STEM analysis was 475 ± 86 kDa ($n = 184$; standard error (SE) = 6 kDa; overall uncertainty = 25 kDa considering the 5% uncertainty of the STEM calibration) for the single complex, 947 ± 140 kDa ($n = 114$; SE = 13 kDa; overall uncertainty = 49 kDa) for the double form and 1357 ± 108 kDa ($n = 30$; SE = 20 kDa; overall uncertainty = 71 kDa) for the triple form (Figure 4A).

Figure 3 Detergent-induced oligomerization of ClyA_{red}. **(A)** ClyA_{red} (5 μ M) was incubated in 0.1% DDM at 15°C. Samples were taken at the times indicated and subjected to analytical gel filtration in 0.1% DDM at 4°C. For comparison, the elution profile of soluble, monomeric ClyA is shown (left panel). On addition of DDM, monomeric ClyA_{red} immediately associates with detergent, leading to a shift in the elution volume from 15.1 to 12.3 ml. The detergent bound monomer then slowly converts to oligomeric ClyA_{red}, eluting at 9.6 ml. Arrows indicate retention volumes of soluble mass standard proteins. v_0 , void volume. Peak areas of monomeric ClyA_{red} (right, upper panel) and oligomeric ClyA_{red} (right, lower panel) were plotted against time and fitted with a monoexponential function (black line). **(B)** Crosslinking analysis of the oligomerization of ClyA_{red}. Samples identical to those in (A) were mixed with DSP and incubated for 5 min at 25°C. The reaction was stopped by addition of Tris/HCl, and samples were applied onto a 16% polyacrylamide-SDS gel (left panel). The bands of monomeric ClyA_{red} were quantified by densitometry, the fraction of monomers was plotted against incubation time (right panel), and fitted with a monoexponential function (black line). **(C)** Electron-micrograph of negatively stained ClyA_{red} particles taken from the peak eluted at 9.6 ml in (A). **(D)** Top view class average of 145 negatively stained ClyA_{red} particles. **(E)** Side view class average of 205 negatively stained ClyA_{red} particles. The scale bars correspond to 40 nm in (C), and 5 nm in (D) and (E).

Considering the overall uncertainty of the measurement and the presence of associated DDM, these data indicate that the oligomeric ClyA complex comprises 13 ClyA monomers (mass expected without detergent, 448 kDa), consistent with the symmetry of negative stain top view averages (Figure 3D, Supplementary Figure 4).

Images recorded by STEM from the same ClyA sample after negative staining showed that the single complexes associate at their pointed ends (Figure 4B), implying that the cap is the main hydrophobic region. The side views also frequently exhibited six or seven longitudinal striations

compatible with the presence of 13 ClyA monomers in the complex (Figure 4B).

3D Model of oligomeric ClyA

Cryo-EM was used to establish the 3D structure of the ClyA_{red} complex, since quick-freezing of particle suspensions ensures the preservation of the undistorted, native structure (Dubochet *et al*, 1988). In all, 2699 images of top- and 3620 images of side views selected from cryo-electron micrographs taken in plane (Figure 5A) and 2132 particles recorded at 15° tilt angle were used to reconstruct the 3D structure. Class

Figure 4 STEM analysis of the ClyA complex. (A) As shown by the histogram, the masses measured for the unstained ClyA preparation fell into three distinct classes (1, 2, and 3) that on visual inspection of the particles (galleries) could be assigned to single complexes with a mass of 475 ± 86 kDa ($n = 184$; overall uncertainty = 25 kDa), two associated complexes, mass 974 ± 140 kDa ($n = 114$; overall uncertainty = 49 kDa) and three associated complexes, mass 1357 ± 108 kDa ($n = 30$; overall uncertainty = 71 kDa). (B) Dark-field STEM images of the measured ClyA preparation after negative staining. Left, side views of the single complex. Centre, two complexes associated at their tips. Right, three complexes associated at their tips. The high contrast of the STEM allows the individual monomers making up the complex to be distinguished. Either six or seven subunits can be counted on the side views, consistent with 13-fold symmetry. The scale bar corresponds to 20 nm in (A) and (B).

averages of the particles were calculated as described above (Figure 5A, insets). A starting 3D model was calculated from selected top and side views using the EMAN software (Ludtke *et al*, 1999). A 13-fold cyclic symmetry was imposed, since both negative stain EM and STEM indicated that the complex has 13 ClyA subunits. Refinement of the alignment by projection matching in SPIDER (Frank *et al*, 1996) and omitting projections with low correlation coefficients compared to respective back-projections resulted in a final 3D model containing 8429 particles. The 0.5 Fourier shell correlation criterion indicated a resolution of 12 Å (Supplementary Figure 6). Figure 5B displays the surface rendered 3D map of the ClyA complex with characteristic bottom and top features, while the central cross-section through the 3D map is contoured in Figure 5C. The model shows that the ClyA complex is a cylindrical channel that converges into a cap at one end. It

has a length of 160 Å, a maximum outer diameter of 120 Å and a widest inner diameter of about 70 Å. The outer diameter of the cylindrical cap is 40 Å, and its height is 20 Å.

The apparent hydrophobicity of the cap region (see above) suggests that this cap may be critical for membrane insertion. The observed cap may correspond to a 26-stranded β barrel (Figure 5D) to which each protomer contributes two strands. The observed diameter of the cap agrees well with the calculated diameter of a 26-stranded barrel with vertically oriented β strands. The two-stranded β tongue in the structure of monomeric ClyA (Figure 1) is an attractive candidate for assembling such a barrel for the following reasons: the β tongue is hydrophobic, approximately 20 Å long, located at one end of ClyA, and sensitive to mutations that abolish the hemolytic activity of ClyA (Wallace *et al*, 2000).

Cryo-EM of lipid-embedded ClyA

To determine the position of the ClyA pore complex with respect to the lipid bilayer proteoliposomes were prepared by adding monomeric ClyA to brain lipid vesicles. Figure 6A and B show cryo-EM images of membrane bound oligomers associated with a mixture of spherical liposomes and continuous bilayers. Projections of membrane incorporated ClyA range from top views over different side view angles to tangential views through the edges of the 3D liposomes. In total, 1101 side views and 431 top views were selected and analyzed as described above. Density maxima in the gallery of side view averages (Figure 6D–G) allow the detergent-induced pore complex (Figure 6H) to be aligned with respect to the bilayer, indicating that ClyA inserts via its cap region into the membrane. The CTF corrected top view average of reconstituted complexes exhibits a central mass density (Figure 6C). Side view averages have a length of 140–150 Å, depending on the projection angle, and a diameter of 120 Å. The cap seen in the detergent-induced pore complex is not visible in the averages of lipid embedded ClyA (Figure 6D–G), resulting in a 10–20 Å shorter length than the ClyA complex in detergent (Figure 6H). Although the cap is not visible in side view averages of lipid embedded ClyA complexes, this feature is occasionally seen in single complexes (white arrowheads in Figure 6A), together with cap-free conformations (black arrowheads in Figure 6A). Proteoliposomes generated by Biobeads-driven reconstitution of detergent-induced pores (data not shown) exhibited the same morphology as the proteoliposomes in Figure 6, suggesting that the conformation of the detergent-induced pore complex may be a precursor of the lipid bound form.

Spectroscopic analysis of structural changes during ClyA assembly

As shown above, rapid insertion of monomeric ClyA_{red} into detergent micelles precedes the comparably slow assembly of ClyA pore complexes (cf. Figure 3). We used fluorescence and far-UV circular dichroism (CD) spectroscopy to explore the kinetics of the structural changes involved. The tryptophan fluorescence emission of ClyA_{red} increases 1.5-fold upon assembly in DDM (Figure 7A). The signal difference at 330 nm was used to follow the time course of detergent-induced assembly of ClyA_{red} under conditions identical to those in Figure 3A. The kinetic trace is characterized by a very fast and a slow phase with positive and negative amplitudes, respectively (Figure 7B). The fast phase reflects

Figure 5 Cryo-EM of vitrified ClyA complexes and model of the ClyA oligomer. (A) Overview image of frozen hydrated particles. The scale bar corresponds to 50 nm. Top and side view class averages, containing 302 and 796 particles each, are shown in the insets. The top view reveals some structure in the channel center while the cap at one end of the pore complex is seen in the side view. The scale bar in the insets corresponds to 2.5 nm. (B) Isocontour representations of the reconstructed single particle density, showing the prominent cap region as well as the open end of the pore. (C) Side by side comparison of the 3D EM reconstruction of the pore with the X-ray structure of monomeric ClyA. The 3D EM isosurface representation is cut open to expose the cavity as well as the inner contour lines of the protein density. (D) Model of a 26-stranded β barrel, the suggested cap structure. The barrel is formed by the β tongues of each of the 13 monomers.

Figure 6 Cryo-EM images of ClyA in lipid vesicles. (A, B) High-resolution micrographs of ClyA incorporated in lipid vesicles in the absence of detergent. Upon contact with brain lipid vesicles, monomeric ClyA forms pore assemblies appearing as ring-like structures with a central density in top view and as spikes protruding from the lipid bilayer in side view. The scale bar corresponds to 50 nm. (C) CTF corrected top view class average of lipid induced ClyA pore assemblies. The scale bar is valid for all averages and corresponds to 5 nm. (D–G) Gallery of side view averages of lipid induced ClyA pore complexes. Each average contains between 25 and 140 projections. The different orientations of the particles with respect to the spherical vesicles result in variations of the particle's length in projection (140–150 Å). Each image shows three distinct spots of higher density (marked with bars in (G)). (H) Side view average of the detergent induced ClyA complex aligned with respect to the membrane bound pore in (G).

Figure 7 Conformational changes during assembly of ClyA_{red} followed by fluorescence and CD spectroscopy. **(A)** Fluorescence emission spectra of ClyA_{red}. Spectra of the soluble monomer (in PBS), the monomeric intermediate I₁ (in PBS, 0.1% DDM), and oligomeric ClyA_{red} (in PBS, 0.1% DDM) are shown. **(B)** Kinetics of assembly of ClyA_{red} in DDM monitored by fluorescence. Oligomerization of ClyA_{red} (5 μM) induced by 0.1% DDM was followed at 330 nm (15°C). The line shows the fit of a monoexponential function omitting the first 100 s. The arrow indicates the signal of monomeric ClyA_{red} in PBS at 330 nm. **(C)** Far-UV CD-spectra of monomeric and oligomeric ClyA_{red}. Spectra of the soluble monomer (in PBS) and oligomeric ClyA_{red} (in PBS, 0.1% DDM) are shown. **(D)** Kinetics of assembly of ClyA_{red} in detergent and in erythrocyte membranes monitored by far-UV CD. Oligomerization of ClyA_{red} induced by 0.1% DDM (upper panel) or erythrocyte membranes (lower panel) was followed at 225 nm (15°C). The lines show the fit of monoexponential functions omitting the first 100 s. The arrows indicate the signal of monomeric ClyA_{red} in PBS at 225 nm. **(E)** Proposed mechanism of assembly of ClyA. Monomeric ClyA quickly associates with detergent (upper row) or the membrane (lower row) and undergoes a structural change leading to the monomeric intermediate I₁. A second and much slower conversion yields the monomeric and assembly-competent intermediate I₂, which rapidly assembles into the oligomeric pore complex. The detergent-induced pore complex (with cap) converts to the membrane-embedded pore complex (without cap) upon detergent removal in the presence of lipids.

the formation of a hyperfluorescent intermediate (intermediate I₁ in Figure 7A) upon association of monomeric ClyA_{red} with detergent. The slower kinetic phase is described by a monoexponential function with a half-life of 940 s. This half-life agrees with that measured for the disappearance of the monomer and the appearance of the oligomer (Figure 3A and B), suggesting that the observed slow phase reflects a conformational change in ClyA_{red} that coincides with association of the individual subunits.

Figure 7C shows far-UV CD spectra of monomeric and oligomeric ClyA_{red}. Both spectra display minima at 208 and 222 nm, typical for proteins with a high content of α helical secondary structure. Oligomeric ClyA_{red} exhibits increased

signal intensity, indicating a conformational change during assembly. As the overall shape of the spectrum is retained, we conclude that no large α to β transition occurs during oligomerization of ClyA. Similar to the above fluorescence kinetics, we exploited the signal difference at 225 nm to follow the time course of detergent-induced ClyA_{red} assembly (Figure 7D, upper panel). The trace is also characterized by a very fast phase with positive amplitude and a slow phase with negative amplitude. As in the fluorescence kinetics, the slower phase is described by a monoexponential function, yielding a half-life of 1000 s. This process thus coincides again with the disappearance of the monomer and the appearance of the oligomer (Figure 3A and B). The fact that a mono-

exponential function accurately describes oligomerization suggests that the rate-limiting step for assembly is uni-molecular under the chosen conditions. To test this model, we repeated the assembly reaction at different initial protein concentrations (Supplementary Figure 7). The half-life of the slow phase was indeed independent of the concentration of ClyA_{red}. Consequently, a slow conformational change converts the monomeric intermediate I₁ to an assembly-competent intermediate I₂, and this conformational change is rate-limiting for the assembly of the complex. It should, however, be noted that association of assembly-competent subunits is per definition concentration-dependent. Therefore, at very low protein concentrations, assembly itself will become rate-limiting.

To test whether similar structural changes take place upon insertion of ClyA_{red} into erythrocyte membranes, we repeated the CD measurements after addition of erythrocyte membranes to monomeric ClyA_{red} (Figure 7D, lower panel; Supplementary Figure 8). The kinetic trace was very similar to that observed after addition of DDM. Again, a fast phase and a slow phase were detected with positive and negative amplitudes, respectively. Fitting the slower phase yields half-lives of 80 s at 15°C and of 45 s at 37°C. Assembly of ClyA_{red} in erythrocyte membranes is thus about 10-fold faster compared to assembly in DDM. Nevertheless, the assembly kinetics in erythrocyte membranes is slower than the kinetics of hemolysis (Figure 2C). This can be well explained by the fact that a small fraction of functional pores suffices to attain full cell lysis at the chosen hemolysin to cell ratio (cf Figure 2B).

Discussion

Role of the redox state of ClyA for activity and assembly

Atkins *et al* (2000) reported that ClyA_{ox} has no hemolytic activity whereas Wai *et al* (2003a) detected activity of ClyA_{ox}, albeit 16-fold weaker compared to ClyA_{red}. Controversial results were also obtained on the oligomeric state of ClyA in solution. Whereas ClyA_{ox} is consistently found to be monomeric, ClyA_{red} has been described to be monomeric (Wallace *et al*, 2000), partially oligomeric (Atkins *et al*, 2000), or homogeneously oligomeric (Wai *et al*, 2003a). Our data confirm the observation of Wallace *et al* (2000) that both ClyA_{red} and ClyA_{ox} are monomeric in solution. Furthermore, we find that ClyA_{red} and ClyA_{ox} oligomerize quickly upon contact with lipid or detergent and that both proteins display equal activity. From these results and the activity measurements of ClyA_{ox} by Wai *et al* (2003a, b), we conclude that a movement of helix G away from the main body of the molecule is not required for assembly of ClyA into functional pore complexes as suggested by Atkins *et al* (2000). The fact that we have obtained results that differ strikingly from those of other groups can be explained by the previously unknown high tendency of ClyA_{ox} to form inactive aggregates (Supplementary Figure 2). If this phenomenon is not taken into account, the concentration of monomeric ClyA_{ox} cannot be reliably determined, which in turn leads to large errors in activity assays.

We did not observe a role of the disulfide bond in the intrinsic ability of ClyA to assemble in target membranes *in vitro*. However, it seems likely that the disulfide bond plays a crucial role during export of ClyA from the bacterial cell,

because Wai *et al* (2003a) observed that *E. coli dsbA*[−] and *dsbB*[−] strains display a ClyA-dependent hemolytic phenotype on blood agar plates. DsbA and DsbB are required for efficient disulfide bond formation in the periplasm and could influence the vesicle-mediated export of ClyA by directly oxidizing ClyA and/or introducing disulfide bonds into components that participate in the formation of OMVs. In this context it should also be considered that integrity of the outer membrane may depend on proteins containing disulfide bonds (Braun and Silhavy, 2002; Wu *et al*, 2005). The absence of DsbA or DsbB could thus lead to destabilization of the outer membrane, which in turn could facilitate the release of OMVs.

Structure and assembly of the ClyA complex

The increase in total length upon transformation from soluble monomer (110 Å) to detergent induced ClyA complex (160 Å) and the significant changes observed in tryptophan fluorescence indicate that large structural transitions take place in the helix bundle of ClyA, as seen for many other PFTs (Song *et al*, 1996; Zakharov and Cramer, 2002; Czajkowsky *et al*, 2004; Tilley *et al*, 2005). As both tryptophans, W37 and W86, are located within the bundle and shielded from solvent in the structure of the monomer (<6 and <1% solvent accessible, respectively, Figure 5C), the change in tryptophan fluorescence reflects a transition in tertiary structure, rather than surface contacts with detergent or neighbouring protomers. Far-UV CD spectra show that the high content in helical secondary structure is retained during assembly, suggesting that the final membrane channel is built mainly from α helices.

While the overall structure of the ClyA pore complex is the same in detergent and in the lipid bilayer, the cap region appears to be sensitive to changes in the environment of the pore complex. The cap may emerge as the hydrophilic edges of the β tongues of different subunits face each other to form one large 26-stranded β barrel during membrane association and insertion (Figure 5D). Concomitant with the conformational change of the β tongue, adjacent amphipathic helices might rotate to expose their hydrophobic surface and to facilitate complete insertion of the pore complex in the bilayer. This conformational change might in turn induce register shifts in the four-helix bundle, leading to markedly elongated protomers. A similar mechanism is known for other α helical toxins such as colicins and diphtheria toxin, where amphipathic helices are packed around a central hydrophobic helical hairpin that is thought to mediate the insertion into the membrane (Choe *et al*, 1992; Parker and Pattus, 1993). The distinct cap region is barely visible in membrane-embedded ClyA pores, suggesting further conformational changes upon formation of the final pore. However, individual top views often show a central mass density and occasionally side views exhibit a distinct cap. Therefore, the possibility should be considered that the cap functions as a flexible domain of the molecule that can be inverted and exposed again to mediate docking of OMVs to the target cell membrane.

Based on our data we propose a simple model for ClyA assembly (Figure 7E). Monomeric ClyA associates in a very fast step with the lipid bilayer or the detergent molecules, and undergoes a first conformational change to intermediate I₁ (fast phase in Figure 7B and D). The rate-limiting step for

assembly is the second, uni-molecular conformational transition, which generates assembly-competent monomers (intermediate I₂) that rapidly associate to the oligomeric complex. It remains elusive at which stage the marked elongation of the protomer from 110 to 160 Å occurs and what the molecular basis for the increase in length is. The detailed elucidation of the large conformational changes during assembly will require a high-resolution structure of the pore complex.

Materials and methods

Expression and purification of ClyA

The genetic sequence of ClyA was amplified by PCR from genomic DNA of *E. coli* W3110, and introduced into pET11a (Novagen). This plasmid was used to create the (His)₆-tagged ClyA variant (QuikChange, Stratagene), yielding plasmid pClyA. This plasmid promotes cytoplasmic expression of N-terminally (His)₆-tagged ClyA.

ClyA was expressed in the cytoplasm of *E. coli* Tuner (DE3) (Novagen) harboring plasmid pClyA, and purified by chromatography on Ni-NTA agarose and hydroxyapatite. The protein was fully reduced as shown by modification with Ellman's reagent (5,5'-dithio-bis(2'-nitrobenzoic acid)). Edman Sequencing and MALDI-TOF mass spectrometry confirmed the integrity of the protein. For preparation of ClyA_{ox}, 22 μM ClyA_{red} were incubated in 50 mM Tris/HCl, 1 mM CuCl₂, pH 8.0 for 3 h at 25°C. Aggregated ClyA_{ox} was removed by size exclusion chromatography in phosphate-buffered saline (PBS) (Dulbecco's PBS, pH 7.3) at 4°C. Protein concentrations were determined by the extinction coefficients at 280 nm (ClyA_{red} in PBS: 30 300 M⁻¹ cm⁻¹, ClyA_{ox} in PBS: 30 400 M⁻¹ cm⁻¹).

Analytical gel filtration

Gel filtration was performed at 4°C in PBS or PBS, 0.1% DDM using a Superdex 200 10/300 gel filtration column (GE Healthcare). To initiate assembly, ClyA (5 μM) was mixed with DDM and incubated in PBS, 0.1% DDM at 15°C. At different times, samples were withdrawn and subjected to chromatography.

Transmission electron microscopy

Oligomerization of monomeric ClyA (5 μM) was induced by the addition of 0.1% DDM. The sample was incubated at 15°C for 2 h. ClyA particles were examined by adsorbing the sample on glow discharged 400 mesh carbon-coated Parlodion grids and negative-staining with 2% (w/v) uranyl acetate. Images were recorded at × 50 000 on Kodak SO163 film using a Hitachi H-8000 microscope operating at an acceleration voltage of 200 kV. For cryo-EM the same sample was applied to 400 mesh copper grids with a thin continuous carbon film and quick frozen in liquid ethane. Grids were transferred into a Philips CM200 FEG electron microscope operating at 200 kV. Cryo-electron micrographs were recorded on Kodak SO163 film in plane and at a tilt angle of 15°C.

Image processing

Negatives were digitized on a Heidelberg PrimescanD 7100 drum scanner at a resolution of 4 Å/pixel for negatively stained samples and 2 Å/pixel for vitrified samples at the specimen level. The EMAN boxer program (Ludtke *et al*, 1999) was used to select 2699 images of top and 3620 images of side views as well as a total of 8451 particles from electron micrographs including all orientations present on tilted and untilted images. Projections of negatively

stained preparations were subjected to reference-free alignment (Penczek *et al*, 1992) and classification by multivariate statistical analysis (Frank *et al*, 1982) in SPIDER (Frank *et al*, 1996). Rotational power spectra of top view class averages were calculated with the Xmipp software (Sorzano *et al*, 2004) to determine the particle symmetry. EMAN was used to generate a starting volume from top and side views of the frozen-hydrated sample and for an initial refinement enforcing C13 symmetry. For further refinement by projection matching the SPIDER package was employed. The CTF was estimated using the ctffind and ctftilt programs (Mindell and Grigorieff, 2003) and corrected for phases and amplitudes applying a Wiener filter in SPIDER. The resolution of the refined volume containing 8429 particles was estimated by Fourier-shell correlation. All visualization was performed with DINO (<http://www.dino3d.org>), a structural biology visualization program developed by one of the authors (AP).

Formation of proteoliposomes

Brain lipids (Avanti Polar Lipids) were solubilized at a concentration of 5 mg/ml in PBS (Sigma) containing 1% *n*-octyl-β-D-glucopyranoside (OG). The mixture was dialyzed against PBS to remove all detergent from the lipid vesicles. Monomeric ClyA was added to the vesicles to give a final lipid-to-protein weight ratio of 5 and incubated at 4°C for 2 h. Samples were then adsorbed to EM grids and analyzed with negative stain and cryo-EM. Alternatively, the detergent-induced pore complexes were reconstituted by mixing them with solubilized lipids and removing the detergent with Biobeads.

CD- and fluorescence spectroscopy

The ClyA concentration (monomer) was 5 μM in all experiments. Emission spectra were recorded on a Hitachi F-4500 fluorescence spectrometer at 22°C, using an excitation wavelength of 280 nm. Spectra of oligomerized ClyA_{red} were recorded after incubation in PBS, 0.1% DDM for 2 h at 15°C. A spectrum of the monomeric intermediate I₁ was recorded after 100 s of exposure to DDM at 15°C. For fluorescence kinetics, the signal change at 330 nm upon addition of 0.1% DDM to ClyA_{red} was followed at 15°C. Far-UV CD spectra were recorded at 22°C using a Jasco J-810 spectropolarimeter. For Far-UV CD kinetics, the signal change at 225 nm upon addition of DDM (0.1%) or erythrocyte membranes to ClyA_{red} was followed at 15°C. Erythrocyte membranes were prepared as described by van Leengoed and Dickerson (1992). Membranes from approx 9 × 10⁷ erythrocytes were used for each experiment (400 μl total volume). Spectra and kinetic traces were corrected for buffer contributions and converted to mean residue ellipticity.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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