Published in final edited form as:

Biochim Biophys Acta. 2010 February ; 1804(2): 332. doi:10.1016/j.bbapap.2009.09.006.

Proton Transport in Carbonic Anhydrase: Insights from Molecular Simulation

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Summary

This article reviews the insights gained from molecular simulations of human carbonic anhydrase II (HCA II) utilizing non-reactive and reactive force fields. The simulations with a reactive force field explore protein transfer and transport via Grotthuss shuttling, while the non-reactive simulations probe the larger conformational dynamics that underpin the various contributions to the rate-limiting proton transfer event. Specific attention is given to the orientational stability of the His64 group and the characteristics of the active site water cluster, in an effort to determine both of their impact on the maximal catalytic rate. The explicit proton transfer and transport events are described by the multistate empirical valence bond (MS-EVB) method, as are alternative pathways for the excess proton charge defect to enter/leave the active site. The simulation results are interpreted in light of experimental results on the wild-type enzyme and various site-specific mutations of HCA II in order to better elucidate the key factors that contribute to its exceptional efficiency.

Keywords

Carbonic Anhydrase; Proton Transfer; Molecular Dynamics; Multi-State Empirical Valence Bond; Proton Transport; Simulation

1.0 Introduction

Many biological processes depend critically on proton transport (PT) events, but it can be unclear how the complex biomolecular environment facilitates the PT process. The prevailing evidence suggests that PT events are strongly influenced by environmental electrostatics and proton donor-acceptor dynamics, but the molecular-level details of these factors are often unresolved, particularly with regard to the dynamical interactions between the relevant proteins and the water solvent. Unraveling the underlying physical features that drive PT in such systems is of great importance to the resolution of many biological and chemical questions. One class of such questions involves the fundamental acid-base chemistry of proteins, including that related to their enzymatic activity. The importance of PT in biomolecular systems is evidenced by the breadth and volume of studies examining various aspects of this problem in systems

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such as cytochrome c oxidase [1,2], the M2 proton channel in influenza A [3], bacteriorhodopsin [4], and ATPase [5].

One family of biologically relevant enzymes that greatly facilitate the PT process is the carbonic anhydrase (CA) family. CAs are mostly zinc-metalloenzymes that are structurally classified into five families: α , β , γ , δ , and ϵ . The α -class of CA, which includes human carbonic anhydrase II (HCA II), has been extensively characterized. HCA II is a relatively small protein of 260 residues that catalyzes the reversible, two-step conversion of carbon dioxide to a bicarbonate ion and an excess proton via the “ping-pong” mechanism.[6,7] The first step of the conversion is the nucleophilic attack of a carbon dioxide molecule by a zinc-bound hydroxyl group, resulting in a zinc-bound bicarbonate. This product is subsequently displaced by a water molecule:

The second step involves the reversible transport of the proton from the zinc-bound water to His64, then from His64 to the surrounding solvent environment:

Extensive research indicates that the PT step (eq. 2) is rate limiting and that His64, a residue residing at the mouth of the active site, is the prominent proton donor-acceptor [8-13]. The PT step in HCA II proceeds with a second-order rate constant ($k_{\text{cat}}/K_{\text{m}}$) approaching the diffusion controlled limit and a maximal turnover of 10^6 s^{-1} , which for an enzyme system is near catalytically perfect. This observation suggests that the enzyme may be specifically optimized for PT. An elucidation of how HCA II facilitates this mechanism may therefore be quite valuable for understanding PT in other biomolecular systems.

A crucial aspect of the HCA II PT system appears to be the flexibility of His64, which can adopt two distinct orientations: the imidazole ring of His64 buried in the active site (‘in’), or rotated outward and exposed to the solvent (‘out’). The relatively large distance between the zinc-bound water solvent and His64 (8 to 10 Å) rules out direct donor-acceptor proton transfer between these groups, implying that an intramolecular water cluster is the conduit for the long-range PT event.[11] Both orientations of His64, ‘in’ ($R_{\text{Zn-N}} \approx 8 \text{ Å}$) and ‘out’ ($R_{\text{Zn-N}} \approx 10 \text{ Å}$), are indeed connected via a water cluster to the zinc-bound solvent.[14,15] This crucial water cluster is stabilized by several active-site residues, including Thr199, Thr200, Tyr7, Asn62 and Asn67, each of which appears to play a significant role in the PT process (Figure 1).[16-18] These residues line the conically shaped active site and form stabilizing hydrogen bonds with the intramolecular water cluster in order to create an environment conducive to PT. This enzyme-stabilized intramolecular water cluster is thought to facilitate the PT event via the Grotthuss mechanism.[19-25]

While the importance of the key enzyme active site residues, the intramolecular water cluster, and the orientation of His64 has been demonstrated by kinetic and x-ray structural data [16-18], the complete molecular-scale picture as to how these factors cooperate to create a fast and efficient enzyme is not well understood. For the wild-type (WT) enzyme and several site-specific mutants, computer simulations have therefore been employed to probe the complex interplay between the moieties.[14,26-44] A combined (and synergistic) computational-experimental approach thus provides the most effective means of delving into the underlying features of the PT reactions in HCA II.

This review discusses the certain results from recent molecular simulation studies that have helped to reveal the important roles of the stabilizing active-site amino acids, the intramolecular water cluster, the orientation of His64, and the ability of the enzyme as a whole to facilitate PT in HCA II. The following sections of this review will focus on computational studies that have shed light on the complex interplay between the excess hydrated proton and the enzyme active-site environment prior to, during, and immediately after the rate-limiting PT step.

2.0 Computational Methodologies

In recent years there have been several reactive [33-44] and non-reactive [14,15,26-32] molecular simulation studies of HCA II that have explored both static and dynamical properties of the enzyme. Non-reactive simulations are more useful for investigating slow processes such as conformational changes in protein residues and water clusters. Reactive simulations, computationally expensive but capable of investigating bond breakage/formation, are typically utilized to investigate the nucleophilic attack of the EZnOH^- on CO_2 (eq. 1) and the rate-limiting PT event (eq. 2). The following section provides a brief description of the computational methods employed to investigate the various aspects of HCA II.

2.1 Non-reactive Molecular Simulation Methods

Non-reactive molecular simulations, including those employing the Molecular Dynamics (MD) method, have the benefit of computational efficiency (relative to quantum mechanical or *ab initio* approaches) that allows them to reach increasingly long time scales for systems of considerable size and complexity. Such simulations are crucial for exploring the role of the slow and cooperative enzyme fluctuations as well as the (comparatively more rapid) intramolecular water cluster dynamics. Non-reactive simulations are also invaluable to help characterize the broad features of the environmental (e.g., solvation) effects on the PT process. In this regard, MD simulations have revealed the orientational preferences of His64, as well as the characteristics of the water cluster within the WT HCA II systems and several mutant systems [14,15,26-30]. With the aid of experimental x-ray structural data, hydrogen bonding characterization algorithms and free energy methods have also been used to evaluate possible water-cluster network PT pathways into and out of the active site. Such strategies have been applied to both WT HCA II [30,32] and several of its single and double mutants [31].

In general, non-reactive molecular simulations are able to determine the properties of the end states of reactions (such as the $\text{EZnH}_2\text{O}^{2+}$ -His and EZnOH^+ -HisH⁺ states of eq. 2) as well as features of the problem relevant to the PT pathways, but they cannot describe the actual (explicit) PT process. The latter requires a reactive molecular simulation method.

2.2 Reactive Molecular Simulation Methods

Quantum mechanical (QM) methods have been relatively widespread in the study of HCA II [33-44]. While QM methods allow in principle for an accurate representation of a given reaction (including PT), their success is constrained by their high computational cost, so that a statistically meaningful free energy barrier to reaction cannot be calculated, as well as the inherent uncertainties and inaccuracies in the underlying level of the electronic structure calculation. Generally speaking, only gas-phase model problems of significantly reduced complexity can be studied at a high level of electronic structure theory. A number of early studies instead used approximate approaches such as the partial retention of diatomic overlap (PRDDO)[45,46] approximation of the self-consistent field molecular orbital calculations at the minimum basis set level and Austin Model 1 (AM1)[47] semi-empirical methods based on the neglect of diatomic differential overlap approximations. These were followed by studies employing a more rigorous theoretical foundation, but for relatively small systems, including Hartree-Fock (HF), 2nd order Moller-Plesset perturbation theory (MP2), and density functional

theory (DFT). Despite their focus on probing a highly reduced system (in which the larger enzyme environment was ignored), many of these QM studies were able to reveal a number of interesting features of PT in HCA II such as the role of water molecules in mediating the PT energetics from donor to acceptor. However, over time it has become increasingly clear that more accurate and efficient methods are required to capture the complex chemical environment and the dynamic nature of the enzyme.

A step forward in the reactive molecular simulations of enzymes in general (and HCA in particular) has come with the advent of various quantum mechanical/molecular mechanical (QM/MM) methods.[37,48,49] These methods allow the environment, typically modeled by an empirical force field, to influence a strictly delineated QM reactive region in an enzyme. The advent of the QM/MM approach meant that significantly more complex (and more physically realistic) enzymes systems could be simulated. Moreover, such methods are significantly more efficient than a purely QM calculation of a similarly sized system, so there is at least hope that statistically meaningful free energy calculations can eventually be carried out through an MD implementation of the QM/MM methodology.

One particular incarnation of the QM/MM methodology involves the semi-empirical self-consistent charge density functional tight binding (SCC-DFTB) method, which couples with an empirical CHARMM force field [50] (SCC-DFTB/CHARMM). The current SCC-DFTB method [51,52], which is based on a second-order expansion of the Kohn-Sham total energy, has improved on its earlier versions by including the process of charge density relaxation. For small organic molecules, the SCC-DFTB method has been shown to yield energies, geometries, and vibrational frequencies in good agreement with higher-level DFT calculations [52]. The QM/MM SCC-DFTB method has also been used to study HCA II[34-38,44,53], including the prediction of an unusual “proton hole” pathway involving a hydroxide anion transport instead of the widely accepted PT mechanism in HCA II [34]. Aside from the inherent challenges faced by QM/MM methods in general (such as a physically reasonable description of the QM/MM “boundary” problem), the application of the SCC-DFTB QM/MM method to enzymes such as HCA II is limited by the relatively inaccurate level of electronic structure incorporated into the methodology, a lack of validation of the approach for various other PT processes such as hydrated proton solvation and migration in bulk water, and the method's relatively low computational efficiency (although an efficiency much greater than many other purely QM or QM/MM methods).

Another reactive molecular simulation framework that allows full environmental effects to be incorporated into it is a class of methods that rely on what can best be called “multi-configurational” or “multi-state” molecular force fields. Within the context of biomolecular simulations and enzyme studies in particular, perhaps the most well-known of this class of methods is the Empirical Valence Bond (EVB) approach [54-57], including applications of this methodology to study HCA[55,58-62]. It should be noted, however, that multi-configurational empirical and semi-empirical force fields in general have a long history, some of which dates back to the origins of chemical dynamics theory, as has been discussed recently [63]. Nevertheless, this point of view has been vigorously challenged.[64]

In order to better describe the full physics of hydrated proton solvation and transport in various aqueous and biomolecular systems, our group has developed a multi-state generalization of the simpler EVB approach, called the multi-state empirical valence bond (MS-EVB) method. [65-67] This approach has been successfully used to simulate proton solvation and transport in a variety of systems, including bulk water, water clusters, water-alcohol mixtures, water-vapor interfaces, lipid bilayers, polymer electrolyte membranes, and a number of different proteins, including HCA II. For two reviews of this body of work, including a discussion of its connection to related efforts, see Refs. [24,25].

Simulating reactive processes in the MS-EVB framework is achieved through deterministic dynamical (Newtonian) evolution of the system nuclei variables on a potential energy surface that is defined as the lowest energy solution to the MS-EVB Hamiltonian matrix. This matrix is expressed in terms of a dynamically adaptive basis set of valence bond states, $|i\rangle$, which form the MS-EVB Hamiltonian matrix elements from the operator

$$H^{EVB}(\vec{r}) = \sum_{ij} |i\rangle h_{ij}(\vec{r}) \langle j| \quad (3)$$

The diagonal elements of the EVB Hamiltonian, h_{ii} , are defined from an underlying classical force field for a given bonding topology of the system with a corresponding potential energy of each EVB state $|i\rangle$. The off-diagonal elements h_{ij} represent the coupling between EVB states $|i\rangle$ and $|j\rangle$. The diagonalization of the MS-EVB matrix for each configuration of the system nuclei provides a mixing of the EVB basis states and hence an evolution of the overall system bonding topology. The Hellmann-Feynman Theorem allows derivatives (force) to be easily calculated for the ground state of the MS-EVB Hamiltonian, and hence a reactive MD algorithm can be implemented for this methodology. Often overlooked is the critical need to enumerate all relevant EVB basis states in a dynamical fashion for any given timestep within the MS-EVB algorithm. If this is not done properly, then the system energy will not be well-conserved and the resulting molecular dynamics will begin to seriously lose its meaning. Thermostating such meaningless non-conserving energy dynamics in an attempt to maintain a constant kinetic temperature will not yield more meaningful dynamics (in fact, it could be even less so). This feature of the methodology is not merely a technical point, because the dynamical enumeration of all relevant EVB states to ensure good energy conservation in the dynamics is physically equivalent to the inclusion of all possible contributions to the proton charge defect delocalization phenomenon.

There are key physical and algorithmic differences between the static few-state EVB [56,57] and dynamically determined MS-EVB [24,25,65-68] methods. While static two-state or few-state EVB methods are adequate for many traditional ‘donor-acceptor’ systems, the ability to accurately represent delocalized charge defects such as the hydrated proton require many more dynamically determined states (sometimes as many as 30-40). Moreover, in the latter case there is a continual interplay between the excess proton transfer between the molecules and the molecular diffusion (in the case of PT, a process that involves the underlying water molecules). In turn, this physics will influence the magnitude and character of the potential of mean force (free energy profile) for the charge defect migrating in between donor and acceptor through an environment such as an enzyme active site. This physics is critical on both the energetic front (through the effect of charge delocalization) and on the entropic front (via molecular exchange and configuration space ‘freedom’).

When dealing with a delocalized charge defect such as a hydrated excess proton, as is the case for the MS-EVB method, it is therefore important to represent the defect as a localized pseudo-particle. That is, the charge defect can be represented in terms of the so-called center of excess charge (CEC), which is the MS-EVB coefficient-weighted sum over the centers of charge (COC):

$$\vec{r}_{cec} = \sum_{i=1}^N c_i^2(\vec{r}) \vec{r}_i^{COC} \quad (4)$$

The COC are described by

$$\vec{r}_i^{COC} = \frac{\sum_k^{(i)} |q_k| \vec{r}_k}{\sum_k^{(i)} |q_k|}, \quad (5)$$

where the summations are performed over all the atoms (with partial charge q_k) contributing to the i th EVB state. The position of the CEC tracks the most probable position of the charge defect associated with the excess hydrated proton (or more correctly, associated with all protons in the system via the Grotthuss proton shuttling mechanism). The CEC is perhaps the ‘best way’ to characterize a constantly and dynamically changing entity, and the CEC coordinate provides a convenient means to, e.g., follow the charge defect's diffusion and to sample a free energy profile (barrier) for a given complex PT event such as occurs in HCA II.

The MS-EVB approach and approaches like it are in essence ‘‘multi-scale’’ methods that take both quantum mechanical and empirical data on key molecular interactions and then construct, from ‘‘bottom-up’’, a highly efficient, but approximate, description of the exact reactive molecular process.

3.0 Non-reactive Simulations of His64 and the Active-site Water Clusters

In order to computationally study the impact on the PT rate of the His64 orientation and the dynamical nature of the intramolecular water cluster, there are several factors that must be taken into account. The rate-limiting step (eq. 2) contains two limiting forms of the enzyme: $\text{EZnH}_2\text{O}^{2+}\text{-His}$ and $\text{EZnOH}^+\text{-HisH}^+$. These forms may have different ‘in/out’ distributions of His64 and its associated internal water cluster. These orientational distributions and water cluster characteristics are thought to be vital—not just for understanding the rate-limiting PT event, but also for being able to understand the influence of amino acid mutations on the observed rate. At the atomistic level, several computer simulations have probed the orientational preference of His64 (and the resulting characteristics of its intramolecular water cluster) in both limiting forms of the enzyme, for the WT and several site-specific mutations (Y7F and N67L) [14,26,28,30]. Mutations at amino acid positions 7 and 67 are particularly interesting because they appear to directly stabilize the intramolecular water cluster and lie in close proximity to His64 (Figure 1). The following discussion will therefore focus on the orientational preference of His64 and the structure of the associated intramolecular water cluster (bridging the zinc-bound water and His64) in the WT enzyme, as well as the Y7F and N67L mutants.

3.1 WT HCA II Enzyme

The way in which the HCA II enzyme creates an environment conducive to PT is thought to be a key factor governing its fast catalytic rate. Two components of this mechanism are the orientation of His64 and the corresponding intramolecular water cluster that connects the zinc-bound water solvent to His64 in the presence of thermal fluctuations (both large and small) of the enzyme. These constant fluctuations mean that active site water molecules must be able to arrange into clusters of various sizes for an appreciable duration in time. Extensive MD simulations of the reaction end points (eq. 2, the $\text{ZnH}_2\text{O}^{2+}\text{-His}$ and the $\text{ZnOH}^+\text{-HisH}^+$ systems) averaged over many dynamical fluctuations of the enzyme–solvent system (for both His64 orientations) indicate that stable water clusters are indeed present for all end states examined. [14,15,29] It is through these and similar computer simulations that a molecular-scale interpretation of the role of the orientation of His64 and the intervening water clusters has been obtained.

In the $\text{EZnH}_2\text{O}^{2+}$ -His 'in' configuration of the enzyme (the initial configuration for the hydration reaction) MD [26] and x-ray data [27] indicates that His64 will adopt a ratio of ~ 80/20 of 'in' vs. 'out' orientations. Analysis of the 'in' orientation reveals a variety of water clusters inside the active site (Figure 2A). Of these, the water clusters four in size are the most common [14,15,29]. However, the coexistence of several cluster sizes indicates that the water clusters are dynamic and capable of adapting to fluctuations in their environment while continuing to form continuous hydrogen-bonded networks between the proton donor and acceptor pair. The significance of the four-water clusters is clearly seen when simulations are compared to the x-ray crystal structure of the WT enzyme, both the 2CBA[69] and the newer higher resolution 2ILI[27] structures (Figure 1A). Both the simulation and x-ray data point to the coexistence of three-water ($\text{ZnH}_2\text{O} \rightarrow \text{W1} \rightarrow \text{W2} \rightarrow \text{His64}$) and four-water ($\text{ZnH}_2\text{O} \rightarrow \text{W1} \rightarrow \text{W2} \rightarrow \text{W3a} \rightarrow \text{His64}$) clusters [14,15]. These results are also encouraging because they indicate that x-ray data in the case of HCA II seems capable of approximating the solution phase structure of the active site. Furthermore, the MD simulations show that the water-clusters are reasonably robust, as indicated by water-cluster lifetimes in the picosecond (ps) time range (Figure 2D, with 3ps for four water clusters)[14]. These lifetimes are also in agreement with NMR data that indicates the important water molecules have relatively long residence times in the active site [70]. On the other hand, the ps lifetimes of the water clusters indicates that, at least on the reaction time scale, the enzyme environment is best characterized by a distribution of water cluster sizes rather than a single static water structure (or "water wire").

When His64 is in the 'out' position, however, the water cluster size distribution favors larger clusters: five- and six-molecule configurations are most probable (Figure 2B) [14]. This change is expected given that the distance between the zinc-bound water and His64 is larger. Furthermore, the reduced water cluster lifetimes relative to the $\text{EZnH}_2\text{O}^{2+}$ -His 'in'[14] system are indicative of a very dynamic water environment (Figure 2D, E). In this case, a more "classical" excess proton diffusion mechanism for PT may be more likely in the presence of such diverse and short-lived water clusters. This trend is also seen for the dehydration reaction, ZnOH^+ -HisH⁺ (Figure 2C, F), in which His64 remains predominantly in the 'out' orientation due to the like-like electrostatic interactions between the zinc bound hydroxide and His64H⁺, as seen in MD simulation [26, 29, 31, 44] and x-ray data [9] (conducted at low pH).

Computer simulations have therefore helped to define our current understanding of the WT HCA II active site. Once seen as a static, rigid structure (in which the His64 rests primarily in one orientation and active-site waters are likewise localized and static), it is now more evident that the active site should be viewed as a dynamic and fluctuating system (in which His64 shunts between the 'in' and 'out' orientations and water clusters of varying sizes are constantly formed and broken). This behavior is modulated by surrounding amino acids (such as Asn62 and Asn67) [14,30]. The active-site-stabilized water clusters, despite having average atomic positions (water occupancy) in good agreement with x-ray data, is now known to not be static at all [70]. In fact, the active site environment is able to compensate for enzyme thermal fluctuations and differing His64 orientations. It is also evident that the three- and four-water clusters seen in x-ray data are major contributors to the structure connecting 'in'-His64 to zinc-bound water. However, it should be noted that they are not the only water-clusters to form and participate in such a connection. The orientational preference of His64 for the differing end states also suggests that His64 may accept a proton from the zinc-bound-water while in the 'in' conformation and then donate it to the bulk environment by shifting to the 'out' orientation.

3.2 Mutant HCA II Enzymes

The roles of specific amino acids in the complex active site (through their effect on His64 conformation and the intramolecular water clusters) have been investigated by comparing

structural differences between WT and mutant-variants. Two site-specific mutants, Tyr7Phe and Asn67Leu, have been compared to the WT baseline in this manner in order to probe their contributions to the PT reaction.

3.2.a Tyr7Phe Mutant—The effects of the Tyr7Phe site-specific mutation on His64 orientation and the associated intramolecular water-clusters have been studied by both experimental [17] and computational [14] studies. Kinetic results for the Tyr7Phe mutant indicate that PT is 7 times faster in this mutant than in the WT. This is especially interesting because it is rare that mutations away from the native WT sequence (and corresponding structure) can actually increase the rate of an enzymatic reaction. X-ray data (2NXX) for this case reveal that His64 predominantly occupies the inward orientation [17]. MD simulations around the endpoint states of the Tyr7Phe mutant show that this mutation increases the orientational flexibility of His64 about the χ_1 dihedral, resulting in an almost 50/50 distribution of 'in' versus 'out' His64 orientations for the $\text{EZnH}_2\text{O}^{2+}$ -His system. In the WT and Tyr7Phe mutant enzymes, the EZnOH^+ -HisH⁺ system favors the outward orientation. It is evident from these results that orientational mobility (as predicted by simulations) and/or predominance of the inward orientation (as seen in x-ray data) couple to increase the maximal dehydration rate ($\sim 7 \mu\text{s}^{-1}$).

Studies of the intramolecular water clusters associated with the $\text{ZnH}_2\text{O}^{2+}$ -His system reveal significant insight into the mechanism behind the sevenfold rate increase (Figure 2A, B). As with the x-ray data [17], the MD simulation data [14] indicate a preponderance of three-molecule water clusters ($\text{ZnH}_2\text{O}^{2+} \rightarrow \text{W1} \rightarrow \text{W2} \rightarrow \text{His64}$). The simulation study suggests that the Tyr7Phe mutation impedes the stabilization of W3a and results in a linear, non-branched water cluster forming approximately 80% of the time, and lasting for approximately 5 ps (Figure 2A, D). By comparison, the WT four-water cluster forms approximately 40% of the time and lasts approximately 3 ps.[14] This result suggests that linear, non-branched water clusters can facilitate the PT. As will be discussed in section 6.1, a linear, non-branched water cluster favors a concerted reaction and yields a significantly lower barrier to PT. The difference in behavior is thought to explain the Tyr7Phe mutant's sevenfold increase in PT rate [39].

3.2.b Asn67Leu Mutant—The Asn67Leu mutant, in contrast to the Tyr7Phe mutant, yields a fourfold reduction in the observed maximal PT rate. X-ray structural data on this mutant indicates that the 'out' orientation of His64 predominates. [17] MD simulations reveal that the Asn67Leu mutant (like the Tyr7Phe mutant) favors neither His64 orientation in the $\text{ZnH}_2\text{O}^{2+}$ -His system, resulting in an approximately 50/50 distribution of 'in/out' positions while in the ZnOH^+ -HisH⁺ system His64 is predominantly 'out'. At first glance, this finding appears to contradict the x-ray data. [17] However, it is possible that this disagreement is an artifact of the experimental conditions, that is, the crystallization pH favors the ZnOH^+ -HisH⁺ system. The occupancy data from the MD simulations for intramolecular water molecules and amino acid side chains is in excellent agreement with the x-ray data, supporting the idea that the x-ray structure is indeed the ZnOH^+ -HisH⁺ system, and that the Asn67Leu mutant increases His64 orientational flexibility.

The reason for the reduced rate in Asn67Leu, even though the mutant positively affects His64 orientational flexibility, likely lies with the specific water cluster characteristics. A study of the $\text{ZnH}_2\text{O}^{2+}$ -His 'in' system reveals that its water occupancies are quite similar to those of the WT enzyme, but hydrogen bonding analysis reveals that the water clusters are actually rather unstable (Figure 2A) [28]. It appears that the N67L mutation disrupts the directionality of specific waters within the cluster, resulting in shorter lifetimes and a higher variability in cluster size (Figure 2A, D) [28]. Thus, disruption of the water clusters may reduce the efficiency of PT when His64 occupies the 'in' orientation. As a result, the Asn67Leu mutant achieves more efficient PT when His64 is in the 'out' orientation, which has similar intramolecular water

cluster characteristics to the WT enzyme (Figure 2B, C, E, F) [14, 28]. Therefore, a model of the PT event with only the ‘out’ orientation of His64 in the WT enzyme could, in principal, be used to describe the overall reaction for the Asn67Leu mutant.

These site-specific mutations have helped unravel the orientational effect of His64, the importance of the intramolecular water clusters, and the effects of active-site amino acids. While the Asn67Leu mutant helps to determine the role of position 67 on the orientational preference of His64, it also yields clues as to the impact of the ‘out’ orientation on the PT event. The ability of the active-site water clusters to compensate for the longer donor-acceptor distance when His64 is in the outward conformation is remarkable, though PT proceeds with a reduced rate compared to the WT, indicating an elevation in the PT barrier. As seen in the WT enzyme, water clusters are still able to form in the ‘out’ orientation, but with reduced probability and shorter life spans (Figure 2A-D). This fact also implies that PT in this case may rely on a diffusive event rather than a concerted (or partially concerted) reaction, as the latter requires a more structured and smaller water cluster.

The interesting differences between the Tyr7Phe and Asn67Leu mutants highlight the subtle and complex interactions at play in the enzyme active site. While general trends in the rate based on the orientation of His64 can be discerned ($k_B^{in/out} > k_B^{in} > k_B^{out}$) [10,17,18], the role of the intramolecular water clusters is also important. Yet, these insights alone are not enough because the explicit PT process has not been directly studied via reactive MD simulations, which will now be described.

4.0 Reactive Simulations of the Rate-limiting Proton Transport Event

The empirical MD simulations summarized in the previous sections do not explicitly treat the excess proton transport process in HCA. Instead, they focus on elucidating the orientational characteristics of His64 and the intramolecular water clusters, both of which are important for the PT process in an indirect fashion. While non-reactive empirical MD simulations can yield important information about the end states and plausible pathways for the excess proton charge defect, they do not explicitly describe its translocation. This section therefore highlights the insights gained into the rate-limiting PT process through more challenging reactive MD simulations, which probe explicit bond breaking/forming events such as Grotthuss proton shuttling that are not accessible to standard empirical MD techniques. Several reactive MD simulation techniques have been applied to the HCA II system as mentioned in Sec. 2. Here, in addition to summarizing the results of early ab initio studies on reduced cluster systems, the results of MS-EVB [33] simulations of the rate-limiting PT step in the full WT HCA II enzyme are more fully described, along with SCC-DFTB [34,35,37,44] studies of the same process.

4.1 Proton Transport in WT HCA II

4.1.a Cluster Calculations—The rate-limiting proton-transfer event in HCA II has been investigated with several different levels of ab initio theory. Some of the first computational studies of the PT event examined greatly reduced cluster model systems using semi-empirical methods such as PRDDO[40] and AM1[41]. The PRDDO simulations explored the use of ammonia and water molecules as ligands, considering various numbers of bridging water molecules [40]. These studies provided two important insights. First, ammonia is a better ligand than water, and, second, the primary reaction barrier to proton transfer is related to deprotonation of the zinc-bound water. PT through the subsequent waters was found to be facile as the terminal ammonia (a substitute for the His64 group) attracted the excess proton. These calculations, while reproducing the observed trends of PT, were more qualitative than quantitative, e.g., the reported PT barrier values were three to four times larger than those observed in experiments. Subsequent AM1 calculations later replaced the ammonia molecules

with imidazole molecules, and found PT barriers closer to the experimental value. However, the persistent lack of agreement with the experimental values strongly indicated that more realistic ligands for the Zn^{2+} center were required.

Subsequent work revisited the PT event on a higher level of ab initio theory (HF and the MP2), with imidazole as the zinc ligands and an explicit His64 group [42]. These simulations predicted a PT barrier of 8-10 kcal/mole, in good agreement with experiment. However, this work also showed that higher levels of theory and larger basis sets would be required to accurately model the reaction. Furthermore, the simulations indicated the existence of a stepwise reaction, at least three waters were required to form a cluster, and the presence of a proton 'sink' in the form of His64 is vital. Subsequent computations focusing on the stepwise (or concerted) nature of the reactions at relatively high levels of theory yielded additional information about the PT event [39,43]. Shorter water wires, it was found, are more conducive to PT than longer wires, as might be expected. Proton shuttling through water molecules can proceed in a concerted mechanism in the former case but in a stepwise fashion in the latter scenario.

These same reduced model ab initio studies suggest that the PT barrier increases with the number of waters in the cluster. As a result, water clusters of four or five molecules (including the zinc-bound water) yield better agreement with experiment and facilitate partially concerted or stepwise transfer, respectively. It was also found that the location of the barrier to PT of the five-water system did not correspond to deprotonation of the zinc-bound water, but instead was located at the center of the water cluster. The PT barrier value of the five-water cluster was closer to experiment (~ 6 kcal/mole with a zero point energy correction) than that of the four-water cluster, despite the fact that computational kinetic isotope effect (KIE) studies of the smaller water clusters (where concerted reactions prevail) are more in line with experimental KIE values [38,39]. However, certain factors such as the water wire formation free energy [29] and additional solvation energy of the water cluster from the protein environment [43] were not considered in these calculations.

While the reduced cluster ab initio calculations explored the differences between water clusters of various lengths, they did not consider the effect of an outward orientation of His64, the protein environmental effects, or multiple water cluster configurations and their associated entropy. By design, reduced gas-phase cluster calculations neglect the electrostatic stabilizing effects of nearby amino acids (Thr199, Thr200, Asn62, Asn67, and Tyr7) in the active site and the dynamical properties of the aqueous environment. (It should also be noted that these calculations define energy differences, not *free* energy differences.) Clearly, to definitively characterize the PT event on a free energy level, all of these aspects of the problem must be incorporated into the simulation.

4.1.b SCC-DFTB (QM/MM) Results—Recently, the SCC-DFTB method has been used to study the rate-limiting reaction while taking into account environmental effects via a combined QM/MM method coupled to a generalized solvent boundary potential (GSBP) [44] (SCC-DFTB/CHARMM-GSBP). These simulations suggest that water molecules in the active site influence the energetics of the PT reaction, specifically those that solvate the water cluster connecting the zinc-bound solvent and His64 [37]. Minimum energy path calculations of a three-water cluster also revealed that, for the $\text{EZnH}_2\text{O}^{2+}$ -His PT event, the transition state (TS) corresponds to a proton in close proximity to His64. (This finding is in agreement with earlier work suggesting that the TS does not coincide with deprotonation of the zinc-bound water [42]) The results of these simulations indicate that smaller water clusters, particularly those with a size of three, are more conducive to PT which was also found utilizing an adiabatic mapping with a collective variable reaction coordinate [36].

More recent SCC-DFTB/CHARMM-GSBP simulations have, however, suggested the idea that a hydroxide ‘proton hole’ mechanism [34,35] can account for transport of the charge defect. While hydroxide transport was observed when the SCC-DFTB nitrogen parameters for His64 are modified in the simulation, proton transport was seen when the original nitrogen parameters were utilized [34]. It was noted by the authors that with the modified parameters the ‘proton hole’ mechanism may be artificially favored over the hydronium mechanism due to unbalanced descriptions of the proton affinity and solvation energy [34,35]. Proton ‘hole’ (hydroxide transport) nevertheless appears to be plausible, with a predicted maximal barrier of ~12-13 kcal/mole, in line with the WT experimental results (Figure 3). The water molecules and the polar amino acids in the active site (Thr200, Asn62, Asn67, and Gln92) were observed to stabilize the hydroxide, suggesting that the active site could accommodate such a negatively charged ion. While earlier SCC-DFTB simulations indicate a concerted PT reaction, the use of a flexible, collective reaction coordinate predicted in this case a stepwise mechanism for both hydroxide and hydronium transport.

From the SCC-DFTB simulations it was also suggested that the position and height of the PT barrier is largely determined by the electrostatics of the active site [35], in agreement with earlier work done by Warshel and co-workers [58,59,62]. This result implies that the orientation of His64 does not influence the rate-limiting step and that the intramolecular water cluster (as determined by x-ray data) is either a poor indicator of the PT pathway or insignificant to the PT barrier. In other words, the presence of distinct favorable water clusters was predicted in these studies to have minimal effect on the observed rate. These results, while intriguing, are not fully supported by experiments [17,18]. The N62X mutations, for example, indicate that the orientation of the His64 group affects the observed PT rate in the presence of a complete water cluster [18]. Another example is the Y7F mutant, which yields a sevenfold increase in the observed rate when His64 is in the ‘in’ conformation and in the presence of a highly stable, linear, non-branched water cluster [17]. It should also be noted that the SCC-DFTB method has never been validated for the hydrated excess proton or the hydroxide anion in bulk water, so it is unknown whether this computational methodology describes these species accurately and in agreement with experimental results for those systems. As a result, there are several unanswered questions about the contributions of stepwise and concerted PT reactions, the possibility of a ‘proton hole’ mechanism, the role of the orientation of His64, and the importance of metastable active site water clusters from these SCC-DFTB studies.

In addition to the WT HCA II system, the SCC-DFTB/CHARMM-GSBP methods have been used to study the H64A mutant. These simulations predict that the PT barrier is ~21 kcal/mole (Figure 3) [34,35]. While this result was originally used as an argument for the ‘proton hole’ mechanism [34], it may alternatively reflect the features of the His64Ala environment. The removal of His64 as the primary proton donor-acceptor (shuttling) residue in the H64A mutant could allow other, less favorable PT pathways to become viable. The ~20 kcal/mole barrier from the simulation may reflect an actual unfavorable transport pathway for the excess proton into or out of the active site. Other HCA II residues, as yet unknown, may act as new proton shuttling groups in the absence of His64, a result which may be suggested by an experimentally determined PT barrier value (~11-12 kcal/mole) that is much less than the SCC-DFTB simulation result.

4.1.c MS-EVB Results—In contrast to the SCC-DFTB results, MD simulations using the MS-EVB methodology described in Sec. 2.2 suggest that the His64 orientation affects the observed rate. The ‘in’ orientation has a PT barrier of ~10 kcal/mole and a rate of ~1 μs^{-1} (slightly faster than the experimental value of 0.8 μs^{-1}), while the ‘out’ orientation has a PT barrier of ~11.4 kcal/mole and a rate of ~0.09 μs^{-1} (Figure 3) [33]. These results are based on a calculation of the potential of the mean force (PMF) experienced by the excess proton charge defect (its CEC) in the enzyme environment. The PMF incorporates thermally averaged

enzyme and water clusters conformations and hence is a free energy profile for the PT reaction. The His64 'in' configuration has a lower PT barrier due to collective effects between the zinc-bound water solvent, the intramolecular water clusters, and His64. Furthermore, after the CEC has progressed through the intramolecular water cluster to His64, the value of the PMF indicates that His64 has a lower pK_a value in the 'in' position than in the 'out' position, a behavior that is also observed in kinetic experiments [17]. The barrier to rotation once His64 is protonated is also significantly lower than the reverse PT reaction, suggesting that His64 is driven to rotate into the 'out' orientation when shuttling the excess proton out of the active site. Once in the 'out' orientation, the free energy difference between the zinc-bound water and His64H⁺ is near zero. The thermo neutrality of the reaction, the similar free energy difference between the zinc-bound water and His64H⁺ free energy minima, and the similar pK_a values (as determined by similar free energy differences between the bulk and the stable minima) are all in agreement with experimental kinetic results [10]. A simulation of the zinc-bound water dissociation also yields a pK_a value of 7.1, in close agreement with the experimental result of 6.9 [33], as well as previous computational work [53]. An examination of the water clusters shows that three-water and four-water clusters (as seen in x-ray data) are most important for the PT process, the four-water clusters being somewhat more prevalent. The PT is also seen to proceed in a step-wise fashion. All of these results are consistent with experimental results and high-level ab initio calculations. On the other hand, the MS-EVB model used in these studies did not include the hydroxide anion state or water autoionization process in the model, so the possible contribution of a 'proton hole' mechanism was not explored.

It should be noted that the pK_a of His64 in the 'in' and 'out' orientation was not explicitly calculated for the MS-EVB simulations. While it has been suggested by other authors in the present issue [71] that the MS-EVB simulations indicate His64 in the 'in' conformation has an apparent pK_a lower than the zinc bound water by almost 4.4 pK_a units, this interpretation of the PMF and the resulting pK_a is incorrect, perhaps because those authors seem unaware of the proper procedure for calculating a pK_a value from statistical mechanics. As outlined in Ref 68 and paper cited therein, the proper way to calculate the pK_a is to radially integrate the PMF corresponding to the protonation event of the ionizable moiety of interest. For the MS-EVB PMF for the rate-limiting WT PT event, the point of reference is the zinc ion and not the ionizable His64. Therefore, the ability to identify pure product and reactant with respect to His64 and to clearly identify the transition state for the de-/protonation event becomes unclear for obvious reasons. Nevertheless, if one uses the χ_1 dihedral and corresponding $R_{Zn-N\delta}$ data from Ref 26, the appropriate regions of the PMF corresponding to the His64 'in' orientation may be identified. Integrating this region of the PMF to determine His64's pK_a , which should be stressed here is not ideal, gives a pK_a value of 5.8, not 2.7 (the latter is a value determined in error by taking ΔF values from the PMF for the dissociation of the zinc-bound water). The pK_a value of 5.8 for His64 in the 'in' orientation is very similar to the observed pK_a value of 6.0 for the Tyr7Phe mutant, which x-ray data indicates largely occupies the 'in' orientation [17]. To accurately calculate the pK_a of His64, a separate PMF for the proton (CEC) dissociation event originating on the His64 would be required to clearly identify the reactant and product configurations, as well as the transition state.

A full molecular-level picture of the HCA II PT process can be obtained from the MS-EVB simulations by taking into account the His64 orientations, the intramolecular water clusters, the stabilizing active-site amino acids, and an explicit treatment of the excess proton charge defect (CEC). In the WT HCA II system, His64 in the 'in' orientation favors both water 'wire' and water 'cluster' PT events [33]. It has been shown that PT through a water wire is considerably more facile than PT through a fully solvated bulk water environment (or, in this case, a water cluster). Taken together, these results suggest that the beginning of the water cluster is most often a wire formation (near the zinc) in which Thr199 and Thr200 stabilize the zinc-bound water and W1 respectively (see Fig. 1). These two molecules are not fully solvated

by the surrounding water (one hydrogen bond is donated to the active-site amino acids, and the other is donated to the next water in the wire). Only at W2, which is fully solvated by surrounding water, does a more cluster-like environment arise. When His64 is in the 'in' orientation, W2 acts either as the donor of the excess proton to His64, or as the branching point from which the excess proton CEC may proceed to W3a or W3b. In most instances His64 is hydrogen bonded to W3a, and because His64 is the proton 'sink', it draws the CEC through W3a. It is believed that the barrier to this particular PT event lies somewhere between W2 and W3a, as the CEC moves from a fully solvated to a partially solvated water (W3a is hydrogen bonded to Tyr7 and His64).

This balancing act between efficient water-wire PT and slower water-cluster PT may have influenced the evolution of the HCA II enzyme and its optimization of the rate-limiting PT event. Indeed, *ab initio* simulations had already predicted that increasing the solvation in the vicinity of the water wire would cause the PT barrier height to increase as well [43]. The case of the Tyr7Phe mutant provides additional support for this concept. This mutant (as observed in x-ray and MD simulated data) favors the formation of a linear, non-branched water structure ($\text{EZnH}_2\text{O} \rightarrow \text{W1} \rightarrow \text{W2} \rightarrow \text{His64}$) that is more like a water wire than a cluster. The lower PT barrier of this system results in a sevenfold increase in the rate. It is of interest to note that this mutant is not observed in Nature. Its absence suggests that evolutionary pressures did not dictate the need for such a fast enzyme nor was Tyr7Phe's instability at low pH favorable to evolution [17].

In MD simulations allowing only the 'out' His64 conformation, longer, more dynamic water clusters are observed. This result points to a diffusive nature of the excess proton CEC translocation and suggest a possible cause for the elevated PT barrier when His64 is in the 'out' orientation. When His64 is in this orientation, the beginning of the PT event from the zinc-bound water still proceeds through the highly efficient pseudo water wire, but when the proton CEC reaches W2 via Grotthuss shuttling, it proceeds through W3b and then into a more bulk-like environment of water molecules. A pathway through W3a is not present in statistically relevant configurations, thus forcing the CEC to follow less favorable pathways. This phenomenon is clearly visible in the PT PMF (Figure 3), where the free energy profile for the CEC translocation continues to rise as it moves through bulk-like water environment until it begins to interact with His64. From this analysis, the complex interplay between active-site residues, intramolecular water clusters, and His64 begins to become evident. In all cases the active-site amino acids are crucial in stabilizing the water molecules and/or excess proton CEC. While Thr199 and Thr200 are important for the PT events with His64 both 'in' and 'out', Tyr7 seems to play a larger role in the 'in' orientation. Likewise, Asn62 and Asn67 are more important in the 'out' orientation. Furthermore, Tyr7, Asn62, and Asn67 play a supporting role throughout the PT event by stabilizing water molecules that fully solvate W2 (thereby creating the crucial branching point in the PT event).

The MS-EVB results for PT with His64 in the 'out' configuration can also be used to interpret, to a degree, the effects of several site-specific mutations. For example, the N67L mutant (as discussed previously) may have a PT mechanism with His64 primarily in the 'out' configuration because the water cluster network associated with the His64 'in' orientation is significantly disrupted. Therefore, if the WT MS-EVB results for the His64 'out' configuration are used to describe the N67L mutant, a rate of $\sim 0.09 \mu\text{s}^{-1}$ is predicted for the mutant. The experimentally determined rate for N67L is four times slower than that of the WT (i.e., $0.2 \mu\text{s}^{-1}$) in the same ballpark. The aforementioned approximation only utilizes the PT rate results with His64 in 'out' orientation, but there may be some productive transport when the 'in' orientation of His64 exists in conjunction with the disordered water cluster. So, in this case, one would expect a slightly higher rate than that estimate solely from the WT His64 'out'

orientation, as is found experimentally. This line of reasoning can also be used to analyze the N62D mutant, which has a rate of $0.043 \mu\text{s}^{-1}$ [18].

5.0 Various Proton Transport Pathways

Up to now we have mainly discussed the active site PT pathway connecting the zinc-bound water solvent to His64. For the HCA II enzyme to function, however, pathways must exist for the excess proton to reach the bulk environment. Even in the absence of His64 (e.g., the His64Ala mutant), there must be pathways connecting the active site with the surrounding bulk water solvent. This section discusses pathways between the zinc-bound water solvent and His64, His64 to bulk solvent, as well as additional pathways from the active site to bulk solvent. Reactive MD simulation studies of an excess hydrated proton reveal that the proton CEC utilizes several pathways (as identified by hydrogen bond and probable-pathway algorithms [22,35-37]). The MS-EVB simulations, for example, confirm that proton transport takes place in the active site via the $\text{EZnH}_2\text{O}^{2+} \rightarrow \text{W1} \rightarrow \text{W2} \rightarrow \text{His64}$ and $\text{EZnH}_2\text{O}^{2+} \rightarrow \text{W1} \rightarrow \text{W2} \rightarrow \text{W3a} \rightarrow \text{His64}$ pathways (long believed to be the dominant pathways). (It is important to note, however, that the most probable pathway in the MS-EVB simulations is $\text{EZnH}_2\text{O}^{2+} \rightarrow \text{W1} \rightarrow \text{W2} \rightarrow \text{W3a} \rightarrow \text{His64}$.)

After PT from the zinc-bound water to His64, the His64 likely rotates from the 'in' orientation to the 'out' orientation. During this movement there is little evidence that His64 loses the excess proton. That is to say, when His64 is in the 'in' orientation there does not seem to be a free energy driving force for water clusters to translocate the excess proton to the bulk. It is found that the re-orientation of His64 is the lowest free energy path. However, when His64 is in the 'out' orientation, there exist lower free energy pathways for the excess proton to leave His64 and migrate to the surrounding bulk environment. The main pathway sampled by MS-EVB simulations involves traveling through water molecules at the solvent interface that resides between the two loops at the bottom of the active site.

While these results support the proposal that the transient water-clusters connect the zinc-bound solvent to the probable proton donor-acceptor His64, they do not explain how the zinc-bound water solvent might translocate protons to the surrounding bulk environment without utilizing His64. Identifying these pathways may reveal plausible routes of proton transport into and out of the active site in mutants such as His64Ala. MS-EVB simulations that probe alternate PT pathways find that the excess proton CEC utilizes three distinct pathways into and out of the active site (Figure 4) [33]. The most probable pathway utilizes $\text{ZnH}_2\text{O}^{2+} \rightarrow \text{W1} \rightarrow \text{W2}$, or to a lesser extent $\text{ZnH}_2\text{O}^{2+} \rightarrow \text{W1} \rightarrow \text{Wx}$, (where Wx denotes water molecules other than W2 or W3a, b) to travel between the zinc-bound-water and Val139 (a hydrophobic residue residing on the rim of the active site above the zinc-bound solvent). The second most probable pathway is similar to the above pathway but, before reaching Val139, the excess proton CEC diverges from the path and moves towards Glu69 and Asp72 (the x-ray-determined binding site of the chemical 'rescue' agent 4-methyl-imidazolium, 4MI). The results for the first two pathways are in close agreement with hydrogen-bonded network algorithms that predicted very similar paths.[30-32] A third, least probable, pathway utilizes $\text{ZnH}_2\text{O}^{2+} \rightarrow \text{W1} \rightarrow \text{W2} \rightarrow \text{W3a, b} \rightarrow \text{Wx}$ as the excess proton moves toward residue 64 and finally out of the active site.

6.0 Concluding Remarks

This article has reviewed existing kinetic, x-ray, and computer simulation data on the PT mechanism in HCA II as well as several interesting mutants. Connections of the PT behavior have been made to the orientation of the His64 residue, the nature of the stabilizing active-site residues, and the formation of transient water clusters, all of which appear to act in concert to facilitate the rate-limiting PT event. Specific attention has been given to the WT enzyme, as well as the Tyr7Phe and Asn67Leu mutants. What has emerged is a dynamic picture of the

active site, where His64 can switch its orientation. In turn, the distribution of His64 orientations is influenced by residues in the active site, which can also stabilize active site water molecules. To a first approximation, the orientational preference of His64 provides a qualitative basis for estimating the PT rates expected of mutations observed in x-ray and/or simulations. However, there may be several outlier mutants that do not conform to this picture, indicating that more information will be needed, such as the characteristics of the intramolecular water cluster. While x-ray data is vital in these studies, it does not contain the information needed to probe the water cluster dynamics and their influence on the PT process.

MD simulations of the molecular level have been able to determine water cluster size distributions and lifetimes in the active site. This understanding of water cluster characteristics has helped to create a more complete picture of the rate-limiting PT event. For example, the simulations suggest that the WT enzyme utilizes three and four-water 'wires/clusters' by capitalizing on the nature of the bridging molecules. The existence of these pathways is in turn related to the stabilizing influence of the active-site amino acids. Longer water pathways are less conducive to PT and typically lead to a diffusive transport of the excess proton. This latter PT process is believed to be less favorable than that seen in smaller water clusters, resulting in elevated barriers similar to those seen in kinetic studies when His64 is in the 'out' orientation. This provides additional evidence that the orientation of His64 and the formation of appropriate intramolecular water cluster (or clusters) are both important factors in defining the rate-limiting PT event in HCA II.

MD methods such as MS-EVB and SCC-DFTB that explicitly describe the hydrated excess proton and the associated PT process in HCA II have built upon the insights provided by the non-reactive empirical MD simulations. The MS-EVB simulations have highlighted the importance of His64 conformation and the role of transient water clusters, in agreement with the available experimental data. These simulations have also revealed pathways that the excess proton may utilize when traversing into and out of the enzyme active site. These pathways point to chemical rescue mechanisms via the binding of molecules such as 4MI, as well as other possible proton shuttling amino acid residues that may become operational in mutants with non-ionizable substitutions of the His64 group.

Taken as a whole, molecular-scale computer simulations of HCA II and its mutants, when combined with a variety of key experimental results, have contributed greatly to our understanding of this important enzyme. Nevertheless, important experimental questions remain, and the computational methods that have been developed to help address these questions do not always yield the same results. The future therefore presents us with a collection of key challenges, especially in terms of refining the accuracy and rigor of the computational approaches, as well as their continuing impact on experimental carbonic anhydrase research.

Acknowledgments

This work was supported by the United States National Institutes of Health (R01-GM53148)

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Figure 1. Active site of WT (A), Tyr7Phe (B), and Asn67Leu (C) HCA II, depicting an intramolecular water cluster and critical amino acids. Coordinates were taken from the 2ILI PDB [27], 2NXR PDB [17], and 2NWY PDB [17] structures for the WT, Tyr7Phe, and Asn67Leu, respectively. Figure was rendered with the VMD software [72].

Figure 2.

Characteristics of the smallest continuous hydrogen bonded water cluster that connects the zinc-bound water/hydroxide and the protonated N_δ of His64. Water wire size distributions for (A) EZnH₂O²⁺-His in the 'in' orientation, (B) EZnH₂O²⁺-His in the 'out' orientation, and (C) EZnOH⁺-HisH⁺ in the 'out' orientation. Water wire lifetime distributions for (D) EZnH₂O²⁺-His in the 'in' orientation, (E) EZnH₂O²⁺-His in the 'out' orientation, and (F) EZnOH⁺-HisH⁺ in the 'out' orientation. All plots have data from the WT (no fill), Tyr7Phe (light grey fill), and Asn67Leu (dark grey fill) HCA II.

Figure 3.

Various computed free energy curves (PMFs) for the proton translocation in the HCA II enzyme. The PMFs are plotted as a function of the radial distance of the CEC to the catalytic zinc, $R_{\text{Zn-CEC}}$, for the MS-EVB simulation. The SCC-DFTB simulation reaction coordinate (originally a value of zero to one) was converted to $R_{\text{Zn-CEC}}$ based on the location of the zinc-bound solvent minimum and the His64H⁺ minimum (depending on the orientation of His64). The SCC-DFTB simulations are for their proposed ‘proton hole’ hydroxide transfer mechanism [35], while the MS-EVB simulations represent a hydrated excess proton transfer [33]. Also shown are the SCC-DFTB His64Ala mutant PMF results for hydrated excess proton transport. [35]

Figure 4. Spatial occupancy density plot of the excess proton CEC superimposed on the x-ray structure of the WT HCA II enzyme (2CBA). The grey volume represents the isosurface within which the excess proton CEC resides 98% of the time.