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Classical And Ab Initio Qm/mm Simulations Of Bacterial Enzymes

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CLASSICAL AND AB INITIO QM/MM SIMULATIONS OF BACTERIAL ENZYMES

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DEDICATION

To my parents

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CHAPTER 1

INTRODUCTION AND OVERVIEW

Enzymes are catalysts that speed up reactions in biological systems. Without enzymes, many chemical reactions would occur too slowly to sustain life. This thesis describes various types of studies carried out on three enzyme systems: glutamine-dependent amidotransferase (GatCAB), inorganic pyrophosphatase (PPase), and histone deacetylases (HDACs). This chapter gives a brief introduction to each enzyme system in terms of the various types of analyses and the computational methods used to study the system. An overview of the theoretical aspects of the computational methods that were used has been also given at the end.

1.1 GatCAB system

The process of aminoacylation is vital in the accurate translation of the genetic code into amino acids that form proteins. The aminoacylation reactions are catalyzed by aminoacyl tRNA synthetase enzymes that are specific to a given amino acid. In the case of glutamine, the corresponding enzyme that catalyzes the aminoacylation is glutaminyl-tRNA synthetase (GlnRS). Many bacteria and all archaea lack GlnRS enzyme. In these organisms, the aminoacylation of glutamine happens in an indirect mechanism (the reactions involved are described in Chapter 2, Scheme 1). A non-specific glutamyl-tRNA synthetase converts the glutamine residue to Glu-tRNA^{Gln}.[2-5] GatCAB, a heterotrimeric enzyme composed of three subunits GatA, GatB, and GatC, is instrumental in many bacteria for the conversion of Glu-tRNA^{Gln} into Gln-tRNA^{Gln} (Chapter 2,

scheme 1).[6-10] The presence of Gln in the form of Gln-tRNA^{Gln} is essential for the incorporation of Gln into bacterial proteins. As described in Chapter 2 (scheme 1), this conversion happens via three steps (reactions 2,3, and 4 in Chapter 2, scheme 1). The first step of the conversion of   Glu-tRNA^{Gln} into Gln-tRNA^{Gln} , is the hydrolysis (reaction 2) of Gln at the GatA active site. This ammonia is utilized at GatB active site for the conversion of ɣ-phospho-Glu-tRNA^{Gln} into Gln-tRNA^{Gln}.[7]

GatCAB belongs to a family of enzymes known as glutamine-dependent amidotransferases (GATs).[11] As other enzymes in the GAT family,[12] the above-mentioned ammonia formed at the GatA active site is transported to the GatB active site via an intramolecular ammonia tunnel.[1] Intramolecular ammonia tunnels in most GATs are lined with hydrophobic amino acid residues and are poorly solvated.[12-15] In contrast, the GatCAB ammonia tunnel is lined with many hydrophilic amino acid residues and bound to these residues are many, ordered solvent water molecules.[1] Although as not hydrophilic as GatCAB ammonia tunnel, another example of a hydrophilic ammonia tunnel is that of carbamate phosphate synthetase (CPS). In CPS the occurrence of hydrophilic residues lining the tunnel is occasional and the majority of the residues lining the tunnel are hydrophobic.[16]

In light of the fact that the GatCAB ammonia tunnel is lined with hydrophilic ammonia residues, Nakamura *et al.* proposed that ammonia undergoes a series of protonation and deprotonation reactions as it traverses the tunnel in which the hydrophilic amino acid residues act as corresponding general

acids and bases.[1] Protonation would result in the formation of ammonium ion (NH_4^+) and subsequent deprotonation would return it back to ammonia. Exiting the tunnel at GatB active site would happen in the form of ammonia which would combine with ɣ-phospho-Glu-tRNA[Gln] to form Gln-tRNA[Gln].

In 2012, Kang *et al.* [17]proposed a modification to the tunnel proposed in 2006 by Nakamura *et al.* (tunnel1).[1] The intramolecular tunnel including this modification is referred to in this thesis as tunnel2. As described in more detail in Chapter 4, this modification introduces a new entrance point for the ammonia near GatA active site form where it is released by glutaminase activity. The newly suggested region to the tunnel is lined with hydrophobic amino acid residues in contrast to the previously proposed tunnel (tunnel1).

Chapter 2 discusses experimental and computational evidence that support the existence of a communication pathway between two subunits of the *Helicobacter pylori* (*H. pylori*) GatCAB.[18] Molecular dynamics (MD) simulations were carried out to sample the configuration space of the GatCAB system. Snapshots of the MD simulations were used to carry out two types of analyses: average correlation difference analysis, and average RMSD difference analysis.

Chapter three delves more into the mechanism of transfer of ammonia through the aforementioned intramolecular tunnel. This study involved the analysis of the D185[(A)] residue of *H.pylori* GatCAB as a probable catalytic residue in the ammonia delivery mechanism. Experimental evidence that suggests the action of D185(A) as a catalytic acid/base in the transfer of ammonia is discussed. As in Chapter 2, MD simulations and were used to

sample the configuration space of the mutants and the wild-type GatCAB. The snapshots of the MD simulations were used to carry out average correlation difference studies and pK_a analyses.

Chapter four discusses the computational analysis carried out on two putative intramolecular tunnels in *Staphylococcus aureus* (*S. aureus*) GatCAB.[19] The purpose of the analysis was to ascertain the more thermodynamically favorable tunnel out of two putative tunnels. This analysis involved the calculation of intramolecular tunnels and sampling along the tunnels using the umbrella sampling technique.[20] The biased sampling distributions that were obtained were converted to an optimum unbiased sampling distribution using the weighted histogram analysis method (WHAM)[21-23] which uses this optimum distribution for the calculation of free energy along the tunnels. Further characterization of the interactions of the ammonia molecule with the surrounding amino acid residues lining each tunnel were analyzed by calculating the electrostatic potential around ammonia using the linearized Poisson-Boltzmann equation and by energy decomposition analysis (EDA) which calculates the non-bonded interactions between ammonia and all other protein residues.

1.2 PPase system

Inorganic pyrophosphatase enzyme (PPase) has been known to catalyze three reactions: oxygen exchange between inorganic phosphate (P_i) and H_2O,[24-27] inorganic pyrophosphatase (PP_i)-P_i equilibration,[27-30] and the hydrolysis of PP_i.[25, 29] The first reaction, is catalyzed mainly by the PPase of yeast. The catalysis of the hydrolysis of a PP_i molecule into two P_i ions by PPase is a highly exothermic

reaction. It is of utmost importance in biological systems as the energy released can be used to drive kinetically unfavorable biological reactions to completion. It is also important in removing the inhibitory effects on enzymes caused by PP_i.[31, 32] The action of PPase is essential for the survival of bacteria and fungi.[33-35] PPase needs the presence of divalent metal cations at its active site to perform catalysis. The relative efficiency of catalysis has been shown to vary with the divalent metal cations as $Mg^{2+} > Zn^{2+} > Co^{2+} > Mn^{2+}$.[36, 37] In most Family 1 PPases, which is the type present in most eukaryotes, archaea and bacteria, the active site is composed of 13 functionally important residues and three to four Mg^{2+} cations.[38-40] Family 2 PPases, which are present only in some bacteria, archaea, and primitive eukaryotes,[41-44] are mainly catalyzed by Mn^{2+} at the active site. They are an order of magnitude higher in catalytic activity when compared to Family 1 PPase activity.[45] The catalytic action of PPase has been found to be inhibited by Ca^{2+},[30, 46-48] and F^-.[49-51] The precise mechanism of PPase action remains uncertain. The study of the mechanism of PPase in bacteria is important as it gives access to the structures of transition states the knowledge of which can be utilized in the creation of potential drugs in the form of transition state analogs.

As described in detail in Chapter 5, based on the studies of isolated crystal structures of *E. coli* PPase, a mechanism was previously proposed to describe the PPase catalyzed PP_i hydrolysis.[51] Although this mechanism suggests nucleophilic attack by an activated water molecule (W_c) resulting in the hydrolysis of the phosphoanhydride bond in PP_i, such a water had not been seen

in any of the crystal structures isolated thus far. Chapter five presents a quantum mechanical/molecular mechanical (QM/MM) study of the mechanism of PPase action in *Mycobacterium tuberculosis* (*M. tuberculosis*) based on a set of novel crystal structures in different stages of the reaction ranging from apo, to Pi bound to PPi bound which contains a water molecule at the exact position predicted by the previous mechanism in order to carry out a nucleophilic attack. MD simulations were carried out to sample the configuration space of PPase. MD snapshots in which a catalytic water (W_c) is properly oriented for hydrolysis, were used for the QM/MM calculations. After optimizing the reactant and product structures using the QM/MM method, the path between the optimized reactant and the product structures was optimized using the quadratic string method (QSM).[52] The structure that corresponds to the highest point in the QSM calculated path was utilized as the guess structure for transition state optimization.

1.3 HDACs

Chromosomal DNA in eukaryotic cells is densely packed into structures known as chromatin. Chromatin consists of DNA wound tightly around histone proteins. The strength of attraction of DNA to the histone proteins is a key factor in deciding the compactness of chromatin. More compact chromatin is more transcriptionally silent when compared to less compact/relaxed chromatin. Tails of histone proteins are mainly positively charged due lysine and arginine residues. Acetylation of these residues by histone acetyltransferase enzymes (HATs) lead to charge neutralization, and subsequent chromatin expansion[53] that

provides greater accessibility of nucleosomal DNA for genetic transcription.[54, 55] Histone deacetylase enzymes (HDACs) are a class of enzymes that catalyzes the removal of acetyl groups from above-mentioned acetylated lysine and arginine residues in histone proteins. This leads to increased positive charge that results in stronger attraction between the histone proteins and the DNA backbone that leads to more compact, transcriptionally silent chromatin. This modification of gene expression without a change in DNA sequence is an ideal example of epigenetic control.

Eighteen mammalian HDACs have been identified so far and have been divided into four classes based on sequence similarity.[56] Class I contains the HDACs 1,2,3, and 8. HDAC's in this class typically have a nuclear localization[57] and are involved in the regulation of cellular proliferative activity.[58] Class II contains HDACs 4,5,6,7,9, and 10. Class II is further divided into Class IIa (HDACs 4,5,7, and 9) and Class IIb (HDACs 6, and 10) with the primary distinction being the central domain of class IIb containing a zinc finger motif while class IIa contains a structural regulatory zinc binding domain.[59] Class IIa enzymes shuttle between nucleus and the cytoplasm while Class IIb enzymes are mainly found in cytoplasm. With regard to the activity inside the brain Class IIa HDACs are more important than Class IIb enzymes due to their increased expression in the brain. With respect to other HDACs, Class IIa HDACs have weak but measurable direct deacetylase activity and execute inhibition of gene transcription via interaction with transcription factors like RUNX2.[60, 61] Class III HDACs are known as silent information regulators or sirtuins (SIRTs). In contrast

to HDACs of other classes, the SIRTs are NAD$^+$ dependent in their catalytic action. Currently, seven mammalian SIRTs have been identified (SIRT 1-7).[62] Their localization in the cell is also varied among different SIRTs. In terms of activity, SIRT 1 has been found to be more efficient in deacetylase action with respect to SIRT 5 which shows weak deacetylase activity.[63, 64] Some SIRTs like SIRT4 and SIRT6 don't show deacetylase activity at all and are mono-ADP-ribosyl transferases.[65, 66] HDAC Class IV contains HDAC 11. HDAC 11 is normally expressed in brain and testis cells. HDAC11, is considered to be separate from other classes due to its biological actions.[67]

Development of HDAC inhibitors have shown a lot of promise in therapy in curing neurological diseases mediated by the epigenetic modifications catalyzed HDACs. Analysis of the pharmacodynamic profiles of HDAC inhibitors is of great help in studying the HDAC expression *in vivo*. One way of visualizing the HDAC expression-activity is through non-invasive imaging techniques like positron emission tomography (PET) scanning. Visualization of HDAC expression-activity through PET scanning can be achieved by the use of radiolabeled substrates that bind to HDAC active sites. Chapter six, describes a computational analysis of the binding affinity of a set of novel substrates for HDAC Class IIa enzymes. These set of novel substrates are being experimentally tested as possible radiotracers that help visualize the activity of HDAC Class IIa enzymes in vivo. The substrates differ from each other depending on the number of fluorine atoms. The study involves the docking of the substrates to HDAC4 and HDAC8 which are two members of the HDAC Class IIa enzymes. The purpose of the

docking is to identify the variation of the affinity of the substrates to HDAC4 and HDAC8 and also to identify the underlying interactions that can be used to describe mechanistic details of HDAC catalytic activity.

1.4 Computational methods

Given below is an overview of some of the computational methods that were used in the research projects described in this thesis. They have been described in the context in which they were used.

1.4.1 Molecular dynamics (MD)

Molecular mechanics is based on Newton's equations of motion. It views the atoms and the bonds between atoms as balls and springs respectively. This picture of chemical systems may be adequate as long as the systems being analyzed do not involve chemical reactions where insight at the electronic level is required. The iterative application of molecular mechanics equations to sample the configuration space of a system of interest is generally referred to as molecular dynamics (MD). In the context of the research described in this text, MD has been utilized as a means of sampling the configuration space of the large enzyme systems that were described above.

There are several stages in setting up an MD simulation. A force field that is parameterized for handling the type of system we are interested in has to be chosen. A force field used in MD typically includes an equation for calculating the energy of the system in terms of bond stretching, angle bending, torsional (dihedral) energies, and non-bonded interaction energies, which include

electrostatic and Van der Waals interactions. All the MD simulations discussed in this thesis, have been performed using the amber99SB force field as implemented in AMBER11 and AMBER12 software suites.[68] The basic form of the amber force field (without specifically referring to various versions) as discussed by Cornell *et al.*[69] in 1995 is given below:

$$E_{tot} = \sum_{bonds} K_r (r - r_{eq})^2 + \sum_{angles} K_\theta (\theta - \theta_{eq})^2 + \sum_{dihedrals} \frac{V_n}{2} [1 + \cos(n\phi - \gamma)] + \sum_{i<j} \left[\frac{A_{ij}}{R_{ij}^{12}} - \frac{B_{ij}}{R_{ij}^6} + \frac{q_i q_j}{\varepsilon R_{ij}} \right] \quad (1)$$

where E_{tot} is defined as total energy, K_r is the force constant for the rth bond, K_θ is the force constant for θ^{th} angle bending, n is the order (fold) of rotation, V_n is defined as the barrier height for an n-fold rotation, ϕ is the torsion angle, γ is the phase factor, R_{ij} the distance between any two atoms, $A_{ij} = 4\varepsilon_{LJ}\sigma^{12}$, $B_{ij} = 4\varepsilon_{LJ}\sigma^6$ where ε_{LJ} is the depth of the potential well, and σ is the finite distance at which the inter-particle potential is zero, q_i and q_j are the electrostatic charges of the ith and j^{th} atoms, and ε is the permittivity of the medium.

Once a force field is chosen, the initial coordinates for the system can be obtained from experimental data (e.g. crystal structure data or NMR data) or from a theoretical model. The initial velocities for each atom can be assigned from a Maxwell-Boltzmann velocity distribution at the temperature of interest:[70]

$$p(v_{ix}) = \left(\frac{m_i}{2\pi k_B T} \right)^{1/2} \exp\left[-\frac{1}{2} \frac{m_i v_{ix}^2}{k_B T} \right] \quad (2)$$

where $p(v_{ix})$ is defined as the probability of particle i having a velocity v in the x direction, m_i is the mass of the ith particle, T is the absolute temperature, and k_B is the Boltzmann constant. The distribution is populated using a random number

generator that generates random numbers between 0 and 1 which are used as input to create the probabilities between 0 and 1. It would be impossible to calculate a continuous MD trajectory as that would involve an infinite number of calculations. Therefore, for all practical purposes, the number of MD calculations for a given MD simulation is defined using a time step (δt). At each time step an iteration of MD calculations will be performed. The value of δt, is decided upon according to the fastest motion of the system, because, if δt is bigger than the fastest motion, that motion cannot be sampled properly using the simulation. Usually the vibrational degrees of freedom of bonds involving hydrogen atoms are restrained to achieve a bigger time step. In all our simulations, a δt of 1 fs was used. With the initial coordinates ($\mathbf{r}$(t)) and velocities ($\mathbf{v}$(t)) at hand, the forces for each atom are generated by calculating the negative gradient of the force field (equation 1) for each atom. The corresponding accelerations ($\mathbf{a}$(t)) are obtained by dividing the force on each atom, by its mass. The new velocities and coordinates can be calculated with a variety of integration methods. One popular method is the velocity-Verlet algorithm (Swope *et al.* 1982)[71]. This method has four steps:

1) Calculation of coordinates at (t+ δt)

   $\mathbf{r}$(t+ δt) = $\mathbf{r}$(t) + $\delta t\mathbf{v}$(t) + (1/2) $\delta t^2\mathbf{a}$(t)

   where $\mathbf{r}$(t+ δt) are the coordinates at (t+ δt)

2) Calculation of velocities at (t+ (1/2)δt)

   $\mathbf{v}$(t+ (1/2)δt) = $\mathbf{v}$(t) + (1/2) $\delta t\mathbf{a}$(t)

   where $\mathbf{v}$(t+ (1/2)δt) are the velocities at (t+ (1/2)δt)

3) Calculation of new forces from the current positions, thus obtaining $\mathbf{a}(t+ \delta t)$

4) Calculation of velocities at $(t+ \delta t)$

$\mathbf{v}(t+ \delta t) = \mathbf{v}(t+ (1/2)\delta t) + (1/2)\ \delta t\mathbf{a}(t+ \delta t)$

where $\mathbf{v}(t+ \delta t)$ are the velocities at $(t+ \delta t)$

The same set of calculations are performed for the next iterations as well until satisfactory sampling of the configuration space is achieved. In place of the velocity-Verlet method, other integration algorithms like, Verlet algorithm[72], leap-frog algorithm[73], and Beeman algorithm[74] can also be used.

1.4.2 CAVER tunnel calculations

The CAVER[75] program/tool as implemented in the PyMOL[76] visualization package was used for the intramolecular tunnel calculations described in Chapter 4. This program creates a three dimensional grid of nodes. The protein in which the intramolecular tunnels have to be calculated, is placed inside this grid. The atoms of the protein are represented as hard spheres defined by Van der Waals radii. The protein surface ("convex hull") is approximated using Convex Quadratic Approximation (CQA)[77]. All the nodes outside the convex hull are discarded and not included in further calculations. Every node in the convex hull are tagged with a "cost" calculated using the node dependent cost function C(x):

$$C(x) = \frac{1}{\left(r_{\max}(x)+\varepsilon\right)^2} \qquad (3)$$

where $r_{max}(x)$ is radius of the maximally inscribable sphere at node x, and ε is a parameter that is given a value in cases where $r_{max}(x)$ tends to zero, to avoid singularities. Tagging the nodes by costs, creates a mathematical object known as a vertex-weighted graph. Depending on the node specified as the starting

point of the tunnel calculation by the user, the vertex-weighted graph is traversed

using a modified version of Dijkstra's algorithm[78-80] to find the cheapest tunnels.

1.4.3 Umbrella sampling[20]

We often have to calculate free energy surfaces along a chosen set of

coordinates. The coordinates could come from a Monte Carlo[81] trajectory or from

an MD trajectory. The relationship between the probability distribution of the

sampled coordinates and the free energy is logarithmic:[70]

$$A(r) = -k_B T \ln[\rho(r)] + \text{constant} \quad (4)$$

where A(r) is defined as the Helmholtz free energy for the position vector **r**, and

ρ(r) is defined as the probability of being in the bin with **r** as the center. The

logarithmic relationship indicates that for a relatively small change in A(r), the

change in corresponding probability distribution should be an order of magnitude

larger. Unfortunately standard MD sampling at nano-second and micro-second

time scales cannot achieve adequate sampling of higher energy regions on the

free energy surface, and therefore tend to give wrong estimations of free energy

for those regions. One method for achieving better sampling of these

energetically unfavorable regions is umbrella sampling. Umbrella sampling uses

a modified potential function V(r^N)′, where the modification W(r^N), is added as a

perturbation to the original potential function V(r^N):[70]

$$V(r^N)' = V(r^N) + W(r^N) \quad (5)$$

The perturbation W(r^N), is usually a harmonic potential, which has the shape of

an umbrella, hence the name of the method:[70]

$$W(r^N) = k_W (r^N - r_0^N)^2 \quad (6)$$

where k_W is defined as the force constant corresponding to the biasing potential $W(r^N)$ and r_0^N is the equilibrium configuration defined by the N coordinates in the absence of the biasing potential. During umbrella sampling, configurations further away from r_0^N, will be subject to a higher biasing potential than configurations closer to it, thereby yielding a non-Boltzmann distribution. In 1977, Torrie and Valleau showed that Boltzmann averages for properties can be extracted from the non-Boltzmann distribution resulting from umbrella sampling:[20, 70]

$$\langle A \rangle = \frac{\langle A(r^N) \exp[W(r^N)/k_B T] \rangle_W}{\langle \exp[W(r^N)/k_B T] \rangle_W} \quad (7)$$

where angle brackets indicate average values, and the subscript W indicates that the average is based on the biased probability distribution $P_w(r^N)$.

As described in Chapter four, umbrella sampling was used to achieve even sampling of the configuration space of the ammonia molecules that were positioned along each intramolecular ammonia tunnel in GatCAB. Due to the application of the biasing potential, the resulting sampling distribution is a biased sampling distribution which had to be unbiased and optimized in order to be used for further analyses.

1.4.4 Weighted histogram analysis method (WHAM)

The biased sampling distributions obtained using the umbrella sampling technique described above, was converted into an optimum unbiased sampling distribution using this method. This is done by solving the following two equations self-consistently.

$$\rho^{(u)}\left(\xi_j\right) = \frac{\sum_{i=1}^{N_w} N_{ij}}{\sum_{i=1}^{N_w} \frac{N_i \exp\left[-\beta V_i\left(\xi_j\right)\right]}{\mu_i}} \qquad (8)$$

$$\mu_i = \sum_j \exp\left[-\beta V_i\left(\xi_j\right)\right]\rho^{(u)}\left(\xi_j\right) \qquad (9)$$

$\rho^{(u)}(\xi_j)$ is the unbiased probability of bin j; N_w stands for total numbers of umbrella sampling simulations (windows); N_i is the number of snapshots from the ith simulation; N_{ij} is number of snapshots from simulation i that fall into bin j; $V_i(\xi_j)$ is the harmonic potential applied on bin with center (ξ_j) with respect to minimum of window i; μ_i is the normalization constant for window i; β stands for ($1/k_BT$).

All μ_i values are set to 1 at the outset. Equation (2) is used to calculate a set of $\rho^{(u)}(\xi_j)$ values for each bin. This set of $\rho^{(u)}(\xi_j)$ values are used in equation (3) calculate a new set of μ_i values. New μ_i values and old μ_i values are used to calculate a convergence criterion. If the convergence criterion falls below a tolerance value, the calculation is stopped and the set of $\rho^{(u)}(\xi_j)$ values calculated by that time is taken as the set of optimum unbiased probabilities for each bin. If the convergence criterion is not below the tolerance value, the new μ_i values are used to calculate another set of $\rho^{(u)}(\xi_j)$ values using equation (2) which are again substituted in equation (3) to calculate another set of μ_i values. The cycle continues until the convergence criterion falls below the tolerance value. The optimum unbiased probability distribution obtained in this way is used to calculate the free energy along the two tunnels.

1.4.5 QM/MM hybrid method

First introduced in 1976 by Warshel and Levitt,[82] the QM/MM approach combines the accuracy of quantum mechanics and the speed of molecular mechanics into one. This method is specially used in analyzing chemical reactions that occur in macroscopic systems like proteins and nucleic acids. This method has proved highly successful and grabbed the attention of the scientific community in general with the award of the 2013 Nobel Prize in chemistry for Arieh Warshel, Michael Levitt, and Martin Karplus for their work involving QM/MM methods in the development of multiscale models for chemical systems. In the QM/MM hybrid method, the portion of the system that is involved in the chemical reaction of interest, is included in the QM subsystem and is treated quantum mechanically while the rest of the system is addressed using molecular mechanics. The QM treatment of the reactive subsystem yields a more accurate picture at the electronic level which is compulsory for the analysis of chemical reactions and to divulge mechanistic information. For the rest of the system (MM subsystem), for which the accuracy at the electronic level is not a requirement, a compromise in accuracy has been made to gain an advantage in calculation speed by applying the balls and springs picture of atoms and bonds used in molecular mechanics. Many implementations of the QM/MM hybrid method use a two-zone (i.e. QM and MM subsystems) approach as described above. In 1996 Morokuma *et al.*[83] described a multi-layered approach for the QM/MM method, which is now widely known as ONIOM. The two-zone implementation, leaves the user with only two

choices in dividing up the system in terms of the accuracy of the calculations needed. ONIOM allows the user to divide the system into several layers allowing each layer to be addressed with a different level of theory. The outermost layer is usually treated by an MM force field. The level of theory increases when going from the outer layers to the inner layers. In a typical example of ONIOM with three layers, the innermost core might be treated with a B3LYP density functional approach,[84-87] the intermediate layer using Hartree-Fock method,[88] and the outermost layer with MM3.[89]

In the simpler two-zone implementation, the total energy E_{TOT} of the system is given by:[70]

$$E_{TOT} = E_{QM} + E_{MM} + E_{QM/MM} \quad (10)$$

where E_{QM} is the energy of the QM subsystem, E_{MM} is the energy of the MM subsystem, and $E_{QM/MM}$ is the energy of interaction between the QM subsystem and the MM subsystem. As explained by Karplus et al. in 1989,[90] for the calculation of the total energy E_{TOT} of the system, an effective Hamiltonian $\hat{H}_{eff}$, is defined which is a combination of the QM Hamiltonian $\hat{H}_{QM}$, MM Hamiltonian $\hat{H}_{MM}$, and the QM/MM Hamiltonian $\hat{H}_{QM/MM}$. E_{TOT} is calculated using the time-independent Schrödinger equation:

$$\hat{H}_{eff} \psi(r, R_\alpha, R_M) = E_{TOT}(R_\alpha, R_M) \psi(r, R_\alpha, R_M) \text{ (11)}$$

where the wavefunction ψ is a function of the electronic coordinates **r**, that parametrically depends on both QM nuclei positions R_α, and the MM atom

positions R_M. The nature of the expressions for $\hat{H}_{QM}$, and $\hat{H}_{MM}$, depend

respectively on the type of QM method and the MM force field used. In a typical

example where all the interactions between the QM and the MM subsystem arise

from the non-bonded interactions the expression for $\hat{H}_{QM/MM}$ would be written as:

$$\hat{H}_{QM/MM} = -\sum_{iM} \frac{q_M}{r_{iM}} + \sum_{\alpha M} \frac{Z_\alpha q_M}{R_{\alpha M}} + \sum_{\alpha M} \left\{ \frac{A_{\alpha M}}{R_{\alpha M}^{12}} - \frac{B_{\alpha M}}{R_{\alpha M}^{6}} \right\} \quad (12)$$

where the subscripts i and α correspond to QM electrons and nuclei respectively

and M correspond to MM atoms. The first term on the right hand side of equation

12, corresponds to the electrostatic interaction between MM atoms and the QM

electrons, the second term to the electrostatic interaction between MM atoms

and QM nuclei, and the third term the Van der Waals interactions. But in cases

where the QM/MM boundary cuts through covalent bonds, $\hat{H}_{QM/MM}$ should be

able to account for bonded interactions as well. There are three main schemes

that have been developed, to address the situations where a covalent bond is cut

by the QM/MM boundary: the link atom scheme,[90, 91] the pseudobond scheme,[92-

94] and the localized orbital scheme.[95-99] In the link atom scheme, an additional

atomic center called a 'link atom', which is not part of the real system, caps the

dangling bond of the QM atom, thus saturating its free valency. QM calculations

are performed on the system consisting of the inner QM subsystem and the link

atoms. The clipped bond is described at the MM level. Addition of a new atomic

center that is not part of the real system gives rise to problems. Three main

problems are, the introduction of three artificial structural degrees of freedom with

each link atom, over-polarization of the MM point charge of the MM atom

involved in the clipped bond due to its close proximity to the QM electron density, and chemical and electronic incompatibility of the link atom when compared to the group/atom it replaces.

The pseudobond scheme tries to remove the problems associated with the link atom scheme by replacing the MM atom of the clipped bond with a free-valence boundary atom which has a parameterized effective core potential. The effective core potential has been designed in such a way as to reproduce the bond length, and electronic properties of the cut-bond. In the QM/MM calculations discussed in this thesis, the pseudobond scheme has been adopted to treat the covalent bonds that get severed by the QM/MM boundary.

The localized orbital scheme places frozen hybrid orbitals at the QM/MM boundary to saturate the dangling bonds by capping the QM atoms involved in the cut-bond.

As described in Chapter five of this text, the QM/MM hybrid method has been used in analyzing the mechanism of PPase action in *M. tuberculosis*. The reactive subsystem which encompasses the substrate, attacking water, etc. have been included in the QM subsystem while rest of the system has been included in the MM subsystem.

1.4.6 Molecular docking

Molecular docking is a process which finds dominant binding modes (poses) of a ligand molecule (e.g. a drug molecule) to a target receptor molecule (e.g. an enzyme) with the help of scoring functions that rank the binding modes depending on the number/nature of favorable binding interactions between the

ligand and the receptor. Docking programs could be evaluated according to the number of degrees of freedom they ignore when suggesting probable binding modes. Earliest docking programs treated both the ligand and the receptor as rigid molecules and searched only the six translational and rotational degrees of freedom. The docking program DOCK in its earliest form,[100] is such an example. Since then docking programs have evolved to take into account the flexibility of both the ligand and the active site of the receptor, when suggesting probable poses. Modern docking programs adopt various approaches in tackling the docking problem which is essentially a search for conformational degrees of freedom of both the ligand and the receptor that give rise to stable ligand-receptor complexes. Some of these approaches are, Monte Carlo method combined with simulated annealing,[101] genetic algorithms[102-104], incremental construction of the ligand[105-107], and molecular dynamics of the ligand-receptor complex.[108, 109]

Chapter six describes the use of molecular docking as a means of calculating and comparing the binding affinity of a set of novel substrate (ligand) molecules in binding to two members of the HDAC Class IIa enzymes: HDAC4 and HDAC8. The LeadIT program[110] was used for all the docking calculations, which implements a form of the FlexX algorithm.[107] FlexX algorithm is an incremental construction algorithm. It first creates interaction geometries and interaction centers for all groups in the ligand and the receptor that can be thought to have interactions. The ligand is fragmented by severing at all acyclic single bonds. Base fragments are created using single fragments or

combinations of fragments. Base fragments are placed at the active site of the receptor to achieve best overlap of interaction centers in both the ligand and the receptor. The base fragments are built iteratively in agreement with the torsional databases to construct the complete ligand.

CHAPTER 2

ANALYSIS OF THE COMMUNICATION BETWEEN GATA AND GATB

SUBUNITS OF THE ENZYME GLUTAMINE-DEPENDENT

AMIDOTRANSFERASE (GATCAB) IN *HELICOBACTER PYLORI* (*H. PYLORI*)

2.1 Introduction

Aminoacylation of tRNA involves the reaction of amino acids with specific tRNA molecules to produce aminoacyl-tRNAs (aa-tRNAs).[111] This process is catalyzed by specific aminoacyl-tRNA synthetase enzymes (aaRSs). In eukaryotes like humans, the aminoacylation of tRNA^{Gln} is catalyzed by glutaminyl-tRNA synthetase (GlnRS) producing Gln-tRNA^{Gln} .[112] In all archaea and most bacteria GlnRS is not present.[11, 12, 113] The absence of GlnRS should theoretically deprive these organisms of making Gln-tRNA^{Gln}, thus making it impossible for most bacteria to incorporate glutamine into their proteins. In these bacteria, incorporation of glutamine into proteins is achieved by an indirect mechanism.[113] In a first step, a non-specific enzyme called glutamyl-tRNA synthetase (GluRS) catalyzes the misacylation of glutamate with tRNA^{Gln} producing Glu-tRNA^{Gln} (reaction 1).[6, 112, 114] This reaction is followed by conversion of the misacylated tRNA to the correctly acylated tRNA.

In bacterial cells, the conversion of Glu-tRNA^{Gln} to Gln-tRNA^{Gln} requires three reactions (see Scheme 1) catalyzed by the glutamine-dependent amidotransferase (GatCAB).[1, 4, 11, 12, 115, 116] GatCAB is a heterotrimeric enzyme that consists of three subunits: GatA, GatB, and GatC (Figure 1). The hydrolysis of glutamine (reaction 2) happens at the GatA active site resulting in ammonia and glutamate. At the GatB active site the misacylated product Glu-tRNA^{Gln} is activated by phosphorylating Glu-tRNA^{Gln} at the ɣ position of the glutamate (reaction 3). The resulting ɣ-phosphoryl-Glu-tRNA^{Gln} is subsequently transamidated (reaction 4) into Gln-tRNA^{Gln} using the ammonia produced by the hydrolysis of glutamine in reaction 1.[1, 9, 117] Structurally, the GatA and GatB active sites are separated by ≈ 30 Å. The exact mechanism for the transfer of ammonia from the GatA active site to the GatB active site is poorly understood.

tRNA^{Gln} + Glu +ATP → Glu-tRNA^{Gln} + AMP + PP$_i$ (Rxn 1, GluRS)

Gln + H$_2$O → Glu + NH$_3$ (Rxn 2, GatA)

Glu-tRNA^{Gln} + ATP → ɣ-phospho-Glu-tRNA^{Gln} + ADP   (Rxn 3, GatB)

ɣ-phospho-Glu-tRNA^{Gln} + NH$_3$ → Gln-tRNA^{Gln} + P$_i$ (Rxn 4, GatB)
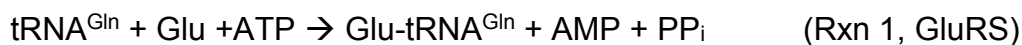
Scheme1: Reactions catalyzed by GluRS (reaction 1) and GatCAB (reactions 2-4).
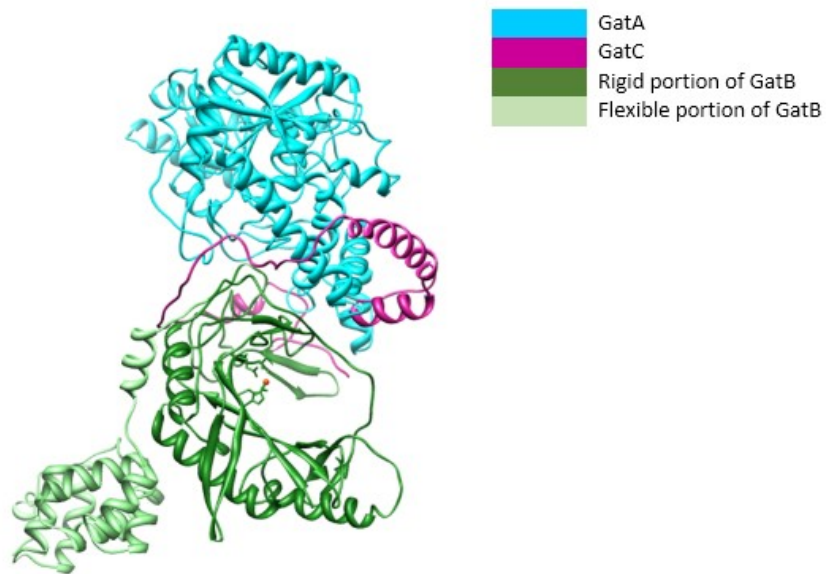
Figure 1: Structure of GatCAB enzyme from *Staphylococcus aureus*[1] (PDB ID: 2F2A)

In order to explain the transport of ammonia from the GatA active site to the GatB active site through the enzyme, a putative tunnel (Figure 2) was proposed by Nakamura *et al.* which will be referred to in this text as tunnel1.[1] Ammonia produced at the GatA active site has to be consumed at the GatB active site at a matching rate to regulate pH homeostasis suggesting a communication pathway between the two subunits.
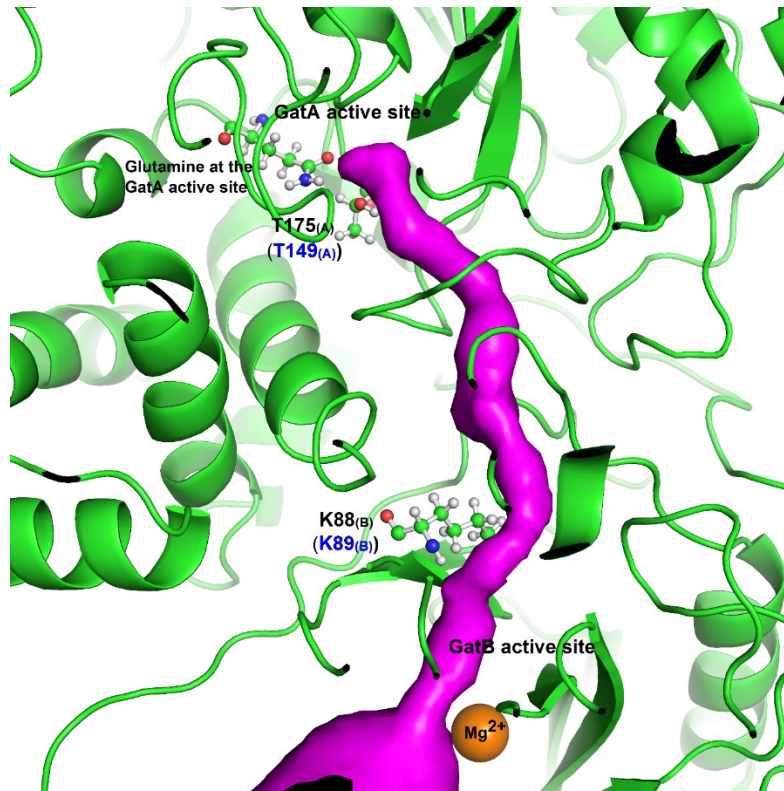
Figure 2: The putative tunnel proposed by Nakamura *et al.* in 2006. Residue numbers in black correspond to *Staphylococcus aureus* notation. The residue numbers in blue correspond to their *Helicobacter pylori* equivalents.

2.2 Experimental findings

This study was performed in collaboration with Professor Hendrickson's group at the department of chemistry in Wayne State University. Prof. Hendrickson's group created mutants of 13 conserved amino acid residues lining tunnel1 in *H. pylori* and measured the rates of the phosphorylation reaction (reaction 3 in Scheme 1) at the GatB active site (shown in Figure 2). The highest changes in these rates were found for T149$_{(A)}$V and K89$_{(B)}$R mutants, where the letters (A)

and (B) in subscript refer to chain names in GatCAB. The fact that a mutation at the GatA active site and at the GatA-GatB interface results in a corresponding change in the phosphorylation rate at the GatB active site suggested a communication pathway between the GatA and GatB subunits as well as the possibility of T149$_{(A)}$ and K89$_{(B)}$ playing major roles in that communication pathway.

2.3 Computational methods and analyses

As there is no crystal structure information for the GatCAB enzyme in *H. pylori,* the crystal structure of the GatCAB enzyme of *Staphylococcus aureus* (*S. aureus*) (PDB ID:2F2A) was used for all the computational studies due to its high sequence identity and similarity with *H. pylori* GatCAB. The mutations T149$_{(A)}$V and K89$_{(B)}$R in *H. pylori* correspond to T175$_{(A)}$V and K88$_{(B)}$R in *S. aureus*.

2.3.1 Molecular dynamics (MD) simulations

MD simulations were performed on eight systems: wild-type with (wild+Gln) and without Gln (wild-Gln) at the GatA active site, T149$_{(A)}$V with (T149$_{(A)}$V+Gln) and without Gln (T149$_{(A)}$V-Gln) at the GatA active site, K89$_{(B)}$R with (K89$_{(B)}$R+Gln) and without Gln (K89$_{(B)}$R-Gln) at the GatA active site, E125$_{(B)}$D with Gln at the GatA active site (E125$_{(B)}$D+Gln), and E125$_{(B)}$Q with Gln at the GatA active site (E125$_{(B)}$Q+Gln) using the PMEMD program in AMBER11 software suite with the amber99SB force field.[68] The E125$_{(B)}$D+Gln and E125$_{(B)}$Q+Gln systems were used as controls. The SHAKE algorithm was applied for bonds between heavy atoms and hydrogen.[118] All MD simulations were carried out with periodic

boundary conditions. The smooth particle Ewald method was used for calculating long range interactions.[119, 120] Systems were minimized using sander with 50 steps of steepest descent and 450 steps of conjugate gradient with a restraint of 500 kcal/mol/Å^2 on the protein atoms. Neutralization of the systems was done by adding Na$^+$ ions using the xleap program in AMBER11. The neutralized systems were solvated with TIP3P water in boxes of size 125 Å×96 Å×142 Å and were minimized using sander as described above. Densities of the systems were equilibrated at 1g/cm^3 in the NPT ensemble with pressure being held constant by anisotrophic pressure scaling. The systems were gradually heated to 300K under constant volume conditions with a 500 kcal/mol/Å^2 restraint on the protein atoms. The restraints were gradually removed. Temperature was kept constant using a Berendson thermostat.[121] Non-restrained production MD was performed for 20 ns for each system with snapshots saved each 1 ps.

2.3.2 Residue-wise correlation studies

Residue-wise correlations were calculated using the *correl* function of the AMBER11 software suite using the trajectory files of the final 10 ns of each simulation (Figure 3). To ascertain the effects of the mutations, correlation differences were calculated for the mutants T149$_{(A)}$V, K89$_{(B)}$R with respect to the wild-type (Figure 4). Correlation patterns of V149$_{(A)}$ (in T149$_{(A)}$V mutant), R89$_{(B)}$ (in K89$_{(B)}$R mutant) with all the other protein residues were compared against the patterns of T149$_{(A)}$ (wild-type), K89$_{(B)}$ (wild-type) with all the other protein residues (Figure 5).

2.3.3 Residue-wise root mean square deviation (RMSD) studies

Average RMSD values for each residue were calculated for the mutants and the wild-type structures with respect to the initial crystal structure, using the MD snapshots of the final 10 ns in each MD simulation. The average RMSD value for each wild-type amino acid residue was subtracted from the average RMSD value of each corresponding residue in the mutant structures to find the RMSD differences. These residue-wise RMSD differences were mapped onto the mutant GatCAB structures to visualize the residues that show high geometrical disturbance due to mutation (Figure 6). For each of the mutant structures, the 300 residues that showed highest percentage RMSD differences were selected. From these groups of 300 residues, the amino-acid residues common to all the mutant structures were selected and mapped onto the structure to visualize their positions (Figure 7).

2.4 Results and discussion

2.4.1 Residue-wise correlation studies

Residue-wise correlation plots are shown below in Figure 3 followed by the correlation difference plots in Figure 4. Correlations have been calculated for wild-type GatCAB and the mutants $T149_{(A)}V$, and $K89_{(B)}R$ with and without Gln at the GatA active site.

Positive correlation values (values greater than zero) correspond to movement of the two residues in the same direction whereas negative correlation values (values less than zero) correspond to movement in opposite directions. Correlation plots are divided into regions that correspond to protein subunits in

GatCAB as shown by the dashed lines on the correlation plots in Figure 3G. As the correlation plots are symmetrical along the main diagonal, the regions labeled as GatA to GatB and GatB to GatA represent the same correlations of GatA residues with GatB resdues. Correlation values along the diagonal are the highest as they represent correlations of residues with themselves.
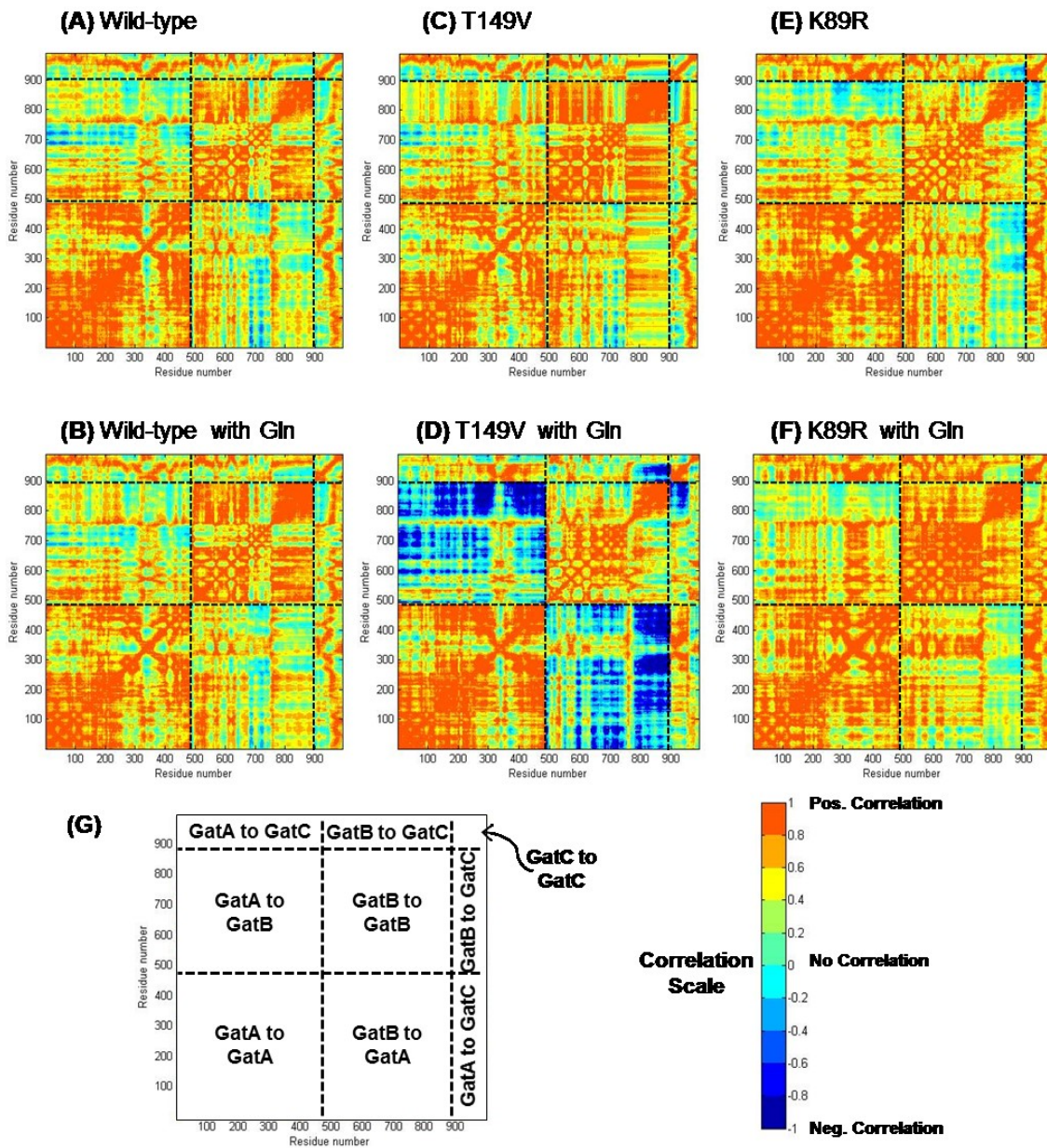
Figure 3: The correlation plots for (A) wild-Gln; (B) wild+Gln; (C) T149$_{(A)}$V-Gln; (D) T149$_{(A)}$V+Gln; (E) K89$_{(B)}$R-Gln; (F) K89$_{(B)}$R+Gln; (G) Map showing different regions marked by dash lines.

The correlation difference plots (Figure 4) show that when compared to the correlation difference patterns of the controls (4(A) and 4(B)) there is a change in the correlation pattern between GatA and GatB residues in the mutants with respect to the wild-type. Without the presence of glutamine at the GatA active site, this change is smaller than the change in the correlation pattern in the presence of glutamine at the GatA active site. This phenomenon is shown by the presence of yellow regions (correlation difference ≈ 0.5) in Figure 4(E) and 4(F) when compared to the presence of dark blue (correlation difference ≈ -1) and orange regions (correlation difference ≈ 0.8) in Figure 4(C) and 4(D) respectively. These changes in the correlation pattern between the GatA and GatB subunits suggest that the effect of the mutation T149$_{(A)}$V at the GatA active site has propagated to the GatB subunit. This observation is in accordance with experimental results which show that the rate of phosphorylation of the misacylated Glu-tRNA^{Gln} (reaction 3, Scheme 1) at the GatB active site is greatly reduced in the T149$_{(A)}$V mutant with respect to the wild-type GatCAB. Furthermore, the changes in correlation pattern as shown by 4(E) and 4(F) suggest that the mutation K89$_{(B)}$R at the GatB subunit can also disrupt the communication pathway between the GatA and GatB subunits which in combination with the 4(C) and 4(D) results gives a sense of bi-directionality to the communication pathway between the GatA and the GatB subunits.
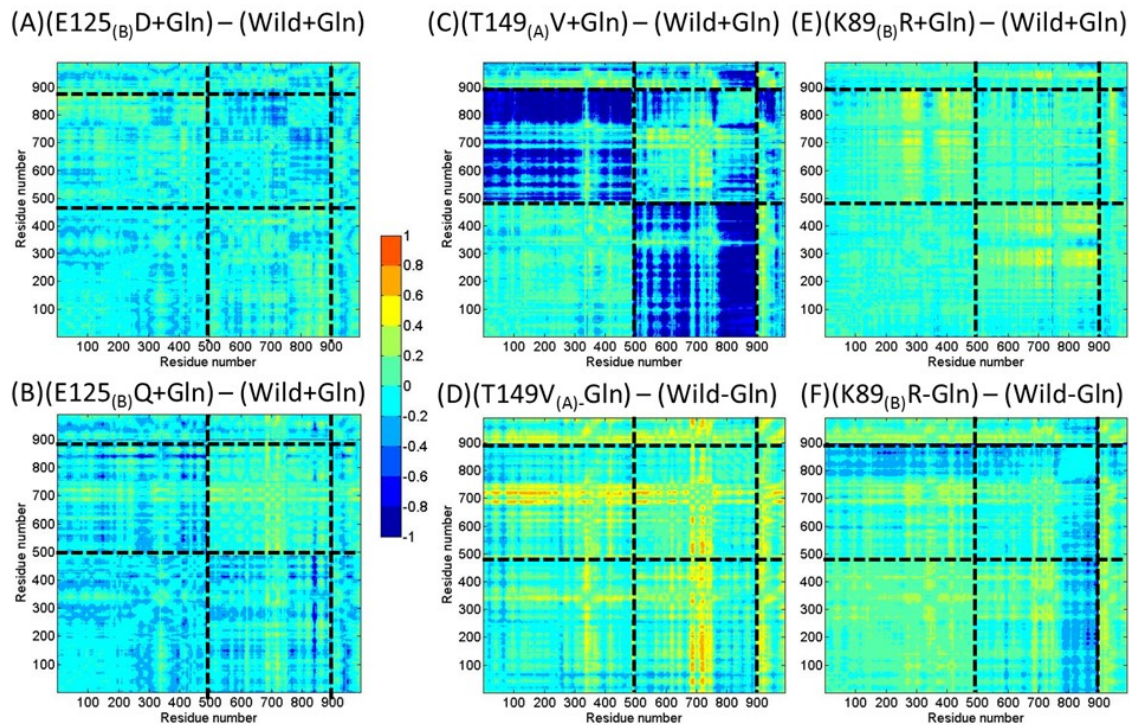
Figure 4: The correlation difference plots for (A) wild+Gln correlation matrix subtracted from E125(B)D+Gln correlation matrix; (B) wild+Gln correlation matrix subtracted from E125(B)Q+Gln correlation matrix; (C) wild+Gln correlation matrix subtracted from T149(A)V+Gln correlation matrix; (D) wild-Gln correlation matrix subtracted from T149(A)V-Gln correlation matrix; (E) wild+Gln correlation matrix subtracted from K89(B)R+Gln; (F) wild-Gln correlation matrix subtracted from K89(B)R-Gln

In Figure 5, the correlation patterns of the T149(A) and K89(B) positions (in wild-type GatCAB) with the other residues in the protein are compared against the correlation patterns of V149(A) (the corresponding position in the T149(A)V mutant) and R89(B) (the corresponding position in the K89R mutant) with all the other amino acid residues.
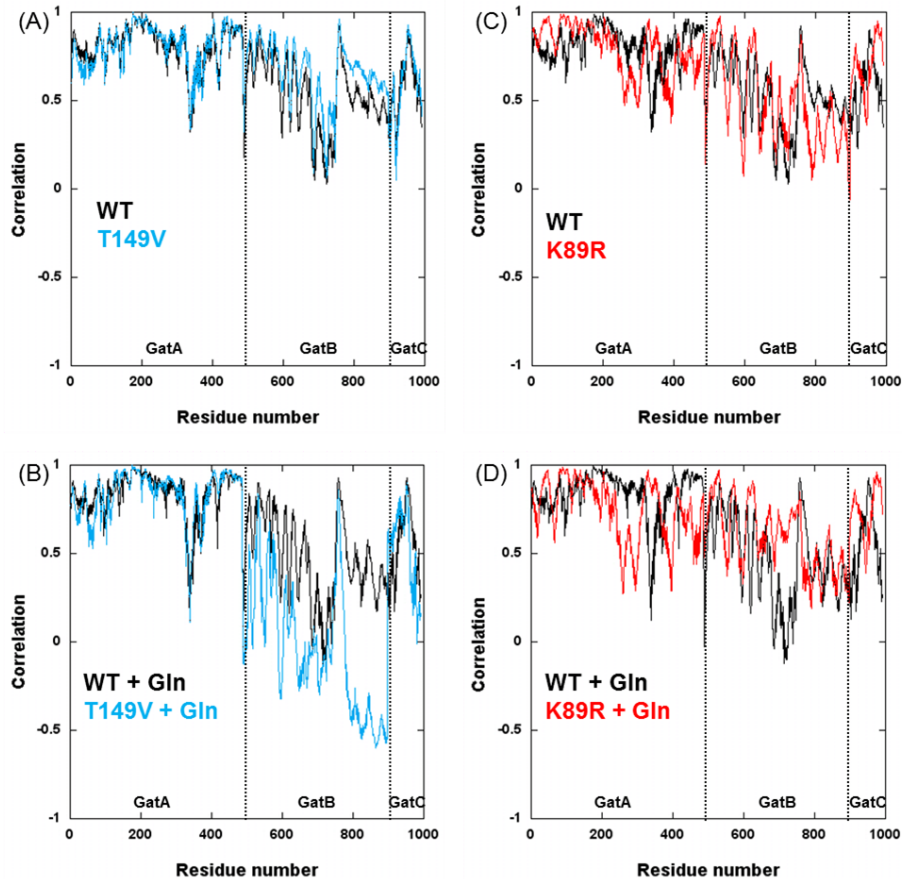
Figure 5: Correlation between each enzyme residue and the T149(A) and R88(B) positions. (A) comparison of correlation pattern of T149(A) and V149(A) (i.e. for the mutant) with all other residues (without Gln at the GatA active site). (B) comparison of correlation pattern of K89(B) and R89(B) (i.e. for the mutant) with all other residues (without Gln at the GatA active site) (D) comparison of correlation pattern of K89(B) and R89(B) (i.e. for the mutant) with all other residues (with Gln at the GatA active site).

In Figure 5A a significant change in the correlation pattern is seen in the GatB region of the T149(A)V-Gln mutant when compared to the correlation pattern of

the wild-type. The mutation T149$_{(A)}$V in the GatA active site has caused a change in the correlation pattern in the GatB region. This observation further supports the idea that a communication pathway exists between the GatA and GatB subunits. This change in the correlation pattern is enhanced in the presence of Gln at the GatA active site as shown in Figure 5B. The exact reason for this enhancement is not clearly understood yet, although it suggests a controlled communication pathway that is made more efficient by the presence of glutamine at the GatA active site. In the case of the K89$_{(B)}$R mutation there is a change in the correlation pattern throughout all three subunits GatA, GatB, and GatC. K89$_{(B)}$ residue is located at the interface of GatA and GatB subunits. The nature of the change in correlation pattern suggests that the effect of the mutation at the interface has propagated in the direction of GatA as well as GatB. This observation further adds to the bi-directionality of the communication pathway between the GatA and GatB subunits. Comparison of Figure 5(C) with 5(D) shows again that the change in the correlation pattern is enhanced in the presence of glutamine at the GatA active site.

2.4.2 Residue-wise root mean square deviation (RMSD) studies

Residue-wise RMSD values were calculated for the wild-type GatCAB and the two mutants using the MD trajectories in the final 10 ns of the simulations. RMSD differences were calculated by subtracting the residue-wise average RMSD values of the wild-type, from those of the mutants as shown below.

$$\Delta RMSD_{ave} = mutant\ RMSD_{ave} - WT\ RMSD_{ave}$$

These RMSD differences were mapped onto the structures to visualize the residues with significant deviations. (Figure 6).
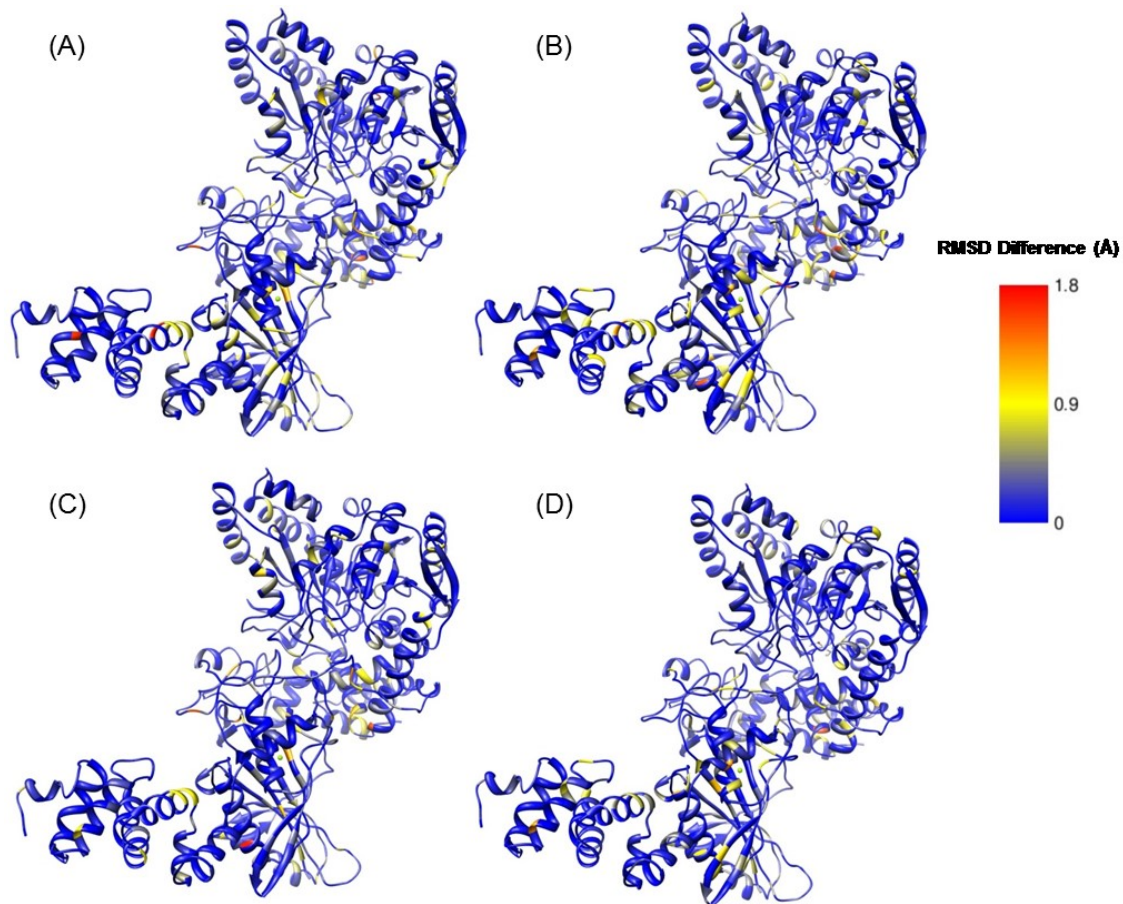
Figure 6: ΔRMSD$_{ave}$ values mapped onto the corresponding simulated GatCAB structures (A) ΔRMSD$_{ave}$ between T149$_{(A)}$V+Gln and wild+Gln. (B) ΔRMSD$_{ave}$ between T149$_{(A)}$-Gln and wild-Gln. (C) ΔRMSD$_{ave}$ between K89$_{(B)}$R+Gln and wild+Gln. (D) ΔRMSD$_{ave}$ between K89(B)R-Gln and wild-Gln.

Higher deviations suggest larger structural perturbations due to the mutations which would facilitate in identifying the amino acid residues involved in inter-domain communication.

The percentage differences in RMSD values were calculated by dividing ΔRMSD$_{ave}$ by WT RMSD$_{ave}$. Analysis of these changes showed that in all four mutant structures (i.e. T149$_{(A)}$V+Gln, T149$_{(A)}$-Gln, K89$_{(B)}$R+Gln, and K89$_{(B)}$R+Gln) approximately 300 amino acid residues showed percentage changes $\geq$ 20%. These four groups that showed higher percentage change in RMSD, contained 59 amino acid residues that were common. This group of 59 amino acids were mapped onto the GatCAB structure to visualize their locations (Figure 7).
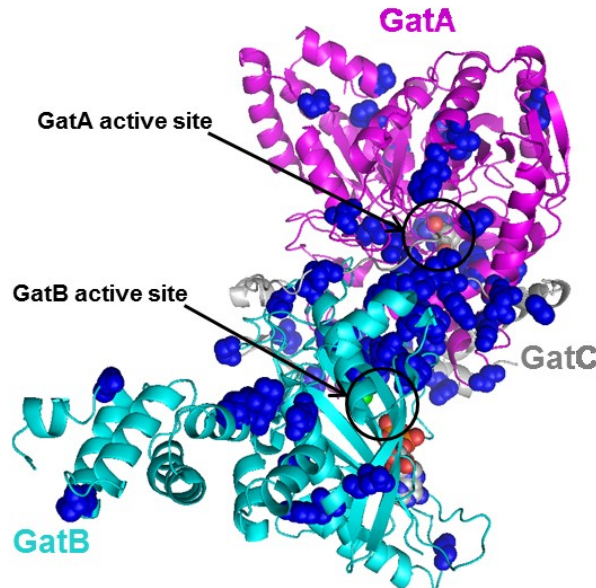
Figure 7: Locations of the 59 common amino acid residues (shown as blue spheres) that show a percentage RMSD change $\geq$ 20%

The fact that these 59 residues are common to all four mutant structures suggests that they may be involved in the communication pathway between the GatA and GatB subunits. The 59 residues are mainly positioned around the two

active sites of GatA and GatB as well as between them. This positioning adds further to the probability of them being candidates to the formation of a communication pathway. Some residues out of this set belong to the GatC subunit which indicates a possible involvement of GatC in communication. Furthermore, contribution of these 59 residues to the inter-domain communication suggests the existence of a common pattern of correlation in the two mutants as well as in the wild-type GatCAB.

To check for a possible correlation pattern, a correlation analysis (Figure 8), as well as a correlation difference analysis (Figure 9), was carried out in the same way as described in sections 2.3.2 and 2.4.1 for these 59 common residues. As shown in Figure 10, the 59 residues in the mutants show low to moderately high correlation differences with respect to the wild-type, which further supports the fact that the correlation that exists between these 59 residues has been disrupted with respect to the wild-type, by the two mutations. These results as a whole suggest the participation of these residues in an extensive network of interactions that enable inter-domain communication between GatA and GatB subunits.
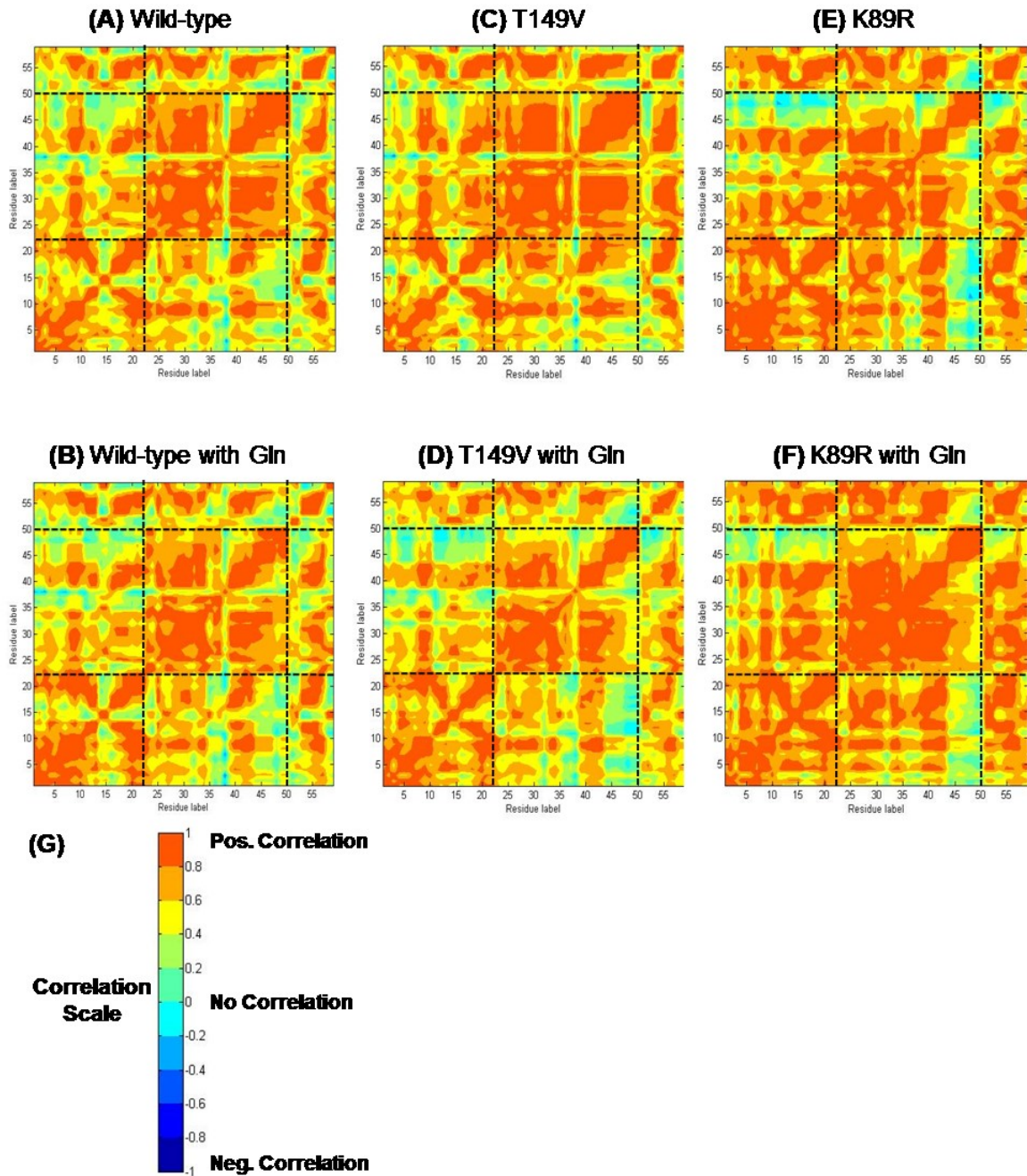
Figure 8: The correlation plots for the 59 common amino acid residues. (A) Wild-Gln; (B) Wild+Gln; (C) T149(A)V-Gln; (D) T149(A)V+Gln; (E) K89(B)R-Gln; (F) K89(B)R+Gln.
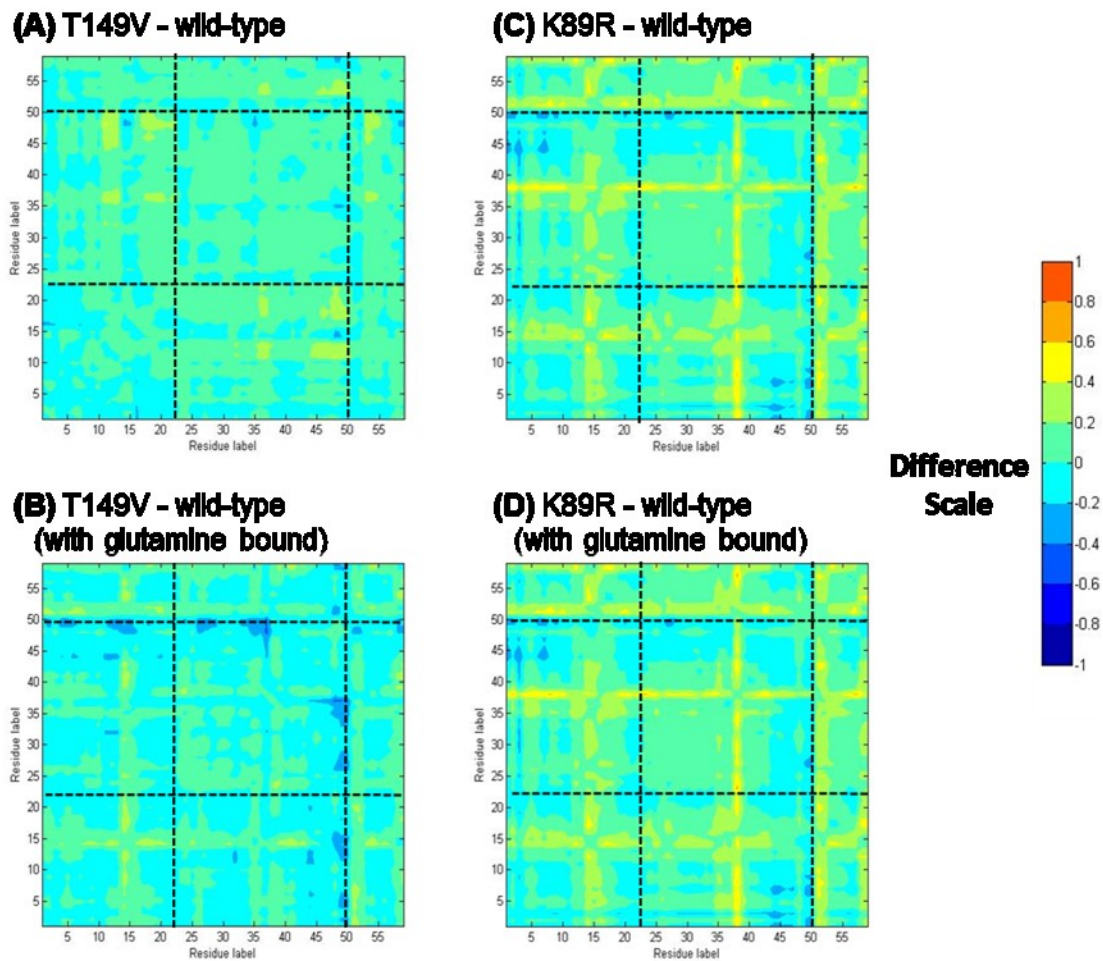
Figure 9: The correlation difference plots for the 59 common amino acid residues. (A) wild-type-Gln correlation matrix subtracted from T149$_{(A)}$V-Gln correlation matrix (B) wild-type+Gln correlation matrix subtracted from T149(A)V+Gln correlation matrix (C) wild-type-Gln correlation matrix subtracted from K89$_{(B)}$R-Gln (D) wild-type+Gln correlation matrix subtracted from K89$_{(B)}$R+Gln

2.5 Conclusions

MD simulations have been performed on wild-type *S.aureus* GatCAB (PDB ID:2F2A) and two mutants, T149$_{(A)}$V and K89$_{(B)}$R, to investigate the existence of a possible communication pathway between the GatA and GatB subunits. MD snapshots were subjected to residue-wise correlation analyses. The correlation differences between the mutants and the wild-type GatCAB show that the T149$_{(A)}$V mutation at the GatA subunit significantly changes the correlation pattern between the GatA and GatB subunits, which supports the existence of a communication pathway between the GatA and GatB subunits. The changes in the global correlation pattern of the K89$_{(B)}$ position due to K89$_{(B)}$R mutation suggest that the communication pathway is bidirectional. Average RMSD difference analyses were able to identify 59 amino acid residues that are common to both mutants that show significantly high changes in percentage RMSD values which are probable candidates as constituent amino acids of the communication pathway. The locations of this subset of residues being mainly around and between the GatA and GatB active sites further adds to their ability to connect the GatA and GatB subunits. The correlation difference plots of these 59 residues show low to moderately high correlation differences in the mutants with respect to the wild-type, which further supports the fact that the correlation that exists between these 59 residues has been disrupted, by the two mutations. This evidence increases the likelihood of these group of residues being directly involved in the inter-domain communication pathway.

CHAPTER 3

ANALYSIS OF D185(A) AS A PROBABLE CATALYTIC RESIDUE IN THE

AMMONIA DELIVERY MECHANISM OF *HELICOBACTER PYLORI*

GLUTAMINE-DEPENDENT AMIDOTRANSFERASE (GATCAB)

3.1 Introduction

As discussed in chapter 1, there is a putative intra-molecular tunnel to describe the transport of ammonia from GatA active site to the GatB active site proposed by Nakamura *et al.* in 2006 (tunnel1). Nakamura *et al.* further went on to show that the tunnel connecting the GatA and GatB active sites was lined with many hydrophilic amino acid residues as well as highly ordered water molecules. Based on this evidence they proposed that the transfer of ammonia from GatA to GatB through the intramolecular tunnel should occur via a series of protonation/deprotonation reactions.[1] This chapter describes an investigation into the possibility of residue D185(A) acting as a catalytic acid/base in the protonation/deprotonation of ammonia released at the GatA active site by glutamine hydrolysis. D185(A) is located close to the GatA active site lining tunnel1 (Figure 10).
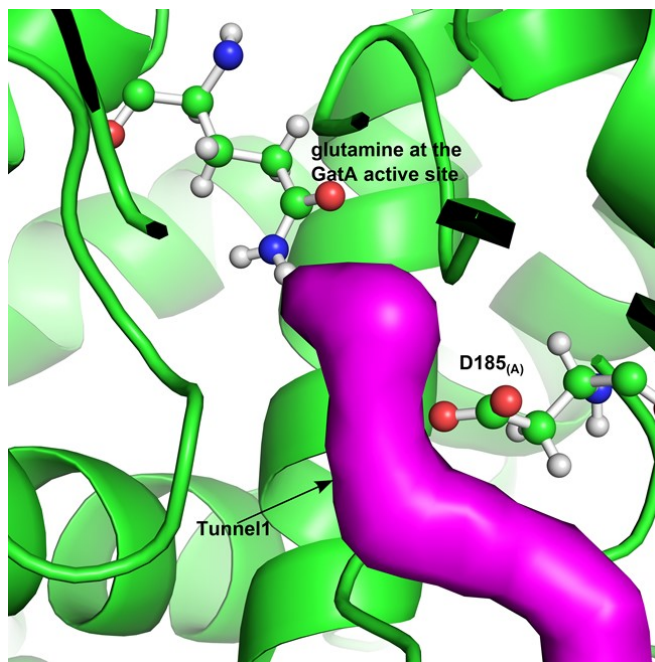
Figure 10: Position of D185$_{(A)}$ relative to the GatA active site and tunnel1

3.2 Experimental findings

This study was performed in collaboration with Professor Hendrickson's group in the department of chemistry at Wayne State University. Prof. Hendrickson and her group created three mutants: D185$_{(A)}$A, D185$_{(A)}$N, and D185$_{(A)}$E, and compared the rates at which they carry out glutamine hydrolysis (reaction 2, section 2.1), phosphorylation (reaction 3, section 2.1), and transamidation (reaction 4, section 2.1). These rates have been compared against the glutamine hydrolysis, phosphorylation, and transamidation reaction rates of wild-type GatCAB. It was seen that the D185$_{(A)}$E mutant retained wild-type like activity for all three reactions. The D185$_{(A)}$A and D185$_{(A)}$N mutants showed 31% and 41% glutamine hydrolysis activity, and 54% and 74% phosphorylation activity respectively. The transamidation activity had noticeably decreased to 2% for

D185$_{(A)}$A and and 6% for D185$_{(A)}$N. As previous studies have shown that the rate-limiting step for GatCAB transamidation of misacylated tRNAs is either ammonia migration through the tunnel or transamidation[9, 117] (as opposed to glutamine hydrolysis and/or phosphorylation), the reductions in rates of glutamine hydrolysis and phosphorylation cannot account for the significant decrease in the rate of transamidation. The only reasons that could describe the decrease in the rate of transamidase activity are either the disruption of the communication pathway between GatA and GatB subunits (which was proven to exist, as described in chapter 2) or the disruption of ammonia migration through the tunnel due to the loss of catalytic activity by the mutations at D185$_{(A)}$. To test the former, computational analyses were carried out, the methods and results of which are discussed below.

3.3 Computational analyses

3.3.1 MD simulations

All MD simulations were carried out using the *S. aureus* GatCAB as described in chapter 2.3. The amino acid residue in *S. aureus* GatCAB enzyme that is equivalent to the D185$_{(A)}$ in *H. pylori* is D211$_{(A)}$. MD simulations were carried out for the wild-type GatCAB and the mutants D185$_{(A)}$A, D185$_{(A)}$N, and D185$_{(A)}$E. The crystal structure for *S. aureus* AdT (PDB ID: 2F2A) was taken from the Protein Data Bank and hydrogenated using MolProbity.[110, 122-124] All structures were capped with acetyl and n-methyl acetamide, neutralized, and solvated with TIP3P water in a rectangular box (approximate dimensions=125 Å × 96 Å ×142 Å) using the tleap program of the AMBER11 software suite.[68, 125] The systems were

minimized with 50 steps of steepest descent and 450 steps of conjugate gradient followed by a density equilibration at 1 g/cm^3 in the isothermal-isobaric (NPT) ensemble and gradual heat up to 300 K in the canonical (NVT) ensemble over 250 ps. Subsequently, each system was simulated for 20 ns under periodic boundary conditions in the NVT ensemble at 300 K keeping the temperature constant using the Berendsen thermostat.[121]

3.3.2 Residue-wise correlation analyses

Correlation analyses were performed via the correl function in ptraj (a program that processes MD trajectories in the AMBER11 software suite) to calculate the correlation matrices of wild-type GatCAB and the mutants at the residue level using the trajectory snapshots from the final 10 ns of each simulation. Correlation differences were calculated by subtracting the wild-type correlation matrix from that of each of the mutants.

3.3.3 pK$_a$ analyses

pK$_a$ calculations were carried out with the PropKa[126, 127] software for all conserved, ionizable tunnel residues in all three mutants and the wild-type GatCAB using the snapshots obtained from the MD simulations.

3.4 Results and discussion

3.4.1 Residue-wise correlation analyses

The correlation difference plots (Figure 11) of the mutants against wild-type GatCAB do not show significant differences in the correlation patterns. This observation supports the hypothesis that the mutations D185$_{(A)}$A and D185$_{(A)}$N at the D185$_{(A)}$ position didn't disrupt the communication pathway between the GatA

and GatB active sites. This conclusion left us with the possibility that the decrease of the transamidase activity at the GatB active site was due to the disruption of ammonia migration through the tunnel caused by the D185$_{(A)}$ mutations. As the D185$_{(A)}$E mutant retained wild-type activity, it suggests that the D185$_{(A)}$A and D185$_{(A)}$N mutations disrupted ammonia transport. This evidence emphasizes the importance of the retention of the carboxylic acid side chain of D185$_{(A)}$ for the acid/base catalytic activity, the loss of which prevents the required protonation/deprotonation activity which subsequently leads to the disruption of ammonia transport.
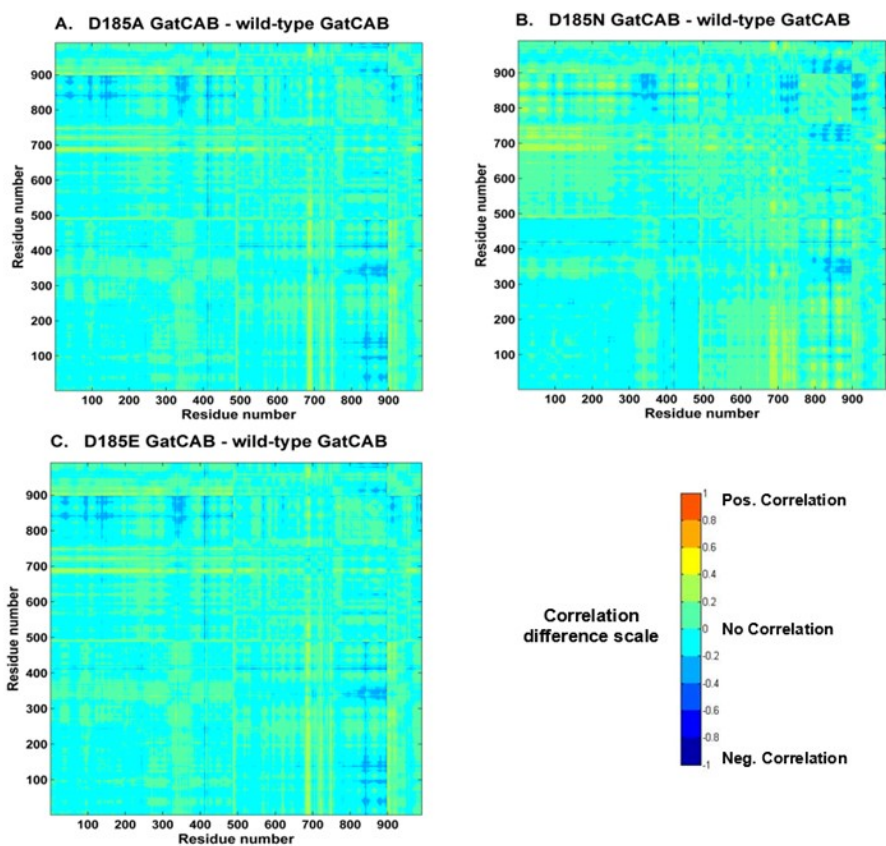
Figure 11: Correlation differences of (A) D185$_{(A)}$A against wild-type; (B) D185$_{(A)}$N against wild-type; (C) D185$_{(A)}$N against wild-type.

3.4.1 pK$_a$ calculations

The calculations demonstrate that several amino acid residues in wild-type GatCAB, including D185$_{(A)}$, show significantly elevated pK$_a$ values (Table 1). This elevated pK$_a$ supports the possibility of D185$_{(A)}$ acting as a base in deprotonating ammonium to ammonia but the possibility of it acting as an acid, protonating ammonia to ammonium, cannot be ruled out.

Residue position	Standard pK$_a$ value*	Calculated pK$_a$ values of amino acid side chain			
		Wild-type	D185$_{(A)}$A	D185$_{(A)}$E	D185$_{(A)}$N
R174	12.48	16.4 ± 0.3	15.9 ± 0.7	16.6 ± 0.5	16.0 ± 0.4
D185	3.86	5.6 ± 0.5	X	5.9 ± 0.5	X
R295	12.48	12.8 ± 0.3	13.3 ± 0.5	12.9 ± 0.3	13.1 ± 0.5
R79	12.48	15.9 ± 0.4	16.4 ± 0.3	16.1 ± 0.4	16.1 ± 0.4
K80	8.95	10.6 ± 0.9	12.1 ± 1.3	10.6 ± 0.7	10.3 ± 0.6
K89	8.95	9.8 ± 0.2	9.8 ± 0.3	9.9 ± 0.3	9.9 ± 0.3
E126	4.25	4.6 ± 0.4	4.5 ± 0.3	4.7 ± 0.3	4.7 ± 0.3
D276	3.86	5.7 ± 0.5	5.7 ± 1.0	5.7 ± 0.6	5.1 ± 1.1

Table 1: pK$_a$ values of conserved, ionizable tunnel residues in AdT. The standard pK$_a$ values are from the *Handbook of Chemistry and Physics*.[128] Values highlighted in grey indicate significant deviations of the calculated pK$_a$ value from the standard value (i.e. ±1 pK$_a$ units)

The evidence given above which suggests that $D185_{(A)}$ most probably acts as a catalyitc base supports the protonation/deprotonation mechanism of ammonia transport that was proposed by Nakamura *et al.* in 2006.

pK$_a$ calculations of the mutants show that there is no significant change in the calculated pK$_a$ values of the ionizable side chains in the tunnel when compared to the wild-type. This consistency suggests that there was no disruption of the chemistry of the other tunnel residues due to mutation at the $D185_{(A)}$ position.

3.5 Conclusions

MD simulations were performed on wild-type GatCAB and the mutants $D185_{(A)}A$, $D185_{(A)}N$, and $D185_{(A)}E$. Correlation difference analyses, and average pK$_a$ calculations were performed using the snapshots of the MD trajectories. Correlation difference analyses show that the mutations $D185_{(A)}A$, $D185_{(A)}N$, and $D185_{(A)}E$ do not disrupt inter-domain communication between the GatA and GatB subunits. This conclusion, combined with the experimental result that the $D185_{(A)}E$ mutant retains wild-type enzyme activity, suggests that the decrease in the transamidase activity at the GatB active site caused by the mutations is due to disruption of ammonia migration through the tunnel. These results further show that the retention of the carboxylic acid side chain of $D185_{(A)}$ is important. The significant increase in the calculated pK$_a$ values of the wild-type $D185_{(A)}$ (with respect to the free amino acid) indicates the possibility of $D185_{(A)}$ acting as a catalytic base, converting ammonium ions to ammonia. To our knowledge, this is the first instance of catalysis inside an intramolecular tunnels, in contrast to

other hydrophobic tunnels found in GATs as well as the partially hydrophilic tunnel found in CPS. These experimental and computational results, supports the previously proposed hypothesis that the transfer of ammonia through the intramolecular tunnel in the GatCAB enzyme happens via a series of protonation/deprotonation reactions.

CHAPTER 4

COMPUTATIONAL ANALYSIS OF AMMONIA TRANSFER ALONG TWO INTRA-MOLECULAR TUNNELS IN *STAPHYLOCOCCUS AUREUS* GLUTAMINE-DEPENDENT AMIDOTRANSFERASE (GATCAB)

4.1 Introduction

Two putative intra-molecular tunnels (Figure 12) have been proposed to explain the passage of ammonia from the GatA subunit to the GatB subunit. The first tunnel, tunnel1, which was already discussed in chapter 2, was proposed by Nakamura *et al.* based on their crystallographic structure.[1] Subsequently, Kang *et al.* proposed an alternative path (tunnel2), based on molecular dynamics (MD) simulations.[17] Tunnel2 was proposed to be the preferred path based on umbrella sampling calculations. The potentials of mean force calculated for both paths were obtained starting from two different points, labeled K1 and K2 in Figure 12, and spanning 4 Å into each tunnel.[17] This chapter presents the investigation of the statistics, structure and energetics of passive ammonia transport through both tunnels based on classical MD simulations. The details for the MD simulations, calculation of tunnel probability starting at three different points along the path, umbrella sampling/WHAM analysis for the energetics of ammonia transport, and energy decomposition and APBS analyses are presented.

Subsequently, the analysis for ammonia transport along both tunnels is presented, followed by concluding remarks.
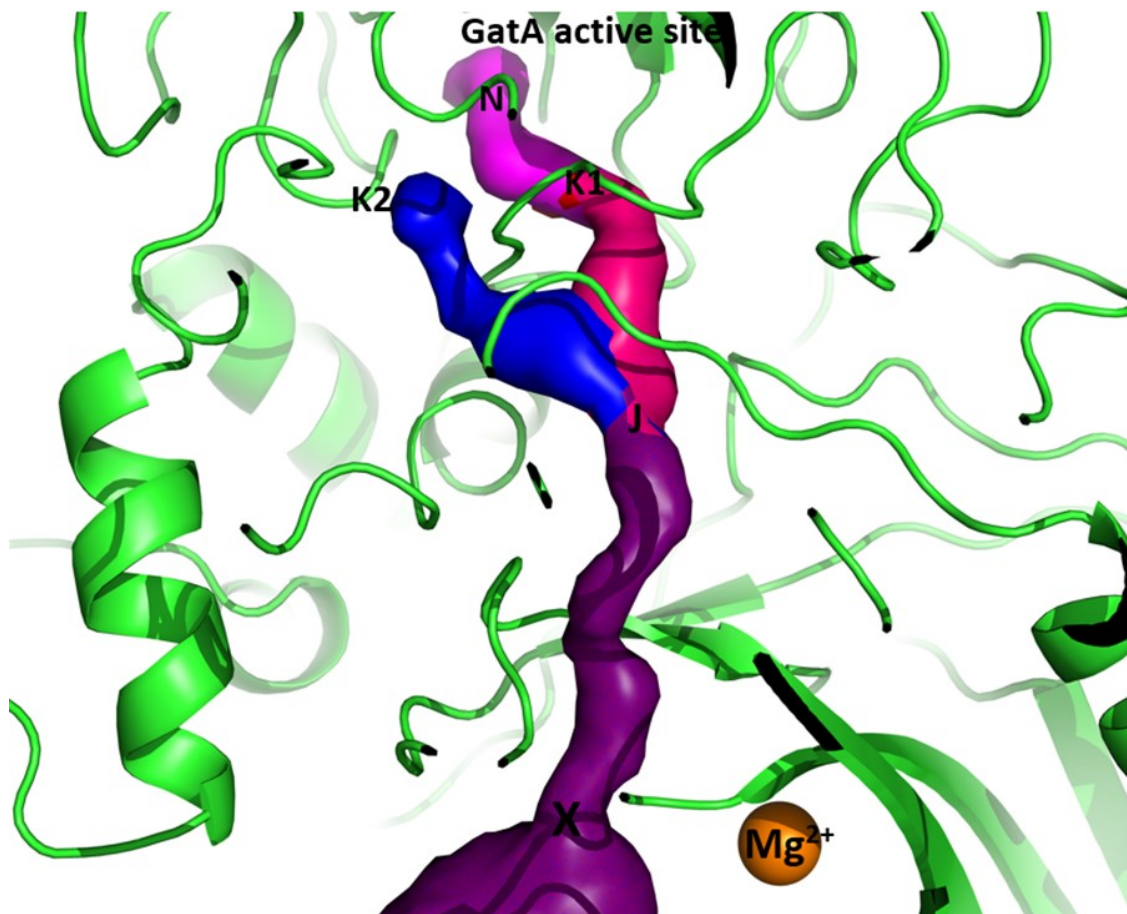
Figure 12: The two tunnels and different starting points for free energy calculations. The path N, K1, J, X denotes tunnel1 and the path N, K2, J, X denotes tunnel2. The junction of the two tunnels is located at the point marked by J. The two entrance points used by Kang *et al.*[17] are K1 and K2. The common exit point of the two tunnels is denoted by X. The Mg^{2+} ion at the GatB active site is also shown.

4.2 Methods

This subsection discusses the computational procedures followed and the subsequent analyses carried out, beginning with the molecular dynamics simulations as described in Subsection 4.2.1 and the four types of analyses: CAVER analysis, WHAM (Weighted histogram analysis method) analysis, energy decomposition analysis (EDA), and the APBS (Adaptive Poisson-Boltzmann solver) analysis described in Subsections 4.2.2, 4.2.3, and 4.2.4 respectively.

4.2.1 Molecular dynamics simulations on wild-type and mutant structures

Eight different systems were considered initially, which involved mutations to two of the subunits. Since GatCAB is comprised of three subunits, each subunit is denoted by a subscript. The eight systems include wild type with glutamine in the GatA active site (Wild+Gln), wild type without glutamine in the GatA active site (Wild-Gln), $T175_{(A)}V$ with glutamine in the GatA active site ($T175_{(A)}V$+Gln), $T175_{(A)}V$ without glutamine in the GatA active site ($T175_{(A)}V$-Gln), $K88_{(B)}R$ with glutamine in the GatA active site ($K88_{(B)}R$+Gln), $K88_{(B)}R$ without glutamine in the GatA active site ($K88_{(B)}R$-Gln), $E125_{(B)}D$ with glutamine in the GatA active site ($E125_{(B)}D$+Gln), and $E125_{(B)}Q$ with glutamine in the GatA active site ($E125_{(B)}Q$+Gln). The mutants were selected based on our previous studies on GatCAB.[18]

All MD simulations were performed using the PMEMD program of the AMBER12 software suite with the ff99SB potential.[68] The SHAKE algorithm was applied to bonds between hydrogen and heavy atoms.[118] Periodic boundary conditions were used for the explicit solvent calculations. Long-range interactions

were accounted for by the smooth particle mesh Ewald (SPME) method.[119, 120] Hydrogens were added to the crystal structure using MolProbity.[123, 124] N termini of the protein were capped with acetyl (ACE) groups and the C termini were capped with N-methyl acetamide (NME) groups. Each system was neutralized by adding Na^+ ions (wild-type GatCAB, T175$_{(A)}$V, K88$_{(B)}$R, E125$_{(B)}$D systems were neutralized by adding 41 Na^+ ions and the E125$_{(B)}$Q system was neutralized by adding 40 Na^+ ions) and solvated in rectangular boxes of TIP3P water, with a 15 Å buffer (final dimensions approximately 125 Å × 96 Å × 142 Å), using the tleap program of AMBER12. The total number of atoms in the final solvated, neutralized wild-type GatCAB system was 183394. All systems were minimized using sander with 50 steps of steepest descent and 450 steps of conjugate gradient while keeping the protein and the substrates restrained with a force constant of 500 kcal mol^{-1}Å^{-2}. The density for each system was equilibrated to 1 g cm^{-3} in the NPT ensemble. The temperature of each system was increased to 300K in ten stages (20 ps at each stage) while keeping the protein and the substrates restrained with a force constant of 500 kcal mol^{-1}Å^{-2}. Subsequently, the restraints were gradually removed over 100 ps, followed by 20 ns of unconstrained MD on all systems while keeping the temperature at 300 K using the Berendsen thermostat[121] in the canonical ensemble.

4.2.2 CAVER analysis

Thirty random snapshots were selected from the final 10 ns of each of the eight trajectories resulting in the selection of 240 snapshots. Tunnels were calculated using CAVER version 2.1.2,[75] for each selected snapshot with K1, K2, and the

GatA active site (see Figure 12) as starting points. K1 was defined by residues Asn81$_{(A)}$, Ala128$_{(A)}$, Val204$_{(A)}$, Ala205$_{(A)}$, Phe206$_{(A)}$. K2 comprises residues Leu203$_{(A)}$, Ala205$_{(A)}$, Phe206$_{(A)}$ and Asp211$_{(A)}$. The GatA active site was defined by residues Lys79$_{(A)}$, Ser154$_{(A)}$, Ser173$_{(A)}$, Thr175$_{(A)}$, and, Ser177$_{(A)}$. Tunnels obtained from the calculation were categorized as either tunnel1 or tunnel2 based on whether they followed the N, K1, J, X or N, K2, J, X paths respectively. Some tunnels could not be categorized as either tunnel1 or tunnel2 due to steric constraints.

4.2.3 Umbrella sampling and WHAM calculations

The initial structure for the potential of mean force (PMF) calculations was selected from a snapshot from the Wild+Gln simulation, which includes both tunnels. The coordinate positions along the tunnels were obtained directly from the CAVER 2.1.2 plugin in PyMOL using the default values. Geometry of the ammonia molecule was optimized at MP2/6-31G* level and the RESP partial charges   -1.0749, 0.3583, 0.3583, 0.3583 were used for the N, H1, H2, and H3 atoms respectively. Once the coordinates for the tunnels were obtained, ammonia molecules were positioned to achieve a spacing between adjacent umbrella windows of 0.3 Å. A window spacing of 0.2 Å was used in some places along the tunnels, considering the curvature, to achieve better sampling. Tunnel1 was populated with 63 ammonia molecules and tunnel2 was populated with 68 ammonia molecules. The umbrella sampling method[20] was used to restrain the positions for each ammonia at selected points along the tunnels. The distance, **R**, between the nitrogen atom of each ammonia molecule and the γ carbon of the

glutamine bound in the GatA active site was chosen as the sampling dimension. The position of the first ammonia (N) corresponds to **R**=5.0 Å. This initial position was chosen due to its close proximity to the bound glutamine, the hydrolysis of which releases ammonia. The distance **R**=5.0 Å at this point was set to **R**=0.0 Å thus making it the reference point for the distance measurement. The PMFs for all systems were calculated for the range 0.0 Å ≤ **R** ≤ 13.5 Å. This range involves the section of the path where the PMF can be described by a 1D coordinate. The remainder of the path (i.e. **R** > 13.5 Å) involves higher dimensional sections, which will be investigated in the future. However, the section studied herein seems to be sufficient to determine which tunnel is energetically favored (see below).

A harmonic potential of 20 kcal mol^{-1}Å^{-2} was applied to restrain the motion of the ammonia molecule in each window. All umbrella sampling simulations were performed using the PMEMD program of the AMBER12 package.[68] Each window was sampled for 1 ns. Sampling was done in both explicit solvent (TIP3P water model)[125] and implicit solvent (Generalized Born model).[129] All simulations were performed at 300K temperature using the Langevin thermostat.[130] The two tunnels have been divided into three regions as denoted in Figure 13. Regions 1 (0.0 Å≤ **R** ≤ 5.2 Å) and 3 (**R** > 16.2Å) are common to both tunnels. Region 2 (5.2Å ≤ **R** ≤ 16.2Å) comprises the section where the tunnels differ.
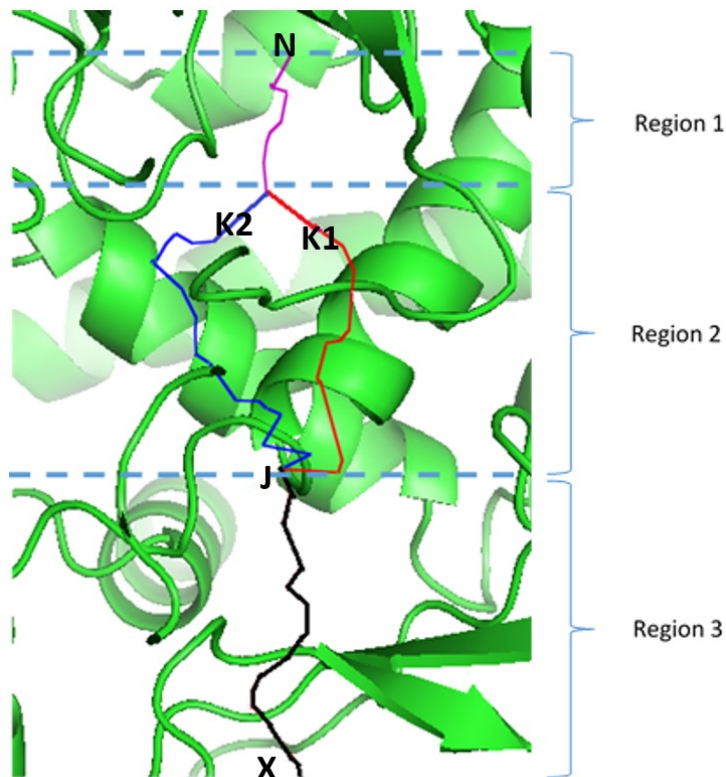
Figure 13: The two proposed tunnels for ammonia transport (represented as lines). The tunnels have been divided into three regions. Region1 (0.0 Å <R < 5.2 Å) and Region3 (R > 16.2 Å) are common to both tunnels. Region2 (5.2Å <R < 16.2Å) is where the two tunnels differ. The letters N, K1, K2, J, and X represent the same points as in Figure 12.

The free energies associated with the ammonia transport along the coordinate **R** were calculated for both tunnels using the 1D-WHAM (one-dimensional weighted histogram analysis method) technique with bootstrapping.[21-23] [131] All 1D-WHAM calculations were performed with the WHAM package.[132]

4.2.4 Energy decomposition analysis and adaptive Poisson-Boltzmann solver calculations

Energy decomposition analysis (EDA) was used to investigate the non-bonded interactions of ammonia with individual residues at selected points along the path. The average non-bonded interaction between a particular ammonia, and every other residue, ΔE_{int}, is approximated by

$$\Delta E_{int} = \left\langle \Delta E_i \right\rangle \quad (13)$$

where i represents an individual residue, ΔE_i represents the non-bonded interaction (electrostatic or Van der Waals) between residue i and the selected ammonia (the interactions between the ammonia and waters or counter-ions are not taken into account), and the broken brackets represent averages over the ensemble obtained from the MD simulations. This analysis has been used previously in QM/MM and classical simulations to investigate interactions between individual residues and ligands or enzyme active sites.[133-144] All energies and energy differences were obtained with an in-house FORTRAN90 program. This program reads the saved snapshots from the MD simulations and the corresponding AMBER parameter set to determine the required non-bonded interactions using the above equation. The adaptive Poisson-Boltzmann solver (APBS) plugin[145] of the PyMOL[76] software suite was used to visualize the electrostatic surface around selected ammonia molecules and the surrounding residues. This plugin uses a parallel focusing method to solve the Poisson-Boltzmann equation in evaluating the electrostatic potential.

4.3 Results and Discussion

This section presents the results obtained from the MD and further analysis for the passive ammonia transport along the two tunnels in GatCAB. In Subsection 4.3.1 the results of the CAVER analysis for selected MD snapshots to investigate the prevalence of each tunnel along the trajectory are discussed. This is followed by the presentation of the free energy profiles obtained from the umbrella sampling and WHAM calculations in Subsection 4.3.2. This subsection also presents the EDA and APBS analysis on different regions of the tunnels to provide further insights on the characteristics of the calculated potentials of mean force (PMFs).

4.3.1 Probability of occurrence of tunnel1 and tunnel2

CAVER analysis was carried out on thirty snapshots for each of the systems studied as explained in Subsection 4.2.1. Based on the CAVER analysis, almost 25% of the tunnels correspond to tunnel2 compared to 5% occurrence of tunnel1 when the starting point of the tunnel calculation is the GatA active site (see Table 2). This shows that tunnel2 has a higher probability of occurrence when compared to tunnel1 based solely on steric constraints.

When the tunnel calculation is started from K2, which is a part of tunnel2, 45.42% of the tunnels fall under the tunnel2 category, compared to 3.75% for the tunnel1 category (Table 4). In contrast, 15.42% of the tunnels correspond to tunnel2 compared to 8.75% for tunnel1 when starting the calculation at K1 (Table 3). This shows that even when starting from a point in tunnel1, there is a higher probability to switch back to tunnel2. There is almost no switching back to

tunnel1 from tunnel2. These results suggest that during a given period of time the dynamic formation of tunnel2 is more probable than that of tunnel1, in agreement with the study by Kang *et al.*[17]

	Tunnel1	Tunnel2
Wild+Gln	6	12
Wild-Gln	0	0
T149$_{(A)}$V+Gln	0	12
T149$_{(A)}$V-Gln	0	0
K89$_{(B)}$R+Gln	0	9
K89$_{(B)}$R-Gln	0	0
E125$_{(B)}$D+Gln	3	9
E125$_{(B)}$Q+Gln	3	17
Total out of 240	12 (5.00%)	59 (24.58%)

Table 2: Categorization of tunnels starting from GatA active site

	Tunnel1	Tunnel2
Wild+Gln	9	12
Wild-Gln	0	0
T149$_{(A)}$V+Gln	0	0
T149$_{(A)}$V-Gln	0	0
K89$_{(B)}$R+Gln	6	12
K89$_{(B)}$R-Gln	0	0
E125$_{(B)}$D+Gln	3	4
E125$_{(B)}$Q+Gln	3	9
Total out of 240	21 (8.75%)	37 (15.42%)

Table 3: Categorization of tunnels starting from K1

	Tunnel1	Tunnel2
Wild+Gln	3	24
Wild-Gln	0	6
T149$_{(A)}$V+Gln	0	15
T149$_{(A)}$V-Gln	0	0
K89$_{(B)}$R+Gln	0	18
K89$_{(B)}$R-Gln	0	16
E125$_{(B)}$D+Gln	3	10
E125$_{(B)}$Q+Gln	3	20
Total out of 240	9 (3.75%)	109(45.42%)

Table 4: Categorization of tunnels starting from K2

In contrast to the number of instances of tunnel1 and tunnel2 in Wild+Gln, there is an overall decrease in the occurrence of both tunnels in $T175_{(A)}V$, $K88_{(B)}R$, $E125_{(B)}D$, and $E125_{(B)}Q$ mutants. The overall decrease in the tunnel formation for the $T175_{(A)}V$ and $K88_{(B)}R$ mutants is in accordance with results from our previous study[18] on *H. pylori* GatCAB enzyme, which showed that the corresponding mutants $T149_{(A)}V$ and $K89_{(B)}R$ have a marked decrease in the rate of phosphorylation (Chapter 2, reaction 3) at the GatB active site.

The CAVER algorithm simplifies the substrate molecule (the whole ammonia molecule) to a sphere. It also simplifies the protein atoms to spheres whose magnitudes are governed by Van der Waals radii. The algorithm determines the existence of the tunnel purely based on steric considerations and it does not consider actual inter-molecular interactions. This steric nature of the calculation prevents any insight into the energetics of transport along the tunnel. Therefore, to gain further insight, umbrella sampling/WHAM calculations were carried out as detailed below.

4.3.2 Free energy profiles for ammonia transport along tunnel1 and tunnel2

The PMFs for the transfer of ammonia in explicit versus implicit solvent are shown in Figure 14 and Figure 15 respectively. It can be seen that the relative shapes of free energy profiles for tunnel1 and tunnel2 look similar in both implicit and explicit solvents. The region 0.0 Å $\leq$ **R** $\leq$ 5.2 Å (region 1 in Figure 13) is common to both tunnel1 and tunnel2. As expected, the free energy profile of both tunnels overlap in this region. The point at **R**=0.0 Å corresponds to point N (Figure 13), which was the starting point of free energy calculations. Bootstrap

analyses[40] (see Figures 16-19) were done to validate the reproducibility of the data for each free energy calculation. To enable the comparison of all calculated PMFs, all free energies were calculated relative to point **R**=2.5 Å (common to both tunnel1 and tunnel2). For tunnel1, the calculated free energy barrier for ammonia transfer is 3.64 kcal/mol in explicit solvent and 1.23 kcal/mol in implicit solvent. For tunnel2, the calculated free energy barrier is to 7.98 kcal/mol in explicit solvent and 2.93 kcal/mol in implicit solvent.

The previous study by Kang *et al*[17] concluded that tunnel2 is preferred over tunnel1. However, the free energy differences calculated in that study were limited to a region spanning 4 Å along an imaginary Z axis that begins at the initial position of ammonia, proceeds to both entrance points (given by K1 and K2 in our study), and connects the initial point with the Mg^{2+} ion at the GatB active site. In our study, the two entrance points K1 and K2 correspond to **R**=6.1 Å along tunnel1 and **R**=6.3 Å along tunnel2 respectively. We have studied the two tunnels from **R**=0.0 Å to **R**=13.5 Å. Our study shows that, although there is a slight preference for tunnel2 over tunnel1 at the entrance points of the two tunnels (5.3 Å ≤ **R** ≤ 6.4 Å), as ammonia moves further along each tunnel, the calculated relative free energy of transport for ammonia is significantly lower for tunnel1.
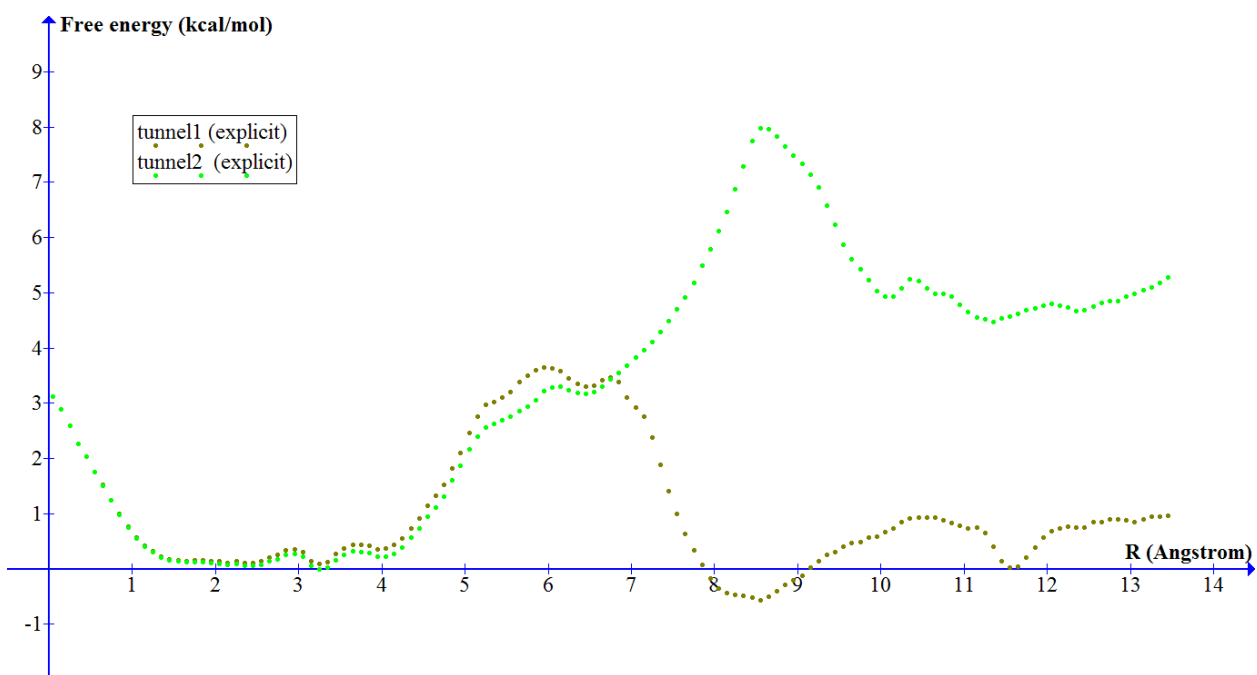
Figure 14: Free energy of ammonia transfer along the two types of tunnels for 0.0 Å ≤ R ≤13.5 Å in explicit solvent.
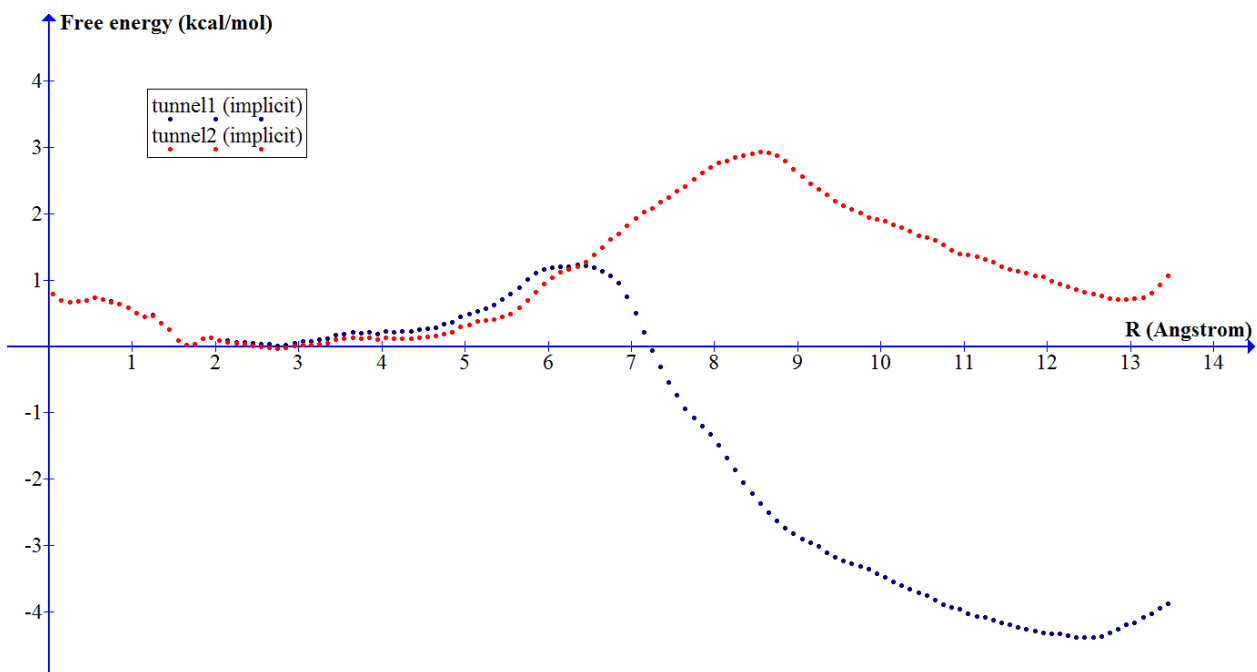
Figure 15: Free energy of ammonia transfer along the two types of tunnels for 0.0 Å ≤ R ≤13.5 Å in implicit solvent.
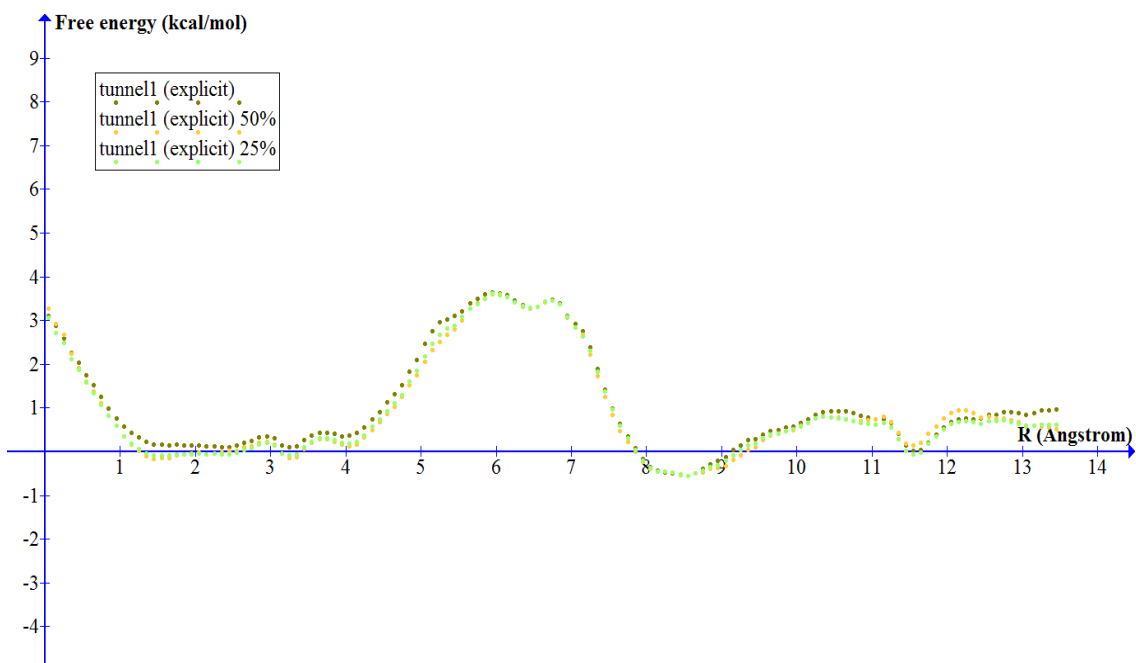
Figure 16: Bootstrapping in tunnel1 with explicit solvent
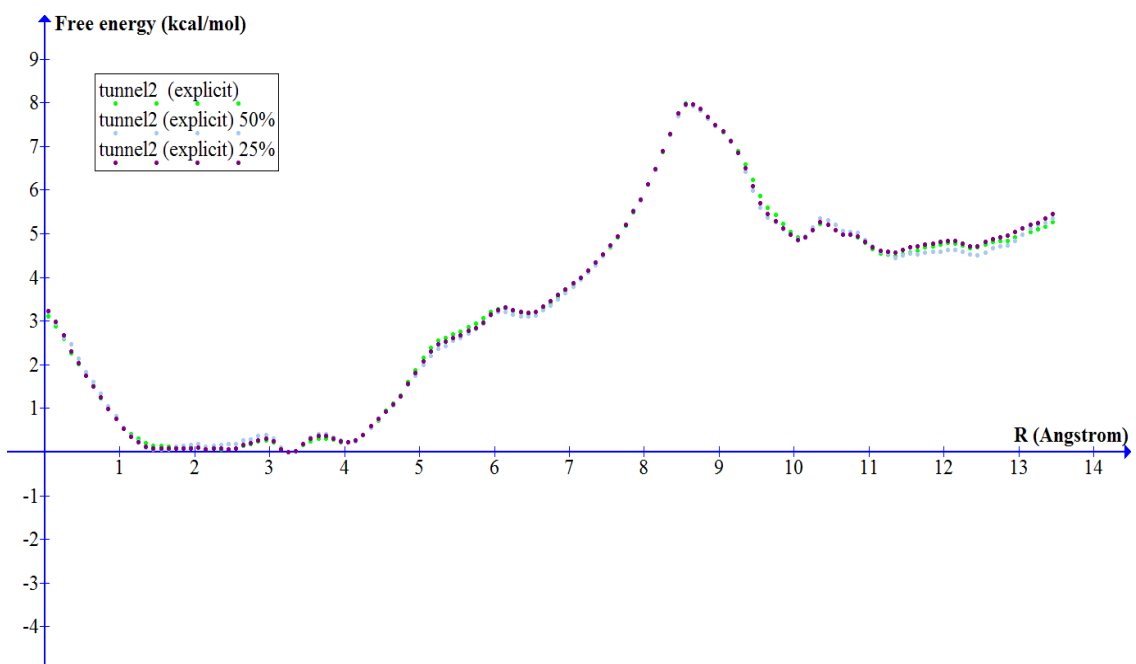
Figure 17: Bootstrapping in tunnel2 with explicit solvent
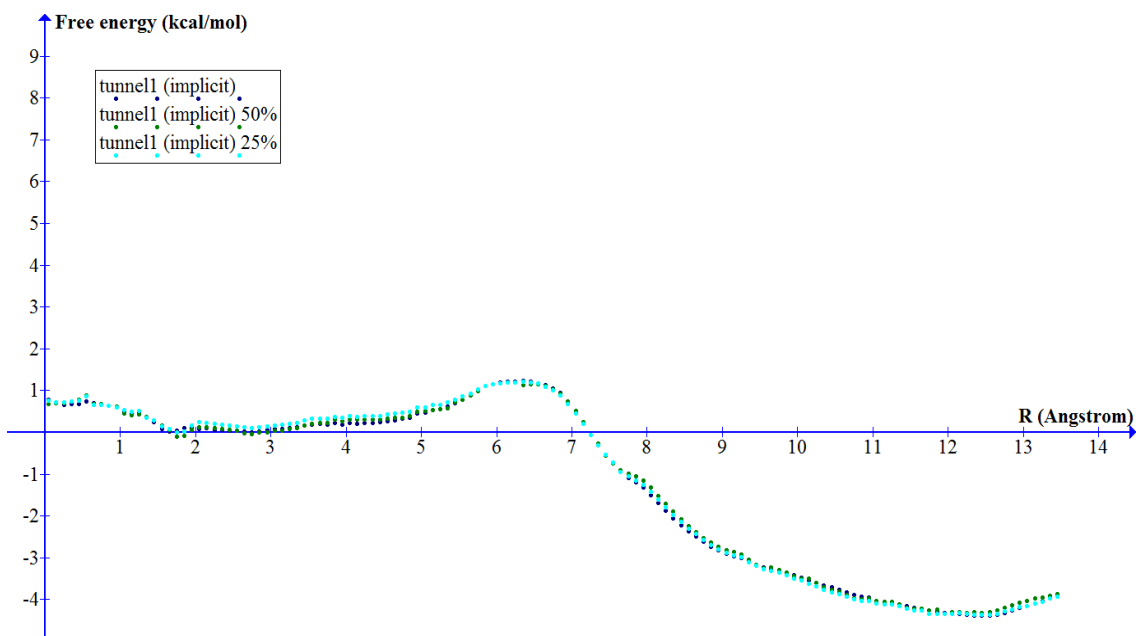
Figure 18: Bootstrapping in tunnel1 with implicit solvent
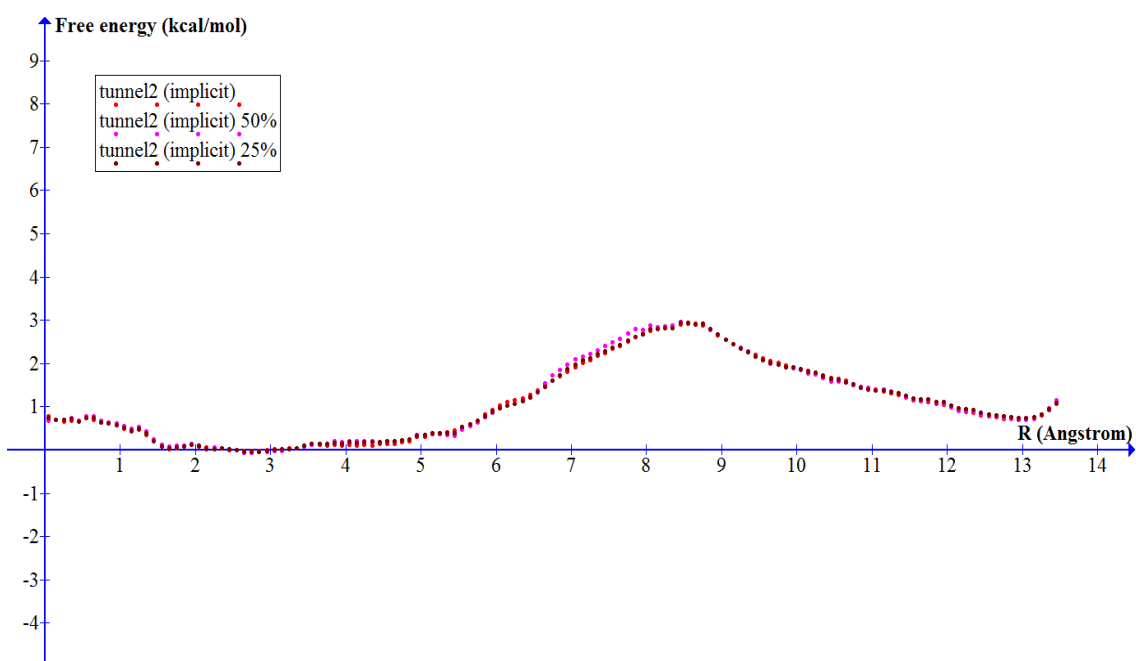
Figure 19: Bootstrapping in tunnel2 with implicit solvent

A rise in free energy at the beginning of the PMF (**R**≈0.0 Å) is observed for the explicit solvent simulations compared with the region around **R**≈1.5 Å. At the beginning of the sampling coordinate, **R**≈0.0 Å, the ammonia molecule exhibits large (attractive) electrostatic interactions with K79$_{(A)}$, A128$_{(A)}$, T175$_{(A)}$, and D211$_{(A)}$ based on the EDA calculations as shown in Figure 20. The attraction between ammonia and A128$_{(A)}$ at **R**≈0.0 Å, as observed by visualizing the trajectory, is due to the formation of a hydrogen bond between the –NH hydrogen atom of the peptide bond and the nitrogen atom in ammonia. Analysis of the MD trajectories at **R**≈1.5 Å, shows that the ammonia moves towards a more polar environment created by K79$_{(A)}$, and D211$_{(A)}$, compared to the environment around **R**≈0.0 Å.

The ammonia molecule shows a significant attraction in both sections of the PMF to K79$_{(A)}$, and D211$_{(A)}$. However, at **R**≈1.5 Å, the attraction is larger than that at **R**≈0.0 Å, leading to greater stabilization of the ammonia at **R**≈1.5 Å. This greater attraction is due to ammonia getting trapped in the interspace between K79$_{(A)}$, and D211$_{(A)}$ making hydrogen bonds with both of them as shown in Figure 21. The difference in the sum of electrostatic and Van der Waals interactions between ammonia and the protein residues in the explicit solvent as calculated by EDA results in approximately an 2.7 kcal/mol lowering in energy at **R**≈1.5 Å with respect to **R**≈0.0 Å. This energy difference agrees with the free energy difference of 3.1 kcal/mol calculated by WHAM as shown in the PMF plot in Figure 14.
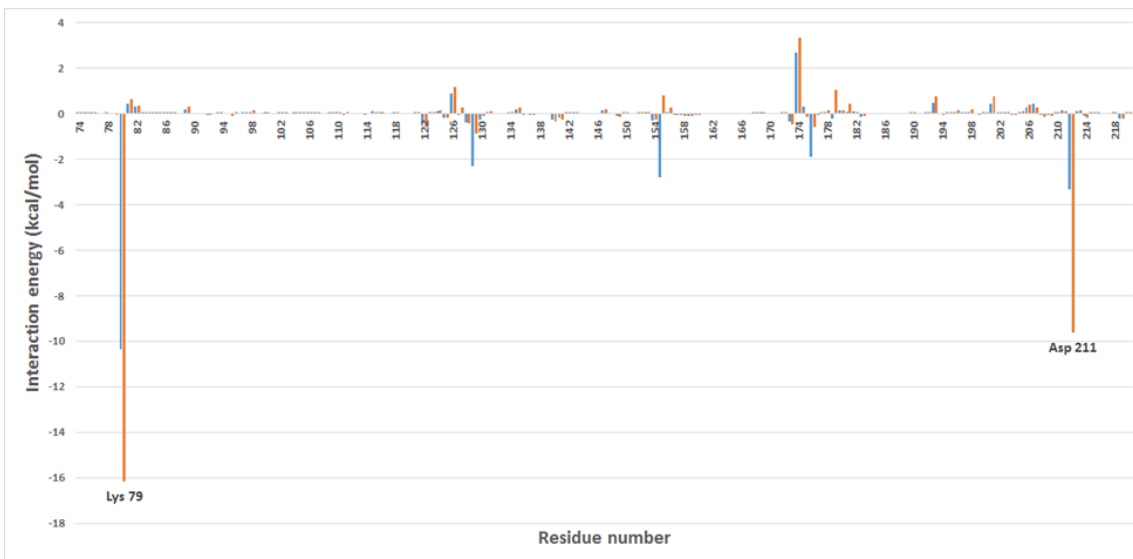
Figure 20: EDA analysis for the explicit solvent simulations at R≈1.5 Å and R≈0.0 Å. Blue denotes interaction energies at R≈0.0 Å and orange indicates interaction energies at R≈1.5 Å. Interaction energies are given in kcal/mol. Only residues with interactions ≥ |1 kcal/mol| were considered.

Ten random snapshots were subjected to APBS analysis to determine the electrostatic potential around ammonia at **R≈1.5 Å**. Residues located within a radius of 4 Å from ammonia were included in the electrostatic potential calculation. In the electrostatic potential surface (Figure 21), the potential at a particular point is the potential energy of a proton placed at that point. The red regions correspond to regions with negative electrostatic potential and the blue regions correspond to regions with positive electrostatic potential. The color key has been defined in energy units of k_BT/e (where 'e' is the charge of an electron). The amino acid residues that showed significant interactions with ammonia in the

EDA analysis have been labeled within the surface to illustrate their location with respect to the ammonia molecule.

As shown in Figure 21, the ammonia molecule is oriented in a way such that the hydrogen atoms are oriented toward regions of negative electrostatic potential and the nitrogen atom is oriented toward regions of positive electrostatic potential. The distance between the ammonia nitrogen and the $K79_{(A)}$ hydrogens as well as the distance between ammonia nitrogen and, Asp $211_{(A)}$ oxygen, together with their relative orientation show that ammonia forms hydrogen bonds with these residues, which support the electrostatic attractions shown by the by the EDA analysis.
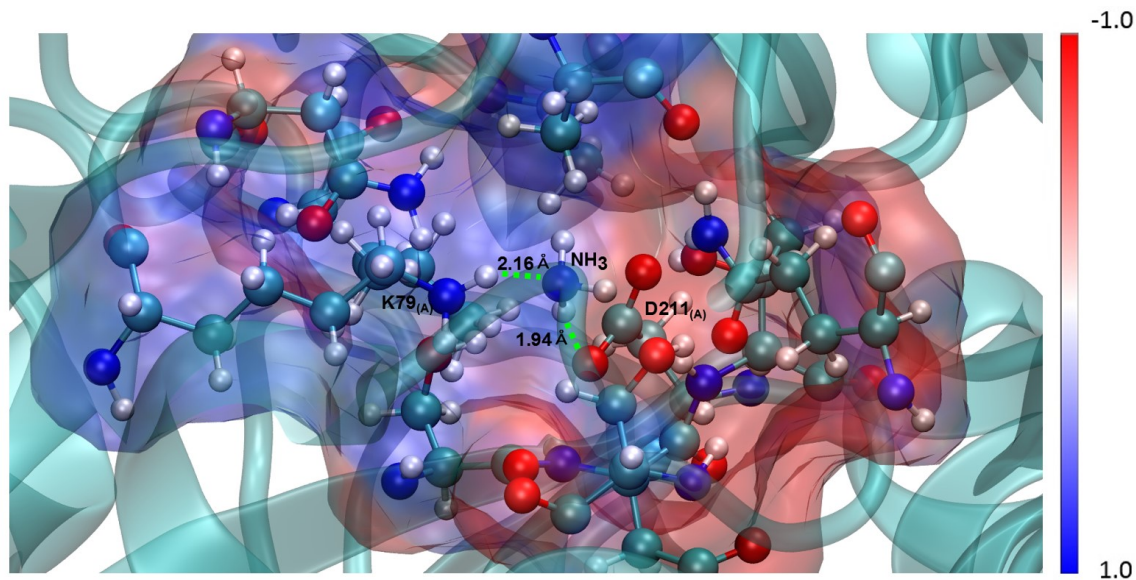
Figure 21: APBS analysis using ammonia, and residues within 4 Å of ammonia at R≈1.5 Å. The color key has been defined in energy units of kBT/e (where 'e' is the charge of an electron).

The free energies for ammonia transfer in both tunnel1 and tunnel2 are comparatively close to each other in the region **R**≈5.4 Å - 6.4 Å as the divergence

into two different tunnels from the branching point at $R \approx 5.2$ Å is minimal in this region. Analysis of the MD trajectory for tunnel1 shows that, as the ammonia molecule progresses from the hydrophilic 1.5 Å $\leq R \leq$ 3.5 Å region to $R \approx 6.0$ Å, it gets closer to a hydrophilic environment created by residues $R200_{(A)}$, $A207_{(A)}$, $D211_{(A)}$ (Figure 22).

Other than the attractive interactions with $R200_{(A)}$ and $D211_{(A)}$, the EDA analysis at $R \approx 6.0$ Å (see Figure 23) also shows attractive interactions with $A207_{(A)}$, which are mainly due to hydrogen bonding between the nitrogen atom of ammonia and the –NH hydrogen of the peptide bond between $A207_{(A)}$ and $F206_{(A)}$. The difference in the sum of electrostatic and Van der Waals interactions between ammonia and all protein residues in the explicit solvent simulation calculated by EDA between $R \approx 1.5$ Å and $R \approx 6.0$ Å (see Figure 23) is around 3.2 kcal/mol, in close agreement with the barrier of 3.6 kcal/mol. This result suggests that the change in non-bonded interactions between the minimum ($R \approx 1.5$ Å) and the highest energy region ($R \approx 6.0$ Å) is mainly due to the reduction of attractive non-bonded interactions in the section corresponding to $R \approx 6.0$ Å in comparison with $R \approx 1.5$ Å. It should be noted that in the proposal of tunnel1 from the original crystal structure, it was suggested that $R200_{(A)}$ and $D211_{(A)}$ could form hydrogen bonds to an ammonia molecule at the same time. Based on our results, it is observed that throughout the simulation, the hydrophilic side chains of $R200_{(A)}$ and $D211_{(A)}$ are pointed away from each other. This orientation of the side-chains diminishes the chances of entrapment of the ammonia molecule in the interspace between $R200_{(A)}$ and $D211_{(A)}$.
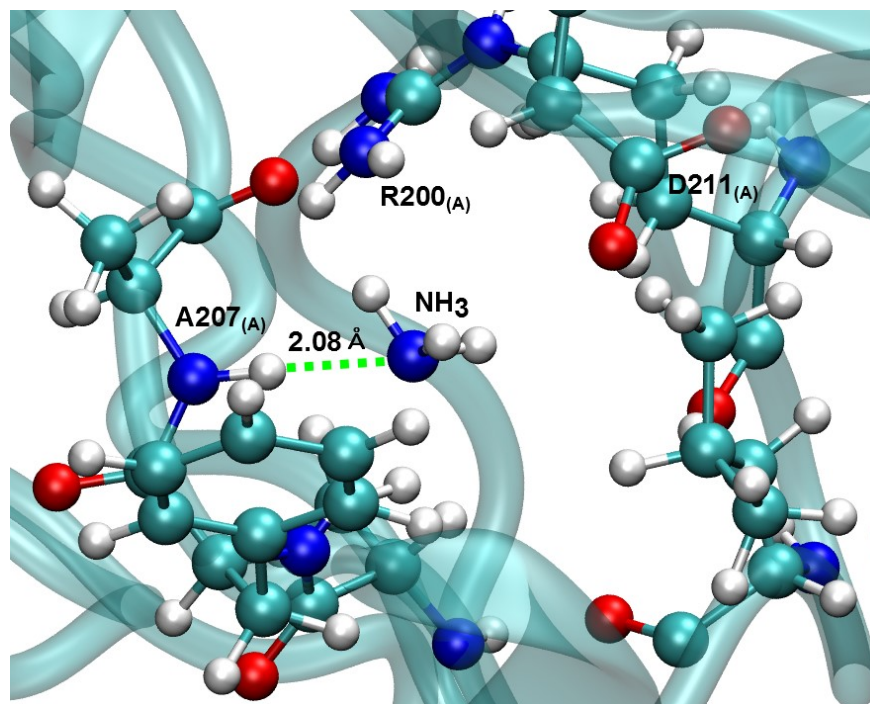
Figure 22: Ammonia in the hydrophilic environment in the region R≈6.0 Å inside tunnel1.
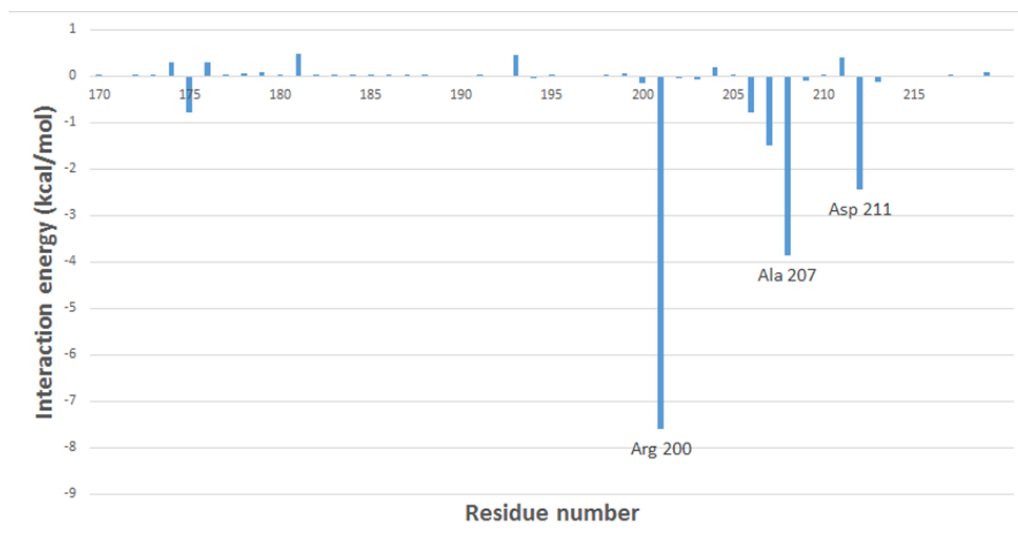
Figure 23: Explicit solvent EDA analysis at R≈6.0 Å in tunnel1 (showing the most prominent peaks)

Immediately after the branching point in tunnel2, in the region $R \approx 6.0$ Å, ammonia enters the hydrophilic environment created by residues $K79_{(A)}$ and $D211_{(A)}$ (Figure 24). Existence of these attractive interactions are further confirmed by the EDA analysis (see Figure 25). The difference in the sum of electrostatic and Van der Waals interactions between ammonia and all protein residues in the explicit solvent simulation as calculated by EDA gives a change in energy of ≈ 2.6 kcal/mol between $R \approx 6.0$ Å and $R \approx 1.5$ Å, which is in close agreement to the free energy barrier of 3.2 kcal/mol increase at $R \approx 6.0$ Å calculated by WHAM. As shown in Figure 24, the ammonia molecule is oriented in such a way that the nitrogen atom of ammonia is pointed towards the hydrogen atoms of the $-NH_3^+$ group of $K79_{(A)}$ and the hydrogen atoms of ammonia are pointed towards the oxygen atoms of the carboxyl group of $D211_{(A)}$, which facilitate formation of hydrogen bonding.
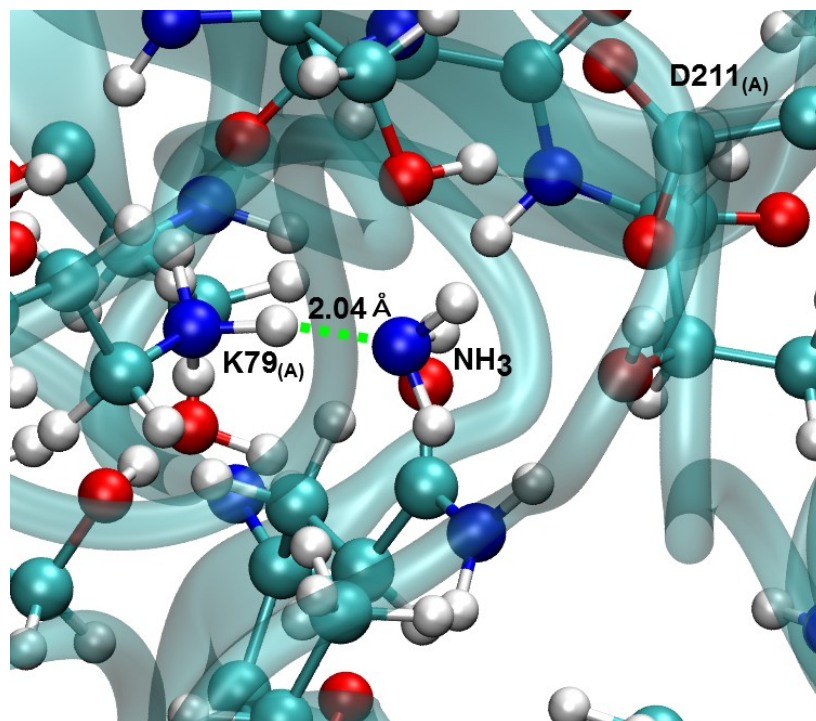
Figure 24: Ammonia in the hydrophilic environment in the region R≈6.0 Å inside tunnel2
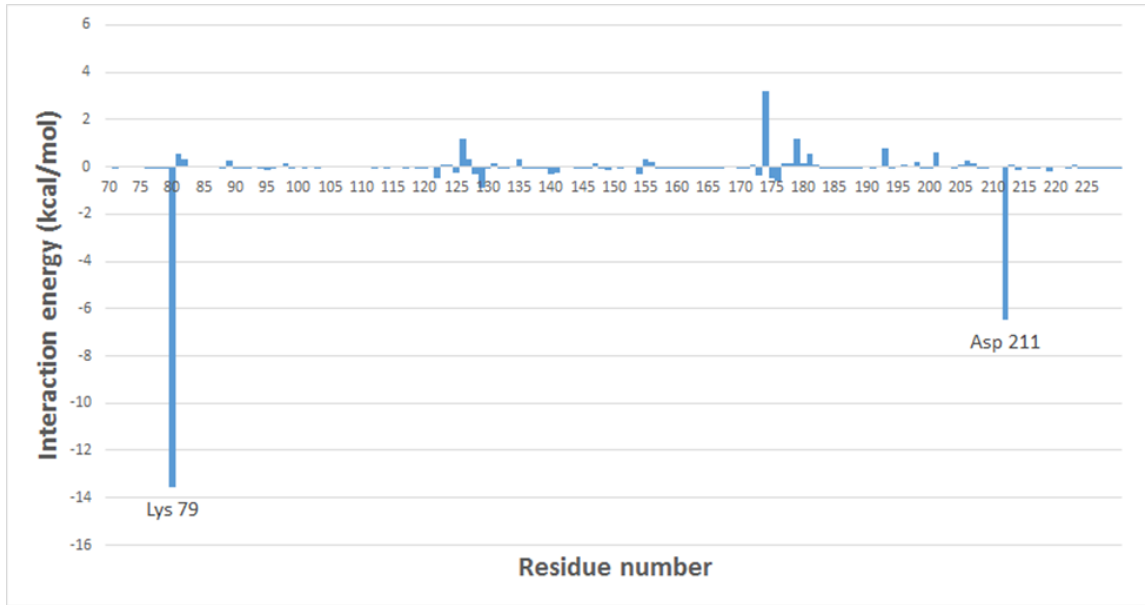
Figure 25: Explicit solvent EDA analysis at R≈6.0 Å in tunnel2 (showing the most prominent peaks)
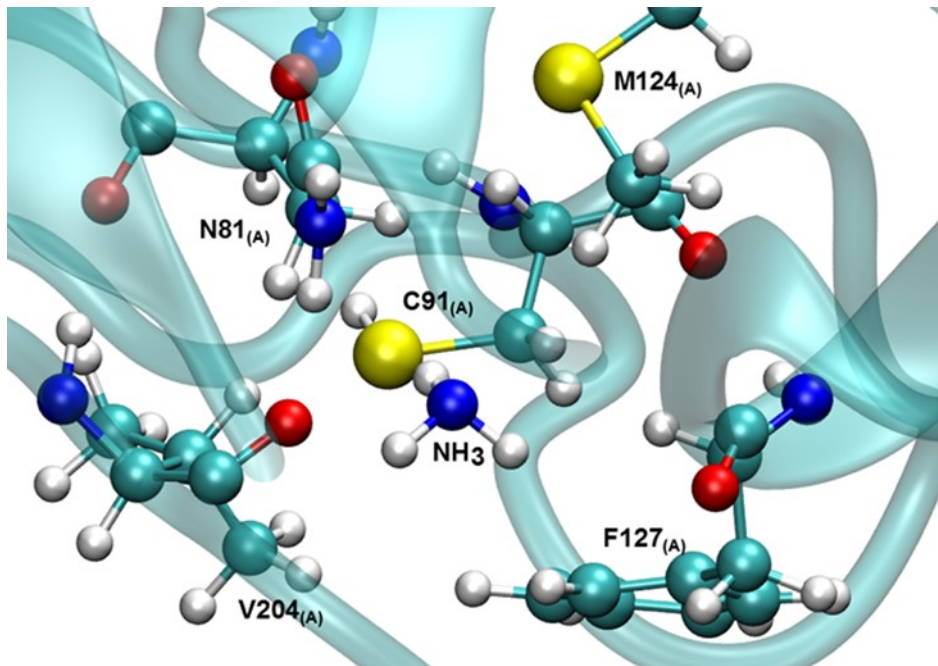
Figure 26: Ammonia in a hydrophobic environment in the region 6.5 Å ≤ R ≤ 8.5 Å inside tunnel2.

Figure 27: Explicit solvent EDA analysis at R≈8.5 Å in tunnel2 (showing the most prominent peaks)

A sharp rise in free energy is observed in tunnel2 between **R=**6.5 Å and **R=**8.5Å. This rise in free energy for tunnel2 can be attributed to the lack of attractive interactions between ammonia and the surrounding residues with respect to the attractive forces experienced in the **R≈**1.5 Å region. The only significant attractive force experienced by ammonia, as shown by the EDA analysis (Figure 27) are due to N81$_{(A)}$ and C91$_{(A)}$. Other than this, the ammonia molecule is in a relatively hydrophobic region (Figure 26) created by V204$_{(A)}$, M124$_{(A)}$, and F127$_{(A)}$, when compared to the hydrophilic region at **R≈**1.5 Å. The difference in the sum of electrostatic and Van der Waals interactions between ammonia and all protein residues in the explicit solvent system (as calculated by EDA) is around 7.5 kcal/mol for the region at **R≈**8.5 Å with respect to **R≈**1.5 Å, close to the calculated

free energy barrier of 8.0 kcal/mol at $R \approx 8.5$ Å calculated by WHAM (see Figure 14).

In contrast to the sharp rise in free energy in tunnel2 between $R=6.5$ Å and $R=8.5$ Å the free energy for transport of ammonia in tunnel1 decreases consistently between $R=6.5$ Å and $R=8.5$ Å. The stabilization of the ammonia in tunnel1 in this section is mostly due to the electrostatic interactions experienced by ammonia in the hydrophilic environment created by the residues $R200_{(A)}$, $S208_{(A)}$, $N320_{(A)}$, and $D274_{(B)}$ (Figure 28). Moreover, $R200_{(A)}$, $S208_{(A)}$, and $D274_{(B)}$ are conserved residues lining tunnel1 as noted by previous studies.[1] The orientation of ammonia and the measured distances show hydrogen bonding interactions between ammonia and all of the above-mentioned residues. The hydrophilic environment created by these polar residues also attracts water molecules as seen in the trajectory snapshots (Figure 28). The presence of water further adds to the attractive interactions experienced by ammonia through hydrogen bonding.
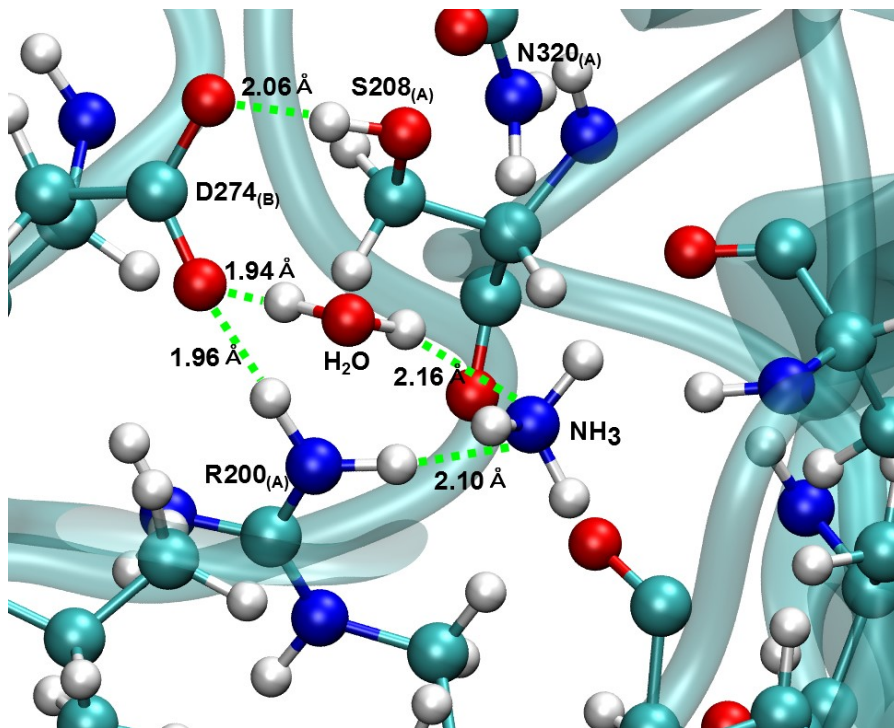
Figure 28: Ammonia in a hydrophilic environment in the region 6.5 Å ≤ R ≤ 8.5 Å inside tunnel1.

4.4 Conclusions

MD simulations have been performed on wild-type GatCAB of *S. aureus* (PDB ID:2F2A) and four mutants, T175$_{(A)}$V, K88$_{(B)}$R, E125$_{(B)}$D, and E125$_{(B)}$Q, to investigate the transport of ammonia along two putative tunnels. MD snapshots thus obtained were analyzed for the intra-molecular tunnels using the CAVER plugin of the PyMOL software suite and categorized as either tunnel1 or tunnel2. This categorization showed that tunnel2 has a higher probability of occurrence than the tunnel1 based purely on steric considerations. Potentials of mean force for both tunnels were calculated using umbrella sampling/WHAM analysis for the wild-type enzyme. Our results point to a significantly lower free energy of transfer

of ammonia through tunnel1. EDA and APBS analyses were used to provide further insight into the differences for ammonia transport between each tunnel. The large increase in free energy observed in tunnel2 after the bifurcation between both tunnels can be ascribed to a highly hydrophobic region around this area. This is in contrast to the previous study by Kang *et al.,* which determined the free energy of transport for a small region after the entrance points to the two tunnels. The present results cover approximately half of the distance for both tunnels, which corresponds to the section of the path that can be described by a one-dimensional coordinate. The remainder of the tunnel requires the use of multi-dimensional WHAM analysis, and will be investigated in a future contribution.

CHAPTER 5

QUANTUM MECHANICAL/MOLECULAR MECHANICAL (QM/MM) INVESTIGATION OF *MYCOBACTERIUM TUBERCULOSIS* INORGANIC PYROPHOSPHATASE (PPASE) MECHANISM

5.1 Introduction

PPase catalyzes the hydrolysis of an inorganic pyrophosphate (PP_i) molecule into two phosphate ions (P_i). The highly exothermic nature of this reaction is of utmost importance in biological systems as the energy released can be used to catalyze kinetically unfavorable biological reactions. The exact mechanism of action for PPase has not been established yet. This, and the fact that PPase is essential for the survival of many bacteria and fungi mark the importance of study of the mechanism of action of PPase.

There are two main families of PPases: Family 1, and Family 2. Although the PPases in the two families are not homologous in sequence or structure they function via a similar mechanism.[43] This chapter discusses a study of the mechanism of action of *M. tuberculosis* PPase, a Family 1 PPase. As with all the other Familiy 1 PPases, *M. tuberculosis* PPase requires divalent metal cations for catalytic activity. The computational analysis described in this chapter has been performed with three Mg^{2+} ions in the active site of *M. tuberculosis* PPase.

Previous biochemical and structural studies performed mostly with the *E. coli* and *S. cerevisiae* PPase have led to a proposed mechanism of Family 1 PPases (Figure 29).[51]
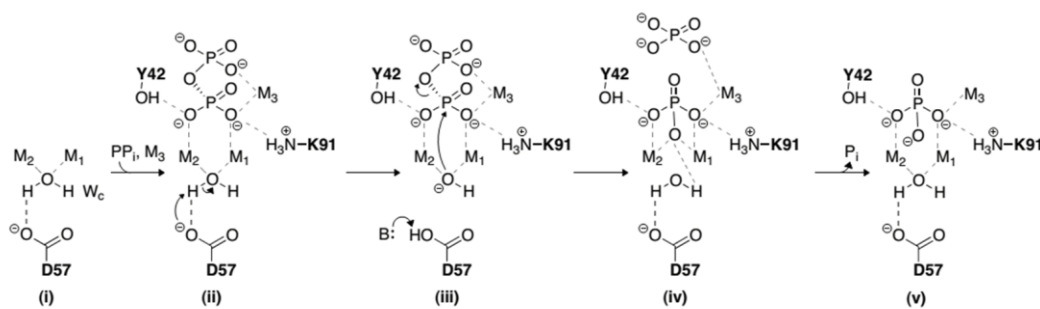
Figure 29: Mechanism for Family 1 PPase action proposed by *Samygina et al.* in 2007.

According to this mechanism, the PP_i bound at the PPase active site is activated by three separate metal ions: M_1, M_2, and M_3. In addition to the activation of the substrate, two of the metal ions, M_1 and M_2, activate a putative water molecule (W_c) by increasing its nucleophilicity. A nearby aspartate residue (D57) is also involved in the activation of W_c. The third metal ion, M_3, is chelated by the PP_i substrate. D57 abstracts a proton from the activated W_c resulting in the formation of a hydroxyl anion which carries out a nucleophilic attack on the proximal phosphorus atom of the PP_i. This nucleophilic attack results in the cleavage of the phosphoanhydride bond producing two phosphate (P_i) ions. In addition to the metal ions, the PP_i substrate is also stabilized by hydrogen bonds and salt bridges with surrounding amino acids. It has to be noted that although the presence of W_c was proposed in the mechanism, crystal structures with W_c positioned as presented in Figure 29 have not been reported.

5.2 Experimental findings

This study was carried out in collaboration with Professor Oleg Tsodikov's group at University of Kentucky. They have used Ca^{2+} ions in the crystallization solution alongside P_i and PPase to support the backward reaction in which Pi ions react with each other to form PP_i ions. In this process, they were not only able to capture PP_i bound to PPase but also the predicted catalytic water molecule (W_c) shown in Figure 29, in a precatalytic position. They were also able to isolate crystal structures of PPase bound to P_i (the product of the forward reaction) and for apo-PPase.

In the isolated crystal structures, W_c was found to be bridging two metal ions and in close proximity to the surrounding amino acids D57 and D89. This supports the existence of the structures (ii) and (iii) from the previously proposed mechanism (Figure 29). W_c is not observed in the P_i-bound or apo-PPase which suggests that W_c binds to PPase in concert with PP_i and leaves the active site with dissociation of the first P_i in contrary to the previously proposed mechanism, which suggested that W_c was already bound to the active site of PPase before PP_i is bound.

It was observed that apo-PPase bound to Ca^{2+} contains only one metal ion at the M_1 position and apo-PPase bound to Mg^{2+} contains two metal ions at the M_1 and M_3 positions, which suggests that M_1 is a higher affinity site for a divalent metal than M_2 or M_3. Furthermore, in the Pi bound PPase structure, it is observed that the metal ion at the M_2 position is shifted towards the P_i that is left in the active site after hydrolysis. This remaining P_i is located at a position in

which the distal phosphorus of the PP_i bound to PPase is observed. The shift of the M_2 metal ion and the relative position of the remaining Pi at the active site suggests that the first Pi that leaves the active site involves the proximal phosphorus in PP_i, contrary to the previously suggested mechanism, in which the Pi formed of the distal phosphorus atom of the bound PP_i was proposed to leave the active site first.

Based on the above observations Professor Tsodikov's group has proposed a new mechanism that is shown in schematic form in Figure 30.
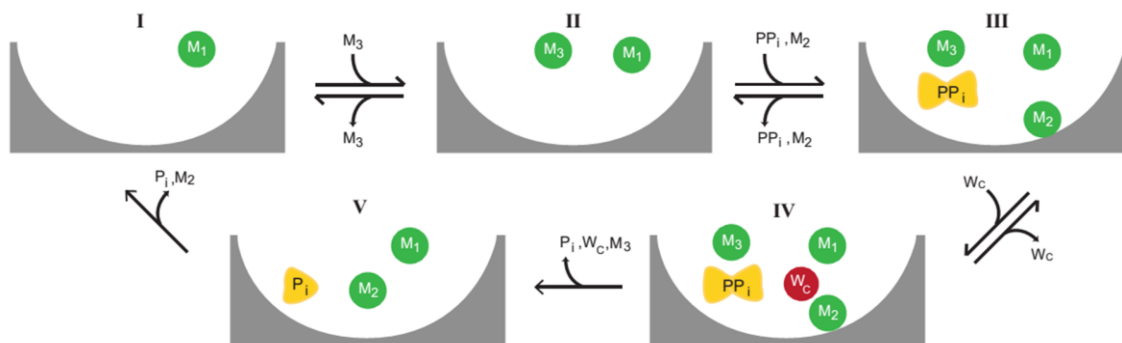
Figure 30: Mechanism proposed by Professor Tsodokov's group to explain the observations.

According to the newly proposed mechanism (Figure 30), M_3 binds to the active site which is already bound to M_1 likely in an equilibrium process. Binding of both M_1 and M_3 facilitates the binding of PP_i and M_2 (which was proposed to bind in a cooperative fashion). Then the binding of W_c occurs followed by deprotonation, formation of the hydroxide anion and hydrolysis of the phosphoanhydride bond giving rise to the two P_i ions. One of the P_i ions leaves the active site along with the M_3 cation. M_2 shifts closer to the remaining P_i and subsequently leaves the

active site with the P_i restoring the initial state of the active site (with only M_1 bound) to start a new catalytic cycle.

5.3 Computational procedure

We have used classical MD and hybrid QM/MM calculations to investigate the hydrolysis of PP_i. This step involves the abstraction of a proton from W_c by one of the surrounding aspartate residues (D57 or D89) followed by a nucleophilic attack on the proximal phosphorus atom of PP_i by the resultant hydroxyl ion. This nucleophilic attack leads to the cleavage of the phosphoanhydride bond (i.e. the P-O-P bond in the PP_i substrate).

The crystal structure of *M. tuberculosis* PPase bound to PP_i, provided by Prof. Tsodikov's group, was used for initial MD simulations. This crystal structure is a dimer of hexamers. For the computational analyses, only one hexamer was used and out of the six corresponding active sites for the hexamer, one active site was used for the QM calculations. The Ca^{2+} ions in the crystal structure were replaced by Mg^{2+} ions. The system was hydrogenated using MolProbity and neutralized by adding 51 K^+ ions using the tleap program of the AMBER12 package.[68, 123, 124] Initial MD simulations run with the neutralized system were shown to be unstable. The ionic strength of the system was increased to 100 mM by the addition of 102 K^+ ions and 51 Cl^- ions using tleap. The system was solvated with TIP3P water with a 15 Å buffer in a final box size of approximately 150 Å×140 Å×147 Å. The total number of atoms in the system is 50396.

MD simulations were performed using the PMEMD program in the AMBER12 software suite with the ff99SB force field.[68] The SHAKE algorithm was applied for bonds between heavy atoms and hydrogens.[118] All MD simulations were carried out under periodic boundary conditions. The smooth particle Ewald method was used for calculating long range interactions.[119, 120] Systems were minimized using sander with 50 steps of steepest descent and 450 steps of conjugate gradient with a restraint of 500 kcal/mol/$Å^2$ on the protein atoms. Densities of the systems were equilibrated at 1 g/cm^3 in the NPT ensemble with pressure being held constant by anisotrophic pressure scaling. The systems were gradually heated to 300K under constant volume conditions with a 500 kcal/mol/$Å^2$ restraint on the protein atoms. The restraints were gradually removed and the temperature was kept constant using the Langevin thermostat.[130] Non-restrained production MD was performed for 20 ns for each system with snapshots saved each 1 ps.

Twenty random snapshots were selected from the MD trajectory. These snapshots were selected based on the relative orientation of the attacking water toward PP_i in the active site. Upon selection of these structures, the ten snapshots for which the angle $\widehat{abc}$ (Figure 31) was closest to 180 degrees were selected from the trajectory for QM/MM calculations.
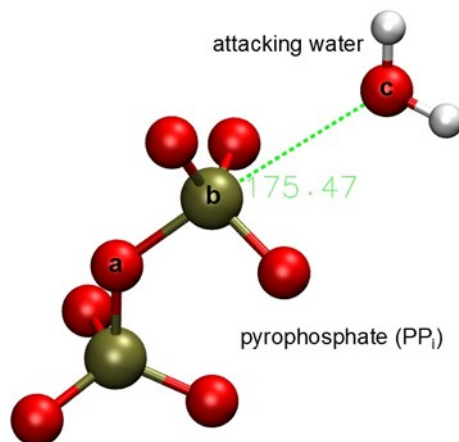
Figure 31: Diagram showing the $\widehat{abc}$ angle used in selecting candidate snapshots

The QM subsystem was selected to include (--CH_2COO^- groups of) the aspartates D57, D84, D89, PPᵢ, and 13 water molecules including the attacking water, and the three Mg^{2+} ions that correspond to M_1, M_2, M_3. All atoms where a covalent bond crosses the QM/MM boundary were treated using the pseudobond approach.[146] All the QM/MM calculations were performed by means of an in-house program that links modified versions of Gaussian09[147] and TINKER[148] to perform additive QM/MM with electrostatic embedding. An iterative method was adopted in the QM/MM optimization.[149] The QM calculations were performed at the B3LYP/6-31G** level with an overall charge of negative one and a multiplicity of zero.

The optimized reactant and product structures were used as starting point for the calculation of the reaction path by using a chain-of-replicas method called Quadratic String Method (QSM).[52] The path was created using 18 structures, including the optimized reactant and the product structures at each end. The MM

subsystem of the optimized reactant was used as the MM subsystem of each of the 16 intermediate structures between the optimized reactant and the product. The MM subsystems of the intermediate 16 structures were subjected to constrained optimization.[150] The constraints were gradually reduced after each QM/MM optimization cycle. The individual QM/MM optimizations for each of the 16 structures were performed using the same in-house program that links modified versions of Gaussian09 and TINKER, which was used for the QM optimizations. The structure corresponding to the highest energy point in the optimized path calculated using QSM was used as initial guess for the transition state optimization. Transition state optimization was performed using the QST3 method.

5.4 Results and discussion

Initial calculations performed using the completely deprotonated form of PP_i gave rise to problems in product optimization, resulting in the dissociation of a nearby metal-coordinated water and the protonation of one of the oxygen atoms in PP_i proximal to W_c. To prevent this dissociation of water, an oxygen in the P_i distal to W_c was protonated. This new system has a charge of zero and a multiplicity of zero and a total of 76 atoms in the QM subsystem.

The structures of the optimized reactant and the product are shown in Figures 32 and 33 respectively. The experimental counterparts are also included for comparison. The optimized reactant shows an overall backbone RMSD deviation of 0.9 Å and an RMSD deviation of 0.6 Å for all atoms in the active site.

The optimized product shows an overall backbone RMSD deviation of 0.9 Å and an RMSD deviation of 0.8 Å for all atoms in the active site.
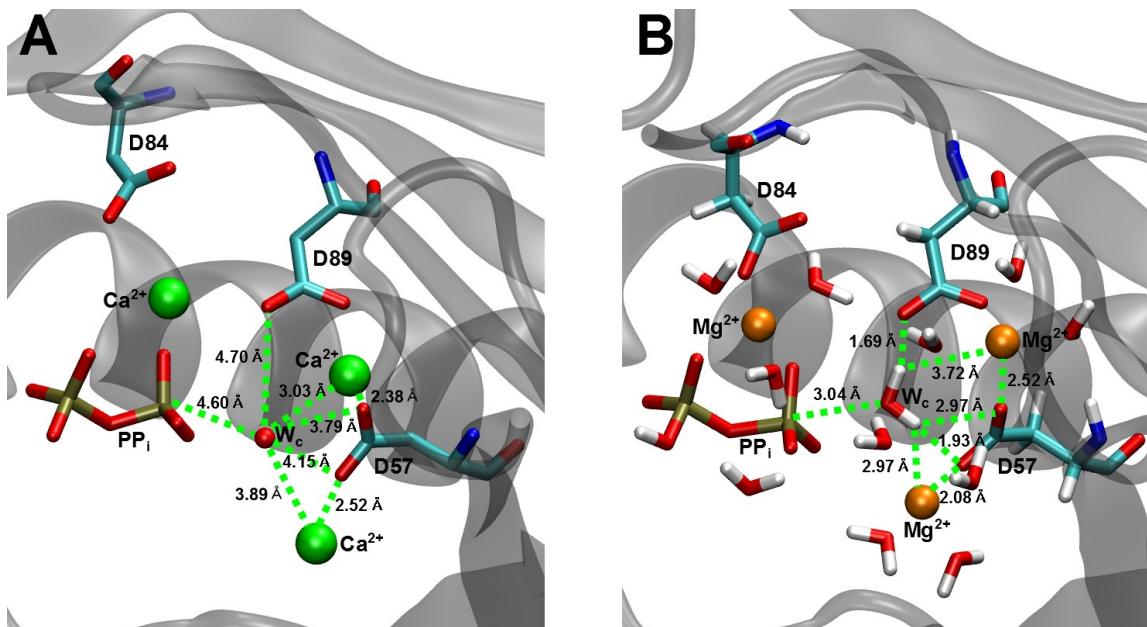
Figure 32: Comparison of experimental (A) and optimized reactant (B). The attacking water (W_c) is labeled as "catalytic water".
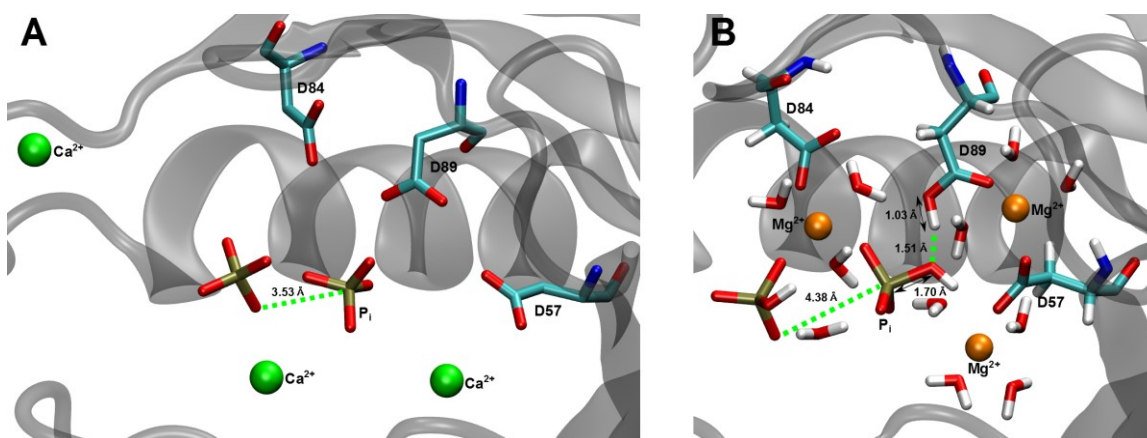
Figure 33: Comparison of experimental (A) and optimized product (B). The PPi has been hydrolyzed by W_c breaking the phosphoanhydride bond.

The relatively small RMSD deviations of the optimized reactant and product structures show that the calculated structures have not been significantly perturbed in comparison to the experimental structures. The optimized product structure is 11.24 kcal/mol lower in energy with respect to the optimized reactant structure.

The optimized transition state structure was characterized with a single imaginary frequency of 240.8i cm^{-1}. The visualization of the corresponding vibration showed that the vibration is along the reaction coordinate that describes the abstraction of the proton from W_c and the corresponding cleavage of the phosphoanhydride bond. The calculated reaction barrier for the reaction is 6.6 kcal/mol. Although many theoretical studies have been carried out in the past involving different types of phosphatases and hydrolysis of phosphates,[150-156] to our knowledge there haven't been any theoretical studies carried out on inorganic PPase action other than a DFT study involving a model system that analyses the inorganic pyrophosphatase mechanism in *E. coli*.[157] The authors of this study reported a calculated barrier (for a cluster model with continuum solvation) of ≈ 9 kcal/mol. This barrier is close to our calculated barrier of 6.6 kcal/mol, especially taking into account that the present results explicitly include the entire enzyme and solvent in the calculation.

After replacing the three Ca^{2+} atoms in the active site and subjecting the structure to MD and QM/MM optimization, a shift in the active site is observed. In the optimized reactant structure (Figure 32B), the change in cations results in a

tighter active site as expected with closer distances between the aspartic acid side-chains and the metals. For example, the distance between D57 and M_1 changes from 2.52 Å to 2.08 Å. This tightening promotes a rearrangement of the active site, which results in D89 no longer coordinating M_3. In addition, the attacking water is observed to form hydrogen bonds to both D89 and D57. The hydrogen bond between the attacking water, W_c, and Asp89 is slightly stronger than to D57 as observed by the distances (see Figure 32B).

The oxygen from D89 abstracts the proton from W_c, since it becomes both basic and properly positioned with respect to W_c. In contrast, both oxygens of D59 remain coordinated to divalent metal ions. Based on the minimum energy path, the nucleophilic water transfers a proton to D89 to yield a hydroxyl anion and almost concomitantly carries out the nucleophilic attack on the proximal P atom of the PP_i.
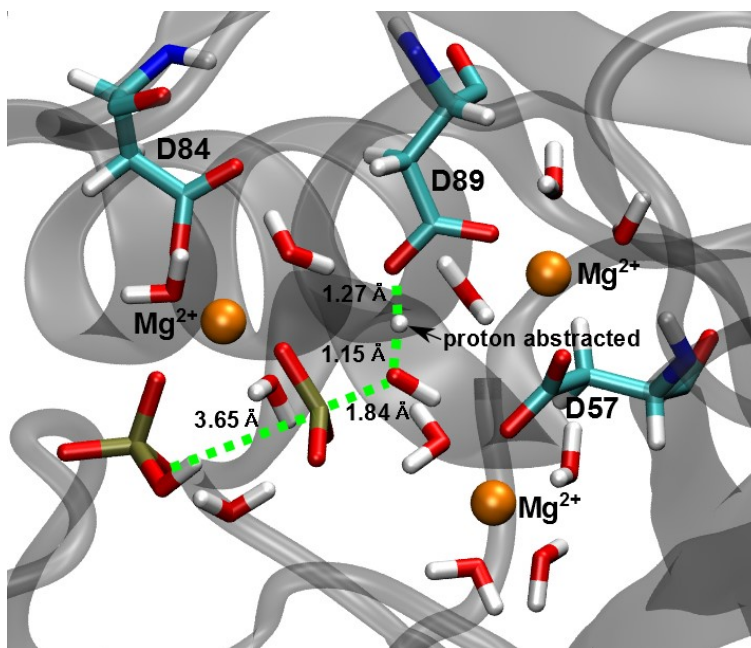
Figure 34: The optimized transition state showing the proton being abstracted by D89 and the formation of hydroxyl anion which carries out the nucleophilic attack.

5.5 Conclusions

In this study we have analyzed the PPase reaction mechanism in *M. tuberculosis* based on a novel set of crystal structures. One of these crystal structures contained a previously predicted catalytic water molecule (W_c) that had not been observed previously. Study of the characteristics of the novel structures has led to the proposal of a new reaction mechanism of the PPase action in *M. tuberculosis*.

Computational studies were carried out using the QM/MM hybrid method to analyze the PPi hydrolysis step of the proposed mechanism. The presence of W_c bridged by the divalent metal ions M_1 and M_2, along with its close proximity to D57 and D89 yields evidence for the catalytic steps leading to species (ii) and (iii) in Figure 29. As suggested by the experimental structures and supported by the calculated reaction path, the bridged water with a decreased pK_a can be readily deprotonated by the nearby carboxyl group of Asp89, and the newly formed hydroxide ion can then more readily attack the PP_i. This is in contrast to the previous studies that suggest the deprotonation of W_c is D57 rather than by D89. Our studies show D89 is more suited for proton abstraction from W_c in terms of orientation and the strength of attractive forces between W_c and carboxyl groups of D57 and D89. However, since our calculations only take into consideration one of 12 possible active sites, each of which has slightly different arrangements, our

simulations do not positively discard D57 as a possible general base. The calculated reaction barrier for the central catalytic step is 6.6 kcal/mol.

CHAPTER 6

COMPUTATIONAL ANALYSIS OF THE BINDING AFFINITY OF NOVEL

SUBSTRATES FOR HDAC CLASS IIA ENZYMES

6.1 Introduction

Eukaryotic chromosomes exist in the form of densely packed structures known as chromatin.[158] Chromatin is composed of DNA molecules tightly wound around histone proteins. The strength of attractive force between the DNA molecules and the histone proteins, which results in relaxed or compact chromatin, has been found to play a vital role in the expression of genes.[159-162] Histone deacetylases (HDACs) are a class of enzymes that catalyze the removal of acetyl groups from ε-N-acetyl lysine amino acid residues in histone proteins.[163-165] This removal results in an increase in the positive charge of the histone proteins which bind more tightly around the DNA backbone that is composed of negatively charged phosphate groups leading to the formation of more compact and transcriptionally silent chromatin.[58, 59, 159] This form of control in gene expression without an actual change in the nucleotide sequence is an ideal example of epigenetics.[164] Due to their ability to epigenetically regulate gene expression, they have become important targets in areas like cancer therapy where silencing of genes whose expression promotes cancer growth is required.[59, 163, 164, 166-170] Targeting HDACs in specific organs and tissues *in vivo* can be greatly enhanced by the capability to visualize their activity. Therefore there is a pressing need for the development of HDAC class-specific radiotracers

which would enable the non-invasive and repetitive imaging of HDAC activity through techniques like PET imaging.

This chapter describes a computational study of the binding affinity of a set of novel substrates that have been experimentally tested for the imaging of HDAC class IIa enzymes.

6.2 Experimental findings

This study was carried out in collaboration with Professor Juri Gelovani's group at Wayne State University. They have synthesized a set of novel substrates: 6-(fluoroacetamido)-1-hexanoicanilide (FAHA), 6-(difluoroacetamido)-1-hexanoicanilide (DFAHA), 6-(trifluoro acetamido)-1-hexanoicanilide (TFAHA), the structures of which are shown in Figure 35. These substrates differ by the number of fluorine atoms in the acetyl group.

6-(fluoroacetamido)-1-hexanoicanilide (FAHA)

6-(difluoroacetamido)-1-hexanoicanilide (DFAHA)

6-(trifluoro acetamido)-1-hexanoicanilide (TFAHA)
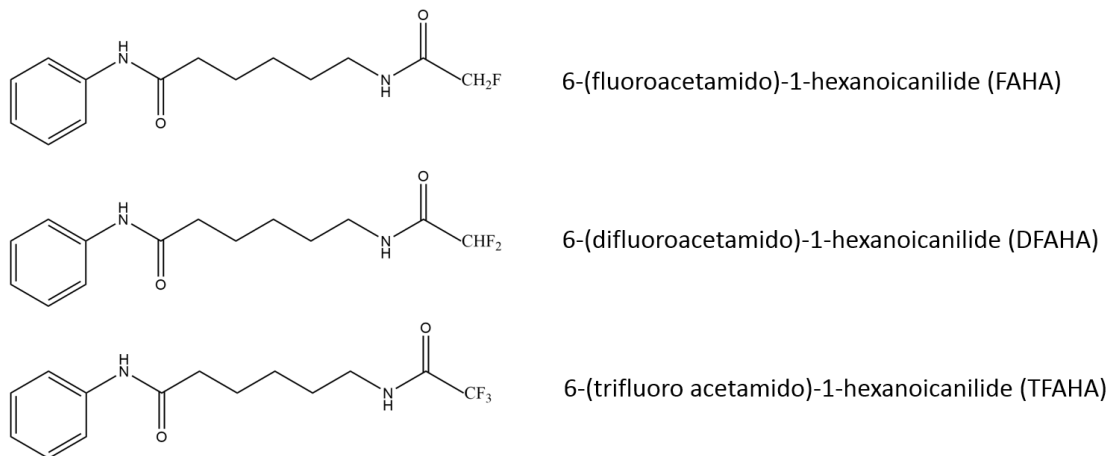
Figure 35: Structures of the novel substrates to be used as HDAC class IIa specific radiotracers synthesized by Professor Gelovani's group

The above mentioned substrates have been made radioactive by labeling them with radioactive fluorine [^{18}F] thus giving rise to [^{18}F]FAHA, [^{18}F]DFAHA, and [^{18}F]TFAHA. They have been used as radiotracers in selected regions of rat brains that are known to have higher HDAC class IIa activity. Subsequent PET/CT scanning of these regions showed higher [^{18}F]TFAHA–derived radioactivity than [^{18}F]DFAHA-derived radioactivity or [^{18}F]FAHA-derived radioactivity of the same regions. Quantitative analysis of the PET/CT scanning images by AMIDE Software (Source Forge)[171] has shown that the selectivity and accumulation of the above radiotracers in regions that show HDAC class IIa activity varies as TFAHA>DFAHA>FAHA. These experimental results suggest that the relative affinities of the above radiotracers to HDAC class IIa enzymes are as follows: TFAHA>DFAHA>FAHA.

6.3 Computational procedure

For *in silico* modeling purposes, the crystal structures of one member of the HDAC class IIa (HDAC4) and one member of the HDAC class 1 (HDAC8), were used. The crystal structures for HDAC4 (pdb id: 2VQJ)[172] and HDAC8 (1T69)[173] were downloaded from the pdb data bank.[122] For HDAC8, the missing residues 1-13 and 85-90 were added using MODELLER.[174] The residues 85-90 are involved in the formation of a loop that is in close proximity to the inhibitor binding region of HDAC8. Due to this potential importance, the loop formed by these MODELLER-added residues was further refined using Chimera.[175] The two crystal structures were hydrogenated using MolProbity.[123, 124] Docking calculations were performed using the FlexX algorithm[107] as implemented in the

LeadIT software package.[110] For both HDAC8 and HDAC4, native ligands in the crystal structure (namely SHH for HDAC8 and TFG for HDAC4) were used as reference ligands for the docking calculations. A grid volume that covered amino acids within 20 Å from each reference ligand was used. All the waters within the grid volume were taken as freely rotatable and displaceable. Pharmacophore rules based on the knowledge of ligand-receptor binding were used to guide the ligands towards the binding site. For both HDAC8 and HDAC4 docking was performed with the ligands FAHA, DFAHA, and TFAHA. Docking scores for the best 20 poses were calculated.

6.4 Results and discussion

The calculated docking scores for the best docking poses are given in Table 5 below.
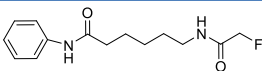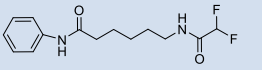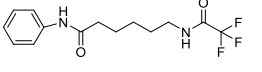
Compound Name	Compound Structure	HDAC 4 Score	HDAC 8 Score
FAHA		-8	-20
DFAHA		-10	-20
TFAHA		-11	-17

Table 5: Docking scores of the substrates for HDAC4 and HDAC8

The docking scores shown for FAHA, DFAHA, and TFAHA do not follow the trend in affinity predicted by the experiment. This trend is to be expected as HDAC8 is not a class IIa enzyme. As described in 2013 by *Lobera et al.*,[176] the

replacement of a key HDAC class I and HDAC class IIb tyrosine residue in the active site by a histidine residue in HDAC class IIa enzymes changed their catalytic activity and enables them to receive bulkier molecules at the active site in comparison to other HDACs. So the decrease in binding affinity shown by HDAC8 when going from DFAHA to TFAHA can be described as being due to its increased inefficiency in accommodating bulkier molecules at its active site.

In the case of HDAC4 we see the same trend in the variation of binding affinity of the substrates as seen in the experiment. Going from FAHA to TFAHA increased the negative value of the docking score which is an indication of increased affinity between the substrate and the corresponding HDAC active site.

As reported by *Wu et al.* in 2010,[177] there are two previously suggested mechanisms for class I/II/IV HDACs and HDLP. One of these mechanisms is a general acid-base catalytic pair mechanism[178] and the other one is a proton-shuttle catalytic mechanism.[179] In both these mechanisms, it can be seen that the activation of the active site moieties, including the carbonyl carbon of the acetyl group of the substrates and the Zn^{2+}, play a vital role in catalysis. When going from FAHA to TFAHA, each hydrogen in the methyl portion of the acetyl group gets replaced by a fluorine atom. Fluorine being the most electronegative of all the elements withdraws C-F bond electrons towards itself thus increasing the electrophilicity of the carbonyl carbon in the acetyl group making the carbonyl carbon more susceptible to nucleophilic attack. As predicted by the two mechanisms stated above, this nucleophilic attack is carried out by a hydroxyl anion that is formed by the abstraction of a proton from a water molecule by a

histidine residue in the HDAC4 active site. Furthermore, the presence of fluorine in the vicinity of the Zn^{2+} ion polarizes the Zn^{2+} due to the electron withdrawing nature of the fluorine. As a consequence, the strength of interaction between the Zn^{2+} ion and the carbonyl oxygen of the acetyl group increases the electrophilicity of the carbonyl carbon even more.

The above arguments account for the increase in affinity of the substrate in the order FAHA<DFAHA<TFAHA for the HDAC4 active site.

6.5 Conclusions

We have performed docking studies on three novel substrates: FAHA, DFAHA, and TFAHA that are currently being tested as potential radiotracers for imaging HDAC class IIa enzyme activity. The pattern in the change of affinity for HDAC4 is the same as that obtained by the experiments. These combined experimental and theoretical results have led to the conclusion that the affinity of the substrates towards HDAC4 changes as FAHA<DFAHA<TFAHA. The trends in affinity of the three compounds for HDAC8 don't show the same pattern as shown for HDAC4. This trend is to be expected as TFAHA, DFAHA, and FAHA are meant to be specific for HDAC IIa enzymes whereas HDAC8 is a member of HDAC class I enzymes.

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ABSTRACT

CLASSICAL AND AB INITIO QM/MM SIMULATIONS OF BACTERIAL ENZYMES

by

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Advisor: Professor Andres Cisneros

Major: Chemistry (Physical)

Degree: Doctor of Philosophy

This thesis describes analyses performed on three enzyme systems. Chapter 2, 3, and 4 involve studies carried out on the GatCAB enzyme of *H. pylori* and *S. aureus*. Since information at the electronic level was not required for these studies, sampling of the configuration space carried out at the molecular mechanics level was adequate. In Chapter 2, the snapshots sampled using MD were used as input for average correlation difference calculations and average RMSD difference calculations to ascertain the existence of a communication pathway between two subunits of GatCAB. Experimental and computational results obtained, suggest the existence of a communication pathway between the GatA and GatB subunits of *H. pylori* GatCAB. In Chapter 3, the snapshots sampled using MD were used as input for average correlation difference calculations and pK_a calculations to analyze the possibility of an amino acid residue acting as a possible catalytic acid/base at the entrance of an intramolecular tunnel that transfers ammonia from one active site to another.

Experimental results that compare glutaminase, kinase, and transamidase activity for wild-type *H. pylori* GatCAB with the mutants D185$_{(A)}$E, D185$_{(A)}$A, and D185$_{(A)}$N as well as our computational results support the possibility of D185$_{(A)}$ in *H. pylori* GatCAB acting as a catalytic acid/base in protonation/deprotonation of ammonia. In Chapter 4, MD snapshots were used to calculate intramolecular tunnels inside *S. aureus* GatCAB along which the free energy for the transfer of ammonia was calculated using the WHAM method to identify the tunnel that was thermodynamically more favorable. EDA and APBS analyses were utilized for further characterization of the tunnels in terms of non-bonded interactions between the ammonia molecule and the surrounding amino acids lining the tunnel. The results of the free energy calculations using WHAM as well as the energy differences calculated using EDA showed that tunnel1 (proposed in 2006 by Nakamura *et al*.) is thermodynamically more favorable for transfer of ammonia compared to tunnel2 (proposed in 2012 by Kang *et al*.)

Chapter 5 discusses the inorganic pyrophosphatase (PPase) mechanism in *M. tuberculosis* which requires information at the electronic level. The QM/MM hybrid method was used in which the reactive moieties were included in the QM subsystem to achieve accuracy at the electronic level while the rest of the system was treated at the molecular mechanics level. Based on a set of newly isolated crystal structures and the results of the QM/MM calculations, a new mechanism for the PPase catalyzed PP$_i$ hydrolysis was proposed and an energy barrier of 6.6 kcal/mol was calculated at the B3LYP/6-31G(d,p) level of theory.

In Chapter 6, molecular docking was used to dock a set of novel substrates that are being experimentally tested as potential radiotracers, onto HDAC4 and HDAC8 enzymes to calculate/compare their relative binding affinities. The trends in the increase in affinity, of the novel substrates for HDAC4 and HDAC8 active sites calculated by molecular docking, agreed with the experimentally shown trends.

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Publications

1. Dewage S.W., Cisneros G.A.,"Computational analysis of ammonia transfer along two intramolecular tunnels in Staphylococcus aureus glutamine-dependent amidotransferase (GatCAB)", *Journal of Physical Chemistry B,* 2015. 119(9): p. 3669-77.

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