INFLUENCE OF 2 FLUOROHISTIDINE ON PORE FORMATION IN THE ANTHRAX PROTECTIVE ANTIGEN

A Thesis by

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I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirement for the degree of Master with a major in Chemistry.

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ABSTRACT

*Bacillus anthracis* secretes a toxin which consists of three proteins that is the cause of the anthrax disease symptoms leading to death. They are called the protective antigen (PA), edema factor (EF) and lethal factor (LF). The three proteins self-assemble into toxic complexes after PA binding to its receptors present on host cells. The toxin receptor complexes are then internalized and acidic endosomal pH triggers pore formation by PA and translocation of the LF and/or EF into the cytosol. In this study, we labeled PA with 2-fluorohistidine, an analog of histidine with a dramatically reduced side-chain pKa, in order to test the hypothesis that histidine protonation triggers the pH dependent change from a prepore to a pore. We have analyzed its functional properties. It can be cut by furin or trypsin and it can also bind with one of its receptor, the VWA domain of CMG2 and form a heptamer as wild type PA. However, the pore formation can be blocked by this labeled protein when bound to CMG2. Independent experiments show that 2F-His labeled PA can also block translocation. By modifying the protein with 2F-His, we show that histidine in PA and CMG2 does not play as a pH-sensitive trigger in the pore formation process. We provide hypotheses for these findings.
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<tr>
<td>A.A.</td>
<td>Amino acid</td>
</tr>
<tr>
<td>Asp (D)</td>
<td>Aspartic acid</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ATR</td>
<td>Anthrax toxin receptor</td>
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<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BisTris</td>
<td>2-[bis (2-hydroxyethyl) amino]-2-(hydroxymethyl) propane-1, 3-diol</td>
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<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CMG2</td>
<td>Capillary morphogenesis protein 2</td>
</tr>
<tr>
<td>Cys (C)</td>
<td>Cysteine</td>
</tr>
<tr>
<td>DEPC</td>
<td>(DEP) Diethyl pyrocarbonate</td>
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<tr>
<td>EF</td>
<td>Edema factor</td>
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<tr>
<td>EFn</td>
<td>N-terminal fragment of EF</td>
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<tr>
<td>ETx</td>
<td>Edema toxin</td>
</tr>
<tr>
<td>Glu (E)</td>
<td>Glutamic acid</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-(2-hydroxyethyl)piperazine-N’-3-propanesulfonic acid</td>
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<tr>
<td>His (H)</td>
<td>Histidine</td>
</tr>
<tr>
<td>I domain</td>
<td>Integrin-like domain</td>
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<tr>
<td>Ile (I)</td>
<td>Isoleucine</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>LB</td>
<td>Lauria–Bertani</td>
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<td>LF</td>
<td>Lethal factor</td>
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xiv
<table>
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<tr>
<td>LFn</td>
<td>N-terminal fragment of LF</td>
</tr>
<tr>
<td>LRP6</td>
<td>Low-density lipoprotein receptor-related protein 6</td>
</tr>
<tr>
<td>LTx</td>
<td>Lethal toxin</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>Mek</td>
<td>MAPK kinases</td>
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<tr>
<td>MES</td>
<td>2-(N-morpholino)ethanesulfonic acid</td>
</tr>
<tr>
<td>MIDAS</td>
<td>Metal ion dependent adhesion site</td>
</tr>
<tr>
<td>MTS-ET</td>
<td>Methanethiosulfonate ethyltrimethylammonium</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>PA</td>
<td>Protective antigen</td>
</tr>
<tr>
<td>PA63</td>
<td>63-kDa C-terminal fragment of PA</td>
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<td>PA20</td>
<td>20-kDa N-terminal fragment of PA</td>
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<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate saline buffer</td>
</tr>
<tr>
<td>Phe (F)</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions per minute</td>
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<tr>
<td>RT</td>
<td>Room temperature</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<tr>
<td>TEM8</td>
<td>Tumor endothelial marker 8</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris Hydroxymethyl aminoethane</td>
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Tyr (Y)  Tyrosine
UV      Ultraviolet
VWA     Von Willebrand factor A
WT      Wild type
CHAPTER I
INTRODUCTION

1.1 Anthrax Toxin

1.1.1 Research History

Anthrax toxins are virulence factors of *Bacillus anthracis*, which is a gram-positive rod-like bacterium that causes anthrax. *B. anthracis* is large spore-forming rod and it is around 1-1.2μm wide and 3~5μm long with square or concave ends [1]. *B. anthracis* is able to produce aerobic endospores so that they are highly resistant to dessication and capable of surviving for decades in soil. The natural reservoir for *B. anthracis* is soil. Under favorable circumstances, anthrax spores enter a vegetative phase and multiply to levels which are high enough to infect grazing herbivores.

Anthrax affects primarily livestock but can sometimes be transmitted to people who come in contact with infected animals or animal products.

Virgil first described anthrax as a plague affecting humans and cattle in eastern Alps [2]. Also there were some people speculated that anthrax was the cause of two Egyptians plagues described in the Old Testament, the appearance of boils and the death of cattle. Anthrax played a vital role in the history of bacteriology. Koch studied *B. anthracis* and used it in 1876 to prove his famous hypothesis. He was able to induce disease by growing the bacteria *in vitro* and challenging animals with culture of the bacterium, which disproved the theory of spontaneous generation and introduced the notion of germs. It was Koch who, for the first time, observed the life cycle of *B. anthracis* and showed that the bacteria could form highly resistant spores. That was 126 years ago and at that time, anthrax mainly damaged domesticated animals but in humans was fairly rare. The risk of infection was about 1/100,000. Today because of the terrorist attacks shortly after 9.11, anthrax is well known for being used as an important agent of biological terrorism and it threatens to people’s lives [3].

1.1.2 Symptoms of Toxin

The stability and infectivity of *B. anthracis* along with its ability to produce a rapidly lethal pneumonia has the potential to be a weapon of mass casualties [4, 5]. Anthrax occurs when spores of *B. anthracis* enter host tissues. In animals usually this occurs by ingestion, whereas in human beings, the infection is acquired from
infected animals, and so usually it is an occupational disease of farmers, woolworkers, slaughterers, etc. Over 95% of cases the infection is dermal. The commonest form of the disease is anthrax of the skin.

Large inhaled doses of spores can produce serious pneumonia to humans even though they are less susceptible to anthrax than most herbivores. Finally the inhaled spores devastate blood-borne infection. The inhaled particles are between one and five microns in diameter. They can lodge in the lungs.

Illness usually occurs two or three days after exposure. If under mild degrees of exposure, the incubation periods can be longer. For the first few hours, the symptoms are like influenza with aches and pains, fever and increasing cough as well as shortness of breath. Then the symptoms progress rapidly to severe cough, collapse and respiratory failure, often with a fatal outcome in two or three days [1].

The estimated inhalation dose for anthrax spores in humans is 8,000 - 50,000 spores based on the data from studies on primates [6] the aerosol particles larger than 5 microns quickly subside and form a possibility of a secondary aerosol formation. Smaller aerosol particles (1 - 5 microns in diameter) behave as gas and persist in the environment without settling. The reported incubation period for inhalation anthrax ranges from 1 to 43 days [7]. The lethality in untreated inhalation anthrax reaches 86 - 97% [6].

1.1.3 Pathogenesis and Role of the Toxins

No matter what the route of entry is, spores are phagocytosed by macrophages where they germinate [8]. Immune-system macrophages in the lungs ingest *B. anthracis* spores when they are inhaled. Such invaders are usually destroyed by macrophages; however, in this case the spores kill the macrophages instead. By an unknown mechanism, the bacterium escapes destruction and lyses the macrophages to gain access to host tissue. Bacterium then finds its way into the bloodstream, where it secretes the three-component anthrax toxin [1].

In its host, *B. anthracis* appears as a large encapsulated, non-motile rod often found in long chains. During infection the bacteria replicates to high titers of up to 10^9 cfu/ml of blood. This exceptional infectivity is due to two main virulence factors coded by genes present on two large plasmids, plasmids pXO1 and pXO2. Plasmid pXO2 carries genes directing the synthesis of a polyd-glutamic acid capsule that was shown to inhibit phagocytosis of the planktonic bacteria [9]. Plasmid pXO1 carries genes coding for toxins.

Subsequent research proved that the toxin consists of three polypeptides: protective antigen (PA), edema factor (EF) and lethal factor (LF). EF and LF are enzymes which are delivered to the host cell’s cytoplasm by PA.
LF is a protease that cleaves members of the mitogen-activated protein kinase kinase (MAPKK) family of signal transduction proteins close to their amino termini. Shutting down this signal transduction pathway can inhibit proliferation of the macrophages when they attempt to mount an immune response to the anthrax bacteria. The LF component is a metalloenzyme that has a Zn$^{2+}$ atom at its active site. LF comprises four domains. Domain I, or LFn, (residues 1–263) is the N-terminal PA binding domain. Domains II, III and IV work together to create a long, deep groove which holds the N-terminal tail of MAPKK before it is cleaved [11]. The crystal structures for the LF component, bound and unbound with N-terminus of MAPKK are shown below.
substrate. (Right) unbound structure of LF. LF is with same color as left. The Zn\(^{2+}\) in the active site was shown to be ligated by His 686, His 690, Glu735, and the activate water which serves as the nucleophile in the cleavage of the peptide bond is shown as blue [10].

EF catalyzes the conversion of ATP to cyclic-AMP (cAMP):

Figure 3. EF catalyzes the reaction of ATP conversion [10].

EF is necessary for the edema causing toxin activity. EF from \textit{B. anthracis} is an 89 kDa protein including 800 amino acids [12]. The activated EF is able to generate cyclic AMP. Too much cAMP can block them from performing their normal role of sending out cytokines to alert the immune system to a bacterial invasion [13]. EF is known to be an inherent adenylate cyclase, which leads to an impairment of host defenses. The edema toxin rises when bound with PA (PA+EF) and this complex can induce edema [14].

It has been shown that these three proteins that constitute anthrax toxin assemble into two distinct toxins with different physiological effects. A mixture of PA and EF forms edema toxin (ETx), which causes edema when injected subcutaneously in laboratory animals. A mixture of PA and LF forms lethal toxin (LTx), which causes death when injected intravenously in laboratory animals.

Many evidences demonstrate that the toxins play a key role in the pathogenesis of anthrax. Work in recent years has improved our understanding of the structure, delivery, and cellular activity of the toxins [15, 16, 17].
However, some molecular details of the intoxication still remain unclear. More importantly, the in vivo mechanism of behavior of the toxins is very little understood.

1.1.4 Anthrax as a Bioweapon

The use of *Bacillus anthracis* as a biological weapon can be traced from the period between World War I and II. German agents tried to disseminate *B. anthracis* among animals in Argentina, Romania, Spain and USA during World War I. From 1932 to 1945 in China, anthrax was in the biological arsenal of Japan Unit 731. The first experiments with anthrax were carried out on Chinese prisoners in occupied Manchuria. It is estimated that 2,000 prisoners and 700 civilians died from anthrax attacks [18]. In 1941 the British government tested the release of anthrax spores on an island near Scotland [19, 20]. The island remained a hazard all of following years until tons of seawater and formaldehyde were used to sterilize its soil in 1986. Hitler's Germany conducted offensive anthrax research in a secret laboratory near Poznan. Fortunately, the end of the war came earlier than results could be applied in practice [21]. In former Soviet Union, an epidemic occurred in Sverdlovsk in 1979 [22, 23]. Ninety-six people were reported ill and 64 died. Even though the authorities claimed that it was an outbreak in cattle which was transmitted to humans by consumption of contaminated meat. Later it was shown that an accidental release of an aerosol from the military facility which was near the Sverdlovsk was the criminal. Iraq was convicted of offensive research, production, weaponizing and storage of anthrax in rockets and bombs [24]. These facilities were destroyed after the Gulf War. Also Aum Shinrikyo, a Japanese who attempted several times to kill people by anthrax aerosol in different places in Japan between 1990 and 1995. Luckily, these attempts failed [25]. In the USA, terrorists misused anthrax after 9/11. The first inhalation case was reported on Oct. 4th in 2001, and the last one on Oct., 31st, in 2001. This attack resulted in one death from 22 confirmed cases of anthrax exposure [26].

One of the challenges of anthrax is that at the beginning of infection it is difficult to distinguish between influenza-like illnesses and inhalation anthrax. Once symptoms develop, it is often too late to undertake an effective treatment. Antibiotics can eradicate the bacteria, but the huge load of toxins can still kill the victim at last. The only applicable course of action today is aggressive supportive care. The outbreak in 2001 showed that this way reduced the mortality rate from around 100% to a little bit below 50% [27].

1.2 Protective Antigen (PA)

Protective antigen (PA) is a 735-residue long, 83-kDa protein. The name for PA comes from its use in producing vaccines against anthrax. The structure of PA was solved not only as a monomer but also as a trypsin-
activated, purified soluble heptamer of PA63 [28]. The soluble heptamer structure is thought to be the same as to that of the heptamer assembled on the cell surface prior to endocytosis. Hence it was termed “prepore” to differentiate it from the structure of the membrane-inserted “pore” at low pH. And also, there is little structural difference between the two forms of PA63, which is in the monomer, and the prepore. PA has four domains (Fig. 4). The function of each domain is described below.

**Figure 4. Crystal structure of PA.** PA monomer and the four domains are in different colors: domain 1 in yellow; domain 2 in red; domain 3 in grey and domain 4 in green [29].

### 1.2.1 Domain 1

Domain 1 (residues 1–249) contains the furin cleavage site at position 167 of the N-terminal. The domain therefore has two subdomains, PA20 domain 1a (residues 1–167) and domain 1b (residues 167–249). Domain 1 has two calcium ions tightly bound and most likely play a structural role [30, 31]. There is little structural difference between the two forms of PA63 in the monomer and the prepore confirms the idea that the only role of PA20 is to sterically prevent oligomerization of PA63. The release of the PA20 fragment allows the remaining PA63 to form heptamers. The removal of the PA20 fragment also exposes a hydrophobic surface on PA63, which is believed to be site where LF and EF bind [32].

### 1.2.2 Domain 2
Domain 2 (residues 249 to 488) has a β-barrel core structure and makes up most of the lumen in the prepore structure. Domain 2 was shown to possess a chymotrypsin-sensitive region critical in the translocation process [33]. This region, corresponding to the loop joining the 2β2 and 2β3 strands, is disordered and unresolved in the PA structures, suggesting that it is highly flexible. It was hypothesized that this flexible loop might insert into membranes at low pH [28]. The loops of all the monomers would then form a β-barrel channel in the membrane. The structure of the pore form of the heptamer remains elusive, but various approaches were developed to test this hypothesis.

The insertion of the flexible loops would require a major rearrangement in the rest of domain 2 [28]. The structural rearrangement taking place upon pore formation is likely responsible for the sodium dodecyl sulfate (SDS) resistance of the pore, whereas the prepore is SDS sensitive [34]. The identification of the residues that provoke this conformational change upon a drop in pH, the “pH trigger,” remains elusive. It was postulated that histidine residues, which have a protonation state likely to change between pH 7 and pH 5, might be involved [28]. In domain 2, histidines are located at the top of the flexible loop or in a loop with a pH-sensitive mobility. No confirmation that these residues are indeed part of the pH trigger has been obtained.

In the cysteine-scanning study, most of the mutations that abolished the activity of PA were found in domain 2 and clustered in the region forming the lumen of the prepore. This region seems therefore important. Most of these mutants were unable to make pores and translocate LFn [35].

![Figure 5. Crystal structure of domain 2. a) Domain 2; b) the loops in domain 2 which plays a role in pore formation [10].](image)
1.2.3 Domain 3

Domain 3 (residues 488–595) has a ferredoxin-like fold. In a random mutagenesis approach undertaken to elucidate the function of this domain, inactive mutants were found to be unable to form heptamers [36]. The primary role of domain 3 therefore seems to be the oligomerization of PA63.

1.2.4 Domain 4

Domain 4 (residues 595–735) has an initial hairpin and helix, followed by a \(\beta\) -sandwich with an immunoglobulin-like fold. Mutations in domain 4 affect the binding of PA to target cells [37, 38, 39] and monoclonal antibodies binding to this domain affect PA binding [40]. This proves that domain 4 is the receptor-binding domain. The crystal structure below shows contact between domain 4 of PA with the VWA domain of the receptor of CMG2 [41].

![Figure 6. PA63 heptamer subunit bound to VWA domain of CMG2. PA63 domain 4 (red) and 2(blue) are at the top. The VWA domain is at the bottom. The metal ion dependent adhesion site (MIDAS) on the VMA domain interacts with Asp 683 from domain 4 of PA [10].](image)

1.3 Receptors for PA

The first step in the intoxication is the binding of PA to its cellular receptor what was called the Anthrax Toxin Receptor (ATR) [1]. ATR is expressed in a variety of tissues. PA is able to bind to one kind of receptor named the tumor endothelial marker 8 (TEM8) [42]. PA is also able to bind to another receptor named the capillary morphogenesis protein 2 (CMG2) [41]. Both of these two receptors are transmembrane receptors and
share a sequence identity of 60% [43]. The von Willebrand factor A (VWA) domain on the receptor is where the PA component binds.

Recent studies show that there is approximately one pH unit difference of the pH threshold for conversion of the PA prepore to the pore and for translocation depending on whether TEM8 or CMG2 is used for entry. Furthermore, PA will dissociate from receptor before pore formation [44].

1.3.1 TEM8

An 80-kDa cellular protein was found to be associated with PA by using cross-linking experiments [45]. After a decade, the gene coding for the receptor for the toxin was found by a genetic method. Then, a complementing cDNA was identified by screening a library which named tumor endothelial marker-8 (TEM8). TEM8 is expressed in many tissues. TEM8 contains a VWA domain, also called integrin-like domain (I-domain). Usually these kinds of domains mediate protein–protein interactions. The VWA domain of TEM8 is the PA binding site [42], and it was found that a MIDAS motif within the domain was important for toxin binding. The divalent cations like Mg$^{2+}$ or Mn$^{2+}$ are vital for ligand binding.

1.3.2 CMG2

The second receptor for PA was encoded by capillary morphogenesis protein 2 (CMG2) [46]. CMG2 is widely expressed and dependent on the I-domain and its ion-binding motif like TEM8 and the I-domain of CMG2
is highly homologous to that of TEM8. In CMG2 the binding of PA was like that of TEM8. The most distinctive feature of these I-domains is that a structural motif forming MIDAS, where a divalent metal ion is needed by the receptor and at the same time is recognized by a negatively charged amino acid from its ligand. Asp683 from domain IV of PA has been pinpointed by mutagenesis studies and genetic analysis to bind the metal ion at the MIDAS site of the CMG2 VWA domain [47, 38]. The affinity between PA and CMG2 is at least 1000 fold higher [48] than that of a typical integrin-ligand complex [49], which indicates that additional interactions in PA-CMG2 binding must exist [41].

1.4 Toxin Assembly and Endocytosis

Once bound to a receptor, PA can be cleaved by furin or a furin-like membrane endoprotease [50]. The cleavage results in the release of an N-terminal 20-kDa fragment (PA20). This fragment is not play a further role in the intoxication. The remaining 63-kDa fragment (PA63) oligomerizes quickly into a heptamer[51], a step which was prevented sterically by PA20 before cleavage. Cleavage of PA can also be achieved in solution using trypsin [33].

Figure 8. PA63 monomer and heptamer. a) PA63 subunit. Domains 1, 2, 3, and 4 are colored in pink, green, yellow and blue; b) PA63 heptamer. The heptamer is viewed from the bottom. Domains 2 (green) and 4 (blue) from the 3.6Å (PA63)- structure, as viewed from the bottom. The domain 2 insertion loop (red) projects out to bind the neighboring monomer in a groove between domain 2 and domain 4 [52].

Once PA63 assembled into a heptamer, it can associate with LF and EF. Then the fully assembled toxic complex was yielded. The PA-binding domains of LF or EF are in what is called LFn or EFn, which are
approximately 250 residue-long N-terminal domains [53]. The saturating concentrations of enzymatic moieties are three ligand molecules (either EF or LF) per heptamer [54].

Prior to cleavage, PA bound to its receptor that is found in detergent-soluble parts of the plasma membrane. As monomers, receptor molecules are transferred slowly into detergent-insoluble parts, also called lipid rafts. After the heptamer forming, the endocytosis is becoming rapid [55]. The mechanism for governing endocytosis is still unclear. After endocytosis, the toxic complex is delivered to an acidic compartment [32].

Wei and coworkers have shown that the low-density lipoprotein receptor-related protein 6 (LRP6) is needed for the endocytosis of anthrax toxin. LRP6 works as a coreceptor which will form a complex with TEM8 or CMG2. This coreceptor makes the endocytosis process much easier to happen [56, 57].

1.5 Translocation of EF/LF

The low-pH environment in the acidic compartment triggers the conformational change in the prepore that leads to formation of the toxin pore. The low pH simultaneously causes a partial unfolding of the lethal and edema factor proteins, preparing them for transporting through the pore. The pore has the shape of a mushroom with its stem penetrating the membrane of the target cell. The current model of the stem is a 14-stranded barrel with a diameter of 15 Å. This is the exact size to allow the passage of an unfolded polypeptide [58].

![Figure 9. Conversion of prepore to pore](image-url)
By cysteine-scanning mutagenesis, Krantz et al. modified Phe427 and Ser429 to Cys which were two neighboring residues in the pore and they found the ion conductance of the pore then was blocked. Further studies showed that the seven Phe427 residues from the seven subunits move close to each other during the prepore-to-pore transition and presumably form an aromatic ring which they called “φ-clamp” that constricts the pore. The translocation rate through the pore decreases tremendously when Phe427 is replaced by a small residue Ala and removal of the “φ-clamp” makes a less efficient pore for translocation. This suggests that the “φ-clamp” is not only constricting the pore but also play an active role in the translocation process. Krantz et al. suggest that the “φ-clamp” serves a chaperone-like function and it grabs the successive hydrophobic segments in the lethal and edema factor proteins when they are pulled through the pore. This chaperone prevents refolding of the protein and it helps to facilitate the conversion of LF and EF to a translocation-competent form [59].

![Figure 10. Translocation model. The model shows how hydrophobic segments in the unfolded protein bind in succession to φ-clamp and promote translocation of LF and EF into the host cell cytoplasm [58].](image)

There are still lots of questions challenging such a model for the translocation step. Since the lumen of the heptamer can only permit one polypeptide to be translocated at a time, how these three molecules can be translocated one after another is not clear. It is easier to imagine the translocation happens on the sides of the heptamer instead of through its lumen [32].
The three proteins attack human cells as a team. Once assembled, the lethal factor can enter the cell. These cell-killing machines once inside the cell, they hop from one molecule to another, destroying each in turn. Their efficiency is so high that in some cases an entire cell can be killed by a single molecule [1].

Figure 11. Intoxication mechanism of anthrax toxins. (1) The intoxication starts with the binding of PA to the TEM8 and/or CMG2. (2) PA is cleaved by membrane endoproteases of the furin family, releasing an N-terminal fragment, PA20. (3) The remaining C-terminal fragment, PA63, oligomerizes into heptamers. (4) The heptamers associate with EF and/or LF. (5) The assembled toxic complexes are endocytosed and transferred to an acidic compartment. (6) Low pH triggers a conformational change resulting in the β strand peels away from domain 2 of PA. (7) The formation of a pore. (8) translocation of unfolding EF/LF though φ clamp. (9) LF is a zinc-dependent endoprotease which cleaves Meks. (10) EF is a calcium- and calmodulin-dependent adenylate cyclase which affects the process of ATP circulation.

1.6 In Vivo Effects of Anthrax Toxins
Low doses of LTx would induce a lot of cytokine released upon lysis in the macrophages which ultimately induce a shock [60]. Little is known about the mechanism of edema formation by ETx in vivo. By inactivating the genes coding for EF or LF in *B. anthracis*, it is clear that both genes are required for full virulence of infection [61]. This suggests that ETx and LTx act synergistically during this process. It is usually assumed that ETx cause an increase in cAMP in cells and an efflux of water. However, the nature of the cells that are affected by ETx during an infection is undetermined [32].

1.7 Inhibition of Anthrax Toxins

The bacteria can be destroyed from the host by treating with antibiotics; however, because of the continuing action of the toxin, such therapy is almost meaningless once symptoms have become evident. Thus, a specific inhibitor of the toxin's action might prove to be adjunctive to antibiotic therapy [1]. There are a number of steps in the intoxication process that can be targeted, so there can be various types of inhibitors can be used to block them. For example, inhibitors for receptor binding, furin activation, assembly, translocation, as well as enzymatic activities can be designed [32].

1.8 Using Anthrax Toxins

Numerous advances in recent years in the anthrax toxin field have also made possible the use of therapies in cancer treatment and vaccines.

Since LFn was found to be sufficient for binding to PA63 and for delivery of recombinant polypeptides in the presence of PA [62], a cytotoxic immune response against human immunodeficiency virus (HIV) could be achieved using this delivery system [63].

When LF was shown to have an effect of inhibiting MAPK signaling [64], scientists recognized that LTx could be used as an antitumor therapeutic. Further research can be done by preventing recombinant PA binding to normal cells; instead, by redirecting it specifically towards tumor cells, antitumor effect could be achieved [32].

1.9 Remaining Problems

There are still have much remains to be learned about anthrax toxin. The structure of the pore formed by PA63 heptamers upon exposure to low pH remains elusive. The same is true about the conformational changes taking place during the transition from prepore to pore. The fundamental question of how EF and LF are translocated is also unanswered. We know that EF and LF require some unfolding and that PA heptamers are making pores in the membrane. We do not know whether EF and/or LF use the channel as a conduit for their
translocation or if they can cross the lipid bilayer around the channel. We also do not know if cellular proteins help in the translocation process or in the refolding of EF and LF. These questions remain a major challenge. And, lastly, we must not forget that EF and LF act synergistically when assessing the roles and action of the toxins. The need for anti-toxin drugs to counter the use of *B. anthracis* spores as weapons and the potential applications of toxin-based new therapeutics has made this research even more essential [32].
CHAPTER 2
RESEARCH BACKGROUND AND GOAL

2.1 Research Background of Pore Formation and Translocation

2.1.1 Importance of PA

*Bacillus anthracis*, the microbe that causes anthrax, produces three proteins that combine to form a toxin. The proteins attack human cells as a team. One protein-protective antigen (PA) - binds to a receptor on the cell surface and is cleaved by enzymes there. From the 83 kD PA molecule a domain 20 kD in size is cleaved off by a host protease, furin, to a 63-kDa fragment forms (PA63). Seven molecules of the PA toxin subunit bind to one another to form a pre-pore on the cell's surface [65, 66]. The other toxin subunits, LF and EF, bind to the pre-pore with high affinity. After internalization by receptor-mediated endocytosis, the complexes are transported to the endosome. In the acid environment of the endosome, the pre-pore inserts into the membrane to form a pore and mediates translocation of EF and LF to the cytosol, where they destroy the cell [67]. This deadly triumvirate makes blood pressure plummet, causes hemorrhaging, and can lead to coma and death. From these findings we can see PA plays a very important role in the killing process of anthrax. Therefore, having a clear picture of character of PA could lead to an antidote to the anthrax toxin and help clarify the mechanism by which it kills.

2.1.2 Prepore to Pore Conversion

PA forms a heptameric prepore in its activated 63 kDa form, which converts to a pore in endosomal membranes at low pH. It has been proposed that a conformational rearrangement of a loop (residues 302-325) is involved in this prepore-to-pore conversion, in which loops from the 7 subunits combine to form a transmembrane 14-stranded β barrel.
To test this model, Benson and coworkers generated Cys substitutions in 24 consecutive residues of the loop, formed channels with each mutant in artificial bilayers. After adding the reagent methanethiosulfonate ethyltrimethylammonium (MTS-ET), changes in channel conductance were examined. The rationale for this Assay is that reaction of MTS-ET with Cys residue adds a positively charged group and then reduce channel conductance if the residue were facing the ion-conducting lumen.
Among the residues 302-325, they found alternating reduction and absence of reduction of conductance in consecutive residues on the loop and residues 312-315 keeping have a reduction of conductance which indicates a turn region. This pattern is consistent with the model below in which alternating polar and apolar residues of the loop facing the pore and the bilayer, respectively.

Figure 14. Model of two antiparallel $\beta$ strands [68].

The filled boxes indicate residues that have reduction of channel conductance. The open boxes indicate residues with little or no effect upon MTS-ET addition. These findings gave strong evidence of the model of the prepore to a 14-stranded $\beta$ barrel pore conversion and gave us a solid foundation for following studies to understand the mechanism of pore formation and translocation of anthrax toxin [68].

2.1.3 The Reasons for Histidine Being Targeted

It was found that a lower pH was required for the prepore to pore conversion in the presence of receptor [52].

18
In experiments above, SDS resistance was used as a reporter for pore formation [69]. At pH 7 or higher, the heptamer dissociated in SDS to monomer, but it converted to an SDS-resistant form that is correlated with pore formation at pH 7 or below. This conversion occurred only around pH 6 or lower in the presence of CMG2 which indicated that the interaction between PA and CMG2 regulates pH-dependent pore formation [52].

The pH profile of membrane insertion is consistent with the titration of histidine residues [70] (See the table below).
The seven of the nine histidines within PA63 cluster located at or near the interface between domain II and IV and His 121, which presents in both receptors and located at the domain II binding interface could be implicated in the mechanism of pore formation [71].
The β2–β3 loop and flanking regions of PA domain II are in red. The histidine residues within PA and CMG2 are colored cyan [29]. These residues could act as the pH sensor so that upon histidine protonation at acidic pH, domain II would separate from domain IV and enable rearrangement of the pore-forming β-hairpin. The role of the receptor as a pH-dependent clamp for pore formation is strongly supported by shifts the pH dependence of the conformational change towards acidic values in the presence of CMG2 [30]. These pH dependent changes are consistent with the protonation of histidine residues.

However, it has been found that multiple environmental factors would affect pKa values of histidine. To test the hypothesis that acidic histidine pKa values are related to burial within proteins, pKa for histidines in different environment were surveyed [72].
TABLE 2

pKa OF 37 HISTIDINE RESIDUES FROM 13 PROTEINS [72]

<table>
<thead>
<tr>
<th>Titratable histidines</th>
<th>Residue number</th>
<th>pKa</th>
<th>PDB code</th>
<th>NMR référence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barnase</td>
<td>102</td>
<td>6.3</td>
<td>1A2P</td>
<td>Sali et al., 1988</td>
</tr>
<tr>
<td>Barnase</td>
<td>18</td>
<td>7.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine</td>
<td>57</td>
<td>7.3</td>
<td>2TGA</td>
<td>Markley and Ilanetz, 1978</td>
</tr>
<tr>
<td>Chymotrypsinogen</td>
<td>40</td>
<td>4.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HP</td>
<td>75</td>
<td>6</td>
<td>1POH</td>
<td>Kalbitzer et al., 1982</td>
</tr>
<tr>
<td>Lysozyme Hen Egg</td>
<td>15</td>
<td>5.5</td>
<td>6LYZ</td>
<td>Takahashi et al., 1992</td>
</tr>
<tr>
<td>Lysozyme human</td>
<td>78</td>
<td>7.1</td>
<td>1LZ1</td>
<td>Takahashi et al., 1992</td>
</tr>
<tr>
<td>metaMb equine</td>
<td>81</td>
<td>6.6</td>
<td>1YMB</td>
<td>Cocco et al., 1992</td>
</tr>
<tr>
<td>metaMb equine</td>
<td>36</td>
<td>7.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>metaMb equine</td>
<td>113</td>
<td>5.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>metaMb equine</td>
<td>115</td>
<td>6.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>metaMb equine</td>
<td>119</td>
<td>6.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P1-phospholipase c</td>
<td>92</td>
<td>5.4</td>
<td>1GYM</td>
<td>Liu et al., 1997</td>
</tr>
<tr>
<td>P1-phospholipase c</td>
<td>82</td>
<td>6.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P1-phospholipase c</td>
<td>32</td>
<td>7.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P1-phospholipase c</td>
<td>227</td>
<td>6.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIBONUCLEASE A</td>
<td>12</td>
<td>5.8</td>
<td>7RSA</td>
<td>Markley, 1975</td>
</tr>
<tr>
<td>RIBONUCLEASE A</td>
<td>105</td>
<td>6.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIBONUCLEASE A</td>
<td>119</td>
<td>6.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>STAPHYLOCOCCAL NUCLEASE</td>
<td>45</td>
<td>5.7</td>
<td>1STG</td>
<td>Alexandrescu et al., 1988</td>
</tr>
<tr>
<td>STAPHYLOCOCCAL NUCLEASE</td>
<td>8</td>
<td>6.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>STAPHYLOCOCCAL NUCLEASE</td>
<td>124</td>
<td>6.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xylanase</td>
<td>156</td>
<td>6.5</td>
<td>1KNB</td>
<td>Plesniak et al., 1996</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Untitratable histidines</th>
<th>pKa limit</th>
<th>pKa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclophilin</td>
<td>54</td>
<td>4.2</td>
</tr>
<tr>
<td>Cyclophilin</td>
<td>92</td>
<td>4.2</td>
</tr>
<tr>
<td>FKBP</td>
<td>25</td>
<td>3.6</td>
</tr>
<tr>
<td>metaMb equine</td>
<td>24</td>
<td>4.8</td>
</tr>
<tr>
<td>P1-phospholipase c</td>
<td>81</td>
<td>3</td>
</tr>
<tr>
<td>P1-phospholipase c</td>
<td>61</td>
<td>3</td>
</tr>
<tr>
<td>Ribonuclease A</td>
<td>48</td>
<td>6</td>
</tr>
<tr>
<td>Xylanase</td>
<td>149</td>
<td>2.3</td>
</tr>
</tbody>
</table>

This large standard deviation from average pKa value of histidine reflects differences due to the specific environment of each residue. The pKa can be reduced due to highly positively charged binding pocket [73]. It is demonstrated that His 24 of apomyoglobin [74] and His 149 of xylanase [75] are not charged at pH values as low as 4.8 and 2.3, respectively. This may provide a possible explanation for the lower pH requirement for the preopore to pore conversion in the presence of CMG2.
Thus, even though the pKa of different histidine in PA and CMG2 may be different and the pKa value may not be constant in the whole pore formation process; histidine protonation still can provide a plausible reason to be a trigger for the release of domain II from CMG2 in the acidified endosome [29].

2.1.4 Finding an Appropriate Way to Modify Histidine

Histidine can get a proton in the low pH, but it can lose this function after modification by some chemical regent like DEP, etc.

![Protonation reaction of histidine](image)

**Figure 17. The protonation reaction of histidine.**

**DEP modification of His residues**

DEPC is also called DEP (Diethylpyrocarbonate). It is a compound that specifically reacts with histidine residues (see Figure 18).

This reaction can be reversed using hydroxylamine at pH 7.0. Side reaction with Lys also occur which are not reversible. So if an enzyme’s activity is affected by treatment with DEP and it regains activity after hydroxylamine treatment, it probably has an important His residue in its active site. Recently, DEP was successfully used to modify histidine in the protein of vesicular stomatitis virus-induced membrane fusion [77], and modification of histidine in sarcoplasmic reticulum Ca\(^{2+}\) -ATPase by DEP can cause some inhibition of phosphoenzyme formation [78]. However, the histidyl residues in PA have not been researched by DEP modification until now. We try to use DEP as one way to see if histidine is the key to the pore formation process of PA.
Figure 18. Reaction of histidine side chains with DEP [76].

2-fluorohistidine modification of His residues

The fluorine nucleus is small and it is only a little larger than the hydrogen nucleus. Although the dipole moment of fluorine and hydrogen is different, the perturbation to the structure, stability and functionality is minimal [79]. Amino acids modified by fluorine are convenient molecular probes for conducting research on protein structure and dynamics.

The pKa of the side chain of 2F-His has been measured previously, and is decreased from approximately 6.0–7.0 to 1 [80]. Because of this, this analogue provides a means for verifying the role of native His in pH-dependent processes. So if a protein’s function changes a lot by 2F-His substitution, the His residues may play an important role in the protein.
Recent studies have successfully shown the site-specific labeling of histidine on PapD (R200H) with 2F-His [81]. The expression and purification of 2F-His PapD are not affected by this modification and 2F-His PapD can be used to do $^{19}$F NMR for studies of both protein structure and protein folding. Fluorine labeled protein can exhibit NMR peaks only to the label and it is very sensitive to its environment. This sensitivity makes $^{19}$F NMR suited for protein studies, especially for higher molecular weight protein than proton NMR [79].

All of these gives us a clue that 2F-His could be used in the labeling of histidine in PA and CMG2 in the research of pH-dependent pore formation process.

2.1.5 Stability and Affinity of PA-CMG2 Complex

Krantz and coworkers decided the molar ratio of binding between PA and CMG2 by fluorescence equilibrium stoichiometry titration assays [82].
Molar ratio is defined as \([\text{CMG2}] / [\text{PA}]\). Additions of the acceptor CMG2 increased the apparent fluorescence resonance energy transfer (FRET) signal until all binding sites between PA and CMG2 were saturated. Fluorescence equilibrium binding titration of PA83 monomer (left) reveals a stoichiometry of 1.0 ± 0.1 CMG2 moieties per PA83, whereas heptamer (right) binds 7.1± 0.3 CMG2 moieties. So for monomer PA, it needs a stoichiometric equivalent of CMG2, whereas the heptameric prepore needs 7 equivalents of CMG2 [82].

However, these binding curves could not give us any clues of the stability and affinity between PA and CMG2, which reflect the character of protein. In order to address this issue, we developed a means to observe these parameters by native gel and urea gradient gel.

**Native gel Electrophoresis**

Native gels are sensitive to any process that alters either the charge or the conformation of a protein. They are excellent tools for detecting things such as:

- Changes in charge due to chemical degradation
- Unfolded or other modified conformations
- Oligomers and aggregates (both covalent and non-covalent)
- Binding events (protein-protein or protein-ligand)

Starting with commercial pre-cast gels and use the buffers needed. We develop a protocol for use of this native gel in the research on the affinity between PA and CMG2 in our lab.

**Urea Gradient Gel Electrophoresis**

Although urea gradient gel provides only qualitative or semi-quantitative information, it offers several advantages for studying protein folding/unfolding transitions, since they are:

- Easy to implement, not expensive.

- Require only small amounts of protein (typically 50 micrograms).
- Can analyze complex mixtures.

- Able to measure conformational or covalent heterogeneity in protein samples.

So the urea gradient gels can be used in the research of stability between PA and CMG2.

### 2.1.6 Confirm the Pore Formation Blocking by Translocation Assay

The protective antigen component of anthrax toxin forms a pore in the endosomal membrane, creating a narrow passageway for the enzymatic components of the toxin to enter the cytosol. If the conversion of the heptameric precursor to the pore is blocked, as a consequence, the translocation should also be blocked. Collier and coworkers developed a means to supervise this translocation process by detecting the conductance in the lumen \textit{in vitro} [83].

![Figure 21. Conductance records of LF$_N$ translocation [83].](image)

They let PA63 and its F427 mutant protein form channels in planar lipid bilayers. Planar lipid bilayer macroscopic conductance records of LF$_N$ translocation. Translocation (as manifested by the rise in conductance) was initiated by increasing potential. With the most active substitutions at F427, LF$_N$ translocated at rates that
trended as Phe > Leu, Trp > Tyr. Other small hydrophilic substitutions were inefficient at promoting translocation. The most active residue, therefore, was Phe [83].

Figure 22 gives a model for this process. When the pore was blocked by LFn, no conductance was found. Either a change in pH ($\Delta$ pH) or a change in voltage ($\Delta \Psi$) across the bilayer can result in translocation. After the substrate protein was translocated, the conductance reaches its maximum. So we can use translocation Assay to confirm whether a pore formed or not by observing the conductance signal in the process.
2.2 Research Goals

1) Explore the role of histidine in the pore formation process by following experiments:
   a) expressing and purifying PA and CMG2 protein
   b) modifying His of PA and CMG2 proteins using DEP
   c) labeling PA and CMG2 with 2F-histidine
   d) conducting a binding assay with modified protein to see whether the pore formation process is affected or not

2) Determine the affinity between labeled PA and CMG2 by native gel and gradient gel

3) Carry out translocation assay to check the function of labeled protein
CHAPTER 3
MATERIALS AND METHODS

3.1 Expression and Extraction of Protein

3.1.1 Culture the Cells

Bacterial cultures were grown from a single colony picked from a freshly streaked selective plate. Then use sterile rope transfer a single colony into 5 ml LB media containing 5 µl correspondent antibodies. It was grown with vigorous shaking for 3 hours at 37 °C. Then transfer 500 µl culture cells from above into 50 ml LB media containing 50 µl correspondent antibodies. Shake vigorously for another 3 hours. Then 6 ml above cells was put in 600 ml LB media containing 600 µl correspondent antibodies. They are shaken at 37 °C at 250 RPM until OD$_{600}$ around 0.5~1. Then set the temperature to desired degree for induction.

3.1.2 Induction with IPTG

In order to make the final concentration of Isopropyl β-D-thiogalactopyranoside (IPTG) to be 1 mM, 600 µl freshly made 1 M IPTG was put in the 600 ml cultured flask. Induce for 3 hours before centrifuge.

3.1.3 Extraction of the Periplasm

- Centrifuge the cultured cells, and then sit on ice.
- Weigh bacteria pellet.
- Add 5 ml 20 mM Tris pH 8.0, 20% sucrose (20/20) per gram of bacteria.
- Add 0.1 M EDTA to final concentration of 5 mM.
- Add 10 mg/ml lysozyme stock to a final concentration of 50 µg/ml.
- Inoculate on ice for 10 minutes.
- Add 1 M MgCl$_2$ to final concentration of 20 mM.
- Transfer the sample to polypropylene tubes.
- Centrifuge at 10,000 RPM for 10 minutes at 4 °C.
- Keep the supernatant for purification or gel running.

3.2 Purification of Protein

3.2.1 Q Sepharose Column
Q Sepharose™ Fast Flow is a strong anion exchanger with excellent flow properties and high capacity for proteins of all pI values. Here is the protocol of its daily usage:

- Lamp on for half hours.
- Put two pumps from 20% ethanol to buffer A and buffer B.
- Link onto column at 3 ml/min flow rate.
- System wash.
- 0% buffer B wash the column for 5 minutes per column.
- 100% buffer B wash the column for 5 minutes per column.
- 0% buffer B wash the column for 5 minutes per column.
- Load protein by injection or loading from a pump.
- Wash with buffer A for 10 minutes to establish stable baseline.
- Set gradient parameters to elute protein.
- After the protein came out, set 100% buffer B wash the column until the sharp peak came out.
- End the program.
- Wash system.
- Put two pumps in Millipore water and wash the column at 5 ml/min for 10 minutes.
- Put two pumps in 20% ethanol and wash the column at 5 ml/minute for 10 minutes.
- End the program.
- Take out column and seal it. Keep in 4 °C.
- Link two lines of the machine together.
- Lamp off.

3.2.2 Gel-Filtration

Gel filtration is the simplest and mildest of all the chromatography techniques and separates molecules on the basis of differences in size. Here is protocol for daily use:

- Lamp on for half hour.
- Put two pumps from 20% ethanol to gel-filtration buffer.
- Link onto column at 0.5 ml/min flow rate.
- Equilibrate the column for 8 hours.
- Load protein by injection.
- Elute protein by wash the column by gel-filtration buffer until the protein come out.
- End the program.
- Take out column and seal it. Keep in 4 °C.
- Wash system.
- Link two lines of the machine together.
- Lamp off.

### 3.2.3 Ultracentrifugation

Based upon differences in the size, the protein is put in filters and ultracentrifuged in high speed. After 10~15 minutes, the protein needed is concentrated and purified because the protein and impurities which have a smaller size than the pore on the filter are thrown away from the filter.

### 3.2.4 Dialysis

After a protein has been extracted from the periplasm, the solution contains a lot of residual salt which is bound to the protein. One way to remove this excess salt is to dialyze the protein against a buffer low in salt concentration.

First, the concentrated protein solution is placed in dialysis bag with small holes which allow water and salt to pass out of the bag while protein is retained. Next the dialysis bag is placed in a large volume of buffer and stirred for many hours (16 to 24 hours), which allow the solution inside the bag to equilibrate with the solution outside the bag with respect to salt concentration. After this process of equilibration is repeated several times (replacing the external solution with low salt solution each time), the protein solution in the bag will reach a low salt concentration.

### 3.3. SDS-PAGE Gel, Native Gel and Gradient Gel

#### 3.3.1 SDS-PAGE Gel

SDS-PAGE stands for sodium dodecyl (lauryl) sulfate-polyacrylamide gel electrophoresis. The purpose of this method is to separate proteins according to their size [84].

The purpose of the SDS detergent is to take the protein from its native shape, which is basically a big globule, and open it up into a linear piece.
PAGE is used to provide the proteins with an environment where different sized proteins can move at different rates.

Once an SDS-PAGE gel is run, the proteins in the gel are fixed so they don't come out when the gel is stained. Acetic acid 25% in water is a good fixative, as it keeps the proteins denatured. The gel is typically stained with Coomassie blue dye R250, and the fixative and dye can be prepared in the same solution using methanol as a solvent. The gel is then destained and taken gel pictures.

To make two 12.5% SDS-PAGE gel, follow the below to make stacking gel first.
- Acrylamide (30%) 6.25 ml
- Water 5.55 ml
- Tris-HCl pH 8.8 (1.5M) 3 ml
- SDS (10% w/v) 150 µl
- APS (10%) 50 µl
- TEMED (electrophoresis grade) 7.5 µl

Drop water saturated butanol to make the upper line even. When the gel is polymerized, one can go on to make the running gel above the stacking gel by putting the agents below.

- Acrylamide (30%) 0.8 ml
- Water 3.6 ml
- Tris-HCl pH 6.8 (0.5M) 0.5 ml
- SDS (10% w/v) 50 µl
- APS (10%) 17 µl
- TEMED (electrophoresis grade) 5 µl

Leave the gel in hood until it completely polymerizes, then store in refrigerator for future use.

### 3.3.2 Native Gel

Native gel electrophoresis is run in the absence of SDS. While in SDS-PAGE the mobility of proteins depends primarily on their molecular weight, in native PAGE the mobility depends on both the protein's charge and its hydrodynamic size.

Since the protein retains its folded conformation in native gel, its mobility on the gel will vary with the conformation (higher mobility for more compact conformations, lower for larger structures like oligomers).

### 3.3.3 Gradient Gel

Urea gradient gel electrophoresis [85] is a simple method for monitoring the denaturant-induced unfolding of proteins. Urea gradient gels are slab gels prepared with a horizontal gradient of urea concentration, usually 0 - 8 M. The protein sample is applied across the top of the gel and run in a direction perpendicular to the urea gradient. As the protein is electrophoresed, molecules at different positions are exposed to different urea concentrations. At positions where the urea concentration is high enough to promote unfolding, the mobility of the protein decreases
because of the greater hydrodynamic volume of the unfolded form. When the gel is stained, the electrophoretic pattern can be interpreted directly as an unfolding curve, as shown below [86].

![Unfolded gel](image)

**Figure 25. Urea gradient gel of two forms of bacteriophage T4 lysozyme [86].**

The upper band in the gel contains a mutant form of lysozyme, and the discontinuous lower band contains the WT protein. The wild-type protein was applied to the gel first, and then the mutant protein was applied. The gel was stained with Coomassie blue. The gel reveals two important differences in the unfolding transitions:

- The mutant protein unfolds at lower urea concentrations than the WT protein.
- The WT protein’s interconversion between the native and unfolded forms is slow, giving the discontinuous band, while that for the mutant protein is fast, giving a smooth band.

To make gradient gel, prepare 0M and 8M gel solution first.

**0 M Urea Gel:**

- 11 ml acrylamide (30%)
- 3 ml 10 × buffer (with composition and pH chosen for the particular experiment)
- 1.5 ml sodium toluenesulfinate solution (20mM)
- 1.5 ml diphenyliodonium chloride solution (1mM)
- 11.5 ml water

Degas for 5 minutes and cover by parafilm.
8 M Urea Gel:

- 14.4 g solid urea (Schwarz-Mann Ultra-Pure)
- 7 ml acrylamide (30%)
- 3 ml 10 × buffer (with composition and pH chosen for the particular experiment)
- 1.5 ml sodium tolenesulfinate solution (20mM)
- 1.5 ml diphenyliodonium chloride solution (1mM)
- 4.4 ml water

The mixtures are heated gently to solve the agents inside. Degas for 5 minutes and cover by parafilm.

Wash gel making barrel with 20% (v/v) ethanol, then put 1.5 ml methylene blue stock (2mM) in 0 M urea gel and pour the solution into the left side of the barrel and run it to let the system filled with 0M urea gel solution. Then stop it and suck the left solution from the barrel back in the original flask and combine the whole solution of 0 M urea gel and pour all of them in the left side of the barrel. Put 1.5 ml methylene blue stock (2mM) in 8 M urea gel and mix it thoroughly and then suck 20 ml out to put into the right side of the barrel. Connect the whole system and then run it by put down the electric key button. Now by the rate of 1 ml/minute, it will take around 40 minutes to run out the solution. Set a timer and turn off the light. When it is almost done, stop it and pour all the rest of 8 M urea gel solution into the left side of the barrel and run the system again. When the gel solution line reaches the top, the pump can be stopped. Leave the gel in the hood to polymerize overnight. Clean everything with water and then with 20% ethanol. Turn off the power in the bottom of the pump.

3.4 Detection of Concentration of Protein

There are two ways used in this thesis to measure the concentration of proteins. These are: bicinchoninic acid (BCA) protein assay kit and extinction coefficient.

3.4.1 BCA Protein Assay Kit

Follow the BCA protein assay kit to prepare BCA standards.
TABLE 3

BCA STANDARDS SOLUTION AND CORRESPONDENT CONCENTRATION

<table>
<thead>
<tr>
<th>Vial</th>
<th>Volume of diluents(µl)</th>
<th>Volume and source of BCA</th>
<th>Protein concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>125</td>
<td>375 µl of stock</td>
<td>1500</td>
</tr>
<tr>
<td>C</td>
<td>325</td>
<td>325 µl of stock</td>
<td>1000</td>
</tr>
<tr>
<td>E</td>
<td>325</td>
<td>325 µl of vial C</td>
<td>500</td>
</tr>
<tr>
<td>F</td>
<td>325</td>
<td>325 µl of vial E</td>
<td>250</td>
</tr>
<tr>
<td>Blank</td>
<td>400</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Prepare working regent (WR) by mixing 50 ml εεA regent A with 1 ml BCA regent B.

Use the ratio of sample to WR as 1 to 20 to prepare the standard reaction serious.

- Pipette 100 µl of each standard and 10 µl diluted unknown sample (diluted with 20mM Tris-HCl pH 8.0 buffer) into an appropriately labeled test tube.
- Add 2.0 ml WR to each test tube and mix well.
- Cover and incubate tubes at RT for 2 hours.
- With the spectrophotometer set to 562 nm, measure the absorbance of all the samples within 10 minutes.
- Subtract the average 562 nm absorbance measurement of the blank standard replicates from the 562 nm absorbance measurement of all other individual standard and unknown sample replicates.
- Prepare a standard curve, using it to determine the protein concentration of each unknown sample.

TABLE 4

BCA STANDARDS SOLUTION AND UV ABSORBANCE

<table>
<thead>
<tr>
<th>Vial</th>
<th>Standard sample absorbance</th>
<th>Blank absorbance</th>
<th>Net sample absorbance</th>
<th>Concentration(µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>1.6538</td>
<td>0.1586</td>
<td>1.4952</td>
<td>1500</td>
</tr>
<tr>
<td>C</td>
<td>1.2417</td>
<td>0.1586</td>
<td>1.0831</td>
<td>1000</td>
</tr>
<tr>
<td>E</td>
<td>0.7556</td>
<td>0.1586</td>
<td>0.5970</td>
<td>500</td>
</tr>
<tr>
<td>F</td>
<td>0.4848</td>
<td>0.1586</td>
<td>0.3262</td>
<td>250</td>
</tr>
<tr>
<td>Unknown</td>
<td>read</td>
<td>read</td>
<td>calculate</td>
<td>calculate</td>
</tr>
</tbody>
</table>
According this standard curve, the concentration of diluted protein can be calculated at the unit of µg/ml by the absorbance.

### 3.4.2 Extinction Coefficients

Take 10 µl of protein in 990 µl Tris-HCl pH 8.0 buffers. Scan from 200 nm to 500 nm, medium speed. Read the absorbance at 279 nm. So the actual absorbance is 100 times of the result. Take the ε value of the proteins acquired from the ExPASy ProtParam tool and then the concentration of protein can be calculated by the equation below.

\[
A = \varepsilon b C
\]

### 3.5 Making Related Buffer and Agent

#### 3.5.1 Sodium Phosphate Buffer

To make 50 mM sodium phosphate buffer at different pH values, put the correspondent volume of Na₂HPO₄ (0.5M) and NaH₂PO₄ (0.5M) below in 5 ml Millipore water.

- Phosphate buffer pH 6.0: 60 µl Na₂HPO₄ and 440 µl NaH₂PO₄
- Phosphate buffer pH 6.2: 89 µl Na₂HPO₄ and 411 µl NaH₂PO₄
- Phosphate buffer pH 6.6: 176 µl Na₂HPO₄ and 324 µl NaH₂PO₄
- Phosphate buffer pH 6.8: 231.5 µl Na₂HPO₄ and 268.5 µl NaH₂PO₄
- Phosphate buffer pH 7.0: 288.5 µl Na₂HPO₄ and 211.5 µl NaH₂PO₄
- Phosphate buffer pH 7.2: 342 µl Na₂HPO₄ and 158 µl NaH₂PO₄
- Phosphate buffer pH 7.6: 422.5 µl Na₂HPO₄ and 77.5 µl NaH₂PO₄
- Phosphate buffer pH 8.0: 466 µl Na₂HPO₄ and 34 µl NaH₂PO₄
3.5.2 BisTris and Hepes

BisTris (1M)
- BisTris pH 5.0 was made by putting 10.462 g BisTris in 50 ml Millipore water and adjusted pH value to 5.0.
- BisTris pH 5.5 was made by putting 10.462 g BisTris in 50 ml Millipore water and adjusted pH value to 5.5.
- BisTris pH 6.0 was made by putting 10.462 g BisTris in 50 ml Millipore water and adjusted pH value to 6.0.
- BisTris pH 6.5 was made by putting 10.462 g BisTris in 50 ml Millipore water and adjusted pH value to 6.5.

Hepes (1M)
- Hepes pH 7.0 was made by putting 13.0145 g Hepes in 50 ml Millipore water and adjusted pH value to 7.0.
- Hepes pH 7.5 was made by putting 13.0145 g Hepes in 50 ml Millipore water and adjusted pH value to 7.5.
- Hepes pH 8.0 was made by putting 13.0145 g Hepes in 50 ml Millipore water and adjusted pH value to 8.0.

3.5.3 Buffer I, buffer II and 1 M Tris-HCl pH 8.0

Buffer I
250g sucrose and 2.42g Tris as well as 0.37g EDTA were added into a beaker with DI water around one liter, adjust its pH to 8.0 by pH meter and then transfer the solution into a graduated cylinder. Add DI water to one liter. Seal the solution and keep it in RT before use.

Buffer II
0.6g MgSO4 was put in beaker which was diluted by DI water to around one liter. Transfer the solution into a graduated cylinder. Add DI water to one liter. The buffer is stored at 4 °C before use.

1 M Tris-HCl pH 8.0
To make 20ml Tris-HCl pH 8.0, 2.42 g Tris was put in around 20 ml DI water. HCl was added to adjust the pH value to 8.0.

3.5.4 Buffer A, Buffer B and Gel-Filtration Buffer

Buffer A
To make 1 liter buffer A which is 20 mM Tris-HCl pH 8.0 buffer, 2.42 g Tris was put in one liter DI water, then HCl was added to adjust the pH value to 8.0. Sterile filtered and stored at 4°C before use.
Buffer B

To make 1 liter buffer B which is 20 mM Tris-HCl pH 8.0 and 1 M NaCl, 2.42 g Tris and 58.44 g NaCl were added in one liter DI water, then HCl was added to adjust the pH value to 8.0. Sterile filtered and stored at 4°C before use.

Gel-Filtration Buffer

To make 1 liter 20 mM Tris-HCl, 150 mM NaCl pH 8.0 gel-filtration buffers, 2.42 g Tris, 8.766 g NaCl were added in one liter DI water, then HCl was added to adjust the pH value to 8.0. Sterile filtered and stored at 4°C before use.

3.5.5 Media A1, A2, B1, B2 and Resuspend Solution [79]

Media A1

Media A1 contains following ingredients in 3 liter DI water.

**TABLE 5**

<table>
<thead>
<tr>
<th>INGREDIENT OF MEDIA A1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino Acids</td>
</tr>
<tr>
<td>alanine</td>
</tr>
<tr>
<td>arginine hydrochloride</td>
</tr>
<tr>
<td>aspartic acid</td>
</tr>
<tr>
<td>cystine</td>
</tr>
<tr>
<td>glutamine</td>
</tr>
<tr>
<td>glutamic acid</td>
</tr>
<tr>
<td>glycine</td>
</tr>
<tr>
<td>histidine</td>
</tr>
<tr>
<td>isoleucine</td>
</tr>
<tr>
<td>leucine</td>
</tr>
<tr>
<td>lysine hydrochloride</td>
</tr>
<tr>
<td>methionine</td>
</tr>
<tr>
<td>proline</td>
</tr>
<tr>
<td>serine</td>
</tr>
<tr>
<td>threonine</td>
</tr>
<tr>
<td>tyrosine</td>
</tr>
<tr>
<td>valine</td>
</tr>
<tr>
<td>adenine</td>
</tr>
<tr>
<td>guanosine</td>
</tr>
<tr>
<td>thymine</td>
</tr>
<tr>
<td>uracil</td>
</tr>
<tr>
<td>cytosine</td>
</tr>
<tr>
<td>sodium acetate</td>
</tr>
<tr>
<td>succinic acid</td>
</tr>
<tr>
<td>NH₄Cl</td>
</tr>
<tr>
<td>NaOH</td>
</tr>
<tr>
<td>K₂PO₄</td>
</tr>
</tbody>
</table>
The mixture was autoclaved before use.

**Media A2**

Media A2 contains following ingredients in 600 ml DI water.

**TABLE 6**

**INGREDIENT OF MEDIA A2**

<table>
<thead>
<tr>
<th>Amino Acids</th>
<th>Mass(g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>glucose</td>
<td>18.00</td>
</tr>
<tr>
<td>1M MgSO₄</td>
<td>14.40</td>
</tr>
<tr>
<td>FeCl₃·6H₂O</td>
<td>0.01</td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>0.01</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>0.01</td>
</tr>
<tr>
<td>MnSO₄·H₂O</td>
<td>0.01</td>
</tr>
<tr>
<td>thiamine</td>
<td>0.18</td>
</tr>
<tr>
<td>niacin</td>
<td>0.18</td>
</tr>
<tr>
<td>biotin</td>
<td>0.01</td>
</tr>
<tr>
<td>tryptophan</td>
<td>0.18</td>
</tr>
<tr>
<td>phenylalanine</td>
<td>0.18</td>
</tr>
</tbody>
</table>

Sterile filter it before use.

**Media B1**

Media B1 is prepared like Media A1 except putting in 2F-His instead of His.

**Media B2**

Media B2 is prepared like Media A2.

**Resuspend Solution**

To make 1 liter 0.9% NaCl, a stir bar and DI water are put in a one-liter beaker. Then 9 g NaCl are put in. After that the solution is transferred into one liter graduated cylinder. Add DI water until it reaches one liter; mix it, and then sterile filter into an autoclaved one liter bottle. Put the bottle in 37 °C incubator to warm up before use.

**3.5.6 1×PBS Buffer**

Diluted the 10×PBS buffer by add 900 ml Millipore water in 100 ml 10×PBS buffer to get 1×PBS buffer. Stir up and keep in cold room for future use.

**3.5.7 6× SDS Loading Buffer and 5× Electrode Running Buffer**

**6× SDS Loading Buffer**
For 10 ml solution buffer: 100% glycerol 6ml; 1M Tris-HCl pH 6.8 3 ml; 0.5 M EDTA 240 µl; SDS 1.2g; 2-mercapromanol 600 µl; bromphenol blue “pinch”. Put all above in 10 ml Millipore water. Add SDS last. Vortex the solution, then put the falcon tube in 42 °C to solve well. Separate the solution in Eppendorf tubes and keep them in freezer. Warm it up by hand before use.

5× Electrode Running Buffer

Tris base 90g, glycine 432g and SDS 30g were put in 6 liter DI water. The solution was diluted 5 times before use.

3.5.8 10 mM Reduced Glutathione

Use 1 M stocked Tris-HCl to make 50 mM Tris-HCl with 0 mM reduced glutathione in it. Weigh 0.03688g reduced glutathione and dissolve it in 600 µl Tris-HCl 1 M and add DI water to final volume of 12ml. Vortex it and keep in 4°C.

3.5.9 20% Arabinose

Take out 1.2 g arabinose in 6 ml Millipore water and vortex it.

3.5.10 Trypsin and Trypsin Inhibitor

Prepare trypsin (TrypZean™, recombinant bovine expressed in corn. Sigma Cat#: T-3568) at 1mg/ml in 20 mM Tris and 1 mM CaCl₂ buffer pH 8.0. Prepare trypsin inhibitor (Type II-S: Soybean. Sigma Cat#:T-9128) at 10 mg/ml in the same media as trypsin. Add x micrograms trypsin to x milligrams PA, incubate for 20~30 minutes at RT, then add an equal volume of trypsin inhibitor to stop reaction.

3.6 Making LB Plates

Lauria-Bertani (LB) growth medium was used for reproduction of bacteria. LB medium contains 10 g/l bactotrypton, 5 g/l yeast extract, 5 g/l NaCl in Millipore water. To produce solid medium in Petri plates 15 g/l Agar and antibiotics of choice were added.

25 g LB broth and 15 g agar powder were put in 1 liter DI water. Stir it up for 10~15 minutes in RT, then separated them in two 500 ml bottle. The bottles were autoclaved for 30 minutes. Cool down to touch. Put in 500 µl antibodies wanted. Then pour plates. Let the plates sit on bench overnight, then labeled and put in 4 °C cold room for future use.

3.7 Making Storage Bacterial Cells
For long term storage, aliquots of bacteria culture were supplemented with glycerol up to 20%. 80% glycerol 190 µl and bacterial culture 810 µl were put in a sterile cap tube. Then put the tube in liquid N₂ for 3~4 minutes. Pick it up and then put in -80 °C freezer.
CHAPTER 4
EXPERIMENTS, RESULTS AND DISCUSSION

4.1 Preparation of Proteins

4.1.1 PA83

Express and purify PA83 protein.

- Put one colony from the *E. coli* expression host BL21 (DE3). The vector pET22-b+ (Novagen) containing the PA83 gene. The colony was stringed into 5ml LB broth together with 5 µl 100 mg/ml Ampicillin in a falcon tube.

- Shake at 37° C and 250 RPM for 3 hours.

- When the culture becomes cloudy, transfer 500 µl of it into a flask which includes 50ml LB broth and 50 µl 100 mg/ml Ampicillin shaking at 37°C and 250 RPM for another 3 hours.

- Inoculate eight 600ml LB broth together with 600µl 100 mg/ml Ampicillin and 6ml above culture at 37°C and 250 RPM until OD$_{600}$ around 0.5~1.

- Lower temperature to 30 °C and add fresh 1M IPTG to make the final to be 1mM.

- Induce for 3~4 hours at 30 °C. Monitor growth by observing OD$_{600}$ every half hour.

- Remove the cells by centrifuging at 3500 RPM for 10 minutes at 4° C.

- Discard supernatant.

- Resuspend cell pellet in 1 liter buffer I made in 3.5.3.

- Stir for 15 minutes at RT.

- Centrifuge for 15 minutes at 4° C for 10 minutes.

- Discard supernatant in cold room.

- Resuspend cell pellet in 1 liter buffer II made in 3.5.3.

- Stir for 15 minutes at 4° C.

- Add 20mM Tris-HCl pH 8.0 to get a final concentration of 20 mM.

- Centrifuge for 15 minutes at 4° C at 3700 RPM.

- Keep the supernatant and fill it.

- Overnight loading the filled supernatant at 4° C by applying it to Q sepharose column.
- Wash Q sepharose column with buffer A until stable base line established.
- Elute PA83 protein with 0-30% buffer B at a rate of 5 ml/min over 120 minutes.

**Figure 27. First Q sepharose column of PA83.**

Run 12.5% SDS-PAGE of the fractions.

**Figure 28. SDS-PAGE of PA83 after 1st Q sepharose column.**
Accumulate PA83 fractions, concentrate them and then inject into Gel-filtration column to purify it. Take fractions coming out of the column and run 12.5% SDS-PAGE.

Figure 29. First gel filtration of PA83.

Figure 30. SDS-PAGE of PA83 after first Gel-filtration column.
Accumulate PA83 fractions, concentrate them and then inject into Gel-filtration column to purify it. Take fractions coming out of the column and run 12.5% SDS-PAGE.

Figure 31. Second gel filtration of PA83.

Figure 32. SDS-PAGE of PA83 after second Gel-filtration column.
Accumulate correspondent fractions, run Q sepharose column to purify it again. Then run 12.5% SDS-PAGE.

**Figure 33. Second Q sepharose column of PA83.**

**Figure 34. SDS-PAGE of PA83 after second Q sepharose column.**

Combine the fractions, measure the concentration by using extinction coefficient.
Take 10 µl of protein in 990 µl Tris-HCl pH 8.0 buffers. Scan from 200 nm to 500 nm, medium speed. The absorbance at 280 nm is 0.038. So the actual absorbance is around 4. Take the ε value of PA83 at 280 nm as 77280 or 0.935 ml·mg⁻¹·cm⁻¹, then the concentration of protein can be calculated.

\[ A = \varepsilon b C \]

So the concentration of this batch of PA83 is 51.76 µM (4.3µg/ml)

Figure 35. UV absorbance of PA83.

The concentration of product can also be measured by using BCA Protein Assay Kit.

Follow the BCA Protein Assay Kit in 3.4.1 to prepare BCA standards.

Prepare a standard curve and use it to determine the concentration of unknown proteins.
TABLE 7

BCA STANDARDS SOLUTION AND UV ABSORBANCE OF PA83

<table>
<thead>
<tr>
<th>Vial</th>
<th>Standard sample absorbance</th>
<th>Blank absorbance</th>
<th>Net sample absorbance</th>
<th>Concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>1.6538</td>
<td>0.1586</td>
<td>1.4952</td>
<td>1500</td>
</tr>
<tr>
<td>C</td>
<td>1.2417</td>
<td>0.1586</td>
<td>1.0831</td>
<td>1000</td>
</tr>
<tr>
<td>E</td>
<td>0.7556</td>
<td>0.1586</td>
<td>0.5970</td>
<td>500</td>
</tr>
<tr>
<td>F</td>
<td>0.4848</td>
<td>0.1586</td>
<td>0.3262</td>
<td>250</td>
</tr>
<tr>
<td>PA83</td>
<td>0.6658</td>
<td>0.1586</td>
<td>0.5072</td>
<td>434</td>
</tr>
</tbody>
</table>

According this standard cure in Figure 26 (3.4.1), the concentration of diluted PA83 is 434 µg/ml (The absorbance is 0.5072). Since it is 10 times diluted, so the original concentration of PA83 is 4340 µg/ml.

It can be seen that the concentration of proteins detected by BCA protein assay kit and UV absorbance based on the extinction coefficient of proteins at 279nm or 280nm gave very close results. Since it is more convenient to use the extinction coefficient to measure the concentration, in the following experiments, all the concentration was detected by extinction coefficient if not specially mentioned.

4.1.2 PA63

Follow the protocol in 4.1.1, express PA83 and load onto Q sepharose column to purify it.

Figure 36. SDS-PAGE of fractions of PA83 after Q sepharose column.
Combine the fraction, concentrate and measure the concentration to be 81.26 µM. follow the procedure in 3.5.10. Take 800 µl in Eppendorf tube. Then add 5.36 µg trypsin. Shake at RT for 30 minutes. After that, 53.60 µg trypsin inhibitor is added. Shake for another 5 minutes in shaker, and then load it on Q sepharose column.

Figure 37. Purification of PA63 on Q sepharose column.

Figure 38. SDS-PAGE of PA63 after Q sepharose column.
Collect fractions and concentrate to around 500 µl and measure its concentration. Concentrate the fractions to around 500 µl, then measure it concentration by using its extinction coefficient.

Take 10 µl of protein in 990 µl Tris-HCl pH 8.0 buffers. Scan from 200 nm to 500 nm, medium speed. The absorbance at 280 is 0.2659. Take the extinction coefficient value of PA63 at 280 nm as 45139.7, and then the concentration of protein can be calculated.

\[ A = \varepsilon b C \]

So the concentration of this batch of PA63 is 5.89 µM.

4.1.3 2F-His-PA83

2-F-His has lower pKa than histidine and it should block the pore formation if histidine plays the most important role in this process.

\(^{19}\text{F}-\)labeled histidine contained protein was produced by biosynthetic incorporation of the labeled amino acids [79]. Bacterial cultures were grown in a medium which contained a limited amount of the unlabeled histidine and then were harvested, washed and transferred into a medium containing \(^{19}\text{F}-\)labeled histidine. After adding IPTG for another 3 hours, the procedures used for purifying PA83 are applicable for 2F-His-PA83.

Follow the protocol below to make 2F-His-PA83.

- Prepare the medium by the method described in 3.5.5.
- Combine A1 and A2 together in big barrel and mix it.
- Use autoclaved graduated cylinder transfer 600 ml above media to six different autoclaved big flasks.
- Culture bacterial UTH780 with pQE80 plasmid in above media at 37 °C until OD$_{600}$ around 1~3.
- Centrifuge the culture.
- Wash the cells in resuspend solution made in 3.5.5 for two times.
- Centrifuge and resuspend cells in 10 ml $^{19}$F-labeled histidine media, which was made by combining B1 and B2, made as in 3.5.5.
- Transfer the resuspended cultures to the A2 and B2 media.
- Grown bacteria for another 30 minutes.
- IPTG is added to a final concentration of 1.0 mM.
- Express protein for 3 hours at 26 °C.
- Centrifuge and resuspend in buffer I made in 3.5.3.
- Centrifuge and resuspend in buffer II made in 3.5.3.
- Centrifuge and keep supernatant.
- Overnight load onto Q sepharose column.

![Figure 40. First purification of 2F-His-PA83 on Q sepharose column.](image)
Figure 41. SDS-PAGE of 2F-His-PA83 after first Q sepharose column.

Figure 42. Second purification of 2F-His-PA83 on Q sepharose column.

Figure 43. SDS-PAGE of 2F-His-PA83 after second Q sepharose column.
Accumulate correspondent fractions, run Q sepharose column to purify it again. Then run 12.5% SDS-PAGE.

![Figure 44. Third purification of 2F-His-PA83 on Q sepharose column.](image)

![Figure 45. SDS-PAGE of 2F-His-PA83 after third Q sepharose column.](image)

The fractions look pretty pure. The fractions are concentrated and accumulated and then the concentration was detected by using its extinction coefficient.

Take 10 µl of protein in 990 µl Tris-HCl pH 8.0 buffers. Scan from 200 nm to 500 nm, medium speed. The absorbance at 280 is 0.002. So the actual absorbance is around 0.2. Take the extinction coefficient value of 2F-His-PA83 at 278 nm as 78400, and then the concentration of protein can be calculated.

\[ A = \epsilon b C \]

So the concentration of this batch of 2F-His-PA83 is 2.55 µM (0.21µg/ml).
4.1.4 2F-His-PA83

Use the protocol in 4.1.3 to express 2F-His-PA83. Purify it by loading onto Q sepharose column.

![SDS-PAGE of 2F-His-PA83 after Q sepharose column.](image)

**Figure 46.** SDS-PAGE of 2F-His-PA83 after Q sepharose column.

Cut with trypsin. Follow the procedure in 3.5.10.

![Fractions of 2F-His-PA83 cut with trypsin.](image)

**Figure 47.** Fractions of 2F-His-PA83 cut with trypsin.

The protein was cut successfully by trypsin. Accumulate correspondent fractions and load them onto Q sepharose column.
From the SDS-PAGE gel, the fractions are pretty pure. Concentrate the fractions to around 500 µl, then measure its concentration by using its extinction coefficient.

Take 10 µl of protein in 990 µl Tris-HCl pH 8.0 buffers. Scan from 200 nm to 500 nm, medium speed. The absorbance at 280 is 0.00121. So the actual absorbance is around 0.121. Take the extinction coefficient value of 2F-His-PA63 at 280 nm as 45139.7, and then the concentration of protein can be calculated.

\[ A = \varepsilon b C \]

So the concentration of this batch of 2F-His-PA63 is 2.69µM.

4.1.5 CMG2

Follow the protocol below to express and purify CMG2 protein

- *E. coli* strain BL21-DE3 harboring pGEX-4T1-CMG2 is grown in 5ml LB broth together with 5 µl 100 mg/ml Ampicillin in a falcon tube.
- Shake at 37°C and 250 RPM for 3 hours.
- When the culture becomes cloudy, transfer 500 µl of it into a flask which includes 50ml LB broth and 50 µl 100 mg/ml Ampicillin shaking at 37°C and 250 RPM for another 3 hours.
- Inoculate Six 600ml LB broth together with 600µl 100 mg/ml Ampicillin and 6ml above culture at 37°C and 250 RPM until OD\textsubscript{600} around 0.5~1.
- Add fresh 1M IPTG to make the final to be 1mM.
- Induce for 3 hours at 37 °C. Monitor growth by observing OD\textsubscript{600} every half hour.
- Remove the cells by centrifuging at 3500 RPM for 10 minutes at 4°C.
- Discard supernatant.
- Resuspend cell pellet with 20 ml 1×PBS and 2.25 ml 10 mg/ml lysozyme.
- Sit on ice for 30 minutes.
- Ultrasonic the sample.
- Add DNase and RNase to final concentration of 5 µg/ml. Incubate at 4 °C for 10 minutes.
- Transfer to a centrifuge tube and centrifuge at 10,000 RPM for 10 minutes at 4 °C.
- Keep supernatant and put it on ice. Filter it.
- Transfer to GST FF small column by pumping at 1 ml/min.
- Wash with 1×PBS at 0.3 ml/min overnight to wash out other proteins.
- Use a syringe to push in thrombin into GST Trap™ FF column with another column which can attach to thrombin in the other side of the GST column.
- Sit in RT for one day.
- Use 10 ml 1×PBS to elute CMG2 out.

Figure 49. SDS-PAGE of CMG2 protein.

Measure its concentration by extinction coefficient.

\[ A_{278} = 0.07476601 \quad \varepsilon_{278} = 12727 \text{ or } 0.64. \]

Based on the molecular weight of CMG2 (19875.8 KDa), concentration of CMG2 can be calculated to be 0.1168 mg/ml or 5.87 µM.
Concentrate CMG2 for a little bit and then measure its concentration again.

\[ A_{278} = 0.1699094 \]. So the concentration of CMG2 is 0.2655 mg/ml or 13.35 µM. This concentration was used for binding experiments directly.

**4.1.6 2F-His-CMG2**

Follow the protocol below to make 2F-His-CMG2.

- Prepare the medium by the method described in 3.5.5.
- Combine A1 and A2 together in big barrel, mix it.
- Use autoclaved graduated cylinder transfer 600 ml above media to six different autoclaved big flasks.
- *E. coli* strain UTH780 harboring pGEX-4T1-CMG2 is grown in 5ml LB broth together with 5 µl 100 mg/ml Ampicillin in a falcon tube.
- Culture bacterial of CMG2 in above media at 37 °C until \( \text{OD}_{600} \geq 1 \).
- Centrifuge the culture.
- Wash the cells in resuspend solution made in 3.5.5 for two times.
- Centrifuge and resuspend cells in 10 ml \(^{19}\text{F}\)-labeled histidine media which was made by combining B1 and B2 made as in 3.5.5.
- Transfer the resuspended cultures to the B1 and B2 media.
- Grow bacteria for another 30 minutes.
- IPTG is added to a final concentration of 1.0 mM.
- Express protein for 3 hours at 37 °C.
- Remove the cells by centrifuging at 3500 RPM for 10 minutes at 4° C.
- Discard supernatant.
- Resuspend cell pellet with 20 ml 1×PBS and 2.25 ml 10 mg/ml lysozyme.
- Sit on ice for 30 minutes.
- Ultrasonic the sample.
- Add DNase and RNase to final concentration of 5 µg/ml. Incubate at 4 °C for 10 minutes.
- Transfer to a centrifuge tube and centrifuge at 10,000 RPM for 10 minutes at 4 °C.
- Keep supernatant and put it on ice. Filter it.
- Transfer to GST FF small column by pumping at 1 ml/min.
- Wash with 1×PBS at 0.3 ml/min overnight to wash out other proteins.
- Use a syringe to push in thrombin into GST Trap™ FF column with another column which can attach to thrombin in the other side of the GST column.
- Sit in RT for one day.
- Use 10 ml 1×PBS to elute 2F-His-CMG2 out.

**Figure 50. SDS-PAGE of 2F-His-CMG2.**

Since the fractions are not pure, so go on to purify it.

2F-His-CMG2 was dialyzed in 1×PBS overnight. Then load it onto F column at 1 ml/minute. Wash with 10 ml 1×PBS for 10 minutes by the rate of 1 ml/minute. Then elute the protein by pumping into 10 ml 10 mM reduced glutathione at 1 ml/minute. Take 10 µl out for SDS-PAGE gel running.

**Figure 51. SDS-PAGE of 2F-His-CMG2 after second purification.**
Measure its concentration by extinction coefficient. $\varepsilon_{278}$ is 12727 or 0.64. $A_{278}$ is 0.213. So the concentration of 2F-His-CMG2 is 0.334 mg/ml or 16.74 µM. This concentration was used for binding experiments directly.

4.2 Test Experiments

4.2.1 Trypsin Reacts with PA83 and 2F-His-PA83

Prepare trypsin at 1mg/ml in 20 mM Tris and 1 mM CaCl$_2$ buffer pH 8.0. Prepare trypsin inhibitor at 10mg/ml in the same buffer and same PH. Add X micrograms trypsin to X milligrams PA, and then incubate for 30 minutes at RT. Add an equal volume of trypsin inhibitor to stop reaction.

![Figure 52. Trypsin treatment on PA83 and 2F-His-PA83.](image)

It can be seen from the SDS-PAGE gel that both PA83 and 2F-His-PA83 were cut by trypsin, which give the possibility that the protein are our objective protein. In order to further confirm the product, we use furin to treat both proteins.

4.2.2 Furin Reacts with PA83 and 2F-His-PA83

After Q-column, some fractions sit on similar position on the SDS-PAGE gel. In this case, it is hard to tell which band is our PA83. Furin can be a good detector at this time. Take 10 µl fractions of protein, then measure the concentration of the largest fraction protein, add necessary amount of furin. (1 unit equals 0.5 µl furin, and 1
unit furin can cut 100 µg PA). Put same amount of furin to other fractions at the same time. Let the reaction sit on bench overnight. Then run SDS-PAGE gel.

Figure 53. PA83 fractions cut with furin.

From the picture, it is clear which fraction is our wanted protein by observing which fraction is cut by furin. Then the correct fractions can be accumulated and go to the next step for further purification.

Concentrate 2F-His-PA83 product and cut with furin.

Figure 54. 2F-His-PA83 cut with furin.
From the above SDS-PAGE gel, the protein was cut by furin. So it is confirmed that the fractions are 2F-His-PA83. By this way, PA can be double checked.

### 4.2.3 Immobilized Trypsin React with PA83

Remove 100 µl of resuspended resin, and then wash it with 3×500 µl 20 mM Tris pH 8.0. After adding 400 µl of PA83 (38.82µM), incubate them at RT for one hour. Then centrifuge at 4 °C cold room for 5 minutes. Take 10 µl of the supernatant run the SDS-PAGE gel.

![Figure 55. PA83 treat with trypsin.](image)

SDS-PAGE gel shows that the PA83 was cut by trypsin into PA63 and PA20. So trypsin also can be a good regent to test the existence of PA83. In order to know how many times exactly for trypsin to cut the PA83 completely, the following experiment was carried by letting PA83 sit with trypsin at different times before separating them.

Remove 100 µl of resuspended resin (Immobilized trypsin), and then wash it with 3×500 µl 20 mM Tris pH 8.0. Resuspend it in 200 µl 20 mM Tris pH 8.0 buffers. After adding 400 µl of PA83 (38.82µM), incubate them at RT for different time. Then centrifuge at 4 °C cold room for 5 minutes. Take 10 µl of the supernatant out of the Eppendorf. Resuspend the mixture and repeat the above steps every 5 minutes. Run the SDS-PAGE gel of each fraction.

The gel shows under the circumstances described above. 30 minutes are enough time for trypsin to cut the PA83 completely into PA63 and PA20.
4.2.4 Fluorescence Assay

Take out the 50 mM sodium phosphate buffer made in 3.5.1 to do the following Assay.

Adjust pH value by detecting the pH of solution by pH meter.

<table>
<thead>
<tr>
<th>Phosphate buffer pH (principle value)</th>
<th>Phosphate buffer pH (Actual value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.0</td>
<td>6.0</td>
</tr>
<tr>
<td>6.2</td>
<td>6.23</td>
</tr>
<tr>
<td>6.6</td>
<td>6.59</td>
</tr>
<tr>
<td>6.8</td>
<td>6.78</td>
</tr>
<tr>
<td>7.0</td>
<td>7.01</td>
</tr>
<tr>
<td>7.2</td>
<td>7.17</td>
</tr>
<tr>
<td>7.6</td>
<td>7.56</td>
</tr>
<tr>
<td>8.0</td>
<td>7.94</td>
</tr>
</tbody>
</table>

Take out one batch of PA83 protein. The concentration is 4.3 mg/ml. In order to make the absorbance to be 0.01, diluted 11.6 µl of PA83 protein into 5 ml above 50 mM sodium phosphate buffer.
Set the Fluorescence instrument parameter:

Excitation wavelength: 295 nm

Start wavelength: 305 nm        Stop wavelength: 600 nm

Excitation slit wavelength: 5 nm        Emission slit wavelength: 5 nm

Take the pH 7.01 sodium phosphate buffer as blank, and then measure the fluorescence in 280 nm at 10 °C. Subtract the absorbance value at different buffer of the blank and then draw the picture by overlapping different buffer curves.

![Fluorescence absorbance of PA83 at 10 °C.](image)

Figure 57. Fluorescence absorbance of PA83 at 10 °C.

Put the samples in RT overnight. Then take the pH 7.01 sodium phosphate buffer as blank and measure the fluorescence in 280 nm at 25 °C. Subtract the absorbance value at different buffer of the blank and then draw the picture by overlapping different buffer curves.
Fluorescence assay has demonstrated that PA is stable and behaves the same in different temperatures and different pH values. Consequently, for the sake of convenience, most experiments were carried out at room temperature in fixed buffers.

**4.2.5 $^{19}$F-NMR of 2F-His-PA83**

This assay was carried out by Dr. Bann.

NMR spectra were recorded on a Varian Unity-Plus 500 MHz spectrometer operating at 470.3 MHz, which was equipped with a Varian Cryo-Q dedicated 5 mm $^{19}$F probe. The data shown in Figure 59 represent 1024 transients processed with 20 Hz line broadening.
The concentration of 2F-His-PA83 Protein is 50 µM and they are in 10 mM buffer of Tris, pH 8, Heps, pH 7 and Acetate, pH 5. It can be seen from the Figure above that the chemical shift changes from pH 8 to 7, while there are no further changes from pH 7 down to a pH of 5. The chemical shift change would be due to large changes in the structure or dynamics of the protein by the substitution of chosen amino acid [79]. Since shifting downfield indicates a bigger flexibility of the His side chain, the protein of 2F-His-PA83 is kept in pH8.0 buffer as PA83 in the lab in -20°C before any binding assays carried out.

Since we can see clearly the 2F-His signal in $^{19}$F NMR, we can draw a conclusion that the PA was successfully labeled with 2F-His. Considering the trypsin (4.2.1) and furin (4.2.2) assay done on both PA and 2F-PA giving the same results as well as the similar stability between 2F-His-PA and PA in buffers, we got a clue that there is no apparent destruction of the nature of PA by the labeling.

4.3 DEP Assay

4.3.1 Determine the Concentration of DEP

In order to make a 100 mM DEP solution, a 14.5 µl DEP aliquot from the 6.9 M DEP stock solution was diluted with 985.5 µl ice cold ethanol. Make 100 ml imidazole in 50 mM phosphate buffer at pH 7.5. Prepare three samples at the same time. One is filled with 1000 µl imidazole and 10 µl ice cold ethanol as blank control, the other two were filled with 1000 µl imidazole, 9 µl ice cold ethanol and 1 µl DEP diluted solution. Measure the UV-Vis absorbance of the samples at 240 nm. Then even the two results to get the average concentration of the DEP diluted concentration to be 107.7 mM. ($\varepsilon$=3200 M$^{-1}$cm$^{-1}$ at 240nm).
4.3.2 PA83 Reaction with and without DEP

Prepare a sample by combining the 50 µl DEP diluted solution with PA83 (52 µM) by making the final concentration of PA83 to be 2 µM in the 1 ml reaction. Dilute the sample with necessary amount of 50 mM sodium phosphate buffer. At the same time, another sample was prepared which includes 50µl ice cold ethanol instead of DEP as a control. The reaction was performed at RT for 30 minutes, and then quenches them by adding 5 ml 100 mM imidazole in 50 mM sodium phosphate buffer. Concentrate the reaction solution and wash them three times with 20 mM Tris-HCl pH 8.0. Then transfer into Eppendorf tubes and keep them in refrigerator for further use.

4.3.3 Furin Treatment

Treat PA83 and PA83 modified by DEP with furin by the protocol as following.

- Diafilter two 1ml (5.176 µM) PA83 to 100 µl.
- Diafilter into 50 mM phosphate buffer pH 6.0.
- Concentrate to around 100 µl.
- Diluted into around 1000 µl with buffer above in two Eppendorf tubes.
- Add DEP to a final concentration of 0.2 mM for one tube, the other just put in ice cold ethanol.
- React for 30 minutes in RT.
- Add 200 µl 100 mM imidazole in 50 mM phosphate buffer pH 7.0 to a final concentration of 20 mM.
- Diafilter in Tris-HCl pH 8.0.
- Concentrate to 100–200 µl. Resuspend in Tris-HCl pH 8.0 and concentrate two times.
- Treat with Furin 2 µl at RT overnight (1 unit furin equals 0.5 µl and 10 unit furin equals 1 mg PA83 protein).

It can be seen that both PA83 modified with DEP and without DEP can be cut by furin (Figure 60). So DEP may be used in the following as a probe to explore the function of histidine in the process of pore formation.
Figure 60. Furin treatment to PA83 modified with DEP and PA83 together with ethanol as a control.

### 4.3.4 Trypsin Treatment

Treat PA83 and PA83 modified by DEP with trypsin by the protocol as following.

- Diafilter two 0.5ml (5.176 µM) PA83 to 100 µl.
- Diafilter into 50 mM phosphate buffer pH 6.0.
- Concentrate to around 100 µl, and then transfer to Eppendorf tubes.
- Diluted into around 1000 µl with 50 mM phosphate buffer pH 6.0 in two Eppendorf tubes.
- Add 1µl DEP to a final concentration of 0.2 mM for one tube, the other just put in ice cold ethanol.
- React for 30 minutes in RT.
- Add 100 µl 100 mM imidazole in 50 mM phosphate buffer pH 7.0 to final concentration of 20 mM.
- Diafilter in 20mM Tris-HCl pH 8.0
- Concentrate to 100~200 µl. wash with 20mM Tris-HCl pH 8.0 three times.
- Concentrate into 500 µl.
- Treat with trypsin 10.5 µl at RT for 30 minutes.
- Add trypsin inhibitor 1.5 µl.
- Take 10 µl out run SDS-PAGE.

![Image of SDS-PAGE gel showing bands at 63 kDa and 20 kDa]

**Figure 61. Trypsin treatment on PA83 modified with DEP and without DEP.**

The SDS-PAGE gel shows both PA83 modified with DEP and without DEP can be cut with trypsin. Since the trypsin treatment is faster and more effective, the future PA83 is cut by trypsin if not specifically noted.

Since the phenomenon of above two experiments looks the similar to us, another measure is carried out to show whether the DEP works on PA83 or not. Dilute 10 times of PA83 treated with ethanol and PA83 treated with trypsin. Measure absorbance ratio at 240nm and 280nm.

**TABLE 9**

**ABSORBANCE COMPARISON BETWEEN PA83 MODIFIED WITH AND WITHOUT DEP**

<table>
<thead>
<tr>
<th>Diluted sample</th>
<th>A&lt;sub&gt;240&lt;/sub&gt;</th>
<th>A&lt;sub&gt;280&lt;/sub&gt;</th>
<th>Ratio of A&lt;sub&gt;240&lt;/sub&gt;</th>
<th>Ratio of A&lt;sub&gt;280&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA with ethanol</td>
<td>0.041</td>
<td>0.0245</td>
<td>1.577</td>
<td>1.189</td>
</tr>
<tr>
<td>PA with DEP</td>
<td>0.026</td>
<td>0.0206</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

It can be seen that the ratio of absorbance of diluted sample of PA83 modified with DEP and without DEP are different. So DEP does works and PA83 does modified by DEP. Then we can use DEP as a probe to do the following pore formation experiments.
4.3.5 Performance of DEP Modified PA83 in Pore Formation

Take 4 aliquot 10 µl samples of PA83 treated with ethanol and trypsin and 4 aliquot 10 µl samples of PA83 treated with DEP and trypsin from 4.3.4. Incubate them for one hour in RT with 10 µl of the following buffers: 1 M BisTris, pH 5; 1 M BisTris, pH 6; 1 M HEPES, pH 7.0; 1 M HEPES, pH 8.0. Then the samples were exposed to 2.857 µl SDS at room temperature for 20 minutes. After putting 4.57 µl dyes without SDS, the samples were boiled for 5 minutes at a temperature higher than 80°C. Then the samples were separated on a 4-20% Tris-Glycine gel by loading 24 µl of sample to each well. The first well is pure PA63 in buffer pH 5 as control.

![Figure 62. Pore formation of PA83 modified with DEP and without DEP.](image)

The data shows both PA83 modified with and without DEP can be cut by trypsin and furin into PA63. And both PA63 products can form pore in pH5 and pH6. Since the pore formation didn’t be affected after modified with DEP, it indicates that histidine in PA may not play a key role in pore formation process. Even though the normal pore formation range of pH is below 7 instead of 6[52], here we give the reason of this difference that different gel origin may affect a little bit of pore formation pH range.

4.3.6 Performance of DEP Modified CMG2 in Pore Formation

Twelve aliquots of 25 µl 57.47 µg/ml PA63 were prepared in Eppendorf tubes. Six aliquots of PA63 were added with 5 µl 288 µg/ml CMG2. Six of PA63 were added with 20 mM Tris-HCl pH8.0 as control. Letting the
binding reaction last for 15 minutes at room temperature, then the individual aliquots were incubated for one hour with 30 µl of the following buffers: 1 M BisTris, pH 5.5; 1 M BisTris, pH 6; 1 M BisTris, pH 6.5; 1 M Hepes, pH 7.0; 1 M Hepes, pH 7.5; 1 M Hepes, pH 8.0. Then the samples were exposed to 9.3 µl 10% SDS at room temperature for 20 minutes. After putting 5 µl dyes without SDS, the samples were boiled for 5 minutes at a temperature higher than 80°C. Then the samples were separated on a 4-20% Tris-Glycine gel by loading 17 µl of sample to each well.

![Figure 63. pH-dependent conversion of prepore to pore for PA63 binding with CMG2.](image)

The gel shows that in the present of CMG2, the conversion of prepore to pore for PA63 is below pH6, while without CMG2, it is below 7. So the binding of CMG2 with PA63 lower the pH ranges about 1.

In order to see whether the histidine 121 on CMG2 has any effects on this pore formation process, the above experiment was repeated by using DEP modified CMG2 binding with PA63.

Twelve aliquots of 25 µl 57.47 µg/ml PA63 were prepared in Eppendorf tubes. Six aliquots of PA63 were added with 5 µl 288 µg/ml CMG2 with DEP treatment. Six of PA63 were added with 20 mM Tris-HCl pH8.0 as control. Letting the binding reaction last for 15 minutes at room temperature, then the individual aliquots were incubated for one hour with 30 µl of the following buffers: 1 M BisTris, pH 5.5; 1 M BisTris, pH 6; 1 M BisTris, pH 6.5; 1 M Hepes, pH 7.0; 1 M Hepes, pH 7.5; 1 M Hepes, pH 8.0. Then the samples were exposed to 9.3 µl 10% SDS at room temperature for 20 minutes. After putting 5 µl dyes without SDS, the samples were boiled for 5
minutes at a temperature higher than 80°C. Then the samples were separated on a 4-20% Tris-Glycine gel by loading 17 µl of sample to each well.

The SDS-PAGE shows when CMG2 was modified by DEP, it has same pH range of conversion of prepore to pore (below pH 7) as the pure PA63 without CMG2 receptor. Apparently, it just likes the receptor doesn’t exist. We propose a model below to explain this. The binding situation of WT CMG2 and PA63 is shown in Figure 65 a. Since modified histidine in CMG2 gives a result just like PA forms a pore without binding with it, His121 may have function in binding of receptor with PA (Figure 65 b). Since it is also because of steric effect, that is, it might because the volume of DEP is so big that the modified CMG2 could not bind with PA63 effectively (Figure 65 c), which also gives the same result. Another better small probe for the function of histidine is needed to further explore the role of histidine in this pore formation process.

Next, 2F-His labeled protein was used as a probe of histidine instead of DEP in the process of pore formation.

Figure 64. pH-dependent conversion of prepore to pore for PA63 with DEP modified CMG2.

The SDS-PAGE shows when CMG2 was modified by DEP, it has same pH range of conversion of prepore to pore (below pH 7) as the pure PA63 without CMG2 receptor. Apparently, it just likes the receptor doesn’t exist. We propose a model below to explain this. The binding situation of WT CMG2 and PA63 is shown in Figure 65 a. Since modified histidine in CMG2 gives a result just like PA forms a pore without binding with it, His121 may have function in binding of receptor with PA (Figure 65 b). Since it is also because of steric effect, that is, it might because the volume of DEP is so big that the modified CMG2 could not bind with PA63 effectively (Figure 65 c), which also gives the same result. Another better small probe for the function of histidine is needed to further explore the role of histidine in this pore formation process.

Next, 2F-His labeled protein was used as a probe of histidine instead of DEP in the process of pore formation.
4.4 pH-dependent Conversions by SDS-PAGE

4.4.1 PA63 and 2F-His-PA63

Four aliquots of 10 µl 2.69µM PA63 and Four aliquots of 10 µl 2.69 µM 2F-His-PA63 were prepared in Eppendorf tubes. Individual aliquots were incubated for one hour with 10 µl of the following buffers: 1 M BisTris, pH 5; 1 M BisTris, pH 6; 1 M Hepes, pH 7.0; 1 M Hepes, pH 8.0. Then the samples were exposed to 2.857 µl SDS at room temperature for 20 minutes. After putting 4.57 µl dye without SDS, the samples were boiled for 5 minutes at a temperature higher than 80°C. Then they are separated on a 4-20% Tris-Glycine gel by loading 24 µl of sample to each well.

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Figure 65. Model of PA63 binding with CMG2 and CMG2-DEP.

Figure 66. pH-dependent conversion of prepore to pore for PA63 and 2F-His-PA63.
The gel shows both PA63 and 2F-His-PA63 have a pH range of 7 and below for the conversion of prepore to pore.

4.4.2 PA63 with and without WT CMG2

Four aliquots of 10 µl 2.69µM PA63 were prepared in Eppendorf tubes. Four aliquots of 9 µl 2.69µM PA63 were added with 1 µl 140.8 µM CMG2. Letting the binding reaction last for 15 minutes at room temperature, then the individual aliquots were incubated for one hour with 10 µl of the following buffers: 1 M BisTris, pH 5; 1 M BisTris, pH 6; 1 M Hepes, pH 7.0; 1 M Hepes, pH 8.0. Then the samples were exposed to 2.857 µl SDS at room temperature for 20 minutes. After putting 4.57 µl dyes without SDS, the samples were boiled for 5 minutes at a temperature higher than 80°C. Then the samples were separated on a 4-20% Tris-Glycine gel by loading 24 µl of sample to each well.

![Figure 67. pH-dependent conversion of prepore to pore for PA63 with and without CMG2.](image)

The gel shows in the absence of CMG2, the conversion of prepore to pore takes place at pH 7 and below. In the presence of CMG2, a pH of 6 and below is required. So the present of receptor lower the pH range of pore formation. This result is consistent with the data of references [52].

4.4.3 2F-His-PA63 with and without WT CMG2

Four aliquots of 10 µl 2.69µM 2F-His-PA63 were prepared in Eppendorf tubes. Four aliquots of 9 µl 2.69µM 2F-His-PA63 were added with 1 µl 140.8 µM CMG2. Letting the binding reaction last for 15 minutes at room temperature, then the individual aliquots were incubated for one hour with 10 µl of the following buffers: 1 M
BisTris, pH 5; 1 M BisTris, pH 6; 1 M Heps, pH 7.0; 1 M Heps, pH 8.0. Then the samples were exposed to 2.857 µl SDS at room temperature for 20 minutes. After putting 4.57 µl dyes without SDS, the samples were boiled for 5 minutes at a temperature higher than 80°C. Then the samples were separated on a 4-20% Tris-Glycine gel by loading 24 µl of sample to each well.

![Figure 68. pH-dependent conversion of prepore to pore for 2F-His-PA63 with and without CMG2.](image)

Figure 68. pH-dependent conversion of prepore to pore for 2F-His-PA63 with and without CMG2.

The gel shows in the absence of CMG2, the conversion of prepore to pore of 2F-His-PA63 takes place at pH 7 and below. In the presence of CMG2, the pore formation is blocked. To confirm this result, next experiment is carried out.

### 4.4.4 PA63 and 2F-His-PA63 Binding with WT CMG2

Four aliquots of 9 µl 2.69µM PA63 and four aliquots of 9 µl 2.69µM 2F-His-PA63 were prepared in Eppendorf tubes. Each of them were added with 1 µl 140.8 µM CMG2. Letting the binding reaction last for 15 minutes at room temperature, then the individual aliquots were incubated for one hour with 10 µl of the following buffers: 1 M BisTris, pH 5; 1 M BisTris, pH 6; 1 M Heps, pH 7.0; 1 M Heps, pH 8.0. Then the samples were exposed to 2.857 µl SDS at room temperature for 20 minutes. After putting 4.57 µl dyes without SDS, the samples were boiled for 5 minutes at a temperature higher than 80°C. Then the samples were separated on a 4-20% Tris-Glycine gel by loading 24 µl of sample to each well.
Figure 69. pH-dependent conversion of prepore to pore for 2F-His-PA63 with and without CMG2.

The gel shows in the presence of CMG2, the conversion of prepore to pore of PA63 takes place at pH 6 and below while the pore formation of 2F-His-PA63 is blocked. This repeated result of the disappearance of pore formation confirms the blocking of pore formation after labeling.

4.4.5 PA63 Binding with CMG2 and 2F-His-CMG2

12 aliquots of 8.6 µl 22.86 µM PA63 were prepared in Eppendorf tubes. Six of them were added with 1.4 µl 140.8 µM CMG2. Each of the six samples was added 0.98 µl 192.7 µM 2F-His-CMG2, which was diluted with 0.42 µl 1×PBS buffer. Letting the binding reaction last for 15 minutes at room temperature, then the individual aliquots were incubated for one hour with 10 µl of the following buffers: 1 M BisTris, pH 5.5; 1 M BisTris, pH 6.0; 1 M BisTris, pH 6.5; 1 M Hepes, pH 7.0; 1 M Hepes, pH 7.5; 1 M Hepes, pH 8.0. Then the samples were exposed to 2.857 µl SDS at room temperature for 20 minutes. After putting 4.57 µl dyes without SDS, the samples were boiled for 5 minutes at a temperature higher than 80°C. Then the samples were separated on a 4-20% Tris-Glycine gel by loading 24 µl of sample to each well (Figure 70).

The gel shows in the presence of CMG2, the conversion of prepore to pore takes place at pH 6 and below. In the presence of 2F-His-CMG2, the conversion of prepore to pore also takes place at pH 6 and below. These same results of the pore formation indicate histidine in CMG2 doesn’t affect the pore formation process. So His 121 in CMG2 doesn’t work as a trigger in this process as some former authors mentioned [29].
Figure 70. pH-dependent conversion of prepore to pore for PA63 binding with CMG2 and 2F-His-CMG2.

4.4.6 PA63 Incubate in Other Buffers

Put 5 µl PA63 (4.1.2) in two different Eppendorf tubes. One of them put in 5 µl pH 6 BisTris buffer, the other was incubated in pH 6 phosphate buffer. Let both reactions sit on bench in RT for an hour. Then run SDS-PAGE gel.

Figure 71. pH-dependent conversion of prepore to pore for PA63 at different buffers.

The gel shows PA63 can form pore in both buffers. The pore formation process isn’t affected by the nature of buffer. So the buffer here just provides an acidic environment for the pore formation process.
4.5 Protein affinity Research by Native Gel Experiments

4.5.1 Concentration Counterpart between PA83 and 2F-His-PA83

Since the purity of PA83 and 2F-His-PA83 is different because of the purification process. In order to let the result be more comparable, the following experiment is done to find out the concentration counterpart between PA83 and 2F-His-PA83. Keep PA83 concentration constant at 5 µM, and then change the concentration of 2F-His-PA83 from low to high. Run 4-20% SDS-Page to find out the counterpart concentration.

![Figure 72. SDS-PAGE of PA83 and a series of 2F-His-PA83 with different concentrations.](image)

Since it is hard to tell the concentration range of 2F-His-PA83 from 8µM to 10µM, redo the above experiment by comparing 5 µM PA83 with 8 µM, 9µM, and 10µM 2F-His-PA83.

![Figure 73. SDS-PAGE of PA83 and 2F-His-PA83 with different concentrations.](image)
Choose 8µM 2F-His-PA83 as a counterpart concentration with PA83 to do the following binding experiments.

4.5.2 Concentration Counterpart between CMG2 and 2F-His-CMG2

Since the purity of CMG2 and 2F-His-CMG2 is different because of the purification process. In order to let the result be more comparable, the following experiment is done to find out the concentration counterpart between them. Keep CMG2 be 5 µM constant, then change the concentration of 2F-His-CMG2. Follow the same procedure described in 4.5.1.

![SDS-PAGE of CMG2 and 2F-His-CMG2 with different concentrations.](image)

Figure 74. SDS-PAGE of CMG2 and 2F-His-CMG2 with different concentrations.

Take 10 µM 2F-His-CMG2 as a counterpart concentration to 5 µM CMG2.

4.5.3 PA83 and 2F-His-PA83 Binding with CMG2

Make 10 µl reactions. Let PA83 and 2F-His-PA83 be 5 µM unchanged, and then add CMG2 in from 0 µM to 5 µM. Let the reaction sit in RT for 15 minutes, then incubate in 10 µl Hepes pH 8.0 buffer for one hour in RT. Add 80% glycerol 1.33µl, After that, run 15 well 4-20% prepared SDS-PAGE gel by using buffer without SDS for 75 minutes, then dye the gel overnight.
Figure 75. Native gel of PA83 and 2F-His-PA83 binding with CMG2. The numbers show the molar ratio between CMG2 and PA.

The gel shows there are no big difference of binding affinity between PA83 and CMG2 as well as 2F-His-PA83 and CMG2. However, at the molar ratio of 0.8, the binding affinity of labeled PA looks a little bigger.

Redo the above experiment by using water bath to cool down the system.

Figure 76. Native gel of PA83 and 2F-His-PA83 binding with CMG2 in water bath. The numbers show the molar ratio between CMG2 and PA.

The gel shows the similar result.

4.5.4 PA83 Binding with CMG2 and 2F-His-CMG2

Use a new batch of PA83 (16.22 µM) to do the binding experiment with CMG2 (13.35 µM). Dilute the PA83 to 5 µM, then bind with CMG2 which was diluted to different concentration by Tris pH8.0 buffer.
Figure 77. Native gel of PA83 binding with CMG2. The numbers show the final concentration of CMG2 added to PA.

The gel shows the similar result as 4.5.3.

Use same procedure and same batch of PA83 (16.22 µM) to do the binding experiment with 2F-His-CMG2 (16.74 µM) which has the comparable concentration as the CMG2 above.

Figure 78. Native gel of PA83 binding with 2F-His-CMG2. The numbers show the final concentration of CMG2 added to PA.
Still the labeled protein shows a little bigger affinity. Since this small difference maybe due to the purification content of different proteins, gradient gel is then used in order to enlarge the difference of affinity between PA83 and CMG2 as well as F-His-PA83 and CMG2.

4.6 Protein Stability Research by Gradient Gel

4.6.1 PA83

Use PA83(16.22 µM) 18.65 µl, then add 7.5 µl 10× Tris acetate buffer, 15 µl 5×dye and 33.85µl nuclear free water. Put this totally 75 µl samples into the well of the gradient gel. Run four hours at 10 mA. Dye the gel directly without rinsing it with DI water. Use same way to prepare a control. Both of them run at the same condition at the same time in different side of the cell.

![Two gradient gels of PA83 in two sides](image)

Figure 79. Gradient gel of PA83.

The gradient gel on both side of the cell gives the same gradient curve. So the two side of the same cell are comparable. The following results are run at the same time at the different side of the same cell if not specifically mentioned.

4.6.2 PA83 and 2F-His-PA83

Use PA83(16.22 µM) 18.65 µl, then add 7.5 µl 10× Tris acetate buffer, 15 µl 5×dye and 33.85µl nuclear free water. Put this totally 75 µl samples into the well of the gradient gel. Use same way to prepare a a sample of 2F-His-PA83 (19.73 µM). Run 8 hours at 10 mA. Dye the gel directly without rinsing it with DI water overnight. Both of them run at the same condition at the same time in different side of the cell.
The gradient gel of PA83 and 2F-His-PA83 looks similar. Both of them have two transition states which indicates an intermediate exists. The first transition is slow and the second is fast.

\[ \begin{align*}
U & \leftrightarrow \text{slow} & I & \leftrightarrow \text{fast} & N 
\end{align*} \]

### 4.6.3 PA83 with and without CMG2

Use PA83 (16.22 µM) 18.65 µl, then add 22.66 µl CMG2 (13.35 µM). Let them bind at RT for 15 minutes. After that, 41.31 µl Hepes pH8.0 was added. Incubate at this buffer for one hour in RT. Then 20.66 µl 5 × dyes were put in. Load this sample into the well of the gradient gel. At the same time, prepare a sample of PA83 (19.73 µM) by adding 22.66 µl 20mM Tris-HCl pH 8.0 instead of CMG2. Run 16 hours at 10 mA, 40V, and 20°C. Dye the gel directly without rinsing it with DI water overnight. Both of them run at the same condition at the same time in different side of the cell.

![Gradient gel of PA83 and 2F-His-PA83](image1.png)

**Figure 80. Gradient gel of PA83 and 2F-His-PA83.**

![Gradient gel of PA83 binding with and without CMG2](image2.png)

**Figure 81. Gradient gel of PA83 binding with and without CMG2.**
The gradient gel of PA83 shows two transitions on the denature curve. When combined with CMG2, the first transition disappears, which indicates no intermediate any more. Overlap these two gels together, it looks like below:

![Overlap of gradient gel of PA83 binding with and without CMG2.](image1)

**Figure 82. Overlap of gradient gel of PA83 binding with and without CMG2.**

The gradient gel shows that the denature curve of PA83 is a little different with that of the PA83 binding with CMG2. After binding, the protein moves slower which indicates a more compact structure exists. Maybe there are some conformational changes due to binding.

### 4.6.4 2F-His-PA83 with and without CMG2

Use 2F-His-PA83 (19.73 µM) 30.67 µl, then add 45.33 µl CMG2 (13.35 µM). Let them binding at RT for 15 minutes. After that, 76 µl Hepes pH8.0 was added. Incubate at this buffer for one hour in RT. Then 38 µl 5 × dyes were put in. Load this sample into the well of the gradient gel. At the same time, prepare a sample of 2F-His-PA83 (19.73 µM) by adding 45.33 µl 20mM Tris-HCl pH 8.0 instead of CMG2. Run 16 hours at 10 mA, 40V, and 20°C. Dye the gel directly without rinsing it with DI water overnight. Both of them run at the same condition at the same time in different side of the cell.

![Gradient gel of 2F-His-PA83 binding with and without CMG2.](image2)

**Figure 83. Gradient gel of 2F-His-PA83 binding with and without CMG2.**

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The gradient gel shows after binding, the first transition state also disappears. When overlap these two gels together, it looks like below:

![Image: 2F-His-PA83 + CMG2 and 2F-His-PA83 Overlap]

**Figure 84.** Overlap of gradient gel of 2F-His-PA83 binding with and without CMG2.

The gradient gel shows that there are bigger differences of the denature curve of the 2F-His-PA83 when it binds with or without CMG2 than that of unlabeled PA83. This result indicates bigger conformational changes happen after labeling PA with 2F-His.

Upon the data above, hypotheses for pore formation is proposed.

### 4.7 Hypotheses for Pore Formation

![Image: CMG2 VWA domain binding with domain 2 and domain 4 of PA [52].]

**Figure 85.** CMG2 VWA domain binding with domain 2 and domain 4 of PA [52].
According to the crystal structure above, the PA insertion loop and the contiguous β strands (green) are predicted to peel away from the domain 2 to form a pore [52]. Considering the experimental results, we propose the following hypothesis for pore formation process:
When PA63 was exposed in pH value of 8 (a1, Figure 88b), the charge and force (the attraction force and the intrinsic repulse force) of β loop in PA keep balance and the β loop isn’t peeled away. We suppose that the side chain E302, E308 and D315 on the β loop (b of Figure 89) play a role as a salt bridge, they are attracted by side chains of other positive charged amino acids. After the pH changes to a lower value, the negative charges on E302, E308 and D315 are neutralized a little. Even though pKa of the side chain of both amino acids E (Glu) and D (Asp) are 4.5 in normal situation, the pKa of either E or D has the possibility to surpass 8 in this specific PA environment. Lanyi and coworkers once found the pKa of Asp can reach higher than 8 because of the dependence of this residue on the protonation state of Glu when they form part of the reprotoamination switch of bacteriorhodopsin [87]. Acharya and coworkers found the Glu-43 (β) located in the interface of hemoglobin A could exhibit a high pKa of 7 upon deoxygenation of the protein which reflected an increase in the hydrophobicity of the molecule [88]. In this occasion, E302, E308 and D315 lose some of the attraction force from the salt bridge and the original balance is destroyed. The net force left is intrinsic repulse force. As a result, the β loop peels away from the domain 2 of PA (a2 of Figure 88b). When the pH becomes much lower, the intrinsic repulse force increases and the β loop keeps peeling away from PA (a3 of Figure 88b).

When 2F-His-PA63 (b1 of Figure 88b) was exposed in pH value of 7 (b2 of Figure 88b) or lower (b3 of Figure 88b), the acidic environment also increases the intrinsic repulse force on β loop in 2F-His-PA63. The β loop peels away from the domain 2 of PA even though the net repulse force may different from the WT PA63. When the mixture of PA63 bind with CMG2 (c1 of Figure 88b) is exposed in pH value of 7, there are also some repulse forces produced in PA63 due to the acidic environment, which try to make the β loop peels away from the domain 2 of PA. However, at the same time, the conformation of domain 2 and domain 4 changes a lot due to the binding of CMG2, which makes some hydrophobic groups in domain 2 and domain 4 of PA exposed and these hydrophobic groups give the β loop an big attraction because more than half of the amino acids in β loop is hydrophobic, such as F, I, V and A (see the green part in Figure 89) that is opposite to the repulse force. This attraction force balances the repulse force, so the β loop can not peel away from the domain 2 of PA (c2 of Figure 88b). When the mixture is exposed in pH value of 6 or below, the attraction force mentioned above doesn’t change
much due to the binding. But the repulse force increases a lot because of the lower pH, so the net force makes the β loop peel away (c3 of Figure 88b).

![Figure 87. Amino acids on β loop. a, Hydrophobic groups (green) highlighted on β loop. When His304 and His310 were labeled with 2F-His, the hydrophobic force between them and other hydrophobic groups increases greatly. b, Glu and Asp sites on β loop.]

When the mixture of 2F-His-PA63 bind with CMG2 (d1 of Figure 88b) is exposed in pH value of 7, the attraction between the β loop and hydrophobic groups in PA becomes much higher than WT PA63 because of the increased hydrophobicity due to the 2F-His substitution. So the β loop can not peel away (d2 of Figure 88b). This attraction is so high that when pH lowers to 6 or below, the enhanced repulse force still can not balance it. So the β loop still can not peel away (d3 of Figure 88b). As a result, the pore formation is blocked in all pH ranges.

So it seems the His may not be a molecular switch in this pore formation process. Instead, the Glu302, Glu308 and Asp315 may work as a molecular switch.

The experiments above show 2F-His could be a good probe in the study of PA protein research. By successfully labeling His in PA and CMG2 with 2F-His, it was demonstrated that His does not likely hold the key in the pore formation. This suggests that the pKa of His may not be in a range that affects the pore formation. Titration of pKa of each His in the β loop may reveal the actual pKa range of His in the anthrax toxin environment and double checking the role of His in the PA pore formation. Furthermore, Glu302, Glu308 and Asp315 in
domain 2 of PA can also be modified to measure whether they are truly molecular switches in the pore formation process as was hypothesized (see the position of Glu302, Glu308 and Asp315 in domain 2).

Figure 88. Glu302, Glu308 and Asp315 on β loop of domain 2.
5.1 Translocation Assay

In order to see whether 2F-His labeled PA blocked the translocation process or not, the rate of LF\textsubscript{N} translocation through PA63 channels formed in planar lipid bilayers was measured. LF\textsubscript{N} was added to the cis compartment under a $\Delta\psi$ of 1~10 mV (where $pH\textsubscript{cis} = 5.5$, $pH\textsubscript{trans} = 6.5$). After perfusing to remove unbound LF\textsubscript{N}, $\Delta\psi$ was stepped to 30 mV voltage and monitored the rate of translocation by the increase in channel conductance as LF\textsubscript{N} traversed the pore (Fig. 91). Records are normalized as the fraction translocated.

![Diagram of translocation process](image)

**Figure 89. Model of translocation process.**

![Conductance records of LF\textsubscript{N} translocation through WT and 2F-His Channels](image)

**Figure 90. Conductance records of LF\textsubscript{N} translocation through WT and 2F-His Channels (Carried out in Havard Medical School).**
LFn is not translocated in the presence of 2F-His labeled PA63 ($\Delta \Psi = +30 \text{ mV}, \Delta \text{pH} = 1$). While the rate of translocation is high for pores made of WT PA63.

5.2 Hypotheses of Translocation

![Diagram showing three possibilities for blocking of translocation in 2F-His modified PA.]

**Figure 91. Hypothesis of two possibilities of blocking of translocation in 2F-His modified PA.**

We hypothesized two possibilities for this blocking of translocation. First, because of the labeling of 2F-His, the hydrophobicity around the $\beta$ loop increases so much that the pore formation process is blocked and as a consequence, the translocation is blocked (b of Figure 93). This double confirms the result of the pH dependent prepore to pore transition assay which shows the pore formation is blocked after the PA63 is labeled with 2F-His when binding with CMG2. Second, the blocking of translocation is due to the increasing hydrophobicity in the pore after 2F substitution (C of Figure 93).
Since the compounds with more hydrophobic surface can block the translocation more effectively [83], if the 2F-His PA63 forms a pore in the presence of CMG2, the lumen should be more hydrophobic because of the substitution of poor hydrogen-bond acceptors of organic fluorine [89]. However, this increasing hydrophobic environment doesn’t increase the rate of translocation in the lumen and shows almost no signal of translocation instead. Since the φ clamp recognizes substrates primarily by nonspecific hydrophobic interactions [83], these *in vitro* translocation studies, therefore, give us a clue to provide the hypothesis of translocation because of the hydrophobicity as below.

The φ clamp forms a narrow channel which provides an environment that mimics the hydrophobic core of the unfolding molten globules of the substrate protein. This channel can effectively grasp the unwound leading segment of the translocating polypeptide chain to move through the channel. The lumen surface which has hydrophobicity closer to the core of the unfolding molten globules can gives an optimism translocation rate in the lumen (Figure 94).

![Figure 92. Hypothesis of blocking of translocation because of hydrophobicity.](image_url)
The hydrophobicity of the lumen increases from left to right because of the hydrophobicity of amino residues facing the lumen increases. So the conductance signal increases too. F427 which forms the φ clamp gives hydrophobicity closest to the optimism value in the Figure; as a result, the translocation rate at the point is faster than a, b, or d in Figure 94. After passing the highest point, the conductance of pore will decrease. This is because even when a small portion of 2F-His-PA63 overcomes big hinder and forms a pore, due to the high hydophobicity of the pore, the translocation is also impossible because the φ clamp and the lumen grasp the hydrophobically dense peptide segments so tightly that it is hard for them to be released which is necessary for continuous translocation. So according to this hypothesis, channel-blocking drugs can be developed by changing the hydrophobicity of the residues in the β loop.
CHAPTER 6

CONCLUSIONS

Histidine on β loop in PA and His121 on CMG2 does not likely hold the key in the pore formation process. Pore formation process was blocked when 2F-His-PA63 was put in acidic environment in the presence of CMG2. Translocation assay confirms these results. Gradient gel assay shows there are bigger conformational changes happen in 2F-His-PA when it binds with CMG2, which provides one possible explanation of the blocking. The changes of hydrophobicity on β loop in domain 2 of PA is possible for the other reason of the blocking of pore formation and translocation process. Glu302, Glu308 and Asp315 in domain 2 of PA are likely responsible for triggering the pH conversion. They can be modified to detect their role on pore formation in the future.
LIST OF REFERENCES
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