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Proton Transfer Function of Carbonic Anhydrase: Insights from QM/MM simulations

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Abstract

Recent QM/MM analyses of proton transfer function of human carbonic anhydrase II (CAII) are briefly reviewed. The topics include a preliminary analysis of nuclear quadrupole coupling constant calculations for the zinc-ion and more detailed analyses of microscopic pK_a of the zinc-bound water and free energy profile for the proton transfer. From methodological perspective, our results emphasize that performing sufficient sampling is essential to the calculation of all these quantities, which reflects the well-solvated nature of CAII active site. From mechanistic perspective, our analyses highlight the importance of electrostatics in shaping the energetics and kinetics of proton transfer in CAII for its function. We argue that once the pK_a for the zinc-bound water is modulated to be in the proper range (~ 7.0), proton transfer through a relatively well solvated cavity towards/from the protein surface (His64) does not require any major acceleration. Therefore, although structural details like the length of the water wire between the donor and acceptor groups still may make a non-negligible contribution, our computational results and the framework of analysis suggest that the significance of such “fine-tuning” is likely secondary to the modulation of pK_a of the zinc-bound water. We encourage further experimental analysis with mutation of (charged) residues not in the immediate neighborhood of the zinc ion to quantitatively test this electrostatics based framework; in particular, Φ analysis based on these mutations may shed further light into the relative importance of the classical Grotthus mechanism and the “proton hole” pathway that we have proposed recently for CAII.

I. INTRODUCTION

Due to its relatively small size, robust structure and the availability of a large body of experimental data¹, human Carbonic Anhydrase II (CAII) is a wonderful system for probing factors that limit water mediated proton transfers in a biological context. On the other hand, unlike typical ion channels, the active site of CAII is relatively well solvated, especially when the proton acceptor, His64, adopts the “out” orientation (see Fig.1 for an illustration); therefore, the transfer pathway is not as uniquely defined as, for example, gramicidin A.

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These features pose unique challenges to the study of proton transfers and related problems in CAII at a quantitative level.

In the following, we first briefly summarize methodology used in our studies, especially some unique features of combined quantum mechanical/molecular mechanical (QM/MM) methods that have been developed in our group for the long-term goal of understanding biomolecular proton pumps. Then, we discuss application of these QM/MM methods to specific mechanistic problems in CAII, including how the methods were benchmarked in the context of studying proton transfers. Through these discussions, we illustrate insights that emerged from these computational analysis, especially concerning the possible contribution from a “proton hole” transfer mechanism and factors that dictate the apparent proton transfer barrier *in the context of CAII's biological function*. Finally, we briefly summarize general implication of these findings to proton transfers (pumping) in biological systems.

II. THEORY AND METHODS

In experimental studies, different approaches are used because they report on different properties of interest. Similarly, in a computational study, what methods to use depends on the relevant observable and features of the system in hand. For the analysis of spectroscopic measurements that probe local geometry and electronic structure, an active site model treated with a high level of QM theory is likely sufficient, although sampling local hydrogen bonding patterns can be important. For studying long-range proton transfers, especially in a polar environment, it is crucial to perform sufficient sampling such that structural responses to the long-range charge movement are considered; otherwise, as shown below in Sect.III C, qualitatively incorrect results can be obtained. Accordingly, a lower-level QM based method is likely more appropriate for studying proton transfers, although the method clearly needs to be carefully validated for meaningful applications.

A. Nuclear quadrupole coupling constant calculations

Metal-nuclei based solid NMR studies have emerged as a powerful technique for probing active site properties of metalloenzymes^{2,3}. One central observable in this technique is the nuclear quadrupole coupling constant (C_q), which is related to the electric field gradient (EFG) tensor at the metal nucleus⁴:

$$C_q = q_{zz} \frac{e^2}{a_0^3 h} Q \quad (1)$$

where q_{zz} is the zz (principal) component of the EFG tensor and Q is the quadrupole moment of the nucleus; e , a_0 and h are the standard constants: the electron charge, the Bohr radius and the Plank's constant, respectively. Since q_{zz} can be calculated once the charge distribution of the system is known and all other factors in Eq.1 are constants, it is possible to determine structural information for the metal center by comparing calculated EFG/ C_q to measured values. In several recent studies of zinc-enzymes⁵⁻⁸, such comparison indeed has appeared valuable for discriminating between several coordination states of the metal.

Specifically for CAII⁵, EFG/ C_q measurements have been used to probe the ligation state of the zinc-bound water (see Sect.III A for detailed discussions).

Since EFG/ C_q depend on electron distribution at the metal *nucleus*, an accurate calculation likely requires a QM level that treats metal-ligand interactions accurately along with a large basis set (recall that Gaussian basis functions have incorrect behavior at small electron-nucleus distances). For a series of small zinc compounds in the solid state form, Oldfield et al.⁹ compared calculated and measured EFG/ C_q values. They found that a combination of the popular density function theory (B3LYP^{10–12}) and triplet/quadrupole-zeta quality basis set gives very consistent results compared to experiments. In QM/MM studies of several zinc-enzymes^{6–8}, this combination was also found among the most consistent, although using the simpler local density approximation (LDA) functional for geometry optimization has also been recommended based on the finding¹³ that LDA appears to give better zinc-ligand geometry than B3LYP.

In our study of EFG/ C_q for CAII, we have used the B3LYP functional with several basis sets; test calculations on model compounds found that the combination of B3LYP and a triple-zeta quality basis set (6–311G*) gives reliable results. Geometry optimization of the active site is performed in the QM/MM framework, with the convergence threshold for the geometry optimization set to be 0.01kcal/(mol Å); considering the local nature of EFG/ C_q groups beyond 10 Å from the zinc ion are kept to the crystal structure positions (PDB code 2CBA¹⁴) during the minimization. The QM region in the minimization includes the zinc ion plus all its ligands. For the EFG/ C_q calculations, a larger QM region is used following test calculations, which includes Zn^{2+} , its four ligands and Thr199, which forms a hydrogen bond with the Zn^{2+} -bound water/hydroxide, Thr200 and Glu106, and all water molecules within 6 Å of the zinc. The remaining part of the system is treated with the CHARMM 27 force field¹⁵, and the link-host-group exclusion scheme (EXGR)¹⁶ is used with link atoms added between C_α and C_β atoms of the three Histidine residues coordinated to Zn^{2+} . No cutoff for electrostatic interactions is used, and an energy switch scheme is used to describe van der Waals interactions. The minimizations are carried out using the QCHEM/CHARMM interface¹⁷, and EFG/ C_q calculations are carried out using Gaussian03¹⁸.

An issue that we explore briefly here concerns the importance of sampling in EFG/ C_q calculations, which has not been considered in the literature; previous studies⁹, including those on enzyme systems^{5–8}, employed only geometry minimization that initiated from crystal structure. Although using minimized structure is partially justified by the fact that the experiments are typically carried out at very low temperature of 10–20K, proteins have many local minima and therefore conformational/static heterogeneity (as opposed to dynamic heterogeneity) may contribute significantly to the observed EFG/ C_q .

B. Microscopic calculations for pK_a and proton transfers

As alluded to briefly in Introduction, sampling is essential to computational study of long-range proton transfer in a polar environment. Although impressive progress has been made in *ab initio* based QM/MM methods¹⁹, which makes it even possible to determine free energy based reaction paths for enzymes²⁰, application of these techniques to CAII is not straightforward. As several independent simulations using different potential functions have

shown^{21–25}, the number of water molecules that participate proton transfer fluctuates at rather rapid (10–10² picoseconds) time scales, it is thus critical to characterize the free energy of the proton transfer without using a reaction path that explicitly refers to the number of water molecules. These considerations motivated two lines of development in our group: (1) an effective semi-empirical QM based QM/MM simulations²¹; (2) a generalized reaction coordinate that describes long-range proton transfers with complex three-dimensional pathways²⁶. Moreover, due to the importance of electrostatics in long-range charge transfers, we have also made efforts to (3) improve and systematically benchmark electrostatic treatment in QM/MM simulations²⁴.

Since these technical developments have already been discussed in several recent review articles^{21,27}, we will only briefly summarize the unique aspects of our computational framework. Abbreviated as SCC-DFTB/MM-GSBP, the protocol treats on the order of 100–120 atoms (Zn^{2+} ion, its ligands, His64 and intervening water molecules) at the SCC-DFTB²⁸ level, while the rest using CHARMM force field²⁹; electrostatics interactions are treated using GSBP^{24,30}, which describes bulk solvation and electrostatic contribution from atoms far away from the active site (e.g., beyond 25 Å) in the framework of Poisson-Boltzmann. SCC-DFTB is an approximate density functional theory, and its most significant advantage is its computational efficiency, which is about 10³ times faster than popular density functional theories. The use of GSBP to avoid including a large number of explicit water molecules also helps further increase the computational efficiency; comparison to Ewald based periodic boundary condition simulations²⁵ has shown that GSBP provides a reliable description for atoms not in the immediate neighborhood of the boundary. Without resorting to a high-level of parallel computing, tens of nanoseconds simulations can be routinely carried out with SCC-DFTB/MM, which has been essential to both the PMF and pK_a calculations for CAII. Compared to popular NDDO methods such as AM1, PM3, the SCC-DFTB approach generally gives better structures³¹, especially for metal ions³² like Zn^{2+} and hydrogen bonding interactions, both of which are important to the study of CAII. Another type of quantity of general importance to the study of proton transfers is the proton affinity of donor, acceptor and water molecules. The error in proton affinity is generally very large (~10–15 kcal/mol) with semi-empirical QM methods³³, including the standard SCC-DFTB. With recent extensions³⁴, proton affinity calculated by SCC-DFTB can be significantly improved, although it is *relative* proton affinities that need to be accurately reproduced in proton transfer studies, which remain a non-trivial challenge in general applications (see Sect.III C for additional discussions).

For probing the energetics of proton transfers, both minimum energy path (MEP)²¹ and potential of mean force (PMF)³⁵ simulations have been carried out. MEP calculations are commonly used in small molecule systems and many QM/MM studies of enzyme systems³⁶, including relatively short-ranged proton transfers^{37,38}. For long-range proton transfers in a relatively well solvated environment such as in the CAII active site¹, we show in Sect.III C that local MEP calculations can potentially lead to qualitatively incorrect results. For PMF calculations, the particular reaction coordinate that we use is based on a modified²⁶ version of “center of excess charge” (mCEC)³⁹, typically corresponds to the position of the heavy atom (e.g., oxygen in a bridging water) that adopts a different protonation state compared to the reactant state. In addition to its robustness to variations in the geometry of the proton

transfer pathway, mCEC can reflect the position of either a hydronium or a hydroxide. This unique character of the reaction coordinate and flexibility in the QM/MM potential function are essential to the revelation that a “proton hole” pathway⁴⁰ can potentially be an important mechanism in CAII, a canonical system that has been used to illustrate the “Grotthuss mechanism”⁴¹ in biology.

Highly relevant to proton transfer studies is microscopic pK_a calculation for the relevant groups (e.g., donor, acceptor). A meaningful microscopic pK_a calculation (even for pK_a shift relative to a very similar molecule in solution) requires reliable description of both intermolecular interactions and long-range electrostatics, as well as sufficient conformational sampling^{25,42,43}. Therefore, although pK_a shift calculations can in principle be carried out using pure MM potentials, we have carried out microscopic pK_a calculations²⁵ using the same SCC-DFTB/MM-GSBP protocol for CAII as explicit benchmarks. To ensure that adequate amount of sampling has been carried out in these thermodynamic integration calculations⁴⁴, multiple independent sets of trajectories have been carried out and the statistical errors for the free energy (derivatives) have been systematically monitored. In addition to serving as benchmark, as discussed below in Sect.III B, these pK_a calculations are also useful for revealing interactions in the protein that help shape the energy landscape for proton transfers.

III. RESULTS AND DISCUSSIONS

A. Protonation state of the zinc-bound water: interpretation of solid state NMR measurement

In the study of Ellis et al.⁵, C_q was determined for the ^{67}Zn in CAII as a function of pH. Surprisingly, the measured C_q value remained fairly constant (~ 10 MHz) for a broad range of pH between 5 and 10. Calculations using several molecular models for the active site⁵ found that the C_q value is about 25 MHz when the zinc is bound to a water, while the calculation result is much closer to the measured value when the fourth ligand of the zinc ion is a hydroxide. Therefore, it was concluded that for a broad pH range, the zinc-bound water in CAII is in fact deprotonated! This has significant implication to the catalytic mechanism of CAII, and indirectly supports an (old) mechanism in which bicarbonate participates in the generation of the zinc-bound hydroxide^{45,46}. However, since the models studied in Ref.⁵ do not contain the enzyme environment, it is not clear whether the structural interpretation thereof is robust.

Preliminary results from our study are shown in Table 1. The most interesting trend is that the calculated EFG/ C_q values for the zinc-water case do not show any large variation among different configurations, while those for the zinc-hydroxide case undergo significant fluctuations with values of different signs. Analyses of geometries indicate that the active site does not undergo dramatic changes. The sign change in computed C_q is due to the fact that the electric field gradient tensor for the zinc-hydroxide case has two eigenvalues of similar absolute magnitude but opposite signs, thus the principal component (q_{zz}) may change sign upon relatively small structural variations.

With a small number (ten) of configurations, it remains premature to draw firm conclusions regarding the comparison between calculated and measure EFG/ C_q values; a straightforward average fortuitously leads to a good agreement with measured values (note that the NMR measurement does not determine the sign of C_q) when the zinc ligand is hydroxide. However, it is clear is that conformational sampling can be critical even for spectroscopic observables that report highly localized structural features.

B. Microscopic pK_a analysis of the zinc-bound water

As emphasized by many researchers, especially Warshel and co-workers^{47,48}, microscopic pK_a calculations are powerful benchmark for proton transfer studies. For CAII, we have calculated the pK_a of the zinc bound water for both the wild type (WT) enzyme and a mutant that has been characterized in the literature, E106Q. Since the pK_a of zinc-bound water has been well characterized experimentally, the calculation will be a stringent test on its proton affinity and interaction between this special ligand in different protonation states (H_2O and OH^-) with the enzyme environment. More precisely, the actually computed quantity was the pK_a of the zinc-bound water in CAII relative to an imidazole in solution. This choice was motivated by the fact that the proton transfer in CAII occurs between zinc-bound water and His64, and imidazole is a very good model compound for the latter; reproducing a satisfactory relative pK_a is, therefore, desirable. In this context, we note that using a very different compound as solution reference is not straightforward in conventional MM based pK_a calculations and underlines the advantage of using a QM/MM potential function.

As shown in Ref.⁴⁹, once technical issues such as sampling and link-atom scheme are carefully considered, the microscopic pK_a calculations gave satisfactory results in a reproducible manner for the WT CAII; calculated values ranged between 5.4 and 7.1, which compare favorably to the experimental value of 7.0⁵⁰. Therefore, we concluded that the SCC-DFTB/MM-GSBP protocol provides a satisfactory description of the underlying energetics for at least the proton donor and acceptor groups in CAII.

To gain insights into the contribution of various residues and water to the pK_a value, perturbative analysis is carried out in which the electrostatic contribution to the free energy derivatives is analyzed systematically. The analysis found that the net contribution from water is fairly small, on the order of 2.1 kcal/mol, although the water contribution depends steeply on the coupling parameter λ ; i.e., water molecules reorganize significantly to change in the protonation state of the zinc-bound water. By contrast, the net contribution from the protein is significant, on the order of 60 kcal/mol, although the λ dependence is substantially smaller (by a factor of ~ 2), which is consistent with proteins generally being less polar than water. The decomposition of protein contribution into residual components (Fig.2) indicates that three charged groups (Glu106, Glu117 and Arg246) make the dominant contributions, although those from several polar residues are also substantial; we note that due to the perturbative nature of the decomposition analysis, the magnitude of residual contribution is overestimated. Nevertheless, the key point is that protein residues play an important role in modulating the pK_a of the zinc-bound water, which is critical to the proton transfer energetics (see further discussions in Sect.III C).

Calculations for the E106Q mutant found a significantly reduced pK_a for the zinc-bound water, which is ~ 9 pK_a units lower than the WT. This is qualitatively consistent with the perturbative analysis of residual contributions based on WT simulations (Fig.2), but in striking contrast to experimental analysis based on the pH profile of k_{cat}/K_M for E106Q, which yielded no shift relative to the WT enzyme⁵¹. Considering that there is little structural change between the WT and E106Q mutant⁵², the calculation result suggests that there may be a change in the mechanism for the step manifested by k_{cat}/K_M . As described in Ref.⁵⁰, k_{cat}/K_M is associated with the reaction of the zinc-bound hydroxide with CO_2 in the hydration direction and the dehydration of the zinc-bound bicarbonate in the reverse direction. Therefore, one possible mechanistic change in E106Q is that the bicarbonate plays a more active role and the titration result reflects the pK_a for the total complex of the zinc, the bound water, and the bicarbonate. A similar scenario was proposed⁵³ to explain the behaviors of the cobalt substituted CAII where, unlike the Zn(II)-containing CAII, k_{cat}/K_M depends on the concentration of bicarbonate. In addition, the presence of the acetic acid bound to the zinc in the mutant structure⁵² lends additional support for a zinc ion that strongly favors a negatively charged species in E106Q. Nevertheless, additional investigations such as the titration of k_{cat} as done in Ref.⁵³ should be carried out for the E106Q mutant; in further computational studies, potential change in the titration state of other residues in E106Q relative to WT should be investigated using, for example, continuum electrostatic models⁵⁴. Along this line, the observation that the E117A mutant has WT like pK_a ⁵⁵ also deserves further analysis.

C. Explicit study of proton transfer

In previous discussions of “rescue experiments”⁵⁶, the possibility was raised that water wire formation between the proton donor (zinc-bound water) and acceptor (His64) contributes significantly to the energetic bottleneck of the proton transfer. Subsequent computational analyses^{21–25} using different potential functions found, however, water wire formation is rapid and therefore the corresponding contribution is likely very small. Therefore, to identify molecular factors that dictate the proton transfer kinetics, it is critical to explicitly evaluate the energetics of the proton transfer.

1. Minimum Energy Path (MEP) vs. Potential of Mean Force (PMF)—Since equilibrium simulations have shown that the number of water molecules that bridge the zinc-water and His64 can vary significantly, especially as His64 flips from the “in” configuration to the “out” orientation (which is about 8 and 11 Å from the zinc, respectively, see Fig.1), one interesting question is whether the proton transfer depends critically on the length of the water bridge¹.

As the initial exploration²¹, a large number of MEPs were calculated starting from different snapshots collected from equilibrium MD simulations of different chemical states of CAII; following the nomenclature in the literature, CHOH refers to the state with zinc-bound water/neutral His64 and COHH with zinc-bound hydroxide/H64H⁺. Since the two states differ by the position of a positive charge, it was not surprising that the MEP energetics were found to depend on the origin of the starting structure, reflecting the fact that the active-site residues/solvent respond significantly to the proton transfer. For example, when the starting

structure came from a CHOH equilibrium simulation, the proton transfer from the zinc-water to His64 is largely *endothermic* according to MEPs (on average by as much as ~ 13 kcal/mol). By contrast, when the starting structure came from a COHH simulation, the same proton transfer reaction was found significantly *exothermic* (on average more than -8 kcal/mol). Analysis of interaction energies in the structures along the MEPs found that water molecules in the active site (within 7.5 Å from the zinc), which could reorient more easily than protein residues (dipoles), are largely responsible for this remarkable variation.²¹

As an attempt to capture the “intrinsic barrier” for the proton transfer reaction, which is known to be close to be thermoneutral experimentally,⁵⁷ we generated configurations from equilibrium MD simulations in which protons along a specific type of water wire were restrained to be equal distance from nearby heavy atoms (e.g., oxygen in water or $N\epsilon$ in His64). In this way, the charge distribution associated with the reactive components is midway between the CHOH and COHH states, thus the active-site configuration was expected to facilitate a thermoneutral proton transfer process as confirmed by MEP calculations using such generated configurations as the starting structure. Interestingly, the barriers in such “TS-reorganized” MEPs showed a steep dependence on the length of the water wire; it was small ($\sim 6.8 \pm 2.2$ kcal/mol) with short wires but substantially higher than the experimental value (~ 10 kcal/mol) with longer water wires (e.g., 17.4 ± 2.0 kcal/mol for four-water wires). For a snapshot of a transition state involving long water wire, see Fig.3a.

This steep wire-length dependence is in striking contrast with the more rigorous PMF calculations of the same proton transfer process,^{35,40} in which the wire-length dependence was examined by comparing results with different His 64 orientations (i.e., “in” vs. “out”). As shown in Fig.4, the two sets of PMF calculations produced barriers of very similar values, which suggests that the length of the water wire (or donor-acceptor distance) is unlikely an important factor in determining the proton transfer rate. To understand the origin for this qualitative difference between MEP and PMF results, we analyzed the character of configurations sampled in those calculations by the “excess coordination plots”⁴⁰ (Fig.3b vs. 3d). In such plots, the key information is the protonation state of the heavy atoms (donor/acceptor atoms plus mediating water oxygen) as a function of progress of the reaction; a positive “excess coordination” indicates extra proton (e.g., hydronium) while a negative value indicates “proton hole” (e.g., hydroxide). As shown in Fig.3b, the collection of configurations sampled in the MEP calculations, taken together, yield discrete positive peaks throughout the reaction, which indicates an almost fully *concerted* proton transfer. By contrast, the PMF simulations (Fig.3d) exhibit negative peaks along the anti-diagonal with both the donor/acceptor protonated during most of the reaction; as explained in Ref.⁴⁰, this is indicative of a “proton-hole” mechanism that involves the generation and *sequential* propagation of hydroxide (for a snapshot, see Fig.3; see below for further discussions). While sequential transfers of hydroxide (or hydronium) are not expected to exhibit a strong dependence on the distance of transfer, concerted transfers are expected to be more costly for long-distance transfers because many O-H bonds are broken simultaneously. We emphasize that the concerted nature of the MEPs is unlikely an artifact of the path search algorithm because, for each snapshot, multiple guesses were used and they all converged to the same path. The concerted nature is likely dictated by the protein/solvent configurations used in the MEP calculations. As discussed above, to generate the “TS-reorganized”

configurations, all transferring protons along the wire are constrained to be half-way between the neighboring heavy atoms; therefore, such sampled protein/solvent configurations would favor a concerted over step-wise proton transfers by adjusting to the specific charge distribution. Although *all* atoms in the active site are allowed to move, the local nature of those MEP calculations does not allow collective reorganization of the active site residues/solvent molecules thus the “memory” of the sampling procedure is not erased.

The lack of any significant distance dependence of proton transfer can be understood by the following consideration. As shown in Fig.5, in the limit of stepwise transfer, regardless of a “proton hole” or Grotthuss mechanism, the limiting barrier is expected to be correlated with the largest pK_a drop during the sequence of transfers. In WT CAII, since both the zinc-bound water and His 64 have a pK_a near 7.0, the rate-limiting step is expected to be either the proton transfer from the zinc-bound water to active site waters (Grotthuss) or the proton-hole transfer from His 64 to active site waters. In other words, once either a hydronium or a hydroxide is generated, the species can move without a major barrier (consistent with the high mobility of hydronium and hydroxide in solution⁵⁸) over a long distance and therefore the dependence on the length of the water wire is not expected to be significant, which is reflected in the computed PMFs in Fig.4.

Moreover, we note that this framework of analysis points to an energy bottleneck on the order of 7 pK_a units (the pK_a difference between zinc-bound water and hydronium for Grotthuss, and that between His64H⁺ and neutral water for “proton hole”), which is in fact close to the experimentally observed barrier of ~ 10 kcal/mol⁵⁷. In other words, the rate-limiting proton transfer in CAII is not dramatically accelerated beyond expectation on the basis of pK_a s of the proton donor, acceptor and water molecules. Therefore, the pressure to “fine-tune” the proton transfer rate in CA does not seem compelling. The real evolution pressure from a functional perspective seems to be maintaining similar pK_a values for the zinc-bound water and His 64, as reflected by the significant protein contributions to the elevation of zinc-water pK_a (Sect.III B), such that CAII can work with a similar efficiency in both the hydration and dehydration directions.

Our finding that the proton transfer in CAII lacks a significant distance dependence appears to be in conflict with recent experimental and theoretical studies. As discussed in Ref.³⁵, experimental findings that support a significant distance dependence have alternative explanations. For example, Fisher et al.⁵⁹ found that the H64A/N67H mutant is more effective than H64A/N62H for proton transfer by nearly 1 order of magnitude. The crystal structures of these mutants revealed that while His67 is connected to the zinc-bound water via two water molecules, His 62 is at least three water molecules away from the zinc-bound water. The combination of the kinetic and structural results was interpreted to suggest that the length of the water wire is responsible for the difference in the proton transfer rates in these mutants. However, a closer inspection of the two crystal structures suggests that His 62 is involved in hydrogen bonding interactions with both of its nitrogen atoms and therefore is not in a position to accept a proton. Therefore, the proton transfer rate in H64A/N62H is expected to be similar to that in H64A, which is indeed consistent with the experimental measurement. Similarly, interpretation of rescue experiments⁵⁶ is complicated by ambiguity

associated with the binding site of rescuing agents, and therefore can't be used to argue that the "out" configuration of His64 is unproductive toward proton transfer (also see below).

In the recent MS-EVB analysis of Voth and co-workers⁶⁰, thorough PMF calculations using a somewhat similar CEC coordinate found that the proton transfer to the "in" configuration of His64 has a lower barrier than that to the "out" configuration of His64 by ~ 1.4 kcal/mol (10.0 ± 0.4 vs. 11.4 ± 0.4 kcal/mol); therefore, it was concluded that there *is* a distinct preference of proton transfer to the "in" configuration. We note, however, the lower barrier towards the "in" configuration is accompanied by a significant "acidification" of His64: proton transfer to the "in" His64 is endothermic by as much as ~ 6 kcal/mol, while it is almost thermoneutral to the "out" His64, thus the "apparent" pK_a of the "in" His64 is lower by almost $6/1.37 = 4.4$ pK_a units. Although this trend is qualitatively consistent with the idea that a protonated His64 prefers the "out" configuration while a neutral His64 prefers "in"⁶¹, the magnitude of change appears rather large compared to observed pK_a shifts in CAII mutants⁶², thus the difference between the "in" and "out" PMFs deserves further analysis. For example, in the analysis of the Thr200Ser mutant⁶³, it was found that the mutation has little impact on the intramolecular proton transfer rate even though His64 in the mutant structure was directed away from the active site.

Regardless, the MS-EVB result showed that, compared to the "in" configuration, the proton transfer rate for the "out" configuration of His64 would be similar at low exogenous buffer concentrations and lower by only a factor of $\sim \exp(1.4/0.6) \sim 10.3$ at high exogenous buffer concentrations; therefore, the "out" configuration of His64 is clearly far from unproductive towards proton transfer, which was first pointed out by our QM/MM study³⁵. Moreover, although a factor of 10 difference in rate is certainly not negligible, it's far from being dominant compared to the protein contribution in terms of modulating the pK_a of the zinc-bound water. Therefore, from the functional perspective, organizing protein electrostatics in the active site is likely more important than maintaining a short water wire. In fact, perturbative analysis of QM/MM PMF suggests that many of the same residues identified to make a significant contribution to the pK_a of the zinc-bound water also make large contribution to the proton transfer energetics, and collectively the protein contribution is to *disfavor* proton transfer to His64 by ~ 25 kcal/mol. Although the quantitative values from perturbation analyses are of limited significance, the qualitative nature is robust^{64,65} and highlights the role of the protein in raising the pK_a of the zinc-bound water for function. We hope the qualitative contributions can be investigated systematically by mutation studies, especially those not deep in the active site; a recent study in which Lys 170 has been mutated to an Ala or Glu found that the mutations accelerated the proton transfer (D. N. Silverman, private communication), which is in qualitative agreement with the unfavorable contribution (for proton transfer to His64) we found for Lys 170 in the WT CAII.

2. Further discussion of Grotthuss vs. "Proton hole" mechanisms—As illustrated in Fig.3, our PMF calculations revealed the interesting possibility that proton transfers in CAII proceeds through a "proton hole" mechanism⁴⁰ rather than the classical Grotthuss mechanism⁴¹ *assumed* in all previous proton transfer studies of CAII and related systems. Considering remaining systematic errors in the QM/MM methodology, it is important to consider whether this mechanism makes physical sense. As illustrated in Fig.5,

in the limit of stepwise transfer, whether Grotthuus or “proton hole” transfers dominate depends on the pK_a values of the proton donor, acceptor and intervening water molecules. The somewhat special situation in CAII, which is likely driven by the functional importance of being efficient in both hydration and dehydration directions, is that both proton donor and acceptor have a pK_a close to 7.0. Therefore, *assuming* that the pK_a 's of water and hydronium are similar to their bulk values, the basic energetic bottleneck is similar for the Grotthuus and “proton hole” mechanisms. Further considering that CAII is evolved to catalyze a transformation of bicarbonate, a negatively charged species, it's likely that the electrostatic environment of the CA active site favors a negative species (as shown by Poisson-Boltzmann calculations⁴⁰) and therefore the “proton hole” mechanism is favored.

As pointed out in our original analyses^{35,40}, the relative weight of Grotthuus and “proton hole” transfers depends critically on the pK_a of the intervening water molecules; i.e., whether hydroxide and hydronium is preferentially stabilized by the computational method. Although the relative pK_a 's of the proton donor/acceptor (zinc-water and His64) have been well balanced by our method, as reflected by the pK_a and thermoneutral nature of the PMFs, benchmark calculations on solution model systems (Supporting Materials in Ref.⁴⁰) did point to the possibility that hydroxide is artificially stabilized than hydronium by the current SCC-DFTB method. Whether this artificial stabilization is sufficiently large to rule out the “proton hole” mechanism is not straightforward to determine, which highlights the importance of systematically pushing forward the accuracy of QM/MM methods. It is, therefore, valuable to consider whether there is any experimental signature that can be used to distinguish between Grotthuus and “proton hole” transfers.

As discussed in Ref.³⁵, an interesting possibility is to carry out Φ analysis⁶⁶, which has been routinely used in protein folding studies for characterizing the nature of transition state. This is motivated by the consideration that the charge distributions for the transition state in Grotthuus and “proton hole” transfers are likely distinct. As illustrated in Fig.6, the (simplified) transition state in a Grotthuus mechanism is likely featured with a +1 charge for the zinc-center, +1 charge for the hydronium and zero charge for His64; by contrast, the transition state in a “proton hole” mechanism has +2 charge for the zinc-center, -1 for the hydroxide and +1 for the protonated His64. Accordingly, although mutations of residues in the active site would have the same impact on the net proton transfer, the impact would be rather different on the transition state for the two transfer mechanisms; i.e., the Φ values would be different. With the simplifying assumption that the location of the transition state remains the same upon mutation, a perturbative analysis indeed found qualitatively different patterns in the Φ values for the two mechanisms³⁵. As illustrated in Fig.6, residues close to His64 tends to have larger Φ values in the “proton hole” mechanism while the opposite is true for the Grotthuus mechanism. Therefore, we hope that systematic Φ analysis will be carried out experimentally, which may shed light into the relative importance of these two microscopically distinct mechanisms.

IV. CONCLUSION

In this short manuscript, we have reviewed our recent analysis of mechanistic issues related to proton transfers in CAII using QM/MM calculations.

Regarding factors that limit the proton transfer kinetics, our analyses of both pK_a and proton transfer PMF have highlighted the importance of electrostatics in shaping the energetics and kinetics of proton transfer in CAII for its function. From the functional perspective, it is not useful to reduce the proton transfer barrier to be much lower than the value (~ 10 – 12 kcal/mol) expected based on pK_a considerations, much akin to the situation in many diffusion-controlled enzymes where there is no evolutionary pressure to reduce the barrier for the chemical step below 12 kcal/mol⁶⁷. Therefore, the real evolutionary pressure is for the protein to organize active site electrostatics such that the zinc-water has a pK_a near 7.0, which ensures the reversibility of the reaction that CAII helps catalyze. Once the pK_a for the zinc-water is in the proper range, proton transfer through *a relatively well solvated cavity* towards/from the protein surface (His64) does not require any major acceleration. This is not to say that structural details like the length of the water wire between the donor and acceptor groups do not play any role at all, although our computational results and the framework of analysis suggest that the significance of such “fine-tuning” is likely secondary to the modulation of pK_a of the zinc-bound water.

The finding that the proton transfer in CAII may proceed through a “proton hole” rather than the classical Grotthuss mechanism is an interesting proposal made possible by the flexibilities in our potential function and reaction coordinate. Although the weights of these two microscopically distinct mechanisms are difficult to pin down using currently existing computational methods, the most valuable contribution from the corresponding discussion is to help further highlight the importance of considering pK_a for the proton donor, acceptor, as well as the intervening water molecules for mechanistic analysis of long-range proton transfer. This is in fact not trivial for complex biomolecular proton pumps, which typically involve a large number of groups that adopt unusual titration states and the proton transfer pathways may involve hydrophobic segments. Therefore, we strongly echo the suggestion from Warshel and co-workers^{43,47} that any meaningful computational analysis of long-range proton transfers must start with pK_a analyses for the relevant groups, which not only help benchmark the computational model⁶⁸ but also reveal key interactions that likely modulate proton transfer energetics.

Ultimately, understanding proton transfer kinetics requires going beyond pK_a calculations to evaluate the relevant barriers. Along this line, the comparison between MEP and PMF results has clearly underlined the importance of carrying out proper sampling in studying long-range proton transfers. The case of CAII is perhaps somewhat special in that the transfer pathway is relatively well solvated, which is not universal in proton channels/pumps; for example, some segments of proton transfers in bacteriorhodopsin appear to involve a limited number of water molecules, and therefore MEP studies were found instructive^{37,38}. A useful way to judge the validity of MEP calculations is to compare results starting from configurations collected from MD simulations for the proton transfer reactant and product states; if the results differ by a great degree, as we found for CAII²¹, PMF simulations are required. When the active site is solvated and featured with many alternative hydrogen-bonding patterns, our preliminary EFG analysis indicates that even fairly local spectroscopic observables can be very sensitive to the configuration used in the calculations. Whether the spectroscopic results can be properly interpreted, therefore, may depend on the degree of sampling as well. Considering the increasing popularity of solid state NMR in the

study of metalloenzymes^{2,3}, the importance of sampling should be systematically studied using a broad set of examples.

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FIG. 1:

The active site of CAII rendered from the crystal structure (PDB ID: 2CBA¹⁴). All dotted lines correspond to hydrogen-bonding interactions with distances < 3.5 Å. Glu117 and Glu106 are in close proximity to His119, and Glu106 also interacts with Thr199 through the presumed hydroxyl proton of Thr199 (not shown for clarity). His64 is resolved to partially occupy both the “in” and “out” rotameric states; whether both states are productive toward proton transfer from the zinc-bound water is one of the mechanistic issues under debate¹ (see text).

FIG. 2:

The contribution to the electrostatic component of free energy of deprotonation for the zinc-bound water from protein atoms (only MM atoms are considered) in the WT and E106Q mutant CAII based on perturbative analysis²⁵. Contribution from individual residue is plotted against the distance (Ca) from the zinc ion. Note the striking similarity between the WT and E106Q results, except, apparently, for residue 106.

FIG. 3:

Snapshots and excess coordination number plots⁴⁰ (see text) for PMF and MEP simulations of proton transfers in CAII. (a) Transition state from a MEP spanning four bridging water molecules between the zinc-bound water and His64; (b) the excess coordination number plot for MEPs involving four bridging water molecules, which indicate the concerted nature of the transfer pathways; (c) a snapshot from PMF simulations ($\zeta \sim 0.7$ window) with the “out” configuration of His64, which clearly shows a hydroxide being stabilized by water molecules and polar residues (Asn67, Gln92) in the active site; (d) the excess coordination number plot for the “out”-His64 PMF simulations, which highlights a “proton hole” mechanism.

FIG. 4:

Calculated classical potentials of mean force (PMFs) for the proton transfer between the zinc-bound water and His 64 in the “in” and “out” configurations for the wild type CAII. The x -axis is a generalized coordinate (ζ) that characterizes the progress of the proton transfer based on a modified center of excess charge.^{26,40}

FIG. 5:

A schematics that illustrates the relative energetics of the Grotthus and "proton hole" transfer mechanisms, which involve step-wise transfer of hydronium and hydroxide ions, respectively. When pK_a differences between key groups are large, the basic energetic profile can be estimated based on the pK_a values of the proton donor/acceptor groups (AH, BH) and water (W) as well as hydronium (WH^+); the distance between the donor/acceptor is not an important factor for the proton transfer kinetics (assuming that the process is not controlled by the diffusion of the hydronium or hydroxide).

FIG. 6:

Simplified characteristic charge distribution in the transition state for the proton-hole and Grotthuss mechanisms in CAII. The former is featured with +2 for the zinc-center, +1 for His64H⁺ and -1 for the hydroxide; the Grotthuss mechanism is featured with +1 for the zinc-center, 0 for the neutral His64 and +1 for the hydronium. Accordingly, Φ values for residues in the active site are likely to have different patterns in the two mechanisms (estimated Φ values based on perturbative analysis³⁵ are shown for several residues for an illustration). Thus a systematic Φ analysis can provide experimental insights into the relative importance of the proton-hole and Grotthuss mechanisms in CAII.

TABLE I:

Calculated electric field gradient (EFG) principal component (q_{zz} in atomic unit) and nuclear quadrupole coupling constant (C_q in MHz) for the Zinc ion in CAII with different protonation states for the zinc-bound water^a

Snapshot	EFG (q_{zz})	H ₂ O		OH ⁻	
		C_q	EFG(q_{zz})	C_q	
1	-0.64	-22.7	-0.63	-22.2	
2	-0.88	-31.0	+0.58	+20.4	
3	-0.84	-29.6	-0.77	-27.0	
4	-0.66	-23.3	-0.68	-24.1	
5	-0.48	-16.8	+0.60	21.2	
6	-0.83	-29.1	-0.59	-20.9	
7	-0.65	-22.8	-0.69	-24.3	
8	-0.69	-24.4	-0.65	-23.1	
9	-0.84	-29.7	+0.74	26.1	
10	-0.66	-23.4	-0.68	-23.9	
Avg.		-25.3		-9.8	

^aFor each snapshot, minimization is carried out with a small QM region (zinc plus all ligands); in the subsequent EFG/ C_q calculations, a larger QM region that further includes Thr199, Thr200, Glu106 and all water molecules within 6 Å from the zinc is used. The rest MM atoms are included in both minimization and EFG/ C_q calculations. The method is B3LYP and 6-311G(d), which according to model calculations gives satisfactory results. The experimental value for C_q is about 10 MHz⁵; note that the sign of C_q is not available experimentally.