THE ROLE OF THE CHEMORECEPTOR ZINC-BINDING DOMAIN IN BACTERIAL SIGNAL TRANSDUCTION

BY

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Chemotaxis is the biochemical and cellular process by which bacteria sense particular molecules in their environment and adjust swimming behaviors accordingly. Chemotaxis and other motility alterations in response to endogenous HOCl (hypochlorous acid, also known as bleach) are known to be necessary behaviors by which bacteria locate nutritive niches within the human body, allowing them to survive and thrive. A wide variety of gut-colonizing bacteria, including pathogens such as *Escherichia coli* and *Salmonella. enterica* depend on the Chemoreceptor Zinc-Binding (CZB) domain to sense certain aspects of their environments and alter swimming patterns accordingly. Thus, understanding the biochemistry underlying CZB signal transduction has the potential to set the foundation for future therapeutics while also expanding our understanding of the ways in which bacteria survive the often-hostile context of the human body. While existing research treated the CZB domain as a zinc sensor, my work contends that in certain molecular and cellular contexts the domain acts as an HOCl sensor instead. My work shows that the CZB possesses a highly conserved, bleach-sensitive cysteine (1/2 oxidized at 312 μM bleach) and indicates that

oxidation of this cysteine results in subtle yet observable structural changes to the CZB domain. In the context of *H. pylori*, the CZB domain likely allows the chemoreceptor transducer-like protein D (tlpD) to swim towards sites of inflammation and injury within the stomach, thus allowing the pathogen to reach a “safe harbor” within the human stomach. This behavior illustrates the possible significance of the CZB domain in human disease: pathogens may use the domain to sense sites of inflammation, which in many cases can indicate safe and nutritive niches for these pathogens. In other contexts, CZB bleach sensing may constitute the basis for bleach chemorepulsion, where bacteria may be able to flee this generally harmful molecule. Thus, CZB bleach sensing could underly an important first step in bacterial infection and proliferation, and thus constitute a target for future therapeutic efforts.

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INTRODUCTION

Bacterial Motility

Chemotaxis is the process by which bacteria sense particular molecules in their environment and alter their swimming patterns in response. In the simplest sense, chemotaxis causes bacteria to, in the aggregate, swim towards safer, more nutritive areas, and away from dangerous ones (Adler 1966). Bacterial movement, or motility, is generally determined by the rotation of one or many flagella, or "tails," which propel the bacterium through the fluids in which they reside. Chemotaxis occurs either via maintaining or disrupting the rotation of bacterial flagella. So-called "chemoattractants" encourage undiminished rotation of the flagellum, which keeps the bacteria swimming along a smooth trajectory. Conversely, "chemorepellents," often harmful molecules or molecules indicative of a harmful environment, stimulate biochemical changes within the cell that cause the flagella to rotate in the opposite direction. This has the effect of causing the bacteria to tumble through its environment and choose a new random orientation (fig. 1).

After some time, normal flagellum rotation resumes and the bacteria continues to swim smoothly once more, though the direction in which the bacteria sets off is not necessarily directly away from the chemorepellent. While bacteria randomly "choose" the direction in

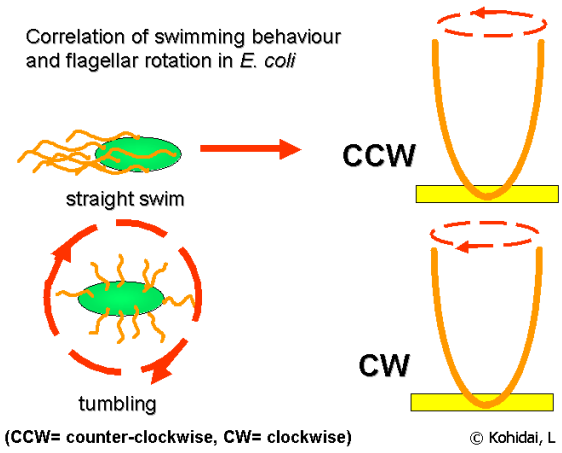


Fig 1. Relationship between bacterial flagella rotation and bacterial swimming pattern. Uninterrupted ccw rotation causes smooth swimming, whereas a sudden reversal to cw spinning causes tumbling and directional reset. Straight swimming is associated with chemoattraction, and tumbling with chemorepulsion.

which it sets off followed chemorepellent-mediated tumbling, in the aggregate, the effect of this smooth-swimming vs. tumbling choice is to direct bacteria away from sources of chemorepellents, and towards sources of chemoattractants (Adler 1975).

Bacterial motility, however, is more sophisticated than merely deciding to swim towards nutrients and away from harmful molecules. Many bacteria can exist in two states: either as free-swimming “planktonic” cells, or as stationary “sessile” residents of a biofilm, or extended conglomeration of bacteria held together by an adhesive matrix of sugars, proteins, lipids, and DNA. Biofilm formation is well-known to increase the tolerance of the bacteria to common disinfectants via a variety of mechanisms (reducing contact with soluble disinfectants, repeated sub-lethal exposures to the disinfectant resulting in biochemical adaptation, differences in metabolism caused by being in a biofilm, etc) (Bridier 2001). As with chemotaxis, the decision to be free-swimming or in a biofilm occurs as a response to the bacteria’s environment: a free-swimming bacteria may be able to flee dangerous zones or better acquire soluble nutrients, but may lack the protection common of biofilms. Analogously to chemotaxis, bacteria make this decision by sensing molecules in their environment: certain molecules encourage the formation of biofilms, while others encourage their dispersal (O’Toole 2000). These behaviors are often critical in the development of bacterial pathogenesis. In the case of *H. pylori*, chemotaxis is necessary for bacteria to locate nutritive niches within the human body, such that mutant strains incapable of chemotaxis cannot survive within mouse hosts (Aihara 2014). Furthermore, the NIH reports that about 65% of microbial infections are associated with the formation of biofilms, generally conferring some degree of antibiotic and immune system resistance to pathogens. Biofilm formation is especially

problematic in the context of medical implants because they often necessitate invasive surgery to clean and replace fouled devices (Jamal 2018). Thus, there is a significant and recognized medical interest in understanding the biochemistry of bacterial motility.

This avenue of inquiry allows us to gain insight into the evolutionary strategies that bacteria use to survive and thrive within the human body; these bacterial behaviors are evolutionarily driven, and therefore likely represent strategies adopted to maximize survivability within their hosts. As a result, to study these interactions is to study the evolutionary playbook that bacteria have developed over the course of millennia to allow them to infect humans and thrive within them. Furthermore, in order to understand motility and sensing with enough detail to develop drugs and other therapeutics against pathogens, it is necessary to understand the biochemistry that underlies these behaviors.

Signal Transduction

The biochemical basis of both chemotaxis and biofilm formation/dispersal begins with a protein sensing the informative molecules in question and transducing the signal to other proteins involved in actuating chemotaxis/biofilm formation (fig 2). In general, proteins can be broken down into smaller building blocks known as "domains," which have discrete functions. For instance, a chemoreceptor protein might

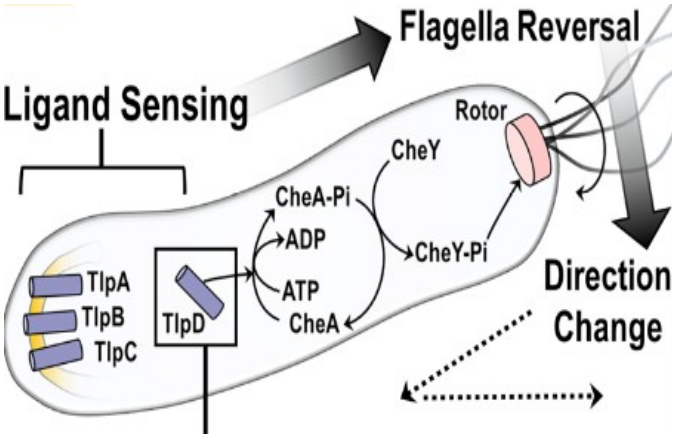


Fig 2. Example of chemosensing signal transduction in the context of *H. pylori*. Transducer-like protein D (tlpD), a protein kinase, acts to phosphorylate CheA, itself a protein kinase. Subsequently, CheA-Pi phosphorylates CheY, and CheY-Pi acts on bacterial rotors to reverse the direction in which the flagella rotate, causing tumbling. Therefore, molecules that inhibit the activity of tlpD encourage smooth swimming, while those that activate it encourage tumbling (Perkins 2019). The phosphorylation cascade indicated serves the purpose of amplifying weak signals, such that even partial activation or inhibition of tlpD can manifest as changes in swimming behavior. Figure adapted from Perkins et al. 2019

consist of two domains: one responsible for sensing and transmitting a signal to other parts of the protein, and another responsible for some enzymatic activity that constitutes transduction of the signal to subsequent members of the signaling network. By studying the means by which the sensing domain of a chemoreceptor protein senses the molecule that triggers motility changes, we can build an understanding of the crucial first step in chemotaxis and other changes in bacterial swimming behavior. Subsequently, understanding the mechanism of sensing on a molecular level can be paired with investigations into the significance of this sensing domain—we might ask ourselves how bacteria behave without this sensing domain, or with small portions of the domain altered. In the grandest scheme of things, we can begin to determine the effect that this domain has on the bacteria's ability to colonize its ideal niches, leading to crucial hints as to the ultimate evolutionary purpose of the sensing domain, and crucially, ways to inhibit the function of this sensing domain as a means of designing new classes of antibiotics.

My research is focused on understanding the function and significance of a sensing domain previously studied as a determinant of biofilm formation, the Chemoreceptor Zinc-Binding (CZB) domain. Work performed by my mentor and I clarified the role of the CZB domain: while it was previously known to regulate chemotaxis and biofilm formation, we worked to understand the domain's ligand sensing properties as well as its mechanism of signal transduction, specifically in the context of bleach sensing. Inflammation caused by tissue damage is known to attract human immune cells known as neutrophils, which use the enzyme myeloperoxidase to combine H_2O_2 and free chloride to produce concentrations of hypochlorous acid of up to

5 mM. Hypochlorous acid, or bleach, is a potent reactive-oxygen species, with great potential for damaging human tissues and killing bacterial cells. The biological purpose of producing hypochlorous acid is likely to decrease the risk of infection at susceptible regions (Winterbourn 2016). Thus, it makes sense that certain bacteria would be equipped with proteins tuned to sense bleach.

Existing Literature

Existing work has characterized the importance of the CZB-containing transducer-like protein D chemoreceptor (TlpD) in the gastric pathogen *Helicobacter pylori*. Research shows that mutant *pylori* that lack this chemoreceptor are unable to effectively colonize their host organisms, suggesting a crucial role for the CZB domain. *H. pylori* leads a precarious existence in the stomach: once ingested, its survival depends on damaging and subsequently burying itself into the stomach's lining. It particularly likes to colonize ulcers and other sites of inflammation within the stomach, which may be easier to enter and may offer easy access to host blood, which the bacterium could then use as a source of iron. Thus, ulcer location and infiltration are crucial to *pylori* because of the protection it offers from stomach acid as well as the access it affords to nutrients. In mouse models, researchers were able to observe aggregation of *pylori* at sites where the stomach wall was damaged using a laser, indicating that damaged tissue releases some sort of diffusible agent that is sensed by *pylori* and which subsequently mediates attraction (Aihara 2014). A potential candidate for this chemoattractant was hypochlorous acid, or bleach, which would be produced at sites of injury.

Nevertheless, *pylori* is known to happily reside in these sites of inflammation, indicating a certain tolerance to hypochlorous acid. Previously, my mentor and I

performed work that showed that tlpD mediates chemoattraction towards bleach in *H. pylori* (Perkins 2019), potentially explaining these swimming pattern (fig 3). We hypothesized that the CZB domain could constitute the basis for bleach-sensing in this system. This was a novel interpretation of the CZB's function. Previous work approached the CZB sensing domains in the context of diguanylate cyclases, which are a class of enzyme that produce the secondary messenger cyclic-di-Guanadine

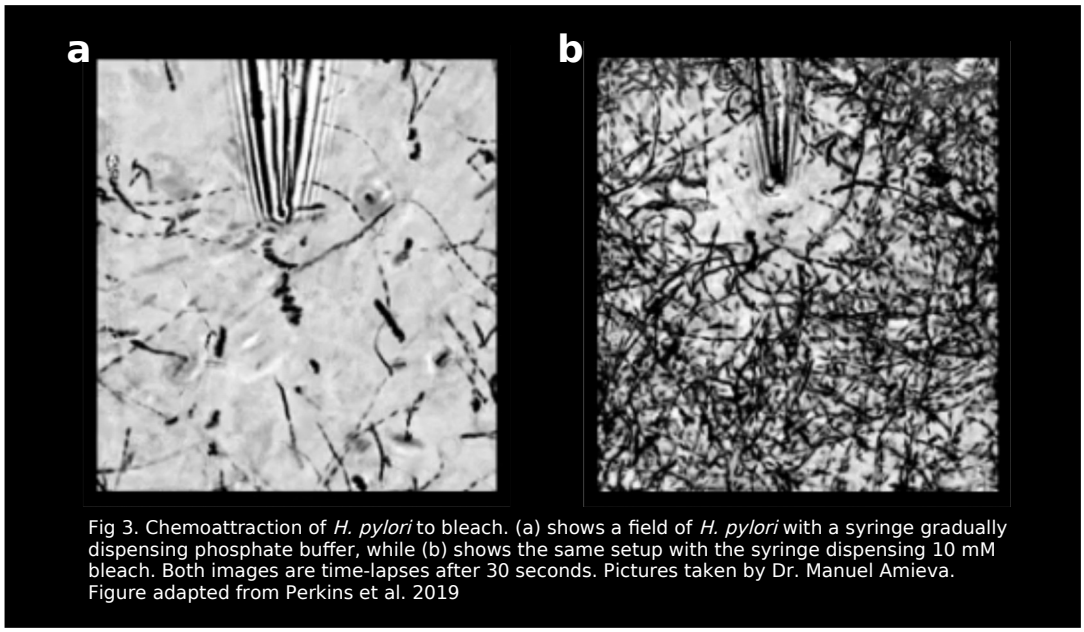


Fig 3. Chemoattraction of *H. pylori* to bleach. (a) shows a field of *H. pylori* with a syringe gradually dispensing phosphate buffer, while (b) shows the same setup with the syringe dispensing 10 mM bleach. Both images are time-lapses after 30 seconds. Pictures taken by Dr. Manuel Amieva. Figure adapted from Perkins et al. 2019

Monophosphate (c-di-GMP), which in turn regulates the dispersal of biofilms and the speeds at which bacteria move. These studies interpreted the CZB domain as a zinc sensor, rather than a hypochlorous acid sensor (Zahringer 2013). This is the context that much of my work found itself situated in: expanding our understanding of CZB domains as bleach sensors as well as zinc sensors depending on the domain's cellular contexts.

Structure, Conservation, and Hypothesis

Crystal structures of the diguanylate cyclase Z (DgcZ, a sensing protein associated with biofilm formation in *E. coli*) CZB, and of the DgcZ full-length protein with a point mutation in the diguanylate cyclase domain have been determined, but neither a full-length tlpD nor a full-length wildtype DgcZ structure have been solved to date. Nevertheless, it is possible to gain crucial structural detail from existing structures

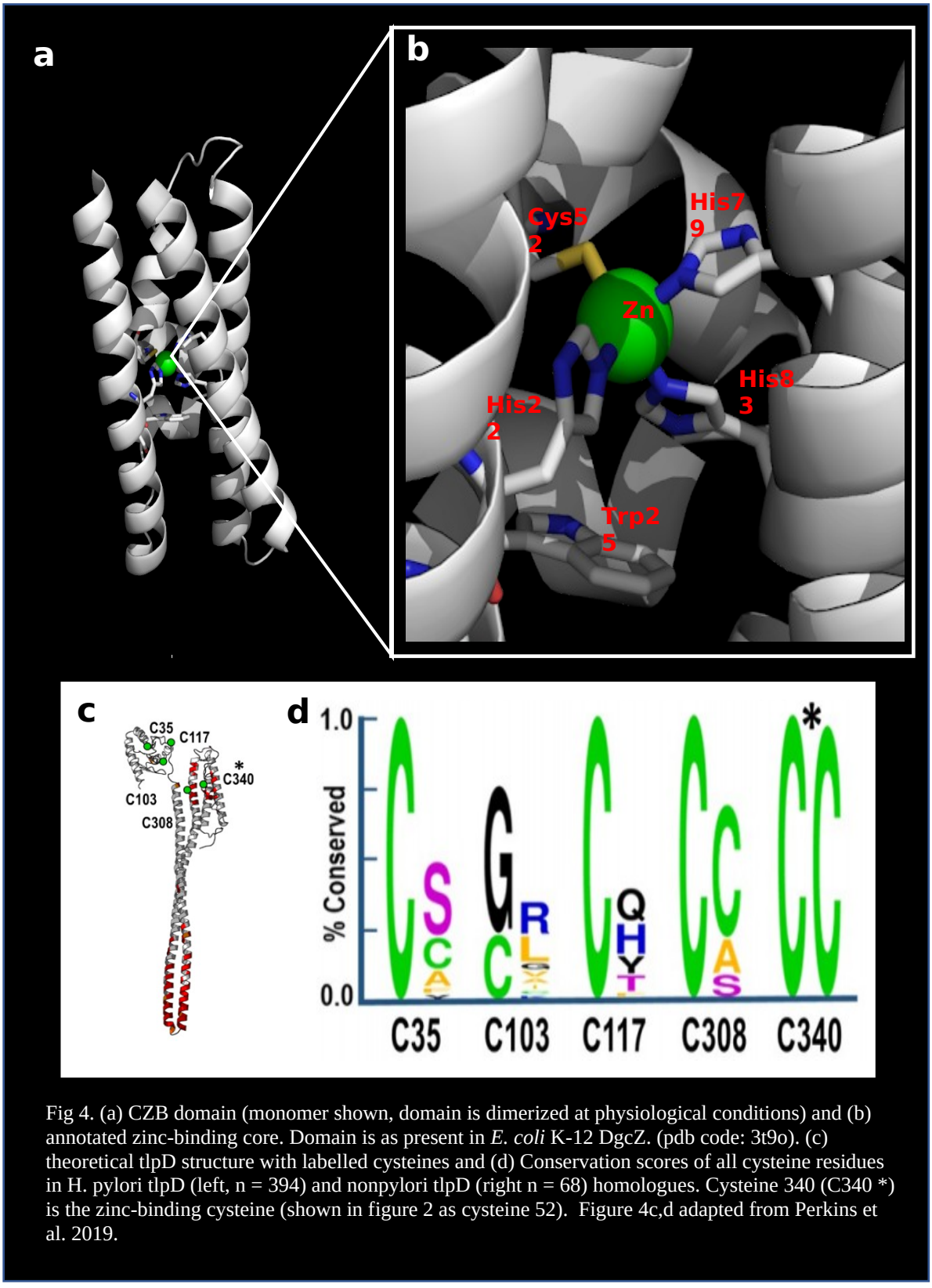


Fig 4. (a) CZB domain (monomer shown, domain is dimerized at physiological conditions) and (b) annotated zinc-binding core. Domain is as present in *E. coli* K-12 DgcZ. (pdb code: 3t9o). (c) theoretical tlpD structure with labelled cysteines and (d) Conservation scores of all cysteine residues in H. pylori tlpD (left, n = 394) and nonpylori tlpD (right n = 68) homologues. Cysteine 340 (C340 *) is the zinc-binding cysteine (shown in figure 2 as cysteine 52). Figure 4c,d adapted from Perkins et al. 2019.

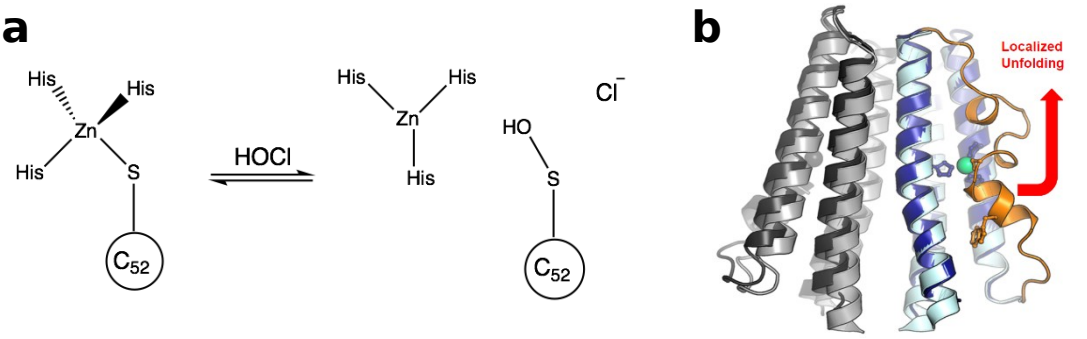


Fig 5. Hypothesized reaction mechanism and structural change. (a) cysteine 52 is bound to the central zinc atom in its reduced form, and oxidation by bleach causes the residue to "let go" of the central zinc atom. (b) This structurally manifests as localized unfolding of one of the alpha helices. Figure 5b adapted from Perkins et al. 2019.

and conservation maps. Structurally, the CZB domain is a bundle of alpha helices connected either n-terminally or c-terminally to the rest of the chemoreceptor (fig 4a). The zinc-binding core of the CZB domain features 3 zinc-binding histidines and a single zinc-binding cysteine (fig 4b). While this cysteine is located in the center of the bundle, there is a small channel that allows the domain's solvent environment to contact the cysteine residue. This cysteine residue possibly "ties" one of the alpha helices to the zinc binding core, rigidifying this alpha helix. Since it is known that cysteine residues are susceptible to oxidation, forming a sulfenic acid group, it is likely that bleach oxidation of the zinc-binding cysteine residue disrupts coordination of the bound zinc atom. This could therefore result in one of the helices "letting go" of the central zinc atom. I hypothesized that this was the basis of CZB bleach sensing: the zinc-binding cysteine residue is oxidized by bleach, which forms a sulfenic acid group. This results in residue Cys52 releasing the zinc core, which results in some form of local unfolding that is then propagated throughout the protein (fig 5).

This hypothesis would explain why the zinc-binding cysteine is universally conserved even when histidine residues would be just as capable of coordinating the central zinc atom (fig 4d); if the domain depends on a zinc-binding residue that can act as a "switch," releasing the zinc residue depending on the environment of the domain, then it makes sense that the cysteine residue is as strongly conserved as it is. This model of CZB function also provided several testable hypothesis: this hypothesis implies that the cysteine residue is sensitive to oxidation, which I subsequently quantified, and that observable and stereotyped structural changes occur to the domain as a consequence of oxidation as readily as does oxidation of the zinc-binding cysteine itself.

METHODS

Protein Purification

In order to study the CZB domain's biochemical properties, I took a reductionist approach, purifying proteins and studying them individually. After isolating the CZB domain itself, I was able to analyze its physical and chemical properties in highly controlled experimental contexts. In order to do this rigorously, I needed to grow, purify, and concentrate the proteins I wanted to study.

Bacteria optimized for protein overexpression and purification (most commonly BL21(DE3) competent *E. coli*) were transformed (using plasmids that confer ampicillin resistance) via electroporation and plated on +ampicillin LB agar plates. These were allowed to grow overnight at 37 °C. In the morning, an individual colony was used to inoculate 25 mL of LB/+AMP cultures, and these cultures were allowed to grow

overnight. The following morning, 5 mL of this overnight cultures was added to each of 4 x 1 L cultures of LB/+AMP, and these larger cultures were allowed to grow shaking at 37 °C until they reached an OD between 0.6 and 0.8. The cultures were induced using 1 mM IPTG, and allowed to grow for several hours if at room temperature (can be varied to optimize depending on the protein and bacteria), or overnight (if using low-temperature-optimized bacteria). The cells were harvested via centrifugation at 5,000 rpm at 4 °C.

To purify the protein, cells were homogenized into ice-cold lysis buffer (10 mM imidazole, 50 mM HEPES, 10% glycerol, 300 mM NaCl, and 0.5 mM TCEP (pH 7.9)). The cells were lysed via sonication while on ice, and then the lysate was collected by centrifuging at 15,000 rpm and collecting the soluble portion. In a 4°C cold room, the lysate was applied to a prepacked gravity column of Ni-NTA agarose beads (Qiagen) equilibrated with the lysis buffer. Lysate was incubated with the beads for 10 minutes, and then allowed to flow through the column. The lysate was passed through the column twice. The column was then washed with lysis buffer until no protein was present in the flow through as determined using a Bradford Assay. Protein was eluted via adding elution buffer to the column (300 mM imidazole, 50 mM HEPES, 300 mM NaCl, and 0.5 mM TCEP (pH 7.9)), incubating the buffer in the column for 10 minutes, and then collecting the flow through in fractions. This was repeated until the collected fractions show no protein via Bradford assay.

To obtain protein lacking the affinity tag, the eluted protein fractions were incubated with 1 mg TEV protease per 20 mL elution volume overnight (New England Biolabs) and re-purified. Samples of each elution fraction were checked for purity via

SDS-PAGE. Fractions containing pure protein were pooled and then concentrated using a centrifugal concentrator at 4 °C.

Cysteine-sulfenic acid quantification

Chemiluminescent antibodies raised against alkylated sulfenic acid groups were used to quantify relative amounts of oxidized cysteine residues under varying concentrations of hypochlorous acid.

Reactions were prepared with purified protein (10 µM) dialyzed into phosphate buffered saline (pH 7.2), 500 µM 5,5-Dimethyl-1,3-cyclohexanedione (dimedone), and additions of HOCl or buffer. Reactions were allowed to proceed for 10 minutes at room temperature, and then quenched with 100 µM L-methionine (this neutralizes any residual hypochlorous acid). 20 µL of each sample was dispensed into 180 µL of phosphoric acid quenching buffer and drawn by vacuum through a Polyvinylidene fluoride membrane in a 96-well slot blotter. The membrane was blocked in a buffer of 5 % milk in 50 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween-201 (TBST) for 10 minutes and incubated overnight at room temperature with rabbit α-cysteine-dimedone antibody (Kerafast) at a 1:5,000 dilution. Subsequently the membrane was washed three times with 20 ml of TBST and incubated with goat α-rabbit-HRP secondary antibody (1:5,000) for 1 H. To analyze, the membrane was washed three times with 20 ml of TBST, and then visualized using an enhanced chemiluminescence substrate that causes the secondary antibody to emit low-intensity light. The light was quantified using a scanner, and the total intensity of light from each sample is taken to be proportional to the total amount of sulfenic acid present in the sample.

Zinc Probe Fluorescence Trials

In order to assess the total amount of free zinc in samples, an organic probe, Zinpyr-1, was used. This molecule fluoresces strongly when it is bound to zinc, and weakly when it is not.

Oxidation reactions were made using 20 µM purified protein, phosphate buffered saline (pH 7.2), and hypochlorous acid at desired concentrations. Ten minutes after addition of hypochlorous acid, each stock was quenched using 15 mM L-methionine. Samples for fluorometer analysis were made using a 1:10 dilution of reaction alongside 50 µM of Zinpyr-1 (Abcam, Eugene, OR) in phosphate buffered saline, and dispensed into a 2 x 2 mm quartz cubette (Starna Cells Inc. Atascadero, CA). Emission spectra were then collected between 450 and 650 nm using a Fluoromax-3 spectrofluorometer (HORIBA Scientific, Austin, TX) (excite at 492 nm; emission slit width: 2nm; excitation slit width: 1nm).

Tryptophan Fluorescence

Samples were prepared using iodoacetamide, a cysteine-alkylating agent used to prevent the formation of CZB dimers. 100 µM CZB was incubated in 20 mM iodoacetamide at room temperature, in the dark, for 1 H. Subsequently, samples of 20 µM iodoacetamide-treated CZB were diluted into purified water buffered by 1x Tris-buffered saline pH 7 (100 mM Tris-Hcl, 150 mM NaCl), alongside variable amounts of bleach. 500 µM of tris(2-carboxyethyl)phosphine (TCEP), a strong reducing agent, was added to negative control samples.

Subsequently, the samples were dispensed into a 2 x 2 mm quartz cuvette (Starna Cells Inc. Atascadero, CA), and fluorescence spectra of the experimental samples were collected using a Fluoromax-3 spectrofluorometer (HORIBA Scientific, Austin, TX) (excite at 295 nm; collect between 300 nm and 400 nm. Scan speed: 50 nm/ min, emission slit width: 4 nm; excitation slit width: 3 nm).

Motility measurements

E. coli and *H. pylori* were grown in appropriate media and diluted into fresh media to an OD of approximately 0.1. For each treatment, 2 µL of diluted, motile bacteria were combined with an equal volume of either chemotaxis buffer (10 mM PBS [pH 7]) or hypochlorous acid diluted into chemotaxis buffer. The samples were mixed gently via pipetting, applied to a glass slide with wells, and immediately observed under a microscope. For each experiment, brightfield videos of bacteria in the appropriate media were recorded using a Nikon Eclipse Ti inverted scope using a 20x objective equipped with an AirTherm temperature-controlled sample chamber set to a constant temperature of 37 °C. For each experiment, 30 seconds of video were recorded at 25 frames per second. Number of motile bacteria recorded per video vary from 0 to 400 bacteria for *E. coli*, and between 0 and 90 motile bacteria for *H. pylori*.

In order to measure the velocity of the bacteria in each video, an in-house MATLAB-based particle tracking software from the Dr. Raghuveer Parthasarathy lab was used to track bacterial swimming paths, with each bacteria being given a location for each frame (Parthasarathy 2012). The difference between bacterial locations between frames, the framerate of the camera, and the magnification of the scope were used to determine the velocity at which bacteria were swimming in each video.

Circular Dichroism Spectroscopy

Samples of oxidized protein were prepared using 500 µM dimedone, purified protein, and desired concentrations of hypochlorous acid. Subsequently, these samples were desalted, either via a prepacked desalting spin column, or via dialysis into pure water. Variable amounts of the protein coming off of the desalting columns were tested until a strong but not overwhelming CD spectrum could be observed using a Jasco J-810 spectropolarimeter (about 50-80 µM of protein), and this amount of protein was used for each subsequent trial to ensure that collected spectra were comparable. The samples were dispensed into 1 mm wide quartz spectrophotometer cells (Starna) and kept at 20 °C using a Peltier temperature control system for the duration of the scan. Each scan ranged between 200 and 250 nm.

Bleach hardiness assay

96-well trays of bacteria in nutritive media at an OD of 0.1 were prepared. To each of these samples, buffered hypochlorous acid to achieve the desired final concentration were added. The plate was subsequently sealed with parafilm, and placed in a temperature-controlled plate reader at 37 °C, and growth curves over 8 hours were recorded using the absorbance of each sample at 600 nm.

RESULTS

The CZB's Zinc-binding cysteine is oxidized by hypochlorous acid

Since the zinc-binding cysteine of the CZB domain is conserved in nearly all instances of this domain in bacteria, we suspected that studying it further would offer crucial insight into the mechanisms by which the domain acts as a sensor. It is known that cysteine is susceptible to oxidation, with one oxidation reaction reversibly converting the thiol (-SH) group into a sulfenic acid (-SOH) group, and subsequent oxidations irreversibly converting the sulfenic acid to sulfinic acid ($-SO_2H$). This posed a practical challenge to my task of measuring the susceptibility of CZB cysteine to oxidation because a single cysteine may be oxidized multiple times, or a cysteine may be oxidized and then spontaneously convert back to the thiol form (fig 6). In order to make it possible to draw stoichiometric equivalences between the total fraction of cysteines oxidized and the concentration of oxidant present in the CZB's environment, I used 5,5-Dimethyl-1,3-cyclohexanedione (dimedone), an alkylating agent that modifies sulfenic acid groups, preventing both subsequent oxidations and reductions back to the

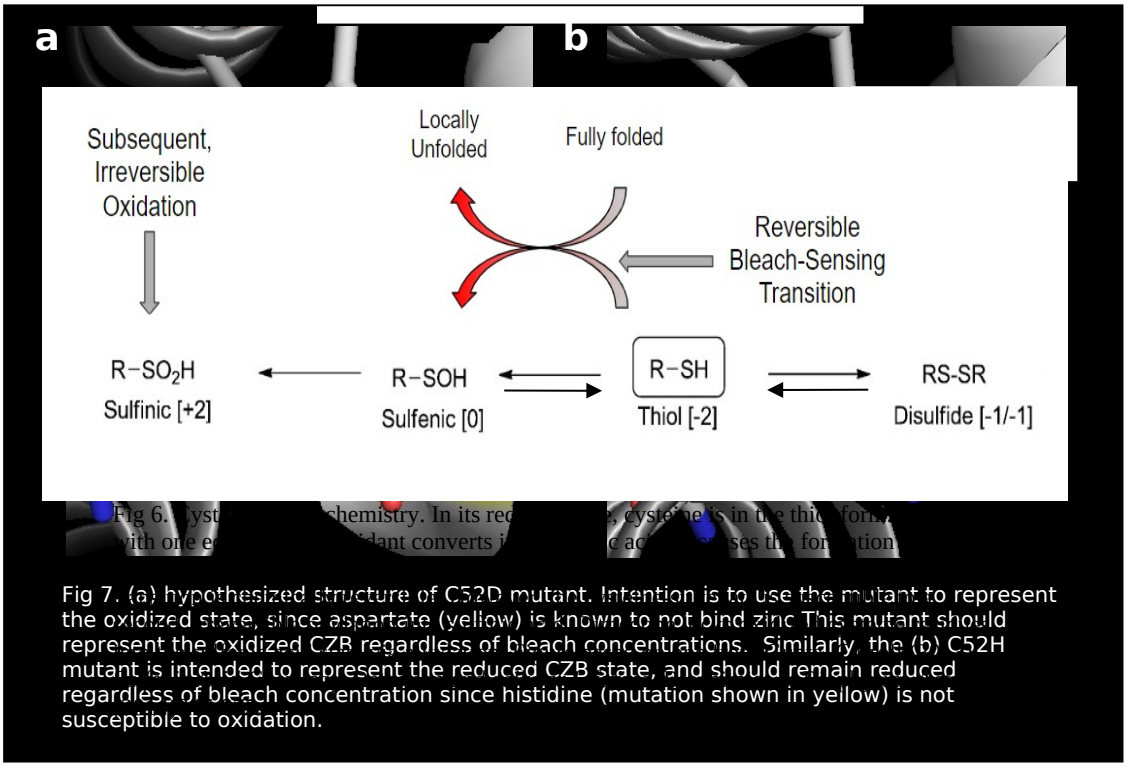


Fig 6. Cyst chemistry. In its red e, cyste ne is in the thio form with one e dant converts i c ac ses the for ation

Fig 7. (a) hypothesized structure of C52D mutant. Intention is to use the mutant to represent the oxidized state, since aspartate (yellow) is known to not bind zinc This mutant should represent the oxidized CZB regardless of bleach concentrations. Similarly, the (b) C52H mutant is intended to represent the reduced CZB state, and should remain reduced regardless of bleach concentration since histidine (mutation shown in yellow) is not susceptible to oxidation.

thiol form. In addition to simplifying the cysteine redox chemistry, this alkylating agent also creates a chemical site for which there is a readily available antibody, allowing antibody-based quantification of the total amount of sulfenic acid in a particular sample. To accomplish this, I exposed a constant amount of protein to concentrations of bleach between 0 µM and 1 mM in the presence of dimedone. I then quantified the total amount of sulfenic acid-dimedone adduct form using the described antibody-chemiluminescence method. In order to hone in on the reactivity of the zinc-binding cysteine, I performed this experiment on 3 protein constructs: the wildtype CZB domain taken from tlpD, a mutant form where the zinc-binding cysteine was mutated to a histidine (termed C52H), and a mutant form where the zinc-binding cysteine was mutated to an aspartic acid (termed C52D) (fig 7). Both histidine and aspartic acid are not susceptible to bleach oxidation. Thus, these constructs are essentially replicates of the wildtypes, minus the zinc-binding cysteine that we are interested in. This allows us to investigate how the presence of the zinc-binding cysteine affects the reactivity of the domain by providing a negative control. This CZB construct has two surface cysteines that have low conservation scores, indicating that they likely do not play a biochemical role that depends on that particular amino acid. Since these two cysteines are still present on the C52H and C52D mutant proteins, we would expect the signal produced by the mutant proteins to be 2/3 the signal of the wildtype CZB (since they have two cysteines rather than 3). Instead, the wildtype domain reactivity curve is often around 5 times the intensity of the C52H mutant, and 15 times the intensity of the C52D mutant (fig 8a, 8b). This indicates that the wildtype CZB domain is far more susceptible to oxidation than the mutants, and since the only obvious difference between the mutants

and the wildtype is the zinc-binding cysteine, it is reasonable to attribute the increased reactivity of the wildtype domain to the zinc-binding cysteine specifically. As a result, this experiment demonstrates that the zinc-binding cysteine is particularly sensitive to oxidation by bleach, to a degree that far exceeds the capacity of a generic cysteine.

This experiment and others like it were repeated using hydrogen peroxide, another reactive oxygen species commonly found within the human body; we found that even using concentrations many times larger than the concentrations of bleach used could not replicate the reactivity curve of CZB in the presence of bleach. In summary, not only is the zinc-binding cysteine tuned to react to much smaller concentrations of bleach than the average cysteine, it also seems to be tuned to sense bleach over other reactive oxygen species. This is to say, not only is the zinc-binding cysteine sensitive to bleach molecules, it is also specific to it, suggesting that the “purpose” of the residue is to sense bleach (fig S1).

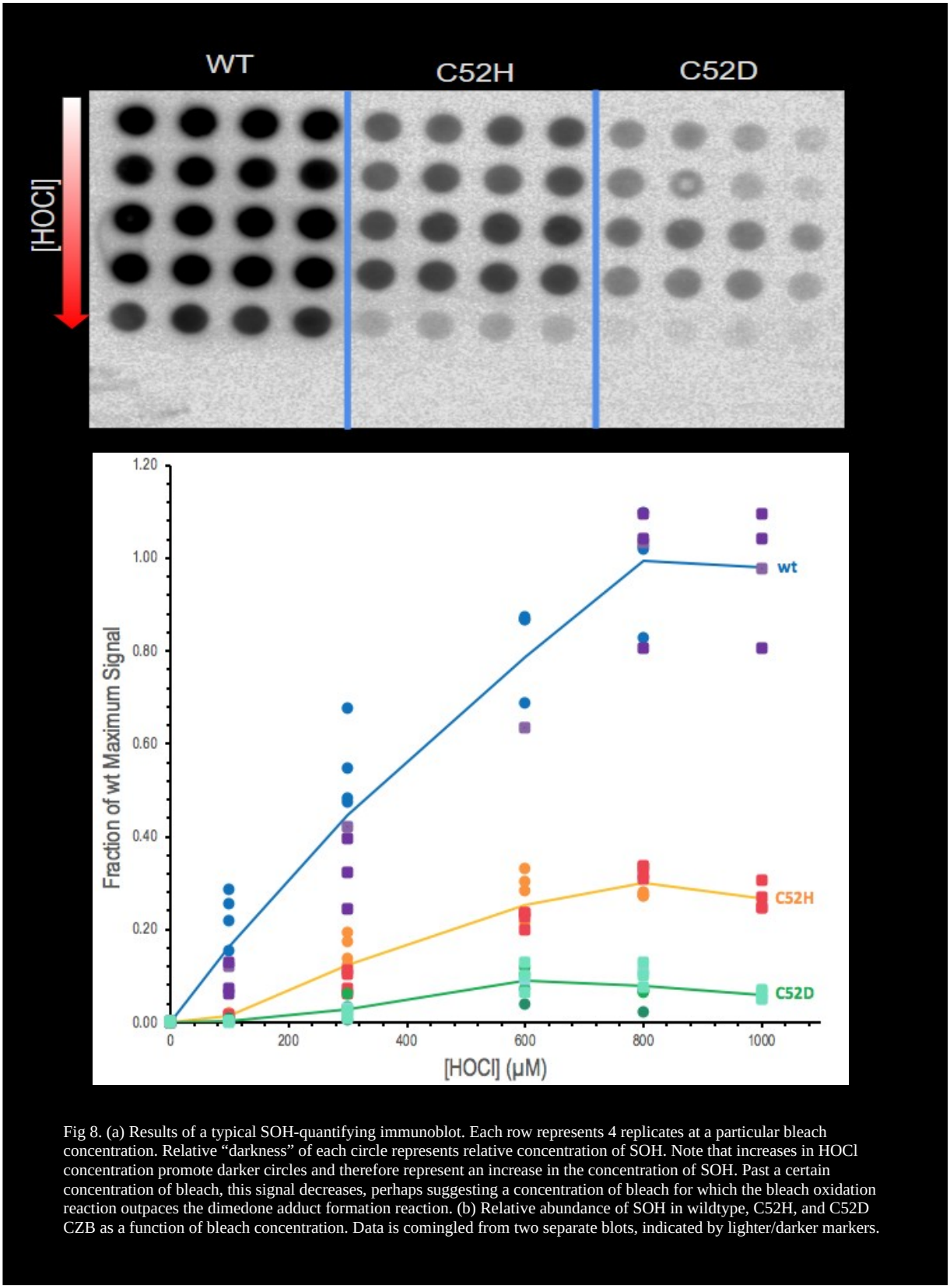


Fig 8. (a) Results of a typical SOH-quantifying immunoblot. Each row represents 4 replicates at a particular bleach concentration. Relative "darkness" of each circle represents relative concentration of SOH. Note that increases in HOCl concentration promote darker circles and therefore represent an increase in the concentration of SOH. Past a certain concentration of bleach, this signal decreases, perhaps suggesting a concentration of bleach for which the bleach oxidation reaction outpaces the dimedone adduct formation reaction. (b) Relative abundance of SOH in wildtype, C52H, and C52D CZB as a function of bleach concentration. Data is comingled from two separate blots, indicated by lighter/darker markers.

The structural change associated with CZB oxidation is not visible under CD

In order to understand the mechanism by which the CZB domains function, it is necessary not just to understand how the domain reacts with oxidants/ligands, but also to understand how the domain transmits the signal to other parts of the protein after sensing its "input." In order to understand the structural changes that occur upon oxidation in broad strokes, I used circular dichroism spectroscopy (CD), a method that grants information on the secondary structure composition of protein samples.

Protein structure can be described on three general levels: primary, secondary, and tertiary structure. Primary structure refers to the sequence of amino acids encoded for by some lifeform's genes; secondary structure refers to the local folds and motifs that quickly develop between amino acids that are (generally) close together. For the purposes of CD spectroscopy, the secondary structures we are concerned with are alpha helices (tight spirals that constitute the most common secondary structure), beta pleated sheets, and disordered coils. Tertiary structure refers to how these secondary motifs are packed together in relation to each other.

CD spectroscopy produces spectra that report on the secondary structure composition of proteins. Its output is a spectrum that can be interpreted to give the relative percentage that each secondary structure comprises in the protein sample. In essence, every CD

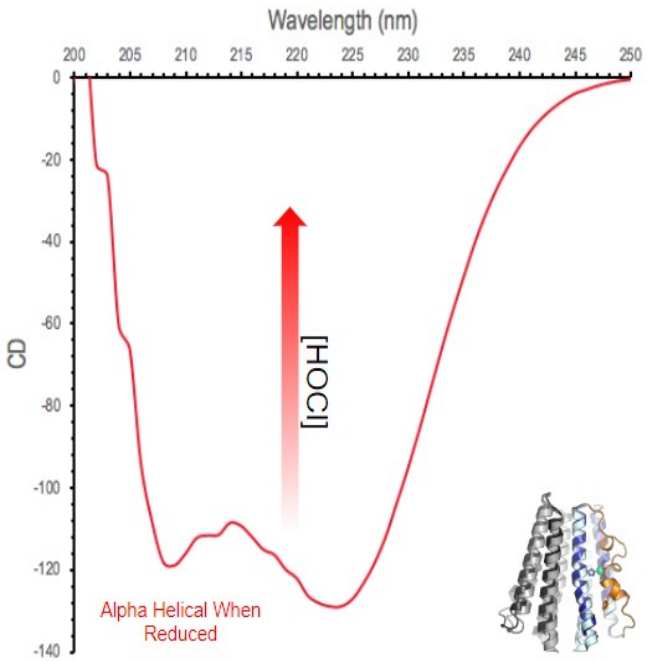


Fig 9. Hypothesized change in CD spectrum upon bleach oxidation. Transition from almost entirely

would “flatten” the spectrum.

spectrum is a combination of the characteristic spectra for alpha helices, beta sheets, and disordered coils. For instance, the more the spectrum looks like the characteristic spectrum for alpha helices, the more the protein is comprised of alpha helices.

Analysis of the CZB domain under CD yielded the expected result—the CZB domain is essentially a bundle of alpha helices with some disordered coils connecting the helices. Correspondingly, the CD spectrum shows a largely alpha helical spectrum (fig 9).

Experimentally, addition of bleach reduces the alpha helical character of the spectrum, which on its own is consistent with a model where oxidation of the zinc-binding cysteine results in local unfolding of an alpha helix (fig 10). However, this unfolding occurs at a bleach concentration greater than that required to oxidize the zinc-

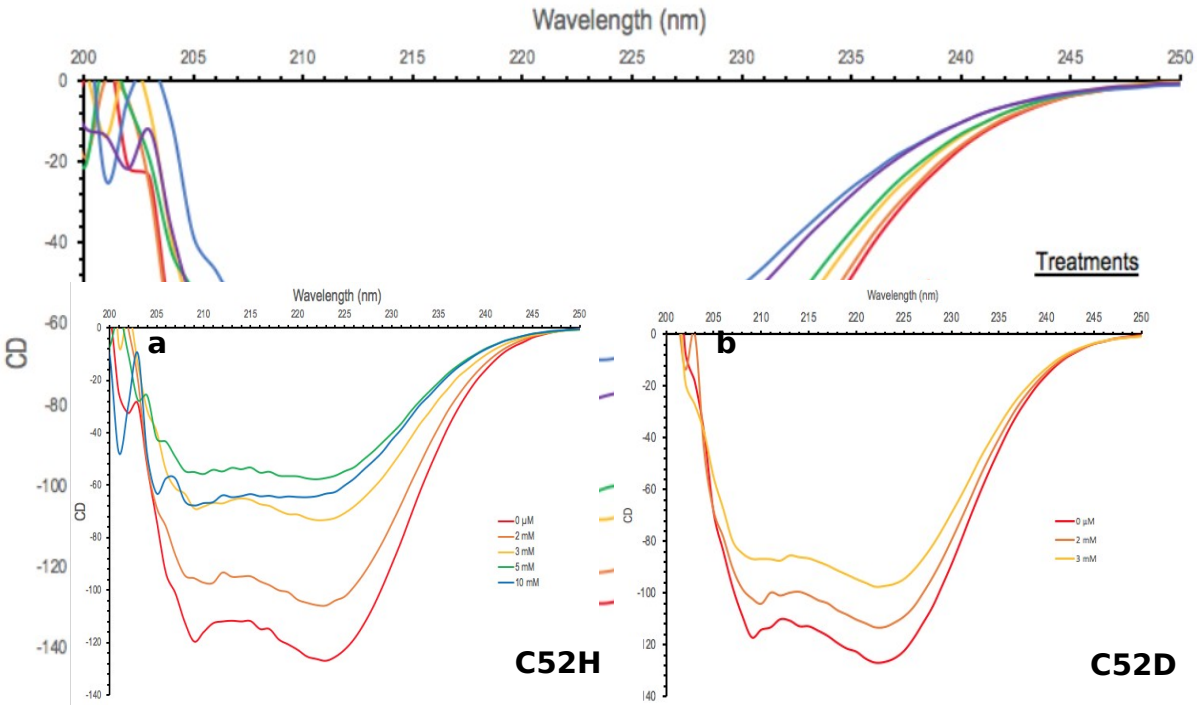
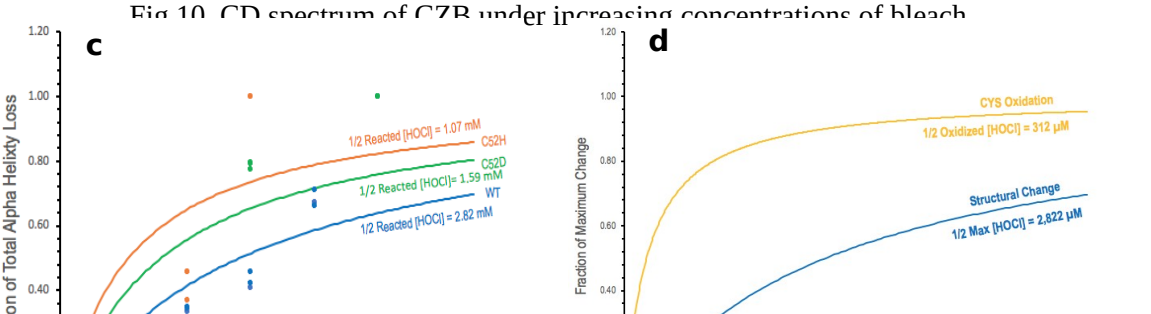


Fig 10. CD spectrum of CZB under increasing concentrations of bleach



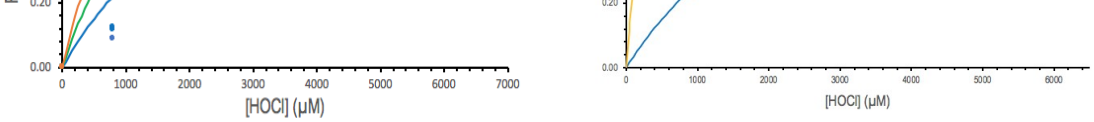


Fig 11. (a) CD spectrum of C52H and (b) C52D CZB under increasing concentrations of bleach. (c) compares the rate at which alpha helicity is lost as a function of bleach concentration between wild-type and mutants to illustrate how all constructs react at similar orders of bleach concentration (if this change reflected a C52 dependent change, then we would expect only the wildtype to react. (d) compartes the rate at which C52 is oxidized to the rate at which the structure of the protein changes according to CD, showing that C52 oxidation is likely not precisely related to the structural change observed under CD, but may instead be due to general degradation of the protein by high concentrations of bleach.

These two facts indicate that the loss of alpha-helicity observed via CD is not related to oxidation of the zinc-binding cysteine specifically, and thus may represent some structural change that is not related to the domain's sensing function, since any sensing mechanism that explains the high conservation score of the zinc-binding cysteine would need to be specific to the wildtype CZB domain, and therefore not occur when this cysteine is mutated out of the domain (as in the C52D and H constructs).

These experiments indicate that the structural mechanism by which oxidation of the zinc-binding cysteine is transmitted to the rest of the protein likely involves a very subtle structural change—perhaps unfolding of a few residues in an alpha helix—in such a way as to be too small to be observable via circular dichroism. This conclusion immediately suggests using more precise methods in structural biology to analyze conformational changes following oxidation, such as protein NMR, rather than the coarse information provided by circular dichroism.

Tryptophan fluorescence suggests that bleach oxidation increases CZB core solvent exposure

Repeated oxidation trials indicate that exposing the CZB domain to bleach generally decreases the fluorescence of the protein at the characteristic tryptophan fluorescence maximum at 350 nm (fig 13). Tryptophan residues fluoresce strongly when sequestered

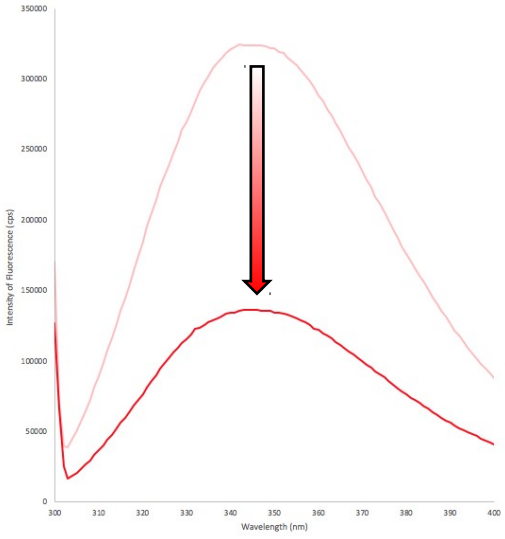


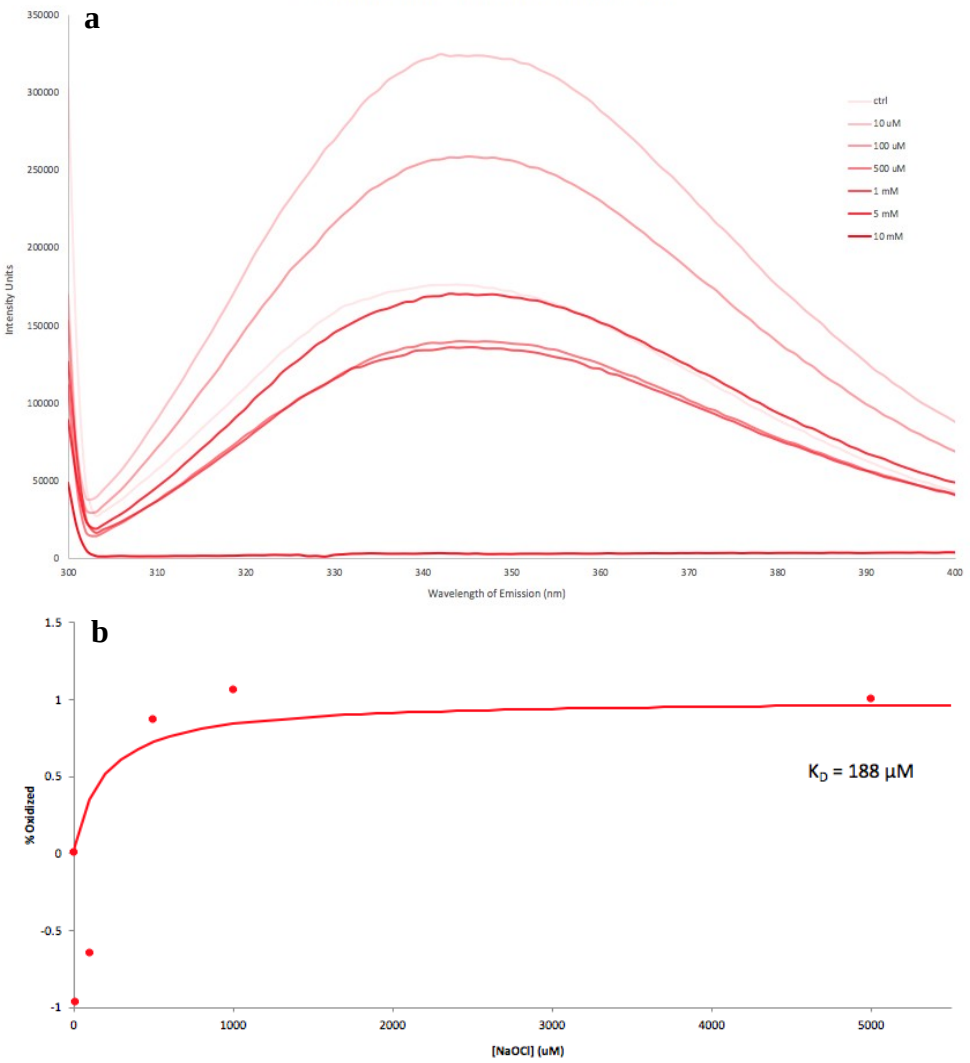
Fig 12. Expected change in tryptophan fluorescence when a tryptophan residue goes

from water, as in the case of a tryptophan residue buried in the hydrophobic core of a globular protein. Exposing tryptophan residues to polar solvents facilitate fluorescence quenching and therefore lower signal intensity (fig 12). This phenomenon is possibly due to tryptophan 25, which crystal structures show is positioned near the zinc-binding core when the CZB domain is in its reduced state. Since the residue is sequestered away from solvent when the domain is in its reduced state, it fluoresces strongly. My hypothesis is that oxidation results in localized unfolding of the domain, which in turn would result in this previously buried residue being exposed to solvent, explaining the decrease in fluorescence intensity following treatment with bleach.

Repeated experiments of this type resulted in data that was not inconsistent, but rather consistently noisy—in general, exposure of the CZB domain to bleach resulted in decreased fluorescence, but sometimes, small initial doses (<100 μM) actually increased the fluorescence intensity (fig 13a). this suggests there may be some phenomenon in addition to the bleach-mediated structural change affecting the fluorescence of tryptophan residues within samples of the CZB. One possible explanation is that bleach oxidation encourages dimerization of the domain. This would be enabled by the fact that the DgcZ CZB used for these trials has a second tryptophan residue located on the exterior of the protein. Furthermore, there is a cysteine residue located nearby. It is therefore possible that oxidation of this solvent-exposed cysteine encourages the formation of disulfide bridges between CZB monomers, thereby stabilizing the dimerized form, and that this reaction occurs more readily than does the hypothesized cysteine 52-mediated structural change. Furthermore, disulfide-mediated dimerization was observed via SDS-PAGE during protein purification. It is possible that

as a result of this dimerization, the second, solvent-exposed tryptophan residue is sequestered. This would compete with the decreased fluorescence of the core tryptophan residue, explaining the noisy but consistent decrease in tryptophan fluorescence seen. Treatment of the protein with iodacetamide, an alkylating agent that prevents the formation of disulfides, appeared to decrease the "noisiness" of the data somewhat.

In order to measure how sensitive this putative conformational change is to bleach, I assumed that the difference in fluorescence intensity from any given trial to the 10 mM (presumably maximally oxidized) trial is proportional to the amount of CZB domain that has undergone the conformational change, and fit the data to a binding



concentrations of hypochlorous acid. (b) Fluorescence data computationally fit to hill equation w/ n = 1 (Θ = [L]/(Kd+[L])) where Θ is the fraction of oxidized CZB, [L] is the concentration of hypochlorous acid in solution, and Kd is an optimized parameter

equation (fig 13b) , resulting in an experimental “bleach unfolding” “half reacted concentration” (analogous to K_D) of 188 μM.

Bleach oxidation promotes zinc release

Previous work approached the CZB domain as a zinc sensor, and described the domain’s role as regulating the activity of diguanylate cyclase activity by binding/releasing zinc. This was supported by the observation that zinc inhibits biofilm formation in *E. coli*—high levels of diguanylate cyclase activity are associated with biofilm formation in *E. coli*, so it makes sense that DgcZ CZB domains would bind zinc as a regulatory mechanism. Previous work by my mentor and I, however, showed that bleach regulates the chemoreceptor tlpD, which also possesses a CZB domain. This begs the question: do CZB domains sense zinc, or do they sense bleach? Remarkably, it may be true that they perform both of these functions, and that sensing bleach and sensing zinc may be connected in some way. Using a zinc-sensitive fluorescent probe, I demonstrated that exposing DgcZ domains to bleach promotes zinc release (fig 14). Exposing the CZB domain to bleach increases the fluorescence intensity of the Zinpyr-1 probe, indicating an increased concentration of free zinc. Furthermore, while this effect is very pronounced in the case of wt CZB (fig 14, grey), it is very minimal for both C52H and C52D (fig 14, red and blue), indicating that cysteine 52 is necessary for CZB’s affinity for zinc to be modulated by bleach exposure.

Fig 14. Zinpyr-1 fluorescent spectrum for CZB wt (grey), C52H (red) and C52D (blue). Wt curves show substantial increases in free zinc concentration as bleach concentration increases, and only very small increases for C52H and C52D. Baseline fluorescence for C52H and C52D also appears to be lower, perhaps suggesting that some portion of the proteins have a chelating effect, removing free zinc from the water.

Bacterial tolerance to bleach varies between species

In order to begin connecting biochemical studies of the CZB domain to its importance to the survivability of bacteria within the human host, I examined how well various bacteria tolerated bleach. After all, if *H. pylori* is readily killed by bleach, it would not make sense that it is attracted towards bleach—this would pose a crucial contradiction that must be detangled.

Preliminary studies observed that at certain concentrations of bleach, bacteria may still be able to swim while not being able to persist in the oxidative medium long term. Conversely, bacteria may be rendered immobile, and yet be able to recover and grow in the long run. In order to understand the characteristics of bacteria that possess CZB sensing domains, we measured swimming speed as a function of bleach concentration for two CZB-possessing bacteria, *S. enterica* and *H. pylori*, as well as a for a bacteria that has no CZB-containing chemoreceptor, *E. coli,* but which has the CZB-containing diguanylate cyclase DgcZ. In order to do this, we collected video of the bacteria under differing bleach concentrations and used a script to determine the speed at which the bacteria were swimming from these videos. Our experiments showed that the two CZB-chemoreceptor possessing bacteria, *S. enterica* and *H. pylori* were hardy swimmers in response to bleach, often continuing to swim in even 5-8 mM bleach. In contrast, *E. coli* ceased to swim at a bleach concentration of 3 mM, which is still very close to the 5 mM maximum concentration of bleach that the human body is capable of generating (fig 15). Thus, while these three CZB-possessing bacteria were fairly hardy with respect to bleach, the ones possessing CZB chemoreceptors were the most apt to retain their motility.

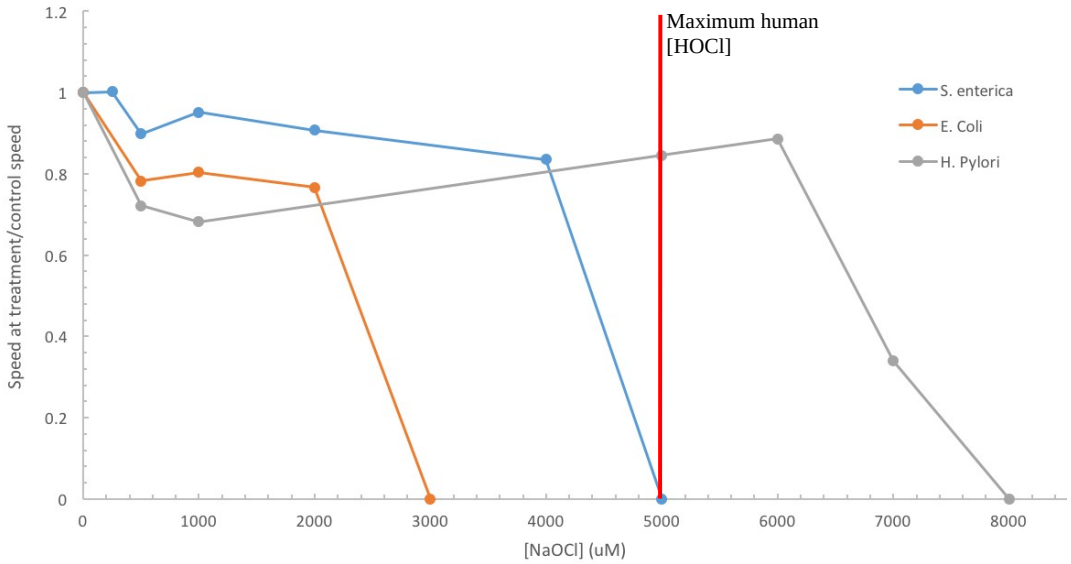


Fig 15. Bacterial swimming speed as a function of bleach concentration relative to untreated speed.

In addition to considering the "motility tolerance" of these bacteria to bleach, defined as the bleach concentration needed to immediately stop the bacteria from moving, I also wanted to determine what concentration of bleach is sufficient to prevent an established (OD ~0.1) population of bacteria from growing. Growth curves were collected for bacterial populations exposed to increasing concentrations of bleach (see supplemental figure S2 for an example), and the concentration of bleach necessary to prevent the bacterial population from growing was plotted alongside the bleach concentration necessary to prevent motility (fig. 16). This analysis revealed both similarities and differences between bacteria; for instance, established populations of *E. coli*, *S. enterica*, and *H. pylori* all fail to grow post treatment with 5 mM bleach. At the same time, *pylori* and *enterica* remain motile at 8 mM and 5 mM respectively, while *E.*

coli stops moving at 3 mM. This information may serve to illustrate differences in the way that bacteria use bleach to orient themselves in their hosts; for instance, it makes

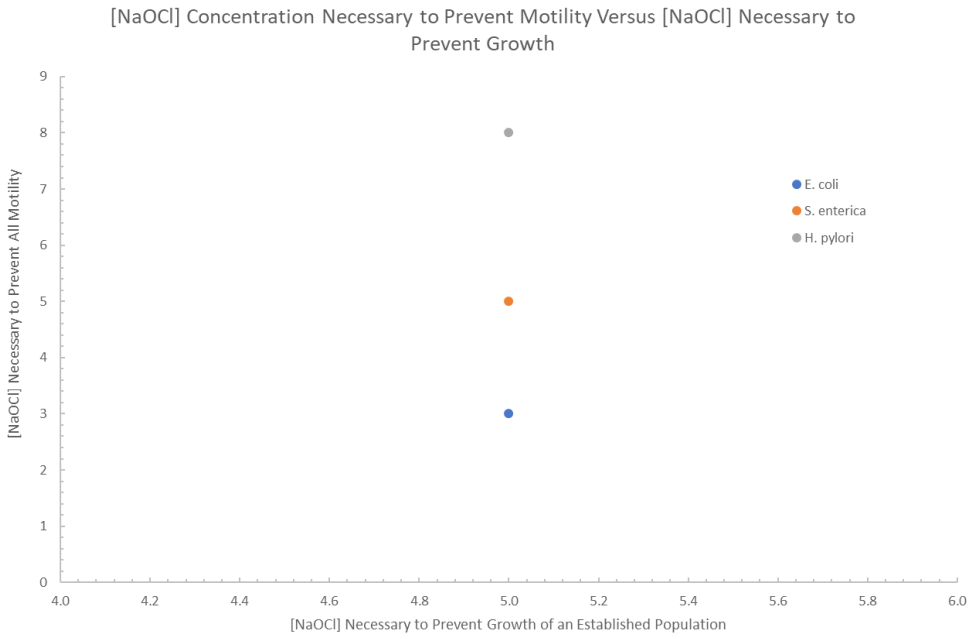


Fig 16. Bacterial bleach resistance with respect to swimming vs. long-term growth. Concentration in mM. Represented values indicate lowest observed value that prevent motility/growth; actual minimum [HOCl] may be lower.

sense that *pylori*, which must swim towards bleach to locate safe niches, would be able to continue swimming even in concentrations of bleach that would not be viable long-term, in order to reach some site within the body that is safer. A similar phenomenon may be at play with *S. enterica.* To contrast, *E. coli*, which does not have a CZB chemoreceptor, does not have a similarly robust bleach swimming capacity.

CONCLUSIONS

Biological Significance

Bacteria are, quite possibly, humanity's most intimate companions and enemies; this companionship is predicated on finely tuned behaviors and biochemical tricks that allow the bacteria to survive and thrive in the context of the human body. In the case of the pathogens involved in this study, this hardiness explains how these bacteria are able to survive in an environment that is actively trying to eliminate them, and thus helps us understand their biological success.

In particular, the work I've done helps elucidate how gut-colonizing bacteria like *Salmonella* and *Helicobacter* are able to sense and respond to endogenous sources of hypochlorous acid, or bleach. This is a crucial capacity for certain bacteria to have: bleach concentrations can reach up to 5 mM at sites of inflammation, and it is known that *H. pylori* mutated to lack the bleach chemoreceptor tlpD is largely incapable of colonizing mice. Our work indicates that *pylori* may use bleach as a signal of host tissue damage, and therefore swims towards these sites of injury, possible in order to scavenge host iron from hemoglobin. While my work focused on the CZB domain in the context of common gut-colonizing bacteria like *S. enterica, H. pylori*, and *E. coli*, the CZB domain is also widely present amongst *Firmicutes, Proteobacteria* and a few *Bacteriodetes*. Furthermore, the key residues on this domain are highly conserved amongst all homologues; this indicates that the gut-colonizing pathogens used in this study likely serve as good model organisms, and the findings of this study may

highlight general and important bacterial-host interactions rather than unique properties of particular bacteria.

Our work identified the CZB-possessing chemoreceptor tlpD as a bleach sensor that allows *H. pylori* to sense and swim towards sources of bleach. In support of this, my results show that *H. pylori* is quite resilient, surviving concentrations of bleach of up to 8 mM. Understanding that *pylori* has both the resilience and means of identifying and thriving within an environment rich in hypochlorous acid, my next objective was to understand the biochemical mechanism by which the hypochlorous acid is sensed in the first place. Of particular interest was residue 52 on the CZB domain, which corresponds to a cysteine residue that aids in coordination of a structurally-important zinc atom. This residue is conserved universally in CZB homologues, and cysteines are known to be sensitive to oxidation. Using antibody-based quantification of sulfenic acid, I was able to demonstrate that cysteine 52 is not only exceptionally sensitive to bleach, but also specific to it; the CZB domain does not appear to react to hydrogen peroxide in any substantial quantity. This is significant because cysteine oxidation has the potential to disrupt zinc coordination. Thus, oxidation of this sensitive cysteine residue likely results in the cysteine residue releasing the structural zinc atom, which is expected to result in some local structural change that constitutes the basis by which the bleach sensing signal input is conveyed to the rest of the chemoreceptor, and thereby into the rest of the cellular signaling network.

Cysteine 52 oxidation and structural change

In order to understand this structural change, I performed circular dichroism spectroscopy on samples of the CZB domain, as well as on mutant constructs of the

domain that replace the zinc-binding cysteine with a histidine or an aspartate respectively. While I was able to observe a loss of alpha helicity via CD as a consequence of oxidation, this change occurred at a much greater concentration of bleach than the cysteine 52 oxidation occurred at. Furthermore, the mutant strains displayed a very similar loss of alpha helicity at similar concentrations of bleach. This indicates that the loss of alpha helicity observed via CD is not due to oxidation of the zinc-binding cysteine 52, but rather due to some more generalized structural change or damage inflicted upon the domain by large concentrations of bleach. This would indicate that a hypothetical structural change caused by bleach oxidation of cysteine 52 is not subtle enough to not be picked up by CD, and must therefore consist of some more subtle shift in the CZB domain—perhaps the unfolding of only a few residues in an alpha helix, or a change in orientation of an alpha helix so as to change the structure of the CZB domain without affecting the secondary structure composition. This interpretation would conclude that the structural changes observed via CD may result from nonspecific damage to the protein caused by the presence of a strong oxidation at high concentrations. Perhaps these destructive concentrations of bleach are never actually reached *in vivo* within the bacteria's cytosol because of reductants within the cytoplasm, or alternative oxidation susceptible targets present within the cell, potentially explaining why high concentrations of bleach were able to damage the CZB domain *in vitro* while not jeopardizing the live bacteria's ability to orient itself and swim *in vivo*. While I wasn't able to observe the hypothesized structural change via CD, tryptophan fluorescence does indicate that treating CZB with bleach increases the solvent-accessibility of the zinc core, and that this transition occurs at the same bleach

concentrations that the zinc-binding cysteine is oxidized at (~100 μM). Tryptophan fluorescence provides a specific window into the chemical properties of the CZB core region, whereas CD offers only a bulk average representation of the secondary structure composition of the CZB sample under analysis. Thus, while the hypothesized conformational change might be invisible under CD, we can still study its effect on the CZB protein using tryptophan fluorescence. Together, the sulfenic acid quantification and tryptophan fluorescence experiments indicate that cysteine 52 is tuned to sense bleach, being more sensitive to it than a generic, solvent-exposed zinc residue, and that this oxidation is correlated with some form of localized unfolding.

Future Questions

While my work has largely focused on analyzing purified CZB domain in solution, it is also true that the cellular and molecular context of the CZB may have a great deal of influence in how it operates. In particular, the CZB domain is hypothesized to act as a zinc sensor in *E. coli*, and my mentor and I showed that it acts as a bleach sensor in *H. pylori*. Is it possible that differences in these biochemical roles stem not from the structure of the domain itself, but rather from the cellular environment that it finds itself in?

E. coli's cytoplasm is characterized by a robust glutathione/glutathione reductase (about 15 mM) system that rapidly acts to neutralize oxidants within the cytoplasm, diminishing the possibility that DgcZ CZB acts to sense bleach in this context (Pittman 2005). In contrast, it is not well-know what reduction systems *H. pylori* has, with evidence pointing to thioredoxin (Windle 2000). Thus, it is possible that *pylori* may not neutralize reactive oxygen species as quickly as does *E. coli*, enabling

the CZB domain to act as a bleach sensor in one context, and a zinc sensor in another. Furthermore, I have shown that oxidation of the domain by bleach promotes zinc release. Does this result in “cross talk” where varying concentrations of zinc and bleach are able to interfere with sensing of one or the other, or are these bacterial systems adapted such that one domain is capable of sensing both? Or perhaps the cellular environment of each CZB-possessing bacteria is evolutionarily optimized to hone in on a single signal input, with strong reductants opposing oxidation such that the CZB domain can solely transduce zinc concentration signals.

With the wide variety of bacteria possessing CZB, this an exciting area of research that promises to elucidate a great deal about how bacteria sense and survive within the human body, providing an interesting view into what is shaping up to be a flexible and biologically important sensing domain. Furthermore, the importance of motility to bacterial pathogenesis suggests that a strong understanding of the structural and mechanistic function of the CZB domain will permit targeting of them in the context of new therapeutics and antibiotics.

APPENDIX

Glossary

Enzyme: a protein that fulfills its biological purpose by accelerating a chemical reaction that otherwise would occur at a slow rate or otherwise so slow as to be functionally stagnated; an enzyme is a biomolecule that performs catalysis.

Murine: pertaining to mice.

Reactive Oxidative Species: Unstable chemical agent that tends to cause oxidation, ie rapid addition of the highly electronegative oxygen atom. Often damaging to biological molecules.

Secondary Messenger: A cell might receive an input from outside of the cell, and in order to relay the signal, produces a second molecule within the cell. This second molecule is the secondary messenger, and is responsible for beginning the biochemical sequences that result in the cell's characteristic response to that signal.

Activity: Macroscopic property representing the rate at which a certain amount of enzyme performs catalysis.

Glutathione: common antioxidant molecule. In its reduced state, it is a potent diffusible antioxidant. In its oxidized state, it forms disulfide bonds with other glutathiones, resulting in the formation of GSSG, or two oxidized glutathiones bound together. This form still retains the capacity to counter oxidation by exchanging disulfide bonds with oxidized sulfurs in a process known as S-glutathionylation.

Wildtype: variant of a gene, protein, or organism most commonly found in the wild/most representative of the species.

Conservation Map: Graphic that demonstrates which parts of a protein are least subject to mutational change across related species. High rates of conservation generally indicate that the particular amino acid is necessary to perform some crucial function.

Alpha helix: Common structural motif that protein chains adopt in which every backbone amino group forms a hydrogen bond with the backbone carbonyl three or four residues earlier.

Homologues: Proteins bearing distinct sequences that serve the same biological purpose.

Affinity: How much two molecules "like" to be bound together. Often expressed in terms of a dissociation constant, Kd, which represents the concentration of ligand (or bound molecule) at which half the protein is in the bound state.

Chelation: Process of binding of binding a metal ion at multiple points using a single molecule.

Stochasticity: the quality of lacking order or predictable behavior.

In vitro: Performed "in a glass," a reconstruction of a biochemical phenomenon to demonstrate an understanding of how its minimal parts fit together. Contrast to *in vivo*, which refers to work done with living systems.

Antibody: Immune protein created by an organism to bind to some structural element characteristic of an invasive, foreign biomolecule. In the context of biochemistry research, generally a protein that binds a particular type of molecule.

Dimedone: 5,5-Dimethylcyclohexane-1,3-dione, an alkylating agent that modifies sulfenic acid (oxidized cysteine), adding an alkyl organic group.

Stochiometry: Referring to discrete ratios of molecules.

Thiol: An organic compound containing a sulfur bound to a hydrogen. Imagine an alcohol but smellier.

Plasmid: A loop of DNA present in bacteria that replicates independently of chromosomes. Useful in laboratory manipulation of bacteria, as bacteria can be made to take up foreign plasmid and began producing the proteins encoded therein.

Overexpression: a process by which a protein encoded by a plasmid is triggered to be produced in a bacterial population at many times the rate a normal protein would be, often coming to represent a significant portion of the mass of the bacteria.

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis. Visualization technique that separates proteins based on their size/mass.

Histidine tail: a string of histidines artificially engineered to the tail of a protein. Provides a unique chemical characteristic that can be used to isolate the protein from a mixture of biomolecules.

Ni-NTA: Ni-Nitrilotriacetic acid, a chemical group, generally bound to agarose beads, which positions nickel, which has a high affinity for histidine, to bind histidine tails, allowing the isolation of particular tagged proteins from a solution.

Supplemental Figures

Fig S1. Oxidation of CZB domains via HOCl and H2O2, determined using immunofluorescence of sulfenic acid. The lower set of lines (yellow) are orange, grey, and yellow overlapping. WT HOCl oxidation curve is ended prematurely because the domain appeared to be fully oxidized (subsequent additions of bleach indicated little change)

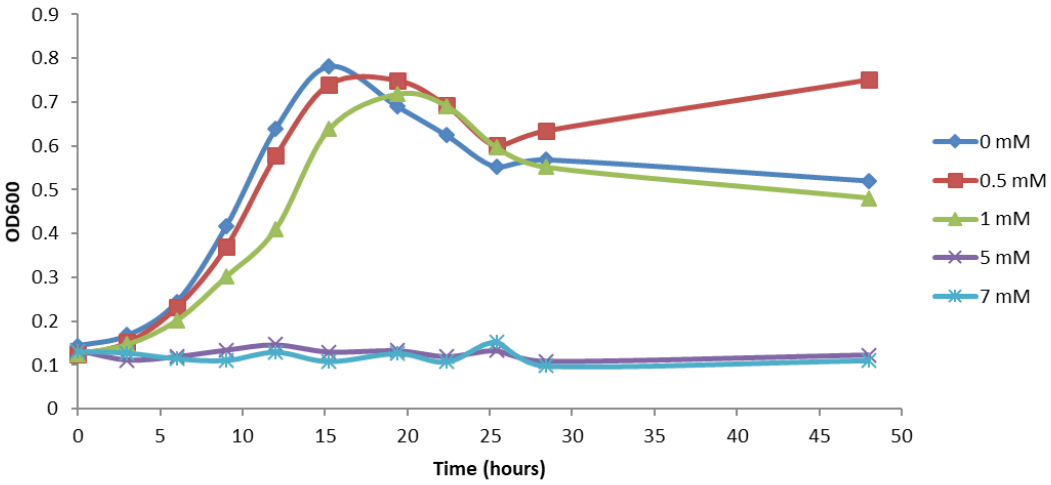


Fig S2. *H. pylori* growth curves following treatment with a given concentration of HOCl (figure legend). Similar curves were produced for *E. coli* and *S. enterica*, and used to determine bacterial tolerance to bleach.

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