Mechanism of the Human Carbonic Anhydrase II Catalyzed Hydration of Carbon Dioxide

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Abstract: The catalytic mechanism of human carbonic anhydrase II (HCAII) has been studied by using a combined high-level ab initio and free energy perturbation approach. We have examined two hydration reaction mechanisms. The first involves hydration of CO_2 followed by loss of bicarbonate. The second, originally proposed by Liang and Lipscomb, involves hydration of CO_2 followed by an internal proton transfer and then loss of bicarbonate. We find that the former mechanism is more likely than the latter. On the basis of our results we have been able to predict the location of the bicarbonate proton in the recently solved X-ray structure of the HCAII-bicarbonate complex. We find that the proton is hydrogen bound to Thr-199, while one of the bicarbonate oxygens is hydrogen bound to the main-chain NH of Thr-199. This hydrogen-bonding pattern is analogous to that seen for sulfonamides, which suggests that these molecules are substrate (or transition-state) mimics. Our calculated free energy barrier for CO_2 hydration is in reasonable accord with experiment, while that for the dehydration of bicarbonate is in poor agreement. The reasons for this disagreement are discussed. The molecular-level details obtained from this study have been used to construct a detailed catalytic mechanism for the mode of action of HCAII.

Introduction

The carbonic anhydrases (CAs; carbonate hydro-lyase, EC 4.2.1.1) are a family of enzymes that are found in both plants and animals whose only known physiological function is the interconversion of CO_2 and HCO_3^- (eq 1).[1] There are now thought

$$CO_2 + H_2O \rightleftharpoons H^+ + HCO_3^- \quad (1)$$

to be at least seven human carbonic anhydrases (HCAs), which have been identified as HCAI-HCAVII.[2] In the present paper we will be concerned with the high turnover HCAII form of the enzyme.

HCAII is a small monomeric protein consisting of 260 residues, with a molecular weight of about 30 kDa. The structure of HCAII has been extensively characterized.[3-7] The native zinc-hydroxide form of HCAII is now known to 1.54-Å resolution,[3] the native zinc-water form is now known to 1.67-Å resolution,[4] and the complex of a mutant HCAII (Thr-200 → His-200) with bicarbonate is known to 1.9-Å resolution.[5] Furthermore, the structures of a number of mutant[6] and inhibitor-bound[7] HCAIIs have been reported. Structural data for HCAI[8] and HCAIII[9] have also been reported.

The key residues in the active site of HCAII are given in Scheme I. The active site is about 15 Å across and 15 Å deep and has a zinc ion positioned at the bottom of it.[3] The zinc ion is essential for catalytic activity[1] and is coordinated to three histidines (His-96, -94, and -119) and to a hydroxide ion at high pH (zinc-hydroxide form).[1,3] At low pH the fourth ligand is a water molecule (zinc-water form), and during turnover the fourth ligand can be a bicarbonate ion (zinc-bicarbonate form).[1,4,5] The zinc-bound ligands are hydrogen bonded to Thr-199, which is itself hydrogen bonded to Glu-106.[1,3] This hydrogen-bonding pattern appears to be important for rapid enzymatic catalysis,[10] governing the pK_a of zinc-bound water,[11] and inhibitor binding.[3,7] Another important feature of the active site cavity is the presence of a hydrophobic pocket, which usually contains the so-called "deep-water",[3] but is now also thought to be important for CO_2 recognition.[6,10,12-14] His-64 is thought to be important for shuttling protons out of the active site.[1] However, recently it was suggested that this residue is not required for high CO_2 hydration activity,[15] but this conclusion was later found to be caused by the use of imidazole-like buffers.[16] Another interesting aspect of His-64 is its conformational flexibility,[4b,6a] but the functional significance of this observation is still unknown. Finally, there are several ordered waters in the active site of HCAII, which are thought to be important for the intramolecular transfer of protons from

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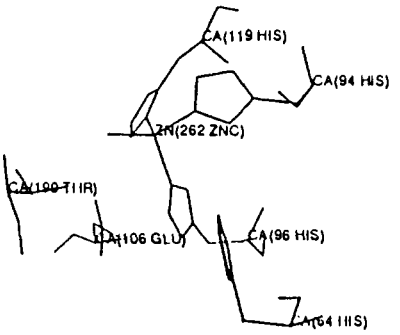
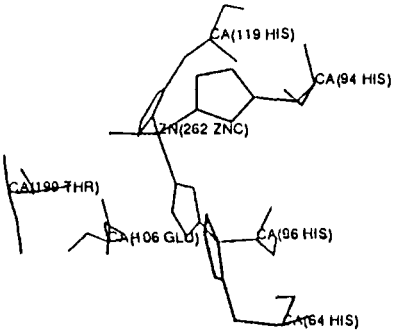
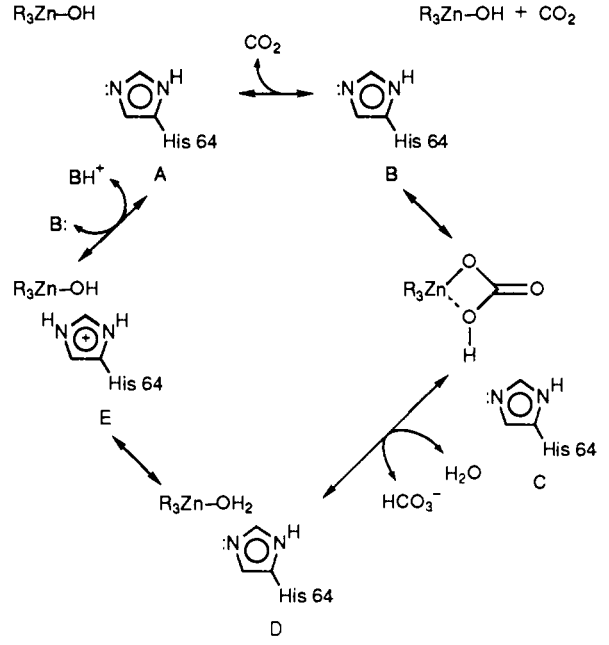
Scheme I

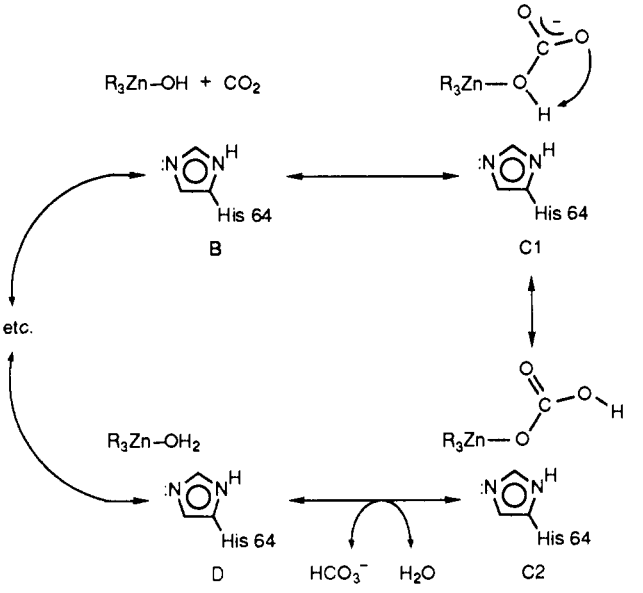




Scheme II



Scheme III



the zinc-bound water to His-64.[3]

HCAII is one of the most efficient enzymes known, with a k_{cat} of 10^6 s^{-1} and a K_m of 8.3 mM for the forward reaction.[1] The kinetic parameters for the reverse reaction are $k_{cat} = 6 \times 10^5$ s^{-1} and $K_m = 32$ mM.[1] On the basis of the k_{cat}/K_m ratio, HCAII appears to be a "perfectly evolved" enzyme.[1]

The most widely accepted catalytic cycle for HCAII is the so-called zinc–hydroxide mechanism, which is presented in Scheme II.[1] The following observations were crucial in developing the catalytic cycle given in Scheme II: (A) The catalysis is dependent on a group whose pK_a is around 7.[1] After much debate it was decided that a zinc-bound water satisfies this criterion.[1] (B) The rate-limiting step for maximal velocity at high buffer concentrations is an intramolecular proton transfer between a zinc-bound water and a residue in the active site. The identity of the residue that serves this function is thought to be His-64.[1,15,16] (C) The intermolecular proton transfer between protonated His-64 and the surrounding milieu depends on the concentration of buffers in the surrounding medium. At low buffer concentrations this step is rate limiting, while at high buffer concentrations this step is not rate limiting and step B above is. These observations suggest that buffers play a crucial role in CA activity.[1] (D) Finally, the mechanism follows ping-pong kinetics where the CO_2 hydration (A → D; see Scheme II) and proton-transfer steps (D → A) are kinetically distinct.[1]

The first step in the mechanism is the introduction of CO_2 into the active site of the zinc–hydroxide form of the enzyme (A → B) followed by the hydration of CO_2 to form the zinc–bicarbonate intermediate (B → C). Water then displaces the bicarbonate ion to form the zinc–water form of the enzyme (C → D), which then undergoes an intramolecular proton transfer to His-64 which forms E. This intermediate next transfers its proton to a buffer or water molecule in the surrounding milieu, which regenerates A. Liang and Lipscomb[17] have proposed a slightly different mechanism which was arrived at through extensive theoretical calculations. This mechanism is summarized in Scheme III. This pathway is very similar to the zinc–hydroxide one except that it requires an internal proton transfer of zinc-bound bicarbonate. Thus, CO_2 is hydrated to form C1, which has the hydroxyl oxygen bound to zinc, and this is followed by a proton transfer to give C2, which then loses bicarbonate to give D. The remainder of the mechanism is similar to that given in Scheme II.

One mechanism that is significantly different from those given above but that cannot be ruled out is the proton shuttle mechanism of Kannan et al.[3d] The zinc ion still has a similar role, but the proton is not shuttled out of the active site via His-64. Instead, this mechanism involves the active site residues Thr-199 and Glu-106 in a proton relay. The one drawback of this mechanism, though, is that it requires that the activity-linked group whose pK_a is around 7 to be Glu-106. The pK_a of a glutamic acid is normally around 4.5, but highly perturbed pK_a values for glutamic acids have been observed, for example, in lysozyme.[18] Recent theoretical work has predicted that the pK_a of Glu-106 should be in the "normal" range, which brings this mechanism into doubt.[19] However, experimental verification of this prediction is still not available. Regardless, we will not consider this mechanism further.

The catalytic mechanism of the CAs has been studied in detail by a large number of theoretical groups. Early ab initio pseu-

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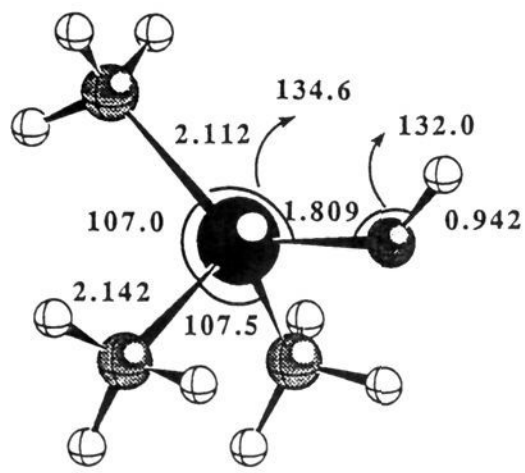
dopotential work by Demoulin and co-workers played a role in supporting the postulate that zinc-bound water has a pK_a of around 7 as well as providing insights into several of the catalytic steps.[20] Allen and co-workers have studied the electrostatic potential in the active site of CA,[21] used ab initio pseudopotential methods to propose a transition state (TS) for the hydration of CO_2,[22] and discussed various aspects of the inhibition of CAs.[22] Clementi and co-workers[23] as well as Jönnson[24] have used Monte Carlo methods to look at the hydration of the active site of CAs. Sokalski and co-workers have used CNDO/2 to study various aspects of CA chemistry.[25]

Liang and Lipscomb[17] have carried out a large number of quantum mechanical calculations (PRDDO and ab initio methods) on the catalysis of CA and have arrived at a mechanism (see Schemes II and III) that fits their results. Semiempirical quantum mechanical calculations have been reported by Merz et al.[26] where they have used the AM1 method to locate the TS for CO_2 hydration and to study the intramolecular proton transfer. In the last two years a series of more sophisticated ab initio calculations have appeared that have addressed several aspects of CA activity. Krauss and Garmer[27] have used ab initio pseudopotential calculations with full geometry optimization to characterize the $(NH_3)_3ZnOH$ (**1**) surface in detail. Furthermore, they addressed



(1)

the effect that Glu-117 (which is hydrogen bonded to His-119) might have on the pK_a of zinc-bound water. Tapia and co-workers have examined the hydration of CO_2 using large basis set ab initio calculations, which include correlation at the MP2 level.[28] These investigators used for their calculations a primitive model for the active site (i.e., only $ZnOH^+$) and looked at hydration by both the zinc–hydroxide and zinc–water forms of the enzyme. Sola et al. have also looked at the hydration of CO_2 on the $(NH_3)_3$-ZnOH surface using 3-21G for Zn, C, O and N and STO-3G for hydrogens and including correlation at the MP2 level.[29] These authors also used self-consistent reaction field (SCRF) calculations to study the role hydration plays in the CA reaction. They conclude that hydration plays an important role and that both of the mechanisms outlined above may be operative (Schemes II and III).

Table I. The ESP Charges for the Molecular Mechanical Models

atom	1	2	3	5	6
N	0.468 463	0.493 855	0.479 310	0.571 042	0.467 066
Zn	0.200 124	−0.037 429	−0.231 169	−0.326 320	−0.188 568
N	0.375 421	0.435 295	0.499 88	0.544 941	0.511 269
N	0.375 407	0.435 137	0.499 531	0.546 983	0.509 832
O	−0.755 828	−0.700 479	−0.390 865	−0.392 232	−0.436 726
H	0.336 413	0.331 589	0.265 070	0.411 684	0.494 326
O		−0.362 687	−0.375 755	−0.515 273	−0.610 255
C		0.802 019	0.675 94	0.789 861	0.978 319
O		−0.397 299	−0.421 696	−0.630 686	−0.725 263

Several recent studies have begun to use all-atom models to study various aspects of CA chemistry. This has become possible with the advent of realistic ways in which to include metal ions into simple molecular mechanical force fields.[30] Merz and co-workers have reported a number of simulations that have addressed inhibitor binding,[31] CO_2 binding,[10,12] and the pK_a of Glu-106 in HCAII.[19] These authors have also presented detailed studies on the uncatalyzed reaction of CO_2 with H_2O[32] and hydroxide,[33] which have yielded insights into the catalyzed hydration reaction in CA. In the CO_2-binding studies two binding sites were located in the HCAII active site. The first, which corresponds with the deep-water pocket, was labeled the A binding site, while another site between zinc and His-64 was labeled the E binding site. The remaining B, C, and D sites were all covalent binding sites around the zinc that have been identified crystallographically.[7c] Recent simulations by Åqvist and co-workers have examined the intramolecular proton transfer in HCAI[34] and CO_2 hydration by hydroxide in aqueous solution and in the active site of HCAI.[35]

All of the quantum mechanical calculations were carried out using small basis sets and/or very simple representations of the reaction center. Furthermore, in all cases the enzyme environment was completely neglected. The end result of this was that the activation barriers etc. varied quite widely among the methods used. Moreover, all of the previous ab initio calculations ignored entropic as well as enthalpic effects on these reactions.

In order to alleviate these deficiencies we have used large basis ab initio calculations to study the $(NH_3)_3ZnOH$ reaction surface. The basis set we are using here is the best used to date and should give an accurate description of this potential surface. Furthermore, we have included thermodynamic contributions as well as correlation corrections out to the MP2 level. To study the effect of the environment we have used free energy perturbation (FEP) simulations to determine the free energy profile for this reaction as predicted using the $(NH_3)_3ZnOH$ active site model. We have also used these calculations to determine which catalytic cycle is preferred (Scheme II or III) and to identify the position of the proton in the zinc–bicarbonate form of the enzyme.

To accomplish our goals we examined the reaction of $(NH_3)_3ZnOH^+$ with CO_2, which was then used to build a molecular mechanical potential to study the effect of the enzyme environment on the ab initio reaction profile.

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Table II. The Calculated Total Energies and Thermodynamic Properties for the Species Studied

compound	E_{tot} (au)	E_{tot} (MP2)	H (kcal/mol)[a]	S (kcal/mol·K)
CO_2	−187.633 517	−188.094 820 8	9.624	50.798
$Zn(NH_3)_3H_2O^{2+}$	−2020.864 419 8		102.778	112.563
1	−2020.585 703 3	−2021.484 094 7	93.761	105.538
2	−2208.234 125 55	−2209.595 418 7	104.975	129.883
3	−2208.225 994 56	−2209.594 569 9	104.408	120.943
4[b]	−2208.246 508 81	−2209.603 98	106.289	114.401
5	−2208.248 490 01	−2209.607 512 8	106.804	118.479
6	−2208.266 722 15	−2209.622 753 9	106.830	119.352
7[c]	−2208.238 458 79			
8[d]	−2208.243 545 93	−2209.600 939 8	106.420	114.355
9[e]	−2208.183 212 82	−2209.561 168 5	103.706	115.369

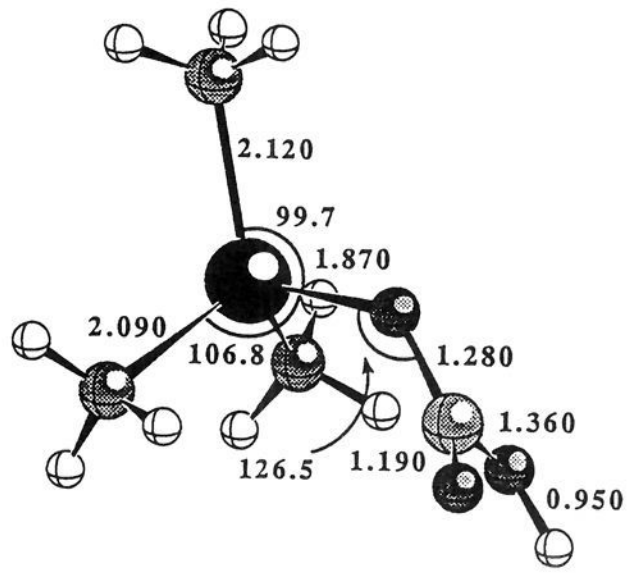
[a] This refers to the translational, rotational, and vibrational contributions to enthalpy. [b] Transition state for rotation around the Zn–O bond. [c] Only HF energy computed. Vibrational frequencies indicate that this is a hilltop. [d] Transition state for the 1,3-H shift. [e] Transition state for rotation around the C–O bond.

Theoretical Procedures

Ab Initio Calculations. The calculations were carried out using the Gaussian 88[36] and 90[37] programs with the general basis set option. For C, O, N, and H the 6-31G* (C, N, and O, (631/31/1); and H, (31)) basis set was used. The Zn basis set is (622211/612/32).[38] The basis set for Zn is the same as that used by Jacob et al.[28] These authors used a (5211/32) basis set for C, N, and O and a (31) basis set for H. Hence, our basis set for C, N, and O is of a slightly better quality in that we include polarization functions. Indeed, previous authors have suggested that polarization functions are essential to obtain reasonable structural data in these systems.[39] All geometries were fully optimized without any assumptions unless otherwise noted. Moreover, all stationary points were characterized by calculating force constants. For a stationary point to be a minimum we require that there be no negative force constants, while a transition state should have one, and only one. Thermodynamic corrections (298 K) were determined using the calculated vibrational frequencies.[40] Electron correlation effects were included via MP2 single-point calculations.[40]

Free Energy Perturbation (FEP) Simulations. The FEP simulations were carried out using the AMBER force field[41] with specific modifications for carbonic anhydrase.[30] The nonbond parameters for CO_2, OH^-, and HCO_3^- were those reported by Peng and Merz.[33]

The initial structures were generated by docking the ab initio structures into the active site of HCAII. These structures were rigidly held in the active site through the use of relatively stiff force constants of 500 kcal/(mol·Å^2) and 100 kcal/(mol·deg^2) for bonds and angles, respectively. The torsional barrier heights were set to 0.[30] The atomic point charge distributions for the ab initio structures were determined using electrostatic potential (ESP) fitting techniques incorporated into Gaussian 88.[42] We docked into the active site the heavy atoms from the ab initio active site model. For example, the zinc–hydroxide model consists of $(NH_3)_3ZnOH$, but we have no use for the hydrogens bound to the ammonia groups. This is because the ammonia groups are replaced by imidazoles in the enzyme active site. To eliminate the ammonia hydrogens we use ESP fitting procedures in which the hydrogen charges are set to 0.[19,42] The resulting fit places charges only on the $(N)_3ZnOH$ fragment. The values for the atomic point charges used in this study are given in Table I. This procedure has been used before, in the context of developing united-atom charge models for amino acid residues.[19] The charge models were then fit into the AMBER force field such that an integral charge was retained. This required modifying the charge model used for the imidazole ring from the histidine residue. The charge on the zinc-bound nitrogen from His-94, -96, and -119 was dispersed evenly around the remaining four heavy atoms of the imidazole ring.



(9, TS)

Figure 1. Calculated geometries for species involved in the reaction of $(NH_3)_3ZnOH$ with CO_2. Bond lengths are in Å, and angles are in degrees.

These structures were then solvated with a 20-Å sphere of TIP3P[43] water molecules, which was centered at the zinc ion in the HCAII active site. Any water molecules that came within 2.4 Å of a protein atom were removed. The water molecules were restrained to stay within this sphere through the use of harmonic restraining forces (0.5 kcal/(mol·Å)), which were applied to any water molecule that strayed out of the sphere. The resulting solvated protein was optimized for 1000 steps followed by molecular dynamics (MD) equilibration. The simulations were kept at 298 K by coupling to a temperature bath.[44] All residues within 15 Å of the zinc ion as well as all water molecules were permitted to move during the course of MD equilibration and FEP simulations. Residues lying outside this region were held fixed. SHAKE[45] was used to constrain bond lengths at their equilibrium values (i.e., the ab initio distances). A time step of 1.5 fs was employed. The nonbonded pair list had a cutoff of 9 Å, and was updated every 10 time steps, and a constant dielectric of 1 was used throughout. The equilibration period was 30 ps. The free energy evaluations were done using the slow-growth procedure.[46] Eight separate simulations were carried out to map the enzymatic reaction profile. The simulation time period was 180 ps each, and the simulations were carried out in the forward (λ = 1–0) and backward (λ = 0–1) directions for a total of 360 ps of simulation time.

From the ab initio calculations we obtain the $\Delta G_{gas\text{-}phase}$ contribution to the reaction, while the FEP simulations give us the ΔG_{soln} contribution. Since we use the gas-phase ab initio free energy for the intramolecular contributions, only intermolecular contributions were accumulated during free energy perturbation calculations. The total free energy for the

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(45) van Gunsteren, W. F.; Berendsen, H. J. C. *Mol. Phys.* **1977**, *34*, 1311.

(46) Kollman, P. A.; Merz, K. M., Jr. *Acc. Chem. Res.* **1990**, *23*, 246. Beveridge, D. L.; DiCapua, F. M. *Annu. Rev. Biophys. Biophys. Chem.* **1989**, *18*, 431. Jorgensen, W. L. *Acc. Chem. Res.* **1989**, *22*, 184. van Gunsteren, W. F. *Protein Eng.* **1988**, *2*, 5. Mezei, M.; Beveridge, D. L. *Ann. N.Y. Acad. Sci.* **1986**, *482*, 1.

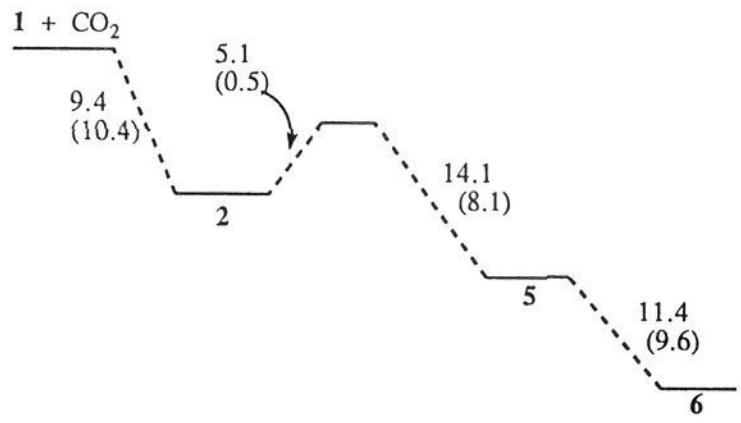


Figure 2. Calculated reaction profile (kcal/mol) for the reaction of **1** with CO_2 at the HF (MP2) level.

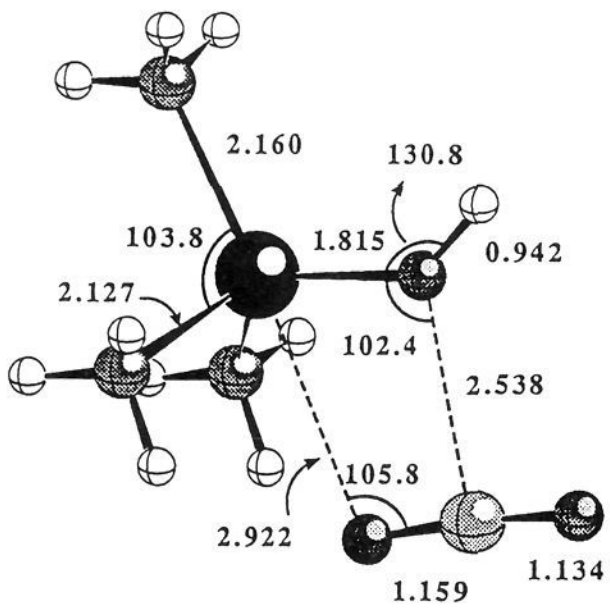
reaction is then given by $\Delta G_{total} = \Delta G_{gas\text{-}phase} + \Delta G_{soln}$. This method has been used by us with great success in previous studies on the solution-phase reactions of CO_2 with hydroxide and water.[32,33] A similar procedure has been used by Blake and Jorgensen in a recent study of the Diels–Alder reaction.[47]

Results and Discussion

The presentation of our results will be grouped into two parts. The first part deals with the $(NH_3)_3ZnOH^+ + CO_2$ reaction in detail. Then in the second part we describe the FEP results. The calculated total energies and thermodynamic contributions for all species involved in this study are given in Table II.

1. The $(NH_3)_3ZnOH^+ + CO_2$ Reaction. First, we calculated the deprotonation enthalpy of zinc-bound water in $(NH_3)_3ZnH_2O^{2+}$ (see Table II). The calculated deprotonation enthalpy using our basis set is 166.5 kcal/mol at 298.15 K. Demoulin and Pullman[20] earlier reported a value of 171 kcal/mol; Krauss and Garmer[27] obtained a value of 182.5 kcal/mol and Merz et al.[26] obtained a value of 183.4 kcal/mol using the AM1 method. All of these values are much smaller than the experimental deprotonation enthalpy for H_2O (367.2 kcal/mol[48]). Clearly, the zinc-bound water is much more acidic than water itself.

Next, we investigated the reaction between $(NH_3)_3ZnOH^+$ and CO_2. The calculated geometries and reaction profile are given in Figures 1 and 2. This reaction profile is very different from the gas-phase reaction of OH^- with CO_2 because a barrier has been introduced along the reaction profile.[33,49] The energetics for complex formation is −10.4 kcal/mol, and the barrier height for the conversion of CO_2 to bicarbonate is 0.5 kcal/mol. The zinc to CO_2 oxygen distance in the complex is found to be 2.922 Å (**2**), which is much shorter than that seen in molecular dynamics



(2, Complex)

simulations, where we found the zinc to CO_2 oxygen distance to

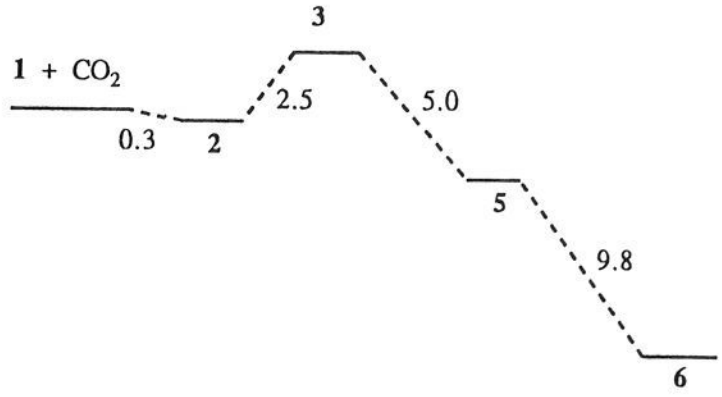
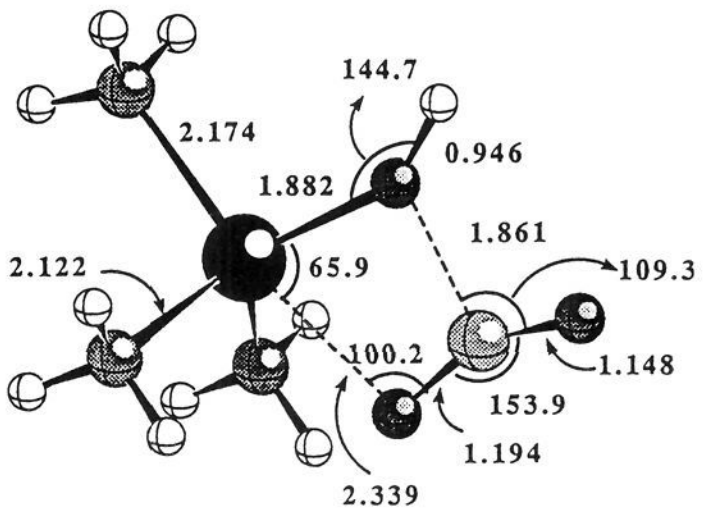


Figure 3. Calculated free energy profile (kcal/mol) for the reaction of **1** with CO_2. Energies were determined at the MP2 level, while the thermodynamic corrections were determined at the HF level.

be 3.85 Å[12] on average. This difference could be due to the use of ammonia ligands instead of imidazole ligands, which would alter the steric picture of the active site model. Furthermore, this could be due to the neglect of the protein environment or the inability of the molecular mechanical model to handle bond formation. Further insights into this are given below. Finally, we also find that CO_2 is only slightly bent in complex **2**.

The calculated energy difference between complex **2** and transition state **3** is 5.1 kcal/mol (0.5 kcal/mol) at the HF (MP2) level. Previous values calculated for this barrier height at the



(3, TS)

HF (MP2) level using different basis sets include 42.6 (36.4)[29] and 7.0 (3.9)[27] kcal/mol. Clearly our values are much lower than those obtained by Sola et al.[29] and are in reasonable accord with those obtained by Krauss and Garmer.[27] Conversion of the total energy surface into a free energy one gives a barrier height of 2.5 kcal/mol (Figure 3). The free energy barrier height for this conversion has been estimated by Behravan et al. to be about 6.5 kcal/mol for HCAII.[50] Hence our calculated value is significantly smaller. However, we have not considered the effect of the enzyme active site (see below).

We find three stationary points on the potential energy hypersurface for the zinc-bound bicarbonate (**4**, **5**, and **6**). In **4**, there is a hydrogen bond between the hydroxide oxygen and the ammonia hydrogen (see Figure 1). Frequency calculation indicated that **4** is a transition state for rotation of the bicarbonate moiety around the Zn–O bond in **5**. Previous work predicted **4** to be a minimum.[27] This observation may be an artifact of the smaller basis set used in the previous study.[27] However, the calculated energy difference between **4** and **5** is only about 2.2 kcal/mol at the MP2 level (1.2 kcal/mol at the HF level), so the potential surface is quite flat in this region. The energy difference between **4** and **5** could be much bigger if the ammonia ligands were replaced by imidazoles, where the intramolecular hydrogen bond would be impossible. As expected, we found **6** to be the most stable form for the zinc–bicarbonate complex.

Although structural information concerning the HCAII–bicarbonate complex is not available at present, an X-ray structure

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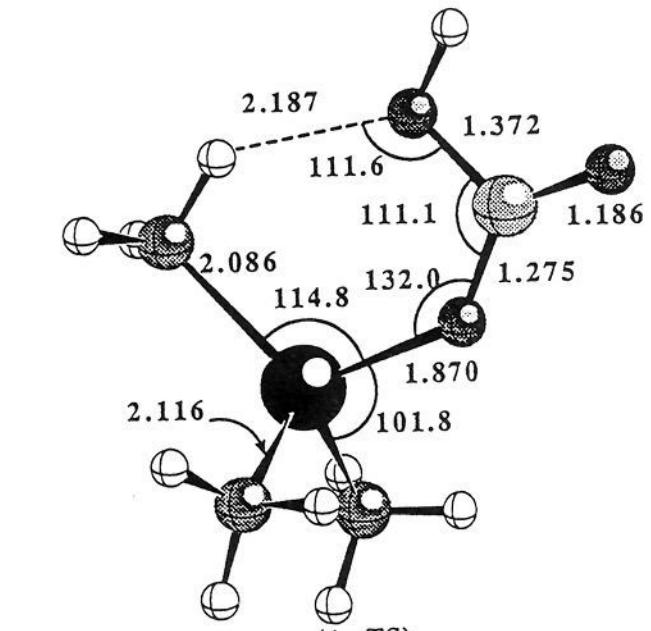
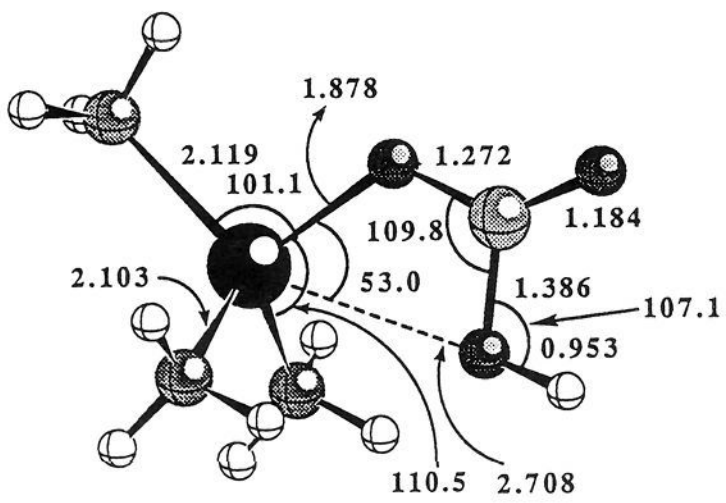
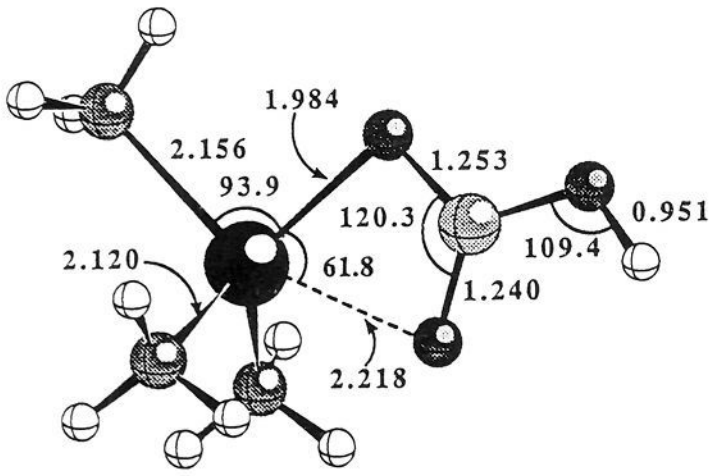
(50) Behravan, G.; Jonsson, B.-H.; Lindskog, S. *Eur. J. Biochem.* **1990**, *190*, 351. The free energy values given in this study assume 1 M standard states, 25 °C, 1,2-dimethylimidazole buffer, pK_a = 8.2, and pH 8.2.



(4, TS)



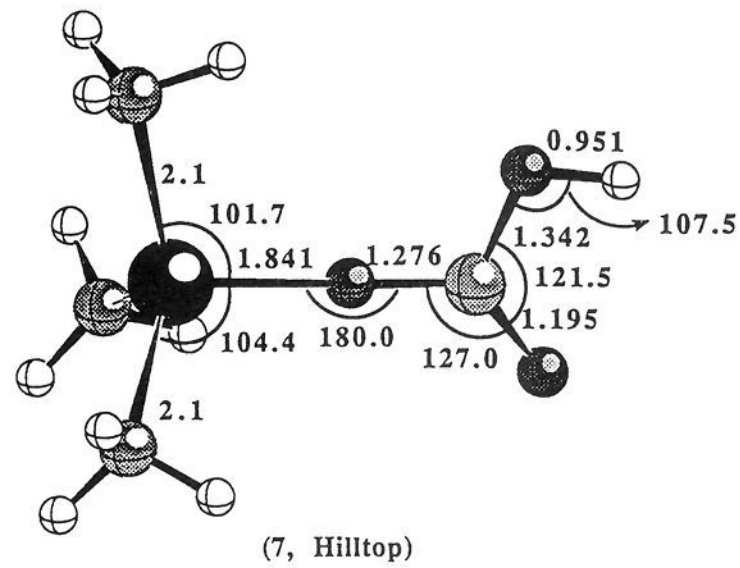
(5, Complex)



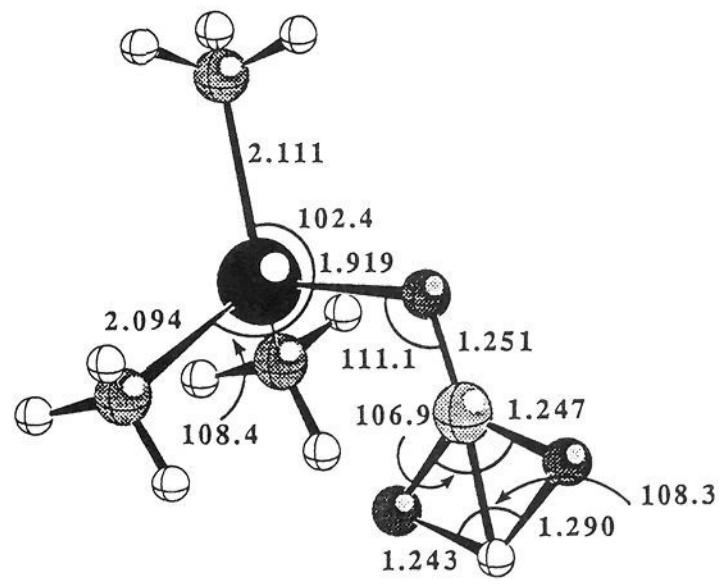
(6, Complex)

of a mutant HCAII–bicarbonate complex has been solved (1.9-Å resolution).[5] The distances between the zinc ion and the two bicarbonate oxygens are 2.23 and 2.54 Å, respectively, while our calculated distances are clearly too short in **6** and are too long for one Zn–O bond and too short for the other in **5** (see Figure 1). This observation is not unexpected because of two factors: First, we use a greatly simplified model of the coordination sphere of zinc (ammonia vs imidazole). Second, we have completely neglected the role of the active site environment might play by altering the geometry of the zinc–bicarbonate complex. For example, the bicarbonate ion might reorient itself in order to optimize hydrogen-bonding interactions between Thr-199 and the hydroxyl hydrogen from bicarbonate. Finally, we note that the X-ray structure does not provide the position of the proton, so we are not certain which structure (**5** or **6**) is present in the crystal structure. Moreover, there is some uncertainty in the zinc–oxygen distances, given the resolution (1.9 Å) of the X-ray structure. This issue is explored more fully below.

Currently, there are two mechanisms proposed to explain the interconversion of the hydroxyl proton among several possible zinc–bicarbonate species. One is the Lipscomb mechanism, which involves a proton shift from one oxygen to another (see Schemes II and III).[17] This hydrogen shift is required by this mechanism since the investigators predict that structure C1 (see Scheme III) is an intermediate along the reaction pathway. We find no evidence in our calculations that indicates that this structure is important. It is well-known that a 1,3-shift between two oxygens occurs quite readily in silicon chemistry (e.g., 1,3-silyl migrations),[51] but it is almost impossible in carboxylic acids except under very extreme conditions. Previous theoretical calculations predicted a very high barrier for the 1,3-hydrogen shift in formic acid.[52] The other alternative is bending of the Zn–O–C angle (Lindskog mechanism).[53] This mechanism has been shown to have a very high activation energy in $ZnCO_3H^+$,[28] but the barrier in the $(NH_3)_3ZnCO_3H^+$ case[27] is relatively small. Our calculated barrier for bending (see **7**) is about 6.3 kcal/mol, while the barrier



(7, Hilltop)



(8, TS)

for the 1,3-hydrogen shift is predicted to be about 29.1 kcal/mol (41 kcal/mol at the HF level). The transition state for the 1,3-hydrogen shift (**8**) is found to be a genuine one with only one negative force constant corresponding to the interconversion of **5** to **6**. However, the bending transition state (**7**) is not a genuine one since it has two negative force constants.

It is interesting to note that the Zn–O bond is trans to the carbonyl group in **5** and cis in **6**. It has been shown experimentally that for carboxylate esters the cis orientation is more stable, and the barrier for interconversion between the trans and cis forms is usually very small (generally under 10 kcal/mol).[54] Hence, a simple pathway for conversion of **5** to **6** could involve rotation around the C–O bond instead of bending of the Zn–O–C bond.

(51) Brook, A. G.; Bassindale, A. R. In *Rearrangements in Ground and Excited States*; de Mayo, P., Ed.; Academic Press: New York, 1980.
(52) Bouma, W. J.; Vincent, M. A.; Radom, L. *Int. J. Quantum Chem.* **1978**, *14*, 767.
(53) Lindskog, S.; Engberg, P.; Forsman, C.; Ibrahim, S. A.; Jonsson, B.-H.; Simonsson, L.; Tibell, L. *Ann. N.Y. Acad. Sci.* **1984**, *429*, 61.
(54) Anet, F. A. L. In *Conformational Analysis of Medium-Sized Heterocycles*; Glass, R. S., Ed.; VCH: New York, 1988.

To test this possibility, we located this TS. In this TS structure (**9**; see Figure 1), the bicarbonate moiety is rotated about 65.9°. The calculated barrier at the Hartree–Fock level is about 3.1 kcal/mol (4.1 kcal/mol at the MP2 level). Frequency analysis shows that this is a true TS with only one negative force constant corresponding to the interconversioin of **5** to **6**. Clearly, this is the most likely pathway for interconversion of **5** to **6**. Further examination of the bending TS proposed by Lindskog (see above), which we found to be a hilltop, indicates that this structure collapses to **9**. Hence, careful exploration of the interconversion potential surface leads us to conclude that the most facile way in which to convert **5** to **6** is via **9** and not through the Lipscomb TS (**8**) or via the Lindskog pathway (**7**).

Our ab initio calculations give no indication that an internal proton transfer of zinc-bound bicarbonate along the lines suggested by Liang and Lipscomb is necessary (C1 → C2). We find that intermediate C1 (see Scheme III) does not exist on the $(NH_3)_3ZnOH$ potential energy surface at the level of sophistication we employ. Any attempt to locate this intermediate results in the formation **5**. Liang and Lipscomb argue that since alkyl carbonates (RCO_3^-) readily bind to CA[55] and show no substrate activity, this indicates that an internal proton transfer of zinc-bound bicarbonate is needed (the implicit assumption is that alkyl carbonates should be CA substrates). This is because, as they show, the barrier for internal alkyl transfer is on the order of 90 kcal/mol. However, the observation that alkyl carbonates are not substrates can be explained by alternative, non-catalytically productive binding of these molecules to CA. For example, we find that **5** is the most stable binding mode for HCO_3^- to CA (see FEP results below), which would require the alkyl group to be placed toward Thr-199 or into the hydrophobic pocket. Hence, we expect that the resulting severe steric contacts would force an alternative binding mode that would not allow CA to turn over an alkyl carbonate to CO_2 and the corresponding alcohol.

It is still possible that the bicarbonate ion might need to rearrange around the zinc ion, and we have studied this process in detail (i.e., bicarbonate binds as in **6** and not as in **5** to the enzyme). We find that the Liang and Lipscomb type pathway for the rearrangement of **5** to **6** is not favored, and the Lindskog type process (opening of the Zn–O–C angle) is not a true TS. We have found an alternative TS (**9**) that simply involves rotation around the O–C bond, which involves a low activation barrier. Hence, we feel that this pathway is the most likely pathway for this rearrangement.

Taken together, our ab initio calculations suggest that the zinc–hydroxide mechanism for the B to C transition given in Scheme II is the favored reaction process for CO_2 hydration. We find no need for alternative mechanisms such as that given in Scheme III for this process. Hence, our evidence indicates that CO_2 binds in the active site[10,12] and is hydrated, yielding a zinc-bound bicarbonate, which is subsequently lost. A crucial remaining uncertainty in this mechanism is the placement of the proton in the zinc–bicarbonate form of the enzyme. The proton could be placed as in **5** or **6**. If the most stable form of HCAII continues to be **6**, as it is in the gas phase, an intramolecular rearrangment of **5** (which is the first formed intermediate) will be necessary, via **9**. This issue is addressed below.

2. Free Energy Perturbation Results. Next, we studied the influence that the enzyme environment has on our ab initio model of the hydration of CO_2 by CA. The ab initio geometries were docked into the active site, and FEP simulations were used to calculate the free energy contributions due to the enzyme environment. Before we could carry out these FEP simulations, we needed to decide upon a working model for the preferred reaction profile which was based on what was known about this reaction from experiment and theory.

On the basis of previous theoretical[12,13] and experimental[6,14] work we expect that CO_2 interacts with the "deep-water" pocket, which implies that the CO_2 approaches opposite to the hydroxyl hydrogen of the zinc-bound hydroxide.[10] The hydrogen bond between the hydroxyl hydrogen and Thr-199 is an important interaction that is retained in all structures determined to date that leave Thr-199 and Glu-106 unaltered.[3-9] Indeed, Liljas and co-workers have termed Thr-199 as "the gatekeeper" for the CA active site.[7] Hence, maintaining contact by this interaction throughout our reaction profile is warranted.

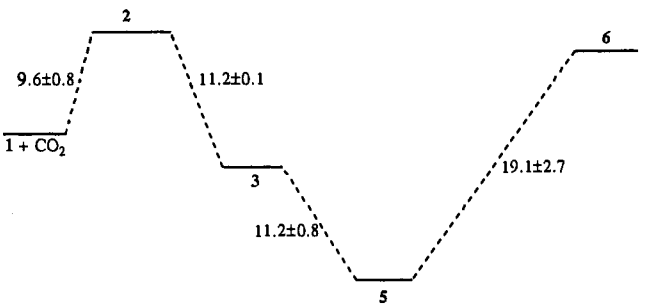


Figure 4. Calculated environmental free energy differences (kcal/mol).

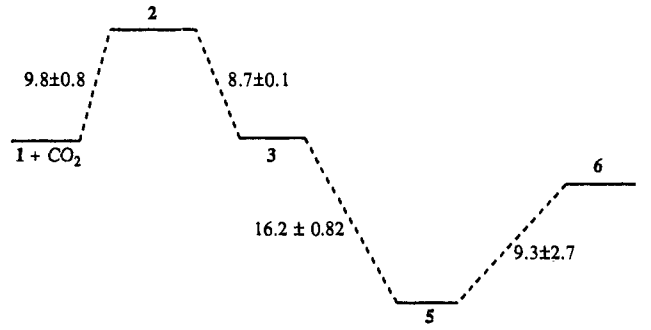


Figure 5. Calculated free energy profile (kcal/mol) for the enzymatic reaction.

Once the TS is surmounted, the zinc–bicarbonate species is formed. There are several possible orientations that need to be considered. First, there are two possible ways in which **5** can be fitted into the active site. One places the bicarbonate hydroxyl hydrogen in such an orientation that it can hydrogen bond with Thr-199 (complex 1). The other places the hydroxyl proton into the deep-water pocket of the CA active site (complex 2). We expect complex 2 to be very unlikely because it places an oxygen in proximity to the hydroxyl oxygen of Thr-199, which would result in a repulsive interaction. The hydroxyl hydrogen of complex 2 can still form a hydrogen bond with an active site water. Complex 1 can form two stabilizing hydrogen-bonding contacts (Thr-199 and an active site water), while complex 2 forms one repulsive interaction (Thr-199) and one attractive interaction (active site water). Furthermore, another hydrogen bond between the main chain NH of Thr-199 and the zinc-bound bicarbonate has been observed.[5] In complex 1 this hydrogen bond is with a carboxylate oxygen, while in complex 2 it is with the hydroxyl oyxgen. Given that the hydroxyl oxygen is less negatively charged than the carboxylate oxygen, it appears that the first orientation is again preferred (see Table II). Hence, we have only examined the orientation that retains the Thr-199 hydrogen bond contact.

There are two possible ways in which to place **6** in the HCAII active site. One is to have the bicarbonate hydroxyl hydrogen point toward Thr-199, and the other is to have it point away. Again, for the same reasons advocated above we have only considered the case where the hydrogen bond contact with Thr-199 is retained.

The first simulation we did was the conversion from **2** to TS **3**. This is a very small perturbation since **2** and **3** have very similar structures. The perturbation is dominated by electrostatic interactions, and the convergence is very good. The forward (from **2** to **3**) and the reverse (from **3** to **2**) runs differ by only 0.2 kcal/mol in free energy, and the average free energy value for this perturbation is 11.2 ± 0.1 kcal/mol (Figure 4). This is a large free energy difference given that the two structures are relatively similar. However, the dipole moments of these two

(55) Pocker, Y.; Deits, T. L. *J. Am. Chem. Soc.* **1983**, *105*, 980.

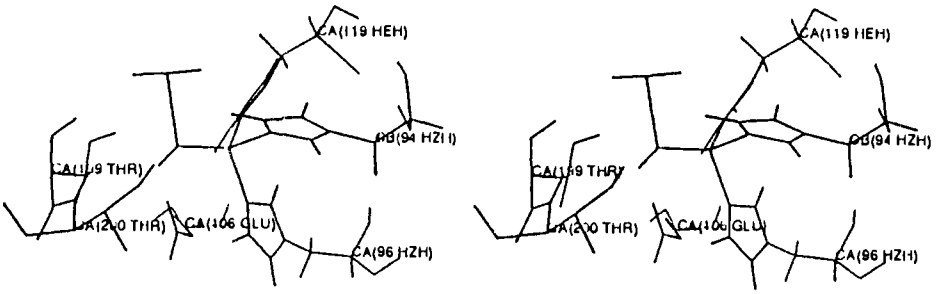


Figure 6. Snapshot of **2** docked in the active site of HCAII. Obtained after 180 ps of FEP simulation from **3**.

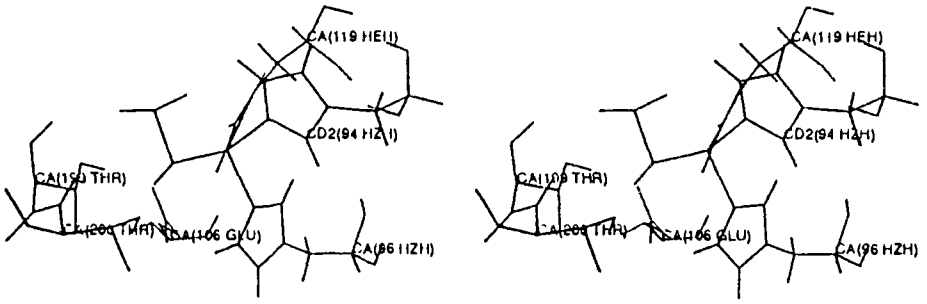


Figure 7. Snapshot of **3** docked in the active site of HCAII. Obtained after 30 ps of MD equilibration.

species are significantly different (**2**, μ = 10.8 D; **3**, μ = 11.5 D), with **3** being more polar than **2**. Combination of the environmental (Figure 4) and gas-phase (Figure 3) free energies gives a free energy difference between **2** and **3** of 8.7 ± 0.1 kcal/mol (Figure 5). This gives the impression that CO_2 hydration is activationless, which is thought not to be the case.[50] Next we considered the stability of **1** relative to **2**.

Stereo plots of **2** (obtained after 180 ps of FEP simulation) and **3** (equilibrated for 30 ps) in the HCAII active site are given in Figures 6 and 7. As mentioned above, there are several contacts that have been identified as being important in stabilizing small molecules in the HCAII active site.[1,3-10] For our purposes three are of particular interest. First, the way in which the hydroxyl oxygen and main-chain NH of Thr-199 interact with our docked intermediates is of interest. Optimal interactions are indicated by hydrogen-bonding distances in the range 1.5–2.5 Å, and less than optimal interactions are longer.[1,3-10] Second, the hydrogen bond between Thr-199 and Glu-106 is an important indicator regarding the orientation of any hydrogen bonds between Thr-199 and any of our docked intermediates. If this interaction is maintained, we feel that this indicates that any hydrogen bond contacts between zinc-bound species and Thr-199 are in an optimal orientation. If not, these hydrogen bond contacts are in a less than optimal orientation. This conclusion is justified because in all crystal structures determined to date for HCAII it has been found that this interaction is retained.[3-9] Again, a hydrogen-bonding distance of anywhere between 1.5 and 2.5 Å is considered in the optimal range for the Thr-199 to Glu-106 hydrogen bond.[10]

The hydrogen bond between the hydroxyl oxygen of Thr-199 and the hydroxyl hydrogen from zinc-bound hydroxide is 3.0 Å long in the case of **2**, while it is 3.4 Å in the case of **3**. This interaction in both cases is clearly very weak. The distance from the main-chain Thr-199 NH to the CO_2 oxygen is 2.4 Å in both **2** and **3**, which indicates that there is some interaction between these two groups. Regardless, we find overall that both **2** and **3** are not well accommodated in the HCAII active site. This is manifested by the data given above and the disruption of the Thr-199 to Glu-106 hydrogen bond in the case of **2** (hydrogen bond distance of 3.4 Å). In **3** this interaction is better retained, being only 1.8 Å long. These results suggest that some geometric relaxation may be necessary in order to better accommodate these structures in the active site of HCAII. This observation is discussed in more detail below.

Next, we did a simulation that annihilated CO_2 from the active site. This involves determining the absolute free energy of binding of CO_2 to HCAII by disappearing the substrate molecule. The method by which this can be done has been described by Jorgensen et al.[56] This method requires that we do two simulations. The first evaluates the free energy of solvation of CO_2 (ΔG_{sol}), and the next involves determining the free energy of binding to the enzyme (ΔG_{enz}). The summation of these two terms gives the absolute free energy of binding (ΔG_{bind}). The scheme used is given in eq 2. Since the solvation free energy for CO_2 in water (ΔG_{sol})

$$\begin{array}{ll} EO \rightarrow ES & \Delta G_{enz} \\ S \rightarrow O & \Delta G_{sol} \\ \hline E + S \rightarrow ES & \Delta G_{bind} \end{array}$$

$$\Delta G_{bind} = \Delta G_{enz} + \Delta G_{sol} \qquad (2)$$

has been reported previously to be 0.54 ± 0.78 kcal/mol,[12] we only needed to evaluate ΔG_{enz}. The average values for the forward and backward simulations are given in Figure 4 (ΔG_{sol} correction not included). Here, this is a relatively large perturbation, so the results do not converge as well as in the previous case. The calculated free energy difference is again quite large (9.6 ± 0.8 kcal/mol), but it is opposite to the dipole moment trend we observed in the previous calculation (this trend is observed in all of the following simulations). The dipole moment of **1** is 7.0 D, versus 10.8 D for **2**. This suggests that the free energy difference is arising due to bad steric contacts between active site residues and the active site model we are using. This is further suggested by the observation that the computed free energies have very large van der Waals contributions. Furthermore, we find that the zinc to CO_2 oxygen distance is far shorter in our quantum mechanical model (~3.0 Å) than in our molecular mechanical one (~3.9 Å).[12] Taken together this suggests that the zinc to CO_2 distance is probably too short in the ammonia model we are using.

Another interesting aspect of this case is the polarization of the CO_2 molecule when it is interacting with the zinc ion (**2**). Previous work has found that the charges of the C and O in free CO_2 are +0.908 and −0.454, respectively.[12] In Table I we find that these charges are reduced to +0.80 and ~−0.38, respectively. Hence, there is significant polarization of CO_2 (in the direction of donating electron density to the zinc ion) when it is coordinated to the zinc ion.

From the first FEP simulation (**2** to **3**) we predict a large environmental contribution. Since the gas-phase difference be-

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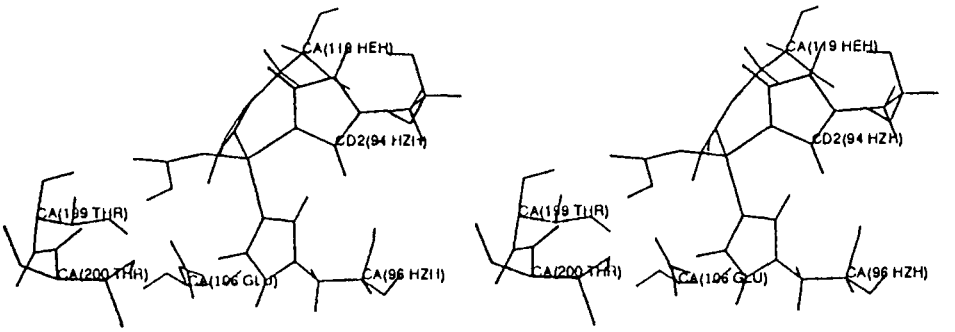

Figure 8. Snapshot of **5** docked in the active site of HCAII. Obtained after 30 ps of MD equilibration.

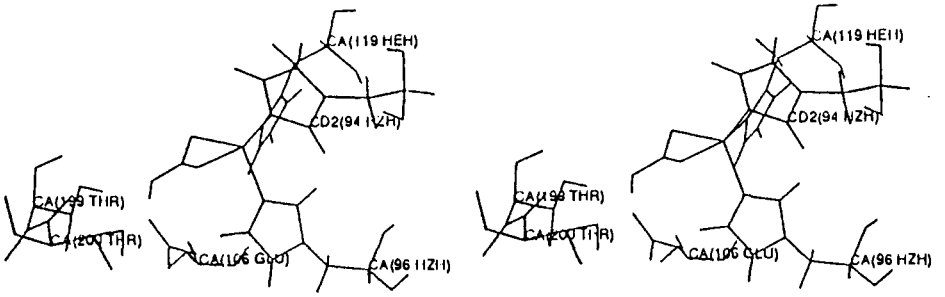

Figure 9. Snapshot of **6** docked in the active site of HCAII. Obtained after 180 ps of FEP simulation from **6**.

tween **2** and **3** is only about 2.5 kcal/mol, the enzyme stabilizes TS **3** more than complex **2**. Therefore, **3** becomes much more stable than **2**. From the second simulation we find that **2** is destabilized by the enzyme compared to the separated species (CO_2 and the zinc–hydroxide form of the enzyme). Hence, from these calculations we find that the location of the transition state has shifted. The reaction barrier for HCAII has been estimated to be 6.4 kcal/mol, and the free energy of reaction is estimated to be ~−2.5 kcal/mol.[30] Our calculated barrier of 9.8 ± 0.77 kcal/mol matches the experimental data reasonably well. Furthermore, we do not find an intermediate in which CO_2 is noncovalently bound to the deep-water pocket (A binding site[12]) in the HCAII active site. This is in contrast to recent MD[12,13] and FEP[12] simulations that predicted there was an intermediate in which CO_2 was noncovalently bound to HCAII in the A binding site with a ΔG_{bind} of −3.4 kcal/mol.[12] On the other hand, the lack of a CO_2 binding site in the deep-water pocket appears to be consistent with recent experimental studies, which indicate that mutating a residue in the deep-water pocket (Val-143 → Gly, His, Phe, Tyr) does affect function, but not to such an extent that a tightly bound CO_2 intermediate is necessary to explain the results.[6,14] However, since we find that the TS has shifted its location and given the model (i.e., ammonia for imidazole) we are using, there is some uncertainty regarding this observation.

The next two simulations involved perturbations from **3** to **5** and from **5** to **6**. We find that **5** is much more stable (−11.2 ± 0.8 kcal/mol) than **3** in the enzyme active site. This observation follows the same dipole moment trend described above (**5**, μ = 13.0 D; **3**, μ = 11.5 D). We also find that **5** is much more stable than **6** in the active site of HCAII (19.1 ± 2.7 kcal/mol). Again the dipole moment trend is followed (**5**, μ = 13.0 D; **6**, μ = 9.5 D). Hence, we predict that structures like **6** are not important in catalysis by HCAII. This suggests that rearrangement of the bicarbonate coordination from **5** to **6** is unnecessary for effective catalysis. From these calculations we find, however, that our free energy of activation in the reverse direction is too large (~25 kcal/mol versus ~8 kcal/mol). Hence, there are still some aspects of our model that are not reproducing the energetics of HCAII catalysis. This is further discussed below.

Stereo plots of **5** (a 30-ps equilibrated structure) and **6** (obtained after a 180-ps FEP simulation) in the HCAII active site are given in Figures 8 and 9. For **5** the interaction between the hydroxyl oxygen of Thr-199 and the hydroxyl hydrogen of the zinc-bound bicarbonate is retained (hydrogen bond distance of 1.5 Å), while for **6** it is not (2.7 Å). Moreover, the main-chain NH of Thr-199 interacts very nicely with the carbonyl oxygen of the zinc-bound bicarbonate (2.3 Å) in the case of **5**. For **6** this is not the case, where this distance is 4.7 Å. Furthermore, the Glu-106 to Thr-199 hydrogen bond contact is very distorted in the case of **6** (2.1-Å hydrogen bond distance). In this case the hydroxyl hydrogen from the zinc-bound bicarbonate is found to interact directly with Glu-106. For **5** this contact is nicely maintained and is not distorted (1.8-Å hydrogen bond distance). Hence, it is clear that **6** is not suited to interact effectively with the HCAII active site, while **5** fits nicely in the HCAII active site.

We have carried out reasonably sophisticated ab initio calculations and have studied this reaction using procedures that have been successful in studying solution-phase reactions;[32,33,46] however, we have not obtained an accurate estimation of the energetics of this reaction. We suspect that there are several reasons for this deficiency. First, we have had to use an approximate model of the reaction center due to computational expense. The past justification for using ammonia as a zinc ligand instead of imidazole came from early ab initio calculations of Pullman and co-workers.[20] They found that ammonia and imidazole donated similar amounts of electron density to zinc and that ammonia was therefore a reasonable model. Merz et al.[26] found that ammonia was a poor model for imidazole in their semiempirical calculations. In the present study we have found that CO_2 is much closer to zinc in our ammonia model than in our previously reported imidazole model.[12] This suggests that ammonia is possibly not as good a steric model for imidazole. Finally, all of the successful studies of reactions in solutions modeled the reactions in the gas phase using all of the required atoms (e.g., the Diels–Alder study used a full representation of the diene and dienophile[46]).[32,33,46] Taken together the data suggests that ammonia, while a computationally prudent model, is not the most realistic representation possible.

We have also observed in this study that the position of the TS changes once it is placed in the enzyme active site. This change of mechanism is due to the constraints imposed by the active site of the enzyme, which only permits certain orientations of the reacting species. This is not as important in the solution phase, where constraints such as those observed in an enzyme active site are not as important. In summary, we feel that the present model is deficient in the proper steric and electronic representation needed to obtain an accurate estimation of the HCAII-catalyzed reaction. To overcome this problem, coupled potential calculations using

an accurate representation of the HCAII active site seem warranted.[57]

Conclusions

High-level ab initio calculations and FEP simulations were used to investigate the HCAII-catalyzed hydration of CO_2 and dehydration of bicarbonate. Thermal and correlation corrections have also been employed, and FEP simulations were used to obtain the environmental free energy contributions.

The ab initio calculations match in many regards the structure and energetics observed in previous studies of this reaction.[20,27,29] However, we have used a better basis set and have fully characterized the potential surface using computated vibrational frequencies. Furthermore, we have assembled the free energy profile for this reaction and have found it to be significantly different from the total energy surface. Hence, consideration of enthalpic and entropic effects is necessary to get an accurate picture of this reaction surface.

The ab initio results combined with the results from the FEP simulations predict a barrier for the forward enzymatic reaction which matches the estimated experimental data reasonably well (~6.5 kcal/mol experimental versus 9.8 ± 0.77 kcal/mol calculated). The reverse barrier height, however, is poorly reproduced (~8.0 kcal/mol experimental versus ~25 kcal/mol calculated). We feel that this problem is due to the inherent limitations of using ammonia as a replacement for imidazole. From our ab initio calculations we find that species bound to zinc can hydrogen bond to ammonia in a manner that would be impossible if imidazole were used as a ligand (e.g., **4**). Furthermore, ammonia does not have the same "steric signature" as imidazole and probably does not have the same electronic characteristics.[26] Finally, we have also found that the TS position has shifted when we place our ab initio structures into the HCAII active site. These results taken together suggest that future work should focus on using a more realistic model of the zinc coordination environment (e.g., imidazole instead of ammonia) and on a realistic representation of the active site environment.

Our ab initio calculations predict that the proton-transfer pathway suggested by Liang and Lipscomb[17] and the angle-bending pathway proposed by Lindskog and co-workers[53] are less likely than a pathway involving rotation around the (Zn–)O–C(–O) bond. Furthermore, from our ab initio and FEP calculations we predict that internal proton transfer of zinc-bound bicarbonate is not necessary to explain HCAII catalysis. Experimental work has shown that there is no rate-limiting proton transfer during CO_2/HCO_3^- interconversion,[58] which is consistent with our results that suggest that no proton transfer is needed at all. The mechanism of CO_2 hydration that the present work suggests is as follows (see Scheme II): First, CO_2 associates with HCAII. The location of the binding site(s) can be either the E binding site[12] or the A binding site.[12] Our previous FEP and MD simulations prefer the A binding site,[12] while the present calculations indicate that the E binding site is more important. Once CO_2 is recognized, it is then hydrated by the zinc–hydroxide form of the enzyme. Next the zinc–bicarbonate form is generated, and we predict that it has a structure like that given in Figure 8. In this structure Thr-199 is hydrogen bonded to the hydroxyl hydrogen of bicarbonate, and the remaining oxygen atom of bicarbonate with the main-chain NH of Thr-199. This orientation is not identical (in structure) to that observed by Xue et al.,[5] but it is possible that when geometric relaxation is introduced, our structure might adopt the orientation seen experimentally. However, there is some uncertainty in the experimental structure given the resolution of the structure determination (1.9 Å), so it is hard to make a definitive comparison between these two structures. Finally, bicarbonate diffuses out and is replaced by a water molecule. Our proposed mechanism is similar to that proposed by Lindskog[59] (ours has more molecular-level details) and Håkansson et al.[3] Thus, it appears that theory and experiment have begun to reach a consensus regarding the molecular-level details of the mechanism by which HCAII hydrates CO_2.

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