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A R T I C L E S

Kinetic Isotope Effects for Concerted Multiple Proton Transfer: A Direct Dynamics Study of an Active-Site Model of Carbonic Anhydrase II

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Abstract: The rate constant of the reaction catalyzed by the enzyme carbonic anhydrase II, which removes carbon dioxide from body fluids, is calculated for a model of the active site. The rate-determining step is proton transfer from a zinc-bound water molecule to a histidine residue via a bridge of two or more water molecules. The structure of the active site is known from X-ray studies except for the number and location of the water molecules. Model calculations are reported for a system of 58 atoms including a four-coordinated zinc ion connected to a methylimidazole molecule by a chain of two waters, constrained to reproduce the size of the active site. The structure and vibrational force field are calculated by an approximate density functional treatment of the proton-transfer step at the Self-Consistent-Charge Density Functional Tight Binding (SCC-DFTB) level. A single transition state is found indicating concerted triple proton transfer. Direct-dynamics calculations for proton and deuteron transfer and combinations thereof, based on the Approximate Instanton Method and on Variational Transition State Theory with Tunneling Corrections, are in fair agreement and yield rates that are considerably higher and kinetic isotope effects (KIEs) that are somewhat higher than experiment. Classical rate constants obtained from Transition State Theory are smaller than the quantum values but the corresponding KIEs are five times larger. For multiple proton transfer along water bridges classical KIEs are shown to be generally larger than quantum KIEs, which invalidates the standard method to distinguish tunneling and over-barrier transfer. In the present case, a three-way comparison of classical and quantum results with the observed data is necessary to conclude that proton transfer along the bridge proceeds by tunneling. The results suggest that the two-water bridge is present in low concentrations but makes a substantial contribution to proton transport because of its high efficiency. Bridging structures containing more water molecules may have lower energies but are expected to be less efficient. The observed exponential dependence of the KIEs on the deuterium concentration in H_2O/D_2O mixtures implies concerted transfer and thus rules out substantial contributions from structures that lead to stepwise transfer via solvated hydronium ions, which presumably dominate proton transfer in less efficient carbonic anhydrase isozymes.

I. Introduction

Medium- and long-range proton transport forms part of many biological processes.[1] It is well recognized that water molecules embedded permanently or transiently in proteins contribute to this transport and may actually provide the dominant pathway.

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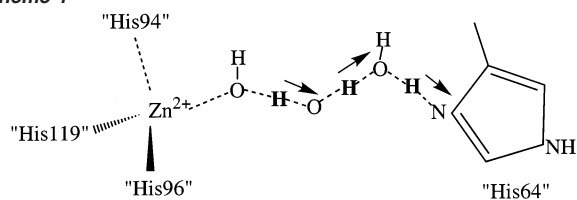† Steacie Institute for Molecular Sciences, National Research Council of Canada.
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In general, however, it has proved difficult to locate these water molecules by the presently available experimental techniques, so that there remains considerable uncertainty about the proton transport mechanism. Although quantum-chemical and molecular mechanics calculations can help locating water wires and their connection to peptide chains, to establish the transport mechanism it will be necessary to consider the facts that protons tend to move by quantum-mechanical tunneling and that their transfer may proceed either stepwise or concertedly. The most powerful tool to sort out these possibilities is the kinetic isotope effect (KIE). KIEs have been observed for several enzymatic reactions and their interpretation in terms of specific transport mechanisms is the subject of many recent papers.[2] In this paper,

Scheme 1

we extend the discussion to multiple proton transfer. Specifically, we investigate the rate-determining step in the reaction catalyzed by the enzyme carbonic anhydrase II (CAII), which is known to involve proton transfer through a bridge of two or more water molecules.

This choice is motivated by the relative simplicity of the enzyme and of its mode of operation. CAII is one of the 14 α-carbonic anhydrases found in humans.[3] These enzymes catalyze the conversion of CO_2 into HCO_3^-. The overall mechanism of this process has been studied extensively and is now reasonably well understood.[4-6] As illustrated in Scheme 1, the active site contains a Zn^{++} ion coordinated with three histidine residues and one hydroxide ion. This hydroxide ion reacts with a carbon dioxide molecule that enters the active site through a hydrophobic pocket to form a HCO_3^- ion, which dissolves in bulk fluids and is replaced as a zinc ligand by a water molecule. The rate-determining step in the overall process is the reconstitution of the active zinc site. This requires removal of a proton from the water ligand to regenerate the hydroxide ion. CAII is one of the α-carbonic anhydrases in which this removal involves "intramolecular" transfer of the proton to another histidine residue, labeled His-64, which is located some 8 Å away from the zinc ion at the opposite end of the pocket. That this proton transfer, which takes about 1 μs, is the slow step in the overall process follows from experiments with isotope-labeled water.[7-9] Given the large distance the proton needs to cross, it is generally accepted that it is assisted by water molecules, possibly in the form of a transient hydrogen-bonded bridge. From His-64 the proton is transferred to the bulk, a process which becomes rate-determining only when insufficient buffer is available. If His-64 is replaced by alanine, which does not act as a proton shuttle, so that the proton is directly released to the solution, the rate is down by more than an order of magnitude. It has been observed, however, that the activity is restored to a level approaching that of the wild-type enzyme if the solution is buffered by imidazole, the histidine part active as a shuttle.[10] In that case imidazole occupies a position in the pocket close to one of the two positions assumed by the histidine residue in wild-type CAII, namely the "out" position.[11] The corresponding "rescue" of the catalytic activity of the mutants

by imidazole and other proton acceptors has been studied extensively by Silverman and co-workers.[12] The rate-determining step of the reaction shows a KIE, defined as the ratio between the proton and the deuteron rate constant, in the range 3-4 when the reaction takes place in heavy water, depending (weakly) on the pH of the buffer solution.[7,8] In H_2O/D_2O mixtures, the rate constant depends exponentially on the D_2O fraction,[13] from which it was concluded that the rate is determined by the movement of more than one proton. In that case, it would appear that the deuterium isotope effect is anomalously weak in view of the cumulative effect of the differences in zero-point energies and the expected tunneling contribution. Although molecular dynamics simulations[14,15] and ab initio quantum-chemical calculations[16] have provided a rough indication of the structural and energetic requirements for rapid proton transfer, they have not directly addressed the dynamics and hence do not allow an assessment of this apparent anomaly.

In this paper, we report a first calculation of the rate constant and the KIE of the rate-determining proton-transfer step of the CAII reaction in buffered aqueous solutions. For practical reasons, this calculation is limited to a model of the active site of the enzyme and does not include the protein. To investigate proton transfer from the zinc-coordinated water molecule to His-64, we use a model in which the zinc ion and its four ligands are linked to an acceptor imidazole ring by a chain of two water molecules arranged in a structure closely resembling that observed by X-ray diffraction. Although the presently available X-ray diffraction experiments[4,6,17,18] do not have enough resolution to locate the water molecules in the pocket unambiguously, the available information suggests that a minimum of 2-3 molecules is necessary to form a connecting bridge. Because there is room in the pocket for more water molecules, the limitation to a single bridge of two waters is clearly an oversimplification. It is more realistic to assume a dynamic equilibrium between several bridging and nonbridging structures. However, one may reasonably assume that short and straight bridges are better proton conduits than long and tortuous ones. Hence, there are good reasons to expect the chosen simple bridge to be representative for the bridging structures that will dominate proton transport. As distinct from the available reaction path calculations,[14-16] our dynamics approach should therefore permit a direct comparison of the calculated KIE with the anomalously low value observed experimentally as well as with the KIEs observed in H_2O/D_2O mixtures.

The potential-energy surface used in these calculations is derived from a recent approximate density functional treatment of the proton-transfer step[19] at the Self-Consistent-Charge Density Functional Tight Binding (SCC-DFTB) level.[20] The SCC-DFTB method is very efficient due to the approximations made in the electron integrals, and has been demonstrated to

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give superior results compared to standard semiempirical approaches such as AM1 and PM3 for model systems of biological interest.[21] Application of the method in a combined quantum-mechanics/molecular mechanics (QM/MM) framework has been shown to give satisfactory results for a number of enzyme systems including triosephosphate isomerase, alcohol dehydrogenase and chorismate mutase.[21a]

The calculation includes (i) the Zn^{++} ion coordinated with three histidine residues, represented by methylimidazole molecules, and a water molecule or hydroxyl ion, (ii) a fourth, similarly represented histidine residue (His-64), which accepts the proton, at the opposite end of the pocket, and (iii) a water bridge consisting of two molecules connecting the zinc-coordinated water with His-64, this residue being oriented in the "in" position: altogether 58 atoms. The four methyl carbons are fixed in positions given by X-ray data. The parametrization of the method was found to reproduce the results of a higher-level MP2/6-31G* calculation on a guanine−water complex in which two water molecules form a bridge for proton transfer between oxo and hydroxo forms.[22] In this complex, as well as in CAII with a bridge of two waters, concerted proton transfer is predicted, as follows from the observation of a single transition state. The observed KIE indicates that proton-transfer rather than bridge formation is rate-determining. Then the transfer rate constant can be calculated by one of the available methods for direct-dynamics multidimensional tunneling transfer, such as Variational Transition-State Theory with Semiclassical Tunneling Corrections (VTST/STC)[23] or the Approximate Instanton Method (AIM).[24] Of these, the latter method is the least time-consuming and has proven to be particularly effective for the calculation of KIEs.[25] We therefore use AIM, as implemented in the DOIT 1.2 program,[26] to calculate rate constants for a wide range of isotopomers and subsequently use Canonical Variational Transition-State Theory (CVT)[23] together with the small curvature approximation (CVT/SCT hereafter)[27] to validate the results for two of them.

II. Dynamics Calculations

To relate the model system to the actual enzyme, we assume that it is one of several configurations of the active site that are in thermal

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equilibrium. The observed rate constant will then be of the form

$$k_{obs}(T) = \sum_j K_j(T) k_j(T) \qquad (1)$$

where K_j is the equilibrium constant of structure j, presumed to be isotope-independent, and k_j is the corresponding proton transfer rate constant. The observed kinetics is consistent with the assumption that the equilibrium is established rapidly on the time scale of the transfer. Because the elimination of the HCO_3^- ion prior to the proton-transfer step studied will presumably disturb any existing water bridge, it is likely that a new bridge is formed during each catalytic cycle. Because the enzyme can accommodate a substantial number of structures j, the individual K_j will be small. This holds in particular for the structure represented by the model system, because the free energy of this configuration is expected to be higher than that of configurations with more hydrogen-bonded water molecules. The model structure can contribute significantly to proton transport only if the rate of transfer is high enough to compensate for this smallness. A good indication for this to be the case is a calculated KIE close to the observed value. In the following subsections, we briefly describe the methods used to calculate $k_j(T)$ at $T = 298$ K from the structural and energetic parameters generated by the SCC-DFTB calculations.[19]

A. AIM Calculations. AIM, as implemented in the DOIT code, is an outgrowth of the instanton theory of tunneling.[28] This quasiclassical theory is based on the recognition that, among the trajectories that connect the reactant state with the product state, there is one, the *instanton path*, that dominates the tunneling rate. Although this trajectory follows the minimal energy path in the vicinity of the equilibrium configurations, when moving toward the transition state, it deviates from that path and follows a "tunneling" trajectory of lower energy. The corresponding tunneling rate is proportional to $e^{-S_I(T)}$, where $S_I(T)$ is the *instanton action* (of dimension energy × time and in units $\hbar$), which is thus the quantity of practical interest. AIM is designed to allow direct application of this methodology to proton transfer in multidimensional systems for which the structure and vibrational force field of the stationary states along the reaction path can be evaluated quantum-chemically.

To calculate $S_I(T)$, AIM separates the tunneling mode, taken to be the mode with imaginary frequency (ω^*) in the transition state, from the other (transverse) modes. The one-dimensional instanton action for the tunneling mode is readily calculated. The effect of the transverse modes is introduced in the form of linear couplings to the tunneling mode, calculated from their displacement between the reactant and the transition state. If the barrier is (approximately) symmetric, then the only displaced modes are those that are either symmetric or antisymmetric relative to reflection in the dividing surface between reactant and product. (For asymmetric barriers normal modes are decomposed in symmetric and antisymmetric components.) The antisymmetric modes have the same symmetry as the tunneling mode and undergo final reorganization during the process, whereas the symmetric modes do not undergo such reorganization. The effect of the skeletal modes on the tunneling rate depends on their frequency and strength of coupling. Qualitatively, high-frequency modes are included directly in the potential, which results in a higher effective mass for the tunneling mode and reduced barrier height, the overall effect on the tunneling rate being a balance of the two. Low-frequency antisymmetric modes contribute a Franck−Condon factor to the rate constant. Hence, they slow the rate of tunneling, the more so the lower the temperature. Low-frequency symmetric modes shorten the tunneling distance and lower the barrier and thus promote tunneling. In technical terms, high-frequency modes are treated in the adiabatic approximation and low-frequency modes in the sudden approximation; which approximation

(28) See, for example, the review Benderskii, V. A.; Makarov, D. E.; Wight, C. H. *Adv. Chem. Phys.* **1994**, *88*, 1 and references therein.

applies to a given mode is determined by a parameter (zeta factor[24,29,30]) that depends not only on the frequency but also on the coupling strength.

Generalizing the exact results obtained as solutions for low-dimensional model systems,[28] we combine these effects in an approximate analytical expression for the instanton action of the form[24,30]

$$S_I(T) = \frac{S_I^0(T)}{1 + \sum_s \delta_s(T)} + \alpha_s \sum_a \delta_a(T) \quad (2)$$

In this expression $S_I^0(T)$ is the instanton action of an effective one-dimensional motion along the reaction coordinate, corrected for adiabatic coupling to high-frequency modes. The correction factors $\delta_{a,s}(T)$ represent the contributions of low-frequency antisymmetric and symmetric modes, respectively, and $\alpha_s \leq 1$ describes the modulation of the antisymmetric correction by the symmetric coupling. The $\delta_a(T)$ give rise to squared vibrational overlap integrals (Franck–Condon factors) and the $\delta_s(T)$ have the general form

$$\delta_s(T) \simeq \frac{1}{2}\left(\frac{\Delta U_0}{U_0}\right)(\omega_s/\Omega)\coth\frac{\hbar\omega_s}{2k_B T} \quad (3)$$

where ΔU_0 equals the amount by which the barrier height U_0 is reduced due to coupling with the symmetric mode ω_s, and Ω is a (scaling) frequency,[24] which is normally close to the imaginary frequency ω^*. To the resulting tunneling rate constant

$$k_{tun}(T) \simeq k_{AIM}(T) = (\Omega_0^R/2\pi)e^{-S_I(T)} \quad (4)$$

where Ω_0^R is the effective frequency of the tunneling mode in the reactant state, we must add the classical rate constant for transfer over the barrier, which is calculated by standard transition-state theory (TST), to obtain the total rate of proton transfer. All these calculations are performed by the DOIT 1.2 code using input parameters read directly from the SCC-DFTB code. For isotopomers new force field calculations are needed but the same potential energy surface applies.

B. VTST Calculations. To validate the AIM results, CVT/SCT calculations were carried out for two isotopomers, namely those with none and those with all of the oxygen-bound protons replaced by deuterons. These calculations are more expensive than the AIM calculations because many points along the reaction path with the corresponding gradients and generalized frequencies are required. However, the method has been thoroughly tested by comparing with accurate full quantum mechanical results for small molecules in the gas phase.[31] Although AIM has been shown to give similar results as VTST for several single proton-transfer reactions in the temperature region of interest,[25b] no comparison of the two methods has been made thus far for concerted proton-transfer reactions such as those relevant for CAII.

The calculations were carried out with an interface between POLYRATE 8.0[32] and the SCC-DFTB program such that the latter provides the necessary information on the potential energy surface and its derivatives to the former. Due to the size of the system, the calculations were limited to the small curvature approximation. This

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Table 1. Coupling Parameters for the CAII Model, Calculated for an Adiabatic Barrier Height $U_0 = 8.1$ kcal/mol and a Temperature $T = 298$ K[a]

	HHH	HHD	HDH	DHH	HDD	DHD	DDH	DDD
Δx^R	1.33	1.47	1.51	1.41	1.61	1.53	1.57	1.66
Δx^P	0.28	0.36	0.37	0.34	0.45	0.42	0.43	0.51
ω^*	603i	533i	508i	567i	461i	507i	483i	443i
Ω_0^R	1924	1705	1494	1789	1386	1621	1414	1334
ω_c	440	431	421	435	417	429	419	415
$\sum_s \delta_s$	0.16	0.18	0.24	0.18	0.22	0.21	0.26	0.26
$\sum_a \delta_a$	1.64	1.37	1.33	1.67	1.30	1.37	1.31	1.29
$m_{eff} - 1$	$4.7x^2$	$3.1x^2$	$2.6x^2$	$3.8x^2$	$2.0x^2$	$2.6x^2$	$2.2x^2$	$1.8x^2$
S_I^0	9.22	10.19	10.35	9.98	10.06	10.88	11.22	11.90

[a] $\Delta x^{R,P}$ denotes the displacements of the tunneling mode in the reactant and product state relative to the transition state, their sum being the tunneling distance. ω_c is the frequency of the most strongly coupled quasisymmetric mode. Frequencies are in cm^{-1}, mass-weighted displacements in Å.amu$^{1/2}$, mass and action in dimensionless units.

approximation was found to be accurate for many reactions at room temperature.[33]

III. Results

A. Results from AIM. The AIM calculations, which include all normal modes of the system, are similar to those reported earlier.[22] From the stationary structures and their vibrational force fields, we calculate the dynamics parameters and the coupling constants $\delta_{a,s}(T)$ listed in Table 1. They show that the coupling is fairly strong as reflected in the low value of the imaginary frequency ($\omega^* = 603i$ cm^{-1}), which indicates that there is a substantial contribution of heavy-atom motion to the reaction coordinate, as illustrated in Figure 1. Coupling to quasisymmetric low-frequency modes, discussed in Section IIA, reduces the distance between the donor oxygen atom bound to zinc and the acceptor nitrogen atom of His-64 by more than 0.4 Å in the transition state relative to the initial state. The strongest coupling of this type is obtained for a mode of 440 cm^{-1} corresponding to a predominantly symmetric stretching vibration that modulates the length of the bridge. This mode and all higher-frequency modes are treated in the adiabatic approximation on the basis of their zeta factors.[24, 29, 30]

The calculated endothermicity $\Delta E = 0.8$ kcal/mol[19a] is small enough to be consistent with the reversibility of the transfer observed experimentally. The adiabatic barrier height is calculated to be $U_A = 8.1$ kcal/mol. These results, and particularly the nature of the transition state found by the SCC-DFTB approach, were verified with B3LYP/6-31G(d) calculations for the saddle point. As shown in Figure 1, critical geometrical parameters (such as the bond distances associated with the transferring protons) are very similar at the two levels; the transition state is highly concerted in both methods. As described in more detail in ref 19, the energetics are also reasonably well described at the SCC-DFTB level, the barrier height at the B3LYP/6-311+G(d,p)//B3LYP/6-31G(d) level being 5.9 kcal/mol. In heavy water, all the protons of the water molecules between the zinc ion and His-64 will be replaced by deuterons. We assume that in D$_2$O/H$_2$O mixtures the D/H ratio will be the same as that of the solvent. It has been found that in CAII with zinc replaced by cobalt, the deuterium content of the cobalt-bound water molecule is about 0.8 of this assumed value,

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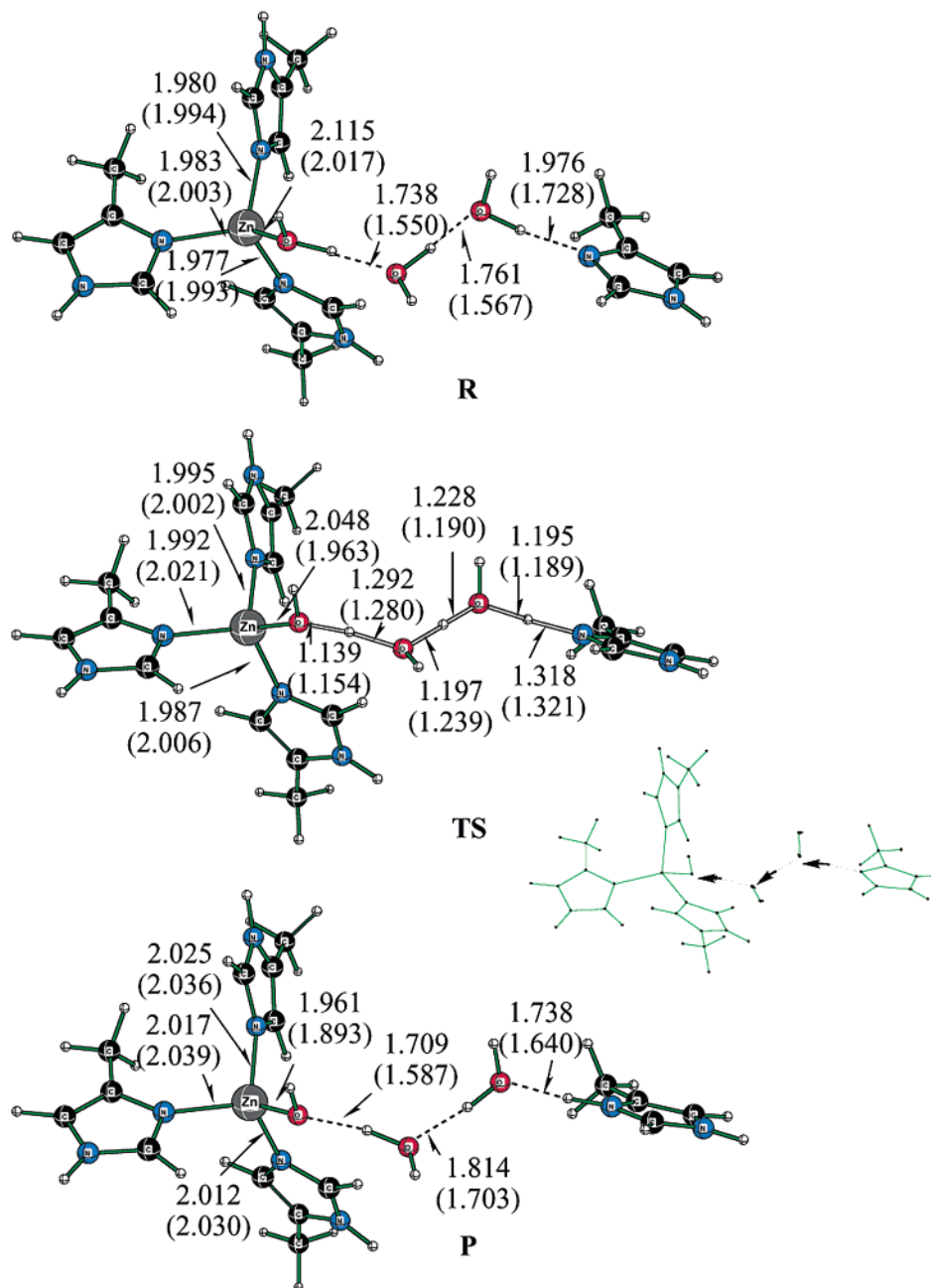
Figure 1. Reactant (R), transition state (TS), and product (P) configurations for the rate-determining triple proton-transfer step of the 58-atom model used to represent the active site of carbonic anhydrase II. The numbers denote bond distances (in Å) calculated at the SCC-DFTB level and (in parentheses) the DFT/B3LYP/6-31G(d) level. The arrows in the side figure represent the tunneling mode and illustrate the degree of synchronicity of the transfer.

whereas that of the more distant waters shows no such fractionation.[34] Because a similar effect in unsubstituted CAII would modify the calculated rate constant by an amount smaller than the reported experimental error, we are neglecting this correction. The reported data combine primary and secondary KIEs, which cannot be separated. To reduce the number of structures for which force fields and rate constants must be calculated, we arbitrarily put the nontransferring protons and deuterons in each water bridge in positions such that the bridge contains only H_2O and D_2O molecules before transfer. This leads to six different isotopomers (DHH, HDD, and their permutations), all of which are included in the calculations.

With these input parameters we obtain the rate constants and KIEs of triple proton/deuteron transfer at 298 K listed in Table 2 and depicted in Figures 2 and 3, where they are compared with the corresponding experimental parameters. The calculated rate constants are higher by more than 3 orders of magnitude, which indicate that the chosen configuration can make a significant contribution to proton transfer only if its equilibrium constant $K_j > 10^{-5}$. Arguments in favor of such a contribution can be derived first from the similarity of the calculated and observed KIEs shown in Figure 3, and second from the exponential dependence of the rate constants on the deuterium concentration shown in Figure 2. Such a dependence is typical for concerted transfer and is not found in the case of stepwise

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Table 2. Comparison of Calculated and Observed Rate Constants (in s^{-1}) and Kinetic Isotope Effects at $T = 298$ K for Triple Proton Transfer in the CAII Model System[a]

	HHH	(H$_2$D)$_{av}$	(HD$_2$)$_{av}$	DDD
k_{AIM}	3.9×10^9	2.5×10^9	1.5×10^9	8.9×10^8
$k_{CVT/SCT}$	6.8×10^8	—	—	1.1×10^8
$(k^H/K^D)_{AIM}$	—	1.6	2.6	4.4
$(k^H/k^D)_{CVT/SCT}$	—	—	—	6.2
k_{TST}	6.6×10^7	2.4×10^7	9.4×10^6	3.7×10^6
$(k^H/k^D)_{TST}$	—	2.7	7.0	18.0
k_{Obs}	6.0×10^5	4.0×10^5	2.8×10^5	1.8×10^5
$(k^H/k^D)_{Obs}$	—	1.5 ± 0.2	2.2 ± 0.2	3.2 ± 0.4

[a] The calculations, based on an adiabatic barrier height of 8.1 kcal/mol, are performed with the Approximate Instanton Method (subscript AIM), Variational Transition State Theory with Tunneling Corrections in the Small-Curvature Approximation (subscript CVT/SCT), and classical Transition State Theory (subscript TST). The observations (subscript Obs) are taken from ref 13. The theoretical values for mixed isotopes represent averages over all possible permutations of the protons and deuterons.
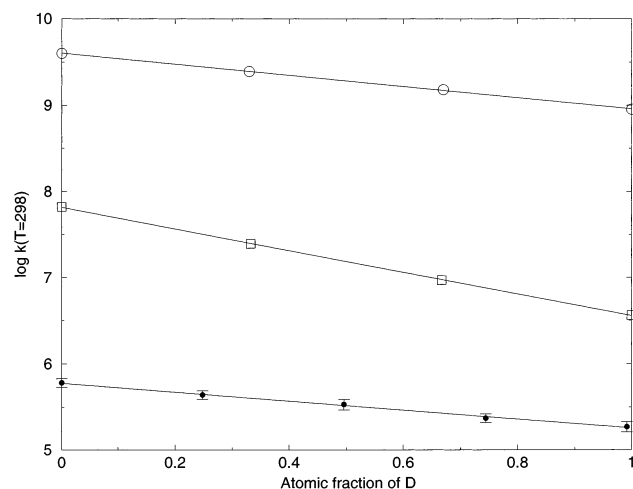

Figure 2. Rate constants in carbonic anhydrase II plotted as a function of the atomic fraction of deuterium in water. The dots represent experimental results taken from ref 13, the circles and squares represent the calculated quantum and classical results, respectively. The exponential dependence shown by the plots is characteristic of concerted multiple proton transfer.
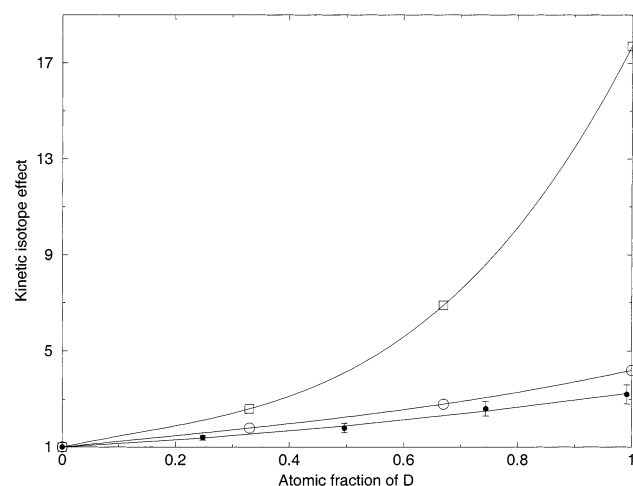

Figure 3. Kinetic isotope effect in carbonic anhydrase II plotted as a function of the atomic fraction of deuterium in water. Symbols as in Figure 2.

transfer, as e.g. in porphine.[25c,35] In fact, model calculations indicate that the concerted transfer must be synchronous or nearly so to show this behavior.[35]

(35) Smedarchina, Z., to be submitted.

B. Comparison with VTST Calculations. Because of the computational cost, the CVT/SCT calculations were limited to all-hydrogen and all-deuterium bridges. The calculated rate constants are listed in Table 2 along with the KIE for full deuteration. Variational effects reduce the CVT rate constants relative to TST rate constants by factors of 0.78 and 0.81 for HHH and DDD, respectively. The variational transition state is close to the classical transition state and reduces the KIE by an insignificant factor of 0.96. This confirms that the anomalous KIE is not due to variational effects, which are not included in AIM, but to quantum-mechanical tunneling of a proton coupled to skeletal vibrations. Compared to the experimental parameters, the CVT/SCT rate constants are higher by 3 orders of magnitude and the KIE is higher by 90%. The values obtained in the AIM and CVT/SCT calculations are within the same order of magnitude and the difference is not out of line considering the complexity of the system and the approximations used in the two treatments. It has been found earlier that AIM tends to overestimate rate constants when the coupling is strong. On the other hand, the limitation of the VTST calculations to the small-curvature approximation, made because strong-curvature calculations would be unwieldy for the present system, may underestimate the effect of strong couplings. The larger-than-observed KIE value suggests that this is indeed the case. Taken together, the results of the two calculations are therefore expected to provide a reliable order-of-magnitude estimate of the rate constants for the chosen model structure. It would imply that this structure can make a significant contribution to the observed transfer rate even if it is present in very low concentrations.

C. Comparison with Classical Results. Rate constants and KIEs calculated from classical TST as implemented in the DOIT 1.2 program are compared with the AIM and CVT/SCT values in Table 2 and Figures 2 and 3. The rate constants are higher by up to 2 orders of magnitude than those observed, but considerably smaller that the tunneling rate constants. They also show the exponential dependence on the deuterium dependence that is typical for concerted transfer. However, the KIEs are much larger than the observed values and the values calculated by the tunneling treatments. In the absence of tunneling, KIEs are governed by the effect of deuterium substitution on the difference in zero-point energy between the initial state and the transition state. According to classical TST, the KIE, to be denoted by η in the equations, equals

$$\eta = \left(\frac{Q^{\ddagger}}{Q^R}\right)^H \bigg/ \left(\frac{Q^{\ddagger}}{Q^R}\right)^D \qquad (5)$$

where Q^R and $Q^{\ddagger}$, the partition functions for the reactant and the transition state, respectively, are given by

$$Q = \prod_j \frac{1}{2\sinh(\hbar\omega_j/2k_BT)} \qquad (6)$$

the product being over all normal modes of the system. If all affected frequencies are much higher than k_BT, the hyperbolic sine can be replaced by an exponential, so that the rate constant assumes the familiar form

$$k_{TST}(T) = (k_BT/h)e^{-\tilde{U}_0/k_BT} \qquad (7)$$

where $\tilde{U}_0$ is the barrier height corrected for zero-point energy changes between the reactant state and the transition state. However, this commonly used approximation, which leads to a KIE of the form

$$\eta_{TST} = e^{(\tilde{U}_0{}^D - \tilde{U}_0{}^H)/k_B T} \qquad (8)$$

is *not* valid if the barrier width and height are modulated by modes with frequencies of the order of $k_B T$ and is not used in the present calculations.

The large KIE calculated for classical triple proton transfer along a water bridge is due to the accumulation of zero-point energy differences and their distribution over many low-frequency modes. The difference in zero-point energy between the initial state and the transition state amounts to 1335, 1166, 988, and 839 cm^{-1} if, respectively, zero, one, two, and three of the H_2O molecules connecting Zn^{++} with His-64 are replaced by D_2O molecules, so that the zero-point corrections to the barrier height increase from 169 via 347 to 496 cm^{-1} when one, two, and three D_2O molecules are introduced. If these corrections applied only to the tunneling mode, their effect on the KIE would be small, but in the case of a hydrogen-bonded chain of water molecules, they are distributed over many vibrations of much lower frequency, which leads to the large KIE calculated classically.

The much smaller KIE for the tunneling mechanism can be traced back to the symmetric coupling of the tunneling mode to low-frequency skeletal modes. Although deuterium substitution increases the tunneling path, the main increase of S_I^0 is due to the doubling of the mass of the tunneling particle, m_{eff}, as S_I^0 is proportional to $\sqrt{m_{eff}}$. This effect is mitigated by coupling of the tunneling mode to skeletal modes that modulate the tunneling distance, which in the AIM formalism of eq 2 is introduced through correction terms δ_s. In the simple case that there is only a single mode coupled symmetrically to the tunneling mode, we obtain from eqs 2 and 4

$$\eta_{AIM} \simeq \sqrt{2}\, e^{S_I^{0,D}/(1 + \delta_s{}^D) - S_I^{0,H}/(1 + \delta_s{}^H)} \qquad (9)$$

where normally $\delta_s^D \geq \delta_s^H$. If the coupling is weak or moderate, then we can write $1/(1 + \delta) \simeq 1 - \delta$, so that

$$\eta_{AIM} \simeq \eta_{AIM}^0 \cdot e^{-\delta(S_I^{0,D} - S_I^{0,H})} \qquad (10)$$

where η_{AIM}^0 represents the KIE in the absence of coupling. The exponential shows how symmetric coupling reduces the effect; for example, if the coupling lowers the barrier by 20%, it reduces the KIE more than 2-fold. Hence, the rate constant no longer depends exponentially on the square root of the tunneling mass, as in the one-dimensional case, but exhibits a weaker mass-dependence, as the transition probability is affected by the slow motion, which is isotope-independent. This effect, which is general and depends only on the strength of coupling, was found earlier by Benderskii et al.[36] from exact solutions for model systems. The significance of this effect was also recognized by Schwartz et al.[37] in their work on proton tunneling in enzymatic

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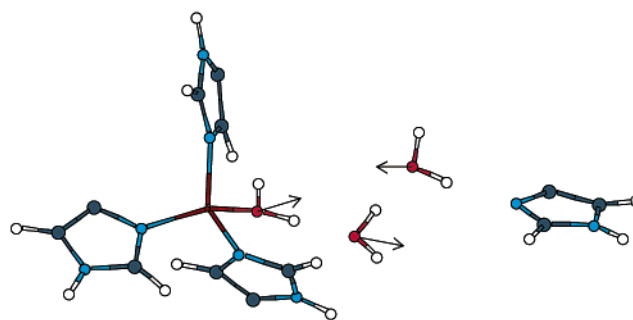
Figure 4. Reactant state of the rate-determining triple proton-transfer step of the model of Figure 1 (with the methyl groups omitted), with arrows depicting a low-frequency mode ($\omega = 337$ cm^{-1}) of the water bridge that is strongly coupled to the tunneling mode.

reactions. Low-frequency quasisymmetric modes, such as the mode depicted in Figure 4, are particularly effective in reducing the KIE, especially at higher temperatures. In CAII the most effective quasisymmetric modes have frequencies in the range 300−400 cm^{-1}, so that their excited levels will contribute significantly to the tunneling. These contributions are responsible for reducing the KIE from 18 to about 6. Coupling to antisymmetric modes results in a further reduction to the calculated value of 4.4. This small KIE contrasts sharply with the much larger value calculated for classical transfer and runs counter to the expectation that a large KIE indicates a tunneling mechanism.

The smaller rate constant calculated for classical transfer compared to tunneling indicates that classical transfer is not competitive for this model and thus does not contribute significantly to transport along the two-water bridge. The calculated classical KIE, which exceeds the observed KIE by a factor of almost 6, as shown in Figure 3, confirms this conclusion. The predominant proton transport mechanism along this bridge is thus concerted quantum-mechanical tunneling. Earlier conclusions that this transport in CAII proceeds by tunneling were based on the observed KIE, which however, as the present calculations show, does not by itself justify such a conclusion.

To what extent the present water bridge contributes to the overall proton transport in the enzyme cannot be established without investigating alternative bridges. In principle, the large value calculated for the rate constant supports the notion that it is a plausible contributor, since its equilibrium constant relative to other water structures in the pocket, represented by $K_j(T)$ in eq 1, need only be of order $10^{-4} - 10^{-5}$ to make its contribution significant. Such an equilibrium constant corresponds to an excess free energy in the range of 3−4 kcal/mol, the energy of a typical hydrogen bond. This suggests that the two-water bridge will be an important pathway for proton transport if the optimum number of water molecules in the pocket is 3−4.

IV. Alternative Models

The calculations suggest that the model two-water bridge is present in low concentrations, so that there must be alternative bridging water structures containing three or more water molecules that transport protons more slowly. The relative energies of such structures have not been explored thus far. However, using molecular dynamics simulations, Voth and Lu,[15]

and Zheng and Merz[38] found that the solvent structure fluctuates considerably. To find alternative stable active-site configurations in a systematic way, it will be necessary to extend the calculations to the amino acid residues that shape the pocket and thus interact with the water molecules inside. Most of these are hydrophobic but Thr-199 may form a hydrogen bond with a water molecule inside.[18] The importance of the interactions follows immediately from the observation that mutants of CAII, in which alanine adjacent to His-64 is replaced by bulky amino acids, show reduced activity.[39] It seems probable that formation of a water bridge is inhibited in these systems. The number of water molecules normally residing in the pocket is not known and may well be variable. It has been estimated to be as high as six,[40] so that it seems highly probable that a wide variety of arrangements can be realized at 298 K. The hydrogen-bonding network in such a water cluster is highly polarizable and may be able to stabilize ionic intermediates.[19,41] This means that in larger clusters transfer may be stepwise rather than coherent. In that case, the rate-determining step would probably be single proton transfer. However, such a mechanism will be difficult to reconcile with the observed experiments in H_2O/D_2O mixtures, which provides evidence for multiple proton transfer.[13] Indeed, model calculations suggest that the observed exponential dependence of the rate constant on the deuterium concentration implies a transition state in which the transferring protons have completed a similar fraction of their respective pathways,[35] as in the present calculation.

It is reasonable to expect that a third water molecule will tend to insert itself into the bridge to optimize hydrogen bonding, while avoiding strong repulsion. Although this may be energetically favorable, given the size of the pocket, such a bridge would be longer and thus probably less efficient for proton transfer than the model bridge considered. Recent calculations suggest that the transfer will still be concerted, i.e., characterized by a single barrier, but less synchronous than that of the two-water bridge, as indicated by the positions of the moving protons and the flatness of the barrier top in the transition state.[19b] Whether a fourth water molecule is more likely to lengthen the bridge further or to solvate the bridge cannot be deduced with confidence without an extension of the model that includes enough additional amino acid residues to simulate a realistic pocket. Clearly, the larger the bridge, the greater the chance that a stable intermediate hydronium ion is formed.[19b] This would break the synchronicity of the transfer implied by the observed effects of partial deuteration. Because solvated hydronium ions presumably are the proton carriers in carbonic anhydrase isozymes without a His-64 receptor, it seems likely that structures leading to such an intermediate share the low activity of these isozymes. These arguments suggest that proton transport in CAII is dominated by short and simple bridges.

V. Conclusion

The present model study represents the first calculation of the rate of proton transfer along a water bridge in an enzyme. One end of the two-water bridge is connected via a third water

molecule to a zinc ion and the other end is connected to a methylimidazole molecule that models a histidine residue. The corresponding triple-proton transfer is found to be concerted, i.e., to proceed by quantum-mechanical tunneling through a single barrier. Strong coupling of the proton transfer to skeletal motions of the bridge results in a KIE that is small, much smaller than predicted by classical TST, where it is associated with cumulative zero-point energy shifts. This situation is the reverse of that encountered in single proton transfer, where tunneling tends to yield larger KIEs than classical motion. Hence, the conclusion that the rate-determining proton-transfer step in CAII proceeds by tunneling is based on the observation that the KIE is abnormally low, not abnormally high, in comparison with classical transfer, as usually assumed. In other words, the earlier conclusion that the protons in CAII tunnel was right but for the wrong reason.

The observation of an anomalously small KIE for concerted multiple proton transfer is not limited to CAII, but can be shown to be a general phenomenon for systems with loosely bound networks of hydrogen bonds at temperatures leading to excitation of soft modes whose frequencies are sensitive to the position of the moving protons.[35] It is due to the fact that phase coherence between the proton motions implies that these motions are coupled through the atoms that serve as proton donor to one hydrogen bond and simultaneously as proton acceptor to another. Because of this coupling the tunneling coordinate acquires a significant heavy-atom component, which reduces the KIE for tunneling transfer. This same coupling distributes the zero-point energy shift resulting from isotopic substitution over several modes, including low-frequency skeletal modes, which increases the KIE for classical transfer. It leads to the "anomaly" that in such systems classical KIEs tend to exceed quantum KIEs for concerted multiple proton transfer. This is obviously an important consideration for the interpretation of KIEs, especially in biological systems.

A second conclusion to be drawn from the calculations is that it is now possible to carry out meaningful proton dynamics calculations on realistic biological model systems with readily available dynamics codes. Although the agreement between the two methods used is not perfect, the same basic transfer mechanism and anomalously low KIE is predicted by both.

These conclusions do not depend on the relevance of the chosen two-water bridge for the actual transport mechanism in CAII or on the precise values obtained for its structure and energetics, which will undoubtedly be modified in a more complete model of the active site. Nevertheless, the calculations make it plausible that simple structures of the type considered can contribute significantly to proton transport in CAII, even when present in low concentrations, because of the efficiency of the transfer. The calculated KIEs, especially those for partially deuterated bridges, support this conjecture. Competing structures based on bridges with more and/or cross-linked water molecules may be present in larger concentrations due to their lower energy and/or higher entropy, but are expected to have lower transfer rates, because these bridges are longer and/or less homogeneous than the model bridge. Bridges leading to stepwise transfer, such that a single-proton transfer step is rate-determining, cannot explain the observed exponential dependence of the rate constant on the deuterium concentration. This applies in particular to structures in which the proton can be trapped as a hydronium

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ion. Their behavior is likely to emulate the reduced activity of carbonic anhydrase isozymes without a His-64 receptor. These observations suggest that the present model gives a reasonable picture of the rate-determining proton-transfer step of CAII.