Designing a Pore-Forming Toxin Cytolysin A (ClyA) Specific to Target Cancer Cells

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DESIGNING PORE-FORMING TOXIN CYTOLYSIN A (CLYA) SPECIFIC TO TARGET CANCER CELLS

A Thesis Presented
by
ALZIRA ROCHETEAU MONTEIRO AVELINO

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

MASTER OF SCIENCE

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Program in Molecular and Cellular Biology
DESIGNING PORE-FORMING TOXIN CYTOLYSIN A (CLYA) SPECIFIC TO TARGET CANCER CELLS

A Thesis Presented

by

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DEDICATION

TO MY CARING GRANDMOTHER
ACKNOWLEDGEMENT

I take this page to thank everyone who was involved directly or indirectly in this project. To my advisor, Dr. Min Chen, who has given me the chance to work under her experienced guidance and supervision. I would like to also thank the rest of my committee members, Dr. Ludmila Tyler and Dr. David Gross for their invaluable input and for being wonderful professors.

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My gratitude goes to every person who helped make this project possible in one way or the other; by supporting with constructive criticism or simply by providing positive energy, which has kept me going. Thanks again; I could not have done it without you.
ABSTRACT

DESIGNING A PORE-FORMING TOXIN CYTOLYSIN A (CLYA) TO TARGET CANCER CELLS

SEPTEMBER 2014

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Directed by: Dr. Min Chen

Cytolysin A (ClyA) is a member of a class of proteins called pore-forming toxins (PFTs). ClyA is secreted by Gram-negative bacteria, and it attacks a number of mammalian cells by inserting into and forming channels within the cell membrane (Oscarsson J et al., 1999). It has been suggested that ClyA binds to cholesterol (Oscarsson J et al., 1999) and thus can insert into the membranes of many different cell types of eukaryotic origin. In our studies we propose to engineer a ClyA protein that can only attack a small subset of cell types. We propose to engineer ClyA that can be only activated when exposed to specific cell-surface proteases produced by a specific cell type. We ultimately want to target breast cancer cells that differentially secrete or express proteases such as matrix-metalloproteases (Stautz D et al., 2012; Zhang, M et al. 2013). To engineer this protein we took advantage of the N-terminus of ClyA. The N-terminus of ClyA, which is highly hydrophobic (Oscarsson J et al), undergoes a conformational change to insert into the target cell membrane (Oscarsson J et al). This conformational change allows ClyA to penetrate the target membrane.
to form a transmembrane domain of ClyA. The hydrophobic nature of lipid membranes makes it highly unfavorable for any charged residues to cross the membrane (Hunt J 1997). With this in mind, we hypothesize that negative charges inserted into the N-terminus of ClyA will inhibit it from inserting into the membrane. Thus, we mutated the N-terminus of the ClyA protein by inserting an inactivation site composed of negatively charged amino acids that we hypothesize would prevent insertion into the plasma membrane of the target cell. Once we confirmed that this construct was an inactive ClyA mutant, we inserted a thrombin cleavage site right after the inserted negative charges. This site should allow us to remove the negative charges once the protein is exposed to thrombin. Once the negative charges are removed, the protein should recover its activity. This approach will allow us to create a version of ClyA that is protease-switchable.
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CHAPTER 1

INTRODUCTION

1.1 Cytolysin A (ClyA) as a potential cancer therapeutic

Finding new treatments for cancer has been one of the top priorities of many research laboratories. There have been many treatments such as chemotherapy and radiation therapy that have been shown to be effective. However, these treatments have many side effects. These treatments often attack rapidly replicating cells in a non-specific manner, and there is always a chance of disease reoccurrence. For this reason it is necessary to develop new treatments that are more effective and specific (Hassan et al., 2010).

New developments combine current therapies with bacterial therapy (BT). Studies have shown that bacteria such as *Escherichia coli* (*E. coli*) accumulate in tumors (Chen D et al., 2010; Kim J. et al. 2008; Anderson, J. C. et al. 2006), and we can use this characteristic to deliver specific anti-tumor treatments.

1.2 Cytolysin A (ClyA)

Cytolysin A (ClyA) is a pore-forming protein secreted by Gram-negative bacteria such as *E. coli* and *Salmonella enterica*, serova, typhi and *Paratyphi A* (Jiang S. et al., 2010; Von Rhein, C. et al, 2009). The shape of ClyA is a long rod, and it is comprised of 81% α-helices and 2% β strands (Wallace et al., 2000). It is a 34 kDa protein that is cytotoxic to cultured mammalian cells and can also cause macrophage apoptosis (Jiang S., 2010). ClyA is transported from the bacteria to the target cell in outer membrane vesicles (OMVs) as an oligomer (Chen et. al,
Once ClyA reaches the target membrane, the N-terminus (Figure 1a in red) undergoes a conformational change and inserts into the membrane (Figure 1b). ClyA is made as a soluble monomer and exported to the periplasm (Chen et al, 2008). To form into an active pore, the protein oligomerizes into a dodecamer (Chen et al, 2008) (Figure 1b) that has a diameter of 35 Å at its narrowest opening and 70 Å at the top of the oligomer (Mueller M. et al., 2009). The X-ray crystal structure of the monomer was solved by Wallace et al. (Wallace AJ. et al 2000 Cell), and the oligomer crystal structure was solved by Mueller et al. (Mueller M. et al., 2009).

Figure 1: Schematic representation of ClyA molecules. (a) Representation of an inactive monomer, with the N-terminus tucked in; (b) The active protomer with the N-terminus inserted into a target lipid membrane. In a and b, the N-terminus is highlighted in red. Structures were drawn in PyMol.

The toxic ClyA pore shown in Figure 1b promotes lysis of the target cell by allowing uncontrollable exchange of materials between the extracellular environment and the intracellular contents.
CHAPTER 2
RESULTS AND DISCUSSION

2.1 Construct design

To make a switchable form of ClyA, we took advantage of its N-terminus, which is responsible for its membrane insertion. The N-terminus of ClyA crosses the target lipid membrane and forms a transmembrane domain. By mutating the N-terminus, we can prevent the membrane insertion.

Charged residues are very energetically unfavorable to cross the cell membrane (Hunt J 1997). Moreover, we hypothesize that negative charges are even more unfavorable because the phospholipid heads that are also negatively charged should repel the negative residues on ClyA. Therefore, we hypothesized that negative charges added to the N-terminus will inhibit ClyA insertion into a target membrane.

To accomplish this, we designed mutant ClyA proteins that contain stretches of negatively charged amino acids (aspartate and/or glutamate) and tested the activity of these modified proteins with hemolytic assays, where we mix the ClyA proteins with erythrocytes and if lysis occurs hemoglobin is released and quantified with the use of a spectrophotometer (540 nm). For more detail, refer to Appendix A. Initially our constructs contained negative charges added at the N-terminus and a hexa-histidine tag (His-tag) at the C-terminus. Later, the His-tag was moved from the C-terminus to the N-terminus in modified constructs.
To make the mutant ClyA a switchable protein, we also designed constructs with a protease (thrombin) cleavage site directly after the negative charges. If exposed to protease (thrombin), the negatively charged amino acids could then be cleaved off and the ClyA protein reactivated. Figure 2 summarizes the designed constructs.

![Figure 2: Protein representation of the designed mutant versions of ClyA.](image)

The figure above describes the location of the inactivating site (red), comprised of varying numbers of aspartates (Asp) and/or glutamates (Glu), the His-tag (blue) and the thrombin cleavage site (yellow). ClyA is indicated in gradient black.

### 2.2 Purification of the inactive mutant proteins

All of the constructs were purified using nitrilotriacetic acid affinity chromatography (Ni-NTA) purification, which takes advantage of specific binding between the His-tag and nickel immobilized in the chromatography column (Figure 3). To further purify ClyA, we used gel filtration chromatography (GFC) that separated molecules according to their stochastic radius. Whereas a monomer of ClyA will elute with buffer at a volume of 15 ml using Superdex 200,
the mutant proteins elute at 17 ml of retention volume (Fig. 4). For more information on this technique, refer to Appendix A.

**Figure 3: Nickel affinity chromatography of His-tagged ClyA.** Graph in black represents absorbance of protein at 280 nm and the graph in red is the percentage of 0.5 M imidazole in the elution buffer. ClyA starts to elute at just under 20% 0.5 M imidazole. The light blue bar represents where the ClyA monomer was collected.

**Figure 4: Gel filtration chromatogram of ClyA monomer.** The pure, negatively charged ClyA mutants elute around 17 ml. The red bar represents where the ClyA monomer was collected.
2.3 Testing the activity of purified samples

To test the activity of the ClyA putatively inactive mutants, we use a liquid hemolytic assay (Fahie, M. et al. 2013; Rowe, G. C. et al.1994). ClyA can lyse erythrocytes, thus releasing hemoglobin that can be quantified by measuring its innate absorbance at 540 nm (Iwasaka, M., et al. 2001). The absorbance value and the release of hemoglobin directly correlate with the number of erythrocytes lysed and thus how active the protein (ClyA) is. Refer to Appendix A for details on how the experiment was performed.

Figure 5 below shows the activity of the mutant ClyA using construct 1 from Figure 2, with different numbers of negative charges. At 6, 10, 12, 15, or 18 negative charges (aspartates and glutamates) the activity of ClyA was not completely inhibited, as indicated by 60% lysis or higher. These findings led us to increase the number of negative charges up to 24 (Figures 5 and 6).
Figure 5: Hemolytic activity of ClyA mutants. Constructs with increasing numbers of negative charges (6-cyan, 10-blue, 12-navy, 15-gray, 18-black) designed from construct 1 (Figure 2) were tested. All samples had a protein concentration of 0.7 μg/ml. From the above figures, we can see how various numbers of negative charges affect the activity of ClyA. All samples were incubated with defibrinated sheep red blood cells at 37°C for 15 minutes. WT: wild-type ClyA, without additional negative charges.
Figure 6: Hemolytic activity of mutant ClyA proteins with larger numbers of negative charges. Twenty-one negative charges (cyan) and 24 negative charges (dark blue) from construct 1 (Figure 2) and WT ClyA (white) with a C-terminal His-tag were tested. All samples had a protein concentration of 0.6 μg/ml. From the above figures we can see how various numbers of negative charges affect the activity of ClyA. All samples were incubated with defibrinated sheep red blood cells at 37°C for 15 minutes.

Even with increasing numbers of negative amino acids, the activity of ClyA was still very high. This result suggests that either 24 negative charges can comfortably cross a bilayer, or that we are not working with the expected construct. However, as mentioned earlier, it is highly unfavorably to insert 24 negative charges across a bilayer (Hunt J 1997). To distinguish between the possible explanations, we moved the His-tag from the C-terminus to the N-terminus directly before the negative charges (construct 2, Figure 2). This allows only the correct mutant protein to be selected by nickel affinity purification.
Figure 7 below shows the activity of the mutant ClyA with different numbers of negative charges and with an N-terminal His-tag (Nhis).

Figure 7: Hemolytic activity of mutant ClyA proteins with different numbers of negative charges and an N-terminal His-tag. 6-Nhis is ClyA with six additional negative charges and an N-terminal His-tag, 12-Nhis has 12 negative charges and an N-terminal His-tag, and 18-Nhis has 18 negative charges and an N-terminal His-tag. All samples had a protein concentration of 4.4 μg/ml. The negative control in black contains blood with no protein added. From the above figure we can see how much each number of negative charges with an N-terminal His-tag affected the activity of ClyA. All samples were incubated with defibrinated sheep red blood cells at 37°C for 15 minutes.

The hemolytic assay shows that with 12 and 18 negative charges after the N-terminal His-tag, the activity of ClyA is increasingly inhibited. Because of this result, we concluded that the mutants based on construct 1 were being post-translationally cleaved to wild type (WT)-like-ClyA.
2.4 Making a switchable ClyA

The next step is to introduce a thrombin cleavage site just after the negative charges so that when thrombin is present it can cleave the negative charges, thus reactivating ClyA (construct 3, Figure 2).

After purification with GFC and confirming that we had obtained the pure mutant protein by performing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 8), the new mutant protein with the thrombin cleavage site and 18 negative charges (18′NhisT) was incubated with thrombin in a cleavage assay and its activity tested (Figure 9). For more information on the thrombin cleavage assay see Appendix A. The expected size of ClyA is 34 kDa. However, the mutants with N-terminal negative charges seem to travel further down the SDS-PAGE gel. We hypothesize that the negative charges may be the cause of this migration pattern, since the additional negative charges in the mutant proteins may make them more strongly attracted to the positive electrode during SDS-PAGE (Figure 8). The WT ClyA band intensity is stronger than the band intensities for the negative mutants, because the purification with nickel columns yields a lower concentration of negative mutant.
Figure 8: SDS-PAGE of purified proteins after GFC. 18-Thr-ClyA is the mutant ClyA with 18 negative charges, thrombin cleavage site and N-terminal His-tag. 18-ClyA lacks the thrombin cleavage site. WTClyA has an N-terminal His-tag, but no additional negatively charged residues.
Figure 9: Hemolytic assay of the mutant 18\textsuperscript{th}r-ClyA protein with N-terminal His-tag. One mole of wild-type or mutant ClyA (18-NhisT) was incubated with 2.5 moles of thrombin overnight at room temperature. All samples had a protein concentration of 4.4 μg/ml. Then the samples were incubated with red blood cells for 15 minutes at 37\textdegree{}C to test for hemolytic activity (WT\textsuperscript{ClyA} Nhis-tag in gray, 18-thr-ClyA in white with vertical stripes. 18-thr-ClyA in black indicates the mutant with no thrombin added, and the cyan bar represents thrombin incubated with blood with no other protein added).

The activity assay of Fig. 9 showed that thrombin treatment did not restore the mutant’s activity, leading us to question whether the thrombin site was accessible to thrombin. Thus, we inserted a longer glycine linker between the inactivating site and the cleavage site (Figure 2, construct 3); the insertion of this type of linker has been shown to improve the cleavage site’s accessibility to thrombin (Hakes, D. et al, 1992).
After obtaining and purifying the constructs with longer G-linkers (from 1 glycine to 6 glycines) named 12’NhisT and 18-NhisT (construct 3, Figure 2), the hemolytic activity of the constructs was once again tested after incubation without and with thrombin (Fig. 10).

**Figure 10:** Hemolytic assay of 12’NhisT and 18-NhisT with a hexa-glycine linker. The samples were incubated with thrombin in a 1:3.5 molar ratio overnight at room temperature. Then the samples were incubated with red blood cells for 15 minutes at 37°C. The concentration of protein present in all the samples was 10.5 μg/ml. The negative control in white just contains erythrocytes in buffer with no added protein. WTNhis ClyA in black is the positive control; 12-NhisT in dark gray with oblique strips is the ClyA mutant with 12 negative charges, thrombin cleavage site and N-terminal His-tag. 18-NhisT differs from the previous construct (12-NhisT) only in the fact that it contains 18 N-terminal negative charges.

From Figure 10, we conclude that the longer glycine linker has improved the accessibility of the cleavage site to thrombin, because when these ClyA
mutant proteins were incubated with thrombin, the percent of lysis had a 30% increase for 18-NhisT, and for 12-NhisT to almost WT status. Although after thrombin treatment 12’NhisT2 regained high activity, this protein is still active (~24% lysis) when thrombin is not present. Ideally, we want to design a switchable ClyA that is completely inactive without thrombin treatment even at high protein concentrations of the ClyA construct, which is the case for 18-NhisT. This latter mutant has very low activity, approximately 2%, in the absence of thrombin, but when thrombin is present the hemolytic activity of 18-NhisT increases to approximately 30%. However this activity increase is still not ideal. Ideally we would want a hemolytic activity increase to 80% or more. A shorter incubation period at 37°C would also be preferable (4 hours or less) and a molar ratio of at least 30:1 (ClyA protein to thrombin) is more desirable for medical application. We ultimately want to be able to activate the switchable protein in a short period of time and with a low concentration of protease, which we hypothesize would mimic human biological conditions.

In this matter, new constructs are being designed in order to increase activity when a specific protease is present and also to retain inactivity when the specific protease is absent. Once the new constructs are tested and satisfy all of these requirements, we will proceed to the next steps, which will be to design metalloprotease cleavage sites into the optimal construct and perform activity experiments with cultured mammalian cell lines.
2.5 Mechanism of inhibition

We hypothesized that adding negative charges inhibits membrane interaction, but activity can also be inhibited by preventing the oligomerization of ClyA. The lack of activity of the mutant 18-Nhis could be due to an inefficient membrane insertion or inefficient oligomerization, since the negative charges on individual monomers may not only repel the phospholipid bilayer but also repel each other. To test whether oligomerization was inhibited, we induced oligomerization with the commonly used detergent n-dodecyl-β-D-maltoside (DDM). We used GFC to detect the various oligomerization states (see Appendix A). According to the elution pattern, we can determine whether ClyA is oligomerized or not. Elution of the protein earlier than 17 ml means that the mutant can oligomerize into various forms such as dimers, etc., but if oligomerization is disrupted, the protein will elute as a monomer around 15 ml or 17ml depending on whether it is a WT or mutant ClyA, respectively.

The mutant used for the oligomerization assay is 18-thr-ClyA (construct 3, Figure 2). This mutant has 18 negative charges at its N-terminus and additionally a thrombin cleavage site immediately after the negatively charged region.

Figure 11 shows GFC chromatograms of the elutions of the mutant (18-thr-ClyA) and WT with the N-His-tag.
Figure 11: GFC chromatograms of elutions of the different mutant proteins with and without 0.1% DDM. After incubation with 0.1% DDM, the different samples indicated above were run through GFC in buffer containing 0.01% DDM. a) represents WT ClyA with no DDM added, b) shows the mutant with the 18 negative charges and N-His-tag with no DDM added. c) represents WT ClyA with 0.1% DDM added and incubated at 23°C for 30 minutes prior injection onto GFC, and d) represents the 18-thr-ClyA mutant with the N-His tag prepared the same way as in graph c. The y-axis represents absorbance at 280 nm (wavelength at which protein absorbs light); the x-axis indicates the retention volume in ml.

According to Figure 11, the mutant 18Nhis cannot oligomerize into a stable dodecamer (chromatogram d), because it still elutes after 15 ml of acquired volume. The WTNhis ClyA forms stable oligomers when incubated with 0.1% DDM at 23°C and elutes as a monomer when not incubated with DDM (Fig. 11a).
According to the data obtained thus far, we can conclude first that 18 negative charges at the N-terminus inhibit ClyA activity by inhibiting oligomerization and second that thrombin cleavage with the optimized linker restores the activity of this mutant. Further research is being performed in order to optimize the percent recovery when appropriate protease is added.
APPENDIX A

MATERIALS AND METHODS

Cloning strategy

All chemicals used were purchased from Fisher Scientific (Agawam, MA) unless specified otherwise.

The negative mutants ClyA were cloned and amplified in a pT7 vector with a C-terminal or N-terminal His-tag. The polymerase chain reaction (PCR) was performed using Phusion Polymerase (New England Biolabs, Ipswich, MA). The PCR products were digested with DpnI to degrade the template DNA. The digestion was performed for 3 hours at 37°C or overnight at the same temperature. The DpnI product was then transformed into Escherichia coli (E. coli) Nova Blue cells. The DNA sequence of the desired products was obtained through DNA sequencing from the Genomics & Bioinformatics Facility (University of Massachusetts Amherst, Amherst, MA). Refer to Table 1, Appendix B for the list of primers and templates used.

Protein expression

To obtain the desired protein, we inoculated E. coli BL21 cells containing the plasmid pT7-ClyA with the specific mutation into 1 L of Luria Broth (LB) medium (Boston Bioproducts, Ashland, MA) containing 0.1 mg/ml of ampicillin. The cells were grown at 37°C with shaking at 200 rpm to an OD_{600} of 0.6. Then 0.5 mM of Isopropyl β-D-1-thiogalactopyranoside was added to induce protein expression. The culture was incubated overnight at 15°C.
Protein purification

After expression, the cell culture was spun down for 30 minutes at 4000 rpm in a tabletop centrifuge. The supernatant was discarded and the pellet was resuspended in 20 ml of 50 mM Tris-HCl, pH 8, 0.2 mg/ml lysozyme, 1 mM ethylenediaminetetraacetic acid- EDTA, and 0.1 mM phenylmethanesulfonylfouride (Sigma, St. Louis, MO). Then the lysate was incubated for 30 minutes on ice. The lysate was then spun down for 30 minutes at 13,000 rpm in a Sorvall RC 6 Plus centrifuge from Thermo Scientific (Waltham, MA). The supernatant then was used for the following techniques.

The ClyA purification involves two chromatographic techniques: Ni-NTA purification and gel filtration chromatography (GFC). For Ni-NTA purification, a His-tag is introduced at the N-terminus or C-terminus of the protein. To do the nickel purification, we used a column packed with beads that contain nickel (Pierce centrifuge column, gravity column, from Thermo Scientific, Waltham, MA). This column has a total volume of 4 ml. At the start of purification the column is washed with 2 column volumes (CV) of 20% ethanol, 2 CV of miliQ water and 2 CV of Buffer A (150 mM NaCl, 50 mM Tris-HCl, pH 8). Tris was purchased from Boston Bioproducts (Ashland, MA). After equilibrating the column with Buffer A, the cell lysate is added and incubated with the nickel beads for 15 minutes on ice with shaking. Then the lysate is allowed to pass through the column at a rate of 10 drops per minute. The rate can be controlled by capping the column, reducing or increasing the airflow through the column. Only after the cell lysate has
completely run through is 1 CV of Buffer A added to wash off unbound proteins. Two more washing steps are conducted with Buffer A’ (150 mM NaCl, 50 mM Tris-HCl, pH 8 and 10 mM imidazole from Thermo GE Healthcare) and Buffer A1 (150 mM NaCl, 50 mM Tris-HCl, pH 8 and 25 mM imidazole). Imidazole will displace the protein of interest, which is collected in fractions. All fractions are collected and run on a 15% SDS-PAGE gel, containing 2,2,2-Trichloroethanol (TCE), to determine which fraction has a higher concentration of pure ClyA using the Gel Doc™ imager from Bio RAD (Hercules, CA). The fraction with pure ClyA is then dialyzed overnight (dialysis membrane from Spectra/Pro, MW cutoff 6-8000) in Buffer A (with EDTA) at 4°C. The second part of the purification involves purification by GFC which separates proteins according to their stochastic radius. For this type of chromatography we use the Superdex 200 column from CE Healthcare (Piscataway, NJ). The column has a volume of 24 ml and elutes soluble WT ClyA monomers at a 15 ml retention volume and elutes the negatively charged mutants at a 17 ml retention volume. Initially the column is washed with 2 CV of 20% ethanol and 2 CV of water, and then the column is equilibrated with 2 CV of Buffer A. The dialyzed protein sample is concentrated to 1 ml using a Centricon from Millipore (Billerica, MA). Then the sample is injected into the equilibrated column. Fractions are collected at the appropriate retention volume and analyzed by SDS-PAGE (Figure 8) to determine the purity of the sample. The protein concentration is determined with a Bicinchoninic acid (BCA)
assay (kit from Thermo Scientific, Waltham, MA), and samples are stored at a temperature of -80°C.

**Hemolytic assay**

To perform the hemolytic assays, we use defibrinated sheep blood from Lampire Biological Laboratories (Pipersville, PA). The blood is washed with milliQ water containing 150 mM NaCl and centrifuged in a tabletop centrifuge from Eppendorf (4,000 rpm, for 5 minutes) to pellet the red blood cells, the supernatant is discarded, and the blood is diluted to 25% with milliQ water containing 150 mM NaCl. There are two main controls for this experiment (positive and negative control). The negative control is blood with buffer but no protein added, and the positive control is 25% blood plus milliQ water brought up to the same volume used in other samples. Twenty five percent blood is added to each sample and the final volume of the protein solution depends on the desired protein concentration (indicated in Figures 5, 6, 7, 9, and 10). The samples are incubated at 37°C for 15 minutes; then the samples are centrifuged in a tabletop centrifuge at 14,000 rpm for 2 minutes. The supernatant contains the hemoglobin released from the cell, and its absorbance is measured at 540 nm. The absorbance values are used to plot a graph indicating the percentage of lysis. The positive control indicates 100% lysis while the negative control acts as a baseline for zero lysis.

**Oligomerization assay**

This experiment is performed to determine whether the mutant proteins, like WT ClyA, can oligomerize in 0.1% n-Dodecyl β-D-maltoside (DDM). Seventy
micrograms of protein is incubated with 0.1% DDM at 23°C (see Figure 7) for 30 minutes and then injected into the GFC column equilibrated with Buffer A containing 0.01% DDM. The elution pattern is observed: if the protein elutes before 15 ml of retention volume, it means that the protein can indeed form various oligomers. However, if the protein elutes around 15-17 ml, it is eluting as a monomer.

**Thrombin cleavage assay**

This assay is performed for mutant proteins that have a thrombin cleavage site. A specific concentration of protein (see Figures 5, 6, 7, 9, and 10) is incubated with thrombin (in a molar ratio indicated in the figure legend) overnight at room temperature (approximately 23°C). After incubation, the samples are subjected to a hemolytic assay to test if the activity is recovered. Thrombin is purchased from MP Biomedicals (Santa Ana, CA).
Table 1: List of all the negative mutants and primers used.

<table>
<thead>
<tr>
<th>Negative mutant ClyA</th>
<th>Primers</th>
<th>Template</th>
<th>Inserted amino acid sequence</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-</td>
<td>F- GATGATGACGATGACGACACTGA AATCGTTGCAG R- GTCATCGTCATCATCCATCTCGAG GAGCCC A TATG</td>
<td>WT-ClyA C-His tag</td>
<td>M-D&lt;sub&gt;6&lt;/sub&gt;-ClyA</td>
<td>Engineered 6-Aspartate tag on ClyA at the N-terminus to Inhibit membrane insertion.</td>
</tr>
<tr>
<td>10-</td>
<td>F- AGAGGAGGACGACGAAGAAGAT ACTGAAATCGTTGCAG</td>
<td>6-</td>
<td>M-E&lt;sub&gt;6&lt;/sub&gt;D&lt;sub&gt;2&lt;/sub&gt;E&lt;sub&gt;2&lt;/sub&gt;D&lt;sub&gt;6&lt;/sub&gt;-ClyA</td>
<td>Engineered 6-Aspartate, 2-Glutamate and 2-Aspartate tag on ClyA N-terminus to inhibit membrane insertion</td>
</tr>
<tr>
<td>12-</td>
<td>F- GAGGAGGAAGGAGGAGACTGAAA TCGTTGCAG</td>
<td>6-</td>
<td>M-D&lt;sub&gt;6&lt;/sub&gt;E&lt;sub&gt;6&lt;/sub&gt;-ClyA</td>
<td>Engineered 6-Asp, 6-Glu tag on ClyA N-terminus to inhibit membrane insertion</td>
</tr>
<tr>
<td>15-</td>
<td>F- GAGGAGGATGACGACGACTGAAA TCGTTGCAG</td>
<td>12-</td>
<td>M-D&lt;sub&gt;6&lt;/sub&gt;E&lt;sub&gt;6&lt;/sub&gt;D&lt;sub&gt;2&lt;/sub&gt;E&lt;sub&gt;2&lt;/sub&gt;-ClyA</td>
<td>Insertion of 5 additional negative charges</td>
</tr>
<tr>
<td></td>
<td><strong>R-</strong> GTCTCGTCATCCTCCTCTTCCTCCTCTCTCTCTCG</td>
<td><strong>F-</strong> GGATGACGAGGAGGAAGATCTGAAATCGTGGACAG</td>
<td><strong>M-</strong> D$_3$E$_6$D$_2$E$_3$D-ClyA</td>
<td>Insertion of 3 additional negative charges</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>18-</td>
<td><strong>R-</strong> TATCTTCTCCTCCTCATCTCTCCTCTTCCTCT</td>
<td><strong>F-</strong> GGATGACGAGGAGGAAGATCTGAAATCGTGGACAG</td>
<td><strong>M-</strong> D$_3$E$_6$D$_2$E$_3$D-ClyA</td>
<td>Insertion of 3 additional negative charges</td>
</tr>
<tr>
<td>21-</td>
<td><strong>R-</strong> AGTCTCATCTCATCTCTCCTCCTCTGTC</td>
<td><strong>F-</strong> GGATGACGAGGAGGAAGATCTGAAATCGTGGACAG</td>
<td><strong>M-</strong> D$_3$E$_6$D$_2$E$_3$D-ClyA</td>
<td>Insertion of 3 additional negative charges</td>
</tr>
<tr>
<td>24-</td>
<td><strong>R-</strong> GTCTCGTCATCTCATCTACCATCTCCTCCTCTCCTCTCGT</td>
<td><strong>F-</strong> GGATGACGAGGAGGAAGATCTGAAATCGTGGACAG</td>
<td><strong>M-</strong> D$_3$E$_6$D$_2$E$_3$D-ClyA</td>
<td>Insertion of 3 additional negative charges</td>
</tr>
<tr>
<td>N-his tag</td>
<td><strong>F-</strong> CACCATCATCACCACCATGATGTGACGATGAC</td>
<td><strong>M-</strong> H$_6^-$ inactive site-ClyA</td>
<td>Insertion of an N-terminal His-tag</td>
<td></td>
</tr>
<tr>
<td>Thrombin site insertion 12-</td>
<td><strong>R-</strong> CATGCTGTGATGATGATGTGCATCGAGAGCCCA</td>
<td><strong>F-</strong> CCTCGTGACCCTGACTGACGAGTATTACATGTACCAGTACCAGGCA</td>
<td><strong>M-</strong> H$_6^-$D$_6$E$_6^-$GSLVPRES-ClyA</td>
<td>Insertion of a thrombin cleavage site</td>
</tr>
<tr>
<td>Thrombin</td>
<td><strong>F-</strong> CCTCGTGACCCTGACTGACGAGTATTACATGTACCAGTACCAGGCA</td>
<td><strong>R-</strong> CATGCTGTGATGATGATGTGCATCGAGAGCCCA</td>
<td><strong>M-</strong> H$_6^-$</td>
<td>Insertion of</td>
</tr>
<tr>
<td>n site insertion 18-</td>
<td>CCTGGTGCCGCCTGTTAGCACTGAAATCGTTGCA</td>
<td>with an N-His tag</td>
<td>( D_6E_6D_2E_3D - GSLVPRS-ClyA )</td>
<td>a thrombin cleavage site</td>
</tr>
<tr>
<td>----------------------</td>
<td>-------------------------------------</td>
<td>------------------</td>
<td>---------------------------------</td>
<td>------------------------</td>
</tr>
<tr>
<td>R- CCACGCAGCACAGGCTGCCATCTCCTCCTCGTC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycine linker insertion</td>
<td>F- AGCGGTGGCCGTTGGGACCTGGTGCCCCTGGTGCTGTTG</td>
<td>12- with an N-His-tag and thrombin site</td>
<td>( M-H_6^{-}D_6E_6^{-}GSG_5SLVPRS-ClyA )</td>
<td>Inserting a 5-glycine linker</td>
</tr>
<tr>
<td></td>
<td>R- CAGGCCACCACCGCCACCGCTGCCCTCCTCTC</td>
<td>18- with an N-His-tag and thrombin site</td>
<td>( M-H_6^{-}D_6E_6^{-}D_2E_3D^{-}GSG_5SLVPRS-ClyA )</td>
<td></td>
</tr>
<tr>
<td>C-terminal His tag deletion</td>
<td>F- CTCTCGAGATGACTGAAATCGTGCGAG</td>
<td>6-, 12-, and 18- not applicable</td>
<td>Deleting the C-terminal His-tag</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R- TTTTCAGTCTCATCTCGAGAGCCCATATGG</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

6-, 10-, 12-, 18-21-, and 24- refers to ClyA mutants with 6, 10, 12, 18, 21, 24 negative charges at the N-terminus respectively and a C-terminal His-tag. N-His tag refers to a Histidine tag at the N-terminal of ClyA. All primer sequences are presented from left to right or 5’ to 3’, respectively. The forward (F) and reverse (R) primers are depicted above for every negative mutant.
REFERENCES


