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Computational Analysis of Ammonia Transfer Along Two Intramolecular Tunnels in *Staphylococcus aureus* Glutamine-Dependent Amidotransferase (GatCAB)

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Abstract

Most bacteria and all archaea misacylate the tRNAs corresponding to Asn and Gln with Asp and Glu (Asp-tRNA^{Asn} and Glu-tRNA^{Gln}). The GatCAB enzyme of most bacteria converts misacylated Glu-tRNA^{Gln} to Gln-tRNA^{Gln} in order to enable the incorporation of glutamine during protein synthesis. The conversion process involves the intramolecular transfer of ammonia between two spatially separated active sites. This study presents a computational analysis of the two putative intramolecular tunnels that have been suggested to describe the ammonia transfer between the two active sites. Molecular dynamics simulations have been performed for wild-type GatCAB of *S. aureus* and its mutants: T175_(A)V, K88_(B)R, E125_(B)D, and E125_(B)Q. The two tunnels have been analyzed in terms of free energy of ammonia transfer along them. The probability of occurrence of each type of tunnel and the variation of the probability for wild-type GatCAB and its mutants is also discussed.

Graphical abstract

1. INTRODUCTION

Aminoacylation of tRNA involves the reaction of amino acids with specific tRNA molecules to produce aminoacyl-tRNAs (aa-tRNAs).¹ This process is catalyzed by specific aminoacyl-

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Supporting Information

CAVER analysis data, Bootstrap plots, APBS analysis for ten random snapshots, EDA analysis for $R \approx 6.5$ Å and $R \approx 8.5$ Å, and calculated RESP partial charges. This material is available free of charge via the Internet at <http://pubs.acs.org>

Notes

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tRNA synthetase enzymes (aaRSs). In eukaryotes such as humans, the aminoacylation of the amino acid glutamine is catalyzed by glutamyl-tRNA synthetase (GlnRS) producing Gln-tRNA^{Gln}.² In all archaea and most bacteria GlnRS is not present.^{3–5} The absence of GlnRS should theoretically deprive them 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.⁴ In a first step, a nonspecific enzyme called glutamyl-tRNA synthetase (GluRS) catalyzes the misacylation of glutamate with tRNA^{Gln} producing Glu-tRNA^{Gln} (reaction 1).^{2,6,7} This 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} has been found to happen via three reactions (Scheme 1) catalyzed by glutamine-dependent amidotransferase (GatCAB).^{3,5,8–11} 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 γ position of the glutamate (reaction 3). The resulting γ -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.^{8,12,13}

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 still unknown. Two putative intramolecular tunnels (see Figure 2) have been proposed to explain the passage of ammonia from GatA to GatB. The first tunnel, tunnel 1 in the remainder of this work, was proposed by Nakamura et al. based on their crystallographic structure.⁸ Subsequently, Kang et al. proposed an alternative path, tunnel 2 in the remainder of this work, based on molecular dynamics (MD) simulations.¹⁴ Tunnel 2 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 2, and spanning 4 Å into each tunnel.¹⁴

In this contribution we present the investigation of the statistics, structure, and energetics of passive ammonia transport through both tunnels based on classical MD simulations. The organization of the paper is as follows: In the Methods section we present the details for the MD simulations, calculation of tunnel probability starting at three different points along the path, umbrella sampling¹⁵/weighted histogram analysis method (WHAM)^{16–18} analysis for the energetics of ammonia transport and energy decomposition (EDA), and adaptive Poisson–Boltzmann solver (APBS)¹⁹ analyses. Subsequently, the analysis for ammonia transport along both tunnels is presented, followed by concluding remarks.

2. METHODS

2.1. Molecular Dynamics Simulations on Wild-Type and Mutant Structures

Eight different systems were considered initially, which involve mutations on two of the subunits. Because GatCAB is composed of three subunits, we denote each subunit 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.²⁰

All MD simulations were performed using the PMEMD program of the AMBER12 software suite with the ff99SB potential.²¹ The SHAKE algorithm was applied to bonds between hydrogen and heavy atoms.²² Periodic boundary conditions were used for the explicit solvent calculations. Long-range interactions were accounted for by the smooth particle mesh Ewald (SPME) method.^{23,24} Hydrogens were added to the crystal structure using MolProbity.^{25,26} N termini of the protein were capped with acetyl (ACE) groups and the C termini were capped with *N*-methylacetamide (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 183 394. 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 300 K 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 thermostat²⁷ in the canonical ensemble.

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 2.1.2²⁸ for each selected snapshot, with K1, K2, and the GatA active site (Figure 2) as starting points. K1 was defined by residues Asn81_(A), Ala128_(A), Val204_(A), Ala205_(A), and 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 tunnel 1 or tunnel 2 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 tunnel 1 or tunnel 2 due to steric constraints.

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 (see Supporting Information (SI)). Once the coordinates for the

tunnels were obtained, ammonia molecules (see SI Table S4) 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. Tunnel 1 was populated with 63 ammonia molecules and tunnel 2 was populated with 68 ammonia molecules. The umbrella sampling method¹⁵ 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 $0.0 \text{ Å} \leq R \leq 13.5 \text{ Å}$. 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., $R > 13.5 \text{ Å}$) involves higher dimensional sections, which will be investigated in the future. However, the studied section seems to be sufficient to determine which tunnel is energetically favored (see below).

A harmonic potential of $20 \text{ kcal mol}^{-1} \text{ Å}^{-2}$ 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.²¹ Each window was sampled for 1 ns. Sampling was done in both explicit solvent (TIP3P water model)²⁹ and implicit solvent (Generalized Born model).³⁰ All simulations were performed at 300 K using the Langevin thermostat.³¹ The two tunnels have been divided into three regions as denoted in Figure 3. Regions 1 ($0.0 \text{ Å} \leq R \leq 5.2 \text{ Å}$) and 3 ($R > 16.2 \text{ Å}$) are common to both tunnels. Region 2 ($5.2 \text{ Å} \leq R \leq 16.2 \text{ Å}$) comprises the section where the tunnels differ.

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 using bootstrapping.^{16–18,32} All 1D-WHAM calculations were performed with the WHAM package.³³

2.4. Energy Decomposition Analysis and Adaptive Poisson–Boltzmann Solver Calculations

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

$$\Delta E_{\text{int}} = \langle \Delta E_i \rangle \quad (1)$$

where i represents an individual residue, E_i represents the nonbonded interaction (electrostatic or van der Waals) between residue i and the selected ammonia (the interactions between the ammonia and waters or counterions 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.^{34–45} 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 nonbonded interactions using the above equation. The adaptive Poisson–Boltzmann solver (APBS) plugin¹⁹ of the PyMOL⁴⁶ 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.

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 Section 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 Section 3.2. This section 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).

3.1. Probability of Occurrence of Tunnel 1 and Tunnel 2

CAVER analysis was carried out on 30 snapshots for each of the systems studied as explained in Section 2.1. On the basis of the CAVER analysis, almost 25% of the tunnels correspond to tunnel 2 compared to 5% occurrence of tunnel 1 when the starting point of the tunnel calculation is the GatA active site (SI Table S1). This shows that tunnel 2 has a higher probability of occurrence when compared to tunnel 1 occurrence based solely on steric constraints.

When the tunnel calculation is started from K2, which is a part of tunnel 2, 45.42% of the tunnels fall under the tunnel 2 category, compared to 3.75% for tunnel 1 category (SI Table S3). In contrast, about 15.42% of the tunnels correspond to tunnel 2 compared to 8.75% for tunnel 1 when starting the calculation at K1 (SI Table S2). This shows that even when starting from a point in tunnel 1, there is a higher probability to switch back to tunnel 2. There is almost no switching back to tunnel 1 from tunnel 2. These results suggest that during a given period of time the dynamic formation of tunnel 2 is more probable than that of tunnel 1, in agreement with the study by Kang et al.¹⁴

In contrast to the number of instances of tunnel 1 and tunnel 2 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 study²⁰ on *H. pylori* GatCAB enzyme, which shows that the corresponding mutants T149_(A)V and K89_(B)R show a marked decrease in the rate of phosphorylation (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 intermolecular 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.

3.2. Free Energy Profiles for Ammonia Transport along Tunnel 1 and Tunnel 2

The PMFs for the transfer of ammonia in explicit solvent and implicit solvent are shown in Figures 4 and 5, respectively. It can be seen that the relative shapes of free energy profiles for tunnel 1 and tunnel 2 look similar in both implicit and explicit solvents. The region 0.0 Å

$R = 5.2$ Å corresponds to region 1 (Figure 3) which is common to both tunnel 1 and tunnel 2. 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 3), which is the starting point of free energy calculations. Bootstrap analyses⁴⁰ (SI Figures S1–S4) 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 have been calculated relative to point $R = 2.5$ Å (common to both tunnel 1 and tunnel 2). The free energy barrier for ammonia transfer in tunnel 1 is 3.64 kcal/mol (1.23 kcal/mol) in explicit (implicit) solvent. For tunnel 2, the calculated free energy barrier corresponds to 7.98 kcal/mol in explicit solvent and 2.93 kcal/mol in implicit solvent.

The previous study by Kang et al.¹⁴ concluded that tunnel 2 is preferred over tunnel 1. 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 tunnel 1 and $R = 6.3$ Å along tunnel 2, 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 tunnel 2 over tunnel 1 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 tunnel 1.

A rise in free energy at the beginning of the PMF ($R \approx 0.0$ Å) is observed for the explicit solvent simulations compared with the region around $R \approx 1.5$ Å. At the beginning of the sampling coordinate, $R \approx 0.0$ 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 6. The attraction between ammonia and A128_(A) at $R \approx 0.0$ 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 $R \approx 1.5$ Å shows that the ammonia moves toward a more polar environment created by K79_(A), and D211_(A), compared to the environment around $R \approx 0.0$ Å.

The ammonia molecule shows a significant attraction in both sections of the PMF to K79_(A) and D211_(A). However, at $R \approx 1.5$ Å, the attraction is larger than that at $R \approx 0.0$ Å, leading to greater stabilization of the ammonia at $R \approx 1.5$ 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 7. 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 $R \approx 1.5$ Å with respect to $R \approx 0.0$ 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 4.

Ten random snapshots were subjected to APBS analysis (see Figures 7 and SI S5–S13 for all calculated electrostatic potential surfaces) to determine the electrostatic potential around ammonia at $R \approx 1.5$ Å. Residues located within a radius of 4 Å from ammonia were included in the electrostatic potential calculation. In the electrostatic potential surface (Figure 7) 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 $k_B T/e$ (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 7, 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 EDA analysis.

The free energies for ammonia transfer in both tunnel 1 and tunnel 2 are comparatively close to each other in the region $R \approx 5.4$ – 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, resulting in ammonia showing similar free energy differences. Analysis of the MD trajectory for the tunnel 1 shows that as the ammonia molecule progresses from the hydrophilic 1.5 Å $R \approx 3.5$ Å region to $R \approx 6.0$ Å region, it gets closer to a hydrophilic environment created by the residues R200_(A), A207_(A), and D211_(A) as shown in Figure 8.

Other than the attractive interactions with R200_(A) and D211_(A), the EDA analysis at $R \approx 6.0$ Å (SI Figure S14) 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 (SI Figure S14) is around 3.2 kcal/mol, in close agreement with the barrier of 3.6 kcal/mol. This suggests that the change in nonbonded 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 nonbonded 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 tunnel 1 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. On the basis

of 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).

Immediately after the branching point in tunnel 2, in the region $R \approx 6.0$ Å, ammonia enters the hydrophilic environment created by residues K79_(A) and D211_(A) (Figure 9). Existence of these attractive interactions is further confirmed by the EDA analysis (SI Figure S15). 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 by Figure 9, the ammonia molecule is oriented in such a way that the nitrogen atom of ammonia is pointed toward the hydrogen atoms of the $-\text{NH}_3^+$ group of K79_(A) and the hydrogen atoms of ammonia are pointed toward the oxygen atoms of the carboxyl group of D211_(A), which facilitate formation of hydrogen bonding.

A sharp rise in free energy is observed in tunnel 2 between $R \approx 6.5$ Å and $R \approx 8.5$ Å (Figures 10 and SI S19). This rise in free energy for tunnel 2 can be attributed to the lack of attractive interactions between ammonia and the surrounding residues with respect to the attractive forces experienced in the $R \approx 1.5$ Å region. The only significant attractive forces experienced by ammonia, as shown by the EDA analysis (Figure S18) are due to N81_(A) and C91_(A). Other than this, the ammonia molecule is in a relatively hydrophobic region created by V204_(A), M124_(A), and F127_(A), when compared to the hydrophilic region at $R \approx 1.5$ 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 $R \approx 8.5$ Å with respect to $R \approx 1.5$ Å, close to the calculated free energy barrier of 8.0 kcal/mol at $R \approx 8.5$ Å calculated by WHAM (Figure 10).

In contrast to the sharp rise in free energy in tunnel 2 between $R \approx 6.5$ Å and $R \approx 8.5$ Å the free energy for transport of ammonia in tunnel 1 decreases consistently between $R \approx 6.5$ Å and $R \approx 8.5$ Å. The stabilization of the ammonia in tunnel 1 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 11). Moreover, R200_(A), S208_(A), and D274_(B) are conserved residues lining tunnel 1 as noted by previous studies.⁸ 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 11). The presence of water further adds to the attractive interactions experienced by ammonia through hydrogen bonding.

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

intramolecular tunnels using the CAVER plugin of the PyMOL software suite and categorized as either tunnel 1 or tunnel 2. This categorization showed that tunnel 2 has a higher probability of occurrence than the tunnel 1 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 tunnel 1. 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 tunnel 2 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 multidimensional WHAM analysis, and will be investigated in a future contribution.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
Structure of GatCAB enzyme from *Staphylococcus aureus*⁸ (PDB ID: 2F2A).

Figure 2.

Two tunnels and different starting points for free energy calculations. The path N,K1,J,X denotes tunnel 1 and the path N,K2,J,X denotes tunnel 2. The junction of the two tunnels is located at the point marked by J. The two entrance points used by Kang et al.¹⁴ are K1 and K2. The common exit point of the two tunnels is denoted by X. The Mg²⁺ ion at the GatB active site is also shown.

Figure 3.

Two proposed tunnels for ammonia transport (represented as lines). The tunnels have been divided into three regions. Region 1 (0.0 Å $\mathbf{R}$ 5.2 Å) and Region 3 ($\mathbf{R}$ 16.2 Å) are common to both tunnels. Region 2 (5.2 Å $\mathbf{R}$ 16.2 Å) is where the two tunnels differ. The letters N, K1, K2, J, and X represent the same points as in Figure 2.

Figure 4. Free energy of ammonia transfer along the two types of tunnels for $0.0 \text{ \AA} \leq R \leq 13.5 \text{ \AA}$ in explicit solvent.

Figure 5. Free energy of ammonia transfer along the two types of tunnels for $0.0 \text{ \AA} \leq R \leq 13.5 \text{ \AA}$ in implicit solvent.

Figure 6.

EDA analysis for the explicit solvent simulations at $R \approx 1.5 \text{ \AA}$ and $R \approx 0.0 \text{ \AA}$. Orange denotes interaction energies at $R \approx 0.0 \text{ \AA}$ and blue indicates interaction energies at $R \approx 1.5 \text{ \AA}$ (see SI Figure S19 for implicit solvent EDA). Interaction energies are given in kcal/mol. Only residues with interactions $|1 \text{ kcal/mol}|$ were considered.

Figure 7. APBS analysis using ammonia, and residues within 4 Å of ammonia at $R \approx 1.5$ Å. The color key has been defined in energy units of $k_B T/e$ (where “e” is the charge of an electron).

Figure 8. Ammonia in the hydrophilic environment in the region $R \approx 6.0 \text{ \AA}$ inside tunnel 1 (see SI Figure S20 for stereo view).

Figure 9. Ammonia in the hydrophilic environment in the region $R \approx 6.0 \text{ \AA}$ inside tunnel 2 (see SI Figure S21 for stereo view).

Figure 10. Ammonia in a hydrophobic environment in the region 6.5 Å **R** 8.5 Å inside tunnel 2 (see SI Figure S22 for stereo view).

Figure 11. Ammonia in a hydrophilic environment in the region $6.5 \text{ \AA}$ R $8.5 \text{ \AA}$ inside tunnel 1 (see SI Figure S23 for stereo view).

Scheme 1.

Reactions Catalyzed by GluRS (Reaction 1) and GatCAB (Reactions 2–4)