Exploring the Copper (I) and Iron (III) Binding to Recombinant Wild-type FtrB from Brucella abortus: Role of Cu-ion in the FtrABCD Iron Transport System in Gram-Negative Pathogens

Bridget Aidoo

Hons. College Thesis, Fall 2024

Abstract

This work describes experiments that investigate Cu (I) and Fe (III) affinities of recombinant wild-type FtrB from Brucella abortus 2308 and specificity of this protein towards Cu (II) using established biochemical assays

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Dedication and Acknowledgements

This thesis is dedicated to my family and friends, whose unwavering support and encouragement have been my constant source of strength throughout this journey. Your belief in me has been invaluable and has inspired me to persevere.

I would like to express my deepest gratitude to Dr. Sambuddha Banerjee for his exceptional guidance, mentorship, and support throughout this research. His insights and encouragement have been instrumental in the completion of this thesis, and I am truly grateful for his unwavering commitment to my academic and personal growth.

I am also immensely thankful to my lab mates for their invaluable assistance and the supportive environment they have fostered. Your help and collaboration have made this research experience both productive and enjoyable, and I deeply appreciate your contributions.

Additionally, I would like to extend my sincere thanks to the Summer Brody Research Program (SBRP) and the Louis Stokes Alliances for Minority Participation (LSAMP) for the funding that provided me with the opportunity to continue this research. Your support has been crucial in enabling me to pursue and complete this project.

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To whom it may concern

I wish Bridget Aidoo all the best wishes for her future, and I know whatever she decides to do in the future, she will do it with dedication, compassion, strength, and wisdom. I had the greatest pleasure of growing with Bridget since the first year of her college life in ECU and I am grateful for her dedication, commitment, and the ability to prioritize professional responsibilities. But above all, I am grateful to her for being my coworker. To the reader of this work, I humbly request you to give this thesis the time that it deserves as you read it so that you can appreciate the depth of knowledge that Bridget has demonstrated here. I am indebted to all my students and especially to Bridget for answering a significant question in the story about novel redox proteins with a copper ion as their redox cofactor. To perform the predicted oxidation-reduction (redox) reaction, FtrB from Gram-negative *Brucella abortus* is predicted to bind Cu^{2+} and this Cu^{2+}-FtrB is predicted to perform a ferrous-oxidase function (convert Fe^{2+} to Fe^{3+}) and become Cu+-FtrB. Two key elements in this predicted function of FtrB are its ability to stay bound to both Cu^{2+} and Cu^{+} so that the enzyme does not become inactive, and the ability of Cu^{+}-FtrB to keep the oxidized Fe^{2+} bound to avoid toxicity and precipitation of ferric iron as rust. During her undergraduate research, Bridget spend time in learning how to express and purify recombinant proteins and performed biophysical

experiments (ITC, CD, UV-visible) to characterize the binding thermodynamics of a Cu^{+} and Fe^{3+} mimic to recombinant wild-type FtrB. FtrB being a member of novel protein family with no available experimental data, her work is significant and will help future researchers in characterizing the biological function of this family of proteins. She has presented her work on several scientific platforms, showing her proficiency in data presentation to a wider scientific audience. Soon, her work will be included in a manuscript, and she will become a published author from her undergraduate research work. I wholeheartedly certify that she has fulfilled all responsibilities to submit this Honors College Thesis.

With best regards,

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Non-technical Abstract

FtrABCD is a group of four proteins that help transport iron (Fe^{2+}) in certain harmful bacteria, like *Brucella*. These proteins are crucial for the bacteria to survive in environments where iron is scarce. The function of these four proteins in these iron scarce environments is to scavenge any available iron from their environment and then move this nutrient inside the bacterial cells. Iron nutrient entry to the bacterial cells takes place through the pore, FtrC, which is a protein. This protein pore is like a closed door and to move the iron inside the bacteria, the door in FtrC pore needs to open. The energy for opening this FtrC pore gate is predicted to be derived from a special type of reaction (known as redox reaction) performed by FtrB. For FtrB to do this job, it binds another metal ion, copper, and helps in the redox reaction of iron. To test if this idea is correct, I performed experiments that conclusively showed that FtrB can stay bound to Cu^{+} and Cu^{2+}, the two important forms that can enable the iron redox chemistry to take place. I used specialized experiments, like isothermal titration calorimetry (ITC) and circular dichroism (CD) spectroscopy. These experiments are performed on solutions containing molecules like FtrB and copper ions. By monitoring how heat and light (two different forms of energy) interact with these molecules, I was able to “see” inside the molecular world and confirm that FtrB indeed binds to both Cu^{2+}, a mimic of Cu^{+}, and a mimic of Fe^{3+}. My work will pave the way for future scientists to do more experiments that will allow them to “see” the redox reaction between Cu^{2+}/Cu^{+} bound FtrB and other molecules.

Technical Abstract

FtrABCD is a four-component Fe^{2+} active transporter found in several Gram-negative pathogens, including *Brucella* and *Bordetella* spp. Co-expression of these proteins is essential for the survival of these organisms inside the host where these pathogens encounter iron-restricted environment due to host immune response. Based on protein homology and evolutionary data for FtrC, it is hypothesized that this permease uses the free energy (ΔG) derived from Fe^{2+} oxidation to perform the active transport of its cargo. A similar permease, Ftr1p, is found in yeast, and experiments have confirmed that Fe^{2+} oxidation is required for iron utilization by Ftr1p. In this yeast system, Fe^{2+} oxidation is done by a co-expressed multicopper oxidase (MCO), Fet3p. The proteins co-expressed with FtrC (FtrA, FtrB, and FtrD) are not MCOs and the soluble components, periplasmic FtrA and FtrB do not belong to any known families of redox proteins. Phylogenetic studies on FtrB and known single and multidomain copper redox proteins have shown that FtrB is evolutionarily related to Fet3p. Based on this, it was proposed that *Brucella abortus* FtrB is a novel and uncharacterized copper redox protein and can perform the following reaction—

$$Cu^{2+}\text{-FtrB} + Fe^{2+} \Leftrightarrow Cu^{2+}\text{-FtrB-}Fe^{2+}\ (aq) \rightarrow Cu^{+}\text{-FtrB-}Fe^{3+}\ (aq)$$

For FtrB to be an effective redox protein and perform the above reaction, it must bind Cu^{2+}, Cu^{+}, Fe^{2+}, as loss of Cu^{+} from FtrB will terminate its redox activity. As free Fe^{3+} is toxic and insoluble, it is also predicted that Fe^{3+} produced by Cu^{2+}-FtrB must stay bound to reduced FtrB until it gets recognized by the permease, FtrC, and is translocated. The ability of recombinant wild-type FtrB to bind Cu^{2+} and a Fe^{2+} mimic were determined by another undergraduate researcher from my lab. This thesis describes experiments

performed by me and were performed to establish if FtrB can stay bound to Cu^{+} and Fe^{3+}. To investigate if Cu^{+} and Fe^{3+} bind to FtrB, we used Ag^{+} and Ga^{3+} as mimics for Cu^{+} and Fe^{3+}, respectively. These mimics were chosen because Cu^{+} and Fe^{3+} are unstable and can oxidize quickly, making them difficult to work with directly. Ag^{+} and Ga^{3+} provide similar coordination chemistry and binding properties, allowing for accurate modeling of the interactions without these complications. Isothermal titration calorimetry (ITC) was performed by titrating Ag^{+} into apo-FtrB in water at neutral pH, showing strong binding (Kd ~ 3 μM, N ~1). Further ITC experiments with Ga^{3+} and Ag^{+}-FtrB revealed two distinct binding sites for Ga^{3+}. The first site showed a stoichiometry (n) of 2.844, a dissociation constant (Kd) of (1.208 ×10-5) M, a positive change in enthalpy (ΔH) of 28.94 kcal/mol, and a substantial increase in entropy (ΔS) of (1.196 × 102) cal/mol·K. The second site exhibited a stoichiometry (n) of 10, a dissociation constant (Kd) of (1 ×10-3) M, a negative change in enthalpy (ΔH) of -8.378 kcal/mol, and a decrease in entropy (ΔS) of -14.37 cal/mol·K. These findings enhance our understanding of the metal-binding properties of FtrB and its role in ion homeostasis, emphasizing the importance of these interactions for the protein's biological function.

To determine if recombinant wild-type FtrB can bind Cu^{2+} specifically, I performed circular dichroism (CD) spectroscopy on as isolated wild-type FtrB as it is exposed to other divalent transition metal ions during its expression and purification. I used the signature Cu^{2+}-His CD peaks to establish the presence of Cu^{2+} in as isolated wild-type FtrB.

Key words: *Brucella abortus iron-transport,* FtrABCD, ferrous-oxidase, FtrB, Cu^{+} mimic binding, ITC, CD

Chapter 1: Introduction

Introduction

Iron plays a vital role in numerous biological functions, such as DNA synthesis, respiration, and metabolism.[1] The mechanisms of iron transport and regulation are fundamental to the survival and pathogenicity of various bacteria. In iron-limited environments, particularly within a host organism, bacteria must efficiently acquire iron to thrive.[2] Therefore, the ability to transport and utilize iron effectively is crucial for bacterial survival and virulence. Among the mechanisms bacteria use to manage iron, the FtrABCD system, found in several Gram-negative pathogens like *Bordetella*, *Brucella*, and *Burkholderia*, plays a significant role in iron transport. Recent studies have shown that the *ftrABCD* genes in *Brucella* are similar to those in *Bordetella*, suggesting a conserved mechanism across different species.[3] This similarity indicates that these proteins are genetically and functionally alike, aiding in iron transport. Understanding this conserved mechanism is crucial as it highlights potential targets for antimicrobial strategies across multiple pathogens.

The functions of the FtrA, FtrB, FtrC, and FtrD proteins are predicted based on their structural and functional similarities to known iron transport systems. FtrA is a periplasmic protein that binds iron with the help of copper, showing extensive amino acid conservation with other P19-type proteins known for metal-binding capabilities.[3,4] The conserved Cu^{2+}-binding residues in FtrA are crucial for maintaining its stability and functionality, essential for iron transport.[3] Experimental evidence has shown that Cu^{2+} binding stabilizes FtrA, facilitating iron binding and transport.[4]

FtrB, a small periplasmic protein, works alongside FtrA, FtrC, and FtrD, especially under low iron and acidic conditions, and is essential for iron transport in *Bordetella* and

Burkholderia.[3,4] FtrC, a membrane protein, belongs to the oxidase-dependent ferrous iron transporter (OFeT) family and is involved in iron binding and translocation, with conserved motifs crucial for Fe^{3+} binding and transport.[5] FtrD, an integral membrane protein with ferredoxin domains, likely acts as an electron sink during Fe^{2+} oxidation, facilitating iron transport by interacting with FtrB.[3] The electrons required for this reduction could be donated by FtrA or FtrB, which are part of the broader electron transport chain within the cell.

Based on protein homology and evolutionary data, it is hypothesized that Fe^{2+} utilization through this transporter requires iron oxidase activity by one of the components, potentially FtrB, a novel cupredoxin. Significant progress has been made in understanding this system, including insights into the binding properties and functional roles of FtrA and FtrB. Notably, research has demonstrated that the ftrABCD genes are regulated under acidic pH and low iron concentration, suggesting that these gene products are involved in iron uptake. Studies on Brucella abortus 2308 have shown that FtrABCD is responsible for non-heme Fe^{2+} transport, rather than Fe^{3+}. Furthermore, the FtrABCD system operates independently of energy derived from TonB or ATP. The expression of FtrA in *Brucella abortus* 2308 is crucial for infection and survival in a mouse model.[6] These findings collectively underscore the vital role of these proteins in Fe^{2+} utilization, emphasizing their significance in the iron transport system of *Brucella*.

The FtrABCD system is a four-component Fe^{2+} transporter found in several Gram-negative pathogens, including *Brucella* spp. Co-expression of these proteins is essential for the survival of these organisms inside the host in an iron-restricted environment.[6,7] This observation suggests that each protein in this four-component system has some

essential and non-redundant role. Research has demonstrated that a mutant *Brucella* lacking the *ftrA* gene has trouble utilizing iron and growing in a mouse model, highlighting the importance of these genes in bacterial metabolism.[6]

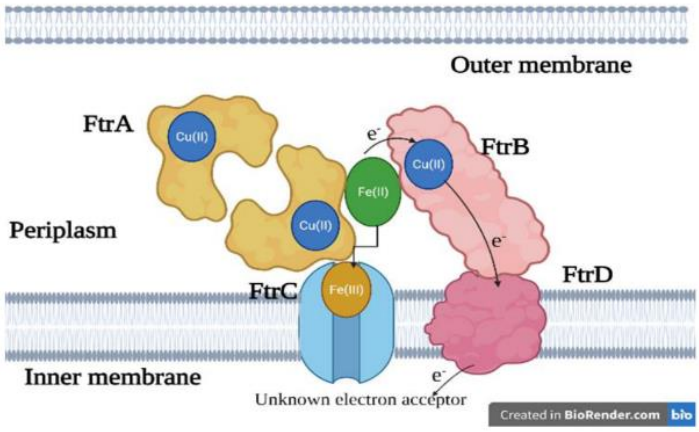

Figure 1: Schematic representation of the FtrABCD system. The figure shows the structural organization and location of each protein within the system. FtrA is a periplasmic protein that binds iron with the help of copper. FtrB, also a periplasmic protein, is hypothesized to have iron oxidase activity. FtrC is a membrane-spanning protein involved in iron translocation, and FtrD is an integral membrane protein with ferredoxin domains, likely acting as an electron sink during Fe^{2+} oxidation.

FtrC, one of the proteins encoded by these genes, stands out because it doesn't use ATP like other iron transporters such as ABC transporters. Instead, FtrC uses specific motifs (ArgGluxxGlu) to bind and transport Fe^{3+} (ferric iron). It is homologous to yeast Fet3p, and both belong to the OFeT family of proteins. Experimental data has confirmed that Fet3p does not require ATP-derived energy for iron translocation. Instead, it uses the free energy released during Fe^{2+} oxidation by its co-expressed protein, Fet3p, to translocate the Fe^{2+}.[8] Similarly, FtrC can work independently, possibly with an unknown

redox protein, which allows it to adapt to varying iron conditions. In addition to its homology with Ftr1p, FtrC also conserves two ArgGluxxGlu motifs. In Ftr1p, these motifs are essential for Fe^{3+} translocation. While direct experimental evidence for FtrC's use of these motifs is lacking, the conservation of ExxE motifs between FtrC and Ftr1p suggests a similar mechanism of iron binding and transport. Based on these similarities, it is proposed that FtrC will function in the same way as Ftr1p, utilizing these conserved motifs for iron binding and transport.

Although it is predicted that FtrC will also need a ferrous oxidase, FtrA, FtrB, or FtrD do not belong to any known ferrous oxidase family. A phylogenetic study and the FtrB evolution paper[3] predicted that FtrB is the missing ferrous oxidase:

$$Cu^{2+}\text{-FtrB-}Fe^{2+}\ (aq) \rightarrow Cu^{+}\text{-FtrB-}Fe^{3+}\ (aq)$$

Before working on this project, previous research demonstrated that wild-type FtrB binds to Cu^{2+} and Mn^{2+} (a Fe^{2+} mimic). As the ferrous oxidase activity requires the enzyme to stay bound to Cu^{+}, my work involves investigating Ag^{+} binding to wild-type FtrB. I also performed ITC to see if Fe^{3+} can stay bound to Cu^{+}-FtrB, as the release of Fe^{3+} in solution can be toxic. Finally, I used visible CD to determine if the as-isolated wild-type FtrB has Cu^{2+} bound to it, using the conserved H121 residue, as the CD signal due to His(N) bound Cu^{2+} d-d transition is known.

Understanding how bacteria transport iron has significant implications for developing new treatments. Iron is a critical nutrient for bacterial pathogens, and their ability to acquire it from the host environment is essential for their survival and virulence. While the FtrABCD system is known to play a crucial role in iron transport, much remains

to be discovered about the specific functions and mechanisms of each component, particularly FtrB. This work aims to address these gaps by investigating the interactions of FtrB with Cu^{+} and Fe^{3+} using isothermal titration calorimetry (ITC) and circular dichroism (CD) spectroscopy. These techniques will help determine whether FtrB functions as a ferric oxidase, a key question in understanding its role within the FtrABCD system. By gaining insights into the functions of FtrA, FtrB, FtrC, and FtrD, we can better understand bacterial iron metabolism and identify potential targets for antimicrobial therapy.

Chapter 2: Materials and Methods

Research questions

As stated in Chapter 1, the Cu^{2+} and Mn^{2+} (a Fe^{2+} mimic) affinities of recombinant wild-type FtrB were already determined by a previous undergraduate in my lab. The aim of my work was to investigate if recombinant wild-type FtrB was able to bind Cu^{+} and Fe^{3+}; and if the observed Cu^{2+} binding to recombinant wild-type FtrB was specific. For FtrB to act as an efficient ferrous oxidase enzyme, it not only needs to bind Cu^{2+} and Fe^{2+} and oxidize the bound Fe^{2+}, but the protein also needs to stay bound to Cu^{+} to continue the enzymatic process. The ability of FtrB to stay bound to Cu^{+} is also important given Cu^{+} is extremely toxic for all living organisms and release of this metal ion after the ferrous oxidase activity can cause detrimental effects. Lastly, I wanted to confirm that the observed Cu^{2+} binding to the wild-type FtrB was specific, that is FtrB can selectively bind to Cu^{2+} even when other similar +2 charged metal ions are present in the medium. For the binding experiments, I used isothermal titration calorimetry (ITC) as it is a well-established method to study protein-ligand interactions, and our lab has access to the departmental TA Instruments Nano ITC machine. Additionally. ITC is an important method also used by pharmaceutical companies to determine drug-drug target interaction and as a prospective medical student, I am interested in learning this method. I used circular dichroism (CD) on as isolated wild-type FtrB to determine its specificity towards Cu^{2+}. I chose this method for the simplicity of instrument operation and data analysis as well as the known d-d band from Cu^{2+} bound to a His sidechain from the literature. *Brucella abortus* FtrB is predicted to bind to Cu^{2+} using a conserved His residue (H121) and conducting CD experiment allowed me to make conclusion about the specificity of this transition metal ion towards the protein. The rationale behind using as isolated FtrB was

that the *E. coli* strain containing the *ftrB* gene (on a vector) was grown using media that contained trace amounts of all essential metal ions and if the CD spectra still showed that FtrB was bound to Cu^{2+}, that would indicate specificity.

Materials and Methods

All buffers used in this work were prepared using the highest purity grade ingredients, as specified by the protocol, and adjusted to the desired experimental conditions with a pH meter (Fisher). For the metal solutions, I weighed out a precise gram amount to create stock concentrations. All experiments were conducted using deionized water from an 18 MΩ Milli-Q water system. The experimental design and data analysis methods for each experiment are detailed in the sections below.

Cloning, expression, and purification of FtrB proteins

The *ftrb* gene was successfully cloned into a pET-28a (+)-TEV vector, resulting in the plasmid pET28-FtrB. E. coli BL21 cells were transformed with this plasmid and cultured in Terrific Broth containing 40 µg/mL kanamycin at 37°C to ensure selective growth of the transformed cells. When the culture reached an absorbance of 1.2, indicating that the cells had reached the desired density (OD 600), protein expression was induced by adding 2 mM IPTG. This induction was carried out to maximize protein production. After 4 hours of induction, the cells were harvested by centrifugation and stored at -80°C to preserve the expressed protein. The cells were then resuspended in His-wash buffer (50 mM sodium phosphate, 300 mM sodium chloride, 10 mM imidazole, pH 7.4) to maintain a stable environment for the protein. They were lysed using sonication, with PMSF added to inhibit proteases and protect the protein during lysis. The

lysate was centrifuged to separate the soluble protein from cell debris, and the supernatant was applied to a HisPur cobalt column for affinity purification of His-tagged FtrB. Before applying the supernatant, the column was washed with autoclaved DI water to remove any residual ethanol, which could interfere with protein binding, followed by His-wash buffer to equilibrate the column.

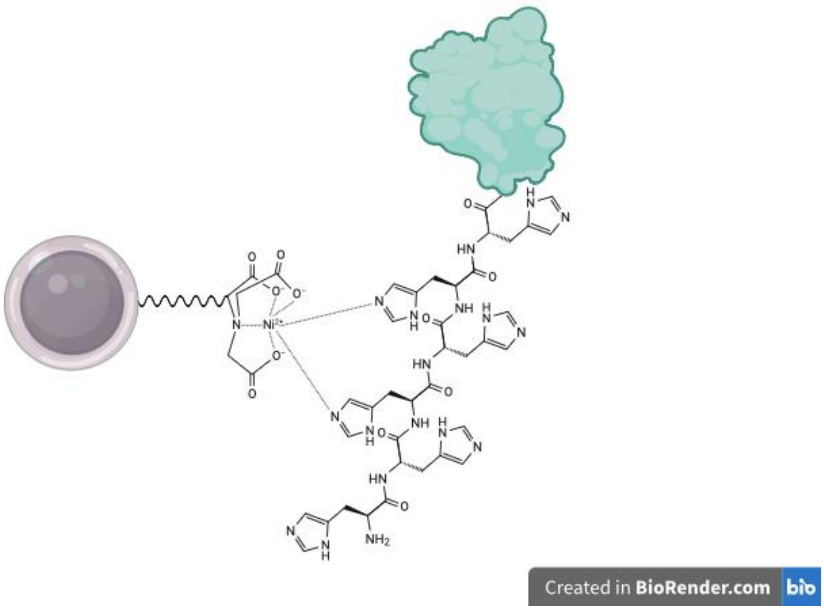

Figure 2. *This figure demonstrates how a His tag (shown in green), which can be genetically engineered at the N-terminus, binds to metal ions in the stationary phase of the chromatography column. In my experiment, I used* Co^{2+}*, although the figure shows* Ni^{2+} *(shown in pink/gray). Both metals work through the same mechanism, effectively binding the His-tagged proteins for purification.*

After applying the supernatant, the flowthrough was collected. The column was then washed with a small amount of His-wash buffer to remove any non-specifically bound proteins. Since the protein has a His tag, it binds strongly to the cobalt column, allowing for selective purification. The protein was eluted using His-Elution Buffer (50 mM sodium

phosphate, 300 mM sodium chloride, 100 mM imidazole, pH 7.4), which competes with the His tag for binding to the cobalt ions. The eluted protein was dialyzed in TEV cleavage buffer (50 mM Tris HCl, 0.5 mM EDTA, and 1 mM DTT at pH 8) to remove imidazole, as TEV protease activity is inhibited by imidazole. This step was crucial to ensure efficient cleavage of the His-tag. Following dialysis, TEV protease digestion was performed to remove the His-tag. The tag-free protein was then applied to the column, now equilibrated with TEV cleavage buffer containing 10 mM imidazole to improve yield by preventing non-specific binding. The flowthrough was collected, containing the purified FtrB protein without the tag.

SDS-PAGE GEL

This technique is used to separate proteins based on their molecular weight. To ensure that the proteins are in a uniform shape and carry a uniform negative charge, they are first denatured. This step is crucial as it allows the proteins to be separated solely based on their size during electrophoresis. After purification, the samples were mixed with SDS loading buffer and heated. Heating the samples denatures the proteins, ensuring they lose their natural structure and are coated with SDS, which imparts a uniform negative charge. This uniform charge is essential for the accurate separation of proteins by size. The samples were then loaded onto a polyacrylamide gel and subjected to electrophoresis. During this process, smaller proteins migrate further down the gel compared to larger ones, due to their size. Following electrophoresis, the gel was stained with a staining buffer to visualize the protein bands. The presence of FtrB was confirmed by comparing the pattern of bands to a known molecular weight standard or ladder. This

comparison is vital to accurately identify the protein of interest among the separated bands.

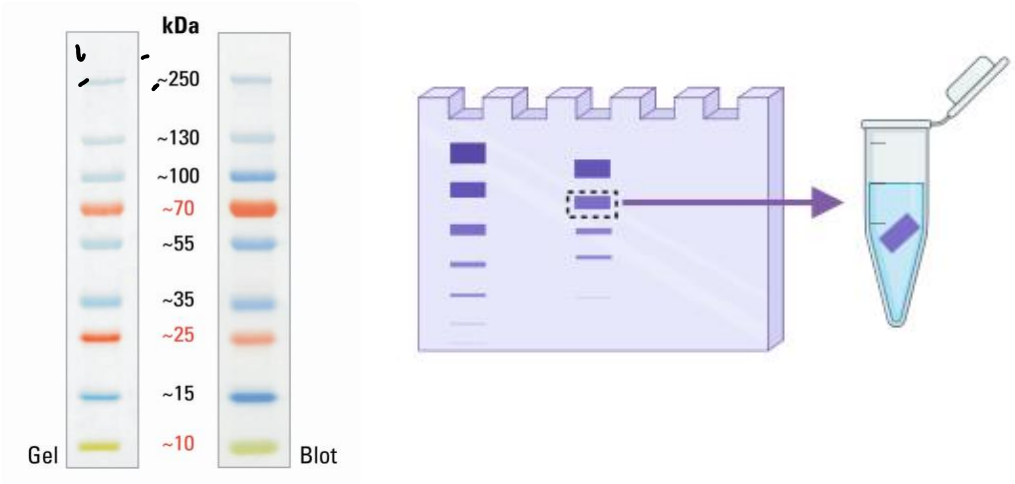

Figure 3. *This figure shows how SDS-PAGE separates proteins based on their molecular weight. The process involves denaturing the proteins to eliminate any charge, ensuring that all samples separate purely by mass. The image highlights a sample tube with a visible protein band, which is then shown on the gel. By using a molecular weight ladder (shown on the left), we can identify which band corresponds to our protein of interest, confirming its size.*

Size Exclusion Chromatography (SEC)

To purify FtrB, which has a molecular weight of approximately 10 kDa, we used Size Exclusion Chromatography (SEC). This technique separates proteins based on their size, which was essential for distinguishing FtrB from larger proteins that are co-expressed during synthesis. In our study, SEC was crucial for isolating FtrB from these larger proteins. Every time we synthesized FtrB, larger proteins were also present,

making SEC an indispensable part of our purification process. We used an SEC column specifically designed for protein purification, which effectively separated small proteins like FtrB from larger contaminants. The process involved preparing the protein sample and loading it onto the column. As the sample passed through the column, smaller proteins like FtrB took longer to elute, while larger proteins eluted earlier. We collected fractions and analyzed them using UV-Vis spectroscopy to measure absorbance at 280 nm, which helped us identify the fractions containing FtrB. Given its smaller size, FtrB was typically found in the later fractions. One of the great things about SEC is that it generates a graph, known as a chromatogram, which visually shows where your protein is eluting. This graph was incredibly helpful in pinpointing the exact fractions that contained FtrB. This method ensured that our final FtrB sample was pure and ready for subsequent analyses, including spectrophotometric and ITC studies. Achieving high purity was crucial for accurately studying the properties and interactions of FtrB without interference from other proteins.

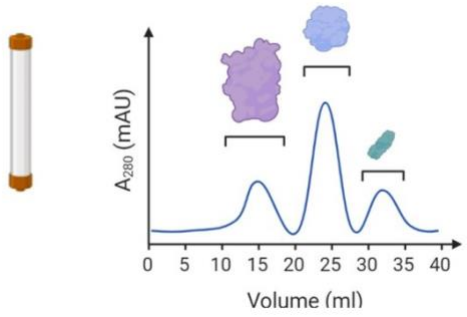

Figure 4. *This graph illustrates the peaks of different molecules separated based on their mass. The smallest molecule, which corresponds to the last peak, elutes last. This visual representation helps us identify the elution profile of our target protein, FtrB.*

Circular dichroism (CD)

We used Circular Dichroism (CD) to determine the secondary structure of the protein and to check for the presence of Cu^{2+} ions in the isolated protein. When proteins fold, they can exist in a chiral environment, which allows them to preferentially absorb left or right polarized light. By measuring the difference in absorbance of polarized light in the 300 to 190 nm range, we can detect the average secondary structure of the protein. Proteins with predominantly beta-sheet structures show a strong negative CD signal around 220 nm, while those with alpha-helix structures show two negative bands at 225 nm and 210 nm.[9] We used CD spectroscopy to verify if the predicted structure of FtrB matches its experimental solution structure. Our lab's predicted structure of FtrB indicates the presence of beta strands. If this structure is retained in solution, we would expect to see a single negative band around 220 nm. The CD experiment was conducted using a CD spectrometer equipped with a Peltier system to control temperature and a 10 mm path length quartz cuvette. To reduce noise from the buffer, the protein was dialyzed against sodium phosphate buffer at pH 7.3 without any salt. Typically, the protein concentration in the CD was around 10 μM, with scans performed in the 300 to 190 nm range. We averaged 8 scans for each sample, and a blank buffer run was performed before each protein scan to create a buffer-corrected CD spectrum.

$$[\theta] = 100\frac{signal}{CnI}$$

Molar ellipticity normalizes the CD data to account for protein concentration and cuvette path length, providing consistent and comparable measurements. This ensures accurate interpretation of the protein's secondary structure across different samples.

Lastly, previous studies have indicated that Cu^{2+} bound to the His side chain has characteristic CD bands in the d-d electronic transition region (400-800 nm) and the presence of such a band can be used to determine if a protein is bound to Cu^{2+} using a His side chain.[10] Experimental and homology data reported by others in my lab have shown that the conserved FtrB residue, H121, is involved in Cu^{2+} coordination. So, I performed CD in the d-d range to detect the presence of Cu^{2+}-His bond in the as isolated wild-type FtrB.

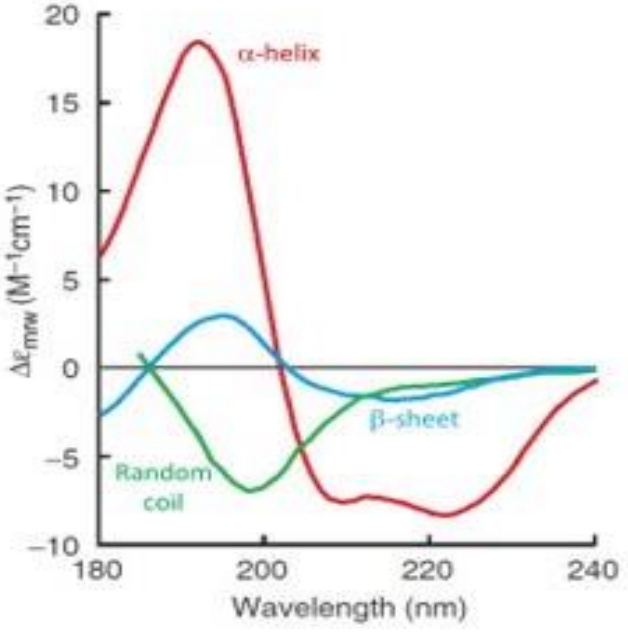

Figure 5. *This graph illustrates the CD spectra of different secondary structures. Alpha-helices are characterized by two negative bands at 225 nm and 210 nm, while beta-sheets show a strong negative signal around 220 nm. Random coils typically display a negative band near 195 nm. By comparing these patterns, we can determine the secondary structure of our protein, FtrB, and confirm its folding in solution*.

Isothermal Calorimetry Titration (ITC)

We used Isothermal Titration Calorimetry (ITC) to investigate the formation of the Ag^+-FtrB complex and to see if Ga^{3+} could integrate into this complex to form a tertiary complex.

The reactions studied were:

$$Ag^+ (aq) + FtrB (aq) \Leftrightarrow Ag^+\text{-}FtrB (aq), K = \frac{[Ag^+ - FtrB]}{[Ag^+][FtrB]}$$

$$Ga^{3+} (aq) + FtrB (aq) \Leftrightarrow Ga^{3+}\text{-}FtrB (aq), K = \frac{[Ga^{3+} - FtrB]}{[Ga^{3+}][FtrB]}$$

$$Ga^{3+} (aq) + Ag^+\text{-}FtrB (aq) \Leftrightarrow Ga^{3+}\text{-}FtrB\text{-}A^{g+} (aq), K = \frac{[Ag^+ - FtrB - Ga^{3+}]}{[Ga^{3+}][Ag^+ - FtrB]}$$

Based on the homology model of wild-type FtrB published by us, it is predicted that Cu^{2+}/Cu^+ binds to this protein using the conserved residues, D118 and H121, and $Fe^{2+/}Fe^{3+}$ binds using E84, E86, and E95 residues. The Cu^{2+}:FtrB stoichiometry determined by our lab is ~1:1, and as I anticipate the Cu^+ mimic (Ag+) to bind in that same location, the Ag^+:FtrB stoichiometry is also predicted to be 1:1. The equilibrium constants (K) and the enthalpy change of the above reactions (ΔHrecation) are directly obtainable from ITC experiments and the data fitting software can convert the K value to ΔG using the relationship, ΔG =-RT*ln*K. Finally, knowing the ΔG and ΔH, allows the calculation of ΔS also possible. I performed the last experiment (titration of Ga^{3+} into Ag+-FtrB) as previous studies from my lab has shown that the Fe^{2+} mimic could only bind wild-type FtrB when it was already bound to Cu^{2+}.

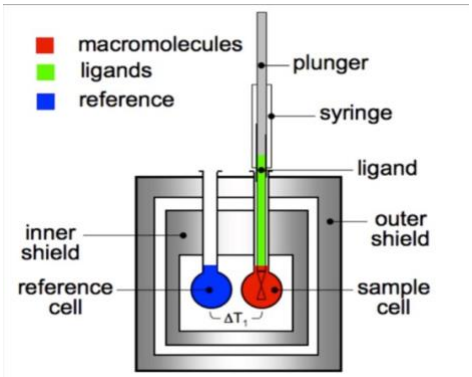

Figure 6. *This illustration shows the key components of the ITC instrument, including the reference and sample cells, plunger, syringe, and ligand. These parts work together to measure the heat changes during molecular interactions, providing valuable insights into binding events.*

A schematic representation of an ITC instrument is shown in Figure 6, and shows two equivalent compartments, the sample and the reference cells. A small molecule/ligand is injected into the sample cell incrementally, usually filled with a solution of the macromolecule, and the hardware of the instrument uses electrical power to maintain both cells at the same temperature (isothermal) throughout. If addition of a small molecule into the sample compartment causes a small heat to change due to reaction, electrical power is sued to compensate for this extra heat and is recorded by the software of the instrument (μwatt). During data processing, this electrical data is converted to heat data (μcal) and is plotted against the concentration of ligand added. The data analysis program can integrate the heat due to each ligand addition step and this integrated heat vs ligand concentration data is used to determine the different binding parameters for the reaction taking place in the sample cell. For good ITC data that can be analyzed, it is crucial that the buffer/solvent used to dissolve the macromolecule (FtrB in this case) is

identical to the solvent used to dissolve the ligand (Ag^+ and Ga^{3+} in this case). As Ag^+ is insoluble in buffers containing Cl^- (forms solid AgCl) and dissolved CO_2, I used a boiled degassed deionized water (DI water) to dissolve the metal ion and the protein (the DI water was boiled to drive out any dissolved CO_2 and then was allowed to cool in an airtight container before adding this to make the solutions.

First, I titrated Ag^+ into FtrB to assess complex formation. If the Ag^+-FtrB complex formed, we then titrated Ga^{3+} into this complex to observe the potential formation of a tertiary complex. To avoid precipitation of Ag^+, the ITC experiment was performed in degassed deionized water without any buffer present. This step was crucial to maintain the solubility of Ag^+ and ensure accurate measurements. I used a Nano ITC instrument from TA Instruments, equipped with a high-sensitivity calorimeter. The Ag^+-FtrB complex was loaded into the cell, and Ga^{3+} was incrementally titrated into this complex. Throughout the titration process, I recorded the heat changes, which provided detailed data on the interactions and the potential formation of a tertiary complex. This method allowed me to precisely measure the binding interactions and understand the thermodynamic properties of the complexes formed.[11] The data obtained from ITC were essential for confirming the formation of the Ag^+-FtrB complex and exploring the integration of Ga^{3+} into this complex.

Chapter 3: Results and Discussion

Results and Discussion

To fully understand the behavior and characteristics of wild-type FtrB, we applied a series of analytical techniques. This section summarizes the key findings from these methods.

Structural Integrity and Thermal Stability of Wild-Type FtrB

The expressed FtrB was buffer exchanged into 10 mM phosphate buffer at pH 7.4 using a 3 kDa MCO spin filter. SDS-PAGE analysis confirmed the presence of FtrB with some impurities. The stock concentration was 302 μM, and the protein in the CD cuvette was diluted to 60.4 μM. Absorbance at 280 nm was measured at 0.0015, confirming the stock concentration. CD scans from 190-300 nm showed a negative band most intense at 217 nm (Figure 7), indicating a well-defined secondary structure in WT FtrB. The CD data acquired by me was compared with the CD data obtained by two other previous students from our lab and all CD experiments on recombinant wild-type FtrB showed identical nature. This showed that the protein purified by me had the same global secondary structure as obtained by others in my lab. This is important as previous students performed the Cu^{2+} and Mn^{2+} binding studies and I wanted to make sure the protein secondary structure did not alter between their and my preparations.

Previous students tried to determine the folding-unfolding energy for wild-type FtrB using differential scanning calorimetry (DSC) in different buffers (ACES, Bis-Tris, HEPES) but no DSC signal was observed for this protein. Stability of protein structure is dependent on the composition of buffer and the pH of the solution due to the hydration ⇔ dehydration

equilibrium of the buffer components as well as proteins in the presence of different buffers and might affect DSC data.[12]

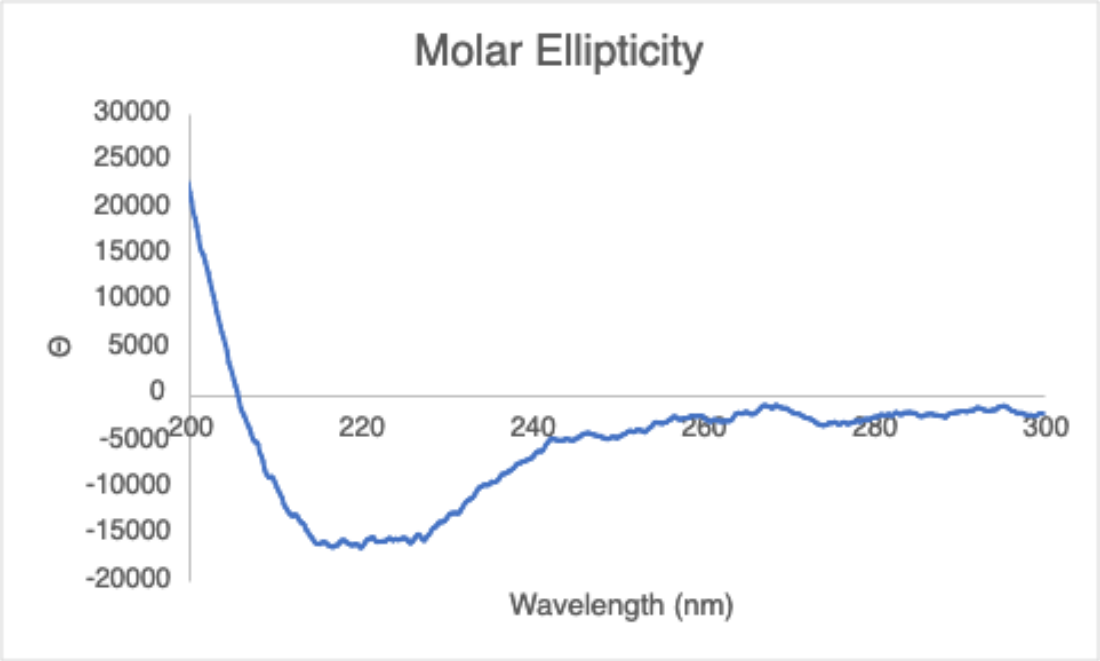

Figure 7. The spectrum displays a distinct negative peak at 217 nm, recorded at a temperature of 25°C. This peak is indicative of the protein's secondary structure, providing insights into its conformational properties under the given conditions.

I used CD to monitor changes in the protein global secondary structure as a function of temperature. Thermal denaturation studies were conducted by increasing the temperature from 25°C to 75°C at 2°C/min in the first trial, and from 40°C to 110°C at 2°C/min in the second trial. The average intensity at 217 nm during the reheating scan was lower than during the initial scan (Figure 8), suggesting potential alterations in the secondary structure upon heating. Although this study did not provide the melting temperature or $\Delta H_{folding}$, it showed that increasing the temperature above 58 ◦C had irreversible and detrimental effect on protein global secondary structure. These structural

studies are crucial because they help us understand the stability and folding properties of WT FtrB, which are essential for its biological function.

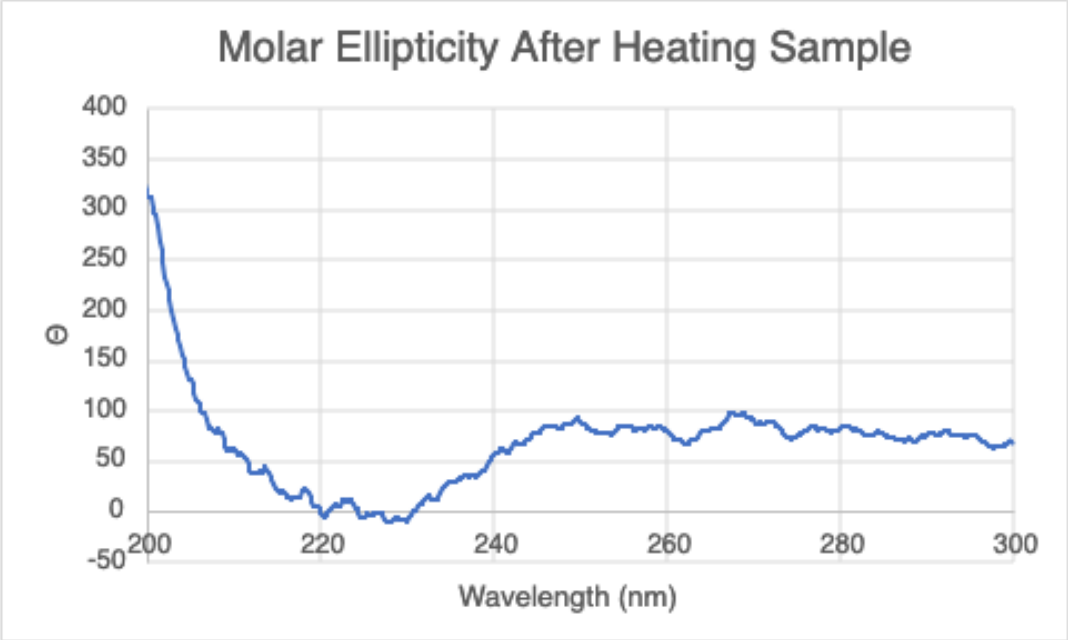

Figure 8. *This graph represents the average CD scan intensity as the temperature of the protein was increased. The temperature was raised from 40°C to 110°C at a rate of 2°C per minute, and from 25°C to 75°C at the same rate. These scans provide valuable insights into the thermal stability and structural changes of recombinant wild-type FtrB under varying temperature conditions.*

Knowing how the protein behaves under different conditions can inform future experiments and potential applications, such as drug design or industrial processes. These results highlight the importance of maintaining consistent experimental conditions for reliable CD measurements. Additional thermal denaturation experiments with controlled buffer conditions could provide more insights into the stability and folding properties of WT FtrB. The observed changes in CD intensity can be attributed to factors

such as protein concentration changes and structural alterations. To maintain consistent conditions during thermal denaturation studies, it is crucial to use sealed cuvettes to prevent evaporation, ensure controlled heating rates, use the same buffer composition, accurately measure protein concentration, perform replicate measurements, calibrate the temperature control system, and normalize CD data. These strategies help ensure that observed changes in CD intensity are due to the protein's response to temperature.

Specificity of Cu^{2+} Binding to FtrB Using CD

Copper ions (Cu^{2+}) are vital cofactors in many biological processes, including the enzymatic activity of FtrB. These ions facilitate redox reactions by cycling between oxidation states (Cu^{+} and Cu^{2+}), which is crucial for the function of numerous enzymes. The binding of Cu^{2+} to FtrB likely influences its structural stability and catalytic properties. Although previous undergraduate researchers from my lab showed that Cu^{2+} can bind to recombinant wild-type FtrB (Table 1), I wanted to determine if this Cu^{2+} binding was specific. That is, if FtrB was present in a solution that contained Cu^{2+} and other di-valent metal ions, such as Mn^{2+}, Zn^{2+}, Co^{2+}, etc., it still preferentially bound to Cu^{2+}. This is important as Cu^{2+} is extremely toxic for bacteria and there is no free Cu^{2+} ion present in the bacterial system. So, Cu^{2+} binding to FtrB must be specific enough that this protein can detect any small concentration of this metal in the periplasm and bind it efficiently. *E. coli* BL21 cells containing the *ftrB* gene (in a plasmid) were grown using LB or terrific broth, both containing Mn^{2+}, Zn^{2+}, Co^{2+}, etc., in addition to Cu^{2+}.[13] Cu^{2+} bound to a His side chain shows characteristic d-d bands which show up in the CD spectra of in the d-d region (400-800 nm).[10] The structural homology of FtrB and ITC experiments on H121A mutant FtrB both suggest that this conserved residue in FtrB is involved in Cu^{2+} binding.

So, if the as isolated wild-type FtrB was specific for Cu^{2+}, it will still show Cu^{2+} bound to this protein and this can be determined by performing CD spectroscopy on the as isolated protein in the d-d region. To explore this, we used Circular Dichroism (CD) spectroscopy to detect Cu^{2+} binding in FtrB. When CD scans were performed on as isolated wild-type FtrB in the d-d range, indeed, signature Cu^{2+}-His peaks in the 400-580 nm range were observed (Figure 9).[10] The presence of these negative CD bands at 560, 545, 527, and 500 nm due to d-d transition from Cu^{2+} bound to His (H121) is significant as it proves that the as isolated wild-type protein was able to differentiate between Cu^{2+} and other divalent metal ions from the growth media.

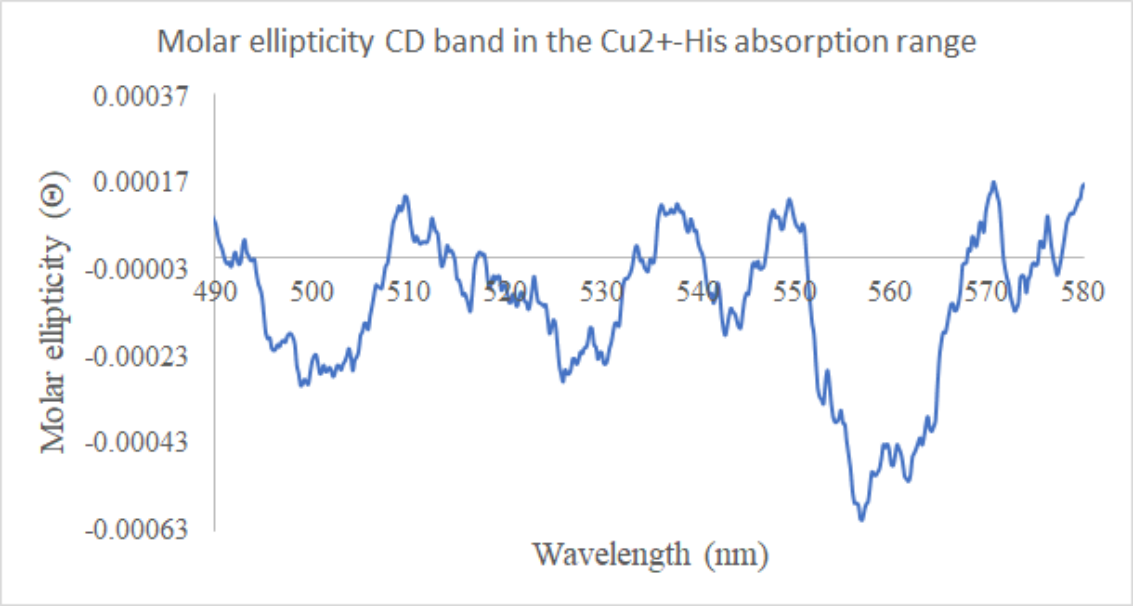

Figure 9. *The spectrum reveals distinct Cu^{2+}-His signature peaks in the 400-580 nm range. These peaks indicate the presence of copper ions bound to histidine residues in the FtrB protein, providing insights into the metal-binding properties and structural stability of FtrB. The* negative CD bands at 560, 545, 527, and 500 nm are due to d-d transition from Cu^{2+} bound to His (H121).

Evaluation of Ag^{+} and Ga^{3+} Binding to FtrB Using ITC

The isothermal titration calorimetry (ITC) experiment aimed to investigate the binding interactions between Ag^{+} and the protein FtrB. The resulting thermogram indicated an exothermic reaction, suggesting that the binding process releases heat. The protein concentration was maintained at 0.05 mM, while the Ag^{+} concentration was 0.8 mM.

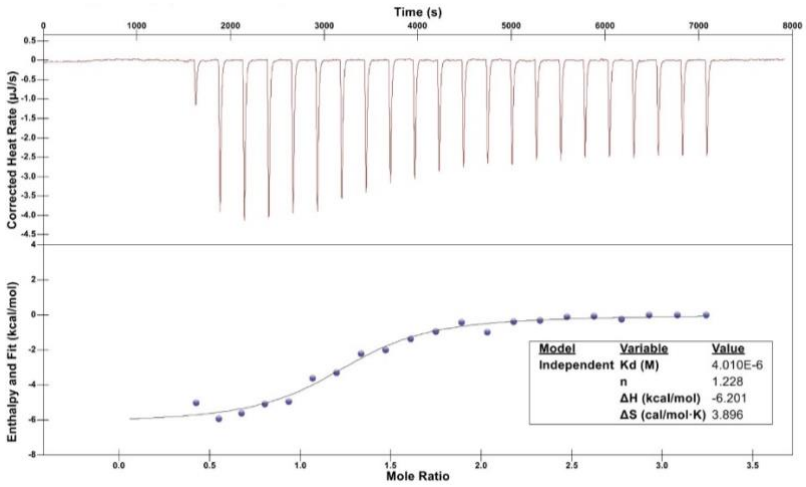

Figure 10: ITC Thermogram of Ag^{+} Binding to FtrB. *The figure shows the ITC thermogram for the binding of Ag^{+} to the protein FtrB. The top panel displays the raw data, with each peak representing a single injection of Ag^{+} into the protein solution. The exothermic nature of the interaction is evident from the downward peaks, indicating heat release upon binding. The bottom panel shows the integrated heat data fitted to an independent model, providing the thermodynamic parameters such as the dissociation constant (K_d), stoichiometry (n), change in enthalpy (ΔH), and change in entropy (ΔS). The high affinity and specific binding interaction between Ag^{+} and FtrB are clearly demonstrated by the fitted curve*.

The ITC data provides clear evidence that Ag^+ binds to the protein FtrB (Figure 10 and Table 1). The exothermic nature of the interaction, as indicated by the thermogram, confirms that the binding process is energetically favorable and releases heat. This suggests a strong and spontaneous interaction between the metal ion and the protein. The dissociation constant (K_d) of (4.010×10^{-6}) M reflects a high affinity between Ag^+ and FtrB. This low μM K_d value indicates that the metal ion binds strongly to the protein, which is crucial for understanding the biological relevance of this interaction. A high affinity suggests that Ag^+ can effectively compete with other potential ligands in the cellular environment, ensuring that it binds preferentially to FtrB. The stoichiometry (n) value of 1.228 suggests that there is roughly one binding site per protein molecule. This value being slightly above one might be due to experimental conditions or minor secondary binding sites. Essentially, this means that each FtrB protein molecule can bind to one Ag^+ ion, forming a stable complex. The change in enthalpy (ΔH) of -6.201 kcal/mol indicates that the binding interaction is exothermic, releasing heat. This negative ΔH value suggests that the interaction is energetically favorable, with the formation of the Ag^+-FtrB complex being driven by enthalpic contributions. The release of heat upon binding is indicative of strong interactions, such as hydrogen bonds or ionic interactions, between the metal ion and the protein. The change in entropy (ΔS) of 3.896 cal/mol·K indicates an increase in disorder upon binding. This positive ΔS value could be attributed to the release of water molecules or other ligands from the binding site, which increases the overall disorder of the system. The increase in entropy, combined with the favorable enthalpic change, provides a comprehensive thermodynamic profile of the binding interaction. The positive entropy change suggests that the binding process is also driven by entropic

contributions, likely due to the displacement of ordered water molecules from the binding interface. In summary, the ITC data clearly demonstrates that Ag^{+} binds to FtrB with high affinity and specificity. The binding is characterized by a favorable enthalpic change and an increase in entropy, suggesting a well-defined and stable interaction. These findings enhance our understanding of metal-protein interactions and may have significant implications for the biological function of FtrB.

Binding of Ga^{3+} to FtrB-Ag^{+} Complex

The isothermal titration calorimetry (ITC) experiment was conducted to investigate the binding interactions of Ga^{3+} with the FtrB-Ag^{+} complex. The thermogram obtained from this experiment showed endothermic peaks, indicating that the binding process absorbs heat. The data was analyzed using a multiple site model, revealing two distinct binding sites with different thermodynamic parameters.

The ITC data (Figure 11, Table 1) for the binding of Ga^{3+} to the FtrB-Ag^{+} complex provides a contrasting picture compared to the binding of Ag^{+} alone. The endothermic nature of the interaction, as indicated by the thermogram, suggests that the binding process absorbs heat, which is a notable difference from the exothermic binding observed with Ag^{+}. For the first binding site, the stoichiometry (n) value of 2.844 indicates that nearly three Ga^{3+} ions bind to each FtrB-Ag^{+} complex. This suggests a higher capacity for Ga^{3+} binding at this site. The dissociation constant (K_d) for this site is (1.208×10^{-5}) M, which is higher than the Kd for Ag^{+} binding, indicating a lower affinity for Ga^{3+} at this site. The positive change in enthalpy (ΔH) of 28.94 kcal/mol indicates that the binding process requires a significant amount of energy, which is absorbed from the surroundings. The

substantial increase in entropy (ΔS) of (1.196×10^2) cal/mol·K suggests that the binding process is driven by entropic contributions, likely due to the displacement of ordered water molecules or other ligands. For the second binding site, the stoichiometry (n) value of 10 indicates that this site can accommodate a larger number of Ga^{3+} ions. The dissociation constant (K_d) for this site is (1×10^{-3}) M, which is significantly higher than the K_d for the first site, indicating an even lower affinity for Ga^{3+}. The negative change in enthalpy (ΔH) of -8.378 kcal/mol indicates that this binding process releases heat, which is a stark contrast to the first site. The decrease in entropy (ΔS) of -14.37 cal/mol·K suggests that the binding process at this site is driven by enthalpic contributions, possibly involving stronger interactions such as ionic bonds. These differences in thermodynamic parameters between the two binding sites suggest that Ga^{3+} interacts with the FtrB-Ag^+ complex in a more complex manner compared to Ag^+ alone. The presence of multiple binding sites with varying affinities and thermodynamic profiles indicates that Ga^{3+} binding is more heterogeneous. The endothermic peaks for the first site suggest that the binding process is driven by entropic contributions, while the exothermic nature of the second site suggests that the binding is driven by enthalpic contributions. In summary, the ITC data for Ga^{3+} binding to the FtrB-Ag^+ complex reveals a more complex interaction compared to Ag^+ binding alone. The presence of multiple binding sites with distinct thermodynamic profiles highlights the versatility of FtrB in accommodating different metal ions. These findings provide deeper insights into the metal-binding properties of FtrB and its role in ion homeostasis, emphasizing the importance of understanding these interactions for the protein's biological function.

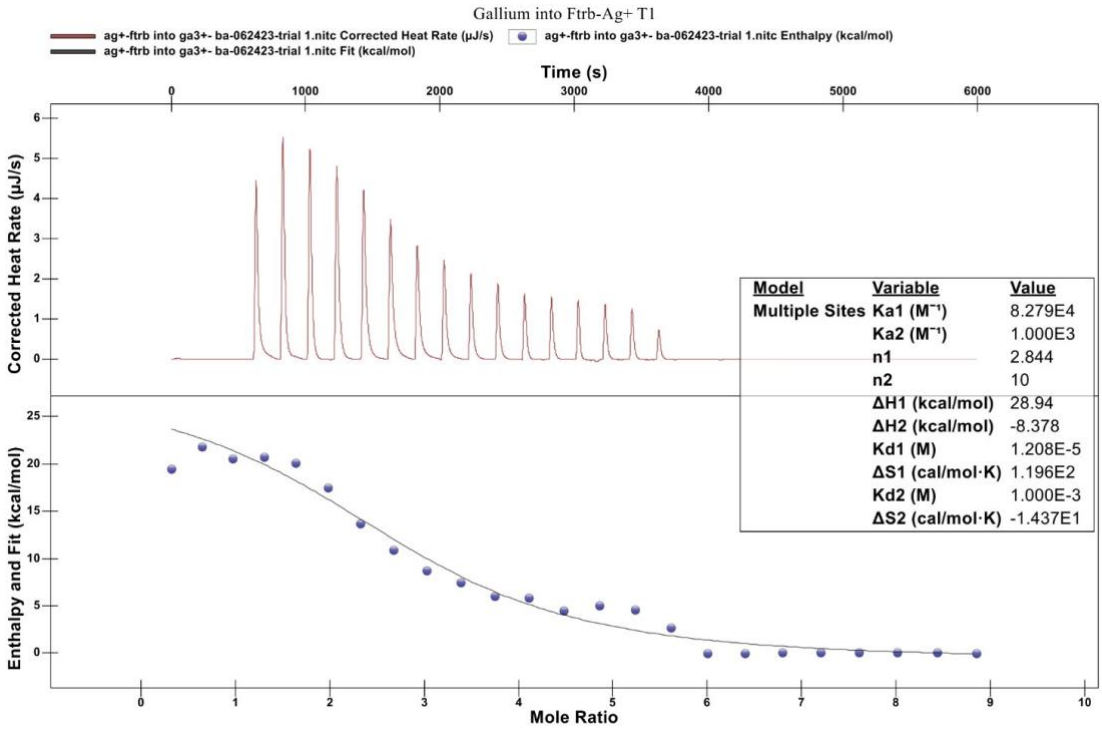

Figure 11: ITC Thermogram of Ga^{3+} Binding to FtrB-Ag^{+} Complex. *The figure shows the ITC thermogram for the binding of Ga^{3+} to the FtrB-Ag^{+} complex. The top panel displays the raw data, with each peak representing a single injection of Ga^{3+} into the protein solution. The endothermic nature of the interaction is evident from the upward peaks, indicating heat absorption upon binding. The integrated heat data, fitted to a multiple site model, is shown with the thermodynamic parameters such as the dissociation constants (Kd), stoichiometries (n), changes in enthalpy (ΔH), and changes in entropy (ΔS) listed to the side. The distinct binding interactions between Ga^{3+} and the FtrB-Ag^{+} complex is clearly demonstrated by the fitted curves.*

Table 1. Thermodynamic data for Cu^{2+}, Mn^{2+}, Ag^{+}, and Ga^{3+} binding to recombinant wild-type FtrB from *Brucella abortus* 2308.

Protein	**Titrated metal**	**K_d (μM)**	**ΔH_{ITC} (kcal/mol)**	**ΔS (cal/Kmol)**	**N**
Wild-type apo-FtrB[a]	Cu^{2+}	23.3 ± 5.1	-11.82 ± 1.01	-18.38±3.76	1.24 ± 0.08
Wild-type apo-FtrB[b]	Cu^{2+}	3.0 ± 1.0	-3.64 ± 0.43	13.99 ± 1.5	2.15 ±0.50
Wild-type Cu^{2+}-FtrB[a]	Mn^{2+}	2.13±0.46	-63.0 ±0.05	-188± 0.03	2.33±0.41
Wild-type apo-FtrB[c]	Ag^{+}	3.47 ± 0.7	-105. ±30	73.2	1.10±0.30
Wild-type Ag^{+}-FtrB[a]	Ga^{3+}	12.08 ± 0.01	28.94	119.6	2.844

Chapter 4: Conclusion and Future Directions

Conclusion and Future directions

This study has significantly advanced our understanding of the FtrABCD iron transport system in Gram-negative pathogens, particularly focusing on Brucella spp. Iron is essential for numerous biological functions, including DNA synthesis, respiration, and metabolism. Efficient iron transport and regulation are fundamental to the survival and pathogenicity of bacteria, especially in iron-limited environments within a host. Therefore, the ability to transport and utilize iron effectively is crucial for bacterial survival and virulence.

Our research confirms that FtrB, identified as a novel cupredoxin, plays a crucial role in the oxidation of Fe^{2+}, a process facilitated by its binding to Cu^{2+}. The detection of Cu^{2+}-His peaks through CD spectroscopy, along with the strong binding affinity observed in ITC experiments, underscores the importance of Cu^{2+} in maintaining the structural stability and enzymatic activity of FtrB. These findings provide valuable insights into the metal-binding properties of FtrB and highlight its essential role in the iron transport and redox reactions that are vital for the survival of Brucella in iron-restricted environments within the host.

To further confirm the role of FtrB as a ferrous oxidase, it is essential to conduct anaerobic experiments. These experiments will help validate the hypothesis by ensuring that the observed oxidation of Fe^{2+} is indeed facilitated by FtrB and not influenced by external oxygen. By creating an oxygen-free environment, researchers can more accurately assess the enzymatic activity of FtrB and its interaction with metal ions.

Exploring the binding properties and biological effects of other relevant metal ions, such as Fe^{3+} and Mn^{2+}, on FtrB could provide further insights into the metal specificity and versatility of the FtrABCD system. This could enhance our understanding of how different metals influence the function and stability of FtrB and the overall iron transport mechanism.

Conducting in vivo studies to observe the physiological relevance of the in vitro findings is also crucial. This includes assessing the impact of metal ion binding on the survival and virulence of Brucella and Bordetella in host environments. Such studies will help bridge the gap between in vitro data and real-life biological scenarios, providing a clearer picture of the role of FtrB in bacterial iron metabolism.

By integrating our in vitro data, which demonstrated the binding of Cu^{2+} and Mn^{2+} to FtrB and the stabilization of FtrA by Cu^{2+}, we can better understand the critical role of FtrB in iron transport. This comprehensive approach not only enhances our knowledge of bacterial iron metabolism but also paves the way for developing new antimicrobial strategies. Understanding these mechanisms is crucial for identifying potential targets for therapeutic interventions in Gram-negative pathogens.

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