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Is a "Proton Wire" Concerted or Stepwise? A Model Study of Proton Transfer in Carbonic Anhydrase

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The energetics of proton transfer reactions in carbonic anhydrase (CA) have been studied with an active site model. Specifically, proton transfer from a zinc-bound water molecule to a histidine residue mediated by a numbers of water molecules was investigated. With two or three bridging water molecules, the proton transfers are fully or nearly fully concerted and only one saddle point exists. With an additional water molecule that forms a ring bridge, an intermediate is formed in which one of the water molecules exists as a hydronium ion. In contrast to previous calculations in which either a low-level of theory was employed or a stepwise mechanism was assumed, the energetics obtained from the current work are approximately consistent with the experimental estimates. In all of the scenarios, the motion of more than one proton is involved in the transition state, which is in agreement with the experimental observation that the reaction rates in H_2O/D_2O mixture have an exponential dependence on the fraction of D_2O in the solvent. For three (W3) or four waters (W4), the proton transfer to the "His 64" model is hardly involved in the transition state, suggesting that the orientation of the proton acceptor is less important than for only two waters (W2). Thus, the W3 and W4 results are consistent with the experimental observation that many kinetic properties of the H64A mutant of CA in well-buffered imidazole solution are similar to the wild type. The barrier height increases, and the barrier frequency (and therefore, the contribution of tunneling) decreases as the number of bridging water molecules increases. Overall, these investigations demonstrate that the proton transfer reaction in CA is sensitive to the nature and structure of the water bridge, which would be influenced by the dynamics of the water molecules and amino acids in the active site of the protein.

I. Introduction

Long-range proton transfer reactions[1] occur in many biological systems. Well-established examples include bacteriorhodopsin,[2] cytochrome *c* oxidase,[3] adenosine 5′-triphosphate (ATP) synthase,[4] and the photosynthetic reaction center.[5] Other interesting systems involving proton transfers are the enzymes carbonic anhydrase (CA),[6] alcohol dehydrogenase,[7] and the transmembrane channel formed by gramicidin (GA).[8] Understanding the detailed mechanism(s) for such long-range proton transfer processes and determining how they are modulated by the protein structure and dynamics are of fundamental, as well as of practical,[9] interest. A popular proposal for the mechanism postulates the existence of proton wires, in which the proton is transported along a chain of water molecules.[10−13] More complicated mechanisms, which include certain protein residues, as well as water molecules, have also been suggested.[14] For example, Warshel and co-workers[14] have done model calculations for pathways involving both water molecules and ionizable protein residues for the photosynthetic reaction center, in accord with the experimental studies of Feher et al.[15] Another issue is whether the transfer, which may involve several sites, proceeds through a concerted or stepwise mechanism. In liver alcohol dehydrogenase, the proton transfer from the enzyme-bound substrate to the bulk solvent through a number of protein residues and a hydroxyl group in the cofactor was found to follow a stepwise mechanism at the PM3 level.[16] By contrast, with an approximate density functional treatment at the self-consistent charge tight binding (SCC-DFTB) level,[17] we found that the proton transfers proceed in an essentially concerted fashion.[18] This highlights the importance of the level of electronic structure calculations in the study of long-range proton transfer reactions. Although kinetic isotope effect measurements can provide insights into the structure of transition states for short-range transfers,[19] it is more difficult to interpret isotope effects for long-range proton transfers due to the large number of atoms involved.[20] In most previous studies involving proteins, the potential energy was described with relatively simple empirical forms such as the empirical valence bond (EVB) model.[14] Calculations with more accurate treatment of the quantitative changes are of interest since, as already mentioned, the resulting mechanism may be sensitive to the quality of the potential energy surface. In this paper, we focus on the water chain mechanism in CA and use a potential energy surface determined by density functional theory (DFT).

CA is an enzyme in which a fairly long-range proton transfer (over a distance of 8 Å) is believed to play an important role. The α class CAs are monomeric zinc-containing proteins with a molecular mass around 30 kDa and are involved in a number of physiological processes, such as respiration and formation

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of secretory fluids.[6] CA catalyzes the interconversion of CO_2 and HCO_3^-.[21] A large number of experimental[21,22] and theoretical[23] analyses suggest that the reaction proceeds in two stages. In the hydration direction (i.e., $OH^- + CO_2 \rightarrow HCO_3^-$), which is the normal direction, the first stage involves the reaction of CO_2 with the zinc-bound hydroxyl ion to form the product HCO_3^-; the HCO_3^- then dissociates from the active site, and the available coordination site of the zinc ion is occupied by a water molecule. In the second stage, a proton transfers from the zinc-bound water molecule to the bulk through a histidine residue (His 64) and, presumably, a number of active site water molecules. This process regenerates the active species, i.e., the zinc-bound hydroxyl ion. The mechanism has been generally accepted, although certain issues such as the kinetics of the substrate binding/dissociation[24] and details of the proton transfer[25] dynamics and kinetic isotope effects[26] are still under investigation. Two types of proton transfer reactions appear to be involved. The intramolecular proton transfer involves the zinc-bound water molecule and a histidine residue on the surface of the protein (His 64 in CA II); the intermolecular proton transfer occurs between the histidine residue and the bulk solvent. Solvent kinetic isotope effects suggest that the intramolecular proton transfer is rate-limiting in solution at pH $\sim$7.[22,27] The proposal that His 64 is the proton acceptor[21d] is supported by the observation that the H64A mutation decreased the catalytic rate by 20-fold[28] and the fact that kinetic properties of the mutant are similar in well-buffered imidazole solution.[29,38] On the basis of solvent kinetic isotope effects and the observation that the acceptor His 64 is rather far from the active site zinc, it was proposed that a number of water molecules function as a bridge or water wire for the proton transfer.[30] The measured rate constant for the proton transfer in the wild-type CA is on the order of 10^6 s^{-1} (the activation free energy for proton transfer in solution is estimated to be 2.4 kcal/mol from NMR relaxation measurement,[31] which corresponds to a rate on the order of 10 s^{-1}), and a substantial effort has been made to understand the correlation between the proton transfer rate and the pK_a difference between the proton donor and the acceptor in the framework of Marcus theory extended to proton transfer reactions.[25a,32] It was found that the kinetic data for a series of mutants under different buffer conditions can be fitted by Marcus theory with a very small intrinsic barrier of 1−2 kcal/mol and a large reorganization term (about 10 kcal/mol) for bringing the solvent and protein side chains into the right conformation for proton transfer.[25a] The precise origin of this reorganization term is not completely clear, although it was suggested that it involves either orienting the bridging water molecules in the active site or a flip of the His64 side chain from an outward to inward orientation.[25a] Molecular dynamics (MD) simulation by Lu et al.[33] indicated that the free energy barrier associated with the water bridge formation is only 2−3 kcal/mol. An alternative proposal introduced an additional intermediate state, which leads to a three state Marcus model;[34] such a model was able to fit the experimental data without the large work term.[25a,34] The energetics associated with the proton transfers in CA has been analyzed theoretically by a number of authors,[25b,34−36] only the recent works of Lu et al.[25b] and Isaev et al.[36] used fairly high levels of theory. It was found[25b] that water molecules assist proton transfer from a model histidine (or a zinc-bound water) to a water molecule by stabilizing the product and thereby lowering the activation barrier. The donor−acceptor distance was found to be critical in determining the barrier height, as pointed out by many authors.[37] The ligand of the zinc was also found to be important; if the Zn−His distances were freely optimized, the barrier for the proton transfer from the zinc-bound water to the next water molecule is more than 20 kcal/mol, while it drops to below 10 kcal/mol if the Zn−N(His) distances are fixed to be 2.20 Å.[25b] However, the proton transfers were assumed to follow a stepwise mechanism in that study, and the present work found that there is no need to stretch the Zn−His distances to obtain sufficiently low barriers. In ref 36, the possibility that the proton transfers occur through a concerted mechanism was considered, and it was found that the stepwise transfers are much more energetically unfavorable and the transition state involves concerted motion of several protons. It was also found that the barrier did not increase much when the length of the water bridge was increased, while the barrier became substantially higher when water molecules were added to the periphery of the bridge because these water molecules tended to localize the transferring proton(s).

Despite the available studies, the detailed character of the proton transfer pathway is not yet clear. For example, no transition state searches were performed in ref 25b; therefore, it is not clear whether the proton transfer assisted by the water chain is stepwise or concerted and if there is a hydronium intermediate.[34] The concerted mechanism was supported by ref 36, but the histidine residues were modeled by NH_3 groups, and all geometry optimizations were performed at a fairly low level of HF/6-31G. Kinetic measurements[38] for the H64A mutant in well-buffered imidazole solution found that many kinetic properties related to the intramolecular proton transfer in the mutant are very similar to those of the wild type; these include the maximum initial velocity, solvent hydrogen isotope effects on the maximal velocity, and the dependence of these isotope effects on the atom fraction of deuterium in the solvent. These observations suggest that the proton transfer is not likely to be controlled by the position and orientation of the proton acceptor (H64 in the wild type and solution imidazole in the mutant). The results were rationalized by assuming that the rate-limiting step is the initial proton transfer from the zinc-bound water to the first bridging water, leading to a hydronium intermediate. This was supported by the model study of Lipscomb and co-worker,[39] who used ammonia to model the histidines at the semiempirical (PRDDO) level. They found that the role of the accepting group ("His 64") is to lower the subsequent proton transfer barriers. The same stepwise mechanism was assumed in the simulation of Warshel et al.[34] based on an EVB parametrization for the proton transfer step. In addition to the approximations in these models, the mechanism does not seem to be fully consistent with the kinetic isotope measurements, which used mixed H_2O and D_2O solvents.[27,30] The result that there is an exponential dependence of the maximum velocity on the D_2O fraction suggests that more than one proton is involved in the transition state of the rate-limiting step. It seems worthwhile, therefore, to investigate the detailed mechanism of the intramolecular proton transfer (i.e., that from the Zn-bound water to His 64) with more a realistic model and level of calculations higher than those employed in the earlier work.[25b,34,36,39]

In the current work, we carry out DFT calculations to investigate the proton transfer reactions in CA with a relatively simple active site model. We were able to determine the saddle points relevant to the proton transfers with different numbers of water molecules involved in the bridge. Interestingly, the mechanism of proton transfer depends rather sensitively on the structure of the water bridge. The current study forms the basis for future work including the entire protein environment in a combined QM/MM framework.

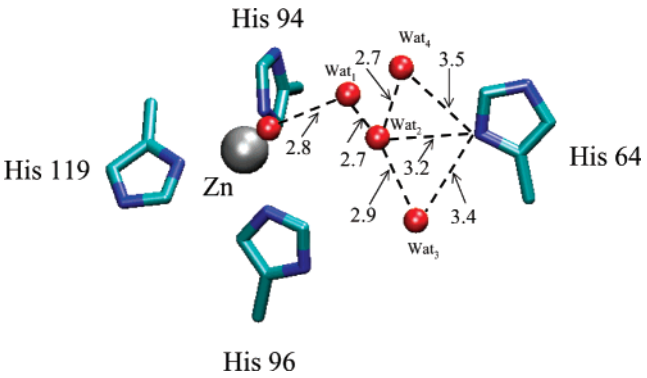

Figure 1. Active site of human CA II (PDB code 2CBA), which shows the three His residues (94, 96, 119) that are bound to the zinc ion and the positions of water molecules bridging the zinc ion and His 64. The structures of reactants in the current models are based on the positions shown (see Methods). The distances are in angstroms.

In Section II, the details of the computational method, including the composition of the active site model, are described. The results are presented in Section III, and the conclusions are summarized in Section IV.

II. Computational Methods

The active site model consists of the zinc ion, the side chains of its three histidine ligands (His 94, 96, and 119), the zinc-bound water (proton donor in the hydration direction), and the proton acceptor, His 64 (e.g., see Figure 1, which shows the X-ray structure with four bridging water molecules). The initial positions of the nonhydrogen atoms were taken from the X-ray structure of CA II (PDB code 2CBA[40]) at 1.54 Å resolution. The "inward" conformer of H64 observed in the X-ray structure, which is appropriate for the proton transfer reaction considered here, was adopted. To maintain the structural similarity to the protein environment in the crystal structure, the positions of the Cβ atoms were fixed in space during the geometry optimization. Several bridging water molecules, ranging from two to four (referred to in the following as W2, W3, and W4, respectively), were included. The initial positions for the bridging water molecules in the W4 cases were taken from the X-ray structure; the starting structure of W3 was obtained by deleting the bridging water molecule W_4 (see Figure 1). For the W2 case, the positions of the bridging water molecules were obtained from a short MD run with a protocol similar to that used in the previous study of the vibrational relaxation of zinc-bound azide in CA;[41] i.e., a stochastic boundary setup[42] for the solvated enzyme was employed.

The entire model system was treated by quantum mechanics. The geometries of stable structures and saddle points were fully optimized at the B3LYP[43]/6-31G(d)[44] level; the energetics were determined at a higher level of B3LYP/6-311+G(d,p).[44] As shown by the test calculations in the Supporting Information, which included comparisons between B3LYP and MP2 for multiple proton transfers in GUA·$(H_2O)_2$ and proton affinity of Zn-bound water molecules, the present B3LYP level of calculation is likely to be sufficient for the proton transfer reactions that we are considering here. Normal mode analysis was carried out for the saddle points to get a qualitative measure of the importance of proton tunneling. All of the calculations were performed with Gaussian98.[45] The effect of zero-point energy (ZPE) for all bound vibrations was included in the energetics based on the B3LYP/6-31G(d) vibrational calculations.

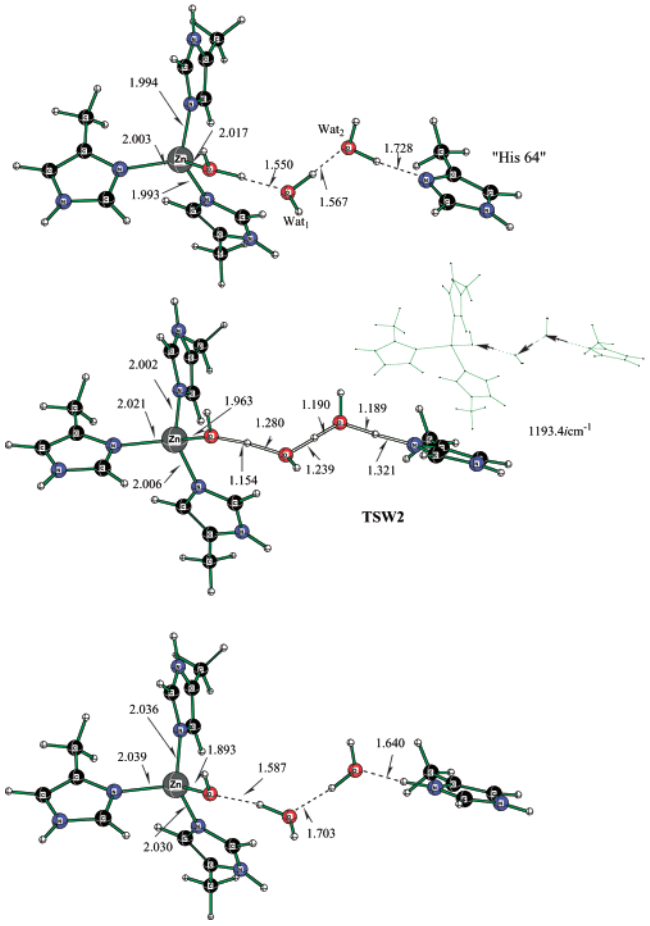

Figure 2. Optimized structures and imaginary mode of the saddle point for the proton transfers in the active site model of CA II with two bridging water molecules at the B3LYP/6-31G(d) level. The distances are in angstroms. Also shown is the imaginary mode at the saddle point.

III. Results

In this section, the results for the three water bridge models (W2, W3, and W4) are presented.

III.1. Two Bridging Waters (W2)—Fully Concerted Proton Transfer. With two bridging water molecules, only one saddle point, which is highly concerted in nature, was located; that is, all three protons are transferred simultaneously (Figure 2). At the saddle point, the donor−proton distances are 1.154, 1.239, and 1.189 Å, and the corresponding proton−acceptor distances are 1.280, 1.190, and 1.321 Å, respectively. Thus, the saddle point has the three transferring protons asymmetrically positioned with the first and third closer to the donor and the second closer to the acceptor, so that the second water is hydronium-like. The eigenvector corresponding to the imaginary mode at the saddle point indicates that the three protons are involved to a similar extent in the reaction coordinate at the saddle point (see Figure 2).

The barrier for the concerted proton transfer is calculated to be 5.9 kcal/mol without ZPE, but it is reduced to 0.6 kcal/mol when ZPE is included. The value is substantially lower than the value of 7.8 kcal/mol from kinetic measurement and about 8−10 kcal/mol estimated from the consideration of pK_a.[25a] It should be noted that the Zn−N_{His} distances of the three His bound to the zinc are optimized freely to be about 2.0 Å in the current work. Therefore, there is no need to elongate the Zn−N_{His} distances to obtain a low proton transfer barrier, as suggested by Voth and co-workers in ref 25b; they found at the level of MP2/4-31G*//HF/3-21G that the first proton transfer

was endothermic by as much as 20 kcal/mol if the $Zn-N_{His}$ distance was optimized and that lengthening the $Zn-H_{His}$ distance stabilized the proton transfer considerably. The difference is likely to be due to the fact that a stepwise proton transfer mechanism was assumed in ref 25b and only the first step (Zn-bound water to the first bridging water) was considered in that study. However, it should be noted that two water molecules hydrogen-bonded to the proton acceptor were included in their model; these two extra water molecules were not between the donor and the acceptor. The exothermicity of the reaction from the present calculations, 4.4 kcal/mol with ZPE, is in reasonable agreement with experimental estimates. The reaction is thought to be nearly thermoneutral in the protein based on the similar pK_a values of the zinc-bound water (estimated to be around 6[32]) and His 64 (p$K_a \sim 7$[46]).

The imaginary barrier frequency is calculated to be 1193 *i*cm^{-1}, which is typical for simple proton transfer reactions; for example, a similar frequency was found in our previous study of an intramolecular proton transfer reaction in triosephosphate isomerase.[47] It is interesting that even though several protons are involved, the barrier remains narrow. Given such a narrow barrier, proton tunneling is expected to make a substantial contribution to the proton transfer rates. An estimate based on an one-dimensional (1D) truncated parabola model[48] gives a tunneling coefficient of 6.3 at 300 K; a more detailed analysis of the tunneling contribution will be reported separately.[49]

III.2. Three Bridging Waters (W3)—Partially Concerted Proton Transfer. With three bridging water molecules, only one saddle point was obtained for the proton transfers, which is quite remarkable considering that four protons are being transferred in the reaction. The transition state is partially concerted (Figure 3). The two middle transferring protons are shared approximately equally between the donor and the acceptor atoms; the relevant donor—proton, proton—acceptor distances are 1.281, 1.147 Å and 1.153, 1.286 Å, respectively. The first proton (from the zinc-bound water) has nearly completed the transfer, and the donor—proton (proton—acceptor) distances are 1.396 (1.079 Å). The last proton is still far from the acceptor nitrogen atom of His 64, and the donor—proton, proton—acceptor distances are 1.068 and 1.525 Å, respectively. No intermediate was found, although the central water molecule is hydronium ionlike; that is, it has three proton distances around 1.15 Å. To confirm the absence of an intermediate, energy minimization starting from an intermediate structure with a hydronium ion in the middle (obtained by deleting the water not directly involved in the proton transfer in the intermediate, which occurs in the W4 model, see Figure 5 and III.3) led directly back to the reactant. Minimization after following the barrier eigenvector beyond the saddle point led directly to the product. We conclude, therefore, that there is no low-energy stepwise path with an intermediate for W3.

The barrier for proton transfer in the W3 case is 8.6 kcal/mol without ZPE and 3.6 kcal/mol with ZPE, significantly higher than that in the W2 case (0.6 kcal/mol with ZPE). The exothermicity of the reaction is 3.9 kcal/mol with ZPE, very similar to the value of 4.4 kcal/mol in the W2 reaction. Therefore, it appears that given that the exothermicity is similar, the "intrinsic proton transfer barrier" becomes higher when the number of bridging water molecules increases from two to three. This is consistent with the observation that W2 has a somewhat larger distance from W1 in the reactant configuration for the W3 case (1.659 Å, Figure 3), as compared to the water—water distances in W2 (~1.55—1.57 Å). Because the positions of all of the water molecules were fully optimized, the longer distance

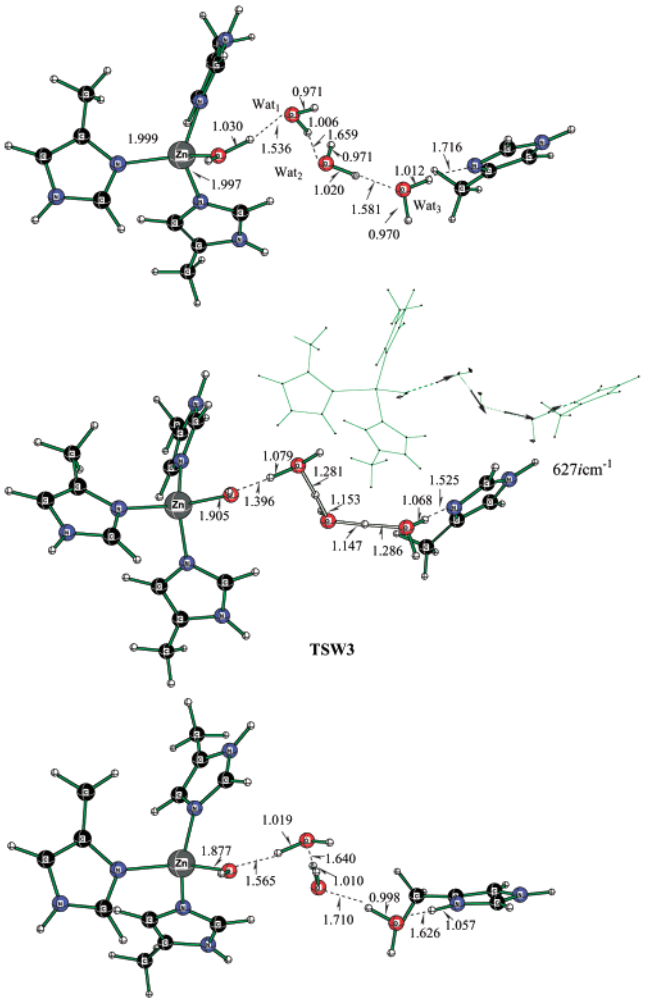

Figure 3. Optimized structures and imaginary mode of the saddle point for the proton transfers in the active site model of CA II with three bridging water molecules at the B3LYP/6-31G(d) level. The distances are in angstroms. Also shown is the imaginary mode at the saddle point.

found in W3 is likely to be a consequence of the structural arrangement of the proton donor and acceptor groups.

Interestingly, the imaginary barrier frequency in the W3 case is substantially lower than that in the W2 case, it is 627 vs 1193 *i*cm^{-1} for W2. This is likely to be due to the fact that the saddle point structure for W3 is less concerted. For such a barrier, a much smaller tunneling contribution to the proton transfer rate is expected; the 1D model gave a tunneling coefficient of 1.5 for W3, substantially smaller than the value of 6.3 for W2.

III.3. Four Bridging Waters (W4)—Stepwise Proton Transfer with a Hydronium Intermediate. When four water molecules are present, and they form a ringlike structure as observed in the X-ray (see Figure 1), the shape of the potential energy surface for proton transfer changes substantially. An intermediate (referred to as INTW4 in Figure 4) is formed after two protons have been transferred, with a water molecule in the middle having the hydronium ion structure. Apparently, the hydronium ion is stabilized sufficiently by the three water molecules through hydrogen-bonding interactions; this corresponds to the nearest-neighbor environment formed in aqueous solution. The smaller number of hydrogen-bonding interactions possible in the case of W2 and W3 is not sufficient to stabilize the hydronium ion, although, as mentioned above, a hydronium ionlike structure appears in the transition state.

The initial proton transfers from the reactant structure to INTW4 are nearly concerted, as represented by the saddle point,

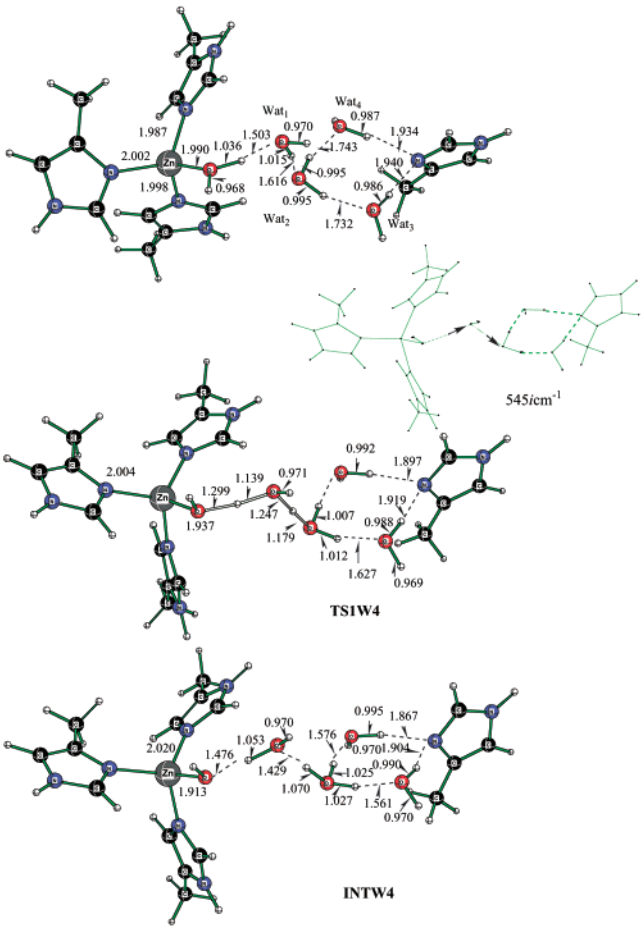

Figure 4. Optimized structures and imaginary mode of the saddle point for the initial proton transfers in the active site model of CA II with four bridging water molecules at the B3LYP/6-31G(d) level. The distances are in angstroms. Also shown is the imaginary mode at the saddle point.

TS1W4. The donor−proton (acceptor−proton) distances are 1.299 (1.139 Å) and 1.247 Å (1.179 Å), respectively. Because two water molecules are close to the Nϵ of His 64 in INTW4 (the distances are 1.867 and 1.904 Å, respectively), the subsequent proton transfer from INT4W to the product can proceed in two ways, when a proton from the hydronium ion gets transferred to the water molecule that transfers its proton to His 64. Both transition states are partially concerted, with one proton shared approximately equally between the two water molecules and another proton closer to the water oxygen atom (∼1.1 Å) than to the nitrogen atom (∼1.4 Å) of His64 (see Figure 5a,b).

As to the energetics, the intermediate (INTW4) is 7.5 kcal/mol above the reactant without ZPE and 6.6 kcal/mol when ZPE is included. The initial saddle point is 7.6 kcal/mol above the reactant without ZPE, so that the intermediate lies in a very shallow potential well. The two subsequent saddle points are 9.2 and 8.1 kcal/mol above the reactant, respectively, without ZPE; they are 5.9 and 4.3 kcal/mol above the reactant when ZPE is included. We note that the effect of ZPE on the barrier heights is smaller in W4 than in the W2 and W3 cases, because the number of protons being transferred at the transition state is smaller in W4 (two protons as compared to three or four protons in W2 and W3, respectively). With ZPE included, the highest energy stationary structure in W4 is INTW4, which is 6.6 kcal/mol above the reactant. This is higher than the barriers in W2 and W3 (Figure 6). The product is also slightly higher in energy than the reactant in W4, which is in contrast with the results found for W2 and W3; in the latter two cases, the product is lower in energy than the reactant by about 4 kcal/mol. This is because W_4 in W4 forms a hydrogen bond network with both "His 64" and the other water molecules in the reactant structure (see Figure 4). This network is broken in the product and the two transition states for the second set of proton transfers (TS2W4 and TS2W4′, see Figure 5) because the Nϵ atom in His 64 is more "dedicated" to the water molecule providing the proton.

The barrier frequencies at the saddle points are all very low, about 500 $i\text{cm}^{-1}$; this trend is consistent with the fact that the barrier heights measured from INTW4 are very small, indicating that the potential energy surface is quite flat in this region. Therefore, quantum mechanical tunneling is not expected to be important for the proton transfer in W4.

IV. Concluding Discussions

Long-range proton transfer reactions participate in many important processes in biology. Often, such reactions involve several water molecules and/or protein residues. One important question that arises in such cases is whether the proton transfer is stepwise or concerted; i.e., whether there are intermediates in the reaction or whether there is a single transition state. This aspect is expected to be sensitive to the number and type of species involved and to the structure of the transfer chain. As an approach to this problem, we have investigated a simple active site model for proton transfer in CA. The identity of the proton donor (Zn-bound water), acceptor (His 64) and the fact that bridging water molecules in the active site play an important role are known from variety of mutagenesis[26,28,32] and kinetic measurements,[21,22,25,27,30,32] as well as from theoretical calculations.[23,24,33−35,39] The detailed characteristics of the proton transfer, however, remain unclear, particularly as to the number of water molecules involved and the concertedness of the reaction. This is due in part to the fact that it is difficult to determine them experimentally and that either only relatively low levels of theory (such as PRDDO, PM3, or EVB) or a simplified description of the proton transfer mechanism (e.g., assumed stepwise behavior)[25b] have been employed in the previous calculations.[23,34,39] In the current work, the stable structures and saddle points of relevance have been determined with geometry optimization at the level of B3LYP/6-31G(d); energetics were obtained from these structures with single point calculations at the B3LYP/6-311+G(d,p) level. According to test calculations (see Supporting Information), this combination of quantum mechanical levels is expected to give reliable structural and energetic results for the present system. Although it will be necessary to include the full protein environment to obtain definitive results for CA, the present analysis provides useful insights concerning possible mechanisms.

The energetics of proton transfer reactions in the active site model for CA with different number of bridging water molecules are summarized in Figure 6. It can be seen that the details depend sensitively on the nature of the water bridge. As the number of water molecules in the bridge increases from two to four, the proton transfer changes from being fully concerted (W2), through partially concerted (W3) to stepwise (W4). An intermediate involving a hydronium ion exists on the potential energy surface (i.e., without ZPE) in the case of W4, because there are enough water molecules to stabilize the ion. Hydronium in a water environment has been the subject of several high-level studies with techniques such as multistate effective valence bond[12a−c] or DFT.[12d,e] Two species, $H_5O_2^+$ (Zundel) and $H_9O_4^+$

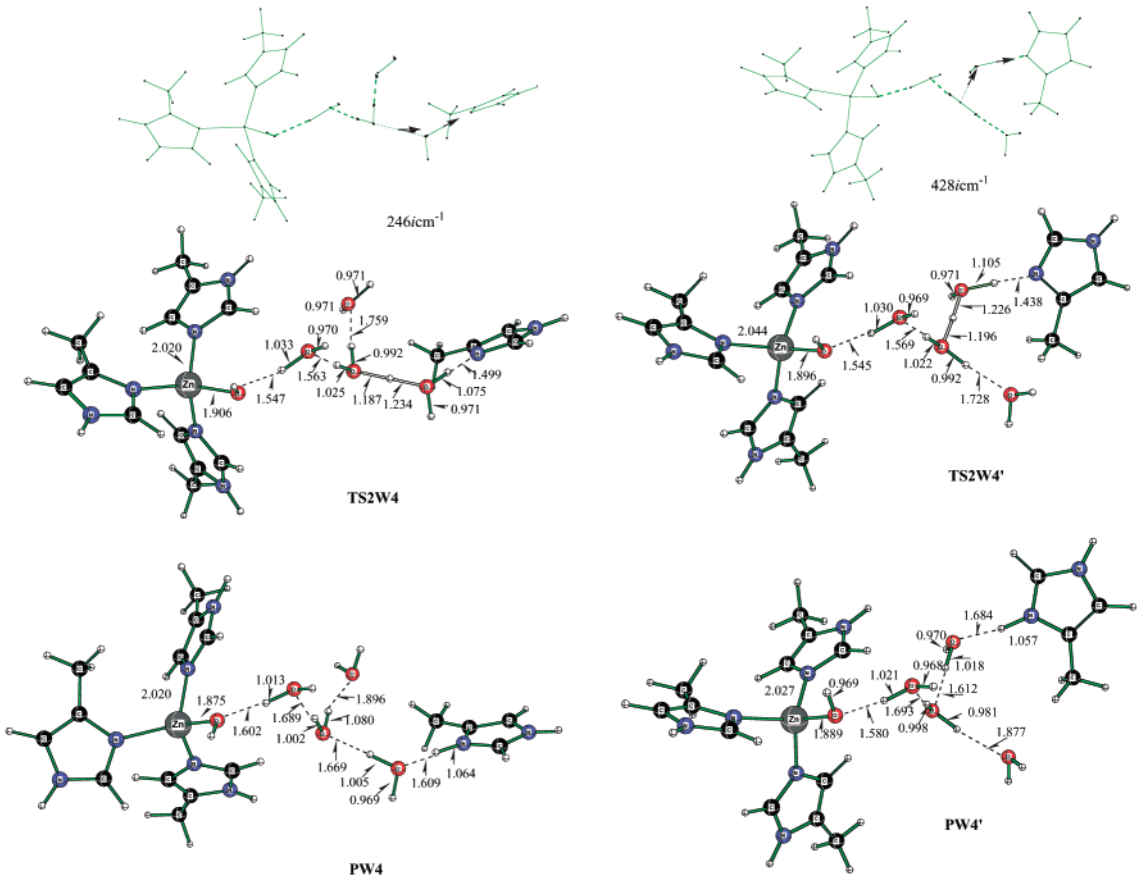

Figure 5. Optimized structures and imaginary mode of the saddle point for the final proton transfers in the active site model of CA II with four bridging water molecules at the B3LYP/6-31G(d) level. Two pathways (a and b) are considered in which different protons in the hydronium ion in INTW4 are transferred. The distances are in angstroms. Also shown is the imaginary mode at the saddle point.

(Eigen), have been proposed as the essential entity, with the latter being more stable relative to a solvated Zundel moiety. Computer simulations also found that quantization of the nuclear degrees of freedom increases the stability of the Zundel cation relative to the Eigen cation, although the latter remains the more stable species.[12a] We note that the four bridging water molecules and the proton in INTW4 in fact correspond to the Eigen model of $H_9O_4^+$ (see Figure 4). In contrast to previous studies at lower quantum levels of calculations,[25b,34,39] the saddle points in all of the cases studied here involve more than one proton moving along the reaction coordinate. They are all in qualitative accord with the hypothesis based on the observation that the reaction rate constant in H_2O/D_2O mixture has an exponential dependence on the fraction of D_2O in the solvent;[27,30] with multiple proton movements in the transition state, a larger fraction of deuterium substitution will influence the rate by a factor that depends exponentially on the ZPE difference caused by the deuterium substitution (assuming that the tunneling contribution is relatively small, as discussed above). From the structures of the saddle points, the barrier for W2 would depend rather sensitively on the orientation of the His 64 residue due to the highly concerted nature of TSW2. In the cases of W3 and W4, by contrast, one would expect that the barrier is less sensitive to the orientation of His 64, because the proton transfer to His 64 has not yet occurred in TSW3 and TS1W4; e.g., for TS1W4, the $O^4\cdots H^4$ and $H^4\cdots N_{His64}$ distances are 1.068 and 1.525 Å, respectively. Correspondingly, the scenario found for W3 and W4 is more consistent with the experimental behavior for CA. It has been observed[38] that many kinetic properties of the H64A mutant in the presence of well-buffered imidazole solution are very similar to the wild type, which suggests that the intramolecular proton transfer is not sensitive to the orientation of the proton acceptor and that the rate-limiting step is associated with the initial proton transfer(s) (see Introduction). However, this conclusion must be considered as tentative because the protein environment has not been included in the current study.

The barrier height for the proton transfers increases as the number of bridging water increases. It is 0.6, 3.6, and ~6 kcal/mol for W2, W3, and W4, respectively, including ZPE. All of those values, however, are lower than the barrier estimated from kinetic measurements or pK_a considerations, which give a value of 8~10 kcal/mol. We note that the values for W3 and W4 are on the order of the experimental estimate. The low barrier found for the W2 case is similar to the intrinsic barrier found in the recent experimental studies of the proton transfer between H64A CA II and derivatives of imidazole and pyridine in solution.[50] Moreover, Lu and Voth[33] found in their classical MD simulation that a value of 2−3 kcal/mol should be added for the organization of the water bridge. This is one aspect of the possible contributions of the protein environment, which was not included in the current analysis. In contrast to the present results, most previous calculations gave barriers that are significantly too high; they are on the order of 20 kcal/mol or higher (e.g., if the Zn−N distances are freely optimized as in ref 22b; see the discussions above). This appears to be due to the use of a low level of quantum mechanical methods (PRRDO[39] or AM1[23]) or to the fact that the stepwise mechanism was assumed.[25b] A notable exception is the value of about 10 kcal/mol obtained at the EVB level by Åqvist et al.,[34] even with

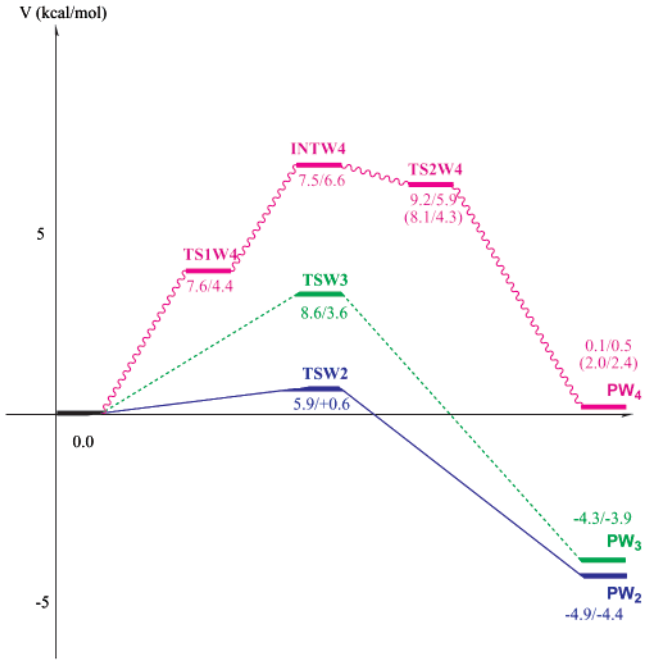

Figure 6. Comparison of potential energy profiles for the proton transfers in the active site model of CA II with a different number of bridging water molecules. The single point energies were calculated at the B3LYP/6-311+G(d,p) level at the B3LYP/6-31G(d) geometries. The values before slash are relative potential energies, and the values after slash include ZPE corrections. For the W4 case, the two sets of values (with and without parentheses) correspond to the two pathways in Figure 5.

the assumption that the proton transfer occurs in a stepwise fashion (they only studied the proton transfer from the zinc-bound water to the next water molecule); although the result might be attributed to the particular EVB parametrization, a more meaningful comparison will be possible only when the enzyme environment and MD calculations are included for the concerted multiple proton transfer pathways. As the current work was essentially complete, an interesting study by Isaev et al.[36] appeared, in which the dependence of barrier height on the water bridge structure was studied with a simpler model (e.g., His residues were replaced by NH_3) at a lower level of description for the geometry (HF/6-31G). As in the current work, it was found that the barrier for proton transfer is very low (nearly zero when ZPE is included) and that "periphery" water molecules can increase the barrier height because these water molecules tend to localize the transferring proton(s).

Because the motion of many protons is involved in the saddle points, a detailed analysis of tunneling is complicated. Nevertheless, the results obtained here suggest that as the character of transition state varies, the barrier frequency changes substantially; it is 1193, 627, and about 500 $i\text{cm}^{-1}$ for W2, W3, and W4, respectively. Therefore, the contribution of quantum mechanical tunneling would be substantial only for W2, and negligible for W3 and W4, due to the sensitivity of tunneling to the width of the barrier (which is partially reflected in the magnitude of the imaginary frequency). A more detailed study is required to reproduce the observed solvent kinetic isotope effect by calculations, especially the variation of KIE as a function of the number of deuterium substitutions in the water bridge.[49] The results for W4 also indicate that the structure for the transition state including quantum mechanical effects could be different from those obtained here; i.e., including the zero-point motions of the protons might make the transition state more concerted such that the proton transfers proceed without an intermediate like INTW4. The possibility could be examined based on a reaction path approach including nuclear quantum effects in the centroid path−integral formalism.[51]

The most important result of the present analysis is that the proton transfer mechanism in this case, and probably in other systems, depends sensitively on the solvent structure of the active site of CA. According to a previous MD simulation,[23c] the number of water molecules involved in the bridge between the Zn-bound water/hydroxide and the His 64 varied from two to six. Mutation experiments on CA III found that k_{cat} correlated with the hydrophobicity of the active site (e.g., mutation at the site of Leu 198[52]), which also points to the importance of active site solvent structure in the proton transfer.[26,40] Furthermore, active site polar residues such as Thr 199 may form hydrogen bonds with the bridging water molecules and therefore can affect the bridge structure and the proton transfer energetics. The fluctuating electric field at the active site due to the protein and solvent environment could also affect the results, as found in the model studies on proton transfer along water wires, in which the protein environment was modeled by a fluctuating electric field.[13] Such a scenario can be compared to autoionization in liquid water,[53] for which the collective fluctuation of the water environment and the hydrogen bond network were found to be essential for permanent charge separation.[53] Although in CA, the charge separation is stabilized by the zinc ion at one end and the histidine residue at the other, the role of the protein and solvent fluctuations (and the resulting electric field) at the active site may still be important.

The current work forms the basis for future studies on proton transfer reactions in the enzyme environment. With the recent development of SCC-DFTB[17] parameters for zinc,[41] which were shown to be able to describe the proton transfers in the active site model of CA at modest computational cost, it is possible to study the detailed mechanism (such as the effect of multidimensional tunneling on kinetic isotope effects[49]) with a combined SCC-DFTB/MM approach. An interesting question raised by one referee is why CA did not evolve to minimize the number of water molecules in the water bridge, given the current finding that the proton transfer barrier seems to increase with the number of peripheral water molecules. The barrier seems to be sufficiently low even with four water molecules (e.g., when the external proton buffer is low in concentration, the rate-limiting step of the entire catalytic cycle is the exchange of proton between the His 64 and the bulk solution[26]), so that there is no evolutionary pressure to further decrease the number of water molecules in the active site. A more definite answer would require additional computational studies, which include the protein environment and consider other steps in the overall reaction.

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Supporting Information Available: Test calculations on a double proton transfer reaction in Gua·$(H_2O)_2$ and proton affinity of Zn-bound water molecule at different quantum mechanical levels. This material is available free of charge via the Internet at http://pubs.acs.org.

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