Conformational Freedom in Tight Binding Enzymatic Transition-State Analogues

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Abstract: Transition-state analogues of bacterial S'-methylthioadenosine/S'-adenosylhomocysteine nucleosidases (MTANs) disrupt quorum-sensing pathways in Escherichia coli and Vibrio cholerae, demonstrating the potential to limit pathogenicity without placing bacteria under intense selective pressure that leads to antibiotic resistance. Despite the similarity of the crystal structures of E. coli MTAN (EcMTAN) and V. cholerae MTAN (VcMTAN) bound to DADMe-Immucillin-A transition-state (TS) analogues, EcMTAN demonstrates femtomolar affinity for BuT-DADMe-Immucillin-A (BDIA) whereas VcMTAN possesses only picomolar affinity. Protein dynamic interactions are therefore implicated in this inhibitor affinity difference. We conducted molecular dynamics simulations of both EcMTAN and VcMTAN in complex with BDIA to explore differences in protein dynamic architecture. Simulations revealed that electrostatic and hydrophobic interactions with BDIA are similar for both enzymes and thus unlikely to account for the difference in inhibitor affinity. The EcMTAN–BDIA complex reveals a greater flexibility and conformational freedom of catalytically important atoms. We propose that conserved motions related to the EcMTAN transition state correlate with the increased affinity of BDIA for EcMTAN. Transition-state analogues permitting protein motion related to formation of the transition state are better mimics of the enzymatic transition state and can bind more tightly than those immobilizing catalytic site dynamics.

Introduction

Enzymatic transition states, determined through a combination of experimental kinetic isotope effects and computational techniques, provide a template for the design of powerful transition-state analogue inhibitors. These chemically stable mimics of the enzymatic transition state are among the tightest binding noncovalent inhibitors known. Transition-state analogues were developed for a class of bacterial enzymes involved in quorum sensing and nucleoside metabolism, the S'-methylthioadenosine/S'-adenosyl homocysteine nucleosidases (MTANs). The relative affinity of these transition-state analogues for MTANs of various species of bacteria revealed two broad classes of transition states for MTANs. Those with an early, partially dissociative transition state include Neisseria meningitidis MTAN, and those with a fully dissociative SN1 transition state include Escherichia coli MTAN (EcMTAN) and Vibrio cholerae MTAN (VcMTAN). Transition-state analogues that are derivatives of Immucillin-A bind preferentially to MTANs with partially dissociated transition states, while the derivatives of DADMe-Immucillin-A bind preferentially to MTANs with fully dissociated transition states.

The role of MTANs in quorum sensing involves the formation of quorum-sensing precursors and the metabolic recycling of S'-methylthioadenosine (MTA) to S'-adenosylmethionine. Disrupting quorum-sensing pathways has the potential to limit bacterial pathogenicity, making MTANs attractive targets. The DADMe-Immucillin-A derivatives are potent inhibitors of both EcMTAN and VcMTAN and have been shown to block quorum sensing without causing growth inhibition or inducing drug resistance.

Both EcMTAN and VcMTAN possess fully dissociative transition states and share virtually identical active site structures (0.4 Å RMSD) when bound to DADMe-Immucillin-A transition-state analogues (Figure 1a). Surprisingly, the two enzymes differ in their affinities for the same transition-state analogue inhibitor, BuT-DADMe-Immucillin-A (BDIA), by 3 orders of magnitude. The equilibrium dissociation constant (K_i*) of BDIA from VcMTAN is 208 picomolar, while the K_i* of BDIA from EcMTAN is 296 femtomolar. Because the affinity of transition-state analogues for their cognate enzymes is proportional to their similarity to the transition state, this result suggests that BDIA more closely resembles the transition state of EcMTAN than that of VcMTAN. Yet, according to transition-state structures based on kinetic isotope effects, transition-state analogue specificity studies, and crystallographic structural analysis, these enzymes...
structures provide equilibrated ground-state structures with motions of the enzyme at the transition state. Likewise, crystal relative to the reactants, they do not provide information about reveal the character of chemical bonds at the transition state crystal structures. While kinetic isotope eff er experiments provide an important starting point for determining hydrogen bonding and ionic interactions based on interatomic distances (Figure 1b).

However, crystal structures reveal only the lowest-energy states averaged over large molecular populations11 without the resolution necessary to provide definitive answers about the presence of particular hydrogen bonding interactions.12

Figure 1. Crystal structures of VcMTAN and EcMTAN in complex with DADMe-Immucillin transition-state analogues. (a) Overlay of the active site residues of VcMTAN (blue) and EcMTAN (red) when bound to the transition-state analogues BDIA (green) and MDIA (orange), respectively (Protein Data Bank (PDB) entries 3DP9 and 1Y6Q, respectively). The oxygen atoms of the proposed catalytic water molecules are depicted in light blue for VcMTAN and pink for EcMTAN. The backbone RMSD of these active site crystal structures is 0.4 Å. (b) Comparison of interatomic distances, in angstroms, between enzyme and transition-state analogue from their respective crystal structures. The distances enumerated in red are from the crystal structure of EcMTAN in complex with MDIA, whereas those in blue are from the crystal structure of VcMTAN in complex with BDIA. The residue names follow the same convention, and purple represents common elements between the two enzymes. The alkane tail of BDIA, which is in complex with VcMTAN, is longer than that of MDIA. The additional carbons of BDIA are also depicted in blue for consistency.

are expected to possess virtually identical transition states.5,8 Their nearly identical static active site structures when DADMe-Immucillin transition-state analogues are bound would be expected to form the same chemical environment for substrate molecules and thus for the formation of the transition state. Therefore, the difference in the binding of the transition-state analogue BDIA to these enzymes must lie beyond the static structures of their active sites.

We explored the mechanism of BDIA binding differences in EcMTAN and VcMTAN by conducting molecular dynamics (MD) simulations of both enzyme–transition-state analogue complexes and compared these results with simulations of the unliganded enzymes. MD simulations provide another dimension to the two-state static results from techniques used to probe the structural elements of the transition-state and crystal structures. While kinetic isotope eff ect experiments reveal the character of chemical bonds at the transition state relative to the reactants, they do not provide information about motions of the enzyme at the transition state. Likewise, crystal structures provide equilibrated ground-state structures with transition-state analogues but not dynamic information.

Analysis of motions associated with formation of the transition state has provided novel information for several enzymes, especially lactate dehydrogenase and purine nucleoside phosphorylase.9,10 The thermodynamic stability of enzymes in complex with a transition-state analogue inhibitor often permits the successful determination of the X-ray crystal structure of these complexes. These crystal structures provide an important starting point for determining hydrogen bonding and ionic interactions based on interatomic distances (Figure 1b).

However, crystal structures reveal only the lowest-energy states averaged over large molecular populations11 without the resolution necessary to provide definitive answers about the presence of particular hydrogen bonding interactions.12

■ METHODS

Computational Approaches. For each enzyme with and without the transition-state analogue (EcMTAN−BDIA, VcMTAN−BDIA, EcMTAN(apo), and VcMTAN(apo)), explicit solvent constant number, temperature, and volume (NVT) dynamics simulations, representing the canonical ensemble, were run using the chemistry at Harvard macromolecular mechanics (CHARMM) program for 5 ns following equilibration.13,14 To better sample conformational space at a given computational cost, a second 5 ns trajectory was run using different starting velocities for each atom.14 To further our sampling of conformational space, five additional 1 ns “derivative” trajectories were created by restarting dynamics from the first trajectory using the position coordinates from that trajectory at 0.5, 1.5, 2.5, 3.5, and 4.5 ns but with new velocities for each atom chosen from a Boltzmann distribution matching the simulation temperature of 300 K. (See Figure S1 in the Supporting Information for a schematic of the trajectory generation process.) The fact that this process led to a greater exploration of conformational space was confirmed by differences in the hydrogen bonding observed in trajectory 1 and trajectory 2 from the same system, for example, as discussed in greater detail below.

An expanded description of the computational methods can be found in the Supporting Information.

■ RESULTS AND DISCUSSION

MD Analysis of Hydrogen Bond Stability. The strength of inhibitor binding is often ascribed chiefly to electrostatic interactions between enzyme and inhibitor. Indeed, hydrogen bonds often play a crucial role in such interactions. A chief aim of our simulations was to observe how H-bond interactions between the active sites and the inhibitors varied through the conformational space of the enzyme−inhibitor complexes. In the CHARMM molecular dynamics software package, hydrogen bonds are handled implicitly: the presence of an H-bond is inferred from the proximity of a suitable H-bond donor and H-bond acceptor. When the hydrogen of an H-bond donor and an H-bond acceptor were within 2.4 Å of one another (corresponding to a donor−acceptor distance of ∼3.4 Å), an H-bond was considered formed. This has been shown to be an accurate criterion for the purpose of comparing relative hydrogen bond stability.17 Our MD simulations of VcMTAN and EcMTAN bound to the transition-state analogue BDIA confirmed many of the hydrogen bonding interactions observed in the crystalline structures of enzyme−transition-state analogue complexes while also revealing H-bond interactions.
not anticipated from these crystal structures. Moreover, differences were observed between the H-bonding of VcMTAN to BDIA and EcMTAN to BDIA (see Supporting Information, Table S1, for a list of all H-bond interactions between enzymes and inhibitors detected by our simulations). However, these differences involved the specific residues and atoms bound to the transition-state analogue at any given moment, not the total amount of hydrogen bonding. Consequently, differences in hydrogen bonding alone cannot account for the thousand-fold difference in affinity for BDIA between EcMTAN and VcMTAN.

MD simulations confirmed that H-bond interactions are maintained from VcMTAN to the purine base of BDIA during the simulation. As shown in Figure 1b, the crystal structure of VcMTAN bound to BDIA (PDB entry 3DP9) indicates that the 9-deazaadenine base forms H-bonds with the main chain atoms of Val153 and the side chain atoms of Asp198. The hydrogen bond between N7 and Oδ2 of Asp198 was present for the entirety of all of our simulations, at both active sites (see Figure 2a), while that between N6 and Oδ2 of Asp198 was present for 99% of the simulation time, also at both active sites.

In the great majority of the sampled conformational changes, an H-bond was observed between N1 of the base and the backbone nitrogen of Val153. A hydrogen bond between the carbonyl oxygen of Val153 and N6 of the purine base was less consistently present, with hydrogen bonds forming between 31 and 81% of the time depending on the specific trajectory (corresponding to an average occupancy of 47% for active site A and 65% for active site B over all trajectories).

The crystal structure shows an oxygen of Glu175 forming a hydrogen bond with the 3′ oxygen of BDIA while the other glutamate oxygen interacts with the proposed catalytic water molecule (labeled W in Figure 1b), which is further stabilized by H-bonds with Glu12 and Arg194. The H-bond interactions between the proposed catalytic water and nearby enzyme side chains were generally less stable than those linking the enzyme to the nucleobase. Notably, as depicted by a dashed orange line in Figure 2a, our simulations support the existence of an additional hydrogen bond not anticipated from the crystal structure. The side chain of Ser76 is within H-bonding distance of the pyrrolidine cationic nitrogen and was present in the majority of sampled conformations. This interaction represents an interaction not apparent in the average of conformers represented by the crystal structure and also may have been missed by a single long trajectory, as this interaction is absent from active site A throughout trajectory 1.

No crystal structure for EcMTAN bound to BDIA has been reported, but the catalytic site similarity when the enzyme is bound to the related transition-state analogue methylthio-DADMe-Immucllin-A (MDIA) permitted the replacement of MDIA with BDIA, which differs from MTA only in the length of its 5′-thioalkyl group, as the starting point for our simulations. While the difference in the length of the inhibitor hydrophobic group affects its affinity for the enzyme, the crystal structure of EcMTAN bound to MDIA indicates that the pattern of hydrogen bonding between the purine and pyrrolidine rings of the inhibitor and the enzyme’s catalytic site is identical to that seen in the VcMTAN–BDIA complex. Nonetheless, our simulations detected differences in the interactions at those two locations between the respective enzymes and the transition-state analogue that were not evident from comparing their respective crystal structures.

Two notable differences were observed in the H-bonding interactions between the pyrrolidine moiety of BDIA and nearby residues of the respective enzymes. In EcMTAN, one of the catalytic sites never formed a hydrogen bond with the N1′ cation via Ser76 (Figure 2b), whereas both did in VcMTAN. In EcMTAN, there was direct H-bonding between Glu12 and the 3′-hydroxyl of the inhibitor in one of the catalytic sites, an interaction absent from the VcMTAN–BDIA simulations. Moreover, in EcMTAN there are greater differences between the two active sites of the same enzyme complex. For example, consider the H-bonding between the active site residues and the pyrrolidine moiety. In our simulations of EcMTAN, certain H-bonds between O3′ of the pyrrolidine moiety and nearby active site side chains were present in one subunit but not in the other. There are more interactions between O3′ and nearby side chains in the EcMTAN–BDIA system, but they are shorter-lived than the single, direct H-bond between the O3′ of BDIA and the active site of VcMTAN. (See Table S1 in the Supporting Information for a complete list of hydrogen bonds detected in our simulations.)
The average total number of hydrogen bonds present between the two molecules of BDIA filling the active sites and VcMTAN (12.85 for both subunits) was extremely close to the average number of hydrogen bonds between the two BDIA molecules and EcMTAN (12.45). The slight differences in total hydrogen bonding observed between the transition-state analogue BDIA and the two enzymes are unlikely to account for the 1000-fold difference in the affinity of BDIA for these enzymes.

**Hydrophobic Interactions.** We also compared the hydrophobic interactions between the butyl tail of BDIA and the hydrophobic regions of the two enzymatic active sites. As hydrophobic “forces” are an entropic phenomenon at room temperature, their influence must be deduced by comparing the actual distances observed between hydrophobic entities. A comparison of the average distances between the four atoms of BDIA’s butyl tail (C20–C23) and the closest atoms of the nearest enzymatic residues shows no significant difference between the two systems VcMTAN–BDIA (4.61 Å) and EcMTAN–BDIA (4.64 Å). (See Table S2 in the Supporting Information for a list of the component distances making up the average enzyme–inhibitor atom distance in the hydrophobic region.) Such a small difference in hydrophobic interactions is unlikely to account for the 1000-fold difference in inhibitor affinity for the two enzymes.

**Differences between VcMTAN and EcMTAN.** Given the similarities in their static catalytic site structures and the overall structures of the enzyme when bound to BDIA (see Figure S2 in the Supporting Information for an overlay of the average structures of the two enzymes), it is not surprising that the two enzyme catalytic site bonding interactions with BDIA are so similar. Many of the equivalent residues in EcMTAN and VcMTAN are in the same position with respect to each other and with respect to the inhibitor molecule. Nonetheless, small differences exist. Specifically, there are more, shorter-lived H-bond interactions in the EcMTAN–BDIA complex than in the VcMTAN–BDIA complex, hinting at a possible explanation for the dramatic difference in affinity: BDIA may be better matched to the flexibility and dynamics of the EcMTAN in its configuration near the transition state.

**Differences in Enzyme Flexibility.** To further evaluate the apparent difference in BDIA’s restraint of EcMTAN’s active site and VcMTAN’s active site, we compared root-mean-square fluctuations (RMSFs) of Cα atoms in both EcMTAN and VcMTAN as well as all heavy (non-hydrogen) atoms of the inhibitor molecules. Calculating the RMSF of each Cα atom allows fluctuations to be spatially mapped by residue. RMSFs were calculated versus a given atom or inhibitor atom) exhibiting fluctuation in its position through the course of a simulation greater than that of its VcMTAN–BDIA counterpart. A negative ΔRMSF, colored blue, indicates that the VcMTAN–BDIA atom exhibits fluctuation greater than that of its EcMTAN–BDIA counterpart. Inset: A close-up of active site A, with residues Val102, Phe105, Tyr107, Pro113, and Glu174 depicted as thin licorice segments.
one, VcMTAN Phe208, is consistently more flexible (i.e., more flexible in both subunits of the enzyme, which suggests an effect that is more robust to different starting conformations) than its equivalent in EcMTAN, Phe207. The Ca RMSF of EcMTAN Phe208 is 9% greater than that of EcMTAN Phe207. In contrast, 15 of the 26 active site residues are consistently more flexible in EcMTAN. Of those 15 active site residues in EcMTAN, 2 are more than 40% more flexible, 4 are at least 30% more flexible, 6 are more than 20% more flexible, and 10 are more than 17% more flexible, on average, than their equivalent active site residues in VcMTAN. (See Figure S3 in the Supporting Information for a residue-by-residue comparison of Ca RMSF in EcMTAN and VcMTAN.) Moreover, the drop in Ca RMSF when BDIA binds to VcMTAN (13.4% less than the unbound enzyme) is greater than that of EcMTAN (11.8% less than EcMTAN(apo)); this difference is greater at the active site (22.2% for VcMTAN versus 17.2% for EcMTAN). (See Table S3 in the Supporting Information for a comparison of average RMSF among enzyme(apo), enzyme–MDIA, and enzyme–BDIA complexes in VcMTAN and EcMTAN.) These differences are not likely to be an artifact of starting conformation as simulations with MDIA, the ligand used to determine the 1Y6Q crystal structure, reveal virtually identical RMSFs (Supporting Information, Table S3); if the greater flexibility of EcMTAN–BDIA were due to modifying the inhibitor of the crystal structure, we would expect that VcMTAN–MDIA would be more flexible than EcMTAN–MDIA.

Visualizing Differences in Flexibility. A “heat map” helps visualize the residues with the greatest absolute difference in RMSF between EcMTAN and VcMTAN enzymes (Figure 3).

Deeper shades of red indicate a larger ARMSF (Ca RMSF of a given residue in EcMTAN minus the Ca RMSF of the equivalent residue in VcMTAN), whereas deeper shades of blue indicate a more negative RMSF. The inset, focusing on active site A, depicts the active site residues with positive ARMSF values that are consistent across both subunits. The deepest red residues in Figure 3, i.e., those with the largest ARMSF values, Val102, Phe105, Tyr107, and Pro113 (corresponding to VcMTAN Ala113), compose the loop that closes over the hydrophobic tail of the inhibitor as it occupies the active site.

This greater RMSF value in the hydrophobic region of EcMTAN surrounding the hydrophobic tail of BDIA is reflected by the increased flexibility in the hydrophobic tail of BDIA itself in complex with EcMTAN than when in complex with VcMTAN (see Figure 4).

Among the active site residues that are involved in reaction chemistry, EcMTAN Glu174 is consistently more flexible (on average, 18%) than its equivalent in VcMTAN, Glu175. This increased flexibility matches the hydrobonding patterns discussed above. In VcMTAN, Glu175 spends the vast majority of the simulation time, 97% at active site A and 88% at active site B, H-bonded to the 3′-hydroxyl of BDIA. In contrast, EcMTAN Glu174 varies which O6′ atom is bound to the 3′-hydroxyl of BDIA, when it is bound at all. As Glu174 is one of the residues that binds the nucleophilic water molecule, this movement may be crucial to the reaction itself. As with the hydrophobic tail, the increased flexibility of the enzyme at this point is reflected by an increased flexibility of adjacent inhibitor atoms (see Figure 4). The dynamic simulations indicate that EcMTAN Glu174 alternately forms a hydrogen bond with O3′.

As a consequence, inhibitor atoms also show increased flexibility when bound to EcMTAN compared to those bound to VcMTAN. The C2′ atom of BDIA is also more flexible in the EcMTAN active site, suggesting that the increased flexibility of Glu174 may also have an effect on the inhibitor via movement of the catalytic water located near this position.

These potentially crucial differences in flexibility at the active site of EcMTAN versus their homologous residues in VcMTAN may be due to differences in the protein sequences of surrounding residues between the two enzymes. Both the series of residues with the largest ARMSF (102, 105, 107, and 113) and the differently behaving Glu174 (with respect to its homologue VcMTAN Glu175) are surrounded by a number of different residues in the two proteins: VcMTAN Iso109 to EcMTAN Tyr109, VcMTAN Met112 to EcMTAN Leu112, VcMTAN Glu115 to EcMTAN Cys115, VcMTAN Val169 to EcMTAN Ala168, VcMTAN Val170 to EcMTAN Iso169, and VcMTAN Ser177 to EcMTAN Thr176.

Figure 4. Root-mean-square fluctuation (RMSF) of individual BDIA heavy atoms when occupying EcMTAN active site (red) and VcMTAN active site (blue). Each value represents the average over all trajectories.

CONCLUSIONS

The tight binding of transition-state analogues represents the conversion of dynamic modes that are crucial to catalysis into a thermodynamic free energy well.2 These structures are far more stable and longer-lived than the Michaelis complex. In the cases of BDIA binding to VcMTAN and EcMTAN, their dissociation constants are in the pico- and femtomolar range, respectively. A counterintuitive mechanism for the tighter binding of the EcMTAN–BDIA complex is suggested by the greater flexibility and increased conformational freedom of the enzyme–transition-state analogue complex. Dynamic motion is required to achieve the transition state, and we suggest that the types of motions conserved in the interaction of the EcMTAN–BDIA complex are those reflecting formation of the chemically competent transition state. The VcMTAN complex is more constrained in these motions, as seen in hydrogen bonding and RMSF studies. The comparative differences in dynamic regions of these proteins are directly involved in catalysis: the 2′ and 3′ positions, involved in stabilizing the catalytic water, and Glu12 and Glu174, which both stabilize the catalytic water and are in position to accept one of its protons.16 Moreover, substrate and inhibitor specificity studies, with transition-state analysis, recognize the required motion of the ribosyl hydroxyl as the
ribosyl group migrates from the purine leaving group to the enzyme-stabilized nucleophile in these reaction mechanisms.\(^7,16\)

We conclude that the specific areas of dynamic motion conserved in the EcMTAN catalytic sites are important in both inhibitor binding and transition-state formation. Dynamic interactions of protein and substrate that normally carry the substrate along the reaction coordinate toward the transition state instead result in inhibitor motions that are compatible with substrate motions during catalysis. Therefore, the increased flexibility of the transition-state inhibitor BDIA when in catalytic site A of EcMTAN, versus VcMTAN, reflects motions compatible with transition-state formation in EcMTAN. These motions must be slightly different in VcMTAN such that the matched flexibility of the EcMTAN catalytic site and inhibitor represents a greater preservation of the protein’s native dynamic modes, resulting in a better dynamic mimic of the transition state and better binding.

A more conformationally free enzyme–inhibitor complex being more tightly bound seems counterintuitive. But there is at least one similar documented example. In the case of transition-state analogue binding to human purine nucleoside phosphorylase (PNP), transition-state analogues that have chemical flexibility to permit a similar range of atomic motion of catalytic site amino acid residues and inhibitor groups bind more tightly and with less entropic penalty than more rigid inhibitors.\(^18\) These findings are consistent with the view that the strength of enzyme–transition-state inhibitor interaction is proportional to the degree that the transition-state inhibitor reflects the actual transition state, which we know to be a dynamic entity.\(^10,19\)

Therefore, the transition-state inhibitor that channels but does not eliminate the dynamic excursions of the enzymatic transition state will be the one that binds most tightly. By preserving the conformational freedom and flexibility of these moieties, the EcMTAN–BDIA complex represents a system closer to the enzyme’s native transition state than the VcMTAN–BDIA system is to VcMTAN’s native transition state. The tightest binding transition-state inhibitors reduce the probability of ligand release through the preservation of the natural protein fluctuations crucial to catalysis.

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### REFERENCES


### ASSOCIATED CONTENT

* Supporting Information
   A schematic of the trajectory generation procedure; a visual comparison of the average structures of EcMTAN–BDIA and VcMTAN–BDIA; an expanded computational methods section; a list of all hydrogen bonds detected in EcMTAN–BDIA and VcMTAN–BDIA simulations; a list of distances in the hydrophobic regions of these two systems; a table comparing RMSFs when the EcMTAN and VcMTAN are unbound, bound to MDIA, and bound to BDIA; and the CHARMM topology and parameter file for BDIA for reference or use. This material is available free of charge via the Internet at http://pubs.acs.org.

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**Notes**

The authors declare no competing financial interest.

