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IDENTIFICATION OF THE DOMAIN(S)
IN PROTEIN DISULFIDE ISOMERASE REQUIRED FOR
BINDING AND DISASSEMBLY OF THE CHOLERA HOLOTOXIN

by

LAURA K. HERNDON

A thesis submitted in partial fulfillment of the requirements
for the Honors in the Major Program in Biomedical Sciences
in the College of Medicine
and in The Burnett Honors College
at the University of Central Florida
Orlando, Florida

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Thesis Chair: Dr. Ken Teter

ABSTRACT

Cholera, caused by the secretion of cholera toxin (CT) by *Vibrio cholerae* within the intestinal lumen, triggers massive secretory diarrhea which may lead to life-threatening dehydration. CT is an AB₅-type protein toxin that is comprised of an enzymatically active A1 chain, an A2 linker, and a cell-binding B pentamer. Once secreted, the CT holotoxin moves from the cell surface to the endoplasmic reticulum (ER) of a host target cell. To cause intoxication, CTA1 must be displaced from CTA2/CTB₅ in the ER and is then transferred to the cytosol where it induces a diarrheal response by stimulating the efflux of chloride ions into the intestinal lumen.

Protein disulfide isomerase (PDI), a resident ER oxidoreductase and chaperone, is involved in detaching CTA1 from the holotoxin. The PDI domain(s) that binds to CTA1 and precisely how this interaction is involved in CTA1 dissociation from the holotoxin are unknown. The goal of this project is to identify which domain(s) of PDI is responsible for binding to and dislodging CTA1 from the CT holotoxin. Through incorporation of ELISA, surface plasmon resonance (SPR), and Fourier transform infrared (FTIR) spectroscopy techniques in conjunction with a panel of purified PDI deletion constructs, this project aims to provide important molecular insight into a crucial interaction of the CT intoxication process.

DEDICATION

For my mentor, Dr. Teter, for your continuous support and guidance throughout my time as an undergraduate researcher. Thank you for giving me the opportunity to experience the rewards of taking part in research.

For Dr. Helen Burress, Carly Bader, and Dr. Lucia Cilenti. I love you.

For my family, for being my “behind-the-scene” reason for success.
You know how much it means to me.

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BACKGROUND

Impact of Cholera

Cholera is a disease that causes severe symptoms similar to food poisoning, such as nausea, fever, and vomiting, as well as massive secretory diarrhea. The copious loss of water, up to a quart per hour, from the extreme diarrheal symptom can lead to life-threatening electrolyte imbalance and dehydration if left untreated (Mayo Clinic, 2014). As a result of electrolyte imbalance, there can be a severe drop in blood pressure and blood oxygen levels leading to hypovolemic shock. Hypovolemic shock can cause death in minutes.

Since 1817, there have been seven cholera pandemics. The disease is currently endemic in over 50 countries (Harris, et al., 2012). The World Health Organization estimates there are approximately 3-5 million global cases of cholera leading to 100,000-120,000 deaths annually (WHO, Cholera Facts Sheet, 2014). Cholera continues to be a serious health problem in Africa, Asia and Latin America (WHO, Cholera Facts Sheet, 2014). In addition to health care concerns, cholera epidemics can also have massive economic consequences. Panic over outbreaks can lead to tourism and travel advisories being put into place, as well as agricultural import restrictions (WHO, Global Epidemics and Impact of Cholera, 2015). These ramifications can cause a devastating blow to the economic structure of affected countries.

The cholera transmission process is human-to-human through the fecal-oral route, usually occurring when the feces of an infected patient comes in to contact with groundwater. Ingestion of this contaminated groundwater then spreads the disease to other individuals. This frequently happens in areas with stressed sewage systems, such as areas exposed to natural disasters, communities subject to major flooding, and cities in underdeveloped countries with high population densities (Harris, et al., 2012). The cholera bacterium, *Vibrio cholerae*, can also persist in the environment in fresh or saltwater, often growing on zooplankton or shellfish. In 1991, shellfish brought from Latin America to the United States was determined to be the cause of five independent cholera cases (WHO, 2002). It has been shown that the environmental distribution of *Vibrio cholerae* is heightened in tropical aquatic environments (WHO, 2002).

Cholera can be diagnosed using darkfield microscopy to examine human stool for *vibrio*-shaped (curved or comma-shaped rod) cells with a darting movement (Harris, et al., 2012). The necessary incubation period for *Vibrio cholerae* is very short, from two hours to five days (WHO, Cholera Facts Sheet, 2014). This brief incubation time of the bacterium enhances the virulence of the disease. A definitive diagnosis for cholera can be achieved through culture-based methods of a stool specimen using thiosulfate-citrate-bile salt sucrose agar plates (CDC, Diagnosis and Detection, 2015). Oral and intravenous rehydration solutions, antibiotics, and vaccinations have been developed, but present cholera control strategies have not proven effective globally (Harris, et al.,

2012). The preferred treatment for cholera is oral fluid replacement therapy (CDC, Cholera- *Vibrio Cholerae* Infection, 2014). Yet, administration and obtainment of the necessary clean, electrolyte-rich rehydration solutions is extremely difficult in developing countries, especially during periods of natural disaster.

Cholera Toxin

Cholera intoxication begins after ingestion of the Gram-negative bacterium, *Vibrio cholerae*, in a dose range of 10^6 to 10^{11} (Krauss, et al., 2003). Two strains of *Vibrio cholerae*, O1 and O139, produce cholera toxin (CT) (Finkelstein, 1996). CT is the virulence factor responsible for the life-threatening secretory diarrhea associated with the cholera disease. CT is produced by the bacterium within the infected intestine and is secreted into the intestinal lumen where it begins its trek into the host epithelial cell by binding to ganglioside GM1 recognition sites on the intestinal plasma membrane (Wernick, Chinnapen, Cho, & Lencer, 2010). The toxin then enters the cell by an endocytosis process and proceeds to travel via a Golgi retrograde transport mechanism from the plasma membrane and the trans-Golgi Network to its ultimately desired destination, the endoplasmic reticulum (ER) (Wernick, Chinnapen, Cho, & Lencer, 2010). CT is an AB₅-subunit toxin with a toxic A domain and a cell-binding pentamer B domain (Figure 1). This pentameric structure of CTB allows the toxin to bind up to five GM1 molecules at once (Wernick, Chinnapen, Cho, & Lencer, 2010). The A subunit of CT is comprised of an A2-chain (CTA2) and an enzymatically active A1-chain (CTA1).

CTA1 relies on a disulfide bond and non-covalent linkages to bridge CTA2, which non-covalently attaches to the central pore of the holotoxin B-subunit (Wernick, Chinnapen, Cho, & Lencer, 2010). CTA2 contains a KDEL amino acid sequence that acts as a target for the migration of CT to the ER (Odumosu, Nicholas, Yano, & Langridge, 2010).

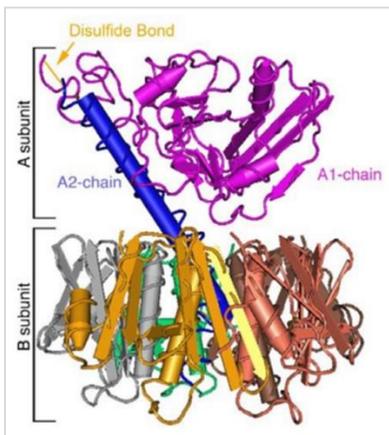


Figure 1: **Cholera Toxin Structure.** Catalytic CTA1 subunit; Linker CTA2 subunit; Cell-binding CTB₅ pentamer. (Wernick, Chinnapen, Cho, & Lencer, 2010)

The target of CT is in the cytosol of the host intestinal epithelial cells. In order for CT to cause intoxication, CTA1 needs to be displaced from the holotoxin and transferred from the ER into the cytosol where it causes the diarrheic response (Wernick, Chinnapen, Cho, & Lencer, 2010). Both the disulfide bond and the non-covalent linkages between the A1 and A2 subunits must be disrupted in order for this displacement to occur (Taylor, et al., 2014). Once CT is exposed to the resident redox state of the ER, the disulfide bond between CTA1 and CTA2 is reduced (Majoul, Ferrari,

& Soling, 1997). However, reduction of the disulfide bond between CTA1 and CTA2 within the redox environment of the ER is not sufficient to cause dissociation of the A1 subunit (Mekalanos, Collier, & Romig, 1979). Even after the disulfide bond is reduced in the ER, CTA1 and CTA2 remain folded together due to the remaining extensive non-covalent bonding network between them. Reduced CTA1 must be physically displaced from the holotoxin by an interaction with a resident ER protein, PDI (Taylor, et al., 2014).

As a thermally unstable protein, when CTA1 is removed from the CTA2/CTB₅ complex, it spontaneously assumes a disordered conformation at 37°C (physiological temperature) (Pande, et al., 2007). This unfolded conformation allows the A1-subunit to be recognized as an ER-associated degradation (ERAD) substrate for retro-translocation into the cytosol (Pande, et al., 2007). The ERAD process acts as a quality control system to remove misfolded proteins from the ER to the cytosol for degradation by the ubiquitin-protease system (Lecker, Goldberg, & Mitch, 2006). Ubiquitin post-translational modification acts a signal for proteosomal degradation of the conjugated protein (Lecker, Goldberg, & Mitch, 2006). This occurs by recognition and binding of exposed lysine residues on the unfolded protein of interest by ubiquitin. CTA1 lacks substantial lysine residues, allowing it to escape degradation by ubiquitin processes in the cytosol (Rodighiero, Tsai, Rapoport, & Lencer, 2002) (Pande, et al., 2007) (Hazes & Read, 1997). An interaction with Hsp90 and lipid rafts allows CTA1 to regain a folded

conformation in the cytosol (Burrell, Taylor, Banerjee, Tatulian, & Teter, 2014) (Ray, Taylor, Banerjee, Tatulian, & Teter, 2012).

Once refolded, the cytosolic A1-chain modifies $G_{s\alpha}$ through ADP-ribosylation to lock the protein in the GTP-bound conformation in order to trigger the continual activation of adenylate cyclase and the rapid production of cAMP (Wernick, Chinnapen, Cho, & Lencer, 2010). The increased production of cAMP leads to cAMP binding of the inhibitory subunits of protein kinase A (PKA) (Fox, 2010). Upon binding of cAMP to PKA, the inhibitory PKA subunits are released from the catalytic PKA subunits allowing activation of the kinase (Fox, 2010). Activation of PKA then causes changes in phosphorylation states of intestinal cellular proteins, most important of which is the cystic fibrosis transmembrane regulator. Phosphorylation of this chloride channel results in the intestinal chloride secretion resulting in the hallmark massive diarrhea of cholera (Fox, 2010). This process is illustrated in Figure 2 below.

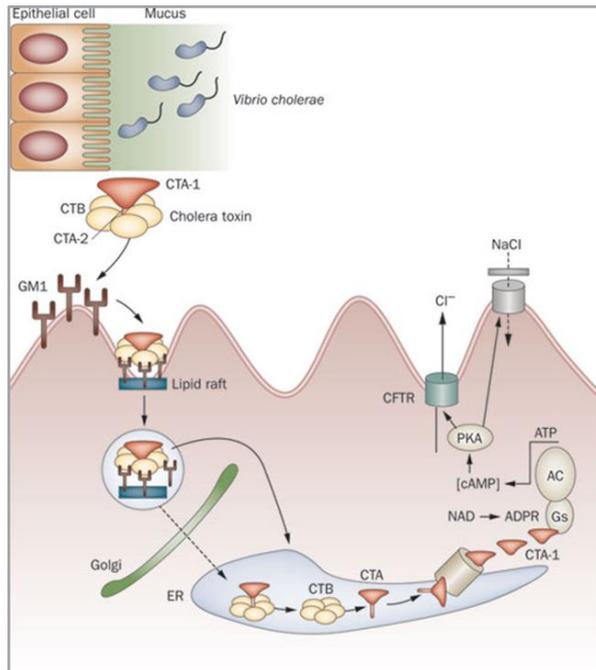


Figure 2: **Cholera Intoxication Process.** Cholera toxin, secreted by *Vibrio cholerae* in the intestinal lumen, binds to GM1 receptors on the plasma membrane of host intestinal epithelial cells. The toxin is internalized and brought to the ER through the Golgi. CTA1 dissociates in the ER and is exported from into the cytosol where the toxin target is located and toxic effects occur. (Clemens, Shin, Sur, Nair, & Holmgren, 2011)

Protein Disulfide Isomerase

The ER-localized host protein, PDI, is responsible for removing reduced CTA1 from the holotoxin. PDI is of the thioredoxin superfamily and a resident ER protein with intrinsic oxidoreductase and chaperone activity. The protein has an abb'xa'c structural orientation comprised of two catalytic domains (a & a') separated by two non-catalytic domains (b & b'), a short x-linker, and a C-terminal extension (c), as shown in Figure 3 (Appenzeller-Herzog & Ellgaard, 2008). The a and a' domains of PDI possess oxidoreductase capability.

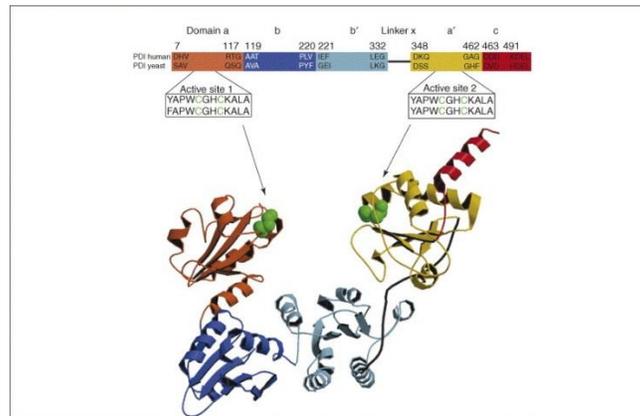


Figure 3. **PDI Structure and Domain Organization.** PDI has an abb'xa'c structural orientation comprised of two catalytic domains (a & a') separated by two non-catalytic domains (b & b'), a short x-linker, and a C-terminal extension (c). The a and a' domains are thioredoxin domains. (Gruber, Cemazar, Begona, Martin, & Craik, 2006)

The linked but independent oxidoreductase and chaperone activities of PDI allow it to facilitate proper folding of newly formed secretory proteins, prevent the aggregation of misfolded proteins, and assist in elimination of misfolded proteins through the ERAD system within the ER (Taylor, et al., 2014).

Functional Interactions Between CT and PDI

Once CT arrives within the ER, an interaction between PDI and the toxin is necessary for successful intoxication. PDI can assist in the reduction of the CTA1/CTA2 disulfide bond (Majoul, Ferrari, & Soling, 1997), but its critical role involves physical displacement of the CTA1 subunit from the rest of the holotoxin (Taylor, et al., 2014). Only after dissociation from the CTA2/CTB₅ complex, can the CTA1 subunit be exported out of the ER to its final destination within the cytosol and

begin its catalytic activity (Wernick, Chinnapen, Cho, & Lencer, 2010). CT disassembly is necessary because CTA1 instability and unfolding is only possible after its removal from the CTA2/CTB complex (Pande, et al., 2007).

The method of removal of CTA1 from CTA2 and CTB₅ begins with the reduction of the CTA1/CTA2 disulfide bond. This reduction, however, is not sufficient for holotoxin disassembly (Mekalanos, Collier, & Romig, 1979). In addition to the disulfide reduction, a crucial interaction between CT and PDI is required which begins by PDI-holotoxin binding. It has been shown that PDI in the reduced state interacts with the folded, holotoxin-associated CTA1 subunit (Taylor, Banerjee, Ray, Tatulian, & Teter, 2011) (Tsai, Rodighiero, Lencer, & Rapoport, 2001). This interaction causes a spontaneous loss of structure in PDI but not CTA1 (Taylor, Banerjee, Ray, Tatulian, & Teter, 2011). This unfolding of PDI is thought to result in an expansion of the hydrodynamic radius of the protein that acts as a type of wedge to physically displace the reduced A1 subunit from the remaining holotoxin (Taylor, et al., 2014), as shown in Figure 4.

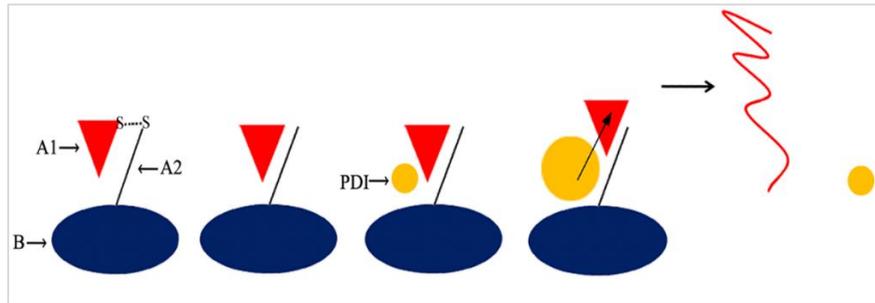


Figure 4: **The Wedge Model of PDI-mediated Toxin Disassembly.** The binding of PDI to CTA1 triggers a spontaneous unfolding of PDI resulting in the expansion of PDI's hydrodynamic radius. This expansion acts in a wedge-like fashion to displace the CTA1 subunit from the CTA2/CTB₅ complex. The release of CTA1 from the holotoxin results in the spontaneous loss of structure. PDI is then able to refold back into its original ordered state (Taylor, et al., 2014). Schematic generously provided by Patrick Cherubin.

PDI with a 400 mM EDC treatment to hold the protein in a folded conformation resulted in binding but no displacement of the A1 subunit (Taylor, et al., 2014).

The unfolding of PDI after contact with CTA1 is related to its chaperone function, reversible, and necessary for holotoxin disassembly as well as cholera intoxication (Taylor, et al., 2014). PDI deficient cell lines, as well as sole loss of PDI's chaperone function but not oxidoreductase function, result in CT resistance (Taylor, et al., 2014) (Taylor, Banerjee, Ray, Tatulian, & Teter, 2011). Upon separation from CTA2/CTB₅, CTA1 spontaneously undergoes a loss of structure and is recognized as an ERAD substrate (Massey, et al., 2009).

Once unfolding of CTA1 occurs, PDI is released from the A1-substrate (Taylor, Banerjee, Ray, Tatulian, & Teter, 2011). It was previously believed that an interaction of PDI with an ER oxidase, resulting in a change in oxidation state, allowed for the release

of its CTA1 substrate binding partner (Tsai & Rapoport, Unfolded cholera toxin is transferred to the ER membrane and released from protein disulfide isomerase upon oxidation by Ero1, 2002). However, more recent studies have shown that PDI does not bind or stay bound to unfolded CTA1, thus indicating that PDI is released at the time of CTA1 unfolding (Taylor, Banerjee, Ray, Tatulian, & Teter, 2011). As shown through techniques utilizing PDI deficient cell lines, PDI does not play a role in the translocation of free CTA1 from the ER to the cytosol (Taylor, Banerjee, Ray, Tatulian, & Teter, 2011). Thus, it seems that PDI's primary role in CT intoxication is in the displacement of CTA1. In addition, studies have shown that PDI only interacts with CTA1 and not CTA2 or CTB₅ (Taylor, Banerjee, Ray, Tatulian, & Teter, 2011). When PDI is in the presence of only CTA2/CTB, there is no binding observed.

Overall, after binding to CTA1, PDI expands its hydrodynamic radius in a way that physically pushes the A1 subunit off of the CTA2/CTB₅ complex in a wedge-like fashion (Taylor, Banerjee, Ray, Tatulian, & Teter, 2011). CTA1 is then able to spontaneously unfold and is recognized as an ERAD substrate for transport into the cytosol (Massey, et al., 2009). Once in the cytosol, CTA1 is then refolded and able to commence its catalytic activity (Wernick, Chinnapen, Cho, & Lencer, 2010). This interaction between PDI and CT within the ER is critical for successful intoxication.

INTRODUCTION

The area of cholera I have researched is the PDI-mediated dissociation of CTA1 from the CT holotoxin. Cholera intoxication happens when CTA1 reaches the cytosol, which is only possible if CTA1 is dissociated from the holotoxin and assumes an unfolded conformation in the ER (Wernick, Chinnapen, Cho, & Lencer, 2010). CTA1 must be physically displaced from the cholera holotoxin (Taylor, et al., 2014). Structural and biochemical studies have shown that PDI, an ER oxidoreductase protein, is actively involved in the CTA1 holotoxin dissociation (Taylor, et al., 2014) (Tsai, Rodighiero, Lencer, & Rapoport, 2001). Furthermore, PDI-deficient cells are completely resistant to CT (Taylor, Banerjee, Ray, Tatulian, & Teter, 2011).

Originally, PDI in the reduced form was thought to actively unfold CTA1 upon binding, causing displacement of the subunit from the rest of the toxin (Tsai, Rodighiero, Lencer, & Rapoport, 2001). It is true that PDI must be in the reduced form in order to recognize the A1-subunit (Taylor, Banerjee, Ray, Tatulian, & Teter, 2011), but recent biophysical analysis reveals that PDI does not actively unfold CTA1 (Taylor, Banerjee, Ray, Tatulian, & Teter, 2011). Rather, recent studies in the Teter lab have found that PDI itself unfolds when bound to the holotoxin associated CTA1 subunit, thus dislodging the subunit from the holotoxin (Taylor, Banerjee, Ray, Tatulian, & Teter, 2011). In addition, PDI's interaction with CTA1 is able to remove the subunit while CTA1 is held in a folded state, indicating that the displacement of CTA1 from the

holotoxin does not involve its active unfolding by PDI (Taylor, Banerjee, Ray, Tatulian, & Teter, 2011). Considering this new observation, it is possible that PDI unfolding upon contact with CTA1 acts as a type of lever to dislodge CTA1 from the holotoxin with the expansion of its hydrodynamic radius, providing a molecular explanation for CT disassembly. In turn, the dissociated A1 subunit unfolds and is recognized as an ERAD substrate for delivery into the cytosol of the host cell.

The exact domain(s) of PDI that binds to CTA1 and precisely how this protein interaction is involved in CTA1 holotoxin dissociation is unknown. The research in this project attempts to address the following outstanding questions: Which domain(s) of PDI assists in the reduction of the CTA1/CTA2 disulfide bond? Which domain(s) binds to CTA1? Which domain(s) of PDI is responsible for dislodging CTA1 from the CT holotoxin, and does this domain unfold? It is possible that the binding domain(s) alone may not be solely responsible for the displacement of CTA1 from the holotoxin. Hence, it is important to determine which domain(s) binds to CTA1, as well as which domain(s) is necessary to displace CTA1.

This project utilizes SDS-PAGE gel electrophoresis, surface plasmon resonance, Fourier transform infrared (FTIR) spectroscopic analysis, and enzyme-linked immunosorbent assay (ELISA) techniques to explore the PDI-mediated disassembly of the cholera holotoxin. Through the results of these assays, we have made strides toward the elucidation of answers to these unresolved questions.

MATERIALS AND METHODS

Materials

CT holotoxin and anti-CTB₅ were purchased from Calbiochem (La Jolla, CA). Anti-PDI was purchased from Enzo Life Sciences (Farmingdale, NY). GSH was purchased from Fischer BioReagents (Fair Lawn, NJ). CTA, PDI, and ganglioside GM1 were purchased from Sigma-Aldrich (St. Louis, MO). Clear, untreated, non-sterile ELISA plates were purchased from MidSci (St. Louis, MO). The anti-CTA1 monoclonal antibody 35C2 (Holmes & Twiddy, 1983) used in this project was a gift from Dr. Randall K. Holmes (University of Colorado School of Medicine). A pOLR130 plasmid encoding N-terminal His₆-tagged, full-length, human PDI was kindly provided by Dr. Lloyd Ruddock (University of Oulu, Finland). The *Escherichia coli* BL21 DE3 strains transformed with DNA plasmid encoding the panel of truncated, N-terminal His₆-tagged PDI deletion constructs used for construct purification in this project were generously produced and provided by Dr. Lucia Cilenti.

PDI Construct Purification

Bacterial recombinant protein expression and Talon affinity chromatography techniques were used to purify full-length PDI and a panel of truncated, His-tagged PDI deletion constructs. *Escherichia coli* strain BL21 DE3 transformed with DNA plasmid encoding a panel of truncated, N-terminal His₆-tagged PDI deletion constructs were inoculated into 10-20 ml of LB broth with kanamycin (40 µg/ml) or ampicillin (50

µg/ml). The cultures were grown overnight at 37°C with shaking. The cultures were then expanded in a 1:100 dilution into 1 liter of LB broth with kanamycin or ampicillin. The cultures are allowed to grow at 37°C with shaking until the O.D.₆₀₀ reached 0.8-1.0. The cultures are subsequently induced with 2 mM IPTG for 4-6 hours. The cells were pelleted at 6,000 rpm and 4°C for 10 minutes. The supernatant was removed and the cells were suspended in 5 ml of extraction buffer containing 1X PBS, 300 mM NaCl, 0.1% Triton X-100, 0.1 µl/ml DNase, 100 µg/ml lysozyme, and 10 µl/ml His-PIC (Sigma Aldrich). The cells were lysed with three 15 minute freeze/thaw cycles at -80°C and 37°C. PDI is a soluble protein, so the supernatant is collected and syringe filtered to eliminate any residual cellular debris.

Preparation of TALON beads were prepared as directed by the manufacturer (Clontech, Mountain View, CA). After the TALON beads were equilibrated with extraction buffer, the collected lysate was added to the bead resin and rotated overnight at 4°C. The resin was centrifuged at 1,500 rpm for 5 minutes and the supernatant was removed. The pellet was resuspended in 10 ml of wash buffer containing extraction buffer with a final concentration of 600 mM NaCl and 0.1% Triton X-100. The resin was agitated at room temperature for 15 minutes and then centrifuged at 700 g for 5 minutes. The wash step was repeated 2 more times with wash buffer. 1 mL of extraction buffer was then added to the washed resin, and the pellet was resuspended. The resin was transferred to a 2 ml gravity flow column and allowed to settle. The

wash buffer was allowed to flow through the resin. 2 ml of extraction buffer containing increasing concentration of imidazole (10, 15, 20, 25, 35, 40 and 50 mM) was used to elute the purified His₆-PDI from the TALON resin. 0.5 ml fractions were collected during each elution and stored at -20°C until needed. SDS-PAGE using 15% polyacrylamide gels and Coomassie Brilliant Blue stain were used to visualize 2 µg samples the His₆-PDI and verify the purity of the samples.

Reduction Assay

To determine the reduction capability of the disulfide bond between CTA1 and CTA2 by the a and a' oxidoreductase domains of PDI, 10 µg of the purified full-length PDI, the abb'x PDI construct, and the bb'xa'c PDI constructs were pre-reduced with 1 mM GSH and PBS in a 20 µl reaction for 30 minutes at room temperature (25°C). 4 µl of each sample was then incubated with 2 µg of disulfide-linked CTA1/CTA2 and PBS in a 20 µl reaction for 1 hour in a 37°C water bath, yielding a final concentration of 2 µg PDI and 250 nM GSH per sample. 250 nM GSH without PDI added to 2 µg CTA1/CTA2 was included in this step as a negative control. Non-reducing sample buffer, lacking β-mercaptoethanol, was then added to the samples, boiled for 5 minutes, and run on a 15% polyacrylamide gel. Coomassie staining was used to visualize the results.

Surface Plasmon Resonance (SPR)

SPR was used to monitor the real-time PDI-mediated disassembly of the cholera holotoxin. The SPR technique utilizes a gold-plated sensor slide that is coated in a protein sample, a flow channel where an additional protein sample in liquid is continuously flowed over the sensor slide, a polarized light source directed toward the sensor slide, and a detector to analyze the changes in the refractive index of the light beam indicating protein-protein interactions. A change in refractive index unit (RIU) is a reflection of change in binding and interaction between the two proteins of interest. Binding is seen as a rise in RIU, indicating an increase of mass bound to the sensor plate. A drop in RIU indicates a loss of mass on the sensor slide, and a drop in RIU below the initial baseline reading can be interpreted as loss of mass of the initial protein coated on the sensor plate. This technique was used with cholera holotoxin-coated sensor slides, and protein disulfide isomerase as the binding protein in the flow channel.

Interactions between the CT holotoxin and the purified PDI constructs were examined with a Reichert (Depew, NY) SR7000 SPR Refractometer. A gold plated SPR sensor slide was coated with ganglioside GM1 and CT holotoxin as previously described in (Massey, et al., 2009). PBST was then perfused over the sensor slide for 10 minutes at 37°C to generate a baseline measurement corresponding to the mass of the bound CT holotoxin on the plate. Each PDI construct was then individually perfused

over subsequent holotoxin coated sensor plates with a final concentration of 0.1 $\mu\text{g}/\mu\text{l}$ in a PBST solution containing 1 mM GSH. PDI samples and 1 mM GSH were allowed to incubate for 10 minutes at room temperature before each experiment. After removal of PDI from the perfusion buffer, antibodies were perfused over the plate in PBST consecutively at the following dilutions to detect the presence or absence of PDI, CTA1, and the CTA2/CTB₅ complex: anti-PDI antibody, 1:200; anti-CTA1 monoclonal antibody, 1:200; and anti-CTB antibody, 1:500. All steps were performed at 37°C with a perfusion flow rate of 41 $\mu\text{l}/\text{minute}$. Control experiments were performed to show that holotoxin disassembly did not occur in the presence of 1 mM GSH without PDI or with PDI in the absence of GSH. All RIU values are relative in these experiments.

Fourier Transform Infrared Spectroscopic (FTIR) Analysis

FTIR is a technique used to analyze protein secondary structure by measuring the wavelength and intensity of infrared (IR) radiation absorption of proteins (Kong & Yu, 2007). FTIR spectroscopy was used to monitor the structural confirmation of PDI in the presence of CTA1. FTIR allows for the determination of two protein structures when in the presence of each other by ¹³C-labeling of one of the proteins. This ¹³C label does not affect protein structure but does allow for the distinction of the individual protein structures by a spectral downshift of only the labeled protein. This downshift allows for both of the protein structures to be resolved in the same spectra. Of the produced spectra, the amide I region (1700-1600 cm^{-1}) absorptions, where peptide bond

carbonyl stretch vibrations are detected, is the most indicative of protein secondary structure (Kong & Yu, 2007).

Protein samples for FTIR measurements were prepared in a D₂O-based buffer containing 10 mM sodium borate pH 7.0. The samples contained either unlabeled PDI, or both unlabeled PDI and uniformly ¹³C-labeled CTA1 at a 1:1 molar ratio. Each sample contained 1 mM GSH. FTIR experiments were performed using a Jasco 4200 FTIR spectrometer at 0.964 cm⁻¹ spectral resolution and a set resolution of 1 cm⁻¹. The absorbance spectra were calculated using the respective buffers as reference and were corrected by subtraction of water vapor contribution, smoothing, and baseline correction in the amide I region. Curve fitting was conducted using the component frequencies from the second derivative spectra, as described in (Tatulian, Attenuated total reflection Fourier transform infrared spectroscopy: a method of choice for studying membrane proteins and lipids, 2003). No appreciable signals from the second derivative spectra were detected in the region above 1700 cm⁻¹.

Enzyme-Linked Immunosorbent Assay (ELISA)

96-well ELISA plates were coated in ganglioside GM1 as described by an ELISA plate GM1 coating procedure (Bech, Jakobsen, & Orntoft, 1994). The coated plates can be stored at 4°C in 0.05% PBST. The plates are then washed three times with PBST, blocked with 1% BSA in PBS for one hour at room temperature, and washed again three times. CT is added to each well at a concentration of 5 ng/μl in PBST for one hour at

37°C, and then washed three times. PDI is added to each well at a concentration of 2.5 ng/μl with 1 mM GSH in PBST for one hour at room temperature or 37°C. The plate is then washed three times. CTA1 monoclonal primary antibody is added at a 1:50 dilution in a 0.1% BSA-PBS solution for one hour at 37°C. The plate is washed, and HRP-conjugated mouse IgG secondary antibody is added at a 1:1,000 dilution in a 0.1% BSA-PBS solution for one hour at 37°C. After an additional wash cycle, TMB at room temperature is added to the plate and the reaction is allowed to incubate for 10 minutes at room temperature. A 2N H₂SO₄-PBS stop solution is then added, and the optical density results are read at 450 nm with a Tecan Microplate Reader.

This process is summarized in Figure 5. The three possible results we expected from this ELISA were: a) the PDI deletion construct would bind and displace CTA1; b) the PDI deletion construct would be able to bind, but not displace CTA1; c) the deletion construct would not be capable of binding or displacing CTA1 (Figure 5).

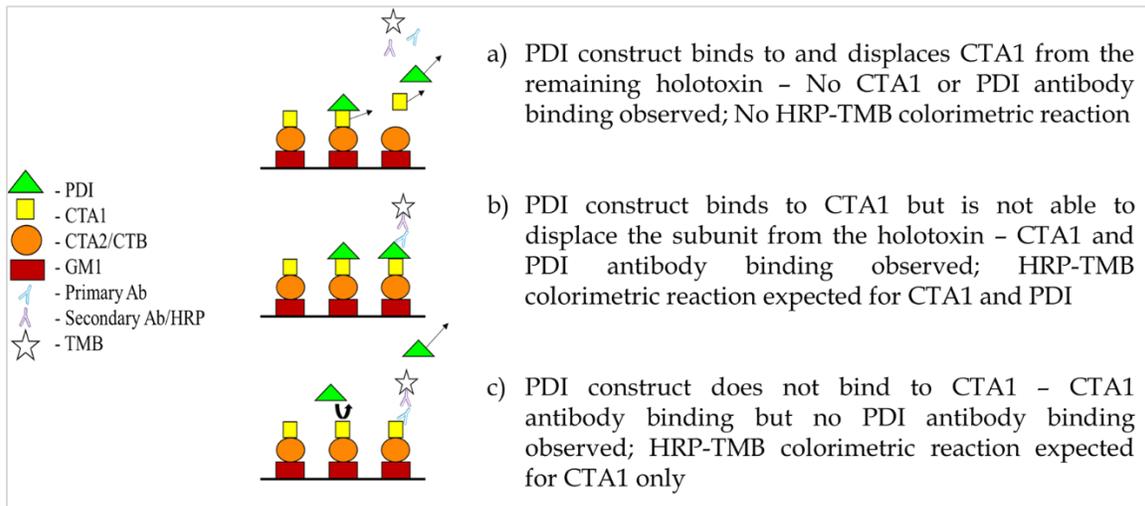


Figure 5: ELISA Method and Possible Results Schematic.

Each condition was performed with 8 replicate samples, and controls were run to show that there is no holotoxin dissociation with 1 mM GSH alone or PDI in the absence of GSH.

RESULTS

Full-length PDI and PDI Construct Purification

Full-length PDI and PDI constructs were purified using the TALON affinity chromatography purification procedure as explained in the methods section. A schematic for the domains of PDI and the deletion constructs can be seen in Figure 5, corresponding to the purification results in Figure 6. SDS-PAGE electrophoresis and Coomassie Brilliant Blue stain were used to verify the purity of the PDI constructs (Figure 6). These constructs were used for the experiments of this project.

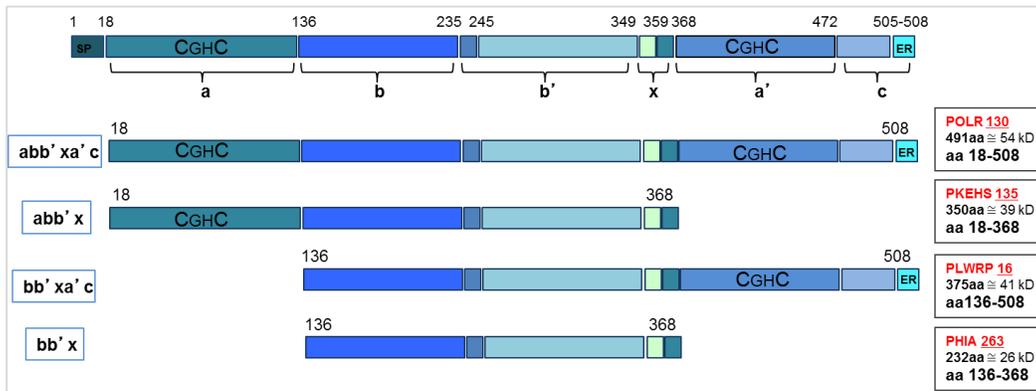


Figure 6: **Schematic of PDI and PDI Deletion Construct Domains.** Domains with oxidoreductase capability are indicated with a "CGHC".

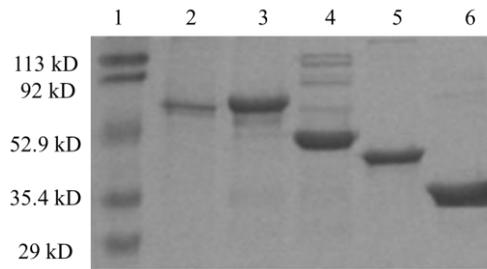


Figure 7: **Purification of Full-length PDI and PDI Deletion Constructs.** Lane 1: Molecular weight markers; Lane 2: Commercially bought PDI; Lane 3: Full-length purified PDI; Lane 4: PDI domain abb'x; Lane 5: PDI domain bb'xa'c; Lane 6: PDI domain bb'x.

PDI a and a' Domains are Able to Assist in the Reduction of the CTA1/CTA2 Disulfide Bond

Before displacement of CTA1 from the CT holotoxin in the ER, the disulfide bond linking CTA1 and CTA2 must be reduced. PDI has two domains that harbor oxidoreductase capability, the a and a' domains. A reduction assay, as described in the methods section, was used to determine if either or both of these domains are able to assist in reduction of the CTA1/CTA2 disulfide bond. Results from this experiment utilizing PDI deletion constructs lacking either the a or a' domains are shown in Figure 7. These results indicate that both pre-reduced a and a' domains are able to take part in this reduction process. A shift in CTA from a higher molecular weight to a lower molecular weight indicates reduction of the disulfide bond. After reduction, addition of non-reducing sample buffer, and boiling, CTA1 (molecular weight: ~25 kDa) can be seen as the band shifts from the 30 kDa CTA1/CTA2 heterodimer to the 25 kDa CTA1 subunit. CTA2 (molecular weight: ~5 kDa) is not visible on the gel. Controls of CTA

alone (lane 2), CTA with GSH only (lane 3), and CTA with pre-reduced full-length PDI (lane 4) are used as negative and positive controls.

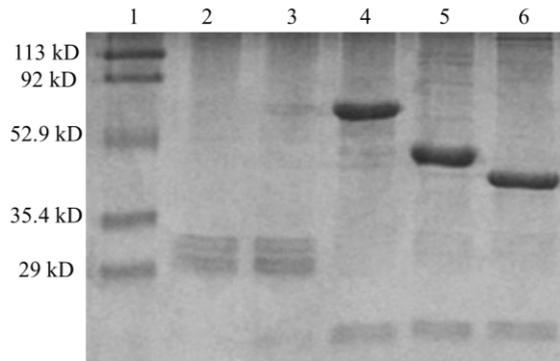


Figure 8: **Reduction of CTA by PDI.** Non-reducing SDS-PAGE and Coomassie staining was used to visualize the reduction of 2) CTA by: 3) 250 ng GSH, 4) Full-length PDI, 5) abb'x PDI construct, and 6) bb'xa'c PDI construct. All constructs were pre-reduced with 250 ng GSH.

The PDI a Domain is Required for CTA1 Disassembly, and the bb'x-Domains are Sufficient for CT Binding.

In order for holotoxin disassembly to occur in the ER, CTA1 must be bound and displaced by PDI. To test which domains are required for binding and disassembly of the CT holotoxin, SPR utilizing the PDI deletion constructs was used to observe the real-time PDI-mediated disassembly of CT. The PDI deletion constructs with 1 mM GSH were perfused over a SPR sensor slide coated with CT. Binding of PDI to the holotoxin is seen as a rise in RIU, indicating an increase in mass bound to the sensor slide. A subsequent drop in RIU below that of the initial baseline measurement (corresponding to mass of the initial bound holotoxin), can be interpreted as a loss of

both PDI and CTA1 from the sensor slide. An increase in RIU after addition of PDI, but no drop in RIU can be interpreted as binding of PDI without displacement. The presence or absence of PDI, CTA1, and the CTA2/CTB₅ complex can be determined with an increase in RIU after the addition of corresponding antibodies.

A positive control experiment was run with full-length PDI with 1 mM GSH perfused over the CT coated slide. A dramatic rise in RIU and then subsequent drop in signal below the baseline value was seen after the addition of full-length PDI (Figure 8A). Addition of anti-PDI, anti-CTA1 monoclonal, and anti-CTB₅ antibodies confirmed holotoxin disassembly by the absence of PDI and CTA1, and the presence of CTB₅. When the abb'x deletion construct in the presence of 1 mM GSH was perfused over the CT-coated sensor slide, a change in RIU signal was detected similar to that of the full-length positive control (Figure 8B). A rapid rise in RIU was followed by a drop in RIU below the baseline after PDI perfusion. This was again followed by an absence of change in signal with the addition of anti-PDI and anti-CTA1 antibodies, but a rise in RIU after the addition of anti-CTB₅ antibodies. These results indicate that the abb'x-domain combination can bind and displace the CTA1 subunit. A repeat of this assay using the bb'xa'c deletion construct shows binding, but no CTA1 disassembly (Figure 8C). The presence of PDI, CTA1, and CTB₅ are all indicated by a rise in RIU after addition of anti-PDI, anti-CTA1, and anti-CTB₅ antibodies. Perfusion of the bb'x deletion construct in the presence of 1 mM GSH produced results similar to that of the

bb'xa'c construct (Figure 8D). Binding was observed but no displacement. Again, PDI, CTA1, and CTB were all confirmed to be present on the plate by a rise in RIU after subsequent addition of anti-PDI, CTA1, and CTB₅ antibodies.

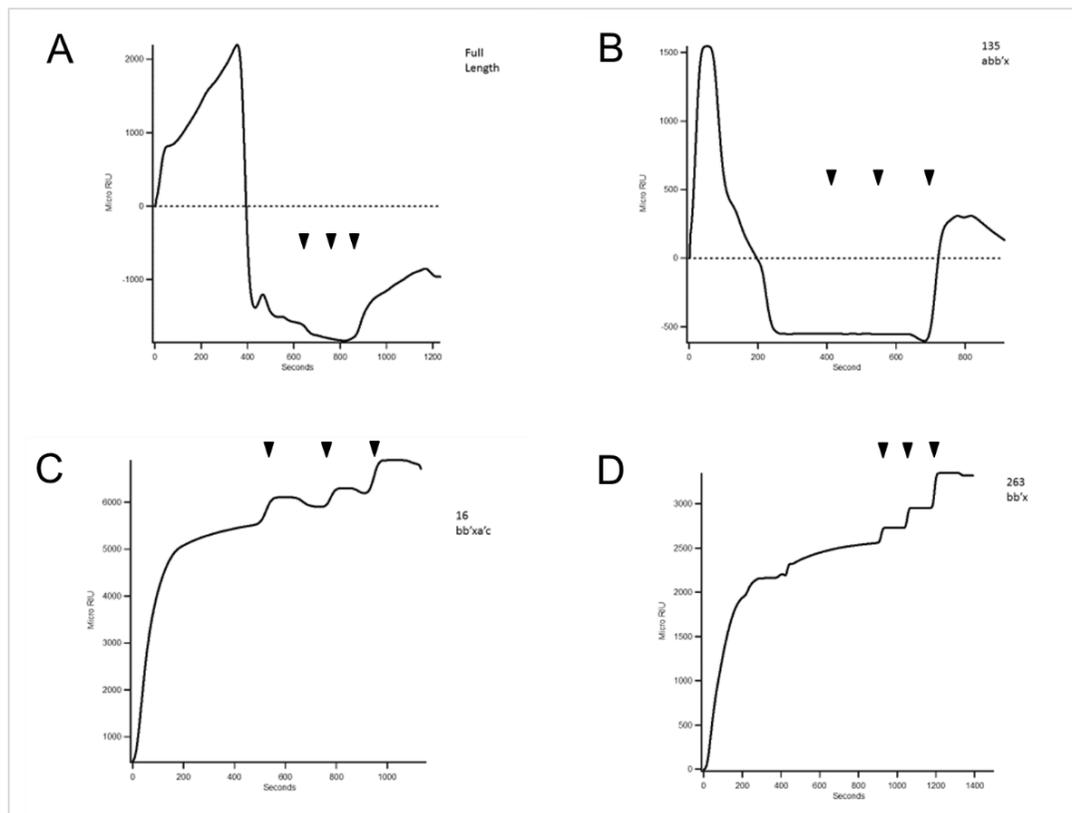


Figure 9: Holotoxin Binding and Disassembly. Real-time holotoxin binding and disassembly by PDI was observed by SPR. Each perfusion buffer contained 1mM GSH. **A)** Full length, **B)** abb'x, **C)** bb'xa'c, and **D)** bb'x PDI constructs were flowed over a CT coated sensor. Anti-PDI, anti-CTA1 monoclonal, and anti-CTB₅ antibodies are added to the perfusion buffer at time points corresponding to the arrowheads. **B** and **C** show that the a domain is required for holotoxin disassembly, and **D** shows that the bb'x domains are sufficient for binding.

A comparison of the abb'x, bb'xa'c, and bb'x deletion construct results can be interpreted as the requirement of the a domain for disassembly and the sufficiency of the bb'x domains for binding of the CT holotoxin. Negative control experiments were

performed to show that holotoxin disassembly did not occur in the presence of 1 mM GSH alone or with PDI in the absence of GSH.

CTA1-PDI Interaction Results in PDI Loss of Structure

To explore the observation that PDI unfolding corresponds to holotoxin disassembly, FTIR spectroscopy was used to monitor the structural confirmation of PDI in the presence of CTA1. FTIR allows for the determination of two protein structures when in the presence of each other by ^{13}C -labeling of one of the proteins. This ^{13}C label does not affect protein structure but does allow for the distinction of the individual protein structures by a spectral downshift of only the labeled protein. This downshift allows for both of the protein structures to be resolved in the same spectra.

All FTIR experiments were run at 10°C, in order to perform the experiments with CTA1 in the folded conformation, and in the presence of 1 mM GSH, unless otherwise stated. Results show that in the absence of CTA1, PDI in the folded confirmation harbored a 33% α -helical, 45% β -sheet, and 19% irregular structure (Figure 9A). These percentages were consistent with the secondary structural content predicted from the crystal structure of PDI (Tian, Xiang, Novia, Lennarz, & Schindelin, 2006) and from previously published FTIR traces for PDI alone with GSH, as well as for PDI with GSH in the presence or absence of CTA1 (Taylor, et al., 2014). Once added to CTA1, the percentage of α -helical and β -sheet content of PDI was reduced considerably to 18% α -helix and 31% β -sheet. This loss of defined structure was replaced by a rise in irregular

structure to 45% (Figure 9B). This shift of PDI from an ordered to a disordered conformation demonstrates an unfolding of PDI after interaction with CTA1. There was no significant difference between the structure of PDI in the presence and absence of GSH alone, and a loss of structure was not observed with PDI in the presence of CTA1 without GSH (data not shown).

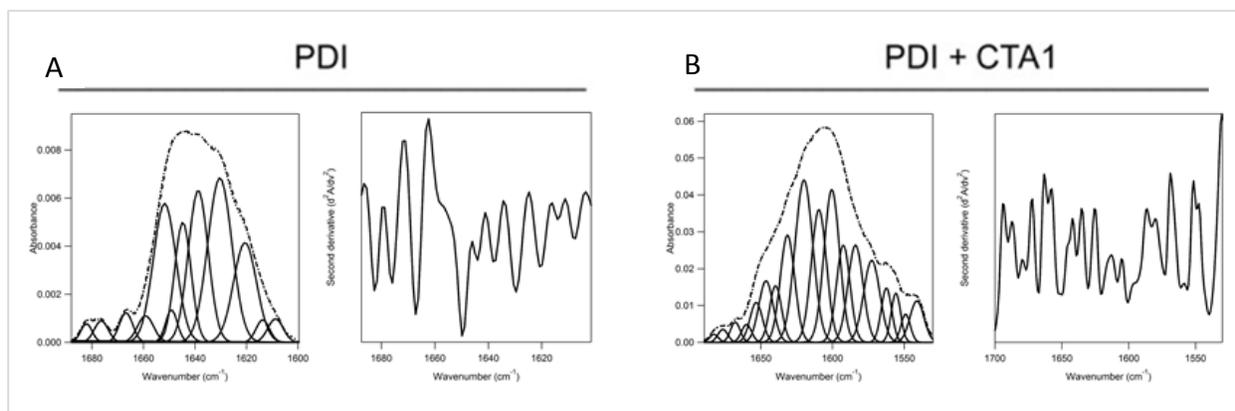


Figure 10: **Effect of PDI-CTA1 Interaction on PDI Structure.** FTIR was used to visualize the changes in structure of PDI when in the absence (A), and presence of CTA1 (B). All experiments performed at 10°C and under reducing conditions with 1 mM GSH.

An ELISA Assay was Generated to Monitor Holotoxin Disassembly

An ELISA assay, as described in the methods section, was produced to monitor PDI-mediated CT holotoxin disassembly. A clear, untreated, non-sterile 96-well plate was coated in GM1 and holotoxin. These CT coated plates are able to be stored in PBST at 4°C. Results are gathered after a colorimetric reaction between the HRP, conjugated

to a secondary antibody, and TMB followed by the addition of a 2N H₂SO₄-PBS stop solution. The presence or absence of proteins of interest are indicated by the intensity of the color from the reaction. The color intensity is proportional to the concentration of the protein of interest present on the plate, as determined by analysis of a microplate reader. Results produced from the addition of purified full-length PDI over a time course of 1-60 minutes is shown in Figure 10A. This assay was run at 37°C with 1 mM GSH. Holotoxin disassembly appears to be time-dependent in this assay, with an increasing loss of CTA1 from the plate with additional incubation time. Results from the addition of PDI in the presence of 30 mM GSH for 1 hour at 25°C (Figure 10B) and at 37°C (Figure 10C) are also shown below. These results show a higher yield of CTA1 dissociation at 37°C for this assay.

Presence or absence of CTA1 on the plate was determined by analysis of the intensity of secondary CTA1 HRP-conjugated antibody colorimetric reactions with TMB. Positive controls of CT with GSH only, and negative controls of wells with no holotoxin were run in all three assays. This protocol is being optimized further for use with the purified PDI deletion constructs.

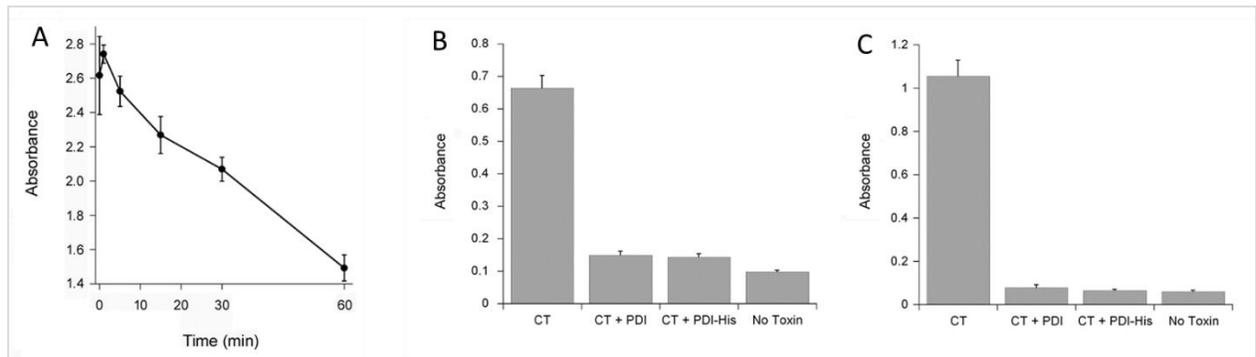


Figure 11: PDI-Mediated Holotoxin Disassembly via ELISA. An ELISA assay was produced to monitor the PDI-mediated CT holotoxin disassembly. GM1 and CT-coated plates were used for an ELISA using a 25 ng/ μ l commercially bought PDI with an incubation time course of 0, 1, 5, 15, 30, and 60 minutes at 37°C (A), and an ELISA using 2.5 ng/ μ l full-length PDI from a commercial vendor (CT + PDI) and purified, His-tagged PDI (CT + PDI-His) with a 1 hour incubation time at 25° (B) or 37° (C), each reduced with 30mM GSH. CT without the addition of PDI was used as a positive control, and wells with no toxin were used as a negative control for each of these experiments. A 1:1,000 dilution of an anti-CTA1 monoclonal antibody was used, and each condition involved 8 replicate samples.

DISCUSSION

In order for a successful intoxication process to occur, CT must travel from the cell surface to the ER. Within the ER, the disulfide bond linking CTA1 and CTA2 must be reduced, and the reduced CTA1 subunit must be displaced from the CTA2/CTB₅ complex by an interaction with PDI. This interaction is described as an expansion in the hydrodynamic radius of PDI, allowing the protein to physically displace CTA1 in a wedge-like fashion (Taylor, Banerjee, Ray, Tatulian, & Teter, 2011). In this work, the domains of PDI that may assist in the reduction of the CTA1/CTA2 disulfide bond, as well as the domains of PDI that are necessary to bind and displace CTA1 during this critical interaction, have been explored.

Using a non-reducing SDS-PAGE, we were able to determine that both the a and the a' domains of PDI may assist in the reduction of the disulfide bond linking CTA1 and CTA2. These two domains harbor oxidoreductase capability and both were shown to successfully reduce the CTA1/CTA2 complex after a 30 minute pre-reduction with 1 mM GSH (Figure 7). The minimal GSH concentration that was still present after the PDI pre-reduction was controlled for with a sample of CTA and GSH only. This minimal amount of GSH was not sufficient to reduce the disulfide bond. The ability of either thioredoxin-like domain to reduce the CTA1/CTA2 disulfide bond suggests both domains are in close proximity to the CTA1/CTA2 disulfide bond when PDI binds to CT.

SPR was used to monitor the real-time disassembly of the CT holotoxin by purified full-length PDI and a panel of various PDI deletion constructs. The results generated by these experiments indicate that the a domain is required for CTA1 displacement, and the bb'x domains are sufficient for holotoxin binding. Our results lead us to the very interesting conclusion that the binding domains alone are not solely responsible for holotoxin disassembly. Holotoxin disassembly was possible only when the a domain of PDI was present in the deletion constructs. In addition, binding was observed by all constructs, leading us to believe that a combination of the b, b', and x domains is responsible for toxin binding.

FTIR spectroscopy was used to show a shift from an ordered to a disordered conformation of full-length PDI after binding to CTA1. It has been previously believed that PDI actively unfolds CTA1, resulting in the dissociation of the subunit from the holotoxin (Tsai, Rodighiero, Lencer, & Rapoport, 2001). In contrast, more recent studies have shown that rather than CTA1 losing structure after PDI interaction, PDI itself acquires an unfolded structural confirmation when bound to CTA1 (Taylor, Banerjee, Ray, Tatulian, & Teter, 2011) (Taylor, et al., 2014). These results were confirmed by the data generated from the FTIR experiment of this project. A significant loss of structure in PDI was observed when in the presence of CTA1, as compared to when CTA1 is absent.

An ELISA protocol has been developed that incorporates a simple and reproducible GM1 (Bech, Jakobsen, & Orntoft, 1994) and CT coating procedure. Results from this present ELISA assays indicate that an increased incubation time of PDI and CT results in a more efficient displacement of CTA1. Increased efficiency is also achieved by performing the PDI-CT incubation at 37°C, rather than at 25°C.

Our work has established further insight into the crucial interaction between PDI and CT within the ER during cholera intoxication. Overall, this work has shown that the a domain is required for displacement of CTA1, the bb'x domains are sufficient for holotoxin binding, and both the a and a' domains may assist in the reduction of the CTA1/CTA2 disulfide bond.

Future Directions

To further examine the results of this project, purification of deletion constructs bb', and b'x are being completed, and application of the SPR assay with these newly purified deletion constructs will be used to further investigate the binding process. Results generated from utilization of these constructs with SPR will indicate whether binding of PDI to the holotoxin requires all three of the b, b', and x domains, or a combination of the three.

In addition, the FTIR assay utilized in this project will be used in conjunction with the panel of PDI deletion constructs in order to determine if a loss of structure or

unfolding of specific PDI domains corresponds to holotoxin disassembly. These results would be used to confirm the SPR findings of this project.

Lastly, continued optimization of the ELISA assay is being explored for use with the panel of purified PDI deletion constructs to further examine the CT binding and disassembly capabilities of specific PDI domains. These results will also be used to support our findings from the SPR and FTIR experiments as well.

In the future, we may be able to use our findings on this critical interaction to design inhibitors that block PDI binding and CTA1 dissociation and the resulting toxic effects in the cytosol.

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