Solvent-Mediated Proton Transfer in Catalysis by Carbonic Anhydrase

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ABSTRACT

Considerable attention has been focused on proton transfer through intervening water molecules in complex macromolecules of biological interest, such as bacteriorhodopsin, cytochrome *c* oxidase, and many others. Proton transfer in catalysis by carbonic anhydrase provides a useful model for the study of the properties of such proton translocations. High-resolution X-ray crystallography in combination with measurements of catalysis have revealed new details of this process. A prominent proton shuttle residue His64 shows evidence of structural mobility, which appears to enhance proton transfer between the active site and bulk solvent. Moreover, the properties of the imidazole side chain of His64, including its conformations and pK_a, are finely tuned by surrounding residues of the active-site cavity. The structure of a network of ordered solvent molecules located between His64 and the active site are also sensitive to surrounding residues. These features combine to provide efficient proton-transfer rates as great as 10^6 s^{-1} necessary to sustain rapid catalysis.

Introduction

The carbonic anhydrases comprise well-studied and distinct gene families (α, *β*, and *γ*) of mostly zinc-metalloenzymes that catalyze the hydration of carbon dioxide.[1–5] It is notable that mechanistic studies of catalysis by enzymes in each of these families can be described by the same overall catalytic mechanism comprising the two separate stages of catalysis shown in eqs 1 and 2, an example of a ping-pong[1,3] or iso mechanism.[6] The first stage is the conversion of CO_2 into bicarbonate by the reaction with zinc-bound hydroxide; the dissociation of bicarbonate leaves a water molecule at the zinc (eq 1). There is no evidence of rate-contributing proton transfer in the steps of eq 1.[7,8] The second stage is the transfer of a proton to solution to regenerate the zinc-bound hydroxide (eq 2); here, B denotes an exogenous proton acceptor from solution or a residue of the enzyme itself that subsequently transfers the proton to solution.

$$CO_2 + EZnOH^- \rightleftharpoons EZnHCO_3^- \underset{H_2O}{\overset{H_2O}{\rightleftharpoons}} EZnH_2O + HCO_3^- \quad (1)$$

$$EZnH_2O + B \rightleftarrows EZnOH^- + BH^+ \quad (2)$$

Seminal work from the lab of Sven Lindskog on human carbonic anhydrase II (HCA II), a member of the α family, used solvent hydrogen/deuterium isotope effects to demonstrate that the maximal velocity of catalysis was limited by intramolecular proton transfer occuring in the second stage of catalysis (eq 2).[9] This proton-transfer system has in the decades since its initial discovery undergone extensive study and is now the best described example of solvent-mediated proton transfer in a protein environment. The investigation of proton transfer in carbonic anhydrase has the advantage that the identities of both the proton donor and acceptor are known and the rate constant for proton transfer is measured in a straightforward manner as the rate-limiting step in maximal velocity[9] and in the exchange of ^{18}O between CO_2 and water.[10] Catalysis by carbonic anhydrase is an excellent model system to understand biophysical properties of proton transfer that can be applied to much more complex proton-transport proteins, such as cytochrome *c* oxidase, bacteriorhodopsin, ATP sythase, and the bacterial photosynthetic reaction center. Recent combination of high-resolution X-ray crystallography combined with kinetic studies have yielded a new level of understanding of the proton-transfer process in carbonic anhydrase. Its description is the aim of this Account.

Histidine 64 as a Proton Shuttle

The results of a number of experiments confirmed the initial hypothesis of Steiner et al.[9] that the imidazole ring of His64 was the internal proton shuttle in HCA II based on its conformation extending into the active-site cavity (Figure 1).[11–13] The side-chain C*β* of His64 is about 7.5 Å from the zinc, and the proton transfer occurs through the intervening solvent.[14] The replacement of His64 in HCA II by Ala (H64A), which cannot transfer protons, caused an approximately 20-fold decrease in the proton-transfer stage of catalysis compared to that of the wild type.[15] Moreover, rescue of the catalytic activity was achieved by the introduction of derivatives of imidazole to solutions containing H64A, supporting a role of His64 as a proton shuttle.[15,16] Studies analogous to these have also identified

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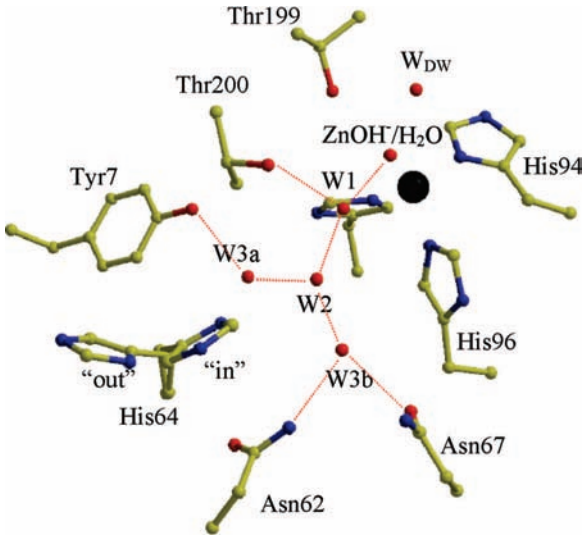

FIGURE 1. Active site of HCA II.[24] Ball-and-stick representation of the active-site residues are as labeled; the zinc atom and water molecules are shown as black and red spheres, respectively. Inferred hydrogen bonds are indicated as dashed red lines.

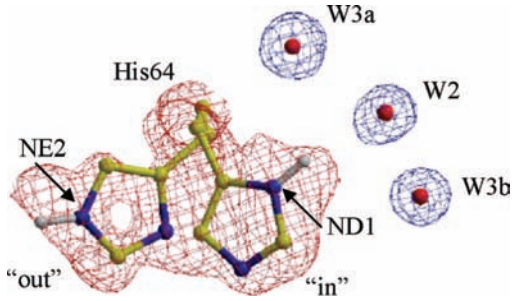

FIGURE 2. Inward and outward conformations of His64.[20] Ball-and-stick representation of His64 as labeled; water molecules are shown as red spheres. The His64 F_o–F_c omit electron-density map is depicted as a red mesh and contoured at 2.5σ. The solvent $2F_o$–F_c electron-density map is depicted as a blue mesh and contoured at 2.0σ. The location of putative hydrogen atoms are as shown.

proton shuttle residues in carbonic anhydrases in both the β[17] and γ[18] classes and therefore provide examples of the convergent evolution of proton-transfer mechanisms.

X-ray structural studies of HCA II determined with crystals equilibrated at pH ranging from 5 to 10 show the side chain of His64 occupying two major conformations, the so-called inward orientation pointing toward the zinc in the active site and the outward orientation pointing away from the active site into the bulk solvent (Figures 1 and 2).[11,12] The side chain of His64 has no apparent interactions with other residues in the active-site cavity, although it may possibly form a π-stacking interaction with the indole ring of Trp5 (not shown) when in the outward conformation. It has been suggested that this observed conformational mobility of His64 is important for efficient proton shuttling between the solution and the zinc-bound solvent (eq 2).

His64 may be an example of a general mechanism for transferring protons to the active site of CA, with other examples being Glu84 in the β-class carbonic anhydrase from *Methanosarcina thermophila*,[18] which shows two rotamers and a chemically modified cysteine residue that acts as a proton shuttle in a mutant of murine CA V, which also shows evidence of multiple orientations.[19] The relation between multiple conformational states of His64 and proton transfer is complex. Several mutations within the active-site cavity have been shown to affect the side-chain conformation of His64 as well as the rate of proton transfer in catalysis.[20] However, the replacement of Thr200 in HCA II with Ser (T200S) caused a change in the side-chain orientation of His64 to a predominantly outward conformation and yet caused no significant change in the steady-state constants k_{cat} that contain rate-contributing proton-transfer steps.[21] Computations show that the rate of rotation from inward to outward orientations is not rate-contributing.[22] In addition, a mechanism has been suggested on the basis of proton tautomerization in the imidazole ring of His64 that does not require a change in the side-chain orientation of this residue.[23]

Solvated Active Site

Within the active site of HCA II, there are several amino acids (Tyr7, Asn62, Asn67, Thr199, and Thr200) that participate in coordinating a solvent network (W1, W2, W3a, and W3b) between the zinc-bound solvent and His64, a network that appears well-ordered in crystal structures at 1.05 Å resolution (Figure 1).[24] Thr199 makes a hydrogen bond to the zinc-bound solvent that, in turn, is hydrogen-bonded to W1. W1 is further stabilized by Thr200 and the next solvent in the chain, W2. The solvent network then apparently branches because W2 is hydrogen-bonded to both W3a and W3b. W3a is further coordinated by the hydroxyl group of Tyr7, while W3b is stabilized by Asn62 and Asn67. This solvent network is conserved over a broad pH range (pH 5.0–10.0) and localizes W2, W3a, and W3b all in close proximity to the side chain of His64 in the inward conformation (Figure 2).[13]

Examination of the structure of HCA II at near atomic resolution reveals aspects not apparent in lower resolution studies.[24] For instance, the solvent molecule W2 (the only ordered solvent molecule in the active site stabilized exclusively by other solvent molecules) is trigonally coordinated with equal distance (2.75 Å) by W1, W3a, and W3b. Within this cluster of solvent molecules, only W2 is in the plane of the imidazole ring of His64 and within hydrogen-bonding distance of ND1 of His64 when in the inward conformation (Figures 1 and 2).

Also, at this resolution of 1.05 Å, the data suggest that the zinc-bound solvent is a water molecule (ZnH_2O of Figure 3) rather than an hydroxide ion. In this geometry, the hydrogen H2 forms a hydrogen bond (2.4 Å) with the solvent molecule W_{DW}, while the hydrogen H1 is free and does not appear to be involved in any interactions. However, it is straightforward to postulate that a small structural perturbation, such as a simple rotation, of the zinc-bound solvent molecule could orientate H1 to be within hydrogen-bonding distance to W1 (Figure 3). The additional solvent molecule (W_{DW}) that has been observed before in other HCA II structures is termed the "deep

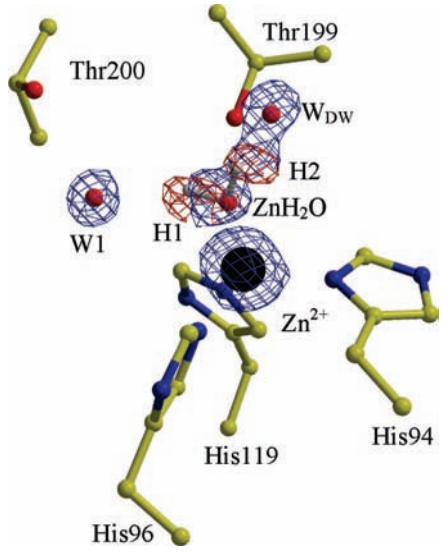

FIGURE 3. Zinc-bound solvent and coordinating ligands.[20] Ball-and-stick representation of active-site residues are as labeled; the zinc ion and water molecules are shown as black and red spheres, respectively. The hydrogen atoms of the zinc-bound water molecule ZnH_2O are shown as red spheres. The $2F_o–F_c$ electron-density map is depicted as a blue mesh and contoured at 2.0σ (showing non-hydrogen atoms), and the $F_o–F_c$ electron-density map is depicted as a red mesh and contoured at 4.5σ (showing the putative position of two hydrogens, H1 and H2). The hydrophobic residues V121, V143, L198, and W209 that surround the deep water O_{DW} are not shown for clarity.

water" and is also well-ordered. It is thought that CO_2 binding in the hydrophobic region (Val121, Val143, Leu198, and Trp209) in the active site displaces the solvent molecule W_{DW} that is hydrogen-bonded to the amide group of Thr199.[25]

Investigations replacing residues in the active-site cavity of HCA II have determined that several of these side chains function to mediate the properties of His64 to maximum efficiency in proton transfer. Replacements in the active-site cavity at Tyr7, Asn62, and Asn67 with hydrophobic residues of about the same size (Y7F, N62L, and N67L) showed in each case altered properties of His64, either in its preferred orientation and/or in its pK_a and often in the altered structure of the solvent in the active-site cavity.[20] For example, the crystal structure of N67L HCA II showed His64 in the outward orientation and the solvent structure disrupted, and this was accompanied by a decrease in the rate constant for proton transfer of up to 6-fold.

The mutant in which Tyr7 was replaced with Phe (Y7F HCA II) had a rate constant for proton transfer at 7 μs^{-1} in the catalyzed dehydration direction, which is greater than the wild type by 7-fold.[20] This enhanced proton transfer is attributed to a number of possible causes. First, the pK_a = 6 of His64 in Y7F is more acidic than that of the wild type (pK_a = 7) and hence is expected to be a more efficient proton donor in catalysis of the dehydration reaction. Second, the predominantly inward orientation of His64 in Y7F may contribute to enhanced proton transfer. This inward orientation may be more significant in measurements of catalysis at chemical equilibrium by ^{18}O exchange between CO_2 and water. In such an isotope exchange experiment, there is no required flux of protons from solution to the active site and the proximity of His64 in the inward orientation provides a nearby protonatable site that participates in catalysis. The predominantly inward orientation of His64 in Y7F may be less significant in steady-state experiments, in which a net flux of protons to the active site is required to sustain catalysis in the dehydration direction. It is in this case that the mobility of His64 may be more pertinent.

Finally, the observed solvent structure was somewhat simplified because the solvent molecule W3a was displaced in the Y7F HCA II crystal structure, reducing the solvent array in Y7F HCA II to an unbranched two hydrogen-bonded network between His64 and the zinc-bound solvent. This simplified solvent network in Y7F HCA II may also contribute to the efficiency of proton transfer, because many *in silico* studies suggest that such a nonbranched array of the solvent enhances proton transfer by avoiding the formation of an "Eigen"-like solvent structure $[H_9O_4^+$, that is $H_3O^+(H_2O)_3]$ in the active-site cavity.[26–28] The stability of Y7F HCA II was found to be considerably less than wild-type HCA II,[20] which may explain why the Y7F variant is not found in nature.

Although the effect of the replacement of residues lining the active-site cavity on the structure and catalysis is complex, the data do lead to a significant conclusion. These hydrophilic residues (Tyr7, Asn62, and Asn67) that line the active-site cavity appear to finely tune the proton-transfer efficiency of His64 (eq 2), while having a much smaller effect on the interconversion of CO_2 and bicarbonate (eq 1), a separate and distinct step which occurs some distance away (7–10 Å) at the zinc. It is notable that these residues are invariant in CA II from a wide range of species from chicken, rodents, bovine, to humans.[29] Moreover, these residues are not conserved in isozymes of carbonic anhydrase in the α class that do not have histidine at residue 64, such as HCA III with Lys64 and Arg67 and HCA V with Tyr64, Thr62, and Gln67.[29]

Mapping Out Proton Transfer in the Active-Site Cavity

The facility with which the proton transfer can be measured between His64 and the zinc-bound solvent molecule suggests mapping out proton transfer in the active-site cavity by placing histidine residues at strategic locations. This approach was first taken by Liang et al.[30] and consists of making a series of double mutants with His64 replaced by Ala and then replacing residues in the active-site cavity with His. X-ray crystal structures show that these substituted residues mostly extend into the cavity and their replacement with His causes minimal structural changes but do however alter the position of the ordered solvent in the active-site cavity compared to the wild-type. The results reflect the efficiency of intraprotein proton transfer. There are very useful controls, one of which is the integrity

Table 1. Rate Constants at 25 °C for Proton Transfer from Various Sites in Human Carbonic Anhydrase II and Site-Specific Mutants

enzyme	donor	distance to Zn[a] (Å)	k_B (ms^{-1})	reference
H64A	4-MI	4.8	inhibitory	46
H64A-T200H	His200	5.2	~300	31
H64A-N67H	His67	6.6	200	13
wild type	His64	7.5	800	13
H64W	4-MI[b]	8.0	130	45
H64A-N62H	His62	8.2	~30	13
H64A	4-MI[c]	12	0	16

[a] Distance between the zinc and N1 or N3 of the imidazole donor. [b] 4-Methylimidazole bound at Trp64. [c] 4-Methylimidazole bound at Trp5.

of the overall X-ray crystal structures of the enzymes. A second control is unaltered values of k_{cat}/K_m, which report the rate of hydration of CO_2 (eq 1), rate constants, which are frequently not affected by replacements of amino acids in the vicinity of His64 or more distant from the zinc.

A series of reports characterize the properties of variants replacing hydrophilic residues with His in the active-site cavity of H64A HCA II. These were done using stopped flow in initial velocity studies[30] or using ^{18}O exchange between CO_2 and water at chemical equilibrium;[13,31] the results of these experiments appear in agreement. His62 and His65 showed minimal proton transfer; however, His67 and His200 showed that histidine at these sites participates in proton transfer. For H64A–N67H HCA II, the rate constant for proton transfer was as great as 25% of the wild type.[13,30] The rate constants and distances between proton donors and zinc are given in Table 1 for these and other results discussed below. A study of variants of HCA II with replacements at residue 64 show that Lys64 or Glu64 can function as proton shuttles; however, their efficiency in proton transfer is much less, typically 5–10% of that of His64 in wild-type HCA II.[32]

It is evident from such data (Table 1) that wild-type HCA II has evolved with the proton shuttle placed at a residue that attains maximal efficiency in proton transfer. However, can we discern from these experiments key factors that account for the efficiency of His64 and His67 compared to His residues at other positions, for example, His62? In X-ray crystal structures, the side chains of both His67 and His62 appear hydrogen-bonded to other side chains (Asn62 and Asn67, respectively). However, there is a feature of these structures that may be a clue to efficiency in proton transfer. The side chains of His64 in the wild type and His67 in H64A–N67H can be connected to the zinc-bound solvent molecule by a hydrogen-bonded chain of two solvent molecules.[13] The distance between the side chain of His62 and the zinc-bound solvent is greater (Table 1) and is spanned by at least three solvent molecules.[13] It may be the closer distance of His67 rather than the specific structure of the ordered solvent that is significant here; however, we believe that the ordered solvent structure is certainly an important clue to the rate of proton transfer. It is interesting that the apparent hydrogen bonds of these residues to nearby side chains do not appear to be an important factor in proton transfer during catalysis, even though these hydrogen bonds could restrict side-chain mobility.

The examination of the variant H64A–T200H HCA II became interesting from several viewpoints. First, His200 is present in wild-type HCA I, which also has His64 and is characterized by a value of k_{cat} at 0.2 μs^{-1}, less than k_{cat} of 1 μs^{-1} for HCA II.[3,33] In addition, X-ray crystal structures show that the side chain of His200 in H64A–T200H HCA II forms a direct hydrogen bond (no intervening solvent molecules) with the zinc-bound solvent.[31] Proton transfer during catalysis by H64A–T200H HCA II measured by the initial velocity of hydration and ^{18}O exchange amounted to about 5–40% of the wild type, depending upon conditions.[30,31] The smaller value of k_{cat} for this mutant compared to that of the wild type may reflect the position of His200 deep in the active-site cavity, too distant from the bulk solution for efficient proton flux to the solution. However, this establishes that a histidine directly hydrogen-bonded with the zinc-bound solvent can adopt a geometry to sustain proton transfer.

Similar studies have been reported, placing histidine residues at strategic sites within the active-site cavity of HCA III. This is of interest because the amino acid residues extending into the active-site cavity are very different in CA III compared to CA II, although their backbone structures are highly homologous.[34–36] However, the proton-transfer-dominated values of k_{cat} for HCA III are 10^3 lower than that for HCA II.[37] First, CA III lacks a proton shuttle residue with Lys64, which does not transfer protons efficiently in HCA III.[38] The difference in catalytic activity compared to CA II is also attributed to very different side chains extending into the active-site cavity, mainly Phe198, Arg67, and Arg91. Here, the placement of histidine residues at position 64 (K64H) and 67 (R67H) in HCA III shows that these histidines are proton shuttle residues[39] (His at residues 5, 7, and 20 were not proton shuttles).[39] Networks consisting of two solvent molecules could be modeled between the side chains of His64 and His67 to the zinc-bound solvent. These studies confirmed the conclusions on the work of HCA II of positions from which proton transfer could occur. The unusual aspect is the range of rate constants for proton transfer. The mutants of HCA III containing His residues had values of these rate constants that were at best 3% of that of His64 in wild-type HCA II, demonstrating that the active-site cavity of HCA III has not evolved for efficient proton transfer. In fact, despite the high abundance of HCA III in skeletal muscle, its function has not been determined.[40]

Properties of Proton Transfer from Small-Molecule Rescue

The term small-molecule rescue or chemical rescue refers to the enhancement of catalytic activity in a mutant enzyme by the addition of exogenous small molecules that take on, at least in part, the role of a replaced residue of the enzyme. The rescue by imidazole of the proton transfer accompanying catalysis by H64A HCA II was observed by Tu et al. in 1989,[15] the first example of rescue

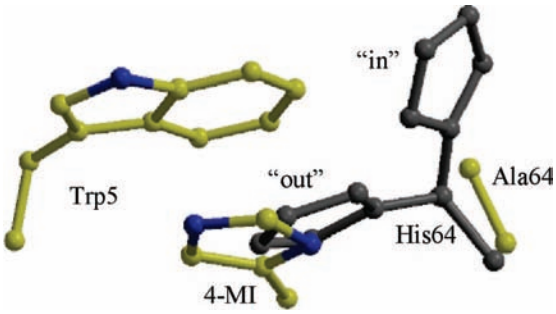

FIGURE 4. 4-Methyl imidazole-binding site.[42] Ball-and-stick representation of residues are as labeled. Superimposed structures of wild-type HCA II showing inward and outward orientations of His64 (gray) and 4-MI bound to Trp5 and Ala64 of H64A HCA II.

by imidazole and the second example of small-molecule rescue of a mutant enzyme. The first was the rescue of a mutant of aspartate amino transferase by Toney and Kirsh.[41] The rescue of H64A HCA II by imidazole and analogues is saturable,[15,42] and at the saturation level, the rate of proton transfer is close to that found in the wild type without the enhancement by imidazole analogues.[42] This observation is explained by two prominent hypotheses of proton transfer in this catalysis: (1) that imidazole binds at different sites in the active-site cavity but the mobility and ensemble of possible solvent structures allow proton transfer at equivalent rates, and/or (2) that imidazole in H64A binds near the side chain of His64 in the wild type. The first of these, proton-transfer efficiency at different sites, was described in the previous section and summarized by the data in Table 1. The second was investigated further by studies carried out to determine the site of binding of the exogenous proton donors/acceptors in the active-site cavity. Earlier studies had used X-ray crystallography to place histamine[43] and phenylalanine[44] in the active-site cavity. The question became whether the binding sites observed for exogenous proton donors/acceptors were productive in proton transfer.

For example, a binding site for 4-methyl imidazole (4-MI) in H64A HCA II was observed by X-ray crystallography at about 12 Å from the zinc, forming a π-stacking interaction with the indole ring of Trp5 (Figure 4).[42] However, the replacement of Trp5 in H64A HCA II with Ala, Leu, or Phe had no significant effect on the enhancement by 4-MI of maximal rate constants for proton transfer in catalysis.[16] This result indicates that 4-MI bound to Trp5 is a nonproductive binding site for proton transfer in H64A HCA II; that is, it does not significantly contribute to the rescue of proton transfer. At this site, the N1 and N3 positions of the bound imidazole ring of 4-MI are 13.4 and 12.1 Å, respectively, from the zinc. A comparison with the distances and rate constants for proton transfer of Table 1 indicates that this is probably too distant for effective proton transfer on the scale of catalysis of the wild type. Another interesting feature of this binding site associated with Trp5 is that 4-MI overlaps the outward orientation of the side chain of His64 in the wild-type enzyme (Figures 2 and 4). This has been interpreted to indicate that the outward position of His64 considered alone is unlikely to make a significant contribution to proton transfer.[16] That is, either the inward position is effective in proton transfer or the side chain of His64 must be sufficiently flexible to occupy both the inward and outward positions.

Repeated failures to find a productive binding site for 4-MI as a chemical rescue agent for H64A HCA II were a motivation to engineer a productive binding site into the active-site cavity. This was performed on the basis of the observed binding of 4-MI to the indole ring of Trp5 and the known role of His64 as a proton shuttle. To this end, a mutant HCA II was constructed with Trp replacing His64 and a second mutant HCA II was constructed also with Trp5 replaced by Ala. This was intended to place the indole ring of Trp, a binding site for 4-MI, closer to the zinc, in the vicinity of the imidazole ring of His64 of the

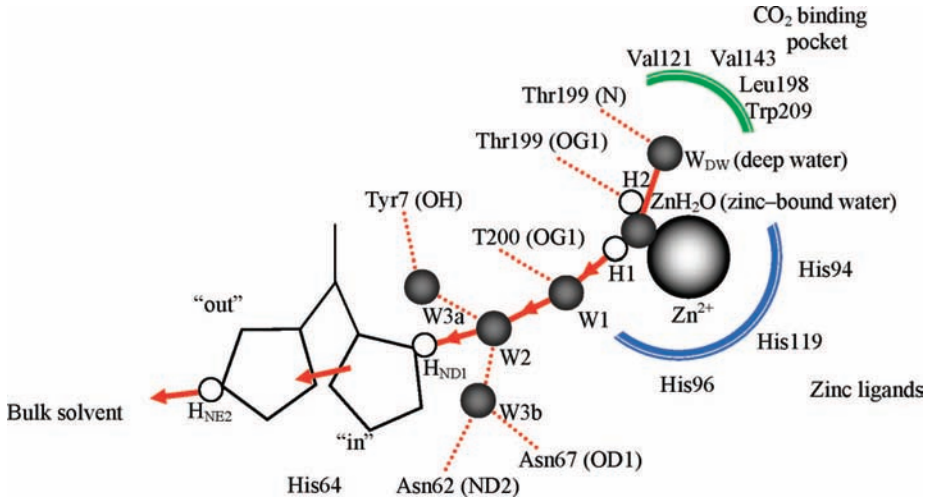

FIGURE 5. Proposed proton-transfer pathway.[20] Shown are the geometry and interactions of the active-site amino acids and solvent arrangement of HCA II. The large black, small black, and open white circles represent the zinc, solvent oxygen, and hydrogen atoms, respectively. The inward and outward conformations of His64 are depicted as open pentagons, and the singly protonated sites are as labeled. The zinc-bound water ZnH_2O and deep water W_{DW} are as labeled. The proposed proton-transfer pathway steps are marked with red arrows. Additional, stabilizing hydrogen bonds are shown as red dotted lines. The zinc-coordinating histidines and hydrophobic CO_2 pockets are shown as open blue and green curved lines, respectively.

wild type. The expected activation was observed.[45] The rate constant for proton transfer during catalysis was enhanced in a saturable manner by small molecules that were imidazole and pyridine derivatives. The saturation level for activation by 4-MI was approximately equivalent to that of the wild-type enzyme.[45] X-ray crystallography showed the binding of 4-MI in a π-stacked interaction with the indole ring of Trp64 and made a hydrogen bond with the side-chain hydroxyl of Thr200 in W5A–H64W HCA II.[45] The bound 4-MI is in a position that appears to straddle the inward and outward positions of His64 in the wild type at ~8 Å from the zinc.

Concluding Remarks

High-resolution X-ray crystal structures of HCA II provide abundant detail on an ordered solvent structure extending between the proton donor and acceptor of catalysis, the conformations of the side chain of His64, and the nature of the zinc-bound solvent molecule. The exact relation of this structure to the pathway for proton transfer is unknown, but certainly, the ordered solvent structure provides strong clues. The zinc-bound solvent hydrogen H2 contributes to a hydrogen bond with W_{DW} (Figure 5). Hence, it would be the proton H1 on the zinc-bound solvent that would be most likely transferred onward to W1, promoting proton transfer out of the active site.

The proximity of His64 to the solvent network, its apparent flexibility, and studies involving mutagenesis and kinetic measurements are all consistent with His64 acting as a proton shuttle. The concept of a shuttle implies the translocation of the proton by picking up a proton when His64 is in the inward position, moving to the outward position, and transferring it to an acceptor in the bulk solvent (Figure 5). The geometry and putative hydrogen atom, H_{ND1}, of His64 in the inward conformation has provided clues to show that W2 is the most likely solvent molecule that satisfies the proton-transfer role. W2 is the only one of the cluster of three waters (W2, W3a, and W3b) to lie in the plane of the imidazole ring of His64, is in close enough proximity to His64 (3.3 Å) to make a weak hydrogen bond, and has the additional electron density of the H_{ND1} of His64 to indicate that it is protonated. This implies that, although there is branching in the solvent network, the additional solvent molecules W3a and W3b play a supporting role for W2. Hence, we propose that the proton transfer from the zinc-bound solvent proceeds in the following manner: Zn–H_2O → W1 → W2 → inward His64(ND1) → outward His64(NE2) → bulk solution (Figure 5).

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