Binding of Bicarbonate to Human Carbonic Anhydrase II: A Continuum of Binding States

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Abstract: Herein, we report molecular dynamics simulations of the enzyme human carbonic anhydrase II (HCAII) complexed with the substrate molecule bicarbonate using a quantum mechanical/molecular mechanical (QM/MM) coupled potential. This study provides novel insights into bicarbonate binding and loss. In particular, we find that a structure related to the so-called Lipscomb binding motif is the global minimum, while the first formed Lindskog binding mode is unstable relative to alternative binding modes. From our simulated results we are able to postulate why Thr-199 destabilizes bicarbonate binding to HCAII (relative to the Ala-199 mutant) and how bicarbonate ion is displace by water to form the zinc−water form of HCAII. This study also demonstrates the capability of QM/MM methods to elucidate structural and mechanistic aspects of enzyme mechanisms.

Introduction

The relationship of enzyme structure to function has been studied in great detail over the years.[1] However, the relationship of enzyme dynamics to enzyme function is poorly understood, but it clearly can have a profound influence on the reactivity of enzymes.[2−4] One promising way in which to address these issues is through the use of theoretical methodologies and in particular, the quantum mechanical/molecular mechanical (QM/MM) methodologies[5] promise to be quite useful in this regard.[6] Purely classical models of proteins have been very useful in obtaining dynamical insights, but they utilize a fixed charge model and are not capable of addressing charge reorganization as a function of geometric variables, *etc*. Furthermore, they incorporate harmonic bonds, which cannot undergo bond breaking or "covalent" reorganization (*i.e.*, going from a four- to five-coordinated state) as a function of time. QM/MM methods, on the other hand, are able to do this to a limited extent. Thus, affects arising from bond fluctuations, *etc*., on an enzyme active site modeled by a QM method can be studied using QM/MM methods.

In order to begin to better understand the relationship between enzyme dynamics and function using computational methods, one needs to work with a system that is well characterized both structurally and biochemically. Human carbonic anhydrase II (HCAII),[7−9] one of seven isozymes of the zinc metalloprotein human carbonic anhydrase (HCA) family,[10] provides just such a system. HCAII is a 259 amino acid residue protein with a mass of ∼29 kDa that contains a single zinc atom in its active site that is necessary for catalytic activity.[7−9] The active site itself lies at the bottom of a deep cavity (15 Å deep) in the protein, which is readily accessible to solvent.[11] The active site cavity is divided into hydrophobic and hydrophilic regions, with a network of hydrogen-bonded water molecules connecting the active site region and the surrounding solvent environment.[11] The catalytically necessary zinc ion lies at the bottom of the active site cleft and is tetrahedrally coordinated by three Histidine residues (His-94, -96, and -119) and a fourth ligand,[11] whose identity is pH dependent. At high pH (>8) the fourth ligand is an hydroxide ion, while at acidic pH, the fourth coordination site is occupied by a water molecule.[7−9]

The catalytic mechanism of HCAII has been studied in detail, yet much still remains unclear.[7−9] Catalysis has been found to depend upon a group with a pK_a of around 7, with the fourth zinc ligand (hydroxide/water) appearing to fulfill this requirement. This consideration, in conjunction with the observed ping-pong kinetics, gave rise to the mechanism shown in Scheme 1.[9] The proton transfer step converting **D** into **E** has been implicated as the rate-limiting step at high concentrations of external buffer, while **E** to **A** is thought to be rate limiting at low buffer concentrations.[7−9] The conversion of **D** to **A** is kinetically distinct from the sequence of steps converting **A** into **D**, *via* **B** and **C**. Although experiments have not completely elucidated the detailed structural changes in the mechanism for catalysis, there is considerable evidence that certain residues are catalytically important (see Scheme 2). These include His-64, Glu-106, Thr-199, and several water molecules near the active site.[7−9] Thr-199 is positioned with Thr-200 on the opposite side of the active site cavity from the zinc atom. These Thr residues, His-64 (located at the entrance of the active site cavity), and Glu-106 combine with other polar residues to constitute the hydrophilic half of the cavity. Thr-199 is an

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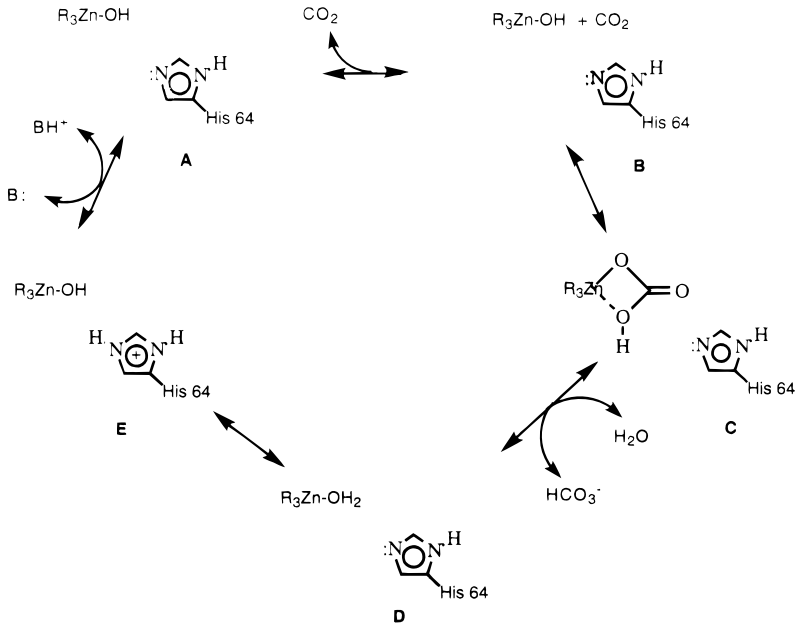
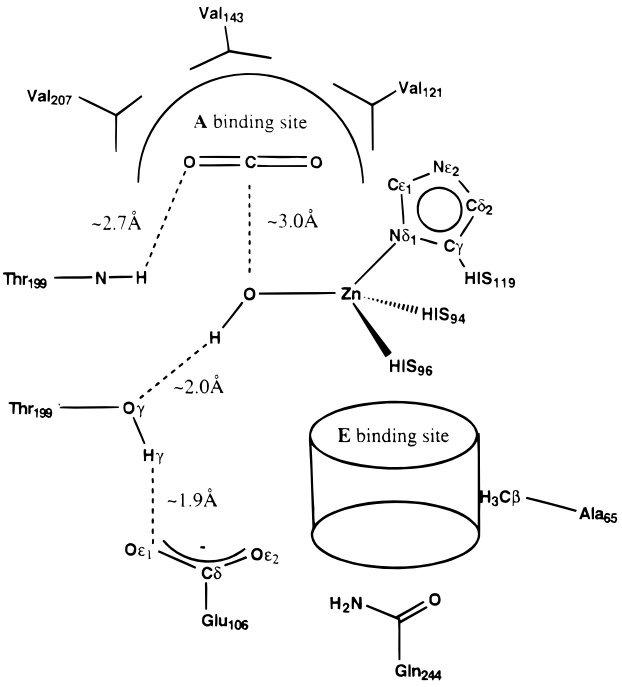
Scheme 1



Scheme 2



important residue which is centered between the hydrophilic and hydrophobic halves of the cavity. It is locked into this position as part of a key hydrogen-bonding network. The hydrogen of the hydroxide/water zinc ligand is donated for hydrogen bonding to the γ-oxygen of Thr-199. The proton of the hydroxyl group on Thr-199 is then in turn donated to an ϵ-oxygen of Glu-106, forming a second hydrogen bond interaction (see Scheme 2). The proximity of Thr-199 to the active site zinc atom and the rigidity of this hydrogen bond network are considered to be crucial for catalysis and inhibitor binding.[11,12] This group of hydrogen bonds may also play an important role in CO_2 binding and catalysis. It has been suggested that this hydrogen bond network serves to properly orient the lone pair electrons of the hydroxide ligand, allowing for rapid addition to CO_2, as the CO_2 molecule approaches from the hydrophobic cavity (**A** binding site in Scheme 2).[12−14] Bordering on this hydrogen bond network is a group of eight water molecules which extends toward bulk water. It has been proposed that these water molecules serve to shuttle a proton out of the active site and into bulk solution *via* His-64.[7]

As alluded to above there is an alternative catalytic pathway to the "Lindskog" mechanism given in Scheme 1. In most of the details the two mechanistic schemes are essentially identical, but when it comes to the mode of binding of bicarbonate, the two mechanisms differ. In this so-called "Lipscomb" mechanism[15] the zinc bicarbonate form initially has the structure given as C1 (Scheme 3), where the carboxylate is pointing into the hydrophobic binding pocket labeled **A** in Scheme 2. This then undergoes a rearrangement to give the structure labeled C2 in Scheme 3 where the carboxylate group of the bicarbonate anion is bound directly to the zinc ion. The weakness in this mechanism is the requirement that a bicarbonate oxygen be in close proximity to the hydroxyl oxygen of Thr-199. This unfavorable electrostatic interaction presumably destabilizes this form of the bicarbonate complex to such an extent that it is not the favored bicarbonate binding mode.[16] However, the suggestion that the azide anion (or bromide ion)[17,18] can bind without forming a hydrogen-bonding interaction with the hydroxyl oxygen of Thr-199 has reopened this debate regarding the catalytic mechanism of HCAII.[17,18]

Besides the controversy surrounding the mechanistic details described above, the favored binding mode of bicarbonate to human carbonic anhydrase II (HCAII) has generated an extensive amount of controversy over the last decade.[8,9,15,16,19,20] Two structures for this complex have been suggested in order to explain HCAII catalysis.[9,15] The first structure is given in

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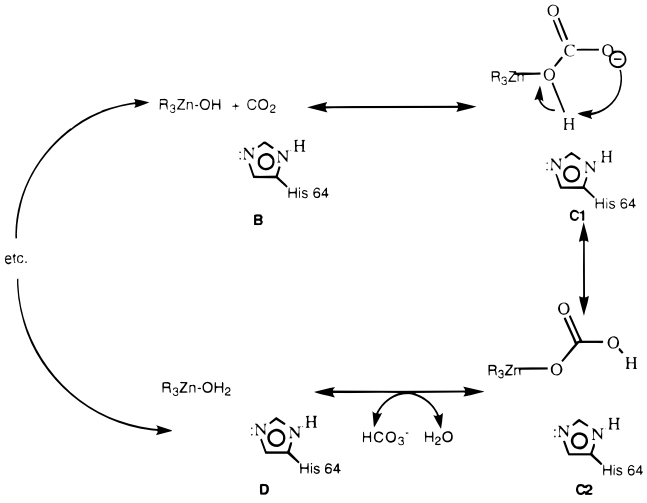
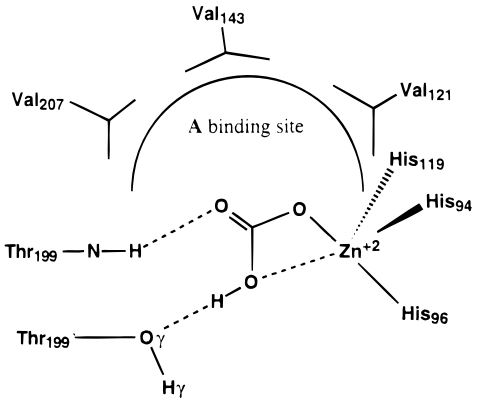
Scheme 3



Scheme 4 and has been termed the Lindskog structure (also see Scheme 1).[9] In this structure one of the carboxylate oxygens is bound to the zinc ion, while the second zinc ligand from bicarbonate is the hydroxyl oxygen. The second structure (Scheme 5) that has been put forward is the termed the Lipscomb structure, and in this complex, both of the carboxylate oxygens are utilized as zinc ligands.[15,19,20] In this binding mode it has been envisioned that Thr-199 does not hydrogen bond with zinc-bound bicarbonate,[21] but forms a close van der Waals contact much like that observed for the HCAII/azide complex.[17,18] An X-ray structure for a mutant HCAII (Thr-200 → His-200)−bicarbonate complex has been determined at 1.9 Å resolution.[22] The structure obtained from this study is given in Scheme 6. These authors used this structure to argue for the Lindskog structure as being the catalytically competent structure in native HCAII. However, in this mutant HCAII, the bicarbonate ion becomes a weak inhibitor which is not the case in native HCAII. Thus, the relevance of this structure to native HCAII can be brought into question. The authors also note that the estimated error in the atomic coordinates is ±0.16 Å, which would readily allow a Lindskog-like structure to be converted into a Lipscomb-like structure.[22] The crystal structure of HCAI−bicarbonate complex[23] has also been determined, and its structure is given in Scheme 6 (these distances are given in parentheses). It is very similar to the Thr-200 → His-200 mutant structure, but interestingly, the zinc−bicarbonate distances are longer in one instance (2.5 → 3.1 Å) and shorter in another (2.2 → 1.8 Å). The complex between bicarbonate and HCAII where the zinc has been replaced by Co(II) has also been reported,[24] but since Co(II) has a different coordination chemistry than Zn(II), it is not clear how relevant this structure is to Zn(II)-catalyzed CO_2 hydration. Thus, it is not unexpected that the Co(II)-substituted HCAII gives a six-coordinated complex around the metal ion (three His ligands, two from bicarbonate and one water molecule). Clearly, in the absence of knowing where the proton is in these structures, it is difficult to definitively identify the binding mode of bicarbonate to the zinc ion in HCAII.

At this point it is generally agreed that the Lindskog structure or something resembling it (*e.g.*, see C1 in Scheme 3) is formed

Scheme 4



LINDSKOG

during the catalytic cycle.[13,14,25−27] The best evidence for this is the importance of the **A** binding pocket to HCAII catalysis.[13,14,25−27] This site appears to be important in CO_2 recognition and subsequent reaction to form the Lindskog bicarbonate structure. Whether this complex then rearranges to arrive at an alternate structure is the critical issue. On the basis of force field calculations, it has been argued by us[16] that the Lindskog structure is the favored bicarbonate binding mode, but this study used fixed geometries derived from gas-phase *ab initio* calculations and, thus, does not allow for geometric relaxation to occur in response to the molecular environment. In order to further understand the structure and dynamics of bicarbonate binding to HCAII requires the use of a method other than a purely classical approach. Thus, in the present manuscript, we use the QM/MM approach in conjunction with MD simulations[6,28] to probe the binding of the bicarbonate ion to HCAII.

Computational Methods

QM/MM Coupled Potential. The theoretical basis of the QM/MM method has already been extensively outlined in numerous publications, so we will only comment upon some significant technical details of our implementation.[29−32] The method we have developed couples together the MD program AMBER 4.0[33] and the semiempirical quantum mechanical program MOPAC 5.0. We have used standard AMBER force field parameters throughout,[34,35] except for the active site region, where we have used the MM parameters of Hoops *et al.* developed especially for HCAII.[36] Similar to the work of others[29,37,38] we have

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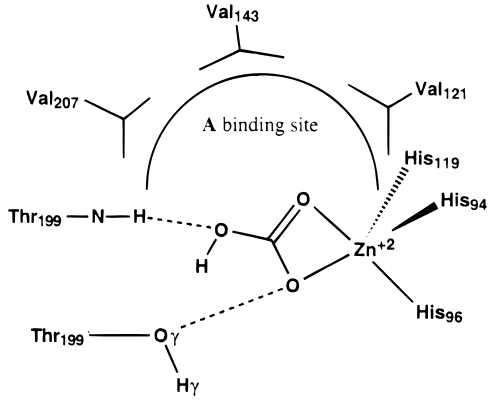
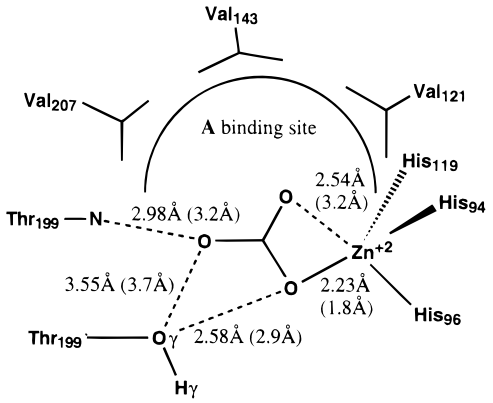
Scheme 5



LIPSCOMB

Scheme 6



EXPERIMENTAL

used link atoms to cap exposed valence sites due to bonds which cross the QM−MM boundary. In this method the QM region of the system is treated as a closed shell molecule with no exposed valence sites. In our system the imidazole rings are capped by hydrogen atoms at the C-γ carbon atom. These carbon atoms (three total) are then bonded to their respective C-β carbon atoms through molecular mechanical bonds (using a standard AMBER carbon−carbon parameter set) between the two carbon atoms. Finally, the nonbonded interactions between the capping atoms and the remainder of the protein molecule are not evaluated.

Computational Protocol. Starting coordinates for the HCAII−bicarbonate complex and the crystallographically located water molecules were taken from the experimental crystal structure of Xue *et al*.[22] TIP3P[39] water molecules were added, where possible, around the active site zinc atom to a distance of 15.0 Å using the EDIT module of AMBER 4.0.[33] All residues were represented using the AMBER united atom model,[34] except for the active site residues His-94, -96, and -119, which were represented as all atom residues. Since the structure of Xue *et al.*[22] was of the His-200 mutant of HCAII, we first altered this residue to Thr to generate the native sequence of HCAII. This was done using computer graphics in conjunction with the known position of Thr-200 from the X-ray structure of the native enzyme.[40] Initial minimization (500 steps steepest descent followed by 1500 steps conjugate gradient) of all atoms was carried out for the Lipscomb and Lindskog forms of the HCAII−bicarbonate complex using an all MM model and a standard version of AMBER 4.0. Following removal of bad contacts by MM minimization, a second set of minimizations were carried out using a modified version of AMBER 4.0, in which the residues His-94, -96, and -119 and the active site zinc atom and its fourth ligand (bicarbonate) were treated as QM atoms using the PM3 Hamiltonian (for a total of 33 QM atoms).[41−43] The junction between the QM and MM regions was made between C-β and C-γ of the His residues. In order to preserve integral charge in the MM region, the partial charges of the β-carbons of the QM His residues and the hydrogens attached to these carbons were changed to −0.080 and 0.048, respectively. For all of the QM/MM minimizations 1000 steps of steepest descent were used followed by 1000 steps of conjugate gradient. In all cases the structures were not fully minimized since we were preparing the system to begin MD simulations and were mostly interested in removing "bad" inter- and intramolecular contacts.

Following the second set of energy minimizations, MD simulations were carried out on the two forms of the zinc bicarbonate complex of HCAII. A 15 Å sphere was defined around the active site zinc atom, and only residues within this sphere as well as the cap water molecules were allowed to move during the MD simulations. The MD simulations covered 150 ps. The temperature was raised from 0 to 300 K during the first 6 ps of simulation, and the temperature was then maintained at 300 K by coupling to a constant-temperature heat bath.[44] A 10 Å nonbond cutoff distance was used, and the nonbond pair list was updated every 25 time steps. The SHAKE algorithm was used to constrain all bonds between pairs of MM atoms.[45] A 1 fs time step was employed during the MD simulations, and over the last 48 ps, coordinates were saved for analysis every 25 (*i.e.*, every 0.025 ps) time steps.

Results and Discussion

Energy Minimization. The first question we addressed is the accuracy of the PM3 semiempirical Hamiltonian[42] to model zinc−bicarbonate complexes. In an earlier publication[16] we reported good quality *ab initio* calculations on both the Lipscomb- and Lindskog-like zinc bicarbonate complexes. These were gas-phase calculations where the zinc ligand was ammonia instead of the imidazole ring observed in the His amino acid residue. Nonetheless, these simpler models afford us the ability to test the quality of PM3. The energy difference calculated using *ab initio* methods was 9.6 kcal/mol[16] (favoring Lipscomb), while that calculated using PM3 is 7.4 kcal/mol, again favoring the Lipscomb structure. Thus, the PM3 Hamiltonian is able to capture the energy difference between the Lipscomb- and Lindskog-like structures reasonably well. There are some structural differences, but the observation of most note is the tendency of PM3 to give slightly longer Zn−O bond distances (Lindskog: *ab initio*, 1.88 and 2.71 Å; PM3, 1.96 and 2.82 Å. Lipscomb: *ab initio*, 1.98 and 2.2 Å; PM3, 2.02 and 2.71 Å.). Overall, though, these observations give us confidence in the accuracy of the results described below using the coupled PM3/MM method. A further PM3 gas-phase minimization was also done where the ammonia ligands were replaced by imidazole rings. From this calculation we find that the Lipscomb-like structure is 10.4 kcal/mol more stable than the Lindskog structure. Thus, whether the zinc ligand is ammonia or imidazole, we find that the Lipscomb structure is favored in the gas phase.

We began our QM/MM calculations by carrying out energy minimization studies on the two previously proposed bicarbonate binding modes. The minimized structure and energy of these two complexes are given in Figure 1. The PM3/MM calculated heat of formation of the Lindskog structure (top panel in Figure 1) is less negative than that obtained for the Lipscomb-like structure (bottom panel in Figure 1) by 8.1 kcal/mol. This

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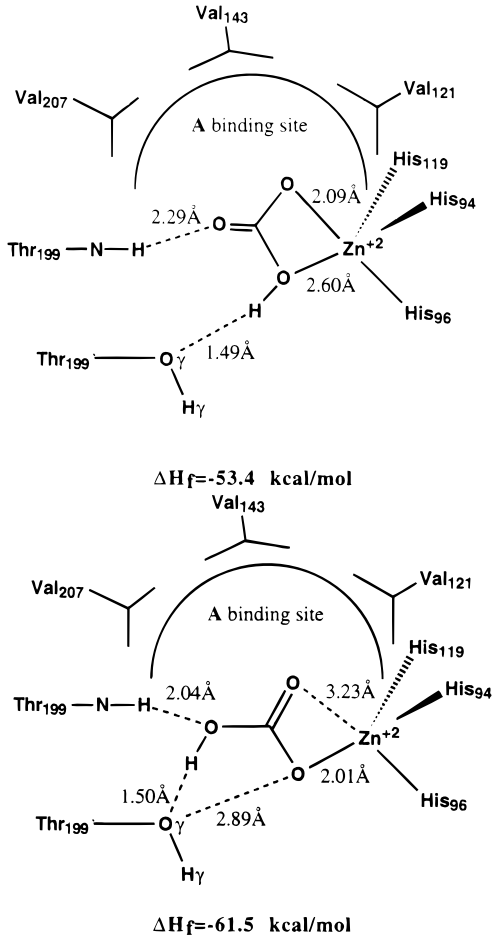


Figure 1. PM3/MM energy-minimized structures for the Lindskog (top panel) and Lipscomb zinc−bicarbonate binding modes.

strongly suggests that the Lipscomb-like structure is favored. The calculated total energies also favor the Lipscomb structure by a large amount; however, since these do not represent fully minimized structures, there is some uncertainty in this conclusion. The MD simulation results give more definitive results as described below. Neither of these structures is in excellent agreement with the experimental structure given in Scheme 6, but the calculated Lipscomb structure gives the best agreement with the trends observed in the zinc−oxygen distances. However, the experimental structure is of a mutant HCAII (Thr-200 → His-200) and the error in the atomic coordinates of the X-ray structure has been estimated to be ±0.16 Å,[22] which gives enough structural "play" to allow either computed structure to come into reasonable agreement with experiment.

While these QM/MM energy minimization studies are unable to provide definitive energetic information they are able to provide some interesting structural observations. Firstly, we do not observe a bicarbonate binding mode related to C1 in Scheme 3. This structure is unstable relative to the Lindskog structure given in Scheme 1 or 4. This is not surprising since C1 contains a free carboxylate, while the Lindskog structure allows the negative charge on the carboxylate group to favorably interact with the zinc ion. The second interesting observation has to do with the observation of a hydrogen bond between Thr-199 and the bicarbonate ion (see Figure 1) in the Lipscomb structure. As mentioned above, it had been postulated[21] that bicarbonate bound in the Lipscomb fashion could not hydrogen bond with the hydroxyl oxygen of Thr-199, but we observe that it can, and indeed, the available experimental evidence can be interpreted in support of this hydrogen bonding interaction.[12,23] This point will be touched on further below.

Molecular Dynamics Simulations. It is well-known that energy minimization studies of enzymes can lead to artifacts associated with local minimum traps.[4] Thus, it is important that MD simulations be done to ensure that the minimum states identified are actually stable and long-lived. We first began an MD simulation on the Lindskog structure. This structure was stable for ∼30 ps, at which time it underwent a conformational change that generated an alternative structure. Clearly, we find that the Lindskog structure is unstable relative to other possible structures. The new structure we observe is given in Figure 2. In this structure the bicarbonate hydroxyl hydrogen interacts with the carboxyl group of Glu-106, which results in the elongation of the hydrogen bond between Thr-199 and Glu-106 from ∼1.6 to ∼3.1 Å. Besides this hydrogen bond, bicarbonate also interacts with the mainchain N-H groups of Thr-199 and Thr-200.

We next carried out an MD simulation where we started with the Lipscomb-like structure. We found that this structure was stable for the entire 150 ps of the MD trajectory, and the average structure (over the last 48 ps) is given in Figure 3a. For the final structure obtained after 150 ps of MD simulation see the stereoplot in Figure 3b. The hydrogen bond between Thr-199 and the bicarbonate hydroxyl hydrogen in this structure is quite reasonable and is consistent with the notion that the hydroxyl oxygen of Thr-199 plays a "gatekeeper" role in selecting what anions bind to the HCAII active site.[46] However, it has not been appreciated that bicarbonate can form a hydrogen bond with Thr-199[21] and it has been thought that bicarbonate may bind in a fashion analogous to that of the azide ion.[17,18] In the HCAII−azide complex, the azide anion does not form a hydrogen bond with Thr-199, but forms close van der Waals contacts with Thr-199, which does not alter the active site structure to a great extent.[17,18] This interaction, however, has been predicted by us to be electrostatically favorable since the central nitrogen of azide was found to bear a positive charge that could interact with the negatively charged hydroxyl oxygen of Thr-199.[28] Thus, we are left to conclude that Thr-199, in the case of bicarbonate, does, indeed, act as a gatekeeper. This leaves the HCAII−bromide complex as the only example where Thr-199 does not play a gatekeeper role.[17]

For the zinc coordination sphere we find that one carboxyl oxygen has a typical zinc−oxygen bond (∼2.0 Å), while the second oxygen bond is quite long (3.6 Å). This pattern is consistent with the experimental structure (see Scheme 6), but the one bond length is too short while the second is a bit longer. The hydrogen-bonding distances also differ from the experimental structure, with some being too short while others are a bit too long. However, by considering the error bars associated with the X-ray structure (∼±0.16 Å), we find that our simulated structure is qualitatively within this uncertainty range.

The two structures given in Figures 2 and 3 are the only two stable structures we were able to locate for the HCAII/bicarbonate structure. In order to determine the relative energy of these two states we calculated the average total energy (TE) of each of the systems. For the structure given in Figure 2 we determined a TE of −8154.3 kcal/mol (the total average potential energy (PE) was −9397.2 kcal/mol), while that for the structure given in Figure 3 was −8175.8 kcal/mol (the total average PE was −9418.2 kcal/mol). The PM3/MM heat of formation of the QM regions give values of −40.1 kcal/mol for the structure of Figure 2 and −43.1 kcal/mol for the Lipscomb structure. The differences in the average total energies predict that the Lipscomb-like structure is 21.5 kcal/

(46) Lindahl, M.; Svensson, L. A.; Liljas, A. *Proteins* **1993**, *15*, 177−182.

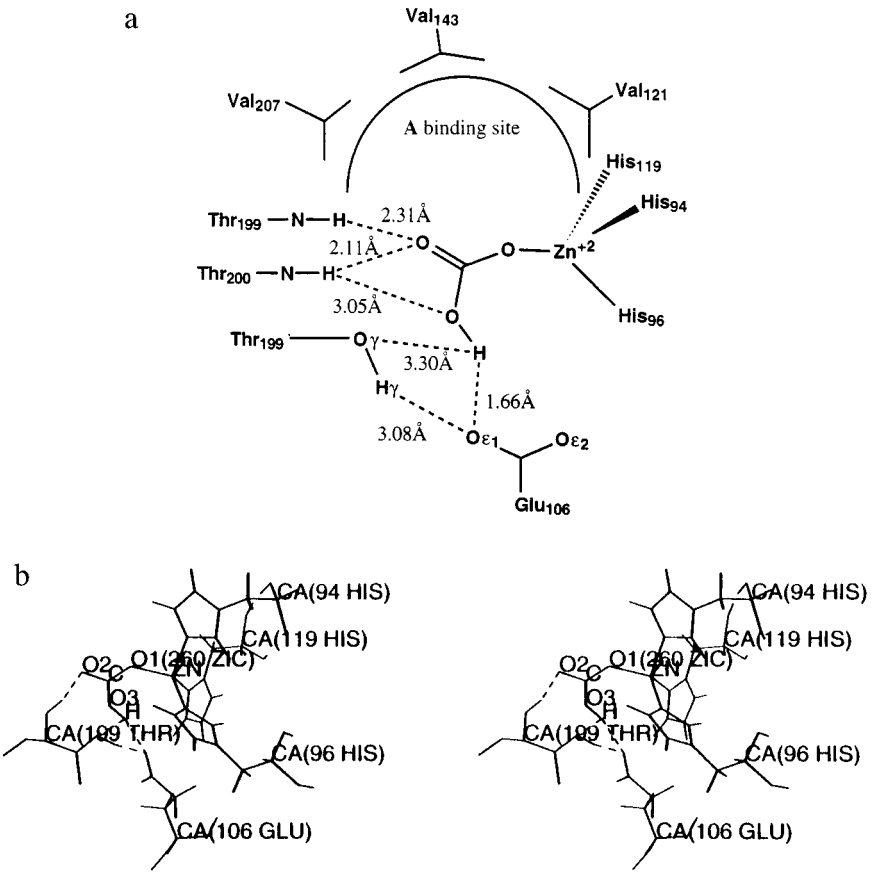


Figure 2. (a) PM3/MM average MD (last 48 ps) structure arising from a Lindskog zinc bicarbonate starting structure. (b) Stereoplot of the structure obtained after 150 ps of MD simulation.

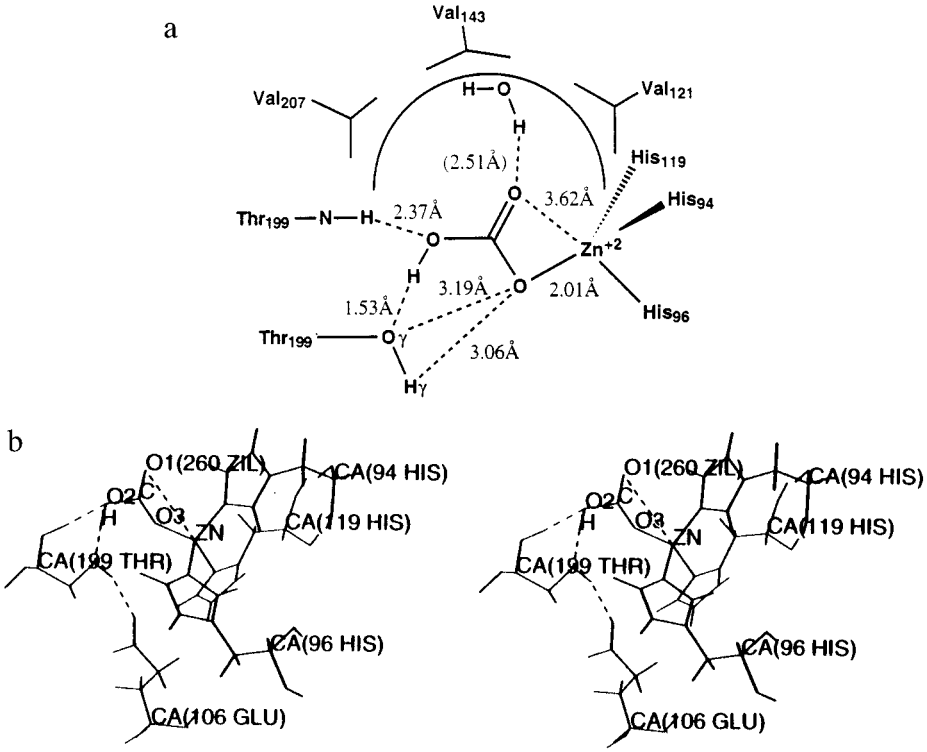
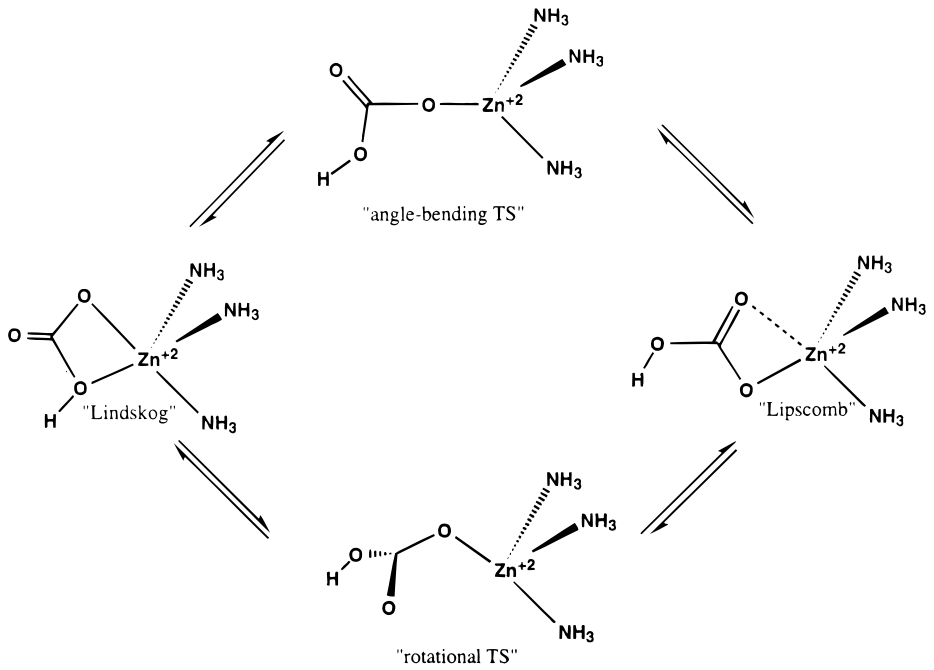


Figure 3. (a) PM3/MM average MD (last 48 ps) structure arising from a Lipscomb zinc bicarbonate starting structure. Distance given in parentheses is over the last 36 ps of the trajectory. This is because the water molecule is exchanging with other surrounding water molecules. (b) Stereoplot of the structure obtained after 150 ps of MD simulation.

mol more stable than the structure given in Figure 2, while the heat of formation difference is only 3 kcal/mol. We believe that the heat of formation energy difference is a better indicator of the energy difference between the two structures. The reason for this is that the total energy contains information on the entire system and, for example, if only a few water molecules are in a slightly different orientation on average during the simulations, we will also capture this energy difference. For the heat of

Scheme 7



EXPERIMENTAL

formation energy difference this only takes into account the fluctuations in the QM region of the system which is of the greatest interest to us. The one real approximation made in working with this energy difference is the fact that the van der Waals component between the QM and MM regions is assumed to be a constant between the two structures. This is not an unreasonable assumption in this case, but this does introduce some error. Nonetheless, we feel that our best estimate for the energy difference between these two structures is obtained from the differences in the average heat of formation (*i.e.*, ∼3.0 kcal/mol). What role might the structure of Figure 2 play in the catalytic mechanism of HCAII? This is described in more detail below, but through a simple opening up of the Zn−O−C angle we can readily generate the Lipscomb-like structure. Thus, this species might be an intermediate along the interconversion pathway from the Lindskog structure to the Lipscomb-like structure.

From the MD simulations it is hard to precisely assess how much it might cost energetically to interconvert between the Lindskog structure and the Lipscomb-like structure. In this case examination of gas-phase calculations would be illustrative in order to get an estimate of this quantity. There are two pathways that we have considered (see Scheme 7). In the first, we open up the Zn−O−C bond angle to generate the angle bending transition state (TS), and in the second, we rotate along the (Zn)−O−C−(O) bond to generate the rotational TS. In earlier work using good quality *ab initio* calculations (with NH_3 as the zinc ligands), we have estimated that the energy cost for the formation of the angle bending TS is ∼6.3 kcal/mol, and using PM3, we estimate that this barrier is ∼11.1 kcal/mol.[16] However, neither of these are true TSs for the interconversion of the Lipscomb and Lindskog structures in the gas phase (*i.e.*, they are so-called hilltops or second-order TSs). When we change the zinc ligands to imidazole the PM3 barrier height is reduced to 8.5 kcal/mol. If this trend holds in the *ab initio* calculations (*i.e.*, 2.6 kcal/mol decrease in barrier height on going from ammonia to imidazole ligands) we estimate that the *ab initio* barrier for this type of transformation would be ∼4 kcal/mol. A rotational TS was identified that interconverted these two structures, and the *ab initio* barrier is 4.1 kcal/mol, while the PM3 value is 2.8 kcal/mol. In the PM3 calculations when we replaced ammonia by imidazole as the zinc ligands the barrier height decreased to 0.2 kcal/mol. Again if this trend is followed by the *ab initio* calculations we estimate that the barrier height would be ∼1.5 kcal/mol. The simulated structure given in Figure 2 would require that the angle bending pathway be followed in order to interconvert to the Lipscomb structure, and from the previous analysis, our best estimate of the barrier would be ∼4 kcal/mol. This barrier height is well below the height of the barrier for the rate-determining step in HCAII catalysis and, therefore, fits into the overall mechanistic scheme. However, as noted above, this pathway does not proceed through a transition state but through a hilltop (*i.e.*, two negative eigenvalues from the vibrational frequency analysis) and this would require that within the enzyme this pathway would become a true transition state. This is not an unlikely possibility, but within the QM/MM method, we do not have a way in which to prove that this is, indeed, the case.

What do our observations predict for the catalytic mechanism for bicarbonate formation and loss. The mechanism we now propose is given in Figure 4. From our work[13,14] and others[21,27] it is clear that CO_2 binds in an hydrophobic pocket close to the zinc ion. This leads to the transition state we have labeled T1 in Figure 4, which in turn leads to the Lindskog binding mode which we have labeled as I1 for intermediate 1. We find no evidence for the intermediate proposed by Lipscomb where a free carboxylate projects into the hydrophobic pocket of HCAII (see C1 in Scheme 3). I1 is unstable (∼8 kcal/mol higher in energy than C2) and another intermediate (transition state?) is formed that we have labeled as I2. We estimate from out MD simulations that this intermediate or transition state is ∼3 kcal/mol higher in energy than C2. Simply by opening up the Zn−O−C bond angle in I2 we can directly form C2 (see Scheme 3), which is the Lipscomb binding mode. We estimate that the barrier for this interconversion is ∼4 kcal/mol. Alternatively, the rotational transition state pathway given in Scheme 7 could be followed, but from our MD simulations, we see no evidence that this occurs. If this pathway was followed we estimate, from

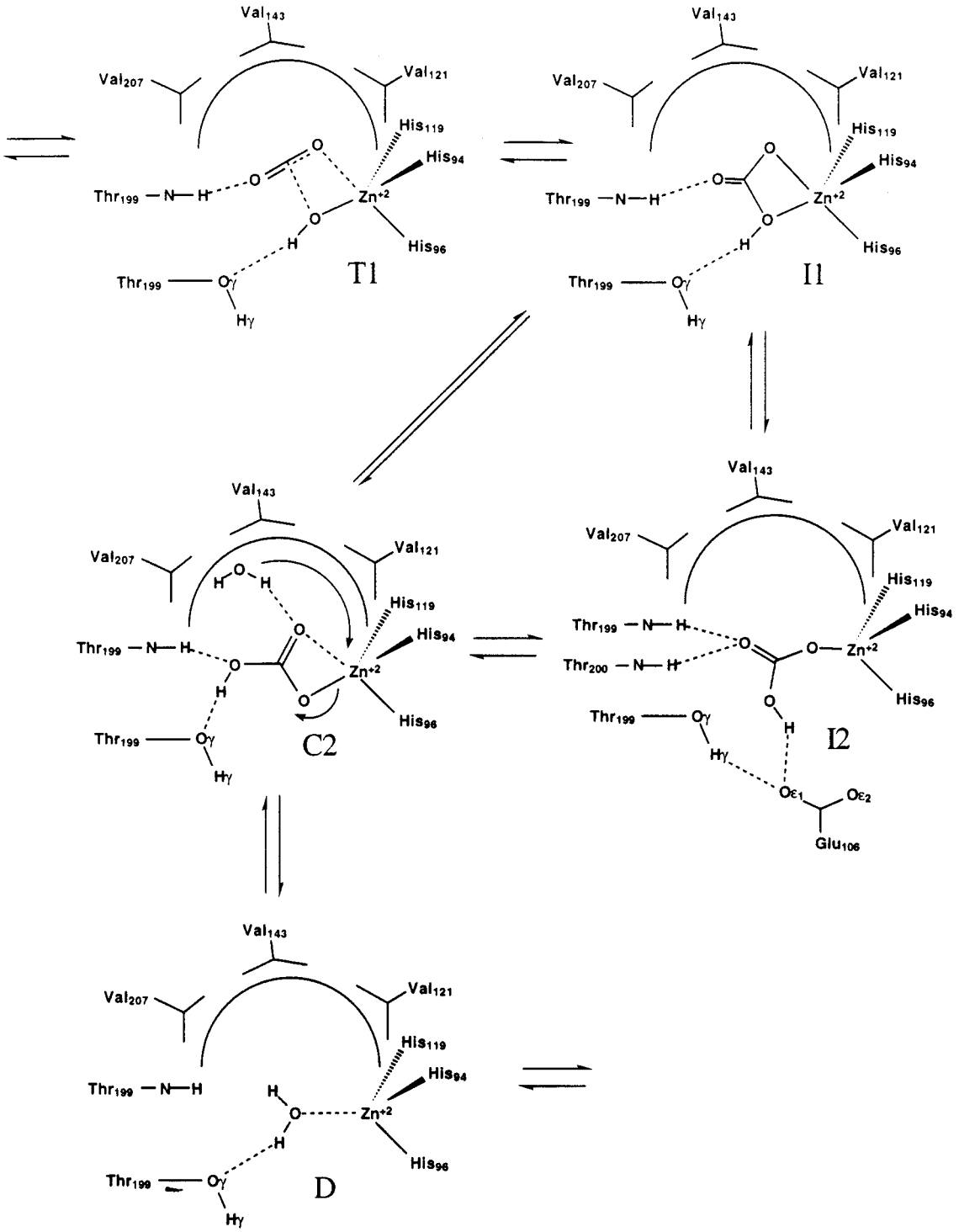


Figure 4. Bicarbonate binding and loss mechanism suggested by the PM3/MM results.

gas-phase calculations, that the barrier height would be quite low. Furthermore, we did not observe the formation of C2 via intramolecular proton transfer (I1 → C2) which has been found to have a large barrier (∼30 kcal/mol[16,19]). This barrier can be reduced when water molecules facilitate the proton transfer.[19] Thus, we cannot rule out this possibility at this time, but we have found a alternative, low-energy rearrangement pathways that can affect this transformation.

Another interesting feature that we observed in our MD simulations is the presence of a long-lived water molecule that resides in the "hydrophobic" pocket and is hydrogen bonded to the zinc-bound bicarbonate (see Figure 3). One of the carboxylate oxygens of bicarbonate has moved away from the zinc ion because of the formation of a hydrogen bond between bicarbonate and Thr-199 (see Figure 3). This then exposes the zinc ion to nucleophilic attack by the water ion which is in a suitable position to allow this to readily occur. This attack (see Figure 4: C2 → D) could result in the loss of bicarbonate directly or the formation of a transiently stable five-coordinated complex which then loses bicarbonate to generate the zinc−water form of the enzyme.

It has been observed[21,47,48] that Thr-199 destabilizes the binding of bicarbonate to HCAII by ∼0.8 kcal/mol (based on the Thr → Ala-199 mutant[47]). Can the new mechanistic model

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we propose explain this observation? From our MD results we believe that the following is occurring: We find that our version of the Lipscomb binding mode is able to hydrogen bond with Thr-199. In order to do this, however, one of the carboxylate oxygens (see Figure 3) must move away from the zinc ion. If this does not occur we retain repulsive interactions between the hydroxyl oxygen of Thr-199 and the other carboxylate oxygen of bicarbonate (see Scheme 5). Ideally, in the gas phase, the bicarbonate ion wants to keep both of the carboxylate oxygens near the zinc ion (our gas-phase *ab initio* results give one distance as 1.98 Å and the other as 2.21 Å[16]) to maintain the strongest electrostatic interactions possible. However, in the HCAII active site, this is not possible and to maintain maximum complimentarity with the HCAII active site one of the zinc−carboxylate oxygen distances increases. We postulate that increasing this bond distance is inherently destabilizing. To estimate how much this might cost we return to PM3 gas-phase calculations (the zinc ligands are ammonia) where we increase the zinc−carboxylate distance from its energy-minimized value (2.71 Å) to the value seen in the enzyme (3.6 Å). This leads to a destabilization of 8.7 kcal/mol, thus supporting this hypothesis. The calculated value is higher than observed experimentally (0.8 kcal/mol), partially due to the fact that the calculated number is not a true free energy, but compensating factors like hydrogen bonding, *etc*., in the enzyme active site will further reduce the net destabilization. These observations lead to the postulate that another function of Thr-199 is to destabilize zinc-bound bicarbonate by pulling the carboxylate oxygen away from the zinc ion and to then expose the zinc ion to nucleophilic attack by a water molecule. The crystal structure of the HCAII−bicarbonate complex for the Thr-199 → Ala-199 mutant supports this hypothesis.[12] In this structure the zinc ion is five-coordinated, with three ligands coming from the His residues, one coming from bicarbonate and the final coming from a water molecule. In the native structure, on the other hand, it is still five-coordinated, but bicarbonate is binding in a bidentate manner. Thus, by the removal of the Thr-199 side chain, the zinc ion can rearrange its ligands in order to provide a structure that is most suitable for the new active site environment.

Conclusions

Through the use of QM/MM MD simulations we have provided molecular-level insights into how bicarbonate binds to HCAII. From these studies, we find that the Lindskog structure, while likely to be the first formed structure, is unstable relative to a Lipscomb-like structure which appears to be the global minimum for the zinc bicarbonate structure. From our MD simulations we predict that Thr-199 continues to play a gatekeeper role by forming a hydrogen bond with the bicarbonate ion. Previously,[21] it had been thought that bicarbonate could bind to HCAII without forming a hydrogen bond with Thr-199 (much like the HCAII−azide complex[17,18]), but we find that this is not the case. Critically, the hydrogen bond interaction between Thr-199 and zinc-bound bicarbonate (in the Lipscomb-like binding motif) appears to destabilize the binding of bicarbonate ion (by increasing one of the carboxylate oxygen to zinc distances), while preparing it for displacement by a proximal water molecule which is hydrogen bonded to the "exposed" carboxylate oxygen (see Figure 4). Besides the Lindskog- and Lipscomb-like structures we observe another binding motif that we suggest is a possible intermediate along the interconversion pathways between these two structures. From these observations we postulate a detailed mechanism for the formation and loss of bicarbonate (see Figure 4). This mechanism is able to explain all available experimental information regarding the interaction of bicarbonate and HCAII.

This study also provides interesting insights into enzyme dynamics and how this might affect function. The binding constant for bicarbonate binding to HCAII is relatively low (77 mM[49]), so it is not surprising that we observe several transiently stable HCAII−bicarbonate structures. The idea of several possible bicarbonate binding modes is also supported by experimental studies of native HCAI[23] and mutant[12,22] and Co(II)-substituted[24] HCAIIs. These structures all show slightly different binding motifs, which support the notion that several low-energy bicarbonate binding modes are available. In our studies, we find that the Lindskog structure, while initially formed is transiently stable relative to the Lipscomb-like structure (or other structures). This appears to arise from the inherent instability of the Lindskog structure over the Lipscomb-like structure in the gas phase. The enzyme, therefore, does very little to discriminate between the two since in both cases the number and types of hydrogen bonds between bicarbonate and the HCAII active site are similar. Given the inherent stability of the Lipscomb-like structure, HCAII has taken advantage of this and, through the suitable placement of a water molecule, provides a means by which a water molecule can readily displace the bicarbonate ion. The interconversion of these two binding modes can take place in a number of ways. The rearrangement can go through a structure like that observed in our MD simulations (see Figure 2) which we have termed as the angle-bending transition state (see Scheme 7). Or alternatively, a rotational transition state can be envisioned, but we have not observed this within the HCAII active site. Both of the pathways provide a low-energy route by which these two structures can be interchanged. The final possible pathway is via an intramolecular proton transfer, but we have not observed this pathway, and in the absence of this process being facilitated by water, the barrier height is quite high.[15,16] Thus, within HCAII, bicarbonate has to adopt a number of conformations to affect catalysis, which suggests that bicarbonate really is binding to HCAII via a continuum of states with the Lipscomb-like structure being the largest contributor.

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