The assembly mechanism of the pore-forming toxin ClyA from Escherichia coli

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The assembly mechanism of the pore-forming toxin ClyA from Escherichia coli

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Abstract

Pore-forming toxins (PFTs) are a widespread class of bacterial and eukaryotic virulence factors that exhibit toxicity by penetration of target cell membranes. All PFTs share the conversion from a soluble, monomeric form to an oligomeric, membrane-embedded complex upon activation. According to the secondary structure elements that form the membrane-spanning region, they are classified as α-PFTs and β-PFTs. The α-PFT Cytolysin A (ClyA) is present in several strains of *Escherichia coli* and *Salmonella enterica* and causes their cytolytic phenotype. ClyA is the first and up to now only α-PFT with known high-resolution crystal structures of both the monomer and the assembled pore complex. The soluble monomer and a subunit (protomer) of the ring-shaped, oligomeric pore complex exhibit major structural differences. The head domain of the monomer (residues 160 – 205) made up of a short β-hairpin (the β-tongue) and two flanking short α-helices is completely restructured upon membrane insertion forming an elongation of the neighboring long α-helices C and F of the tail domain. The N-terminal α-helix A that is part of the five-helix bundle of the tail domain in the monomer swings around by 180°, elongating the flanking α-helix B and leaving a four-helix bundle in the protomer. These conversions result in an elongation of the molecule from 10 nm to 13.5 nm, the formation of the intersubunit interface of the protomer along the tail domain and membrane protrusion of the N-terminal part of α-helix A.

A detailed mechanism of the monomer-to-pore transition consistent with kinetic data of ClyA assembly induced by detergent (unimolecular rate-limiting step of the conformational transition, Eifler et al., 2006) has been proposed (Mueller et al., 2009). The aim of this thesis was to trap and characterize transient intermediates of the conformational transition by site-directed mutagenesis to obtain further insights into the monomer-to-pore transition mechanism of ClyA. Three different strategies were applied to generate trapped assembly intermediates: (i) rigidification of loops flanking the β-tongue by substitution of glycines at positions 180, 184, 201, and 205 (proposed hinge points for swinging-out movement upon membrane insertion) by alanines, valines, or prolines, (ii) covalent attachment of the β-tongue or α-helix A to the tail domain by engineered disulfide bonds, and (iii) inhibition of the protomer-to-pore assembly by introduction of repulsive charges at the intersubunit interface.

While the detergent-induced assembly of ClyA pores was neither inhibited nor retarded when hinge glycines were replaced by alanine or valine, variants with glycine-to-proline substitutions only showed an about 10-fold slower formation of assembly competent
protomers. Consequently, none of the four proposed hinge glycines proved to be essential for monomer-to-protomer transition and pore complex formation, and attempts to trap monomeric transition intermediates via this strategy were not further pursued.

The introduction of artificial disulfide bonds to attach the β-tongue or α-helix A to the tail domain resulted in two redox-controllable ClyA variants (CC50/190 and CC6/264, respectively). The reduced forms of these variants underwent a complete monomer-to-pore transition with kinetics comparable to wild type (wt) ClyA (rate constants of the rate-limiting step of $5.5 \times 10^{-4} \text{s}^{-1}$ and $9.4 \times 10^{-4} \text{s}^{-1}$, respectively, compared to $1.5 \times 10^{-3} \text{s}^{-1}$ for wt ClyA) and formed active pores that were indistinguishable from wt pores in detergent, as judged by negative stain electron microscopy. In contrast, the oxidized (disulfide-bonded) forms of the variants CC50/190 and CC6/264 were trapped in a molten-globule-like intermediate upon addition of detergent, as shown by equilibrium and kinetic experiments recorded with CD and fluorescence spectroscopy. Formation of protomers and functional pore complexes could be completely recovered for both variants upon reduction of either the monomers or the molten-globule-like intermediates by dithiothreitol. The ClyA variants CC50/190 and CC6/264 constitute the first example of an α-pore-forming toxin with a redox switch triggering formation of assembly competent protomers by disulfide bond reduction.

The introduction of repulsive electrostatic charges at the intersubunit interface by simultaneous substitution of K29, G63, and G146 by Glutamate (ClyA variant “protoarrest”) resulted in assembly-incompetent protomers that formed about 2-fold faster than wild type protomers. CD, fluorescence, and limited proteolysis experiments indicate that the protoarrest intermediate exhibits secondary and tertiary structure very similar to that of the wt protomer. The further structural characterization of the protoarrest intermediate should provide insights into the protomer-to-pore transition as final step of ClyA pore assembly.
Zusammenfassung


Die zweite Strategie, nämlich das Einfügen künstlicher Disulfidbrücken, um die β-Zunge oder α-helix A kovalent an die Schwanzdomäne zu binden, resultierte in zwei redox-aktivierbaren ClyA-Varianten (CC50/190 beziehungsweise CC6/264). Die reduzierten Formen vollzogen den vollständigen Konformationsübergang vom Monomer zur Pore mit Ratenkonstanten vergleichbar mit ClyA wt (5.5·10⁻⁴ s⁻¹ bzw. 9.4·10⁻⁴ s⁻¹ gegenüber 1.5·10⁻³ s⁻¹ für wt ClyA). Weiterhin bildeten sie aktive Poren, die im mittels Elektronenmikroskopie nicht von Wildtyp-Poren zu unterscheiden waren. Dagegen wurde der Konformationsübergang nach Detergenszugabe der oxidierten (disulfidgebundenen) Formen von CC50/190 und CC6/264 in einem assemblierungsinkompetenten molten globule – Zustand festgehalten, was mittels Gleichgewichtsmessungen und Kinetiken (Fluoreszenz- und CD-Spektroskopie) gezeigt wurde. In beiden Fällen ermöglichte die Reduktion der künstlichen Disulfidbrücke durch Dithiothreitol entweder im monomerischen oder im intermediären Zustand die Bildung des Protomers und schliesslich der funktionellen Pore. Die ClyA-Varianten CC50/190 und CC6/264 sind die ersten beschriebenen Beispiele für ein α-PFT mit einem Redox-Schalter, um die Protomerbildung auszulösen.

1 Introduction

1.1 Pore-forming toxins (PFTs) – Different origins and a common mechanism

1.1.1 Distribution and relevance of PFTs

Pore-forming toxins (PFTs) are produced by a variety of bacterial and eukaryotic species. They can function as defensive mechanisms or also as a possibility to exploit nutrition sources. PFTs are directly involved in pathogenicity of many bacteria and eukaryotes. Amongst them are some of the most deadly human pathogenic toxins, e.g. Anthrax toxin from *Bacillus anthracis* (Blaustein et al., 1989) or Diphtheria toxin from *Corynebacterium diphteriae* (Choe et al., 1992). Furthermore, many food poisoning diseases are caused by PFTs, e.g. Shiga toxin from enterohemorrhagic *Escherichia coli* (EHEC) (Sack, 1987) or Cytolysin A (ClyA) that is present in pathogenic strains of *Salmonella enterica* (Huang et al., 2010; von Rhein et al., 2009; von Rhein et al., 2006) and *E. coli* (Ludwig et al., 2004).

Besides prokaryotes, different animals also use pore-forming toxins as defensive mechanisms. Well-studied examples are Equinatoxin and Stichiolysin from the sea anemones *Actinia equina* and *Stichodactyla helianthus* (Alegre-Cebollada et al., 2007; Alvarez et al., 2009; Kristan et al., 2004; Rojko et al., 2013). Next to full-length proteins, small peptides can act as PFT. A well-known example therefore is the bee venom melittin, a 26 residue peptide that forms pores in cell membranes without the need for a specific receptor (Ladokhin et al., 1997; Matsuzaki et al., 1997). Animals do not only apply PFTs as defensive mechanism against predators from outside, but also against incorporated parasites. Recently a PFT was discovered in the snail *Biomphalaria glabrata* (Galiner et al., 2013), which is highly toxic against sporocysts of *Schistosoma mansoni*, the pathogen of Bilharziosis. Therefore, this specific PFT could be applied as a treatment against this widespread disease.

1.1.2 A common activation mechanism of PFTs

Pore-forming toxins kill target cells by inserting pores into the cytoplasmic membrane. A common feature of all PFTs is the conversion from a soluble, monomeric form to a membrane-embedded, oligomeric pore complex (Parker and Feil, 2005). Figure 1 shows the distinct steps all PFTs need to undergo during activation. First, the soluble monomeric form of the toxin is exported from the producing cell. Some toxins use standard secretory pathways and therefore possess N-terminal signal sequences that are cleaved before
activation (e.g. *E. coli* α-hemolysin uses the type I secretion system; Gentschev et al. (2002)). Other toxins do not possess a signal sequence and their export is based on intrinsic structural features (e.g. the ClyA type; del Castillo et al. (2001), Ludwig et al. (2010)). After secretion, toxin monomers associate to the cytoplasmic membrane of target cells, either mediated by specific interactions with a cell surface receptor or by unspecific interactions with membrane lipids. The membrane-associated toxin monomers undergo conformational transitions resulting in oligomerization to a prepore complex. Final conformational transitions lead to the integration of the pore complex into the target cell membrane. In some cases the pore itself is the toxic species (e.g. ClyA); in other cases an additional cytotoxic factor is injected into the target cell (e.g. Anthrax toxin). However, in both cases the final result is lysis of the target cell.

**Figure 1:** Common mode of action of pore-forming toxins (PFTs). PFTs are secreted by the (bacterial) cell to the environment, followed by association to the target cell membrane. A conformational transition of the PFTs leads to oligomerization into a membrane-associated prepore complex. Finally, the prepore complex is integrated completely into the target cell membrane leading to membrane protrusion and subsequent target cell lysis.

A prerequisite for oligomerization of the toxin monomers is the formation of intersubunit interfaces. However, oligomerization of the monomers in the cytoplasmic membrane of the toxin-producing cell must be prevented. Therefore, unprotected intersubunit interfaces
cannot be present in the soluble monomers. It has been shown for different types of PFTs that membrane-association of the toxin monomers triggers conformational transitions that are necessary for the formation of the final pore complex (De and Olson, 2011; Eifler et al., 2006; Hotze et al., 2001). An example for an excessive conformational transition is *E. coli* ClyA; its conformational transition involves more than half of all residues of the protein (Mueller et al., 2009).

### 1.1.3 Applications of PFTs

Next to the importance to understand mechanisms of pathogenicity of devastating plagues, the research on pore-forming toxins provides considerable medical and biotechnological applications. Several studies show the ability of different types of PFTs to function as drug delivery systems. Cholesterol-dependent cytolysins (CDC) form huge pores (inner diameter of 26 nm) made up of at least 40 subunits (Tilley et al., 2005) and therefore enable the transport of large compounds into cells. An engineered CDC (intermediyysin from *Streptococcus intermedius*) was successfully used to deliver whole drug-carrier liposomes into living cells (Tabata et al., 2012). As viable cells have the ability to repair lesions formed by CDCs dependent on the presence of Ca\(^{2+}\), cell membrane permeation is reversible (Walev et al., 2001). Smaller pores can exhibit a more specific transmembrane transport capacity. ClyA pores (3.5 nm inner diameter, Mueller et al. (2009)) in combination with a specific receptor were shown to function as transport systems for particular proteins, while other proteins are rejected (Soskine et al., 2012). Furthermore, ClyA has been successfully applied to suppress the growth of solid tumors (Jiang et al., 2010; Ryan et al., 2009).

The antimicrobial effect of some PFTs enables their usage as possible new types of antibiotics (Parker and Feil, 2005). Some *E. coli* strains kill other *E. coli* strains and closely related bacteria, which are competitors for resources, with a variety of complex three-domain PFTs termed Colicins (Lakey and Slatin, 2001).

The possibly best known examples for biotechnological application of PFTs are the insecticidal *Bacillus thuringiensis* Cyt (cytolytic) and Cry (crystalline) toxins (Prieto-Samsonov et al., 1997). Transgenic crop plants that recombinantly express one or more forms of Cyt or Cry exhibit a resistance against affliction by insects resulting in a decreased need for pesticides (Kumar et al., 2008). The different forms of Cry are toxic for specific orders of insects: the Cry1 group is toxic for Lepidoptera, Cry2 for Lepidoptera and Diptera, Cry3 for Coleoptera, and Cry4 for Diptera (Hofte and Whiteley, 1989). Differences in the three-dimensional structure of the receptor domains of Cry implicate the selective toxicity against target insects (Grochulski et al., 1995). The high insect-specificity makes them also
usable as a pesticide, e.g. against Lepidopterae in coniferous forests (Bravo et al., 2007). In contrast, Cyt toxins kill insects with less specificity (Hofte and Whiteley, 1989). However, next to the considerable beneficial environmental effects, the problems of the release of transgenic plants into the environment also need to be considered (Gatehouse et al., 2011).

1.2 Classification of PFTs according to their protein structure

1.2.1 α-PFTs form a transmembrane channel by α-helices

Pore-forming toxins can be classified according to the secondary structure elements that form the pore channel (Gouaux, 1997; Parker and Feil, 2005): α-PFTs span the membrane with α-helices, β-PFTs with β-strands. Various prokaryotic and eukaryotic organisms produce different classes of α-PFTs. The high-resolution structures of some examples are shown in Figure 2. The previously mentioned Bacillus thuringiensis Cry toxins are α-PFTs that specifically kill different orders of insects (Hofte and Whiteley, 1989). The high-resolution crystal structure of Cry1A(a) (Figure 2A1) shows a three-domain protein. Only domain I (shown in blue in Figure 2A1) is made up of α-helices, while domain II (receptor-binding domain) and domain III consists of β-strands. All types of Cry toxins (cf. section 1.1.3) share this common fold (Boonserm et al., 2005). As domain I undergoes membrane interaction and forms the pore channel (Li et al., 1991), Cry toxins are α-PFTs although a major part of their secondary structure is not α-helical. Other examples of bacterial PFTs are Pseudomonas aeruginosa Exotoxin A (Figure 2A2, Wedekind et al. (2001)), which also contains a considerable amount of β-strands, and the Colicins from E. coli (represented by Colicin1a in Figure 2A3). The great overall length of 210 Å of Colicin 1a corresponds to its mode of action. The protein spans the periplasm of target cells and simultaneously effects the outer membrane and the cytoplasmic membrane (Wiener et al., 1997).

Up to now, E. coli ClyA is the only α-PFT with known high-resolution structures of both the soluble monomer (Wallace et al., 2000) and the active pore complex (3.6 nm inner diameter) that consists of 12 subunits (Figure 2B, Mueller et al. (2009)). The knowledge of both structures enables detailed studies on the conformational change of the protein upon pore formation. Monomer and a pore subunit (termed protomer) show a high degree of difference both on the levels of secondary and tertiary structure, implicating a vast conformational change involving more than 50% of all residues as a prerequisite for pore formation (Mueller et al., 2009). The conformational change and the monomeric and pore structures of ClyA are described in more detail in the following sections.
Figure 2: High-resolution structures of selected α-PFTs. A: Different α-PFTs with known high-resolution structures in their soluble monomeric forms. The domains that are responsible for membrane interaction are highlighted in blue. (1): 2.25 Å crystal structure of *Bacillus thuringiensis* insecticidal toxin Cry1A(a) (Grochulski et al., 1995). (2): 1.62 Å crystal structure of *Pseudomonas aeruginosa* Exotoxin A (Wedekind et al., 2001). (3): 3.00 Å crystal structure of *Escherichia coli* Colicin 1a (Wiener et al., 1997). B: Crystal structures of *Escherichia coli* Cytolysin A (ClyA) in the monomeric form (2.00 Å, left; Wallace et al. (2000)) and the dodecameric pore complex (3.29 Å, right; Mueller et al. (2009)). The channel-forming N-terminal helix (residues 1-36) and the β-tongue in the head domain (residues 184-195) are highlighted in blue. One protomer is highlighted in red. C: Crystal structures of *Actinia fragacea* Fragacatoxin C in the monomeric form (2.00 Å, left; pdb ID 3VWI) and the nonameric prepore complex (1.80 Å, right; Mechaly et al. (2011)). The channel-forming α-helix A (residues 16-26) is highlighted in blue. One protomer is highlighted in red. All figures were illustrated in PyMOL (Schrodinger, 2010).
Next to bacteria, sea anemones produce α-PFTs. The toxins from different species share the same fold with two central β-sheets flanked by two α-helices (Athanasiadis et al., 2001; Mancheno et al., 2003; Mechaly et al., 2011) with the N-terminal α-helix forming the pore channel (shown in blue in Figure 2 C). In the case of Fragaceatoxin C from Actinia fragacea, the high-resolution structures of the monomer (pdb ID 3VWI, unpublished data) and the yet inactive prepore complex (4.5 nm inner diameter) made up of nine subunits (Mechaly et al., 2011) have been described (Figure 2 C). In contrast to E. coli ClyA, the structures of the soluble monomer and a prepore subunit are nearly identical (root mean square deviation of Cα of Equinatoxin II monomer and Fragaceatoxin C protomer of 0.5 Å; Mechaly et al. (2011)). However, the formation of the final, active pore species needs a conformational change of the N-terminal α-helix with a swinging-out movement away from the compact β-sheets to insert into the target cell membrane (Rojko et al., 2013).

1.2.2 β-PFTs form a transmembrane channel by a β-barrel

Pore-forming toxins which form the membrane-spanning channel with β-strands are termed β-PFTs. Just as described for α-PFTs, different folds enable the same function, the insertion into target cell membranes. Next to the Cry toxins (cf. Figure 2 A1), Bacillus thuringiensis secretes a further type of insecticidal proteins, the Cyt toxins belonging to the group of β-PFTs. They have a simpler three-dimensional structure than the three-domain Cry toxins: a central β-sheet responsible for membrane insertion is flanked by two layers of α-helices (Figure 3A1). The suggested pore formation mechanism implies a swinging-away movement of the α-helical layers from the β-sheet, exposing a putative lipid interaction site localized on the β-sheet (Cohen et al., 2011). Further, well-studies examples of β-PFTs are cholesterol-dependent cytolysins, which form large pores with diameters above 25 nm (Tilley et al., 2005). The three-dimensional structure of the 500 residue CDC Perfringolysin O from Clostridium perfringens (Rossjohn et al., 1997) consists of four domains that are mainly made up of β-sheets (Figure 3A2). However, the proposed transmembrane parts in domain 3 are α-helical (colored blue in Figure 3A2), meaning an α-to-β-conversion has to precede membrane integration (Shatursky et al., 1999). Another multidomain β-PFT is Aeromonas hydrophila Aerolysin, whose elongated monomeric form Proaerolysin consists of four domains (Figure 3A3; Parker et al. (1994)). Domain 4 contains a cleavable C-terminal elongation that functions as intramolecular chaperone to prevent the monomer from oligomerization (Iacovache et al., 2011). Domains I and II are receptor binding domains, domain 3 is responsible for membrane integration (Degiacomi et al., 2013). The proaerolysin fold can also be found in eukaryotes, as shown for Biomphalaria glabrata Biomphalysin (Galinier et al., 2013).
Figure 3: High-resolution structures of selected β-PFTs. A: Different β-PFTs with known high-resolution structures in their soluble monomeric forms. The domains that are responsible for membrane interaction are highlighted in blue. (1): 2.19 Å crystal structure of Bacillus thuringiensis toxin Cyt1A(a) (Cohen et al., 2011). (2): 2.20 Å crystal structure of Clostridium perfringens Perfringolysin O, a Cholesterol-dependent cytotoxin (Rossjohn et al., 1997; Shatursky et al., 1999). (3): 2.8 Å crystal structure of Aeromonas hydrophila Proaerolysin (Parker et al., 1994). B: Crystal structures of Staphylococcus aureus α-Hemolysin in the monomeric form (3.36 Å, left; Foletti et al. (2013)) and the heptameric pore complex (1.90 Å, right; (Song et al., 1996)). The channel-forming β-hairpin (residues 111-147) is highlighted in blue. One protomer is highlighted in red. C: Crystal structures of Anthrax toxin protective antigen in the monomeric form (2.10 Å, left; (Petosa et al., 1997)) and the heptameric prepore complex (3.60 Å, right; (Lacy et al., 2004)). The predicted membrane-interacting region (residues 303-322) is highlighted in blue (loop not resolved in the monomer). One protomer is highlighted in red. All figures were illustrated in PyMOL (Schrodinger, 2010).
α-Hemolysin (HlyA) from *Staphylococcus aureus* was the first PFT ever of which the high-resolution structure of the active, oligomerized species was solved by X-ray crystallography (Song et al., 1996). The toxin is present in different bacteria, and recently more structures of assembled pores have been published (De and Olson, 2011; Savva et al., 2013). The heptameric, mushroom-shaped pore protrudes the target cell membrane with a β-barrel that consists of 14 strands (two per subunit) and forms an inner diameter of 2.7 nm (Figure 3 B). Recently the structure of the soluble monomer of the same toxin was solved in complex with an antibody (Foletti et al., 2013). The overall fold of the soluble monomer and a pore subunit do not differ in the same extent as in the case of ClyA (cf. Figure 2 B). The only major difference is the insertion hairpin, which is attached to the central β-sheet in the soluble monomer and membrane-integrated in the pore subunit (shown in blue in Figure 2 B).

In contrast to the other β-PFT described here Anthrax toxin is not a sole pore-forming toxin. It consists of a pore-forming species, the Anthrax protective antigen (PA), and a cytotoxic protease that is implanted into target cells mediated by interaction with PA (Pannifer et al., 2001; Petosa et al., 1997). High-resolution structures of the monomer of PA and a heptameric prepore complex (3.1 nm inner diameter) are known (Figure 3 C). The 63 kDa PA domain contains a flexible membrane insertion loop (only resolved in the heptameric prepore complex, colored blue in Figure 3 C) that is stabilized by interactions with the neighboring subunit in the prepore complex (Lacy et al., 2004). Activation of the prepore complex is mediated by acid-catalyzed proteolytic cleavage of the N-terminus of PA in the endosome of target cells (Qa'dan et al., 2005), leading to the proposed conversion of the flexible loop to a β-hairpin and its membrane insertion, resulting in a barrel formed by 14 β-strands similar to the transmembrane barrel of α-Hemolysin (Petosa et al., 1997).

1.3 ClyA – A bacterial PFT undergoing a large conformational transition upon pore formation

1.3.1 Distribution of ClyA and mechanisms of regulation and export

The 34 kDa pore-forming toxin Cytolysin A (ClyA, also termed Hemolysin E (HlyE) or silent Hemolysin A (SheA)) is an α-PFT existing in various nonpathogenic (e.g. K12), human pathogenic, avian pathogenic *Escherichia coli* and *Salmonella enterica* strains (del Castillo et al., 1997; Huang et al., 2010; Ludwig et al., 2004; Oscarsson et al., 2002; von Rhein et al., 2009; von Rhein et al., 2006). Inactive forms of the clyA gene that have insertions or truncations at various sites have been found in several species of *Shigella* (von Rhein et al.,
Transcription of *clyA* in *E. coli* is regulated by a complex system involving four transcription factors (Hunt et al., 2010) illustrated in Figure 4. The *clyA* promoter has a single binding site for the transcription factors FNR (Fumarate Nitrate Reduction regulator) and CRP (cAMP receptor protein). FNR enhances transcription of *clyA* in response to oxygen starvation, and CRP enhances transcription of *clyA* in response to glucose starvation (Westermark et al., 2000; Wyborn et al., 2004b). The nucleoid-binding protein H-NS inhibits FNR-mediated *clyA* transcription under anoxic conditions, but it slightly enhances CRP-mediated *clyA* transcription in the absence of glucose (Wyborn et al., 2004b). The regulatory protein SlyA, which is expressed in response to amino acid starvation, counteracts H-NS repression and activates transcription of *clyA* (Lithgow et al., 2007; Wyborn et al., 2004b). In *Salmonella typhi*, *clyA* expression is activated by the PhoPQ two-component system, which also activates other genes involved in host infection by *Salmonella typhi* (Hunt et al., 2010).

![Figure 4: Regulation of transcription of *clyA* in *Escherichia coli*. Activation of transcription is indicated by “+”, repression by “−”. The transcription factors FNR and CRP bind to the *clyA* promotor under anoxic or glucose starvation conditions, respectively, and enhance *clyA* transcription. H-NS enhances CRP-mediated *clyA* transcription in the absence of glucose, and inhibits FNR-mediated *clyA* transcription under anoxic conditions (Wyborn et al., 2004b). H-NS repression of *clyA* transcription is downregulated by SlyA, whose expression is activated by amino acid starvation.](image)

The exact export mechanism of ClyA from the bacterial cell through the cytoplasmic membrane is still enigmatic. It does not have any known N-terminal or C-terminal signal peptide sequences for membrane translocation that are cleaved upon export and activation (del Castillo et al., 1997). Therefore, it is not exported via one of the common secretion systems that are described in gram-negative bacteria (type I to IV; Thanassi and Hultgren (2000)), as it is the case for other PFTs like α-Hemolysin (Gentschev et al., 2002). Most likely intrinsic structural properties of monomeric ClyA enable membrane translocation. Deletions of parts of the N- and C-terminal α-helices showed the necessity of both regions for ClyA export (Ludwig et al., 2010). The deletion of at least 10 residues at the N-terminus resulted in the entrapment of ClyA in the *E. coli* cytoplasmic membrane. Mutations affecting the integrity of the C-terminal helix (deletion of at least 12 residues from the C-terminus or insertion of a proline residue (A278P) had the same effect (Wai et al., 2003b), as well as...
the deletion of the hydrophobic part that is located in spatial proximity to the C-terminal helix (residues 89 – 101; Ludwig et al. (2010)). The intrinsic ability of ClyA to pass the cytoplasmic membrane was successfully applied to co-transport genetically merged proteins through the cytoplasmic membrane (del Castillo et al., 2001; Kim et al., 2008).

The export of ClyA from the periplasm to the environment is better understood than its translocation through the cytoplasmic membrane. It has been shown that outer membrane vesicles (OMVs) of a ClyA-expressing *E. coli* strain are enriched with ClyA pores (Figure 5; Wai et al. (2003a)). The OMVs containing ClyA pores were shown to exhibit cytotoxic activity similar to soluble ClyA (Oscarsson et al., 1999; Wai et al., 2003a).

![Figure 5](image_url)

**Figure 5:** Electron micrographs of outer membrane vesicles of a genetically engineered *E. coli* strain with enhanced ClyA expression (Westermark et al., 2000). Left: black arrows indicate stain-filled ClyA pores in top view. Right: The black arrow indicates a pore in side view, and the white arrow indicates a pore in top view. Scale bar: 200 nm. The figure was adapted from Wai et al. (2003a).

### 1.3.2 The high-resolution structures of monomeric and assembled ClyA

By now ClyA from *Escherichia coli* K12 is the only α-PFT of which high-resolution structures of the soluble monomer and an assembled and active pore complex are available. Monomeric ClyA (302 residues without N-terminal Met) is an elongated protein with an overall length of 10 nm (Figure 6 A). It consists of two domains: (i) a 7.9 nm long tail domain consisting of a bundle of one short (G) and four long (A, B, C, and F) α-helices and (ii) a head domain (residues 160 – 205) consisting of two short α-helices (D, E) flanking a hydrophobic β-hairpin (residues 185 – 195) termed β-tongue (Wallace et al., 2000). The “tip” of the β-tongue is attached to the tail domain by “central” Phe 190 undergoing aromatic interactions with four other aromatic residues in the center of the molecule (F 50, Y 54, F159, Y 165; Figure 6 B; Mueller et al. (2009)). Due to the hydrophobic character of the β-tongue, this domain was suggested to function as initial interaction point with the target membrane and was initially incorrectly predicted to form a transmembrane helix (del Castillo et al., 1997). In fact, insertion of charged residues into or partial deletion of the
β-tongue leads to a strongly decreased membrane activity and a non-hemolytic phenotype (Ludwig et al., 2010; Wai et al., 2003b).

Figure 6: Structure of the ClyA monomer, the ClyA protomer, and the assembled pore complex. A: Overall view of the 2.0 Å crystal structure of monomeric wild type ClyA, shown in two orientations rotated by 90°. The tail domain is colored red (helices B, C, F, and G), green (loops) and light blue (helices A1 and A2). The head domain is colored in dark blue (loops and helices D and E) and yellow (β-strands). B: The central residue of the β-tongue, Phe 190, undergoes aromatic interactions with four aromatic residues located in the center of monomeric ClyA (F 50, Y 54, F 159, and Y 165). C: Overall view of the 3.29 Å crystal structure of the dodecameric ClyA pore complex. One protomer is highlighted in the same colors as monomeric ClyA in panel A. D: One protomer from the pore complex, shown in two orientations rotated by 90°. The color code is identical with panel A. E: Vacuum electrostatic maps of the ClyA pore, shown in top view (left), side view (middle), and bottom-to-top view (right). Blue indicates positive charges, red indicates negative charges, and white indicates uncharged regions. All figures and the electrostatic maps were prepared in PyMOL (Schrodinger, 2010).
Soluble, monomeric ClyA forms pores in vitro in the presence of detergents (Eifler et al., 2006; Tzokov et al., 2006). The 3.29 Å structure of the dodecameric pore complex in the presence of the detergent n-dodecyl-β-D-maltopyranoside (DDM) has been solved by X-ray crystallography recently (Figure 6 C; Mueller et al. (2009)). Additionally, electron microscopy studies showed tridecameric pores in the presence of DDM (Eifler et al., 2006), and hexameric and octameric pore complexes in the presence of n-octyl-β-D-glucopyranoside (Tzokov et al., 2006). The crystallized dodecameric pore complex has an overall length of 13.5 nm, an outer diameter of 10.8 nm and an inner diameter of 3.6 nm at the narrowest part formed by the N-termini of the protomers (Figure 6 E). The structural differences of a ClyA monomer and a ClyA protomer are remarkable (Figure 6 A and D). The tail domain’s α-helix bundle consists of one short (G) and three long (B, C, and F) α-helices in the protomer, one α-helix less than in the monomer. Monomer’s N-terminal helix (A1 and A2) forms an elongation of α-helix B (A2). The N-terminal part (residues 2 – 35, A1) which has an amphipathic character forms the pore channel through the membrane. Monomer’s head domain is completely restructured and forms an elongation of the flanking α-helices C and F in the protomer. The conformational rearrangements result in an elongation of ClyA from 10 nm (monomer) to 13.5 nm (protomer). The elongated helix bundle of the tail domain mediates protomer-to-protomer interactions with 13 salt bridges and 25 hydrogen bonds per intersubunit interface (Mueller et al., 2009). Together with spatial movements of parts of ClyA, elements of the secondary structure of ClyA are altered. The two short β-strands of the β-tongue (residues 185 – 188 and 192 – 195) form parts of long α-helices in the protomer (colored yellow in Figure 6 A and D), as well as monomer’s loops 180 – 184 and 201 – 205. The short loop connecting α-helices A2 and B in the monomer is part of α-helix B in the protomer, and loop FG is shifted by five residues towards the C-terminus in the protomer (indicated green in Figure 6 D). The β-to-α-transition of the ClyA β-tongue upon pore formation is a contrast to the α-to-β-transition that has been proposed for CDCs upon pore formation (Shatursky et al. (1999); cf. Figure 3 B).

1.3.3 A model mechanism for the conformational change and pore formation

The extensive structural arrangements of the monomer-to-protomer transition of ClyA resulted in a detailed, sequential model of the conformational transition mechanism proposed by Mueller et al. (2009), which is summarized in Figure 7. The β-tongue that consists almost exclusively of hydrophobic residues functions as initial point of membrane interaction, which is accompanied by a swinging-out movement upon membrane integration (A). The swinging-out movement of the β-tongue triggers the subsequent steps of monomer-to-protomer transition of ClyA, starting with α-helix D and loop CD (residues 160
– 179) forming a linear, C-terminal extension of α-helix C that is partially integrated into the membrane (B). As a consequence, the helix bundle of the tail domain of ClyA is shifted away from the membrane. Subsequently, α-helix A undergoes a 180° turn that results in an N-terminal elongation of α-helix B with αA2 and membrane interaction of the amphipathic N-terminal part αA1 (C). The kink mediated by Pro 36 persists. The resulting gap in the tail domain next to α-helix G is filled by α-helix F (D). The movement of α-helix F enables its C-terminal extension by parts of the β-tongue, α-helix E and the connecting loops (residues 190 – 205). The β-tongue of the monomer (residues 185 – 195) undergoes a β-to-α-transition and is part of a membrane-embedded helix-turn-helix motif in the protomer (E). After the described rearrangements the assembly-competent protomer is formed and oligomerization can proceed. Upon oligomerization, the dense packing of helices αA1 at the membrane surface results in membrane destabilization and finally in wedging of αA1 through the membrane opening the pore channel (F). The final, membrane-embedded pore complex is stabilized by iris-like interlocking arrangements of αA1 (G). Kinetic data support the proposed model with a unimolecular conformational transition as rate-limiting step, followed by fast oligomerization at ClyA concentrations of 1 – 10 µM (Eifler et al., 2006).

The model of Mueller et al. (2009) proposes the necessity of distinct hinge points of the swinging-out movement of the β-tongue and the membrane protrusion of αA1. Several glycine residues in the loops flanking the β-tongue (G 180, G 184, G 201, and G 205) form possible hinge points that enable the swinging-out movement. Indeed, substitution of G 180 by V (in combination with substitution of K 275 by R) results in a significantly decreased hemolytic activity (Atkins et al., 2000). P 36 that forms the kink between αA1 and αA2 is proposed as a second crucial point of action: the helix kink should be necessary for enabling the lateral membrane contact of αA1, while αA2 elongates αB. As ClyA is already partially membrane-embedded, a continuous α-helix A would result in a straight, complete dunk of the N-terminal part into the membrane as a result of the 180° turn of α-helix A. That is energetically impossible, as αA1 contains ten charged residues.
Figure 7: Schematic representation of the ClyA conformational transition and pore formation mechanism as proposed by Mueller et al. (2009). The β-tongue (βt, light green) mediates the initial membrane interaction and is integrated into the membrane (represented as gray shade) by a swinging-out movement (A). α-helix D (αD, green) and loop CD form an elongation of α-helix C (dark green), resulting in partial membrane integration of αD (B). Subsequently, α-helix A (αA1 and αA2, blue) undergoes a 180° turn which leads to an elongation of α-helix B (light blue, C). The amphipathic α-helix A1 attaches to the membrane and α-helix F (αF, orange) closes the gap left by α-helix A next to α-helix G (D). The repositioning of αF enables the formation of a helix-turn-helix motif at its N-terminus that is made up of the β-tongue (light green), α-helix E (yellow), and the connecting loops (gray). The formation of the assembly-competent protomer is finished (E). Oligomerization of the protomers leads to membrane distortions caused by the dense packing of αA1 at the membrane surface. This results in wedging of αA1 through the destabilized membrane (F). The final pore with iris-like, interlocking arrangement of helices αA1 is formed (G). The figure was adapted from Mueller et al. (2009).

1.4 Aim: Discovery of the monomer-to-protomer transition mechanism of ClyA

In contrast to the β-PFT α-Hemolysin, high-resolution structures of monomeric ClyA and an assembly-competent protomer show great structural differences, which means an extensive conformational transition has to precede pore formation (Mueller et al., 2009). The knowledge of both structures gives us the unique possibility for detailed, site-directed
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mutagenesis studies to enlighten the exact monomer-to-protomer transition mechanism as molecular basis for the mode of action of ClyA as a general model for α-PFTs. The aim is to trap and stabilize distinct, transient intermediate forms of the monomer-to-protomer transition and to characterize the intermediates by means of structural biology, spectroscopy, and imaging techniques. The detailed characterization of the intermediates should enable us to complement the existing conformational transition model of ClyA (Mueller et al., 2009) or to propose an alternative model of monomer-to-protomer transition and oligomerization.

The work presented here describes three different strategies to interrupt the monomer-to-protomer transition of ClyA resulting in the arrest of transient intermediates. The introduction of single point mutations affecting the conformational flexibility of both the N-terminal α-helix A and the β-tongue is a promising strategy for trapping intermediates. Several glycine residues (G 180, G 184, G 201, and G 205) have been proposed to ensure the β-tongue flexibility (Mueller et al., 2009); therefore, single or combined glycine substitutions by more rigid residues (alanine, valine, or proline) should interrupt the β-tongue movement upon membrane integration and result in the arrest of conformational transition of ClyA at an early state. In contrast to other published mutation studies of the β-tongue involving the insertions of charged residues or the removal of parts of loops and β-strands (Ludwig et al., 2010; Wai et al., 2003b), our more subtle mutations should not interfere with the membrane interaction of ClyA. Another possible site for trapping an intermediate would be the kink between αA1 and αA2 mediated by P 36, which would result in a further processed intermediate form of ClyA according to the model of Mueller et al. (2009).

The second possible approach is the covalent attachment of flexible regions of ClyA by an engineered disulfide bond. This has been performed successfully for other types of α-PFTs (Duche et al., 1996; Kristan et al., 2004) and also β-PFTs (Hotze et al., 2001; Kawate and Gouaux, 2003). However, wild type (wt) ClyA has two natural cysteine residues at positions 87 and 285, which can form a disulfide bond that has no influence on the hemolytic activity (Eifler et al., 2006). Therefore, a cysteine-free ClyA variant has to be designed as a basis for introducing artificial cysteine pairs in ClyA. The trapping of either the β-tongue or α-helix A by each one engineered disulfide bond should result in redox-controllable ClyA variants that form active pores in the dithiol form and are trapped at different inactive intermediate states in the disulfide form.

The third possibility is to inhibit the final pore formation by disturbing the intersubunit interactions of the protomers, as it has been described for the β-PFT α-Hemolysin from
Staphylococcus aureus (Walker and Bayley, 1995). As the ClyA pore subunits are held together by 13 salt bridges and 25 hydrogen bonds (Mueller et al., 2009), an approach to introduce a pair of repelling charged residues at close contact positions of the protomers should be a more promising strategy than neutralizing the interaction network. The result should be an unassembled ClyA conformational transition intermediate that is structurally similar to a protomer.
2 Materials and Methods

2.1 Materials

Chemicals of highest available purity were purchased from Merck (Germany) or Sigma-Aldrich (Germany). EDTA, glycerol, β-mercaptoethanol and dithiothreitol (DTT) were obtained from AppliChem (Germany). Detergents were purchased from Anatrace (USA), and horse erythrocytes were obtained from Oxoid AG (Switzerland). Oligonucleotides were purchased from Microsynth (Switzerland). Trypsin (EC no. 3.4.21.4) and chymotrypsin (EC no. 3.4.21.4) from bovine pancreas were purchased from Boehringer (Germany).

2.2 Molecular cloning

Variants of the E. coli clyA gene with a single codon substitution were produced by quick-change PCR (Kunkel, 1985) in the context of the previously reported T7 expression plasmid (pET11a derivative) for wild-type (wt) clyA with an N-terminal His$_6$-tag (Eifler et al., 2006). Additionally, the cleavage site for NdeI in the clyA gene was removed by quick-change PCR by introducing a silent mutation. A typical PCR batch (50 µl) consisted of 0.2 µM of each primer, 200 µM of each dNTP, 50 ng of template DNA and 1-2 U of Pfu DNA-Polymerase (Promega, USA) in combination with the supplied reaction buffer. PCRs (30-35 cycles) were performed in a T-Personal Combi thermocycler (Biometra, Germany) with 3 min initial denaturation (98 °C), 45 s denaturation (98 °C), 45 s annealing (52 – 55 °C), and 17 min elongation (70 °C). After PCR, template DNA (dam methylated) was digested by 20 U DpnI (New England Biolabs, USA) in the PCR reaction buffer for at least 1 h at 37°C. After template DNA digestion, the amplified DNA was purified using a Qiagen PCR purification kit (Qiagen, Germany) according to the manufacturer’s protocol. Electrocompetent E. coli DH5α cells were transformed with 10 – 20 µl of the purified PCR product and grown overnight (oN) at 37 °C on LB agar containing 150 µg/ml ampicillin. For plasmid isolation, grown colonies were used to inoculate 5 ml of LB medium containing 150 µg/ml ampicillin and incubated oN at 37 °C. Plasmids were isolated using a GeneJet Plasmid Miniprep kit (Thermo Scientific, USA) according to the manufacturer’s protocol and the presence of the desired point mutation was verified by DNA sequencing (Microsynth, Switzerland).

The same quick-change PCR protocol was applied to introduce a cleavage site for TEV protease (DNA sequence GAAAACCTGTATTTCCAG) between the N-terminal His$_6$-tag and clyA.
2.3 Protein expression, purification and related methods

2.3.1 Protein expression

ClyA expression and purification was performed in *E. coli* as described previously (Eifler et al., 2006) with some modifications. Dependent on the solubility of the respective ClyA variant, expression was performed in 1.5 – 9 l of LB medium containing 150 µg/ml ampicillin. Expression cultures were inoculated with 1/50 volume of an overnight preculture grown from a single colony of the expression strain *E. coli* Tuner DE3 (Novagen, USA) which is a *lacZY*-deficient mutant of BL21 DE3 and allows an adjustable level of protein expression by changing the concentration of the inducer IPTG. Cultures were incubated at 37 °C and shaking at 100 rounds per minute. After growing to an OD₆₀₀ of 0.8 – 1.0, expression cultures were cooled to 20°C and expression of ClyA was induced by addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After protein expression (5 – 6 h at 20 °C), cells were pelleted 12 min at 4800 x g and 4 °C and stored at -20 °C until protein purification.

2.3.2 Metal chelate affinity chromatography

All purification steps were performed at 4 °C. After protein expression, *E. coli* cells were resuspended in lysis buffer (50 mM potassium phosphate, 300 mM sodium chloride, 30 mM Imidazole, 2 mM β-mercaptoethanol, pH 8.0) to a final OD₆₀₀ of about 60 and lysed by passing two times through a cell cracker at 70 psi. Cell debries were centrifuged down at 43000 x g for 30 min. The clarified cell extract was incubated with 10 ml NiNTA superfloal agarose (Qiagen) for 30 min. Subsequently, the cell extract including the NiNTA beads was loaded on a gravity flow chromatography column and the beads were washed with 3 column volumes (CVs) lysis buffer. Elution was performed stepwise by addition of each 2 CVs of lysis buffer containing 70 mM, 200 mM and 400 mM Imidazole. The eluate was collected in 10 ml fractions and subjected to SDS-PAGE. Fractions containing ClyA without major impurities were pooled and dialyzed On against 5 l of hydroxyapatite buffer A (HAA; 20 mM potassium phosphate, 2 mM DTT, pH 6.8).

2.3.3 Hydroxyapatite chromatography

Hydroxyapatite chromatography was performed as described previously (Mueller, 2009). The pooled and dialyzed protein solution from the metal chelate affinity chromatography was loaded on a 30 ml CHT ceramic hydroxyapatite column (Biorad, USA), washed with 2 CVs HAA, and eluted by a linear gradient from 0 to 60% hydroxyapatite buffer B (400 mM
potassium phosphate, 2 mM DTT, pH 6.8) over 9 CVs. The eluate was collected in 5 ml fractions and protein-containing fractions were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Fractions containing ClyA in high purity (> 95%, as judged by SDS-PAGE) were pooled and dialyzed against 5 l of phosphate-buffered saline (PBS; 20 mM potassium phosphate, 150 mM NaCl, pH 7.3) containing 2 mM DTT. The identity of all ClyA variants was confirmed by electrospray mass spectrometry at the Functional Genomics Center Zurich.

2.3.4 Preparative size exclusion chromatography

For protein crystallization and after oxidation of cysteine pairs to artificial disulfide bonds, preparative size exclusion chromatography was applied to separate monomeric ClyA from disulfide-mediated oligomers or aggregates. Solutions of ClyA variants were subjected to a Superdex 200 column of the appropriate size (GE Healthcare, Germany). The column was equilibrated in PBS or, in case of subsequent crystallization experiments, Tris-buffered saline (TBS; 20 mM Tris-HCl, 150 mM NaCl, pH 7.3). ClyA was eluted isocratically using the flow setup recommended by the manufacturer. Fractions containing the ClyA monomer were pooled and used for subsequent experiments.

2.3.5 Storage of proteins

For long-time storage (> 1 week), solutions of ClyA in PBS + 2 mM DTT were flash-frozen in liquid nitrogen and stored at -80 °C.

2.3.6 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

The purity of ClyA preparations after each purification step was estimated by denaturing SDS-PAGE (Laemmli, 1970). Samples were mixed with 1/4 of their volume of 5x SDS sample buffer and incubated at 95 °C for at least 5 min prior to loading on a gel. 5 – 20 µl of samples were loaded on 15% polyacrylamide gels and subjected to electrophoresis at limiting current of 55 mA for 45 min. Gels were stained in a 0.2% Coomassie Brilliant Blue G solution for 20 min. Compositions of buffers and solutions used for SDS-PAGE are shown below.

5x SDS sample buffer: 5 g/l SDS, 25% (v/v) glycerol, 50 mM DTT, 0.25 g/l bromphenolblue sodium salt, 1.25 M Tris HCl pH 6.8

4x upper gel buffer (UGB): 4 g/l SDS, 0.5 M Tris HCl pH 6.8

4x lower gel buffer (LGB): 4 g/l SDS, 3.0 M Tris HCl pH 8.8
SDS-PAGE electrode buffer: 1 g/l SDS, 50 mM Tris, 190 mM Glycine

Stacking gel (20 ml, sufficient for 7 gels): 5 ml 4x UGB, 3 ml 30% acrylamide solution (AppliChem, Germany), 12 ml H₂O, 200 µl ammonium persulfate (APS), 12 µl tetramethylethylenediamine (TEMED)

15% separating gel (40 ml, sufficient for 7 gels): 10 ml 4x LGB, 20 ml 30% acrylamide solution, 10 ml H₂O, 300 µl APS, 18 µl TEMED

Coomassie staining solution: 50% (v/v) methanol, 10% (v/v) acetic acid, 0.02 g/l Coomassie Brilliant Blue G

2.3.7 Cleavage of affinity tag by TEV protease

The autolysis-resistant catalytic domain of tobacco etch virus (TEV) protease (Phan et al., 2002) containing an N-terminal His7-tag was purified as described previously (Finder et al., 2010). TEV protease cleaves target proteins after its recognition sequence ENLYFQ with differences in efficiency depending on the following residue (Kapust et al., 2002). In the case of ClyA, the cleavage site is followed either by Thr (wt, CC6/264 (2-303), CC50/190) or by Cys (CC6/264 (6-303)). Both residues enable a theoretical cleavage efficiency of at least 75% (Kapust et al., 2002).

After the initial metal chelate affinity purification (2.3.2), the ClyA containing fractions were dialyzed against 5 l of cleavage buffer (20 mM Tris HCl, 50 mM NaCl, 2 mM β-mercaptoethanol, pH 7.8) and concentrated to at least 20 µM using an Amicon Ultra 10k concentrator (Millipore, USA). TEV protease was added to a final concentration of 2 µM and the cleavage reaction was performed for 3-4 h at room temperature, followed by an incubation at 4 °C. Subsequently, cleaved ClyA was separated from uncleaved ClyA and TEV protease by an additional metal chelate affinity chromatography and the purities and fractions of cleaved and uncleaved ClyA were estimated by SDS-PAGE.

2.3.8 Determination of protein concentration

Concentrations of ClyA variants were determined by absorption spectroscopy using the molar absorption coefficient at 280 nm (ε₂₈₀) calculated from the number of Trp, Tyr and Cystines (Gill and Vonhippel, 1989). ε₂₈₀ values were 30370 M⁻¹ cm⁻¹ for ClyA variants without a disulfide bond and 30495 M⁻¹ cm⁻¹ for ClyA variants containing one disulfide bond, respectively. Measurements were performed with a Cary Varian 100 absorption spectrophotometer (Agilent, USA) or with a NanoDrop spectrophotometer (Thermo Scientific, USA).
2.3.9 Oxidation of ClyA variants

Two ClyA variants containing each one cysteine pair which is able to form an artificial disulfide bond were designed. ClyA CC50/190 contains two Cys residues at positions 50 and 190 with the ability to covalently attach the β-tongue to α-helix B. ClyA CC6/264 contains two Cys residues at positions 6 and 264 with the ability to attach α-helix A to loop FG. In both cases, the artificial disulfide bonds were formed by Cu$^{2+}$-mediated air oxidation, as described previously for ClyA wt (Eifler et al., 2006) with some modifications. 20 – 30 µM of ClyA were incubated with 0.5 mM CuCl$_2$ in PBS (pH 7.3) at 25 °C for 4 h. Subsequently, the sample was dialyzed oN against PBS (pH 7.3) containing 2 mM EDTA at 4 °C, followed by size exclusion chromatography (2.3.4) to remove aggregates and disulfide-mediated oligomers. The absence of free thiol groups in the oxidized protein samples was verified by Ellman’s assay (Ellman, 1958) under denaturing conditions (30 min incubation in buffer containing 4 M guanidinium hydrochloride, pH 7.3) and reversed-phase HPLC (2.5.4).

2.4 Preparation of erythrocyte membranes

Membranes of horse erythrocytes were prepared as described previously (Dodge et al., 1963). All steps were performed at 4 °C. 10 ml of erythrocytes were washed three times in PBS pH 7.3 and a 5% cell suspension (v/v) was prepared and incubated for 30 min. Afterwards, cells were pelleted at 3200 × g for 10 min and hemolysis was performed by resuspending the sedimented cells 1:15 in 7 mM potassium phosphate, pH 7.3 (lysis buffer). 30 min after hemolysis, the erythrocyte membranes were sedimented at 25000 x g for 30 min and subsequently washed 3 times in lysis buffer. Finally, the membranes were washed in pure H$_2$O and lyophilized oN. After mass determination, the erythrocyte membranes were resuspended in PBS to a final concentration of 2 mg/ml and sonicated three times for 30 s in a water bath sonicator. The preparation was stored at -20 °C.

2.5 Biophysical and biochemical characterization of ClyA variants

2.5.1 CD spectroscopy

The kinetics of conformational transition and assembly of ClyA can be followed by observing the far-UV circular dichroism (CD) signal change (Eifler et al., 2006). After triggering the assembly reaction by addition of detergent, the monomer (M) rapidly forms an intermediate (I), which forms the assembly-competent protomer (P) in the rate-limiting step.
Manual mixing kinetics were recorded at 225 nm using a temperature-controlled J715 CD spectrometer (Jasco, Japan) at 22 °C. In a typical experiment, 0.3 mg/ml monomeric ClyA (wt or variant) in PBS pH 7.3 were pre-incubated in a 0.1 cm cuvette for 5 min before starting the conformational transition by addition of n-dodecyl-β-D-maltopyranoside (DDM) to a final concentration of 0.1% (w/v). Samples of reduced ClyA additionally contained 2 mM DTT. Far-UV CD spectra of monomeric ClyA before the assembly reaction and assembled or conformational trapped intermediate ClyA after the assembly reaction, respectively, were recorded from 260 to 195 nm and corrected for the CD signal of the buffer. Near-UV CD kinetics at 280 nm and spectra were measured in the same way with the exceptions that a 10 cm cuvette was used with 0.1 mg/ml ClyA. Fast CD kinetics were obtained using a temperature-controlled (22 °C) PiStar CD stopped flow spectrometer (Applied Photophysics, Great Britain) with 0.2 mg/ml ClyA end concentration and variable concentrations of DDM or membranes. All measured CD data (mdeg) were converted to mean residue ellipticity \( \theta_{\text{MRW}} \) (deg cm\(^2\) dmol\(^{-1}\)) according to Equation 1.

\[
\theta_{\text{MRW}} = \frac{\theta_{\text{mdeg}} \cdot MW}{10 \cdot c \cdot d \cdot n}
\]

**Equation 1:** Calculation of mean residue ellipticity \( \theta_{\text{MRW}} \) (deg cm\(^2\) dmol\(^{-1}\)) from measured ellipticity \( \theta \) (mdeg), molecular weight \( MW \) (g/mol), concentration \( c \) (mg/ml), cuvette thickness \( d \) (cm) and number of amino acids \( n \).

For comparison of conformational transition kinetics of different variants, the second, rate-limiting step (I \( \rightarrow \) P) was fitted according to a first-order mechanism as described previously (Eifler et al., 2006). Kinetics of the whole conformational transition upon DDM addition were evaluated according to the simplest possible mechanism, namely two consecutive irreversible first-order reactions: M \( \rightarrow \) I \( \rightarrow \) P (Crespo et al., 2012) (Equation 2).

\[
s = a \cdot e^{-k_1 t} + b \cdot \frac{k_1}{k_2 - k_1} \cdot (e^{-k_1 t} - e^{-k_2 t}) + c \cdot \left(1 + \frac{1}{k_1 - k_2} \cdot (k_2 \cdot e^{-k_1 t} - k_1 \cdot e^{-k_2 t})\right)
\]

**Equation 2:** Consecutive fit of two irreversible first-order reactions A \( \rightarrow \) B \( \rightarrow \) C, where \( s \) is the observed signal, \( a, b, c \) are the signals of the individual species A, B and C, and \( k_1 \) and \( k_2 \) are the rate constants of A \( \rightarrow \) B and B \( \rightarrow \) C, respectively.

### 2.5.2 Fluorescence spectroscopy with ANS

8-Anilino-1-naphthalenesulfonic acid (ANS) is a fluorescent dye which binds noncovalently to lipids and hydrophobic regions of proteins. Upon binding to a hydrophobic environment, the fluorescence emission shows a blue shift of the emission maximum and a significant increase of intensity (Slavik, 1982). Due to the reversible binding and the strong signal
change ANS fluorescence is well suited to study conformational transitions proceeding via partially folded, molten globule-like intermediates (Poklar et al., 1997; Uversky et al., 1996).

All ANS fluorescence measurements were performed in PBS (pH 7.3) at 22 °C and were recorded with a temperature-controlled Quantum Master 7 fluorescence spectrometer (PTI, USA) at an excitation wavelength of 370 nm and an emission wavelength of 475 nm. Spectra were recorded from 400 – 600 nm with an excitation wavelength of 370 nm. The respective ClyA monomer (5 µM, reduced, sample containing 20 mM DTT) was pre-incubated with ANS (20 µM) and the conformational transition to the protomer was initiated by addition of DDM (final concentration: 0.1% w/v). The oxidized variants CC50/190 and CC6/264 were pre-incubated with ANS and 0.1% DDM and the conformational transition was started by addition of DTT (final concentration: 20 mM). Data were fitted according to a consecutive mechanism with two irreversible reactions (Equation 2).

The apparent dissociation constants of the complex between ANS and the trapped intermediate states of oxidized ClyA CC50/190 and CC6/264 and the binding stoichiometries were determined with fluorescence titration. ANS (20 µM) was incubated with 0.1% DDM and different concentrations (0 – 28 µM) of ClyA for at least 1 h. The fluorescence at 475 nm of each sample was averaged over 60 s and plotted against the respective ClyA/ANS ratio. Data were fitted according to a binding equilibrium with independent (identical) binding sites according to Equation 3.

\[
f(x) = y_0 + 0.5 \cdot (y_\infty - y_0)(1 + \frac{x + K_D}{p} - \sqrt{(1 + \frac{x + K_D}{p})^2 - 4 \cdot \frac{x}{p}})
\]

**Equation 3:** Binding equilibrium of ANS and ClyA assuming independent and identical binding sites. \( f(x) \) is the fraction of sites occupied, \( x \) is the concentration of ClyA, \( y_0 \) and \( y_\infty \) are the fluorescence signals of free and bound ANS, respectively, \( K_D \) is the apparent dissociation constant and \( p \) the stoichiometry of ClyA per ANS.

### 2.5.3 Hemolysis kinetics

Kinetics of lysis of horse erythrocytes were measured as described previously (Rennie et al., 1974) by observing the decrease of optical density at 650 nm upon cell lysis. Defibrinated horse erythrocytes (stored at 4 °C for one month at longest) were washed three times in PBS buffer at 25 °C immediately before usage. Erythrocyte suspension with an OD_{650} of 0.75 (corresponding to approximately 2 x 10^6 cells/ml) were pre-incubated 5 min at 37 °C before starting erythrocyte lysis by addition of ClyA to end concentrations of 2 – 100 nM. Samples with reduced ClyA (wt or variants) contained 2 mM DTT. To demonstrate the reversibility of ClyA inactivation by introduction of artificial disulfide bonds in CC50/190 and CC6/264, oxidized ClyA variants (20 nM each) were pre-incubated with...
horse erythrocytes and the reaction was started by addition of 10 mM DTT. All measurements were performed in PBS (pH 7.3) and 37 °C using a stirred cuvette and a temperature-controlled Cary Varian 100 absorption spectrometer (Agilent).

Data were evaluated by linearly fitting i) the pre-transition baseline and ii) the data points in the middle of the lysis reaction between 35% and 75% of the initial optical density. The lag phase of hemolysis was defined as the time point where the two linear fits intersected, and the maximum lysis velocity was defined as the slope of the linear decrease in optical density between 35 and 75% of the initial cellular density.

2.5.4 Reversed phase high performance liquid chromatography (HPLC)

The amounts of reduced and oxidized ClyA (wt and variants CC50/190 and CC6/264, respectively) in reduction assays of oxidized ClyA were quantified by separation of the reduced and oxidized species by reversed-phase HPLC and peak integration. Oxidized ClyA (wt or variants, 5 µM) was incubated with DTT (10 mM) in the presence or absence of DDM (0.1%) at 22 °C or 37 °C in PBS buffer (pH 7.3). After distinct time intervals, samples were taken and the disulfide bond reduction was stopped by acid quenching (addition of 1/6 volume of 98% formic acid). Samples (50 µl) were analyzed via reversed-phase HPLC using a Zorbax 300SB C8 column (Agilent) by elution on a water/acetonitrile gradient (30 – 80% acetonitrile over 30 min at 1 ml/min). Water and acetonitrile additionally contained 0.1% trifluoracetic acid. The absorption signal was recorded at 220 nm and peaks of oxidized and reduced protein were integrated using the data evaluation module of the Agilent HPLC software. The decay of the amount of the oxidized species was fitted according to a pseudo first-order mechanism.

2.5.5 Analytical size exclusion chromatography

Analytical size exclusion chromatography (SEC) was performed on the one hand in order to check if oligomerization of specific ClyA variants in various detergents occurs and on the other hand to check the binding ability of ClyA variants to erythrocyte membranes. Typically, 0.3 mg/ml ClyA variant in PBS (pH 7.3) were incubated with detergent (0.1% DDM, 0.5 or 1.0% β-D-octylglucopyranoside (OG) or 0.1% n-decyl-β-D-maltopyranoside (DM), respectively) or 1.0 g/l erythrocyte membranes for at least 1 h at 25 °C prior to SEC. Samples of reduced ClyA additionally contained 2 mM DTT. After centrifugation (16100 x g, 20 min, 25 °C), samples of were analyzed using a ProSEC 300S 4.6 mm (Agilent) or a Superdex 200 10/300 column (GE Healthcare). Both columns were equilibrated in PBS and
the runs were performed according to the manufacturers' protocols. 20 or 200 µl of sample were injected, respectively.

2.5.6 Limited proteolysis

Limited proteolysis was applied to compare the solvent exposure of intermediate states of non-assembly competent ClyA variants with pores formed by ClyA wt. 10 µM of ClyA (wt or the respective variant) were pre-incubated in 0.1% DDM at 22 °C and pH 7.3 for 2 h and subsequently subjected to proteolysis by increasing concentrations (0.05 – 1.0 µM) of trypsin or chymotrypsin at 37 °C in PBS pH 7.3. ClyA digestion was stopped after 4 h by addition of the serine protease inhibitor phenylmethanesulfonyl fluoride (PMSF; Gold & Fahrney (1963); end concentration: 1 mM), and the cleavage patterns of ClyA wt or variants in the presence or absence of DDM were analyzed via SDS-PAGE.

2.6 Negative-stain Transmission electron microscopy

300 mesh carbon coated copper grids (Quantifoil, Germany) were glow discharged using an Emitech K100X with negative polarity (25 mA) for 45 s immediately before usage. 3 µl of ClyA wt or variants (5 µM) in PBS (pH 7.3) pre-incubated in 0.1 % DDM for at least 1 h were adsorbed to the prepared grids for 10 s. After drying excess liquid with a filter paper, the samples were stained immediately by placing the grid on top of a drop of filtered 2% uranyl acetate solution. The grid was stained for 5 min, blotted dry, and the staining procedure was repeated two times with fresh drops of uranyl acetate solution. Finally, the stained grid was blotted dry and dried completely next to a hot lamp. Electron micrographs were recorded at 50000 x magnification by a KeenView CCD camera using a Morgagni 268 electron microscope (FEI, USA) operating at an acceleration voltage of 100 kV.

2.7 Protein crystallization

2.7.1 Crystallization of monomeric ClyA variants

To show the presence of the respective artificial disulfide bond, the oxidized ClyA variants CC50/190 and CC6/264 were crystallized in the monomeric state. In both cases, after removal of His₆-tags by TEV protease, more, bigger, and more uniformly shaped crystals were formed. After oxidation (2.3.9), ClyA variants were transferred into TBS (pH 7.3) by size exclusion chromatography (2.3.4) and concentrated to 4 – 6 mg/ml using a stirred Amicon device with a 10 kDa cutoff cellulose membrane (Millipore, USA). Initial
Materials and Methods

Crystallization conditions were discovered by using standard screens in MRC3 sitting drop crystallization plates at the Protein Crystallization Center of the “National Center of Competence in Research (NCCR) Structural Biology”. Conditions, where crystals were observed were refined using 24-well Cryschem sitting drop plates (Hampton Research, USA) by varying the pH value and the concentrations of the precipitant and/or salts. The volume of the mother liquor reservoir amounted to 300 µl, and crystallization drops were set with 1.0 – 1.5 µl protein solution and 1.5 – 2.0 µl mother liquor to yield an initial drop size of 3.0 µl.

Crystals of CC50/190 grew at 4 °C with 1.8 – 2.0 M Li₂SO₄ as precipitant and 0.1 M Tris-acetate (pH 6.2 – 6.6) in the presence or absence of 3 or 10 mM CoCl₂. The initial protein concentration in the crystallization drops varied from 1.3 – 2.8 mg/ml.

Crystals of CC6/264 grew at 20°C with 16 – 21% PEG 3350 as precipitant and different buffers (0.1 M Tris-acetate, 0.1 M MES, or 0.1 M sodium malonate, pH 6.0 – 7.0) in the presence or absence of 10 mM CoCl₂. The initial protein concentration in the crystallization drops varied from 2.0 – 6.5 mg/ml. Single bar-shaped crystals grew at low protein concentrations (2.1 – 2.3 mg/ml). Crystal morphology was further improved by microseeding (Obmolova et al., 2010). A seed stock was prepared by resuspending crystals (3 wells) in 100 µl mother liquor and vortexing for 3 min. Seeding was performed using a Hampton seeding tool according to the manufacturer’s protocol. Conditions with PEG 3350 as precipitant and Tris-acetate buffer were seeded (iterative seeding).

2.7.2 Intermediate formation of ClyA

Assembly-incompetent ClyA variants were crystallized in the intermediate state in the presence of detergent (DDM or OG). 0.2 mg/ml of protein solution in TBS (pH 7.3) were incubated with 0.1% DDM or 1.0% OG oN at 4 °C and subsequently concentrated to 4 – 6 mg/ml using a stirred Amicon device with a 100 kDa cutoff cellulose membrane for DDM and a 30 kDa cutoff membrane for OG, respectively. Samples were centrifuged (16100 x g, 20 min, 4°C) before crystallization.

2.7.3 Crystallization of ClyA conformational transition intermediates

Crystallization of the trapped pore formation intermediate of ClyA K29E_G63E_G146E was performed in the presence of detergent (DDM or OG). Crystals were grown using the ClyA variant with or without the N-terminal His₆-tag. Initial crystallization conditions were discovered by using standard sitting-drop vapor diffusion screens at the Protein Crystallization Center of the NCCR Structural Biology. The initial refinement of
crystallization conditions was performed just as described for the monomeric variants (2.7.1). Crystallization in the presence of DDM occurred at 20 °C in conditions containing 26 – 32% Methylpentanediol (MPD) as precipitant and 0.1 M sodium acetate buffer (pH 4.1 – 5.0) in the presence of 0.2 M NaCl and/or 20 mM CaCl$_2$. Crystal morphology and diffraction was improved by iterative seeding. Crystallization in the presence of OG occurred at 20 °C in conditions containing 0.8 – 1.3% 1,6-Hexanediol as precipitant and 0.1 M sodium acetate buffer (pH 4.0 – 4.4) in the presence of 10 mM CoCl$_2$.

2.7.4 Cryoprotection of protein crystals

Crystals that grew under conditions containing no inherent cryoprotectant were gradually cryoprotected by stepwise addition of cryoprotection solution (CS; well solution containing 25 % glycerol). At the first step, 3 µl of CS were added to the protein crystallization drop, followed by incubation for 10 min after resealing the well. The procedure was repeated with 6 µl, 12 µl and finally 24 µl of CS. In the end, the drop contained 24 % glycerol as cryoprotectant. Crystals were fished with cryoloops (Hampton Research) and flash-frozen in liquid nitrogen.

2.8 Protein structure determination by X-ray crystallography

2.8.1 Data collection

Diffraction data were collected at the Swiss Light Source (SLS) X06SA (PX I) or X06DA (PX III) beamlines at the Paul Scherrer Institut, Villigen (Switzerland). PX I was equipped with a Pilatus 6M detector, PX III with a Pilatus 2M detector (Dectris Ltd., Switzerland), respectively. Data were collected at 77 K at an X-ray wavelength of 1.00 Å. To minimize radiation damage, the beam intensity was set to a maximum of 10% when collecting data of a variant containing an artificial disulfide bond.

2.8.2 Processing of diffraction data

The obtained crystal diffraction intensities were indexed and integrated using the program XDS (Kabsch, 2010). The XDS input file for the Pilatus 6M detector was obtained from the SLS (http://www.psi.ch/sls/pxi/ComputingEN/XDS.INP.P6M.X06SA) and modified according to the applied data collection strategy. After the initial round, processing was repeated with unit cell dimensions fixed to the obtained values and the values for reflection range and beam divergence were updated as suggested in INTEGRATE.LP. The resolution range was changed to obtain I/σ of at least 2.0 and a CC1/2 value greater than 0.5 for the highest
resolution shell in CORRECT.LP, as suggested in Evans (2011) and Karplus and Diederichs (2012).

Scaling was performed using the program XSCELE (Kabsch, 2010) with FRIEDEL'S_LAW=TRUE and MERGE=TRUE. The resolution shells were applied from the CORRECT.LP output file. Scaled data were converted to a CCP4 reflection file (mtz file) by XDSCONV (Kabsch, 2010). A subset of free reflections (R-free) was created using the REFLECTION_FILE_CONVERTER from the PHENIX program suite (Adams et al., 2010). 5% of all reflections were defined as R-free. The space group and unit cell of the obtained mtz file were validated by the programs POINTLESS (Evans, 2006) and PHENIX.XTRIAGE.

2.8.3 Molecular replacement

The phase problem was solved by molecular replacement using the program Phaser (McCoy et al., 2007). For crystal structures of monomeric CC6/264 (2-303) and (6-303), a modified coordinate file (pdb) of ClyA monomer (pdb ID 1qoy) with introduced mutations A6C, V264C, C87A, C286A and a truncated N-terminus (protein chain starting at Thr 2 or Cys 6, respectively) was used as search model. The identity was set to 90 %, and search was performed with six ClyA chains per asymmetric unit.

2.8.4 Refinement

After molecular replacement, the resulting pdb file was refined against the initial mtz file using PHENIX.REFINE. In the first three cycles of refinement, simulated annealing was carried out with a start temperature of 8000 K. In the case of CC6/264 Thr2, TLS refinement was applied with 3 to 4 TLS groups per ClyA chain defined by the TLSMD web server (Painter and Merritt, 2006). Geometry restraints for existing disulfide bonds were added. The bond length was set to 2.05±0.08 Å, and the bond angles were set to 109±5 °. Iterative cycles of automated refinement using PHENIX.REFINE and manual model building using COOT (Emsley and Cowtan, 2004) were performed until $R_{\text{free}}-R_{\text{work}}$ reached its minimum.
3 Results

3.1 Expression and purification of ClyA variants

All described ClyA variants (see Table 1) were expressed in *E. coli* Tuner DE3 cells grown in LB medium at 20 °C. The volume of the expression cultures was varied from 1.5 – 9 l dependent on the solubility and the expected yield of the respective ClyA variant.

In the first purification step, the His$_6$-tagged ClyA variants were subjected to metal chelate affinity chromatography (IMAC). The stepwise elution with buffers containing 70 mM, 200 mM, and 400 mM imidazole led to a purity of 70 – 90% dependent on the expression level and solubility of the respective ClyA variant, as estimated by SDS-PAGE (Figure 8, left panels). The major fraction of ClyA was eluted with 200 mM imidazole, while most impurities were eluted at the beginning of the 70 mM imidazole step. Protein-containing fractions (70_2, 200_1, 200_2 and 400 mM imidazole in the case of both ClyA variants shown in Figure 8 (wt and CC50/190)) were pooled and subjected to subsequent hydroxyapatite chromatography. Fraction 70_1 showed much more impurities in both cases, which was more pronounced for the less soluble variant CC50/190 and therefore it was discarded.

IMAC was followed by hydroxyapatite chromatography to remove remaining impurities. ClyA was eluted from the HA column by application of a linear gradient from 0 – 60% HA buffer B. Elution occurred at 25 – 45% buffer B, which corresponds to 110 – 180 mM potassium phosphate. As observed previously (Mueller, 2009), the HA elution profile of ClyA wt (Figure 9) shows two peaks with the second peak forming the major fraction (~80%). The same was observed for all ClyA variants. Only the protein from the second peak was used for all experiments because it had been shown that protein from the first peak does not crystallize (Mueller, 2009). Furthermore, fractions eluting at higher phosphate concentrations were less contaminated (Figure 8, right panels). Both in case of high-yield wt and low-yield CC50/190, the purity of ClyA after HA chromatography was at least 95%, as judged by SDS-PAGE. In case of subsequent crystallization, preparative gel filtration as additional purification step was performed. Otherwise, ClyA was used for experiments.
**Figure 8:** SDS-PAGE gels of IMAC and hydroxyapatite (HA) purifications of ClyA wt (top) and variant CC50/190 (bottom). 5 µl of pellet (P), supernatant (Sp) and flowthrough (Ft) fractions were loaded. 20 µl of all elution fractions (70_1 – 400 for IMAC and “gradient” for HA) were loaded. M: Protein molecular mass standard.

**Figure 9:** Hydroxyapatite chromatography elution profile of ClyA wt. 10 ml of protein solution after IMAC were subjected to a 30 ml HA column and eluted by application of a linear gradient of HA buffer B (0 – 60% B, 9 column volumes).
Table 1 shows an overview of the purification yields of all ClyA variants used in this work. Protein production using BL21 DE3 instead of Tuner DE3 decreased the protein yield due to weaker expression. In all but one case, mass spectrometry showed the expected mass within the accuracy of ESI-MS. In the case of G201P, the observed mass difference of 70 Da could be explained by re-sequencing the expression plasmid, which showed an additional mutation in the C-terminal region of Glu to Gly at position 299.

**Table 1:** Purification yields (mg protein per 1 l of medium) and mass spectrometry results of all ClyA variants described in this work.

<table>
<thead>
<tr>
<th>ClyA variant</th>
<th>yield (mg/1l)</th>
<th>theoretical mass (Da)</th>
<th>observed mass (Da)</th>
<th>remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>80</td>
<td>34581</td>
<td>34582</td>
<td></td>
</tr>
<tr>
<td>wt TEV Thr2</td>
<td>12</td>
<td>33627</td>
<td>33627</td>
<td></td>
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<tr>
<td>P36A</td>
<td>17</td>
<td>34555</td>
<td>34556</td>
<td></td>
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<tr>
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<td>12</td>
<td>34505</td>
<td>34506</td>
<td></td>
</tr>
<tr>
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<td>8.8</td>
<td>34595</td>
<td>not determined</td>
<td></td>
</tr>
<tr>
<td>G201A</td>
<td>6.6</td>
<td>34595</td>
<td>34597</td>
<td>BL21 DE3</td>
</tr>
<tr>
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<td>9.3</td>
<td>34595</td>
<td>n.d.</td>
<td>BL21 DE3</td>
</tr>
<tr>
<td>G180A G184A</td>
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<td>34609</td>
<td>n.d.</td>
<td>protein spilled</td>
</tr>
<tr>
<td>G201A G205A</td>
<td>5.3</td>
<td>34609</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>G205V</td>
<td>49</td>
<td>34623</td>
<td>34625</td>
<td></td>
</tr>
<tr>
<td>G180P</td>
<td>82</td>
<td>34621</td>
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<tr>
<td>G201P</td>
<td>52</td>
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<td>34551</td>
<td>additional IMAC after TEV cleavage</td>
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<tr>
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<td>34621</td>
<td>34621</td>
<td></td>
</tr>
<tr>
<td>G180P G201P</td>
<td>26</td>
<td>34662</td>
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<tr>
<td>G180P G205P</td>
<td>83</td>
<td>34662</td>
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<td></td>
</tr>
<tr>
<td>C87A C286A (&quot;wt&quot;)</td>
<td>27</td>
<td>34517</td>
<td>34521</td>
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</tr>
<tr>
<td>C87A C286A A6C V264C (&quot;CC6/264&quot;)</td>
<td>30</td>
<td>34553</td>
<td>34557</td>
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<tr>
<td>CC6/264 TEV Thr2</td>
<td>22</td>
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<td>33600</td>
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<tr>
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<td>33157</td>
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<tr>
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<td>3.1</td>
<td>34429</td>
<td>34431</td>
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<tr>
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<td>33476</td>
<td>additional IMAC after TEV cleavage</td>
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<tr>
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<td>34726</td>
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<td>33772</td>
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Purification of ClyA variants containing a TEV cleavage site between His6-tag and protein sequence needed an additional IMAC purification step after cleavage to remove uncleaved protein, cleaved tags, and TEV-protease. Cleavage efficiency of TEV protease after 4 h at RT and oN at 4 °C was between 50% and > 95%, as judged by SDS-PAGE (Figure 10). IMAC was also followed here by HA chromatography because the fraction containing cleaved protein still contained impurities. In most cases, the purification yield of ClyA variants with a TEV protease cleavage site was decreased compared to the corresponding variants without the cleavage site. This was not only caused by incomplete cleavage (e.g. the yield of ClyA variant protoarrest was decreased to 6% although cleavage had approximately 100% efficiency), but also by decreased protein solubility caused by the introduction of the TEV cleavage site, as reported previously (Kurz et al., 2006).

Figure 10: SDS-PAGE gels of IMAC purifications of ClyA variants CC50/190 and protoarrest after cleavage of the N-terminal His6-tag by TEV protease. In case of CC50/190, 20 µl of supernatant after cleavage (Sp), flowthrough (Ft), and wash fraction with 400 mM Imidazole were loaded. In case of protoarrest, 10 µl of all fractions including a sample of uncleaved protein (unc) were loaded. The positions of uncleaved (unc) and cleaved (cl) ClyA on the gel are indicated. M: Protein molecular mass standard.

3.2 Attempts to trap ClyA conformational transition intermediates by introducing point mutations in flexible regions

Comparison of the high-resolution structures of monomeric ClyA (Wallace et al., 2000) and a ClyA pore subunit (protomer) (Mueller et al., 2009) reveals major changes in secondary and tertiary structure. Mueller et al. (2009) proposed an extensive conformational transition mechanism from the monomer to the assembly-competent protomer, in which the hydrophobic β-tongue (residues 185 to 195) and the N-terminal α-helix A play key roles. The structural changes of both parts of ClyA give several possibilities to introduce point mutations that possibly will lead to the trapping of the ClyA conformational transition at a distinct intermediate state. It has already been shown that mutations of hydrophobic to
charged residues within the hydrophobic β-tongue of ClyA eliminate the hemolytic activity of ClyA completely (Ludwig et al., 2010; Wai et al., 2003b; Wyborn et al., 2004a) by inhibition of insertion of ClyA into membrane structures (Ludwig et al., 2010; Wallace et al., 2000). However, it is unlikely that the mutation from a bulky hydrophobic residue to a charged residue can rigidify the flexible loops flanking the β-tongue and therefore block the conformational transition. Figure 11 depicts a comparison of structures of ClyA monomer and protomer. The monomer’s β-tongue is flanked by loops containing each two glycine residues (180, 184, 201, and 205) which can function as “hinge points” for the proposed swinging-out movement of the β-tongue as initial step of the conformational transition. In the protomer, all four glycines are part of α-helices. The mutation of one or more of the glycine residues to alanine is thought to rigidify the flexible loop regions and prevent the complete swinging-out movement of the β-tongue leading to an assembly-incompetent, membrane-bound intermediate form structurally similar to the monomer. The second possibility to trap a conformational transition intermediate by a single amino acid exchange is a mutation of Pro 36, which forms a kink in α-helix A dividing it in two parts (αA1 and αA2). According to the proposed mechanism (Mueller et al., 2009), the last step of pore formation is the “opening” of the pore by wedging of the amphipathic helix αA1 through the membrane upon oligomerization. The helix interruption by Pro 36 is thought to function as a hinge for the membrane protrusion of αA1. The removal of Pro 36 would lead to a partially or completely oligomerized intermediate that is inactive because of an incomplete membrane protrusion.

![Figure 11: Details of crystal structures of ClyA monomer (left) and protomer (right) depicting the positions of glycine residues 180, 184, 201, and 205 in the head domain working as possible “hinges” for initiation of ClyA conformational transition, as suggested previously (Mueller et al., 2009). Cα atoms of Gly are shown as spheres. Phe 190 forming the central “latch” of the β-tongue and Pro 36, which forms a kink dividing α-helix A into two parts, are shown as stick representations. Figures were illustrated in PyMOL (Schrodinger, 2010).]

Therefore, ClyA was subjected to single and consecutively combined point mutations and the conformational transition rates and hemolytic activities of the obtained variants were
determined. The kinetics of the conformational transition from the monomer proceeding via an intermediate to the protomer were followed by far-UV CD spectroscopy as described by Eifler et al. (2006). After the rapid formation of an intermediate with less CD signal intensity at 225 nm relative to the monomer, the protomer that exhibits an increased CD signal intensity is formed in the second, rate-limiting step (Figure 12 A). The rate-limiting steps of the kinetics of different ClyA variants were fitted according to a first-order reaction (Figure 12 B and Table 2). Only the single or double exchange of Gly vs. Pro residues decreased the obtained rate constants by one order of magnitude, while Gly vs. Ala exchanges had a less pronounced effect or even opposite effect (G201A), as summarized in Table 2 and discussed below.

Hemolytic activities of ClyA variants were measured by observation of the decrease of turbidity of a horse erythrocyte suspension and evaluated as illustrated in Figure 12 C. The lag phase before hemolysis and the linear part of the lysis phase (35 – 75% of maximum OD) were fitted by two straight lines, yielding $t_{\text{lag}}$ (time point of intersection of both extrapolated lines) and $v_{\text{lysis}}$ (slope of fit line of $v_{\text{lysis}}$). The single amino acid exchange of three glycine residues proposed as hinge points (G 180, G 201, G 205) with alanine did not lead to a significant decrease of hemolytic activity compared to wt, as shown in Figure 12 D and summarized in Table 2. G201A even caused a slightly increased hemolytic activity, together with a 3.2-fold faster conformational transition in DDM (Figure 12 B, Table 2). G205A caused the greatest decrease of hemolytic activity, but still 52% of wild type activity was left. To increase the possible effect, a more bulky residue (valine) was chosen for glycine substitution. However, no greater activity decrease was observed (64% of wt activity in the case of G205V), although the in vitro conformational transition triggered by DDM was slowed down to 50% of G205A. Also the combined exchange of both glycine residues in the loops flanking the $\beta$-tongue N- and C-terminally (G180A_G184A and G201A_G205A, respectively) did not cause a more pronounced decrease of activity. To gain a further increase of rigidity of the loops, the glycines were substituted with prolines. Indeed, all three single Gly vs. Pro mutants exhibited a more pronounced decrease of hemolytic activity (6 – 22% of wt activity) compared to all other glycine substitutions, together with a 3.5 – 9.1-fold decrease of the conformational transition rates. Double Gly vs. Pro mutants (G180P_G201P and G180P_G205P) did not cause any hemolysis within 45 min at 37 °C. Their conformational transition rates in DDM are in the same order of magnitude as the ones of the single Gly vs. Pro mutants.
Figure 12: Comparison of the conformational transition kinetics and hemolytic activities of ClyA wt and glycine substitution variants. A: Far-UV CD spectra of 0.3 mg/ml ClyA wt as monomer, intermediate (40 s after triggering the conformational transition with 0.1% DDM), and protomer (4000 s after DDM addition). Kinetics in panel B were measured at 225 nm (dashed line). B: Kinetics of conformational transition of ClyA wt and selected glycine substitution variants (0.3 mg/ml each) upon starting the assembly reaction by addition of 0.1% DDM. The second, rate-limiting phase was fitted according to a unimolecular reaction (solid lines). Rate constants are summarized in Table 2. All CD measurements were performed in PBS (pH 7.3) at 22 °C. C: Kinetics of lysis of horse erythrocytes (2 × 10⁶ cells/ml) by 10 nM ClyA wt (black solid line). Kinetics were recorded via the decrease in optical density (OD) at 650 nm as a consequence of erythrocyte lysis. Two parameters were used for quantification of the hemolytic activity of ClyA: the lag time, which is the time point of intersection of the extrapolated lines (dashed) of the lag phase and the linear part of the OD decrease between 35 – 75% of the initial OD, and the lysis velocity, which is the slope of the linear part of OD decrease. D: Hemolysis kinetics of ClyA wt and selected glycine substitution variants (10 nM each). Rates of hemolysis are summarized in Table 2. All hemolysis measurements were performed in PBS (pH 7.3) at 37 °C.

Interestingly, the G201A mutant showed the highest hemolytic activity and conformational transition rate of the single Gly vs. Ala mutants, while G201P had the lowest hemolytic activity and conformational transition rate of the single Gly vs. Pro mutants. The opposite is true for G205A and G205P, respectively. The positioning of both residues in the protomer could give a possible explanation for that, at a first glance, contradictory fact. The loop G 201 – G 205 in the monomer forms a membrane-embedded α-helix in the protomer (cf. Figure 11 and Mueller et al. (2009)). Alanine has an increased propensity to be positioned in an α-helix compared to glycine (Zhu and Braun, 1999) in contrast to proline, which...
interrupts α-helices (e.g. Pro 36 in ClyA). The presence of a proline at position 201 might interrupt the α-helix already in the membrane-embedded part and therefore disturb membrane interaction leading to the loss of the hemolytic phenotype. This fact was observed for the introduction of Pro at position 16 in the N-terminal, membrane-spanning helix, which eliminates the hemolytic activity of ClyA completely (Ludwig et al., 2010). In contrast, the G201A mutation could lead to a faster formation of the transmembrane helix and result in a faster conformational transition rate and hemolysis, as it is shown here. A helix interruption at position 205 is less critical, as it is flanked by two charged residues (Glu 204 and Lys 206, respectively), and therefore Gly 205 cannot be located in the membrane-spanning region.

**Table 2:** Comparison of conformational transition rate constants (monoexponential fits of rate-limiting step) and hemolysis rates of ClyA wt and variants.

<table>
<thead>
<tr>
<th>ClyA variant</th>
<th>CD: intermediate-to-protomer transition rate constant (s⁻¹)</th>
<th>hemolysis: v_lys (∫MD/s)</th>
<th>hemolysis: % of wt v_lys</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>1.38 · 10⁻³</td>
<td>18.3</td>
<td>100</td>
</tr>
<tr>
<td>G180A</td>
<td>1.67 · 10⁻³</td>
<td>16.8</td>
<td>92</td>
</tr>
<tr>
<td>G201A</td>
<td>4.39 · 10⁻³</td>
<td>20.4</td>
<td>111</td>
</tr>
<tr>
<td>G205A</td>
<td>5.53 · 10⁻⁴</td>
<td>9.5</td>
<td>52</td>
</tr>
<tr>
<td>G205V</td>
<td>2.24 · 10⁻⁴</td>
<td>11.7</td>
<td>64</td>
</tr>
<tr>
<td>G180A G184A</td>
<td>1.30 · 10⁻³</td>
<td>10.2</td>
<td>56</td>
</tr>
<tr>
<td>G201A G205A</td>
<td>1.64 · 10⁻³</td>
<td>14.6</td>
<td>80</td>
</tr>
<tr>
<td>G180P</td>
<td>3.92 · 10⁻⁴</td>
<td>2.2</td>
<td>12</td>
</tr>
<tr>
<td>G201P</td>
<td>3.86 · 10⁻⁴</td>
<td>1.1</td>
<td>6</td>
</tr>
<tr>
<td>G205P</td>
<td>1.52 · 10⁻⁴</td>
<td>4.1</td>
<td>22</td>
</tr>
<tr>
<td>G180P G201P</td>
<td>1.76 · 10⁻⁴</td>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td>G180P G205P</td>
<td>2.30 · 10⁻⁴</td>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td>F190A</td>
<td>1.14 · 10⁻³</td>
<td>8.6</td>
<td>47</td>
</tr>
<tr>
<td>P36A</td>
<td>1.07 · 10⁻³</td>
<td>10.3</td>
<td>56</td>
</tr>
</tbody>
</table>

The mutation of Pro 36, which is proposed to function as hinge for the membrane protrusion of αA1 as final pore formation step, to an alanine does not have a major influence on the conformational transition rate and hemolysis velocity (78% and 56% of wt, respectively). Therefore, P36 does not have the proposed hinge function that is necessary for the membrane penetration of α-helix A.

As a consequence, none of the described single or combined point mutants of ClyA could be used for the determination of the high-resolution structure of a stable conformational transition intermediate. Besides both double Gly vs. Pro mutants all variants still had residual hemolytic activity, meaning that functional pores are formed in the end. The double
Gly vs. Pro mutants are most likely inactive due to hindered membrane protrusion because of interruptions in the transmembrane parts of helices formed in the protomer. Therefore, different strategies to trap kinetically stable intermediates were applied, namely the introduction of artificial cysteine pairs (section 3.3), or the prevention of the final pore assembly by introducing point mutations at the intersubunit interface of the protomer (section 3.4).

3.3 Trapping of ClyA conformational transition intermediates by introduction of artificial disulfide bonds

3.3.1 Cysteine-free ClyA as basis for introduction of artificial disulfide bonds in comparison with reduced and oxidized wt

Wild type ClyA possesses two cysteine residues in close spatial proximity (C87, C285) which are able to form a disulfide bond. Despite other published results (Atkins et al., 2000; Fahie et al., 2013; Wai et al., 2003a), the detergent- and membrane-induced conversion of the monomer to the active pore complex is independent of the redox state of this cysteine pair (Eifler et al., 2006), as evidenced by a hemolysis kinetic assay (Figure 13 C). To eliminate the possibility of formation of wrong disulfide bonds in ClyA variants bearing an additional, engineered disulfide bond that traps ClyA in an assembly-incompetent state, a cysteine-free ClyA variant (wt*) was designed, in which the residues Cys 87 and Cys 285 were replaced by alanine residues. A replacement of Cys 87 and Cys 285 by serine residues as reported previously (Atkins et al., 2000) resulted in a seriously decreased expression yield (below 1 mg per liter of expression culture; data not shown), meaning a massive destabilization of the protein fold. In contrast, the purification of the double alanine mutant (wt*) resulted in a reasonable yield of pure protein (27 mg per l of expression culture; cf. Table 1). Like the reduced wild type protein, wt* proved to be fully assembly competent upon addition of the detergent DDM, as judged by negative-stain transmission electron microscopy (Figure 13 A).

In addition, it showed transition and assembly kinetics very close to those of ClyA wt, characterized by rapid formation of an intermediate with less negative ellipticity at 225 nm relative to the monomer, and a slow, rate-limiting conversion to the protomer with increased negative ellipticity (Figure 13 B), which corresponds to the increased \( \alpha \)-helical content of the protomer relative to the monomer (Mueller et al., 2009). In the membranes of red blood cells, ClyA wt* forms functional pore complexes leading to a 2.5-fold decreased \( \nu_{\text{lysis}} \) compared to wild type (5.9 mOD/s vs. 14.5 mOD/s, respectively; Figure 13 C). As both in
*vitro and in vivo* pore formation had been proved for wt*, variants which engineered cysteine pairs could be designed based on this variant.

**Figure 13:** Pore formation and hemolytic activity of cysteine-free ClyA wt* in comparison to ClyA wt. 
A: Negatively stained electron micrographs (2% uranyl acetate) of ClyA wt and wt* (5 μM monomer concentration each) after induction of pore formation with 0.1% DDM at 22 °C in PBS (pH 7.3) and incubation for 1.5 h. Scale bar: 100 nm. 
B: Kinetics of the conformational transition of ClyA wt and wt* (8.9 μM each) initiated by addition of 0.1% DDM in PBS (pH 7.3) and 22°C, recorded with the change in CD signal at 225 nm. The signal intensities of monomeric ClyA at 225 nm are indicated on the y-axis. The second, rate-limiting phase was fitted according to a unimolecular reaction (solid, black lines). The deduced rate constants of the rate-limiting step are average values of three independent measurements. ClyA wt (gray): k = 1.44±0.16 x 10^{-3} s^{-1}; ClyA wt* (green): k = 1.45±0.08 x 10^{-3} s^{-1}. 
C: Comparison of the hemolytic activities of reduced and oxidized ClyA wt and wt* at 37°C in PBS (pH 7.3). Reactions were initiated by mixing horse erythrocytes at a density of 2 x 10^6 cells/ml with the respective ClyA protein (10 nM). Lysis was recorded via the decrease in optical density at 650 nm.

### 3.3.2 Design of ClyA variants with cysteine residues able to form artificial disulfide bonds: CC50/190 and CC6/264

Two variants of wt*, CC50/190 and CC6/264, in which an additional cysteine pair was introduced with the possibility of trapping the monomer in an assembly incompetent state were created. In the first variant, CC50/190, which was designed by manual modeling in PyMOL (Schrodinger, 2010), a disulfide formed by the engineered cysteine pair would fix the β-tongue to the N-terminus of the structurally conserved helix B and prevent the proposed outward swinging of the β-tongue (Mueller et al., 2009) and its membrane insertion (Figure 14 A) The Cβ atoms of the wild type residues (both Phe) are located 5.0 Å apart. The disulfide bond in the second variant, CC6/264, which was predicted by the program “Disulfide by Design” (Dombkowski, 2003) would tie the N-terminus of helix A to the structurally conserved loop between helices F and G (Figure 14 B). The Cβ atoms of the wild type residues (Ala 6 and Val 264) are located 4.3 Å apart. In the protomer, the engineered cysteine residues of CC50/190 and CC6/264 are moved away from each other to theoretical Cβ-Cβ-distances of 27.2 Å and 127.5 Å, respectively, making a formation of protomers impossible when the engineered cysteine residues are covalently linked by disulfide bonds.
Figure 14: A: Positions of the engineered cysteines at residues 50 and 190 to immobilize the β-tongue with a disulfide bond. Models based on the structures of the ClyA monomer (pdb ID 1QOY) and the protomer in the pore complex (pdb ID 2WCD) are shown. The β-strands of the β-tongue are colored yellow, the head domain (residues 160 – 205) is colored blue, α-helix A is colored light blue. B: Position of the engineered cysteines at residues 6 and 264 to immobilize the N-terminal ClyA helix. Models based on the structures of the ClyA monomer and the protomer in the pore complex are shown. Residues 2–7 are not resolved in the crystal structure of the pore complex and were modeled to visualize the position of Cys 6. Figures were illustrated in PyMOL (Schrodinger, 2010).

3.3.3 Formation of artificial disulfide bonds by O₂-mediated oxidation

CC50/190 and CC6/264 were purified in the presence of the reducing agent DTT resulting in sufficient purification yields (cf. Table 1). After removal of DTT, both variants were subjected to Cu²⁺-catalyzed air oxidation to form the artificial disulfide bonds 50-S-S-190 and 6-S-S-264, respectively. After incubation of both variants of ClyA with 0.5 mM CuCl₂ (4 h at room temperature and pH 7.3), precipitate was visible in the reaction tubes. The precipitation was independent of the presence of the hexahistidine tag, hence most likely surface-exposed histidine residues of ClyA are involved in Cu²⁺-mediated protein aggregation. In all cases, the precipitate could be resolved nearly completely after on dialysis against PBS buffer containing 2 mM EDTA. Therefore, the Cu²⁺-catalyzed air oxidation of ClyA does not lead to modification of histidine residues which are responsible for irreversible protein aggregation, as observed elsewhere (Khossravi et al., 2000; Requena et al., 2001). Furthermore, the absence of oxidation products of other amino acids in ClyA CC50/190 ox and CC6/264 ox was proved by mass spectrometry.
After oxidation and dialysis, ClyA variants were subjected to gel filtration on a Superdex 200 column to remove disulfide-mediated aggregates and oligomers. Exactly as their reduced forms, oxidized ClyA CC50/190 and CC6/264 eluted exclusively as monomers with no other detectable peaks (Figure 15 A and B). The elongated form of ClyA causes its elution at a higher apparent molecular weight than expected (ca. 41 kDa instead of 34.6 kDa).

Figure 15: Comparison of elution profiles of size exclusion chromatography (SEC) and reversed-phase HPLC of the reduced and oxidized ClyA variants CC50/190 and CC6/264. A, B: Gel filtration profiles of reduced and oxidized ClyA CC50/190 and CC6/264 applied to a Superdex 200 10/300 column equilibrated with PBS (pH 7.3) at 4 °C, showing the monomeric state of the variants in both redox forms. 200 µl of 0.3 mg/ml protein were injected. The exclusion volume of the Superdex 200 column and the elution volumes of marker proteins are indicated. C: Reversed-phase HPLC elution profiles of reduced and oxidized ClyA CC50/190 and CC6/264 applied to a 300SB C8 column. 50 µl of 50 µM protein were injected and eluted via a gradient of 30-80% acetonitrile at 30 °C.

The quantitativeness of oxidation was verified by Ellman’s assay under denaturing conditions (4 M guanidinium hydrochloride, 30 min, 37 °C, pH 7.3). Additionally, the amounts of oxidized and reduced ClyA CC50/190 and CC6/264 (verified by Ellman’s assay) were quantified via reversed-phase HPLC using a Zorbax 300SB C8 column. The elution profiles of both ClyA variants after oxidation and gel filtration exhibited only one distinct peak, corresponding to the oxidized species (Figure 15 C). CC50/190 in its reduced form (sample of freshly purified protein containing 2 mM DTT) showed one peak that is eluted at higher acetonitrile concentrations than the oxidized form. In contrast, CC6/264 purified in the presence of 2 mM DTT contained a small fraction (10%) of oxidized protein, most likely due to air oxidation of DTT and protein.

3.3.4 X-ray crystal structures of CC6/264 show the artificial disulfide bond

ClyA variants CC50/190 and CC6/264 were crystallized in the monomeric forms to show the presence of the artificial disulfide bonds and their influence on the overall tertiary structure of ClyA. Initial crystallization screens yielded crystals for both variants. Protein crystals of CC50/190 grew at 4 °C with Li₂SO₄ as precipitant at pH values around 6.5. However, crystals never grew to sizes above 50 µm and did not show sharp edges. The removal of the N-terminal His₆-tag resulted in crystals with slightly increased sizes with
sharp edges. The best crystal of CC50/190 grew in space group I222 with 1.3 mg/ml initial protein concentration in 1.9 M Li$_2$SO$_4$, 0.1 M Tris-Acetate pH 6.3, 10 mM CoCl$_2$ for a time period of 9 months and diffracted to 3.0 Å at maximum when cryoprotected with 25% glycerol. Molecular replacement using ClyA wt (pdb ID 1QOY) with point mutations C87A, C285A, F50C and F190C as search model showed one ClyA chain per asymmetric unit. The resulting low-resolution (3.0 Å) electron density map was not suitable to identify the position of the artificial disulfide bond 50-S-S-190 with certainty (data not shown).

The disulfide-bonded CC6/264 variant could be crystallized in two different forms after cleavage of the N-terminal His$_6$-tag, one comprising residues 2–303 (corresponding to ClyA wt where N-terminal residue M1 is cleaved) and an N-terminally truncated form comprising residues 6–303. In both cases, crystals grew with PEG 3350 as precipitant at pH values from 6-7 either as plate stacks predominating at initial ClyA concentrations above 2.5 mg/ml or as single bars with lengths up to 400 µm at lower ClyA concentrations. The crystal of CC6/264 (2-303) used for data collection grew in 21% PEG 3350 and 0.1 M Tris acetate pH 6.5 at an initial protein concentration of 2.0 mg/ml. The crystal of CC6/264 (6-303) used for data collection grew in 19% PEG 3350 and 0.1 M Tris acetate pH 6.7 at an initial protein concentration of 2.1 mg/ml. Both crystals were cryoprotected with 25% glycerol as described in section 2.7.4. By applying molecular replacement using the pdb of ClyA wt (1QOY) with all point mutations present in the variant CC6/264 as search model, the structures of CC6/264 (2-303) and CC6/264 (6-303) were solved to resolutions of 2.12 Å and 1.94 Å, respectively. Crystals were obtained in two different space groups: C222$_1$ (three monomers in the asymmetric unit) in the case of CC6/264 (2-303) and C2 (four monomers in the asymmetric unit) in the case of CC6/264 (6-303) (Figure 16). In contrast, crystals of the published structure of ClyA wt grew in a P4$_3$2$_1$2 unit cell with one monomer in the asymmetric unit (Wallace et al., 2000). Due to conformational flexibility of parts of the ClyA monomers in the crystal packing of CC6/264 (6-303), there is a lack of electron density in the loop regions of the head domain of Chain B. As a consequence, residues 178-183 and 195-201 are not modeled in the final structure (Figure 16 and Figure 17, right panels). The electron density maps shown in Figure 17 clearly indicate the presence of the artificial disulfide bond in at least one of the three protein chains found in the asymmetric unit (chain A) in the case of CC6/264 (2-303) and in at least one of the four protein chains (chain B) in the case of CC6/264 (6-303). The disulfide bonds in the other chains could either not be depicted due to a lack of electron density as a consequence of the flexible N-terminal region (chains A and D; chain C of the 2.12 Å structure) or they were destroyed by X-ray radiation (chain C; chain B of the 2.12 Å structure) (Weik et al., 2000). Besides the local environment of the artificial disulfide bonds and the loops flanking the β-tongue, the
structures of CC6/264 showed no great deviations from that of the ClyA wt monomer, with mean alpha carbon r.m.s. deviations of 0.9 and 0.8 Å (determined by DaliLite (Holm and Park, 2000)), respectively (Figure 17).

Figure 16: Arrangement of ClyA molecules in the crystallographic asymmetric units of CC6/264 (2-303, left) and CC6/264 (6-303, right). The C222₁ unit cell of CC6/264 (2-303) contains three ClyA monomers. The C2 unit cell of CC6/264 (6-303) contains four ClyA monomers. The positions of the intact disulfide bonds in chain A of CC6/264 (2-303) and in chain B of CC6/264 (6-303) are indicated (*). Figures were prepared in PyMOL (Schrodinger, 2010).
Figure 17: Ribbon representations of structural alignments of ClyA wt (pdb ID 1QOY) with chains from crystal structures of ClyA CC6/264 containing the artificial disulfide bond 6-S-S-264 (top). Structural alignments were performed using DaliLite (Holm and Park, 2000). The positions of wild type (87 and 285) and artificial (6 and 264) cysteine residues are indicated. Left: 2.12 Å crystal structure of ClyA CC6/264 (2-303) (blue) aligned with the 2.0 Å crystal structure of ClyA wt (green). Right: 1.94 Å crystal structure of ClyA CC6/264 (6-303) (red) aligned with the 2.0 Å crystal structure of ClyA wt (green). Sections of both structures depicting the artificial disulfide bond (bottom). The electron density maps (contoured at 1.0 sigma) are shown for residues Val 5 - Asp 7 (2-303, left) or Cys 6 - Asp 7 (6-303, right) and Thr 260 - Asp 268. Figures were illustrated in PyMOL (Schrodinger, 2010).
Table 3 summarizes crystallographic data collection and model statistics after the final round of refinement of both crystal structures of CC6/264.

**Table 3**: Crystallographic data collection and model refinement statistics for ClyA CC6/264.

<table>
<thead>
<tr>
<th>ClyA variant</th>
<th>CC6/264 (2-303)</th>
<th>CC6/264 (6-303)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Crystal form</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Space group</td>
<td>C222, (20)</td>
<td>C2 (5)</td>
</tr>
<tr>
<td>Unit cell dimensions a,b,c (Å); α, β, γ (°)</td>
<td>94.92, 125.53, 186.79; α = β = γ = 90°</td>
<td>176.88, 48.44, 152.43; α = γ = 90°, β = 102.34°</td>
</tr>
<tr>
<td><strong>Data collection</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wavelength (Å)</td>
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<td>1.0</td>
</tr>
<tr>
<td>Resolution range (Å)</td>
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<td>40.00 – 1.94</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>63155 (9986)</td>
<td>85704 (12628)</td>
</tr>
<tr>
<td>Completeness (%)</td>
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<td>91.0 (89.2)</td>
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<td>Rmerge (%)</td>
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<td>8.0 (51.8)</td>
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<tr>
<td>I/σ</td>
<td>21.63 (3.10)</td>
<td>11.50 (2.55)</td>
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<td><strong>Model Statistics</strong></td>
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<td>Reflections working/free sets</td>
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<tr>
<td>Allowed (%)</td>
<td>0.81</td>
<td>0.52</td>
</tr>
</tbody>
</table>

3.3.5 Disulfide-trapped variants are non-hemolytic and do not form pores

Next, the behavior of oxidized CC50/190 and CC6/264 upon triggering the assembly by addition of detergent (DDM) was tested and compared with the respective reduced species. The disulfide forms of both CC50/190 and CC6/264 proved to be assembly incompetent upon addition of DDM to the monomers, as evidenced by the absence of pore complexes after prolonged incubation in DDM (Figure 18, right panels). Both CC50/190 and CC6/264 however formed intact pore complexes in DDM in their reduced states (Figure 18, right panels). As observed for ClyA wt (cf. Figure 13 B), their conformational transition from the monomer to the assembly competent protomer proceeded via the transient population of an intermediate state with less negative ellipticity, followed by the rate-limiting, unimolecular transition to the protomer (Figure 18, left panels). The only significant difference between the reduced ClyA wt and the variants CC50/190red and CC6/264red was a 2.6 and 1.5-fold slower, rate-limiting reaction to the protomer, respectively. Like in wt ClyAred, the second, rate-limiting reaction of formation of assembly competent protomers coincided with the
formation of active pores: The electron micrographs of the ClyA CC50/190<sub>red</sub> and CC6/264<sub>red</sub> pores depicted in Figure 18 were recorded after an incubation time when a constant circular dichroism (CD) signal at 225 nm had been reached and showed the same amount of active pores as the reduced wild type at the end of the CD kinetics (cf. Figure 13 A). To conclude, the assembly of pore complexes from assembly-competent protomers is faster than DDM-induced protomer formation and not rate-limiting for formation of pores of any of the investigated ClyA variants at concentrations around 10 µM.

**Figure 18:** Kinetics of the conformational transition of oxidized and reduced ClyA variants CC50/190 (top) and CC6/264 (bottom) (0.3 mg/ml each) initiated by addition of 0.1% DDM at pH 7.3 and 22 °C, recorded with the change in CD signal at 225 nm. Kinetics of the reduced variants were fitted according to a consecutive mechanism (Equation 2); solid, black lines), yielding rate constants for the second, rate-limiting step of 5.5 x 10<sup>-4</sup> s<sup>-1</sup> and 9.4 x 10<sup>-4</sup> s<sup>-1</sup>, respectively. Right panels: Negatively stained electron micrographs (2% uranyl acetate) of oxidized and reduced CC50/190 and CC6/264 subsequent to the kinetics (1.5 h after addition of 0.1% DDM). Scale bar: 100 nm.

The hemolytic activity of the reduced and oxidized variants CC50/190 and CC6/264 was monitored by the decrease in erythrocyte density after addition of ClyA monomers. In their reduced state, both CC50/190 and CC6/264 showed clear hemolytic activity, with a hemolysis time course similar to that of ClyA wt (cf. Figure 13 C). Specifically, the reactions showed an initial lag phase, which was followed by a fast decrease of cell density and a
slow final phase until lysis was completed. As expected, the disulfide trapped variants CC50/190 and CC6/264 showed no hemolytic activity (Figure 19 A). Even after overnight incubation, the erythrocytes were still intact.

With a molar excess of $6 \times 10^6$ ClyA monomers over erythrocytes (equivalent to $5 \times 10^5$ pores per erythrocyte) in our measurements and a minimum of 1000 pores per erythrocyte required for hemolysis (Eifler et al., 2006), it is shown here that less than 0.2% of disulfide-trapped ClyA variants can form functional pore complexes. In comparison, the hemolytic activity of ClyA wt did not change significantly upon oxidation of the natural cysteine pair (cf. Figure 13 C).

**Figure 19:** Hemolysis kinetics recorded after mixing of ClyA variants CC50/190 and CC6/264 with horse erythrocytes ($2 \times 10^6$ erythrocytes/ml) at 37 °C and pH 7.3. Lysis was recorded via the decrease in optical density at 650 nm. A: Hemolysis kinetics recorded for the oxidized and reduced ClyA variants CC50/190 and CC6/264 (20 nM each, corresponding to a $6 \times 10^6$-fold excess of ClyA monomers over erythrocytes). B: Linear dependence of maximum lysis velocity on ClyA concentration as a quantitative measure of hemolytic activity. The following parameters were obtained for the different ClyA variants: wt: 0.96 mOD/(s*nM); wt*: 0.48 mOD/(s*nM) CC50/190: 0.70 mOD/(s*nM); CC6/264: 0.16 mOD/(s*nM). Error bars: standard deviation of three independent measurements.

Subsequently the specific activity of the pores formed by ClyA and its variants was quantified. For this purpose, the kinetics of lysis of erythrocytes was determined as a function of the ClyA monomer concentration. Specifically, the lysis of erythrocytes upon addition of ClyA monomers, recorded via the decrease in optical density at 650 nm, shows a lag phase, followed by rapid, linear decrease in cell density, and a slower, final phase until lysis was completed. The lag phase before start of decrease of optical density and the linear part of decrease of optical density between 70 and 35% of the initial density were fitted by two straight lines. (cf. Figure 12 C) The maximum velocity of cell lysis, defined as the linear decrease in cell density between 70% and 35% of the initial density, proved to correlate linearly with ClyA monomer concentration in the range of 1-100 nM, allowing a quantitative comparison of the hemolytic activity of ClyA wt and variants. The results in
Figure 19 B show that WT*, CC50/190\textsubscript{red} and CC6/264\textsubscript{red} still possess 51, 73 and 16% wild type activity, respectively. The lower activity of the ClyA variants most likely does not only result from their slower conversion to the assembly-competent protomer measured in the presence of DDM, as WT* reacts about 2.6-fold faster to the protomer than CC50/190 (Figure 13 B and Figure 18), but nevertheless has a lower hemolytic activity than CC50/190. The differences in hemolytic activity may thus be a consequence of additional factors, such as different reaction rates to the protomer in lipid environment from those measured with DDM-triggered pore formation, different specific permeabilities of the assembled pores, or differences in protomer association rates (which become rate limiting for pore assembly at the low ClyA concentrations (1-100 nM) used in the hemolysis assays (Stephan Benke, unpublished data)).

### 3.3.6 Reduction reactivates disulfide-trapped CC50/190 and CC6/264

ClyA variants CC50/190 and CC6/264 were inactivated by formation of each one intramolecular disulfide bond, which covalently binds a region with conformational flexibility crucial for pore formation (β-tongue and α-helix A1, respectively) to another part of the protein. Thus, the re-reduction of the artificial disulfide bonds should release the trapped regions and restore the hemolytic activity of ClyA. Therefore, the kinetics of activation of the oxidized forms of CC50/190 and CC6/264 by the reductant DTT was investigated. For this purpose, erythrocytes were pre-incubated with variants CC50/190\textsubscript{ox} or CC6/264\textsubscript{ox}, and the reduction was triggered by addition of 10 mM DTT. Hemolysis kinetics were monitored again just as described before. In both cases the pore-forming activity was recovered leading to quantitative lysis of red blood cells. However, the kinetics of hemolysis upon reduction differed significantly for both variants. The β-tongue bound variant CC50/190\textsubscript{ox} was reduced readily under the chosen conditions leading to a very similar hemolysis lag time (1.4-fold increased) and lysis velocity (1.4-fold decreased) compared to the pre-reduced protein, while the helix A bound variant CC6/264\textsubscript{ox} showed a 6.1-fold increased lag time and an 2.6-fold decreased lysis velocity compared to the pre-reduced protein (Figure 20 A). The results indicate that the disulfide bond of CC6/264\textsubscript{ox} is less accessible for reductants compared to the one in CC50/190\textsubscript{ox}.

The apparent differences in reducibility were quantified by determination of the reduction rates of monomeric CC50/190\textsubscript{ox} and CC6/264\textsubscript{ox} under the same environmental conditions in which the hemolysis kinetics were recorded (37°C, pH 7.3, 10 mM DTT). Samples were taken after distinct time intervals and analyzed via reversed-phase HPLC (shown exemplary for CC50/190 in Figure 20 B) after stopping the disulfide reduction by acid.
quenching (addition of formic acid to an end concentration of 12%). The decay of remaining oxidized species over time was fitted according to a pseudo-first order reaction and yielded apparent rate constants of $1.55 \times 10^{-2} \text{s}^{-1}$ for CC50/190 and $2.41 \times 10^{-4} \text{s}^{-1}$ for CC6/264 corresponding to second-order rate constants of $1.55 \text{M}^{-1} \text{s}^{-1}$ and $2.41 \times 10^{-2} \text{M}^{-1} \text{s}^{-1}$, respectively. A disulfide bond formed by the two natural cysteine residues of ClyA wt (C87, C285) is not reducible even by a 20-fold amount of DTT in the 10-fold time (Figure 20 C). The results correlate well with the measured hemolysis kinetics of reduction of both ClyA variants, where the two orders of magnitude slower reduction of CC6/264 led to the large increase of lag time compared to CC50/190 (cf. Figure 20 A). In the case of CC50/190, the hemolysis lag time is slightly elongated compared to the pre-reduced variant although the reduction half-life with 10 mM DTT is a factor of 5 shorter than the lag time. This is clear evidence for the trapping of an intermediate state before the rate-limiting step of pore formation in contrast to the arrest at a pre-oligomerized intermediate state leading to an almost complete loss of the hemolysis lag phase (Hotze et al., 2001).

**Figure 20**: Regain of hemolytic activity of ClyA CC50/190 and CC6/264 upon reduction. A: Kinetics of activation of the oxidized variants CC50/190 and CC6/264 (20 nM each) by reduction with 10 mM DTT in the presence of horse erythrocytes ($2 \times 10^6$ cells/ml) at $37 \degree \text{C}$ and pH 7.3. Reactions were initiated by addition of DTT. The dashed lines show the hemolysis kinetics of the fully reduced variants (cf. Figure 19). B: Quantification of amounts of reduced and oxidized ClyA CC50/190 by reversed-phase HPLC. CC50/190 was reduced by 10 mM DTT at pH 7.3 and $37 \degree \text{C}$. After the indicated time intervals, disulfide reduction was quenched by acidification with 12% formic acid and samples were analyzed on a 300SB C8 column. C: Kinetics of the reduction of the single disulfide bond in oxidized ClyA CC50/190 and CC6/264 (5 mM each) by 10 mM DTT at $37 \degree \text{C}$ and pH 7.3 in the monomeric form (solid circles) or after 1 h incubation in 0.1% DDM (open squares). Solid lines correspond to second-order fits. The engineered disulfides in monomeric CC50/190 and CC6/264 are reduced with second-order rate constants of $1.55\pm0.02 \text{M}^{-1} \text{s}^{-1}$ and $0.024\pm0.001 \text{M}^{-1} \text{s}^{-1}$, respectively. The rate constants of reduction in the presence of DDM change to $0.86\pm0.01 \text{M}^{-1} \text{s}^{-1}$ (CC50/190) and $1.23\pm0.02 \text{M}^{-1} \text{s}^{-1}$ (CC6/264). The disulfide bond of oxidized ClyA wt is not reduced significantly in a comparable time frame, even with 200 mM DTT (solid gray circles).

In the presence of 0.1% DDM, the reduction rates of both variants are shifted to the same order of magnitude (Figure 20 C). The reduction rate of CC50/190ox is slightly decreased ($0.86 \text{M}^{-1} \text{s}^{-1}$ vs. $1.55 \text{M}^{-1} \text{s}^{-1}$), while the reduction of CC6/264ox is accelerated by two orders of magnitude ($1.23 \text{M}^{-1} \text{s}^{-1}$ vs. $0.024 \text{M}^{-1} \text{s}^{-1}$; Figure S5). The nearly identical reducibilities of both variants in the presence of DDM indicate the formation of trapped intermediate states
with a very similar surface accessibility of the artificial disulfide bonds in the head domain (CC50/190) and the tail domain (CC6/264). The decreased DTT accessibility of the \(\beta\)-tongue of CC50/190 in the presence of DDM compared to the monomer, expressed by a 1.8-fold decrease of the reduction rate (0.86 M\(^{-1}\) s\(^{-1}\) and 1.55 M\(^{-1}\) s\(^{-1}\), respectively) indicates binding of the detergent molecules mainly to the \(\beta\)-tongue around position 190. This is expected because of the accumulation of hydrophobic residues in this region (Mueller et al., 2009).

### 3.3.7 The trapped conformational transition intermediates show molten globule-like properties

The sequential model of the monomer-to-protomer transition of ClyA suggested by Mueller et al. (2009) based on the comparison of structures of ClyA monomer and pore proposes distinct intermediate states that retain major parts of their tertiary structure (cf. Figure 7). According to the model, the initial membrane interaction is mediated by the \(\beta\)-tongue, whose conformational flexibility is blocked in CC50/190\(_{ox}\) by the covalent attachment with the engineered disulfide bond 50-S-S-190. Nevertheless, CD kinetics showed the variant undergoing the first step of the monomer-to-protomer transition with the rapid loss of CD signal intensity (cf. Figure 18) just as the wild type or reduced CC50/190. Additionally, the engineered disulfide bonds of CC50/190\(_{ox}\) and CC6/264\(_{ox}\) have almost the same reducibility and therefore accessibility to DTT (cf. Figure 20 C), although according to the model both intermediate states should be trapped at different states of the conformational transition: CC50/190\(_{ox}\) at the first step of membrane interaction, CC6/264\(_{ox}\) at the second step when the \(\beta\)-tongue is already bound to the membrane (cf. Figure 7).

These inconsistencies of the data shown here with the model of conformational transition suggest that other intermediate states than postulated may form in the presence of DDM. All attempts to crystallize CC50/190\(_{ox}\) and CC6/264\(_{ox}\) in the presence of DDM failed, the crystallization wells of various standard and membrane protein screens showed heavy precipitation already at protein concentrations as low as 2.0 mg/ml. Single molecule fluorescence resonance energy transfer (FRET) studies proved the presence of a transient intermediate with a FRET efficiency of an unfolded chain (Stephan Benke, unpublished data). However, the intermediate retains 75% of monomer’s far-UV CD signal at 225 nm (cf. Figure 12 A and Figure 18). These findings suggest that the conformational transition intermediates of ClyA in DDM are molten globules exposing hydrophobic regions, which can be demonstrated by the binding of the hydrophobic fluorescent dye 8-Anilino-1-naphthalenesulfonic acid (ANS) (Uversky et al., 1996).
Therefore, ANS was applied to demonstrate the surface exposition of hydrophobic regions of ClyA during pore formation. ANS was incubated with ClyA CC50/190 and CC6/264 in the oxidized as well as in the reduced forms in the presence of 0.1% DDM. Figure 21 A, B shows fluorescence emission spectra of ANS together with ClyA pores (wt, wt*, CC50/190_red, CC6/264_red) and conformational transition intermediates (CC50/190_ox, CC6/264_ox). The fluorescence of ANS in the presence of pores of different ClyA variants did not differ significantly, while both intermediates formed by CC50/190_ox and CC6/264_ox increased ANS fluorescence 3.1 and 3.8-fold compared to the pores, respectively. These findings indicate the formation of an intermediate with molten-globule like properties, as the binding of ANS to hydrophobic patches exposed by molten globules causes a fluorescence increase (Poklar et al., 1997; Uversky et al., 1996). Monomeric ClyA in the absence of DDM did not result in a measurable ANS fluorescence increase (not shown).

Next, the time course of ANS fluorescence after triggering the monomer-to-protomer transition with DDM was recorded. In the case of CC50/190_ox and CC6/264_ox, ANS fluorescence increased rapidly with first order rate constants of $9.1 \times 10^{-2}$ s$^{-1}$ and $2.1 \times 10^{-2}$ s$^{-1}$, respectively, and remained at a constant level of increased ANS fluorescence (Figure 21 D). The rates of formation of the intermediate measured by ANS fluorescence increase are comparable to the rates of formation of the intermediate measured by far-UV CD spectroscopy (Figure 18), as summarized in Table 4. That confirms the intermediate causing the increased ANS fluorescence shows residual α-helical secondary structure.

In contrast, the ANS fluorescence of CC50/190 and CC6/264 in the reduced states declined with rates of $7.8 \times 10^{-4}$ s$^{-1}$ and $1.5 \times 10^{-3}$ s$^{-1}$, respectively, after the initial increase of intensity approaching the signal intensity of ANS with 0.1% DDM in the end (Figure 21 F). These findings indicate the formation of a final species without major surface-exposed, hydrophobic regions corresponding to the pore complex embedded in a DDM micelle (Mueller et al., 2009). The results also show that the ANS binding to the intermediate is reversible. Again, the rate constants correspond well with the intermediate-to-protomer rate constants obtained by CD spectroscopy ($5.5 \times 10^{-4}$ s$^{-1}$ and $9.4 \times 10^{-4}$ s$^{-1}$, respectively), indicating the good correlation of conformational transition and reversible ANS binding to the molten globule-like intermediate. The slightly higher rate constants obtained by ANS fluorescence kinetics compared to CD kinetics could be a result of differences in the experimental setups predefined by fluorescence and CD spectroscopy (stirred vs. unstirred cuvette; different surface-to-volume ratios of cuvettes).
Results

Figure 21: Detection and characterization of the intermediate of the monomer-to-protomer transition of ClyA variants CC50/190 and CC6/264 by measurement of changes of ANS fluorescence at 475 nm. All measurements were done with 5 µM ClyA and 20 µM ANS at 22 °C and pH 7.3. A, B: ANS fluorescence emission spectra of ClyA CC6/264 and CC50/190 in the oxidized state (intermediate) and the reduced state (protomer) compared with ClyA wt and wt*. Spectra were recorded after 2 h incubation in 0.1% DDM at 22°C. C: Kinetics of reduction of CC50/190ox and CC6/264ox by 20 mM DTT in the presence of 0.1% DDM. The amounts of reduced and oxidized species at distinct time points were quantified by reversed-phase HPLC (cf. Figure 20 B) and data were fitted according to a second-order mechanism. D: ANS fluorescence kinetics of the monomer-to-intermediate transition of the ClyA variants CC50/190ox and CC6/264ox. Reactions were started by addition of 0.1% DDM. Data were fitted according to a first-order mechanism. E: Intermediate-to-protomer transition of the oxidized variants pre-incubated in 0.1% DDM triggered by addition of 20 mM DTT. Data were fitted according to a consecutive mechanism. F: Monomer-to-protomer transition of the reduced variants triggered by addition of DDM (0.1%). Data were fitted according to a consecutive mechanism. All obtained rate constants are summarized in Table 4.
In a further series of experiments, the disulfide-trapped variants CC50/190$_{ox}$ and CC6/264$_{ox}$ were pre-incubated with 0.1% DDM and the intermediate-to-protomer transition was started by addition of 20 mM DDT, resulting in consecutive kinetics of reduction of the intermediates (I$_{ox}$ → I$_{red}$), followed by the formation of protomers (I$_{red}$ → P$_{red}$; Figure 21 E). The reduction rates of the intermediate forms of CC50/190$_{ox}$ and CC6/264$_{ox}$ in DDM at 22 °C (0.26 M$^{-1}$ s$^{-1}$ and 0.39 M$^{-1}$ s$^{-1}$, respectively) were determined by reversed-phase HPLC (Figure 21 C) just as described before (cf. Figure 20 C). The ANS fluorescence kinetics started by reduction showed an initial lag phase corresponding to the spectroscopic silent reduction of CC50/190$_{ox}$ and CC6/264$_{ox}$, followed by a decrease of ANS fluorescence corresponding to the intermediate-to-protomer transition (Figure 21 E). A consecutive fit with fixed rate constant for I$_{ox}$ → I$_{red}$ described the reaction kinetics well (solid black lines in Figure 21 E) and resulted in rate constants for I$_{red}$ → P$_{red}$ that correspond well with the rate constants measured for the reduced proteins (7.5 x 10$^{-4}$ s$^{-1}$ vs. 7.8 x 10$^{-4}$ s$^{-1}$ for CC50/190 and 1.5 x 10$^{-3}$ s$^{-1}$ in both cases for CC6/264, respectively). To conclude, the transient intermediates of monomer-to-protomer transition of CC50/190$_{red}$ and CC6/264$_{red}$ are identical with the trapped intermediates of CC50/190$_{ox}$ and CC6/264$_{ox}$. An overview of all obtained rate constants is shown in Table 4.

Table 4: Top: Rate constants of disulfide reduction by DTT ($k_{DTT/DDM}$), and rate constants of the monomer-to-intermediate ($k_{MI}$) and the monomer-to-protomer transition ($k_{IP}$) of ClyA wt and variants, obtained by far-UV CD (cf. Figure 18) and ANS fluorescence measurements (cf. Figure 21). Rate constants were obtained at 22 °C and pH 7.3. Bottom: Specific and relative hemolytic activities of ClyA wt and variants and reduction rates ($k_{DTT}$) of monomeric CC50/190$_{ox}$ and CC6/264$_{ox}$ at 37 °C and pH 7.3.

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<td>$k_{MI}$ CD (s$^{-1}$)</td>
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<td>6.4±0.1·10$^{-2}$</td>
<td>9.1±0.1·10$^{-2}$</td>
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<td>2.1±0.1·10$^{-2}$</td>
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<td>$k_{MI}$ ANS (s$^{-1}$)</td>
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<td>9.4±0.8·10$^{-4}$</td>
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<td>$k_{IP}$ CD (s$^{-1}$)</td>
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<td>1.5±0.1·10$^{-3}$</td>
<td>7.8±0.1·10$^{-4}$</td>
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<td>1.5±0.1·10$^{-3}$</td>
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<td>$k_{IP}$ ANS (s$^{-1}$)</td>
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<td>Relative hemolytic activity (% of wt)</td>
<td>1.6 ± 0.1</td>
<td>2.4±0.1·10$^{-2}$</td>
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<tr>
<td>$k_{DTT}$ (M$^{-1}$ s$^{-2}$)</td>
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<td>0.70</td>
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To determine the stoichiometry and the dissociation constant of ANS binding to the disulfide trapped intermediate state of ClyA, a fluorescence titration experiment was performed.
Figure 22 shows the increase of ANS fluorescence upon titration with disulfide-trapped CC6/264 and CC50/190 until saturation of the protein’s ANS binding sites. The fit of a quadratic equation (Equation 3) to the data points yielded a stoichiometry of 1.9 ANS molecules per ClyA chain and an apparent $K_D$ of 1.4 µM for CC6/264$_{ox}$ and 2.0 ANS per ClyA and a $K_D$ of 4.4 µM for CC50/190, respectively. That means both intermediate states are able to bind the same amount of ANS molecules with affinities that are in the same order of magnitude. That is, next to the same far-UV CD signals, an additional hint for the equality of the intermediate states of CC50/190$_{ox}$ and CC6/264$_{ox}$.

![Figure 22](image)

Molten globules are characterized by the presence of secondary structure elements and a lack of tertiary structure (Kuwajima, 1989), therefore they do not exhibit significant near-UV CD signals, but significant far-UV CD signals (Buchner et al., 1991; Schumann and Jaenicke, 1993). In the case of ClyA, the intermediate of the monomer-to-protomer transition shows a residual α-helix content of ca. 75%, as judged by far-UV CD kinetics and spectra (cf. Figure 12 A). To demonstrate the molten globule state of the ClyA conformational transition intermediate in detergent, near-UV CD kinetics of the monomer-to-protomer transition were recorded. Near-UV CD spectra of both the monomer and the assembled pore (1.5 h after starting the assembly reaction with 0.1% DDM at 22 °C and pH 7.3) of ClyA wt showed a well-defined signal (Figure 22 B) indicating a defined tertiary structure. The CD kinetics of monomer-to-protomer transition show a rapid decrease of the signal intensity at 280 nm approaching zero, followed by a slow regain of CD signal intensity (Figure 22 C). The rate constants obtained by a consecutive fit (black solid line in Figure 22 C) are comparable to the rate constants obtained with far-UV CD kinetics (cf.
Figure 13 B: $8.4 \times 10^{-2} \text{s}^{-1}$ vs. $1.5 \times 10^{-1} \text{s}^{-1}$ and $1.2 \times 10^{-3} \text{s}^{-1}$ vs. $1.5 \times 10^{-3} \text{s}^{-1}$ for $k_{\text{MI}}$ and $k_{\text{IP}}$, respectively). That means the intermediate has no defined tertiary structure around its tryptophan residues at positions 37 and 86. The latter one is located close to the C-terminal end of $\alpha$-helix B, which should not be involved in conformational rearrangements according to the model by Mueller et al. (2009). Data shown here suggest that, at least in the artificial membrane-mimicking system DDM, the whole $\alpha$-helix bundle of ClyA undergoes major structural changes upon oligomerization.

In summary, all data together show the intermediate of the DDM-induced monomer-to-protomer transition of ClyA to have residual secondary structure, to bind ANS reversibly, and to have no near-UV CD signal at 280 nm. Two different kinetically trapped intermediate states did not form protein crystals under 864 different crystallization conditions and starting protein concentrations ranging from $1.5 - 4 \text{ mg/ml}$. Altogether, the intermediate formed with a half-life of about 7 s after triggering the conformational transition of ClyA by DDM was shown to have molten globule-like properties without defined tertiary structure.

### 3.4 Trapping of a ClyA conformational transition intermediate by preventing final pore assembly

#### 3.4.1 Design of an assembly-incompetent ClyA variant undergoing the entire monomer-to-protomer transition: ClyA “protoarrest”

Structural studies of an intermediate state of the conformational transition of ClyA could not be performed successfully with both disulfide-trapped intermediates of CC50/190 and CC6/264, as they did not crystallize. To get a more rigid and better defined conformational transition intermediate, a variant of ClyA was designed supposed to be able to undergo the entire monomer-to-protomer transition without the final assembly of a functional pore. The protomer-to-protomer interface is made up of a network of 25 hydrogen bonds and 13 salt bridges (Mueller et al., 2009) resulting in very stable interactions (no complete heat denaturation of the pore at 95 °C, data not shown). Therefore, point mutations at the protomer-to-protomer interface were designed that will introduce repulsive charges on two adjacent protomers opposite to each other resulting in destruction of the intersubunit interface. In the ClyA pore, the backbone of $\alpha$-helix B of one protomer is in close contact with the backbone of $\alpha$-helix C of the neighboring protomer. The backbone-to-backbone distance has a minimum of 4.6 Å between Gly 63 ($\alpha$-helix B) and Gly 146 ($\alpha$-helix C) in the middle of the $\alpha$-helix bundle. These two glycine residues were substituted with Glutamates to introduce efficient electrostatic repulsions (Figure 23). Additionally, Lys 29, forming a salt
bridge with Asp 25 in the neighboring protomer (Figure 23), was replaced by Glutamate to introduce a second point of repulsive forces.

![Diagram of protein structure with mutations](image)

**Figure 23:** Protomer-to-protomer interface in the context of a ClyA pore (pdb ID 2WCD). The N-termini of both protomers are indicated (N 1, N 2). Point mutations that destruct the protomer-to-protomer interface (K29E, G63E, G146E) were designed manually in PyMOL and are depicted as sticks. Residues D21 and D25, which are in close contact to the mutated residue E29, are shown as sticks. The figure was illustrated in PyMOL (Schrodinger, 2010).

The resulting ClyA variant K29E G63E G146E, termed “protoarrest”, was purified as described for ClyA wt (Eifler et al. (2006), and section 3.1) resulting in a sufficient purification yield of soluble protein (33 mg per 1 l of E. coli culture). The introduced point mutations hence do not cause a serious destabilization of the soluble monomeric form of ClyA.

### 3.4.2 ClyA protoarrest undergoes a conformational transition which does not lead to pore formation

The monomer-to-protomer transition of ClyA protoarrest was recorded by CD spectroscopy as described for wild type and all other variants in 0.1 % DDM at pH 7.3 and 22 °C, and the formation of pores of wt and protoarrest was compared by negative-stain transmission electron microscopy. After 1.5 h incubation in 0.1 % DDM, ClyA protoarrest did not show any pore-shaped structures like wt (Figure 24 A). However, far-UV CD kinetics of the monomer-to-protomer transition showed a signal curve comparable to wt with a rapid initial signal intensity decrease, followed by a slower signal intensity increase ending up in a signal intensity that is increased by 16% compared to monomer’s signal (Figure 24 B, C), indicating the formation of a protomer-like species. A consecutive fit of the transition curve of protoarrest resulted in rate constants of $k_{MI} = 1.4 \times 10^{-1} \text{ s}^{-1}$ for the monomer-to-intermediate transition and $k_{IP} = 3.3 \times 10^{-3} \text{ s}^{-1}$ for the intermediate-to-protomer transition (black solid line in Figure 24 C). The latter one is increased by a factor of 2.2 compared to
ClyA wt, while $k_{\text{MI}}$ did not change significantly ($1.4 \times 10^{-1}$ s$^{-1}$ for protoarrest vs. $1.5 \times 10^{-1}$ s$^{-1}$ for wt, respectively). That means the introduced mutations do not have negative influences on both the monomer-to-intermediate transition and the intermediate-to-protomer transition. The increase of $k_{\text{IP}}$ could be a result of an increase of the degrees of freedom of the ClyA chain during the conformational transition, as interactions with adjacent protomers should be minimized because of electrostatic repulsions.

![Figure 24: Comparison of pore formation and monomer-to-protomer transition of ClyA wt (gray) and protoarrest (orange). A: Negatively stained electron micrographs (2% uranyl acetate) of ClyA wt and protoarrest (5 µM monomer concentration each) 1.5 h after induction of pore formation with 0.1% DDM at 22 °C and pH 7.3. Scale bar: 100 nm. B: Far-UV CD spectra of protoarrest (0.3 mg/ml) as monomer (solid line) and after 1.5 h of incubation time in 0.1% DDM at 22 °C and pH 7.3 (dashed line). C: Monomer-to-protomer transition of ClyA wt and protoarrest (0.3 mg/ml each) started by the addition of DDM to an end concentration of 0.1% at 22 °C and pH 7.3, recorded by the CD signal change at 225 nm. Data were fitted according to a consecutive mechanism (cf. Equation 2; black solid lines) and rate constants of $1.5 \times 10^{-1}$ s$^{-1}$ and $1.4 \times 10^{-1}$ s$^{-1}$ for $k_{\text{MI}}$ and $1.5 \times 10^{-3}$ s$^{-1}$ and $3.3 \times 10^{-3}$ s$^{-1}$ for $k_{\text{IP}}$ were obtained for ClyA wt and protoarrest, respectively. D: The same reaction as shown in C was recorded by observing the change of fluorescence intensity of ANS (20 µM) with ClyA wt and protoarrest (5 µM each). A consecutive fit of data (black solid lines) resulted in rate constants of $9.3 \times 10^{-2}$ s$^{-1}$ and $1.0 \times 10^{-1}$ s$^{-1}$ for $k_{\text{MI}}$ and $1.7 \times 10^{-3}$ s$^{-1}$ and $3.5 \times 10^{-3}$ s$^{-1}$ for $k_{\text{IP}}$ for ClyA wt and protoarrest, respectively.]

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**Figure 24:** Comparison of pore formation and monomer-to-protomer transition of ClyA wt (gray) and protoarrest (orange). A: Negatively stained electron micrographs (2% uranyl acetate) of ClyA wt and protoarrest (5 µM monomer concentration each) 1.5 h after induction of pore formation with 0.1% DDM at 22 °C and pH 7.3. Scale bar: 100 nm. B: Far-UV CD spectra of protoarrest (0.3 mg/ml) as monomer (solid line) and after 1.5 h of incubation time in 0.1% DDM at 22 °C and pH 7.3 (dashed line). C: Monomer-to-protomer transition of ClyA wt and protoarrest (0.3 mg/ml each) started by the addition of DDM to an end concentration of 0.1% at 22 °C and pH 7.3, recorded by the CD signal change at 225 nm. Data were fitted according to a consecutive mechanism (cf. Equation 2; black solid lines) and rate constants of $1.5 \times 10^{-1}$ s$^{-1}$ and $1.4 \times 10^{-1}$ s$^{-1}$ for $k_{\text{MI}}$ and $1.5 \times 10^{-3}$ s$^{-1}$ and $3.3 \times 10^{-3}$ s$^{-1}$ for $k_{\text{IP}}$ were obtained for ClyA wt and protoarrest, respectively. D: The same reaction as shown in C was recorded by observing the change of fluorescence intensity of ANS (20 µM) with ClyA wt and protoarrest (5 µM each). A consecutive fit of data (black solid lines) resulted in rate constants of $9.3 \times 10^{-2}$ s$^{-1}$ and $1.0 \times 10^{-1}$ s$^{-1}$ for $k_{\text{MI}}$ and $1.7 \times 10^{-3}$ s$^{-1}$ and $3.5 \times 10^{-3}$ s$^{-1}$ for $k_{\text{IP}}$ for ClyA wt and protoarrest, respectively.
Next, the monomer-to-protomer formation was recorded by observing the fluorescence intensity change of ANS in the presence of ClyA protoarrest in comparison to ClyA wt and fitted according to a consecutive mechanism (Figure 24 D). As observed for measurements with ClyA wt, the fluorescence intensity of ANS in the presence of ClyA protoarrest undergoes a fast signal intensity increase \( (k_{MI} = 1.4 \times 10^{-1} \text{ s}^{-1}) \), followed by a decrease that is 2.1 times faster than in case of ClyA wt \( (k_{IP} = 3.5 \times 10^{-3} \text{ s}^{-1} \text{ vs. } 1.7 \times 10^{-3} \text{ s}^{-1}, \text{respectively}) \). The rate constants correspond very well with the rate constants obtained by CD measurements (see above), indicating the identity of the spectroscopically observed intermediate species. Additionally, ANS fluorescence measurements show a difference in ANS signal intensity caused by the end products of ClyA wt and protoarrest, respectively. The discrete signal intensities of the intermediate species of ClyA wt and protoarrest obtained by the consecutive fit are identical (86 a. u. in both cases), but the end product of protoarrest causes a significantly decreased ANS fluorescence intensity compared to the end product of wt (60 a. u. vs. 68 a. u., respectively). That means the end product of protoarrest, which is most likely an unassembled protomer, exposes less hydrophobic surface areas than the ClyA pore, the end product of the conformational transition of ClyA wt. This could be a result of a more complete embedment of the protomer into a DDM micelle compared to the pore, which causes a shielding of exposed hydrophobic residues against ANS in the solvent. In contrast, the end products of CC50/190\( _{ox} \) and CC6/264\( _{ox} \) are conformationally trapped at a molten-globule intermediate state with increased surface hydrophobicity compared to the pore (cf. Figure 21 A, B).

It could be demonstrated that ClyA protoarrest does not form pores in vitro in the presence of DDM. Next, the formation of pores in a more in vivo-like situation was tested by a kinetic hemolysis assay. ClyA protoarrest did not cause any decrease of optical density of an erythrocyte suspension corresponding to hemolysis within 1.5 h under conditions where ClyA wt leads to quantitative hemolysis within 300 s (Figure 25 A). To prove that the lack of hemolytic activity is a consequence of the assembly-incompetence of protoarrest and not a result of a lack of erythrocyte binding, the binding of ClyA wt and protoarrest to erythrocyte membranes was quantified by gel filtration. ClyA (wt and protoarrest) was incubated with freshly prepared horse erythrocyte membranes (cf. section 2.4, p. 30) at 37 °C and pH 7.3 (conditions of the hemolysis assay) and subsequently the supernatants containing ClyA that is not associated to the membranes were subjected to analytical SEC. The elution profiles of the samples of both ClyA wt and protoarrest did not contain any peak at the retention time of free monomeric ClyA (7.8 min; Figure 25 B), meaning that both wt and protoarrest are completely bound to the erythrocyte membranes. The peak with a retention time of about 9.3 min corresponding to a size of ca. 30 kDa (Figure 25 B) is also present in the
supernatant of the membrane preparation and therefore it is not identical with monomeric ClyA. The peaks of ClyA wt and protoarrest close to the exclusion limit of the column could be a result of the association of the proteins to small liposomes that were not pelleted in the centrifugation step (30 min, 16100 x g) before SEC.

**Figure 25:** Hemolytic activity and erythrocyte interaction of ClyA protoarrest (orange) in comparison to ClyA wt (gray). A: Hemolytic activities of ClyA wt and protoarrest (10 µM each), recorded by the decrease of the optical density of a horse erythrocyte suspension (2 x 10⁶ cells/ml) at 37 °C and pH 7.3. B: Quantitative interaction of ClyA wt and protoarrest with horse erythrocyte membranes, demonstrated by size exclusion chromatography. 0.3 mg/ml of ClyA (wt or protoarrest) were incubated with 1 mg/ml erythrocyte membranes at 37 °C and pH 7.3 for 1 h and centrifuged before subjected to SEC using a ProSEC 300S column. As a control, erythrocyte membranes without ClyA were analyzed (black). The elution retention time of monomeric ClyA (7.8 min; the monomers of wt and protoarrest elute identically) is indicated by the dashed green line. The exclusion limit of the column (V_ex) and the elution times of marker proteins are indicated. No monomeric ClyA is present in both the samples of wt and protoarrest.

All experiments together prove that ClyA protoarrest is not able to assemble both in a detergent environment and in membranes, which results in the absence of pores and therefore of hemolytic activity. By contrast, the association of ClyA to membranes is not inhibited by the introduction of three glutamate residues at sites 29, 63, and 146.

### 3.4.3 Detergent partially protects ClyA protoarrest against protease digestion

It has already been shown that pore formation protects ClyA against digestion by various proteases, as specific cleavage sites are shielded either by detergent or by flanking ClyA pore subunits (Hunt et al., 2008). Therefore, the comparison of limited proteolysis assays of ClyA wt and protoarrest in detergent would provide more insight on the formation of tertiary structure elements and the embedment into DDM micelles of the non-oligomerized ClyA protomers of protoarrest, as both tertiary structure formation and ligand binding should block potential sites of proteolysis. The serine proteases trypsin and chymotrypsin were used for limited proteolysis experiments, as they cleave specifically after a basic residue (trypsin) or an aromatic residue (chymotrypsin) and therefore should result in specific
cleavage patterns of ClyA wt and protoarrest in the presence or absence of DDM. Furthermore, their proteolytic activities can be inhibited completely by PMSF which covalently binds to the active-site Serine (Gold and Fahrney, 1963) and enable a controllable stop of proteolysis.

ClyA wt and variants (protoarrest, and oxidized CC6/264 that forms a molten globule-like intermediate) were pre-incubated with 0.1% DDM for 2 h to ensure quantitative formation of pores or conformational transition intermediates, respectively, before subjected to limited proteolysis by trypsin or chymotrypsin. 10 µM of protein were digested by concentrations of 0.05 to 1.0 µM protease and the reactions were stopped after 4 h at 37 °C by PMSF addition. SDS-PAGE analyses clearly indicate a protective effect of DDM on wt and protoarrest against digestion by trypsin (Figure 26 A). Without DDM (left panel), no full-length ClyA was present anymore already at a trypsin concentration of 0.05 µM (0.5% of [ClyA]). A significant gel band about 3 kDa below the full-length protein remained, indicating a cleavage close to the N-terminus. Also the almost-full length cleavage product completely disappeared at a trypsin concentration of 0.5 µM (5% of [ClyA]). As expected, the ClyA pore (Figure 26 A, 2nd panel) was protected against protease digestion best of all, having still a fraction of about 5% of uncleaved ClyA in the presence of 1.0 µM trypsin (10% of [ClyA]). Additionally, two almost-full length fragments were still present at the highest trypsin concentration. Altogether, the two fragments and the uncleaved chain made up about 90% of the whole sample. Two further distinct cleavage products of about 25 kDa and 15 kDa appeared already at the lowest trypsin concentration of 0.05 µM. ClyA protoarrest with DDM was cleaved more readily than the pore, resulting in the complete disappearance of full-length ClyA in the presence of at least 0.5 µM trypsin, as judged by SDS-PAGE (Figure 26 A, 3rd panel). One almost-full length cleavage product at about 30 kDa remained up to the maximum concentration of trypsin (1.0 µM). Two further prominent gel bands representing cleavage products of about 25 kDa and 15 kDa were present in all digestion samples with at least 0.1 µM trypsin; cleavage with 0.05 µM or 0.1 µM trypsin resulted in two additional cleavage products that are slightly larger than the 30 kDa and the 15 kDa product. The 30 kDa cleavage product was identified as fragment T9 – K290 (exact mass of 31493 Da) by mass spectrometry (ESI-MS). Compared to ClyA protoarrest, ClyA variant CC6/264ox was less stabilized against trypsin digestion by 0.1% DDM (Figure 26 A, 4th panel). Hardly any full-length ClyA was visible on the Coomassie-stained SDS-Gel already in the presence of 0.05 µM trypsin. No cleavage products longer than 15 kDa were visible at all, meaning a clearly increased susceptibility of the intermediate species of CC6/264ox to protease digestion compared to the monomer.
Figure 26: SDS-PAGE gels of ClyA wt and variants protoarrest and CC6/264\textsubscript{ox} subjected to protease digestion by trypsin (panel A, top row) or chymotrypsin (panel B, bottom row) in the presence or absence of DDM. 10 µM of ClyA were pre-incubated in 0.1 % DDM at 22 °C and pH 7.3 for 2 h and subsequently digested by the indicated concentrations of protease (µM) at 37°C in PBS pH 7.3. Cleavage was stopped after 4 h by addition of PMSF (1 mM), and samples of 10 µl were analyzed via SDS-PAGE using a gel with 17% acrylamide.

Comparable results were obtained when ClyA was digested by chymotrypsin instead of trypsin (Figure 26 B). Also here the pore was the most stable species with full-length ClyA visible up to chymotrypsin concentrations of 0.5 µM. Two almost-full length species made up at least 90% of all cleavage products even with 1.0 µM protease (Figure 26 B, 2nd panel). However, the stability difference between the pore and the isolated protomer (protoarrest + DDM, Figure 26 B, 3rd panel) was less pronounced when compared to tryptic digestion. That could be a hint for the accumulation of DDM close to hydrophobic aromatic amino acids that results in a shielding of the respective chymotrypsin cleavage sites. The molten globule-like intermediate (CC6/264\textsubscript{ox}) was not protected against chymotrypsin digestion in the same extent as the intermediate formed by protoarrest. Only a slightly better protection compared to the digestion with trypsin was given with a clearly visible gel band corresponding to full length ClyA when digested with 0.05 µM chymotrypsin (Figure 26 B, 4th panel). Just as observed for tryptic digestion, CC6/264\textsubscript{ox} in the presence of DDM is more susceptible to digestion by chymotrypsin than monomeric ClyA (Figure 26 B, 1st panel), which means the surface exposure of aromatic residues.
In conclusion, the monomer-to-protomer transition intermediate formed by protoarrest in the presence of DDM was significantly better protected against tryptic and chymotryptic digestion than monomeric ClyA. In contrast, the molten globule-like, disulfide-trapped intermediate of CC6/264ox was not protected against digestion at all. The results are a further proof for the conformational transition end product of ClyA protoarrest having a defined tertiary structure. Furthermore, the increased protection level of ClyA protoarrest against chymotrypsin digestion compared to trypsin digestion indicates the association of DDM predominantly to aromatic or bulky hydrophobic residues. The protection level of the pore was not achieved, meaning that there is most likely no oligomerization of ClyA protoarrest.

3.4.4 Crystallization of ClyA protoarrest in the presence of detergent

All previously shown experiments indicate the formation of a monomeric and completely folded end product of the conformational transition of ClyA protoarrest. That makes this variant a more promising candidate for structural determination of a ClyA conformational transition intermediate by X-ray crystallography compared to the disulfide-trapped variants CC50/190ox and CC6/264ox, which failed to crystallize in the intermediate form in the presence of detergent.

Next to DDM, n-octyl-β-D-glucopyranoside (OG) has also been successfully used to form ClyA pores (Tzokov et al., 2006). Therefore, also the possibility of crystallization of ClyA protoarrest into OG micelles and the arrest at a non-oligomerized level are the prerequisites for structure determination of isolated ClyA protomers. ClyA protoarrest pre-incubated in 0.1% DDM (~ 14 x CMC) was eluted from a gel filtration column in one major peak with a higher retention time than assembled ClyA pores and a lower retention time than monomeric ClyA (Figure 27 A, blue elution profile). The same was observed when ClyA protoarrest was pre-incubated in 1.0% OG (~ 1.6 x CMC) under identical conditions (22 °C, TBS pH 7.3; Figure 27 A, orange elution profile). N-decyl-β-D-maltopyranoside was also tried, but ClyA protoarrest precipitated in that detergent (not shown). Compared to DDM, OG leaded to the elution of ClyA protoarrest in a narrower peak, indicating a more homogenous sample. In both cases, the elution time corresponded to an apparent mass of about 230 kDa, which is considerably higher than the sum of masses of monomeric, hexahistidine-tagged ClyA (34.5 kDa) and an average micelle (72 kDa in the case of DDM, Strop and Brunger (2005); maximally 30 kDa in the case of OG, Lorber et al. (1990)). However, the elongated form of
a ClyA protomer (cf. Figure 6 D) could lead to an elution at a higher apparent molecular weight, as proteins used for calibration are globular proteins.

Figure 27: A: Size exclusion chromatography of ClyA protoarrest in the presence of DDM or OG, showing the incorporation of the variant into detergent micelles without oligomerization. ClyA protoarrest (0.3 mg/ml) was incubated in 0.1% DDM or 1.0% OG at 22 °C and pH 7.3 for 1 h and subsequently subjected to SEC using a ProSEC 300S column. As controls, gel filtration runs of ClyA wt as monomer or pre-incubated in 0.1% DDM were performed. B: Long-term incubation of ClyA protoarrest in DDM, showing the stability of the non-oligomerized, detergent-embedded species. ClyA protoarrest (0.3 mg/ml) was incubated in 0.1% DDM at 22 °C and pH 7.3 for the indicated time intervals, and samples of 20 µl were subjected to SEC (ProSEC 300S). Again, assembled ClyA wt (0.3 mg/ml) is shown as a control. The elution time of monomeric ClyA is indicated by the black dashed line. The exclusion limit of the column (V(ex)) and the elution times of marker proteins are indicated. No oligomerization of ClyA protoarrest was detected within 8 days.

To exclude the final formation of pores of ClyA protoarrest in the presence of DDM after long-term incubation in a crystallization experiment, the trapping of ClyA protoarrest at a non-oligomerized level was checked after 1 h, 15 h, 39 h, and 190 h incubation time in 0.1% DDM at 22 °C and pH 7.3. The SEC elution profiles of ClyA protoarrest after all incubation time intervals clearly showed no occurrence of a peak at an elution time of 6.4 min corresponding to the ClyA pore (Figure 27 B). Additionally, the size of the single peak did not decrease over time, indicating a long-term (at least 8 days) stability of ClyA protoarrest in 0.1% DDM without precipitation. Altogether, crystallization of ClyA protoarrest in the presence of detergent should result in crystallization of isolated protomer-like species and not of pores.

Initial crystallization screening of ClyA protoarrest in 0.1% DDM or 1.0% OG was performed at the Protein Crystallization Center of the NCCR Structural Biology using Triple-Drop 96-well sitting-drop vapor diffusion plates. Altogether, 1056 predefined conditions were tested for protoarrest in DDM and 768 conditions in OG. The protein starting concentrations in the crystallization drops amounted to 1.8 – 3.1 mg/ml; crystallization drops consisted of protein/detergent solutions in TBS pH 7.3 and mother liquor from the crystallization well (2:1, 1:1, and 1:2 mixes). In contrast to ClyA CC50/190ox and CC6/264ox, protein crystals
showed up after 1 – 3 weeks under various conditions. All observed crystalline structures were imaged in a UV fluorescence microscope to ensure that the crystals contain protein (not shown). Crystals of ClyA protoarrest grew in the presence of DDM or OG and with different types of precipitants (PEGs, salts, and organic solvents, cf. Table 5). The maximum size of crystals grown in the analytical 96 well plates accounted to about 80 µm.

Table 5: Initial conditions of crystal growth of ClyA protoarrest in the presence of detergent and the maximally achieved resolutions, as judged by the visibility of protein diffraction spots.

<table>
<thead>
<tr>
<th>detergent</th>
<th>concentration of ClyA (mg/ml)</th>
<th>buffer</th>
<th>precipitant</th>
<th>salt/ additive</th>
<th>crystal form</th>
<th>reproducible in 24 well plates</th>
<th>diffraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDM</td>
<td>1.8 - 2.7</td>
<td>NaOAc pH 4.6</td>
<td>30% MPD</td>
<td>0.2 M NaCl</td>
<td>hexagons or cubes</td>
<td>yes</td>
<td>9 Å</td>
</tr>
<tr>
<td>DDM</td>
<td>2.7</td>
<td>NaOAc pH 4.6</td>
<td>30% MPD</td>
<td>0.02 M CaCl₂</td>
<td>spotty hexagons</td>
<td>yes</td>
<td>10 Å</td>
</tr>
<tr>
<td>OG</td>
<td>2.1 - 3.1</td>
<td>NaOAc pH 4.6</td>
<td>1.0 M 1,6-hexanediol</td>
<td>0.01 M CoCl₂</td>
<td>hexagons or plates</td>
<td>yes</td>
<td>20 Å</td>
</tr>
<tr>
<td>OG</td>
<td>2.1 - 3.1</td>
<td>Na-Cacodyl pH 6.5</td>
<td>40% MPD</td>
<td>0.15 M KSCN</td>
<td>rods</td>
<td>yes, only needles</td>
<td>no diff.</td>
</tr>
<tr>
<td>OG</td>
<td>3.1</td>
<td>Glycine pH 9.4</td>
<td>25% PEG 400</td>
<td>0.05 M MgOAc₂</td>
<td>diamonds</td>
<td>no, immediate precipitation</td>
<td>9 Å</td>
</tr>
<tr>
<td>OG</td>
<td>2.1</td>
<td>Tris-HCl pH 8.5</td>
<td>10% PEG 8000</td>
<td></td>
<td>rods</td>
<td>no</td>
<td>no diff.</td>
</tr>
<tr>
<td>OG</td>
<td>3.1</td>
<td>Na-Cit. pH 5.6</td>
<td>1.0 M NH₄H₂PO₄</td>
<td></td>
<td>small hexagons</td>
<td>no</td>
<td>25 Å</td>
</tr>
<tr>
<td>OG</td>
<td>2.1</td>
<td>Tris-OAc pH 7.5</td>
<td>2.0M Na- formiate</td>
<td></td>
<td>hexagons</td>
<td>n.d.</td>
<td>20 Å</td>
</tr>
</tbody>
</table>

The initial screening conditions that enabled protein crystal growth were refined on 24 well preparative scale crystallization plates (ML volume: 300 µl; drop volume: 3 µl) by applying gradients of the pH value and precipitant concentration. pH values were varied in steps of 0.1 – 0.2 to cover the range of 1.0 around the initial condition. Precipitant concentrations were varied in steps of 0.1 M in the case of salts or 1 – 2% in the case of PEGs and organic solvents, respectively, around the initial concentration. Additionally, different salts and additives were tested and combined. Crystal growth of ClyA protoarrest in the presence of DDM was reproducible under acidic conditions (pH 4.2 – 5) with MPD as precipitant and different salts (NaCl, CaCl₂, MgCl₂). Crystals were predominantly shaped hexagonal and grew as stacks of plates with diameters up to 500 µm (Figure 28 A). Several different conditions enabled crystal growth of ClyA protoarrest with OG, but only two of six could be reproduced in a preparative scale (cf. Table 5). Conditions with 1,6-hexanediol as precipitant and acidic pH values (pH 4.1 – 4.4) enabled the growth of three-dimensional, hexagonal crystals up to 600 µm in diameter (not shown). Slightly acidic conditions (pH 6.0 – 6.5), MPD concentrations around 40%, and the presence of 0.1 – 0.2 M KSCN led to
the growth of needle-shaped crystalline structures that were not applicable for structure determination.

If possible, crystals grown on the analytical plates were fished and used for data collection at beamline X06DA (PXIII) at PSI (Villigen, CH). Conditions with MPD concentrations above 25% and PEG 400 concentrations of at least 25% showed no necessity for cryoprotection. In the other cases, the respective mother liquor was mixed with the cryoprotectants glycerol or ethylene glycol (end concentration: 25% each) and the mix was added in a fivefold excess to the drop containing protein crystals (end concentration of cryoprotectant: 20%). Crystals grown in the analytical plates diffracted to a maximum resolution of about 9 Å (cf. Table 5). Crystals of ClyA protoarrest with DDM grown with MPD, NaCl and/or CaCl₂, and NaOAc pH 4.2 – 5.0 in 24-well plates (Figure 28 A) showed a regular diffraction pattern with visible spots up to 7 Å (Figure 28 B). The bigger the crystals grew, the better they diffracted. In contrast, the large crystals of ClyA protoarrest with OG grown with hexanediol, CoCl₂, and NaOAc pH 4.1 – 4.5 showed no diffraction spots at resolutions better than 12 Å (data not shown). Therefore, optimization was focused on the DDM/MPD condition.

![Figure 28: A: Crystals of ClyA protoarrest, grown at 20 °C in a sitting drop made up of 1.5 µl protein solution (4.5 mg/ml in TBS pH 7.3, 0.1% DDM) and 1.5 µl mother liquor (0.1 M sodium acetate, pH 5.0, 0.2 M sodium chloride, 26% MPD). Crystals grew within 3 d to their maximum size when microseeded with fragments of crystals that had grown under the same conditions. Crystals grew as stacked hexagonal plates, shown in top view (indicated by +) and in side view (indicated by -). Scale bar: 200 µm. B: Diffraction image of the best-diffracting crystal of ClyA protoarrest grown under conditions as indicated above, recorded with a Pilatus 2M pixel detector (exposure time: 0.3 s, detector distance: 0.595 m) at beamline X06DA (PSI, Villigen). The crystal showed visible diffraction spots up to a resolution of 7.0 Å (red box).](image-url)

Next to variation of pH and precipitant concentrations, further strategies to improve crystal quality and size were applied. In contrast to monomeric CC6/264_ox (cf. section 3.3.4), the removal of the N-terminal hexahistidine tag by TEV protease resulting in ClyA protoarrest...
Results

(2-303) did not lead to the formation of better diffracting crystals; the crystals from the cleaved variant formed spotty and heterogenous cubes or hexagons. Iterative seeding led to a rapid crystal growth at 20 °C: first crystalline structures were visible 1 h after setting up the experiment, and the maximum size was achieved within three days (e.g. the crystals and the diffraction pattern shown in Figure 28 A, B resulted from iterative seeding experiments). In contrast, at 4 °C crystals of a reasonable size did not grow within two months, no matter if the protein drop had been seeded or not.

The highest-resolution dataset of ClyA protoarrest in DDM (diffraction pattern shown in Figure 28 B) was collected with a 500 µm crystal grown in a drop consisting of 1.5 µl of protein solution (5 mg/ml) in TBS + 0.1% DDM and 1.5 µl of mother liquor (28% MPD, 0.2 M NaCl, 0.1 M NaOAc, pH 4.7) after iterative seeding. With an exposure time of 0.3 s, spots were visible up to a resolution of 7.0 Å (indicated by a red box in Figure 28 B). The obtained data were processed using XDS (Kabsch, 2010). A high-symmetry hexagonal space group was found (P6122) with unit cell dimensions of 194.65 Å x 194.65 Å x 490.20 Å, and angles of 90°, 90°, and 120°. The resolution was truncated at 6.26 Å (I/Σ = 1.88). Molecular replacement using Phaser (McCoy et al., 2007) with up to 30 ClyA protomers as search model did not result in a solution, most likely because of the too low resolution of the dataset. As the unit cell is unreasonably large, it is also probable that the space group solution found by XDS was not true. However, XDS found this solution (error in unit cell lengths of ± 2 Å) independently for all processed datasets (20). Further crystallization optimization needs to be performed to improve the resolution and to get reasonable diffraction data.
4 Discussion

4.1 Evaluation of strategies to trap conformational transition intermediates of ClyA

Three possible strategies were applied to stabilize transient intermediates of the ClyA monomer-to-protomer transition: (1) substitution of residues that mediate conformational flexibility in predicted hinge points of the ClyA conformational transition; (2) covalent attachment of the β-tongue or α-helix A by engineered disulfide bonds and (3) destruction of the protomer’s intersubunit interface by introduction of amino acids with repulsive charges in close spatial proximity (cf. sections 1.4, 3.2, 3.3.2, and 3.4.1). The first strategy failed, but the second and third strategies resulted in altogether three trapped, unassembled intermediates of the monomer-to-protomer transition of ClyA.

The replacement of one or two glycine residues in the flexible loops flanking the β-tongue by alanine or valine did not result in a significant (at least one order of magnitude) decrease of the hemolytic activity of ClyA (cf. Table 2). One possibility could be that the decrease of loop flexibility by glycine-vs.-alanine substitution is counteracted by the higher propensity of Ala to be localized in an α-helix compared to Gly (Zhu and Braun, 1999). Only the replacement of glycine residues by proline resulted in a major decrease of hemolytic activity; in the case of double Gly-vs.-Pro substitutions the hemolytic activity of ClyA was decreased to zero. The propensity of proline to be located in an α-helix is as low as glycine’s α-helix propensity (Zhu and Braun, 1999). Therefore, the synergetic effects of flexibility decrease of ClyA monomer’s “β-tongue hinges” and the interruption of protomer’s transmembrane helices seem to be a prerequisite for a reasonable decrease of monomer-to-protomer transition kinetics and a significant inhibition of hemolysis. To summarize, the effects of glycine substitutions were less pronounced than predicted by Mueller et al (2009), and no intermediate that does not form a functional pore in the end could be trapped with certainty. In contrast, larger modifications of the β-tongue region, namely a complete deletion (residues 181 – 203, Ludwig et al. (2010)), partial deletions (residues 183 – 186, Wai et al. (2003b); residues 183 – 202 del Castillo et al. (2001)), and the insertion of one or more charged residues (Ludwig et al., 2010; Wai et al., 2003b; Wyborn et al., 2004a) were able to knock out the hemolytic activity of ClyA completely.

The strategy of introduction of artificial disulfide bonds to trap transient intermediate states of pore formation has been successfully applied for a variety of α-PFTs and β-PFTs, e.g. actinoporins (Kristan et al., 2004; Rojko et al., 2013), Bacillus thuringiensis Cry toxin (Girard
et al., 2009), different CDCs (Hotze et al., 2001; Pokrajac et al., 2012; Ramachandran et al., 2004; Tabata et al., 2012), the staphylococcal α-hemolysin type (Kawate and Gouaux, 2003; Pelish and McClain, 2009), and aerolysin (Iacovache et al., 2006; van der Goot et al., 1994). In the case of ClyA, the covalent attachment of either the β-tongue (ClyA variant CC50/190) or α-helix A (CC6/264) to the tail domain resulted in a complete inhibition of the hemolytic activity, the formation of pores, and the intermediate-to-protomer transition (the monomer-to-intermediate transition takes place, cf. Figure 18). In both cases, the interruption of the conformational transition of ClyA was fully reversible by reducing the engineered disulfide bonds (cf. Figure 20). However, the intermediates failed to crystallize in the presence of detergent, and biophysical studies (CD spectroscopy, ANS fluorescence; cf. Figure 21 and Figure 22) indicated the arrest of the disulfide forms of ClyA CC50/190 and CC6/264 at a molten globule-like state without defined tertiary structure. Therefore, the intermediates of CC50/190 and CC6/264 cannot provide structural information on the monomer-to-protomer transition of ClyA, but they indicate a different pathway than proposed by Mueller et al (2009). Additionally, the redox-controllable ClyA variants CC50/190 and CC6/264 increase the potential of ClyA for biotechnological or medical applications (Tabata et al., 2012).

An alternative strategy instead of attaching protein domains together by disulfide bonds could be the introduction of binding clusters for divalent cations by introduction of cysteine and histidine residues, as it has been demonstrated in the case of staphylococcal α-hemolysin (Walker et al., 1995). In contrast to the disulfide-trapping strategy no covalent disulfide bond is formed and consequently no reductants are necessary for reactivation (in the case of α-hemolysin, hemolysis was restored by EDTA). Therefore, this particular strategy for trapping intermediates would be employable to redox-sensitive pore-forming proteins. However, attempts to create a closed pore state of ClyA by introducing a histidine trap for divalent metal ions at the pore constriction at the N-terminus (cf. Figure 6 C) failed so far, as no hemolytic inactive oligomeric species could be formed (data not shown).

A conformational transition intermediate of ClyA with defined secondary and tertiary structure was obtained using the third described strategy (destruction of intersubunit interface). ClyA protoarrest exhibits spectroscopic properties of an assembled pore (cf. Figure 24), but no assembly was observed by electron microscopy (cf. Figure 24 A), size exclusion chromatography (cf. Figure 27), and activity assays (Figure 25). Additionally, protection against limited proteolysis is more pronounced for the intermediate of protoarrest in comparison to the molten globule intermediate of CC6/264 (Figure 26), indicating the protection of cleavage sites by a defined tertiary structure. Due to the defined structure the
intermediate also formed protein crystals, which is a prerequisite for ongoing studies of structural biology. To our knowledge, the inhibition of oligomerization by disturbing the intersubunit interface of the active pore is unique for PFTs up to now. This strategy of assembly inhibition is only possible for toxins with a known structure of the active pore complex; therefore, the choice of other possible candidates next to ClyA is limited (the staphylococcal α-hemolysin type (De and Olson, 2011; Song et al., 1996)). In the case of ClyA, intersubunit interface destruction was the only successful way to kinetically trap a pore formation intermediate with defined tertiary structure.

4.2 The trapped intermediate states of ClyA in comparison to trapped intermediates of other PFTs

4.2.1 ClyA CC50/190\textsubscript{ox} and CC6/264\textsubscript{ox}

The monomer-to-pore transition of ClyA in detergent is trapped at a molten-globule state by the engineered disulfide bonds of ClyA CC50/190\textsubscript{ox} and CC6/264\textsubscript{ox} (section 3.3.7). Both are arrested at a monomeric state, which is a difference to most other covalently trapped pore formation intermediates of other PFTs. In the case of another well-studied α-PFT, the actinoporin equinatoxin, a pre-assembled intermediate state has been captured by introducing an artificial disulfide bond that prevents membrane insertion of the N-terminal α-helix and therefore formation of the active pore (Rojko et al., 2013). Similar to ClyA CC50/190\textsubscript{ox} and CC6/264\textsubscript{ox}, the disulfide-trapped prepore intermediate of equinatoxin can be reactivated by reduction. In contrast to ClyA CC50/190\textsubscript{ox}, whose hemolytic activity was restored within 1 min (cf. Figure 20 A), the restoration of the hemolytic activity of eqinatoxin by reduction occurs with a delay of almost 1 h, although reduction itself is not rate-limiting and is practically finished after 3 min in both cases (Figure 20 C and Rojko et al (2013)). That means the monomeric, molten globule intermediate of CC50/190\textsubscript{ox} can continue with the intermediate-to-protomer transition directly, while the prepore state hinders the intermediate of equinatoxin from membrane insertion of the N-terminal α-helix (Rojko et al., 2013). Therefore, it was speculated that the disulfide-trapped prepore intermediate of equinatoxin was a non-natural, off-pathway species (Rojko et al, 2013). On-pathway prepore states that precede membrane insertion have been trapped by artificial disulfide bonds only for β-PFTs so far: staphylococcal α-hemolysin (Kawate and Gouaux, 2003), aerolysin (Iacovache et al., 2006), and the CDC Perfringolysin O (PFO; Hotze et al. (2001)). Reduction of the pre-oligomerized, disulfide-trapped intermediate of PFO results in an immediate lysis of red blood cells because membrane insertion proceeds rapidly after the
rate-limiting formation of the prepore (Hotze et al., 2001). In contrast, the presence of a lag phase of hemolysis in the case of ClyA CC50/190ox (cf. Figure 20 A) indicates that the rate-limiting step of pore formation (the intermediate-to-protomer conversion) still needs to proceed after reduction. In contrast to PFO, another CDC, Pyolysin, has been successfully disulfide-trapped at a monomeric intermediate state of pore formation just like ClyA (Pokrajac et al., 2012), although in both CDCs the identical domains were linked together (domain 2 and domain 3; cf. Figure 3 A). That disparity supports previous studies that indicate the necessity of large conformational changes as a prerequisite for oligomerization of CDCs (Pokrajac et al., 2012; Ramachandran et al., 2004). The possibility of formation of hybrid oligomers of wild type and disulfide-trapped Pyolysin indicates a concerted conformational change and oligomerization mechanism for CDCs (Pokrajac et al., 2012), other than the sequential conformational change and oligomerization of ClyA.

4.2.2 ClyA protoarrest

Next to both disulfide-trapped molten globule intermediates of the ClyA monomer-to-protomer transition, ClyA protoarrest that has been achieved by destruction of the intersubunit interface forms a monomeric intermediate with defined tertiary structure (sections 3.4.2 – 3.4.4). In contrast to all other well-studies PFTs, ClyA has to undergo an excessive conformational transition before oligomerization. As a consequence, it gives us the unique possibility to study a pore formation intermediate that is both monomeric and structurally similar to a pore subunit. In the case of the β-PFT Aerolysin, a conformational transition of a similar extent to that of ClyA has been proposed (Degiacomi et al., 2013). Nevertheless, the already assembled prepore complex of Aerolysin undergoes the major steps of conformational transition upon insertion of the transmembrane barrel by a swirling mechanism, not the Aerolysin monomer before oligomerization. Therefore, the conformational transition intermediate of Aerolysin that has been trapped by site-directed mutagenesis at a non-assembled state resemble the soluble monomer and not a pore subunit (Degiacomi et al., 2013). Is it then a common feature for β-PFTs to assemble without the necessity for preceding conformational changes and for α-PFTs to undergo conformational changes before oligomerization? At least the latter case seems not to be true as a high resolution structure of a prepore complex of the α-PFT Fragaceatoxin (a eukaryotic Actinoporin) demonstrated that the pore subunits are practically identical to the soluble monomer (Mechaly et al. (2011); cf. Figure 2 C).

To our knowledge, no high-resolution structure of a monomeric intermediate of a PFT resembling a subunit of an assembled pore has been shown so far. ClyA protoarrest
exhibits a defined structure and forms protein crystals in the presence of detergent (that means under conditions where ClyA wt forms pores), so the determination of a high-resolution crystal structure of the ClyA pore assembly intermediate is in reach. It will complement our knowledge of the final assembly of ClyA and other related PFTs from human pathogenic bacteria (e.g. the Hbl and Nhe toxins from Bacillus cereus; Ganash et al. (2013)) and could therefore provide us with useful information for possible drug design approaches that inhibit the formation of important bacterial pathogenicity factors.

4.3 A model mechanism for ClyA monomer-to-protomer transition proceeding via a molten globule state

Based on the high-resolution structures of monomeric and assembled ClyA (Mueller et al., 2009) and additional kinetic data (Eifler et al., 2006), a detailed model of ClyA assembly has been proposed (Figure 7; Mueller et al. (2009)). The model suggests sequential major structural rearrangements of α-helix A and the β-tongue together with its flanking loops, minor rearrangements of α-helices D, E, F, and G and no rearrangements of α-helices B and C that form a non-variable backbone. That would mean the overall structural backbone of ClyA is maintained during the whole monomer-to-protomer transition. However, ANS fluorescence kinetics and equilibrium titration experiments (Figure 21, Figure 22 A), the comparison of far-UV (Figure 12 A) and near-UV CD spectroscopy (Figure 22 C), limited proteolysis (Figure 26), and the lack of protein crystal growth of disulfide-trapped intermediates of the ClyA conformational transition suggest an alternative way of monomer-to-protomer transition of ClyA with a molten globule form of ClyA as intermediate state (Figure 29), at least in the in vitro system of a DDM micelle. Additionally, a ClyA fragment composed of α-helices B and C only (residues 55 – 160) also undergoes the first step of conformational transition, namely the rapid far-UV CD signal intensity decrease (data not shown), and single molecule FRET studies show a transient population of a low-FRET species upon monomer-to-protomer transition of ClyA that resembles a non-compact intermediate without defined structure (Stephan Benke, unpublished data).

The monomer-to-protomer transition model of ClyA presented here suggests a rapid formation of the molten globule intermediate (half-life of 5 s in the case of ClyA wt, according to CD and ANS fluorescence kinetics), followed by the formation of the assembly-competent protomer with a half-life of 460 s (Figure 29 A). That means the intermediate becomes maximally populated to an extent of 95% after 30 s, which is in consistence with single molecule data (Stephan Benke, unpublished data). Pore formation
proceeds rapidly at concentrations of ensemble measurements (at least 1 µM). Single molecule studies indicate a delayed pore formation at ClyA concentrations below 0.5 µM (Stephan Benke, unpublished data).

The engineered disulfide bonds of ClyA variants CC50/190\textsubscript{ox} and CC6/264\textsubscript{ox} block the monomer-to-protomer transition reversibly at the molten globule intermediate states, which are formed with half-lives of 8 s and 28 s, respectively, after starting the reaction by addition...
of DDM (Figure 29 B). Structural characterizations of the trapped intermediates were not possible in both cases because protein crystals did not grow. Reduction of the disulfide bonds of both intermediates released the conformational traps and enabled formation of the protomer (half-lives of 1250 s and 730 s, respectively) and finally of active pores. An irreversible conformational trap of the monomer-to-pore formation of ClyA was achieved by disturbing the intersubunit binding interface (introduction of glutamate residues at positions 29, 63, and 146). Just like in the case of ClyA wt, the molten globule intermediate is formed with a half-life of 5 s after DDM addition, followed by a 2.2 times faster protomer formation (half-life of 210 s, Figure 29 C). CD spectroscopy, ANS fluorescence measurements (Figure 24), and limited proteolysis (Figure 26) suggest the formation of a protomer-like species with defined tertiary structure as final state. The absence of oligomers was verified by electron microscopy (Figure 24 A) and size exclusion chromatography (Figure 25 B).

### 4.4 Conclusions and Outlook

High-resolution structures of both the monomer (Wallace et al., 2000) and the assembled pore (Mueller et al., 2009) of the α-PFT ClyA showed large structural differences of a pore subunit compared to the monomer and therefore an extensive conformational transition upon oligomerization. The knowledge of both structures enabled us to rationally design mutations that interrupt the monomer-to-protomer transition at distinct proposed states (Mueller et al., 2009). Attempts to rigidify two loop regions (residues 180 – 184 and 201 – 205) that were proposed to function as flexible hinges for membrane insertion of the central β-tongue by substitution of glycines by alanines, valines, or prolines failed. The covalent attachment of the β-tongue or the N-terminal, membrane-protruding α-helix A to the invariable tail domain of ClyA by engineered disulfide bonds (ClyA variants CC50/190 and CC6/264) resulted in two kinetically trapped conformational transition intermediates that show molten globule-like properties. The disulfide trap is reversible; therefore reduction enables the conformational transition to proceed with kinetics comparable to ClyA wt resulting in active pores that are not distinguishable from wild type pores by electron microscopy. Structural characterization of the molten globule intermediates was not possible by means of X-ray crystallography. A third assembly-incompetent intermediate of the monomer-to-pore transition of ClyA was achieved by destruction of the intersubunit interface (ClyA protoarrest). The protoarrest intermediate proceeds rapidly through the molten globule state and is arrested at a protomer-like form. Its defined tertiary structure enables ongoing structural characterization. All data together indicate a complete loss of
the tertiary structure of ClyA after triggering assembly by detergent, followed by refolding as an assembly-competent protomer and successive pore formation.

Ongoing studies will focus on structure determination of monomer-to-pore transition intermediates of ClyA. Since protein crystals of ClyA variant protoarrest in the intermediate form already grew, further optimization will be performed to improve diffraction to atomic resolution. Optimization of pH and precipitant concentration, microseeding, an additive screen (Hampton Research), and different salts (CaCl$_2$, MgCl$_2$, NaCl) have already been applied with only minor improvement of diffraction (maximum of 6.26 Å, cf. section 3.4.4). The next possible strategy would be the crystallization of a truncated version of ClyA protoarrest to reduce conformational flexibility at the N- and C-terminus and therefore promote a more dense crystal packing that might lead to a better resolution. Limited proteolysis (trypsin) of protoarrest in the intermediate form yielded a well-protected 31.5 kDa fragment corresponding to residues 9 – 290 (cf. section 3.4.3). Cleavage of residues 1 – 8 and 290 – 303 is a hint for high conformational flexibility of both termini that could interfere with dense crystal packing. Therefore, the fragment might form better diffracting crystals.

Next to the described monomeric intermediates of ClyA conformational transition, the high-resolution structure of a “prepore” intermediate could provide us more information on possible structural rearrangements after oligomerization. The introduction of an engineered disulfide bond that attaches α-helices A1 and A2 together could prevent the formation of the final pore that protrudes through the target cell membrane, since α-helix A1 has to delocalize from A2 to form the pore channel (Mueller et al., 2009). Possible cysteine pairs enabling the A1-A2 attachment could be introduced at residues 35 and 37 or 32 and 37 (C$_\beta$-C$_\beta$ distances of 4.9 and 6.8 Å, respectively). If a final, not rate-limiting step of pore formation would follow oligomerization as proposed (Mueller et al., 2009), the intermediate-to-protomer transition of both oxidized ClyA variants would proceed with kinetics similar to ClyA wt and result in assembled, but yet inactive pore complexes. With a good accessibility of the engineered disulfide bonds, reduction should result in an instant activation of hemolysis as demonstrated in the case of the prepore of Perfringolysin O (Hotze et al., 2001). Up to now, no high-resolution crystal structures of both a prepore and a final pore are available for any PFT. Therefore, the comparison of prepore and pore structures of ClyA would not only complement our knowledge of that class of PFTs, but could also be helpful to clarify pore formation mechanisms of many other toxins.
5 References


Kuwajima, K., 1989. The molten globule state as a clue for understanding the folding and cooperativity of globular-protein structure. Proteins 6, 87-103.


References


6 Appendix

6.1 Oligonucleotides

TEV Protease cleavage site (underlined) at N-terminus between His$_6$-tag and Thr2
Forward:
5'-GATATACATATGCATCATCATCATCATCACGAAAAACCTGATTTCCAGACTGAAAATCGTTGCAGATAAAACGGTAG-3'
Reverse:
5'-CTACCGTTTTATCTGCAACGATTTTCAGTCTGGAATAACAGTTTTCGTGATGATGATGATGATGCATATGTATATC-3'

TEV Protease cleavage site (underlined) at N-terminus between His$_6$-tag and Thr2 for CC6/264
Forward:
5'-GATATACATATGCATCATCATCATCATCAGGAAAAACCTGATTTCCAGACTGAAAATCGTTTGCGATAAAACGGTAG-3'
Reverse:
5'-CTACCGTTTTATCTGCAACGATTTTCAGTCTGGAATAACAGTTTTCGTGATGATGATGATGATGCATATGTATATC-3'

TEV Protease cleavage site (underlined) at N-terminus between His$_6$-tag and Cys6 for CC6/264
Forward:
5'-GATATACATATGCATCATCATCATCATCAGGAAAAACCTGATTTCCAGACTGAAAATCGTTTGCGATAAAACGGTAG-3'
Reverse:
5'-CGTTTTTAACCTACTTCTACCCGTTTTATCGCACTGGAATAACAGTTTTCGTGATGATGATGATGATGCATATGTATATC-3'

Removal of NdeI cleavage site (CATATG) from the clyA gene
Forward: 5'-CAGGAAGGAAGCTTATGCGGCTGCCGC-3'
Reverse: 5'-GCCGCCACCGGCATAAGCTCTCCTCCTCCTG-3'

G180A
Forward: 5'-GGAAGCTTATGCGGCTGCCGCAGCCGG-3'
Reverse: 5'-CGCGCTCGCGAGCGCAATTCATGCTCGCCGC-3'

G201A
Forward: 5'-CCTATTTCTATGGCTGCGGCTGCTGTCGGAAG-3'
Reverse: 5'-CCTACCGGCGAATCCGAATACGCTGCTGTCGGAAG-3'

G205A
Forward: 5'-GCGGCGTGCAGTTGGAAGCAGAATTCAGGCCAATAG-3'
Reverse: 5'-CTGGGAATCAGTTGGAAGCAGAATTCAGGCCAATAG-3'
G205V
Forward: 5’-GCGGCGTAGTTGAAGTAAAACTGATTCCAGAATTGAAG-3’
Reverse: 5’-CTTAATCTGGAATCAGGTTTTTACTTTCAACTACGCCCCGC-3’

G180P
Forward: 5’-GAAGCATATGCCCTGCGCAGCCGGTG-3’
Reverse: 5’-CACCGGCTGCAGGCCAGGAATCTGCTTCC-3’

G201P
Forward: 5’-CCTATTCTATTAGTGCGGCCGTAGTTGAAGGAAAACTG-3’
Reverse: 5’-CAGTTTTCCCTTTCAACTACGCCCCGC-3’

G205P
Forward: 5’-GCGGCGTAGTTGAAGTAAAATCGATTCCAGAATTGAAG-3’
Reverse: 5’-CTTCAATTCTGGAATCAGTGGTTTACTTTCAACTACGCCCCGC-3’

G180A G184A
Forward: 5’-GAAGGAAGCTTATGCCGCTGCCGCAGCCGCTGTCGGCTCGGCCGGTCC-3’
Reverse: 5’-GGACCGCGACGACAGCGGCTGCGGCAGCGGCTATAAGCTTCCTTCC-3’

G201A G205A
Forward: 5’-CCTATTCTATTAGTGCGGCCGTAGTTGAAGGAAAACTG-3’
Reverse: 5’-CAATTCTGGAATCAGTGGTTTACTTTCAACTACGCCCCGC-3’

P36A
Forward: 5’-CTCGATCAGGTCATGCCTGGCCAGACC-3’
Reverse: 5’-GGTCTGCGCCAGCCGATGACCGCATCGAG-3’

F190A
Forward: 5’-GTCGTGCGCCGGCTCCAGCTGGAATATCATATTCC-3’
Reverse: 5’-GGAATGATTAAATCCAGCTGGACGCGACGAC-3’

C87A
Forward: 5’-CAAACAGTGTATGAATGGGCTGGTGTTGCGACGC-3’
Reverse: 5’-GCGTCGCAACACCAGCCCATTCATACCTGGTTTG-3’

C285A
Forward: 5’-GCCAAAAATATGGCTAATGCTGATGACTACGCTTTCTGATGGCC-3’
Reverse: 5’-CTTTTCTGATACTCATTAGCGGTGTTAATCATTTTTGGC-3’

F50C
Forward: 5’-CATAAAAGGTTAAGGTCGCTGTAACGAGGATGATTCGACGAGC-3’
Reverse: 5’-GCTGTTGAAATCTCCTGTTTACAGCGACTTACCTCTTTATG-3’

F190C
Forward: 5’-GTGTCGCTGCGCCGGTCATGTGGATTAATCATATTCC-3’
Reverse: 5’-GGAATGATTAAATCCACATGGACCGGCACCACGAC-3’
A6C
Forward: 5’-CATCATCACACTGAAATCGTTTGCATGAAAAACGGTAGAAGTAGTTAAAAACGC-3’
Reverse: 5’-GCCTTTTTAAAATCTACGCGTATGCTGTGATGATGATGAGAATC-3’

V264C
Forward: 5’-CTGAAACAACCAGATTCTACGTTTGTGATTATGATATTTAATGTGT-3’
Reverse: 5’-GAAAGCATTAAATCATCATAATCACAACAGTGAATCTGGTTGTTTCA-3’

K29E
Forward: 5’-AGCATTAGATCTTTATAATGAATATCGATCAGGTCATCC-3’
Reverse: 5’-GGATGACCTGATCGAGATATTCATTATAAAGATCTAATGCT-3’

G63E
Forward: 5’-GCAGCCTCGTTTTAGTGAAGATATTTAATTAACCGGAGGCTGC-3’
Reverse: 5’-TAAGTAAGGTTTTAATATCTTCGACTAAAACGGAGGCTGC-3’

G146E
Forward: 5’-TTCAACACGCTTCCGAGAAACTGCTGGCGTTA-3’
Reverse: 5’-TAACGCCAGCAGTTTCTCGGAAGCGTTGTTTGA-3’

6.2 List of Abbreviations

A absorption
AU absorbance units (UV-Vis spectroscopy)
a.u. arbitrary units (fluorescence spectroscopy)
ANS 8-Anilino-1-naphthalenesulfonic acid
CD circular dichroism
CDC Cholesterol-dependent cytolysin
ClyA Cytolysin A
CMC critical micelle concentration
CS cryoprotection solution
CV Column volume
DDM n-dodecyl-ß-D-maltopyranoside
DTT Dithiothreitol
EC no. enzyme commission number
E. coli Escherichia coli
EDTA Ethylenediaminetetraacetic acid
EM electron microscopy
FRET fluorescence resonance energy transfer
HA Hydroxyapatite
HPLC high performance liquid chromatography
I Intermediate
IMAC Immobilized metal ion affinity chromatography
<table>
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>M</td>
<td>Monomer</td>
</tr>
<tr>
<td>MPD</td>
<td>2-methyl-2,4-pentanediol</td>
</tr>
<tr>
<td>n.a.</td>
<td>not available</td>
</tr>
<tr>
<td>NCCR</td>
<td>National Center of Competence in Research</td>
</tr>
<tr>
<td>OAc</td>
<td>Acetate</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>OG</td>
<td>n-octyl-β-D-glucopyranoside</td>
</tr>
<tr>
<td>OMV</td>
<td>Outer membrane vesicle</td>
</tr>
<tr>
<td>oN</td>
<td>overnight</td>
</tr>
<tr>
<td>ox</td>
<td>oxidized</td>
</tr>
<tr>
<td>P</td>
<td>Protomer</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PFO</td>
<td>Perfringolysin O (a cholesterol-dependent Cytolysin)</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethanesulfonyl fluoride</td>
</tr>
<tr>
<td>PSI</td>
<td>Paul Scherrer Institut (Villigen, CH)</td>
</tr>
<tr>
<td>red</td>
<td>reduced</td>
</tr>
<tr>
<td>r.m.s.</td>
<td>root mean square</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEC</td>
<td>size exclusion chromatography</td>
</tr>
<tr>
<td>sp.</td>
<td>species</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
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<tr>
<td>TEV</td>
<td>Tobacco etch virus</td>
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<tr>
<td>TLS</td>
<td>Translation libration screw motion</td>
</tr>
<tr>
<td>wt</td>
<td>wild type</td>
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