Mode of Action of Carbonic Anhydrase

**Kenneth M. Merz, Jr.,*,†,1 Roald Hoffmann,† and Michael J. S. Dewar‡**

Contribution from the Department of Chemistry, Cornell University, Ithaca, New York 14853, and Department of Chemistry, The University of Texas at Austin, Austin, Texas 78712. Received August 8, 1988

Abstract: AM1 calculations are reported that address the following: (1) the hydration of CO_2 by H_2O and OH^-; (2) the deprotonation enthalpies of zinc-bound and -unbound H_2O and imidazole; (3) the catalytic mechanism of hydration (dehydration) of CO_2 by carbonic anhydrase (CA). The AM1 calculations on the hydration of CO_2 agree well with the ab initio results, in that the hydration of CO_2 by H_2O involves a rather large activation energy in the gas phase, while hydration by OH^- is activationless. However, the heat of reaction for OH^- hydration of CO_2 is found to be too exothermic due to errors in the calculated heats of formation of OH^- and CO_2. Our calculations also show, as have previous ab initio calculations, that zinc-bound water has a lower deprotonation enthalpy than does zinc-bound imidazole. This result agrees with the experimental evidence that suggests that the deprotonation of zinc-bound water initiates the catalytic activity of CA. The detailed calculations on the catalytic mechanism of hydration of CO_2 by CA have uncovered some interesting features as well as corroborated existing experimental results. Our calculations suggest and other workers' experiments have shown that the rate-determining step in the catalytic cycle of CA is the intramolecular proton transfer (PT). We have investigated four mechanisms for this step: (1) PT via a Thr-199, Glu-106 proton relay; (2) PT to water in the CA active site; (3) PT to His-64 via a two-water-proton relay; (4) PT to His-64 via a bicarbonate proton relay. It is predicted that there are two low-energy pathways, namely, the two-water relay and the bicarbonate proton relay. Our calculations also indicate that the hydration of CO_2 by the zinc–hydroxide form of CA proceeds via a trigonal bipyramidal transition state, which forms the zinc–bicarbonate species directly. We also find that the presence of a water molecule inhibits this reaction. Finally, given our results, we have suggested an alternative mechanism for the mode of action of CA.

An important issue in enzyme chemistry is to explain why enzyme reactions are so fast and so specific. To understand these two aspects, it is necessary to know the details of the catalytic mechanism. Unfortunately, such detailed mechanisms for most enzymes are not known with any certainty. This state of affairs is true in the case of the enzyme system studied here, namely, carbonic anhydrase (CA).[2] It is the goal of this research to provide new insight into the catalytic activity of CA.

CA is found in both plants and animals, and its only known biological function is to catalyze the interconversion of CO_2 and HCO_3^- (eq 1). The active site environment, as determined by

$$CO_2 + H_2O \rightleftharpoons H^+ + HCO_3^- \qquad (1)$$
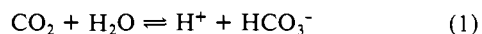
X-ray diffraction,[3] is given for the isoenzymes human carbonic anhydrase B and C (HCAB and HCAC), in Scheme I. In both cases the zinc atom is bound to three histidine imidazole groups, in a distorted tetrahedral array, with the fourth site occupied by either water or hydroxide ion hydrogen bound to Thr-199, which is itself hydrogen bonded to Glu-106. It has been conjectured that these latter interactions may be important for catalytic activity[4] because the Thr and Glu hydrogen-bonding network might serve as a proton shuttle. Ab initio pseudopotential calculations[5] support this proposal; they find that there is no activation energy (E_a) required for the proton relay to occur (i.e., the zinc–water species gives up a proton to Thr, which then gives up a proton to Glu). However, this mechanism requires that the pK_a of Glu-106 be anomalously high, thereby clouding the overall importance of this proton relay to CA activity.[4] The importance of this relay has also been criticized from an experimental point of view.[6] The original X-ray structure determination[7] of HCAC suggests that an ordered array of eight water molecules is in the active site, and recent X-ray studies have further refined the positions of these water molecules.[8] Others have proposed that the presence of these water molecules might be of some functional significance.[2] Another author has suggested that during the course of an enzyme reaction any water molecules that are not important to the catalytic behavior of the enzyme are removed from the active site.[9] It is then concluded that enzyme reactions should be considered as being similar to gas-phase reactions (i.e., occurring in the absence of solvent). We do find that one of the mechanistic steps (hydration of CO_2) is inhibited by the presence of H_2O, but

Scheme I
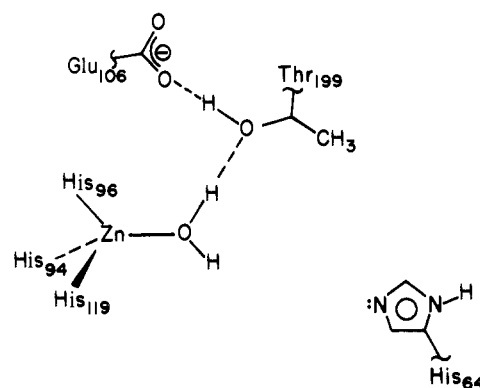
it is generally agreed that the water in the CA active site is of functional importance.[2]

(1) Present address: 152 Davey Lab, Department of Chemistry, The Pennsylvania State University, University Park, PA 16802.

(2) For some recent reviews see: Maikinen M. W. In *Techniques and Topics in Bioinorganic Chemistry*; McAuliffe, C. A., Ed.; Wiley: New York, 1975; p 60. Chlebowski, J. F.; Coleman, J. B. *Metal Ions in Biological Systems*; Sigel, H., Ed.; Marcel Dekker: New York, 1976; vol. 6. Lindskog, S.; Henderson, L. E.; Kannan, K. K.; Liljas, A.; Nyman, P. O.; Strandberg, B. *Enzymes* (3rd Ed.) **1977**, 5, 587. Prince, R. H. *Adv. Inorg. Chem. Radiochem.* **1979**, 22, 349; *Biophysics and Physiology of Carbon Dioxide*; Bauer, C., Gros, G., Bartels, H., Eds.; Springer-Verlag: New York, 1980; *Metal Ions in Biological Systems*; Sigel, H., Ed.; Marcel Dekker: New York, 1983; Vol. 15. Pocker, Y.; Sarkanen, S. *Enzymologia* **1978**, 47, 149. See also ref 6, 8, 13, and 14.

(3) For the HCAB structure see: Kannan, K. K.; Notstrand, B.; Fridborg, K.; Lövgren, S.; Ohlsson, A.; Petef, M. *Proc. Natl. Acad. Sci. U.S.A.* **1975**, 72, 51. See also ref 4. For the HCAC structure see: Liljas, A.; Kannan, K. K.; Bergsten, P.-C.; Waara, I.; Fridborg, K.; Strandberg, B.; Carlbom, U.; Jarup, L.; Petef, M.; Lövgren, S. *Nature New Biol.* **1972**, 235, 131. See also ref 7.

(4) Kannan, K. K.; Petef, M.; Fridborg, K.; Cid-Dresdner, H.; Lövgren, S. *FEBS Lett.* **1977**, 73, 115.

(5) Demoulin, D.; Pullman, A. In *Catalysis in Chemistry and Biochemistry. Theory and Experiment. 12th Jerusalem Symposium*; Pullman, B., Ed.; Reidel: Dordrecht, The Netherlands, 1979; p 51. See also ref 15.
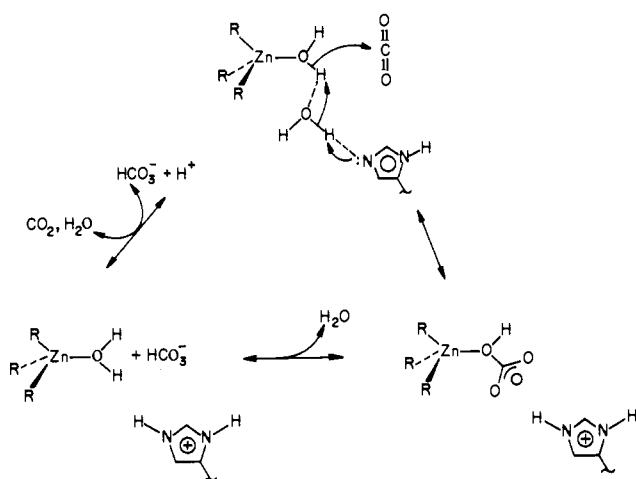
(6) Silverman, D. N.; Vincent, S. H. *CRC Crit. Rev. Biochem.* **1983**, 14, 207.

(7) Kannan, K. K.; Liljas, A.; Waara, I.; Bergsten, P.-C.; Lövgren, S.; Strandberg, B.; Bengtsson, U.; Carlbom, U.; Fridborg, K.; Jarup, L.; Petef, M. *Cold Spring Harbor Symp. Quant. Biol.* **1971**, 36, 221.

† Cornell University.
‡ The University of Texas at Austin.

Mode of Action of Carbonic Anhydrase

J. Am. Chem. Soc., Vol. 111, No. 15, 1989 5637

Scheme II

Scheme III

HCAB has a maximal turnover number for CO_2 hydration of about 1×10^4 s^{-1} at 25 °C, while for HCAC it is about 1×10^6 s^{-1} at 25 °C.[2] The ability of CA to hydrate CO_2 depends on its basic form generated by ionization of an active site group having a pK_a of around 7.[2] Many candidates for this group have been suggested (e.g., zinc-bound imidazole[2]), but in light of the experimental evidence, the consensus is that it is a water molecule bound to Zn(II),[2] which was first suggested by Davis.[10]

Theoretical support also has been given for this conclusion; thus, ab initio calculations have shown that the deprotonation of zinc-bound water is more favorable than either the deprotonation of zinc-bound imidazolium or that of imidazole itself.[11] This result casts doubt on several of the proposed mechanisms because they involve, for example, the deprotonation of imidazolium ion and not zinc-bound water (see Scheme II).[8] Hence, from both the theoretical[11] and experimental[2] evidence, it seems that the role of zinc is to reduce the pK_a of bound H_2O by electrostatic interaction, while at the same time allowing the hydroxyl group to retain significant nucleophilic character to attack CO_2.

There appears to be general agreement among workers studying CA that the catalytic mechanism can be described as follows (see Scheme III):[2] the zinc–water (1) species is first deprotonated, followed by the hydration of CO_2 by the resulting zinc–hydroxide form (3), which forms the zinc–bicarbonate species (5). The bicarbonate anion is then displaced by the water(s) contained in the CA active site, re-forming 1, from which the catalytic cycle continues. This mechanism follows ping pong kinetics with the two half-reactions being the proton transfer (i.e., 1 → 3) and the hydration step (i.e., 3 → 1).[2] From studies of the solvent isotope effect of CA, at high buffer concentrations (i.e., at physiological pH), it has been determined that the likely rate-determining step in this catalytic cycle is the intramolecular proton transfer (PT) from 1 to a proton acceptor in the active site (i.e., 1 to 2).[12] At low buffer concentrations, however, the intermolecular PT is rate determining (2 to 3).[12] The identity of the proton acceptor is possibly water in the CA active site or, more likely, a non-zinc-bound imidazole (His-64). The Thr-199, Glu-106 proton relay, discussed previously, also has been invoked as a proton acceptor,
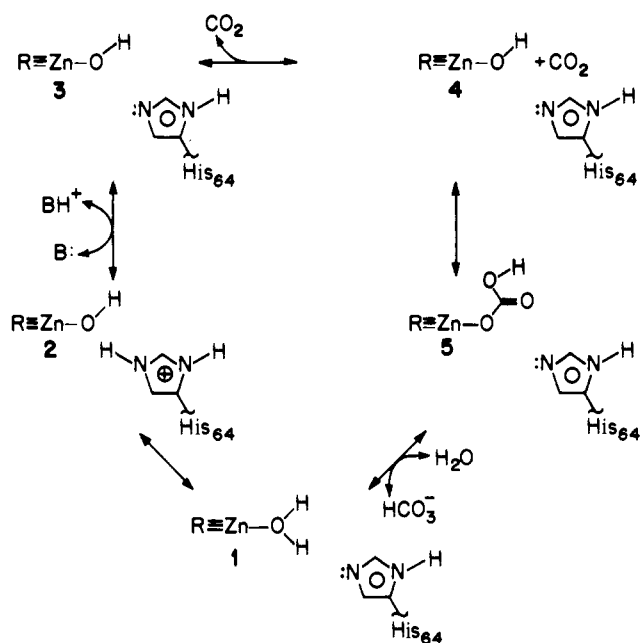
but this suggestion, though, has not been generally accepted.[6] Once the intramolecular PT is complete, the proton is then transferred from the proton acceptor to a buffer in the surrounding medium (step 2 to 3).[2,12] For the formation of the zinc–bicarbonate species several mechanisms have been proposed: for example, one mechanism has the oxygen directly attacking the carbon of CO_2,[13] (the outer-sphere mechanism) while another has the CO_2 oxygen coordinating with the zinc followed by attack by the oxygen of 2 (the inner-sphere mechanism).[14,15] Finally, the loss of the bicarbonate anion is thought to be facilitated by a water in the CA-active site and by a simultaneous loss, from the water molecule, of a proton to a group on the enzyme, which then completes the catalytic cycle by regenerating 1.[2]

Another aspect that has to be considered when studying CA is the dehydration reaction (the reverse of eq 1).[16] Kinetic studies show the bicarbonate anion recombines with CA and undergoes dehydration and not carbonic acid.[16] However, it should be stressed that the identity of the final species released by the hydration reaction (and the starting reactant for the dehydration reaction) is not known, but in light of the aforementioned experimental results, it is generally assumed to be the bicarbonate anion.[16]

Besides the ab initio pseudopotential calculations of Pullman and Demoulin[5,11,15] several other theoretical treatments on CA have appeared.[17-24] Clementi et al.[17] and Jönsson[18] have looked

(8) Eriksson, E. A.; Jones, T. A.; Liljas, A. In Zinc Enzymes; Bertini, I., Luchinat, C., Maret, W., Zeppezauer, M., Eds.; Birkhäuser: Boston, 1986; p 317.

(9) Dewar, M. J. S.; Storch, D. M. Proc. Natl. Acad. Sci. U.S.A. 1985, 82, 2225.

(10) Davis, R. P. J. Am. Chem. Soc. 1959, 84, 5674.

(11) Demoulin, D.; Pullman, A.; Sarkar, B. J. Am. Chem. Soc. 1977, 99, 8498. Demoulin, D.; Pullman, A. Theor. Chim. Acta (Berlin) 1978, 49, 161. Pullman, A.; Demoulin, D. Int. J. Quantum. Chem. 1979, 16, 641. Pullman, A.; Demoulin, D. In Water and Metal Cations in Biological Systems; Pullman, B., Yagi, K., Eds.; Japan Scientific Societies Press: Tokyo, 1980; p 135. See also ref 15.

(12) Tu, C. K.; Silverman, D. N. J. Am. Chem. Soc. 1986, 108, 6065. Kararli, T.; Silverman, D. N. J. Biol. Chem. 1985, 260, 3484. Kararli, T.; Silverman, D. N. Ann. N.Y. Acad. Sci. 1984, 429, 129. See also ref 2 and 6.

(13) Lipscomb, W. N. Ann. Rev. Biochem. 1983, 52, 17.

(14) Lindskog, S. In Zinc Enzymes; Spiro, T. G., Ed.; Wiley: New York, 1983; p 77.

(15) Pullman, A. Ann. N.Y. Acad. Sci. 1981, 367, 340, and references cited therein.

(16) Kalifah, R. G. In Biophysics and Physiology of Carbon Dioxide; Bauer, C., Gros, G., Bartels, H., Eds.; Springer-Verlag: New York, 1980; p 206, and references cited therein.

(17) Clementi, E.; Corongiu, G.; Jönsson, B.; Romano, S. J. Phys. Chem. 1980, 72, 260. Clementi, E.; Corongiu, G.; Jönsson, B.; Romano, S. FEBS Lett. 1979, 100, 313. Clementi, E.; Corongiu, G.; Jönsson, B.; Romano, S. Gazz. Chim. Ital. 1979, 109, 669.

(18) Jönsson, B. In Catalysis in Chemistry and Biochemistry. Theory and Experiment. 12th Jerusalem Symposium; Pullman, B., Ed.; Reidel: Dordrecht, The Netherlands, 1979; p 67.

(19) Sheridan, R. P.; Allen, L. C. J. Am. Chem. Soc. 1981, 103, 1544.

(20) Cook, C. M.; Haydock, K.; Lee, R. H.; Allen, L. C. J. Phys. Chem. 1984, 88, 4875. Cook, C. M.; Lee, R. H.; Allen, L. C. Int. J. Quantum Chem., Quantum Biol. Symp. 1983, 10, 263. For a summary of the research done by Allen group on CA see: Allen, L. C. Ann. N.Y. Acad. Sci. 1981, 367, 383.

(21) Sawaryn, A.; Sokalski, W. A. Int. J. Quantum. Chem. 1979, 16, 293. Sokalski, W. A. Int. J. Quantum Chem. 1981, 20, 231. Sokalski, W. A.; Sawaryn, A.; Cojnacki, H. Int. J. Quantum Chem. QBS10 1983, 321.

at the solvation around the active site of CA via Monte Carlo methods. These workers have suggested that zinc has a coordination number of five or six and that at several angstroms away the water solvation sphere is ordered. Sheridan and Allen[19] have constructed the electrostatic potential field for HCAB and HCAC and have discussed several qualitative features of CA. Cook and Allen[20] using an ab initio approach have located a transition state (TS), involving five-coordinated zinc, for the hydration of CO_2 by CA and have discussed how inhibitors act on CA. The group of Sokalski[21] has used both empirical (CNDO/2) and nonempirical (ab initio) methods to study various aspects of CA activity, while Jean, Lehn, and co-workers have used ab initio methods to study the effect of cations on the hydration of CO_2[22] and the hydration complexes of bicarbonate.[23] Liang and Lipscomb[24] have recently studied CA using PRDDO and ab initio calculations, and we will touch on various aspects of their contributions later.

Since CA hydrates CO_2, it is important to understand how this reaction occurs in the absence of CA both in the gas phase and in solution. Numerous theoretical[25] studies have appeared on the hydration of CO_2 by H_2O and HO^- in the gas phase. These studies have shown that the E_a for H_2O hydration is high (about 55 kcal/mol) while HO^- hydration is activationless. In solution the behavior is, as expected, different.[26] Both hydration reactions now have E_a's, but in the H_2O case the E_a is significantly lower (17.7 kcal/mol) than it is in the gas phase, while for HO^- the E_a is larger than it is in the gas phase (13.1 kcal/mol).

In our theoretical study on CA we decided to address the following: first, we would like to "benchmark" AM1 by studying the gas-phase hydration of CO_2. Next we would like to calculate the deprotonation enthalpies of zinc-bound and unbound H_2O and imidazole, using AM1. Finally, we will study each step of the CA catalytic cycle (except the intermolecular PT step) outlined in Scheme III. We also will consider the ramifications of the formation of carbonic acid as the product in the hydration reaction. In all cases we will compare our results with the available experimental and theoretical results.

Methodology

The calculations were carried out using the standard AM1 method[27] as implemented by the AMPAC program.[28] All geometries were fully optimized with no assumptions made regarding symmetry unless otherwise stated. All reactions will be followed by using the reaction coordinate method[29] with an appropriate internuclear distance as the reaction coordinate. Transition state (TS) found in this way were refined and characterized by the McIver–Komornicki procedure.[30] Several proton-transfer TSs were found by "building" the TS. In this approach symmetry is set between the breaking and forming X–H (X = C, O, etc.) bonds, and a normal Davidson–Fletcher–Powell geometry optimization is carried out. The final point from this optimization is then refined by the McIver–Komornicki procedure.[30] This approach has been found to be a very efficient method in locating proton-transfer TSs in which the forming and breaking bonds are of equal magnitude in the TS. Very early or late proton-transfer TSs are not easily located by using this method.

(22) Jean, Y.; Lehn, J.-M. *Chem. Phys.* **1979**, *39*, 111. Jean, Y.; Volatron, F. *Chem. Phys.* **1982**, *65*, 107.

(23) Jean, Y.; Volatron, F. *Chem. Phys.* **1980**, *53*, 95.

(24) Liang, J.-Y.; Lipscomb, W. N. *Biochemistry* **1987**, *26*, 5293. Liang, J.-Y.; Lipscomb, W. N. Submitted to *Biochemistry*. Liang, J.-Y.; Lipscomb, W. N. Submitted to *Int. J. Quantum Chem.*

(25) (a) Liang, J.-Y.; Lipscomb, W. N. *J. Am. Chem. Soc.* **1986**, *108*, 5051. (b) Nguyen, M. T.; Ha, T. K. *J. Am. Chem. Soc.* **1984**, *106*, 599. (c) Williams, J. O.; van Alsenoy, C.; Schaefer, L. *J. J. Mol. Struct.* **1981**, *76*, 109. (d) Jönsson, B.; Karlström, G.; Wennerström, H. *J. Am. Chem. Soc.* **1978**, *100*, 1658. (e) Jönsson, B.; Karlström, G.; Wennerström, H.; Forsen, S.; Roos, B.; Almlöf, J. *J. Am. Chem. Soc.* **1977**, *99*, 4628. (f) Jönsson, B.; Karlström, G.; Wennerström, H. *Chem. Phys. Lett.* **1976**, *41*, 317.

(26) For hydration by H_2O see: Magid, E.; Turbeck, B. O. *Biochem. Biophys. Acta* **1968**, *165*, 515. For hydration by OH^- see: Pinsent, B. R. W.; Pearson, L.; Roughton, F. J. W. *Trans. Faraday Soc.* **1956**, *52*, 1512.

(27) Dewar, M. J. S.; Zoebisch, E. G.; Healy, E. F.; Stewart, J. J. P. *J. Am. Chem. Soc.* **1985**, *107*, 3902.

(28) QCPE Publication 506, Department of Chemistry, Indiana University, Bloomington, IN.

(29) Dewar, M. J. S.; Kirschner, S. *J. Am. Chem. Soc.* **1971**, *93*, 4290.

(30) McIver, J. W.; Komornicki, A. *J. J. Am. Chem. Soc.* **1972**, *94*, 2625. McIver, J. W.; Komornicki, A. J. *Chem. Phys. Lett.* **1971**, *10*, 303.
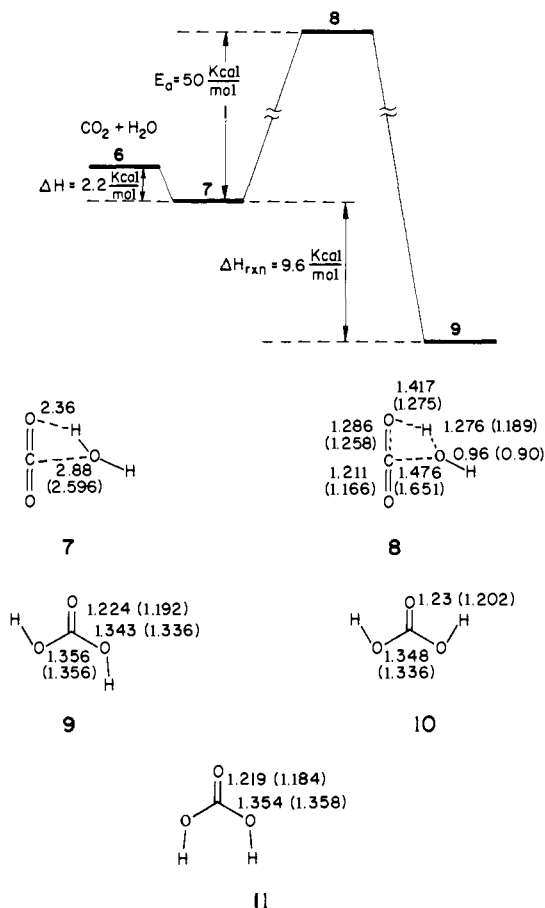

Figure 1. Reaction profile for $H_2O + CO_2 \rightarrow H_2CO_3$ and the AM1 (ab initio) geometries for **7–11** (distances in angstroms).

AM1 has been chosen as our molecular model because it reproduces hydrogen-bond energetics satisfactorily;[27,31] however, the structural features of hydrogen bonding appear to be less well reproduced.[31b,d,g] AM1 was also chosen because it gives E_a's that compare well with experiment,[27] gives proton affinities and deprotonation enthalpies that compare well with experiment,[32] and has been recently parametrized for zinc.[33]

The three-dimensional disposition of the catalytically important groups in the active sites can be obtained from the Brookhaven Protein Data Bank (PDB):[34] The HCAC and HCAB coordinates[3,8] suggest that the zinc coordination sphere is that of a distorted tetrahedron, and we found that our calculations do indeed give this coordination geometry. A further comparison between our model active-site structure and that of HCAC[8] will be given at the beginning of subsection 3. In our first calculations we used the smallest possible model that still realistically mimicked the CA active site (e.g., to model histidine we used ammonia). We found, though, that when we used imidazole to model the histidine imidazoles, the computational results were significantly different (e.g., the E_a's were uniformly higher). Hence, we decided to exclusively use imidazole throughout our calculations, even at the cost of extra computer time, because we felt that this model provides a more reasonable description of the CA active site.

When we studied reactions in which solvation would be important, we initially assembled the solvation sphere around the reacting center using the supermolecule approach.[35] Our implementation of this approach is

(31) See, for example: (a) Donn M. Storch, Ph.D. Dissertation, The University of Texas, 1985, for further discussion. (b) Dannenberg, J. J. *J. Phys. Chem.* **1988**, *92*, 6869. (c) Dannenberg, J. J.; Vinson, L. K. *J. Phys. Chem.* **1988**, *92*, 6869. (d) Williams, I. H. *J. Am. Chem. Soc.* **1987**, *109*, 6299. (e) Galera, S.; Lluch, J. M.; Bertran, J. *J. Mol. Struct. (THEOCHEM)* **1988**, *163*, 101. (f) Buemi, G.; Zuccarello, F.; Raudino, A. *J. Mol. Struct. (THEOCHEM)* **1988**, *164*, 379. (g) Bliznyuk, A. A.; Voityuk, A. A. *J. Mol. Struct. (THEOCHEM)* **1988**, *164*, 343. See ref 9.

(32) Dewar, M. J. S.; Dieter, K. M. *J. Am. Chem. Soc.* **1986**, *108*, 8075.

(33) Dewar, M. J. S.; Merz, Jr.; K. M. *Organometallics* **1988**, *7*, 522.

(34) Bernstein, F. C.; Koetzle, T. F.; Williams, G. J. B.; Meyer, Jr., E. F.; Brice, M. D.; Rogers, J. R.; Kennard, O.; Shimanouchi, T.; Tasumi, M. *J. Mol. Biol.* **1977**, *112*, 535.

Mode of Action of Carbonic Anhydrase

J. Am. Chem. Soc., Vol. 111, No. 15, 1989 5639

Table I. AM1 Calculated ΔH_f's for **6–13** and Experimental ΔH_f's for **9, 12,** and **13**

| compd | ΔH_f, kcal/mol | | compd | ΔH_f, kcal/mol | |
	calcd	exptl		calcd	exptl
6	−139.0		10	−149.8	
7	−141.2		11	−142.9	
8	−91.2		12	−93.9[b]	−128.3[a,b]
9	−150.8	−147.0[a]	13	−179.7	−177.3[c]

[a] From: Pedley, J. B.; Rylance, J. *Sussex-NPL Computer Analyzed Thermodynamical Data: Organic and Organometallic Compounds*; University of Sussex: England, 1977. [b] Calculated by adding together the ΔH_f's of CO_2 and HO^-. [c] Calculated from the experimental ΔH_{rxn} for $CO_2 + HO^-$ to HCO_3^-. See ref 25a.

as follows: we "froze" (i.e., turned off the optimization flags) the geometry of the molecule we wanted to solvate; we then placed a water molecule near the various hydrogen-bonding sites and optimized all of its geometric variables. Upon identification of the lowest energy site for the first water molecule we fixed it at this location and followed the same procedure for the next water molecule. We then followed the reaction using the reaction coordinate method[29] with full geometry optimization at each point along the reaction path. While this approach is not "statistical", we expect that it will be able to allow us to get an idea concerning the effect solvation might have on a particular reaction.
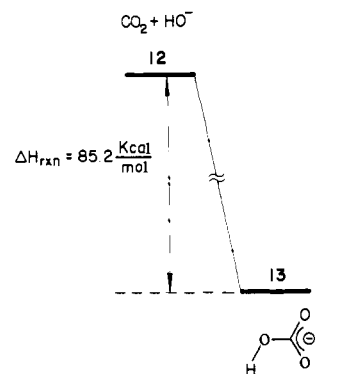
Discussion

This section is broken down into three subsections; the first one describes the hydration of CO_2 by H_2O and HO^-, while the second presents our theoretical determination of the deprotonation enthalpy of zinc-bound water and imidazole. The third section describes our calculations on the mechanism of CA itself.

1. Gas-Phase Hydration of CO_2 by H_2O and HO^-. We first studied the hydration of CO_2 by H_2O. The pertinent geometries are given in Figure 1 (ab initio geometrical parameters in parentheses) along with a schematic reaction profile, while the heats of formation (ΔH_f's) of these species are given in Table I.

The AM1 calculated value for the E_a (50 kcal/mol) is of the same magnitude (49–65 kcal/mol) as that determined by various ab initio methods.[25] The AM1-calculated TS geometry (**8**) is, however, different from the ab initio one. From Figure 1 it can be readily seen that the AM1 TS has O–H bond lengths that are longer than the corresponding ab initio ones,[25b] while the developing C–O bond is shorter. Another discrepancy between the AM1 results and the ab initio results arises when a comparison between the various possible structure of carbonic acid (**9–11**) is made. AM1 predicts the cis–trans form of carbonic acid (**9**) to be more stable than the trans–trans form (**10**), while ab initio calculations predict the opposite.[25b] Both methods predict that **11** is the least stable of the carbonic acid conformational isomers. Regardless of these latter differences the most important thing to remember is that the AM1-calculated E_a for the hydration of CO_2 by H_2O is in good agreement with the ab initio E_a. Finally, AM1 gives a value of 9.6 kcal/mol for the heat of reaction (ΔH_{rxn}) while the best ab initio values are in the range 13–5 kcal/mol.[25]

We next studied the gas-phase hydration of CO_2 by HO^- and found, as have other workers,[25] that this reaction is activationless. Our calculated reaction profile is given in Figure 2, while Table I gives the pertinent ΔH_f's. The AM1 value for the ΔH_{rxn} is −85.2 kcal/mol, which is greater than the experimental value (−49 kcal/mol[25a]) by 36.2 kcal/mol. Almost all of this error is associated with the underestimation of the ΔH_f's of CO_2 and HO^-, by 14.3 and 20.1 kcal/mol, respectively. AM1, on the other hand, gives a good estimation for the ΔH_f's of carbonic acid and bicarbonate ion (see Table I).

In conclusion, AM1 reproduces the potential energy surface of the $CO_2 + H_2O$ reaction reasonably well, while the ΔH_{rxn} for

(35) Pullman, A. In *Quantum Theory of Chemical Reactions*; Daudel, R., Pullman, A., Salem, L., Veillard, A., Eds.; Reidel: Dordrecht, The Netherlands, 1976; Vol. II, p 149. Pullman, A. In *The New World of Quantum Chemistry, Proceedings of the Second International Congress of Quantum Chemistry*; Pullmann, B., Ed.; Reidel: Dordrecht, The Netherlands, 1981; p 1.

Figure 2. Reaction profile for $HO^- + CO_2 \rightarrow HCO_3^-$.

Table II. AM1 Calculated ΔH_f's and ΔH_{dep} for **14, 15,** H_2O, and ImH

HB	B⁻	ΔH_f(HB), kcal/mol	ΔH_f(B⁻), kcal/mol	ΔH_{dep},[a] kcal/mol
14H	14⁻	427.4	243.7	183.4
15H	15⁻	506.7	341.7	202.1
H_2O	HO⁻	−59.2	−14.1	412.3
ImH	Im⁻	50.8	31.3	347.7

[a] Calculated by using the experimental ΔH_f for H^+ (367.2 kcal/mol).[32]

the $CO_2 + HO^-$ reaction is too exothermic by 36.2 kcal/mol. This error, though, is almost entirely due to the errors in the AM1-calculated ΔH_f's of CO_2 and HO^-. We note, however, that while the AM1-calculated ΔH_f for hydroxide ion is off by 20.1 kcal/mol, the ΔH_f's for other anions are reasonably well reproduced (e.g., ethoxide, methoxide, etc.). It is thought that the reason AM1 does well for "substituted" anions and not for the ("unsubstituted") hydroxide ion has to do with the inability of the AM1 Hamiltonian to effectively polarize or spread out the negative charge.[27] This effect is attenuated in the substituted anions due to the presence of more atoms onto which the charge can be shared. This should also be the case for hydroxide that is bound to a zinc ion. Thus, in our subsequent calculations we will concern ourselves only with the fact that AM1 gives a too positive ΔH_f for CO_2.

2. Deprotonation Enthalpies. There was for a number of years quite a bit of controversy surrounding the identity of the active-site group that has a pK_a of around 7, which upon ionization initiated the catalytic activity of CA.[2] Two of the leading contenders were zinc-bound imidazole and zinc-bound water, with the available evidence favoring the latter.[2] One piece of convincing evidence was the ab initio calculation of Demoulin and Pullman,[11] which showed that the complexation of Zn^{2+} with H_2O and imidazole lowers the deprotonation enthalphy (ΔH_{dep}) of the former more than its does for the latter complex. To determine if AM1 gives a similar prediction as the ab initio calculations and to further refine these predictions (AM1 has been recently shown to give ΔH_{dep} values that agree well with the experimental ones,[32] and we are using a more realistic model of the CA active site), we decided to calculate the ΔH_{dep} for the $(Im)_3Zn-OH_2$ (**14H**) and $(Im)_3Zn-Im$ (**15H**) complexes and to compare them to that of uncomplexed H_2O and imidazole.
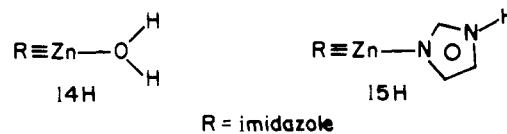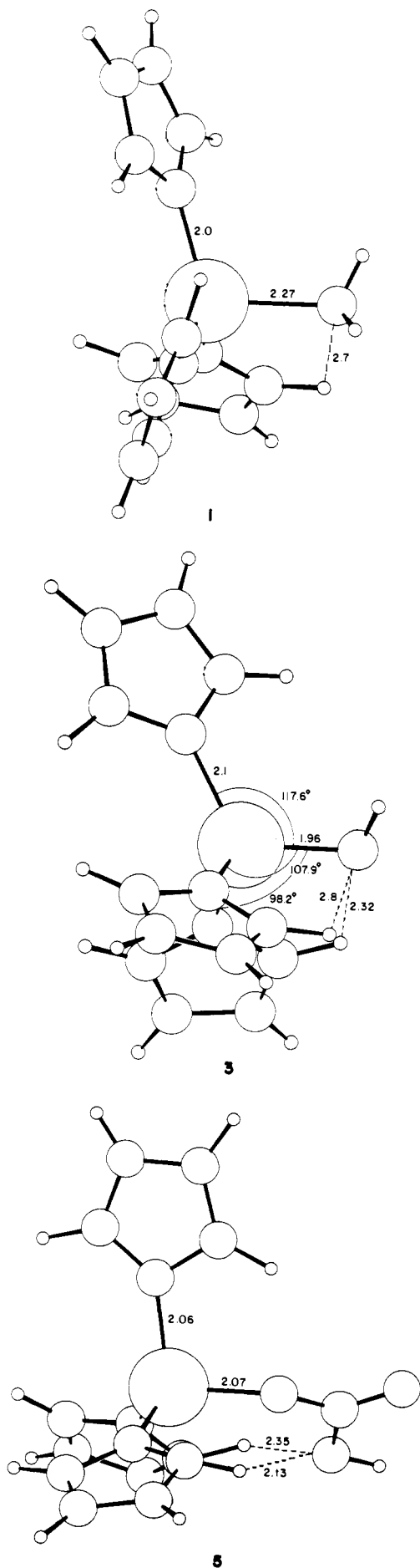

R = imidazole

Table II contains the ΔH_f's for the various species of interest along with our calculated ΔH_{dep} values. The conclusions we arrive at (i.e., the deprotonation of **14H** is favored over that of **15H**) are similar to those of Demoulin et al.[11] Therefore, AM1 also predicts that the zinc–hydroxide mechanism is favored over ones in which the deprotonation of zinc-bound imidazole is necessary. Note, though, that these calculations have not proven that zinc-

Figure 4. Stabilization energies for H_2O + imidazole $\rightarrow$ $H_2O \cdot$ imidazole. Note that each ΔH_{stabil} represents the stabilization resulting from the interaction of *one* water molecule with one site on imidazole.

bound water is the group of pK_a = 7 that controls the activity of CA. Finally, we should note that the ΔH_{dep} calculated for **14H** is to be considered to be the upper limit for this value because the error between the calculated and experimental value for the ΔH_{dep} of H_2O is 21.5 kcal/mol.

3. Mode of Action of Carbonic Anhydrase. There are three distinct mechanistic steps for which we have done calculations: deprotonation step, hydration of CO_2 step, and the loss of bicarbonate ion. We will address each of these in three separate subsections. In the fourth and final subsection we address the possibility of carbonic acid being the final product in the hydration reaction and its possible role as a proton bridge between the active site and His-64. First, though, let us discuss some of the salient geometrical features of the zinc–water (**1**), zinc–hydroxide (**3**), and the zinc–bicarbonate (**5**) species (where R = imidazole).

The geometries for **1**, **3**, and **5** are given in Figure 3. In general the geometries are as expected (e.g., the Zn–O bond shortens on going from **1** to **3**). From the recent HCAC coordinates[8] we are able to compare our zinc–hydroxide structure to that found in the enzyme: the calculated Zn–N and Zn–O bond lengths are 2.1 and 1.96 Å, while the observed values are 2.06–1.99 and 2.12 Å, respectively. Thus AM1 reproduces the bond lengths around the zinc ion reasonably well. However, one unusual feature of these geometries is the short distance between the C–H (α to N and N–H) hydrogen and the lone-pair orbitals present on the oxygen(s), which results in the contraction of the O–Zn–N bond angle. Thus, in **3** we find that this distance is 2.32 Å[36] and the O–Zn–N bond angle is 98.2° (vs 109.2° average bond angle observed experimentally[8]). Similar distances and bond angle contractions are observed in **1** and **5**. The cause of this can be traced to the stabilizing interaction present between the C–H bond and the oxygen lone pairs. This interaction causes a significant distortion in our active-site geometry, so we decided to trace it further to assess whether it would affect any of our conclusions. This is done in Figure 4, where we give the stabilization afforded by a water molecule interacting with various sites on imidazole.

We find that the normal locations for the formation of a hydrogen bond afford the greatest stabilization; however, we also find that the C–H bond α to the two nitrogens can also interact relatively strongly with water. Ab initio calculations have been carried out on the first solvation sphere around imidazole, but they do not address the stabilization afforded by water interacting with the C–H bond,[37a] however, they do provide some geometric information about the N1–H6 bond distance and the C2–N1–H6 bond angle. We note that previous calculations have addressed this question, albeit on molecules other than imidazole, and have demonstrated that strong interactions can be present.[37b] Our calculated value and the ab initio (in parentheses) ones are given

---

(36) It is important to define what AM1 predicts to be a reasonable hydrogen-bonding distance when an oxygen atom is the hydrogen-bond acceptor. In our experience a distance between 2.0 and 2.4 Å (e.g., hydrogen-bond distance in the water dimer is calculated to be 2.16 Å by AM1) signals that a significant stabilizing interaction is present.

(37) (a) Prado, F. R.; Giessner-Prettre, C.; Pullman, B. *Org. Magn. Reson.* **1981**, *16*, 103. (b) Pullman, A.; Pullman, B. *Q. Rev. Biophys.* **1975**, *7*, 505.
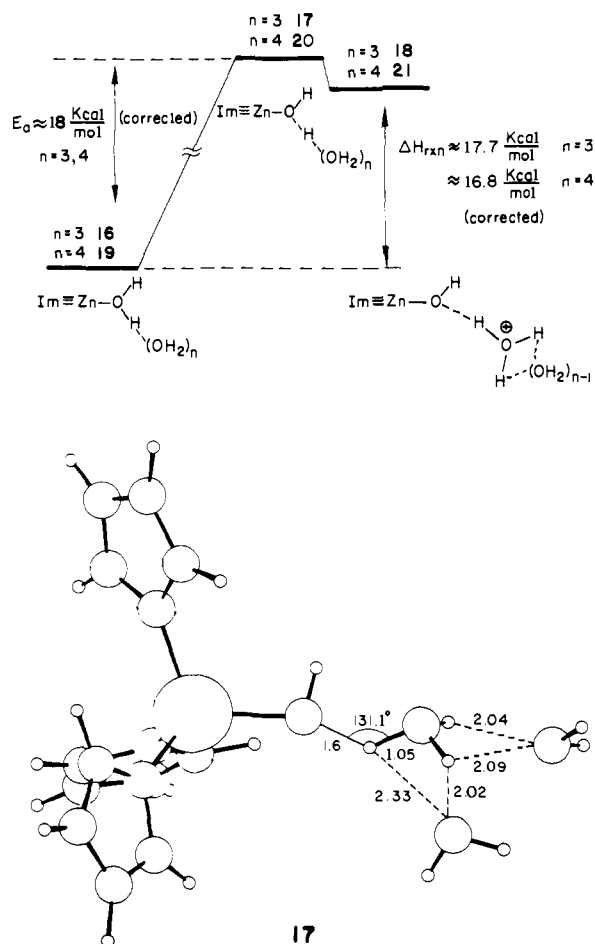

Figure 3. Geometries for **1**, **3**, and **5** (distances in angstroms).

Mode of Action of Carbonic Anhydrase

J. Am. Chem. Soc., Vol. 111, No. 15, 1989 5641

Figure 5. Reaction profile for $Im_3Zn-OH_2\cdot(OH_2)_n \rightarrow Im_3Zn-OH\cdot OH_3^+\cdot(OH_2)_{n-1}$ ($n = 3, 4$) and the geometry of **17** (distances in angstroms).

in Figure 4. Our calculated N1–H6 bond distance (2.74 Å) is shorter than the ab initio distance (3.0 Å), but the major difference is in the C2–N1–H6 bond angle. We predict that this bond angle is 78.5°, while the ab initio value is 107°. The reason for the difference has to do with the strength of the interaction between O11 and H7. In our case this interaction is greater (O11–H7 bond distance is 2.3 Å) than in the ab initio case, but both approaches do indeed predict that some interaction is present between O11 and H7 (otherwise we would expect the bond angle to be about 126°). Finally, we note that we were able to locate a minimum corresponding to a situation in which the O11–H7 interaction is expected to be minimal (i.e., bond angle of 126°) that resulted in only a 0.18 kcal/mol reduction in the hydrogen-bond strength.

In conclusion, while the O11–H7 stabilizing interaction is probably slightly overestimated relative to the ab initio calculations on imidazole,[37a] its effect on what follows should be minimal (e.g., effect on E_a's); therefore, we will not dwell on it further.

A. Deprotonation Step. We will address only three possibilities for this step here. The first involves the transfer of the proton to water that is the active site, the second is the proton relay (Glu-106 and Thr-199), and finally the proton can be transferred to His-64 via a water bridge.[6] In subsection D we will show that carbonic acid can also serve as a proton bridge between the active site and His-64.

In preliminary studies (in which the ligands surrounding zinc were ammonia) we found that in order to get a reasonable E_a for the PT to the surrounding water, we needed at least three water molecules. Hence, in what we report here we have simulated the surrounding water with three and four waters (for the reaction we will be studying see Figure 5). We felt that this number of water molecules was reasonable in that there is evidence that there are about eight water molecules present in the active site of CA.[7]

Table III. AM1 Calculated ΔH_f's for **16–21**

compd	ΔH_f, kcal/mol	compd	ΔH_f, kcal/mol
$n = 3$		$n = 4$	
16	219.0	19	148.9
17	242.0	20	172.0
18	241.7	21	170.8
		24	325.4
		26	323.6

Scheme IV
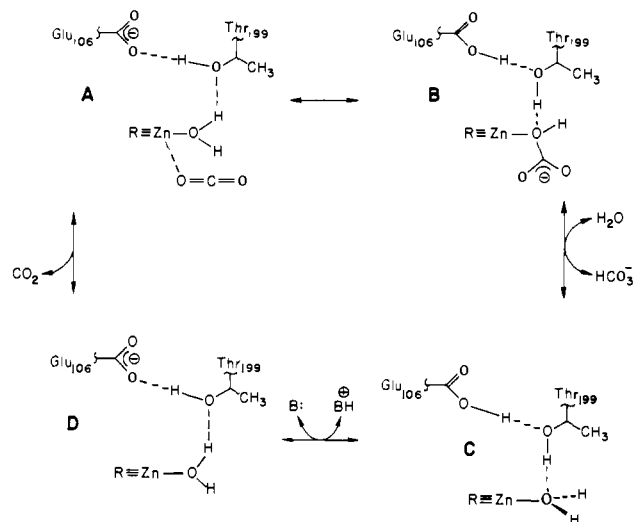
We find that for $n = 3$ (see Table III) the E_a for the PT is 23.0 kcal/mol, while the ΔH_{rxn} for the reaction is 22.7 kcal/mol. Note, though, that the AM1-calculated ΔH_f of the hydronium ion is too positive by 5 kcal/mol.[27] Thus, the calculated E_a and ΔH_{rxn} for this reaction are to be considered as upper limits to the true E_a and ΔH_{rxn}, and more realistic estimates of these parameters are 18 and 17.7 kcal/mol, respectively (both corrected by 5 kcal/mol). The geometry of the PT TS (**17**) is given in Figure 5 along with the reaction coordinate for this reaction, while the pertinent ΔH_f's are given in Table III. The geometry for the PT TS shows that it is best described as being "late", because it closely resembles **18** ($n = 3$). For example, the Zn–O bond is almost completely formed (see **3** in Figure 3), and the hydrogen is almost fully transferred. Another interesting feature of the geometry of **17** is the hydration of the developing hydronium ion. The oxygen atoms of the two water molecules present in the active site form two close contacts with the hydrogens of the nascent hydronium ion. Interactions of this sort allow the water molecules to further stabilize the hydronium ion. For $n = 4$ the results are nearly identical, demonstrating that for even higher n we should not expect a large diminution in the E_a or the ΔH_{rxn}.

We next modeled the PT via the proton relay network. We attempted to locate a minimum corresponding to **22** (with methanol modeling Thr-199, and ionized formic acid modeling Glu-106) that was to model the protonated form of the enzyme; however, no minima could be located that corresponded to **22**.
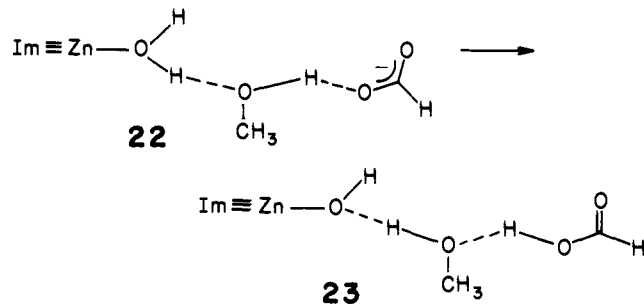
What we found was that **22** rearranged to give **23** without activation. These results are similar to those found by Pullman[15]

using an ab initio pseudopotential approach. This proton shuttle has been invoked in the mechanism outlined in Scheme IV, which has been suggested by Kannan et al.[4] This mechanism proceeds as follows: The CO_2 molecule interacts with the zinc ion, the proton relay shuttles a proton from the zinc-bound water to Glu-106, and subsequently the zinc–bicarbonate form of the enzyme is formed (A → B). Water then displaces the bicarbonate ion from the zinc (B → C), and an external base removes the proton from neutral Glu-106 (C → D). This mechanism satisfies all of the aforementioned criteria for a valid CA mechanism (see introduction); however, detractors of this mechanism,[6] as well as the authors that first proposed this mechanism,[4] point out that Glu-106 would have to have an anomalously high pK_a. It has to be pointed out that the protein environment of Glu-106 may alter the pK_a, but this has not been proven to be the case yet. Our calculations as well as Pullman's[15] do suggest that the zinc–hydroxide form of the enzyme can be formed spontaneously via the proton shuttle (A → B). On the other hand, we have not demonstrated that the pK_a of Glu-106 has been increased, nor will we be able to until the whole protein environment can be accounted for. Thus, while we can support the idea that Glu-106 and Thr-199 can be involved in a proton shuttle (A → B), we cannot address whether Glu-106 does indeed have an anomalously high pK_a or not. This will have to be left to future workers.[38]

An interesting suggestion has been made by Venkatasubban and Silverman[39] concerning a possible mechanism for the intramolecular PT step. In their mechanism two water molecules act as a "water bridge" in the transfer of one proton from the active site to His-64 (see Figure 6). In our calculations on this possibility we were unable to locate a TS (25) for the proton transfer, because once the proton is transferred to the first water molecule in the chain the others simultaneously migrate to give 26. Thus, the geometrical changes that occur at the TS are very large and involve the breaking and formation of a total of six bonds, and any attempt to locate the precise TS geometry was thwarted because of this complexity. The observation that 25 collapses directly to 26 is not an unusual phenomenon when full geometry optimizations are done and is, in this case, the result of the fact that the proton affinity of water (164.5 kcal/mol, AM1; 166.5 kcal/mol, experiment) is much less than that of imidazole (221.7 kcal/mol, AM1; 222.1, experiment). Thus, upon full geometry optimization the lowest energy structure that is obtained is not an intermediate where a water molecule is protonated but one in which the imidazole is (26). We were, however, able to arrive at an approximate E_a of 18 kcal/mol by taking the highest point along the calculated reaction path 24 → 25 → 26 and subtracting 5 kcal/mol for the error in the ΔH_f of H_3O^+. The calculated ΔH_{rxn} is −1.73 kcal/mol. Thus, we predict that this is a viable reaction path for the intramolecular proton transfer. The geometries for 24 and 26 and a reaction profile are given in Figure 6, while the ΔH_f's are given in Table III. When we set up this supermolecule calculation, the initial distance between the zinc ion and the imidazole nitrogen, to which the proton will be transferred, was close to the 8-Å distance observed in the HCAC X-ray structure.[3,8] Upon AM1 optimization of 24 we find that there is not a large deviation from the initial zinc-to-nitrogen distance imposed (this distance is 7.7 Å for 24 and after reaction path following 24 → 25 → 26 this distance in 26 is 10.2 Å).

Our calculated E_a of 18 kcal/mol for the proton-transfer reaction is larger than what is thought to be the activation energy for this reaction (about 10 kcal/mol).[24] Liang and Lipscomb have determined activation energies for this process of 34 kcal/mol (PT from four-coordinated zinc) and 45 kcal/mol (PT from five-coordinated zinc) and demonstrate that if large basis sets, molecular dynamics, and correlation treatments were used, their activation energies would decrease to within experimental range.[24] However, we should stress that our CA model as well as other models used[5,11,15,20,24] have neglected the protein environment, which when

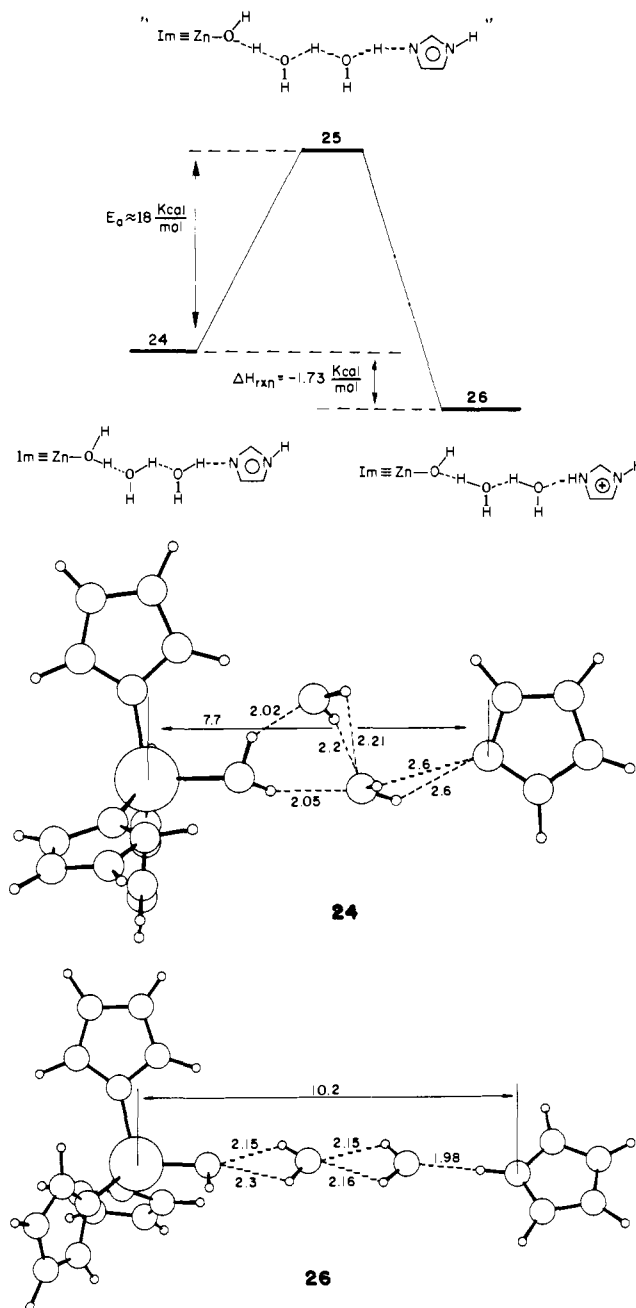**Figure 6.** Reaction profile for the two-water-proton relay and the geometries of 24 and 26 (distances in angstroms).

accounted for can significantly lower the E_a of proton transfers.[40] Another factor that also needs to be considered, which has not been done so here, is to assess what role, if any, that quantum mechanical tunneling might play in reducing the calculated E_a. Thus, our calculated E_a should be considered an upper limit to the "true" E_a.

In conclusion, in terms of the models studied up to this point we favor that of Venkatasubban and Silverman,[39] but we cannot rule out unequivocally the mechanism suggested by Kannan et al.[4]

B. Hydration Step. We first attempted to locate a TS for the direct nucleophilic attack of the zinc–hydroxide oxygen on the carbon atom of CO_2. This TS model has been espoused by Lipscomb.[13] We were, however, not successful and were able to locate only a hilltop (second-order TS) where the second negative eigenvalue of the force matrix suggested that a lower energy TS could be found where the CO_2 coordinated to the zinc atom in

---

(38) Theoretical approaches are now available that can probe this issue: Warshel, A.; Sussman, F.; King, G. *Biochemistry* **1986**, *25*, 8368.

(39) Venkatasubban, K. S.; Silverman, D. N. *Biochemistry* **1980**, *19*, 4984.

(40) van Duijinen, P. T. *Enzymes* **1986**, *36*, 93, and references therein.

Mode of Action of Carbonic Anhydrase

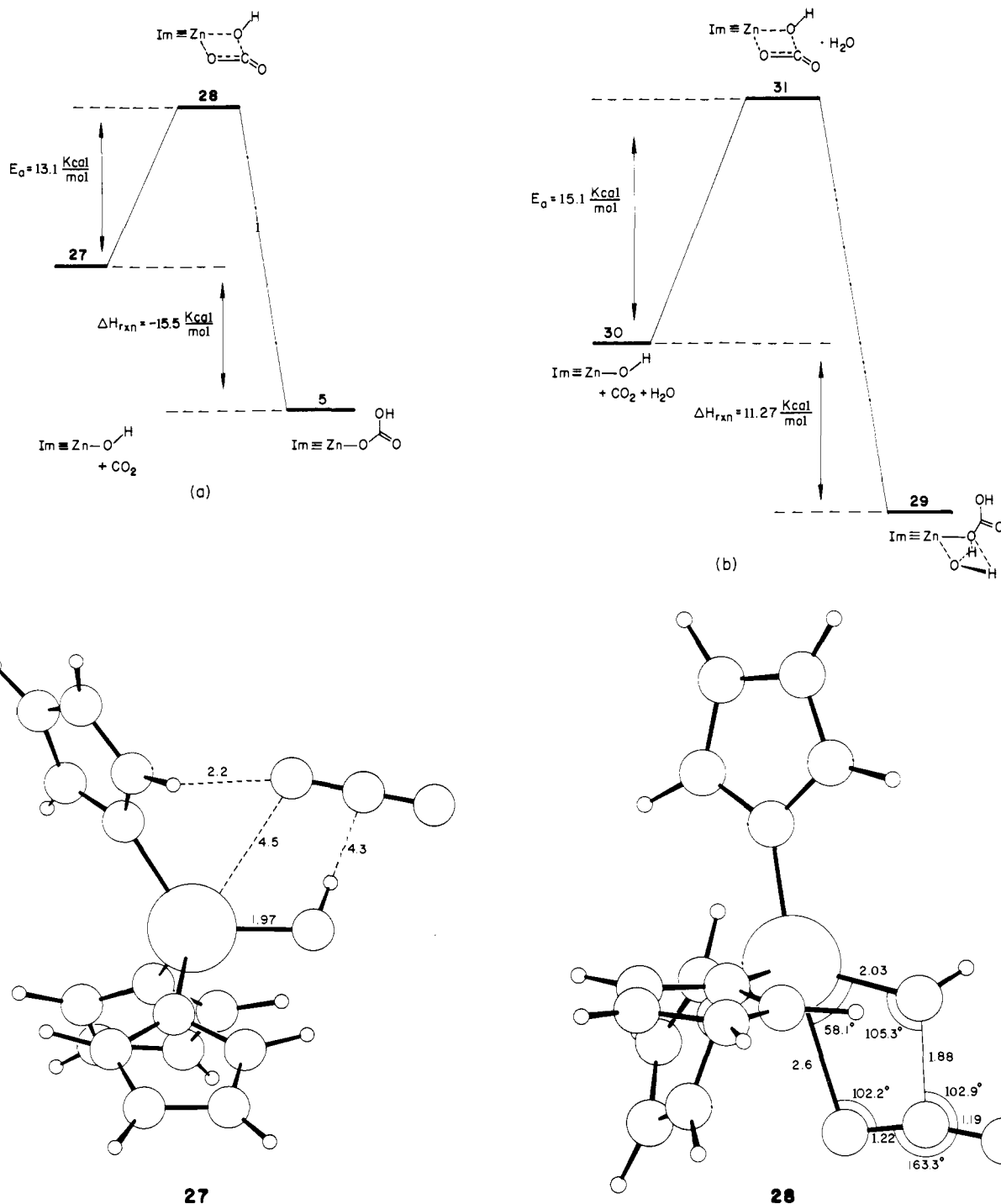*J. Am. Chem. Soc., Vol. 111, No. 15, 1989* 5643

Figure 7. Reaction profiles for the hydration of CO_2 in the (a) absence and (b) presence of water and the geometries of **27** and **28** (distances in angstroms, angles in degrees).

some way. We, indeed, were able to locate a TS where the CO_2 oxygen that was developing a negative charge was forming a zinc–oxygen bond. The geometry of **27** and the TS **28** are given in Figure 7 along with the reaction profile, while the ΔH_f's are given in Table IV. The geometry of **5** (the product of this reaction) is given in Figure 3. In **27** we see that CO_2 is not filling the potential fifth coordination site around zinc, and it is at best loosely interacting with the active site. In the TS (**28**), though, we see that CO_2 has filled the fifth coordination site, resulting in *trigonal bipyramidal* coordination around the zinc. The TS is best described as being "early" because all of the forming and breaking bonds have only partially done so. The calculate E_a is 13.1 kcal/mol, and ΔH_{rxn} is -15.5 kcal/mol; thus, as expected, this reaction should readily occur. Recall, though, that AM1 gave a too exothermic value for the ΔH_{rxn} of $CO_2 + HO^-$ (by 36.2

Table IV. AM1 Calculated ΔH_f's for **5** and **27–31**

compd	ΔH_f, kcal/mol	compd	ΔH_f, kcal/mol
27	164.2	31	108.8
28	177.3	32	90.0[a]
5	148.7	33	−189.0
29	82.5	34	−187.1
30	93.7		

[a] ΔH_f calculated at a fixed zinc–bicarbonate distance.

kcal/mol). Thus, we expect the ΔH_{rxn} in the present case to also be too exothermic. If we remove the obvious error of 14.3 kcal/mol due to the underestimation of the ΔH_f of CO_2, we are now left with a ΔH_{rxn} of -1.2 kcal/mol, which is reasonable. Where did the remainder of the error (21.9 kcal/mol) from the

CO_2 + HO^- reaction go? As mentioned previously we suspect the following: it is well-known that semiempirical methods give ΔH_f's for small charged molecules that are too positive and that the replacement of H for a R group (e.g., methyl) is sufficient to remove this 20 kcal/mol error.[27] Thus, we expect that in the present case where we have replaced H by a zinc ion that this error has been negated and a ΔH_{rxn} of -1.2 kcal/mol is reasonable for this reaction.

We next wanted to study the effect that the presence of a water molecule might have on the hydration reaction. This interest initially came about from the observation that a water molecule nicely sits in the fifth coordination site around zinc (see **29** in Figure 7). In **30**, though, we find that the water molecule does not occupy the fifth coordination site around the zinc ion but is hydrogen bonded directly to the hydroxide ion bound to zinc. The way in which we located the TS in which one water molecule is interacting with **28** is as follows: a series of calculations were done where a water molecule was allowed to interact with various positions on **28** (the geometry of **28** was fixed). The geometry that afforded the greatest stabilization was then taken to be a guessed starting point for the TS, which upon gradient minimization resulted in **31**. The reaction profile is given in Figure 7, and the calculated ΔH_f's are given in Table IV. The structure of **31** is similar to that of **28** except for the addition of a hydrogen-bound water molecule to the CO_2 oxygen that is not forming a bond to the zinc ion. The reason we located **31** in this way is because our standard reaction path approach[30] for the location of TSs failed (i.e., we were able to locate only hilltops). By comparing these calculations with the previous ones where no external water was involved in the hydration reaction, we find that the presence of water *inhibits* the reaction. Thus, the calculated E_a is now 2 kcal/mol higher than it was for the unsolvated TS. We, therefore, envision the hydration step occurring as follows: the CO_2 molecule fills the fifth coordination site and simultaneously is hydrated. The hydration reaction can, therefore, be thought of as a gas-phase reaction. This conclusion agrees nicely with the suggestion that enzyme reactions can be described in some instances as gas-phase reactions.[9]

It is useful at this point to compare our computed E_a of 13.1 kcal/mol to the experimental E_a for the solution-phase reaction of OH^- + CO_2, which has been determined to be 13.2 kcal/mol.[41] Our E_a is only 0.1 kcal/mol lower than the solution-phase E_a, which implies that the enzymatic reaction is only slightly faster than the solution-phase reaction. Again, though, we must keep in mind that the E_a for the CA catalytic cycle is about 10 kcal/mol,[24] and thus the hydration step should be significantly lower than this value since it is not the rate-determining step.[2] The disparity between our computed E_a and the experimental enzymatic E_a (which is <10 kcal/mol) is again probably due to the approximations we have made concerning the protein environment, which if they were incorporated would likely reduce the E_a for this reaction.[40]

Pullman[15] has also studied this reaction using ab initio pseudopotential calculations on a number of selected points along a reaction path that connects **27** and **5** (he used NH_3 instead of imidazole as the zinc ligands). The highest point along the chosen reaction path is only 0.7 kcal/mol above the starting point for the reaction path. Thus, these calculations suggest that the hydration of CO_2 is nearly activationless, which is in better agreement with the experimental E_a (<10 kcal/mol) than our calculations. However, care must be taken in comparing these results to ours since in the former case the "true" TS was never located and proven to be such by determining force constants.

C. Loss of the Bicarbonate Ion. We first examined the case in which **29** loses the bicarbonate ion directly (forming **32**) and found that no TS for this process could be located. The reverse reaction is predicted to be activationless under these conditions, with a ΔH_{rxn} of -7.5 kcal/mol (Table IV contains the pertinent ΔH_f's). To further model this reaction, we decided to see what

(41) Pinsent, B. R. W.; Pearson, L.; Roughton, F. J. W. *Trans. Faraday Soc.* **1956**, *52*, 1512.

Table V. AM1 Calculated ΔH_f's for **35–47**

compd	ΔH_f, kcal/mol	compd	ΔH_f, kcal/mol
35	107.2	42	−139.9
36	87.9	43	−141.3
37	100.6	44	−130.6
38	−174.9	45	−141.6
39	−184.8	46	−132.7
40	−165.4	47	−138.8
41	−141.9		

effect solvation of the leaving bicarbonate might have on the ΔH_{rxn}. We find that if four water molecules are added to this system to form hydrogen-bonding interaction with the bicarbonate ion, the ΔH_{rxn} is lowered to 1.9 kcal/mol (**33** to **34**). Again we were
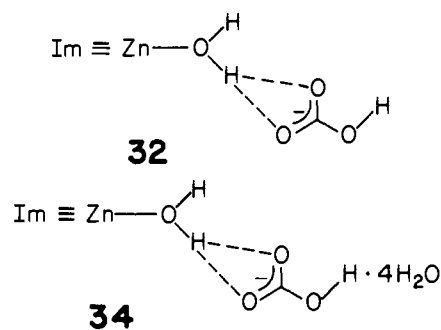
unable to locate a TS, but we did observe an inflection point along the reaction path (the reaction path is very flat). Hence, we find that the loss of the bicarbonate ion is, not unexpectedly, aided by the presence of a number of water molecules. We would again like to stress that from the X-ray data there is evidence for the presence of a number of water molecules,[8] and Clementi et al.[17] have also shown that in the CA active site there is room for three water molecules 2–3 Å away from Zn^{2+} and for four more about 5 Å away. Thus, our model for this reaction is not unreasonable. These calculations suggest that the water molecules in the CA active site are there to aid in the loss of the bicarbonate ion and that in order for the bicarbonate ion to be lost at a reasonable energetic cost a number of the water molecules present must participate. Upon completion of this part of our study we became interested in the possibility that the bicarbonate ion might serve as a "bridge" for the PT step. This is the subject of the next section.

D. Proton Transfer to Bicarbonate Ion. We first investigated the direct formation of carbonic acid from **29** via a 1,3 and 1,5 sigmatropic shift (see Figure 8). Both of the TS were located by "building" the TS as described in the Methodology section. The E_a for the 1,3 TS (**35**) is, not unexpectedly, higher than that for the 1,5 TS (**37**, 24.7 vs. 18.1 kcal/mol), and the ΔH_{rxn} for both is 5.3 kcal/mol. The differences in the E_a are due to two factors: first, **37** is less strained than the **35** and, second, **37** is an aromatic TS (10 e^-) while **35** (8 e^-) is antiaromatic. The pertinent ΔH_f's are given in Table V, while the geometries of **35** and **37** and reaction profiles are presented in Figure 8. The TS geometries both adopt a trigonal bipyramidal coordination around the zinc atom (see Figure 8), with **37** being closer to having this "ideal" structure. Finally, note that the remaining hydrogen on the oxygen atom of the water molecule is almost perpendicular to the plane of O···H···O. This allows for the more effective binding of the developing hydroxide ion to the zinc atom. Since the E_a for the 1,5 TS was found to be similar to that of the two-water-proton relay but the ΔH_{rxn} was found to be unfavorable, we decided to pursue this further by seeing what effect the inclusion of solvent might have.

We next investigated the possibility of the 1,3 and 1,5 shifts occurring from **33**. In the former case the calculated TS (**38**) was significantly different than that obtained in the absence of solvent (**35**). In **38** the bicarbonate ion is released from the zinc atom during the course of the PT, while in the unsolvated TS the bicarbonate ion remained bound to the zinc at the TS. The

Mode of Action of Carbonic Anhydrase

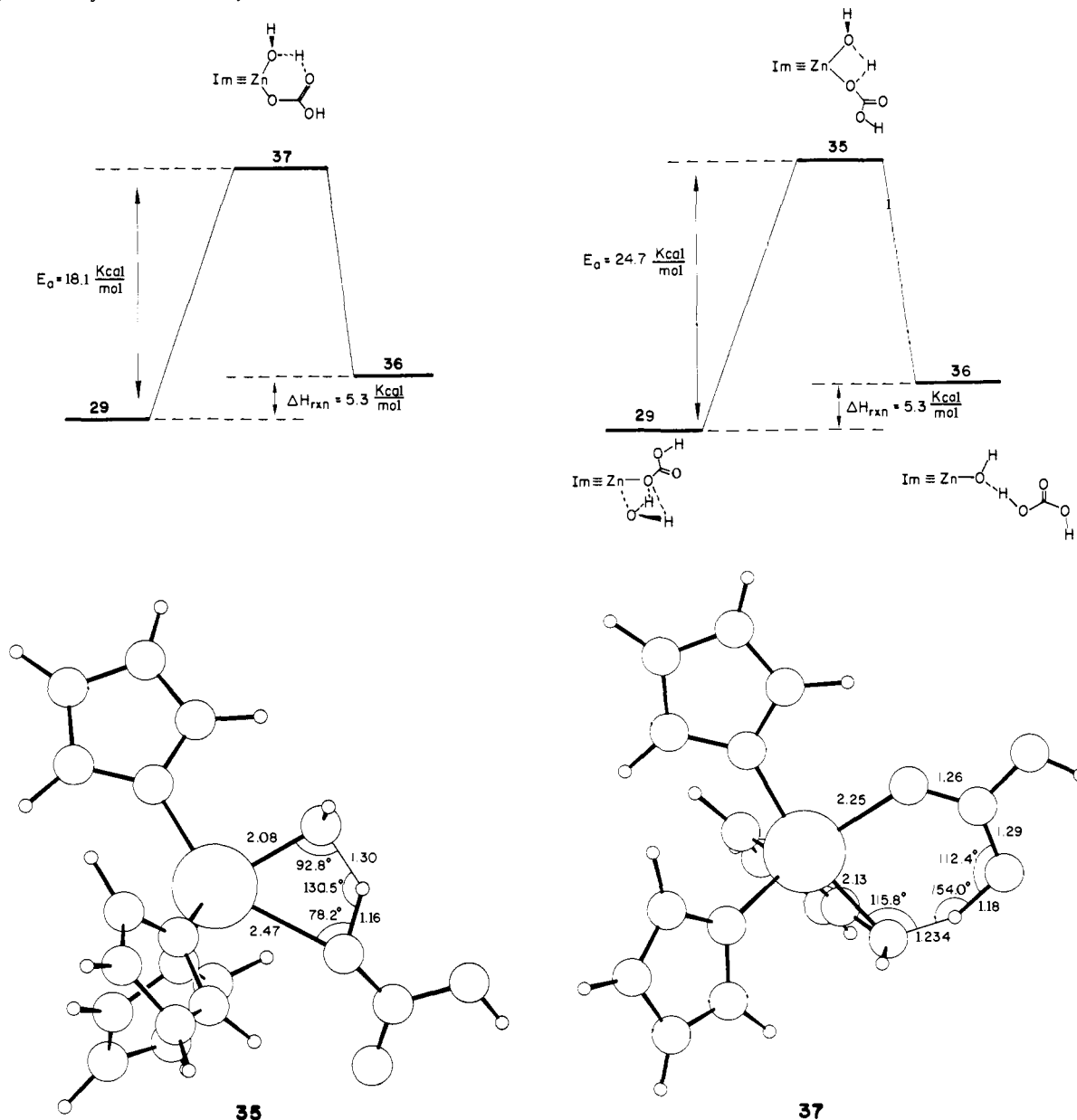*J. Am. Chem. Soc., Vol. 111, No. 15, 1989* 5645

Figure 8. Reaction profiles for the 1,3 and 1,5 sigmatropic shift dehydration reactions and the geometries of **35** and **37** (distances in angstroms, angles in degrees).

calculated E_a for this process is 14.2 kcal/mol, and the ΔH_{rxn} is 4.2 kcal/mol. In the 1,5 shift case we locate the TS **40**, which is formed with an E_a of 23.6 kcal/mol. Thus, the net effect of solvation on the 1,5 sigmatropic shift is to increase the E_a relative to the unsolvated case. This effect could possibly be an artifact of the calculation due to the static model (i.e., supermolecule approach) we are employing; however, the important feature to note is that the solvated "1,3" TS provides us with the lowest barrier we have calculated for the crucial PT step. The necessary ΔH_f's are given in Table V, and the reaction profiles presented in Figure 9.

From the previous calculations we find that the PT to bicarbonate ion is more facile than any of the previous pathways we examined (e.g., water bridge pathway). However, the fact remains that the final product cannot be carbonic acid.[16] To rationalize our data, we developed the mechanism outlined in Scheme V. In this mechanism carbonic acid is treated as the proton bridge between the active site and His-64. Thus, the observed final product is bicarbonate, and the species that undergoes the dehydration is bicarbonate. Hence, this mechanism appears completely reasonable. We also have carried out the necessary calculations to model the steps involved in the PT involving carbonic acid as the proton relay. The active-site model

we used to study this is **41** (see Figure 10). Recall that the distance between the zinc atom and the proton acceptor site (an imidazole nitrogen from His-64) is 8 Å; thus, once again we note that in our initial supermolecule model (**41**) this distance was close to 8 Å before geometry optimization. If our mechanism is reasonable, one criterion that it must satisfy is the active-site geometric constraints, and, therefore, we should expect the zinc-to-imidazole nitrogen distance to be around 8 Å upon optimization and reaction path following (see below). The reaction profile and the geometries of **44** and **46** are given in Figure 10, while the ΔH_f's for **41–47** are listed in Table V.

In subsection C we were unable to locate a TS for the loss of bicarbonate ion, but using the present active-site model, we were able to do so (**42**). The calculated E_a is 2.1 kcal/mol and the ΔH_{rxn} is +0.63 kcal/mol. The next step is the proton transfer from the active site to bicarbonate ion. This is the rate-determining step for this reaction profile with an E_a of 11.4 kcal/mol (relative to **41**). This hydrogen transfer (see **44** in Figure 10) involves a fairly symmetrical TS, which is consistent with the small ΔH_{rxn}. Following the formation of **45**, a proton is then transferred to imidazole ($E_a = 8.9$ kcal/mol), forming **47**. The overall ΔH_{rxn} is calculated to be 3.1 kcal/mol (**41** to **47**), and while we would prefer this number to be negative it is not large enough to inva-
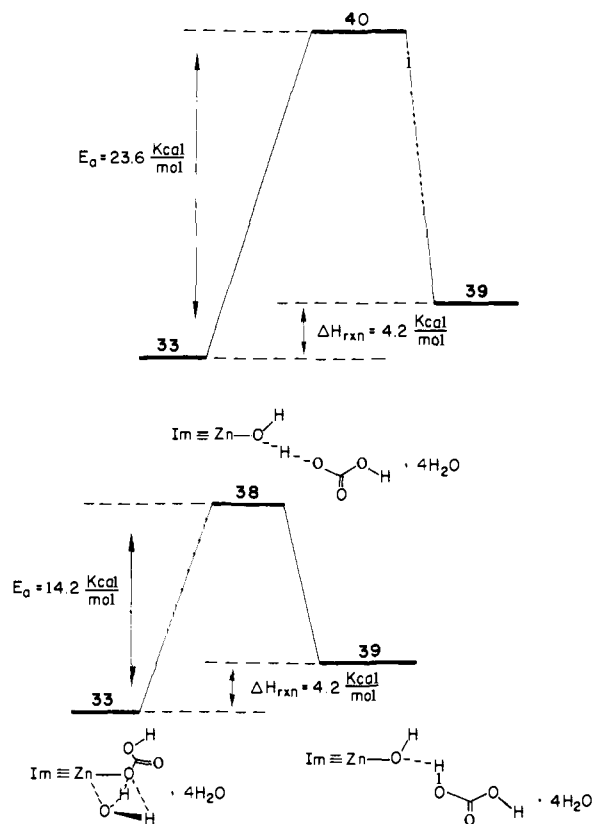
Figure 9. Reaction profiles for the 1,3 and 1,5 sigmatropic shift dehydration reactions in the presence of solvent.

lidate the conclusion that the reaction profile of Figure 10 is a viable one. Again the hydrogen transfer is symmetrical (see **46** in Figure 10), and in **46** the interaction between the transferring proton and a water molecule is not present. Finally, we note that the zinc-to-imidazole nitrogen distances in **41–47** are all in the 8-Å range. Thus, the bicarbonate ion as well as two water molecules can apparently both satisfy the active-site constraints. While our model satisfies the geometric constraints of the CA active site, we should point out that this may be a consequence of our initial supermolecule model (**41**). However, it is reassuring that this distance was maintained throughout all of our reaction path calculations on the rearrangement of **41** to **47**.

Recall that the E_a for the hydration step is 13.1 kcal/mol (see Figure 7), while the E_a for the PT step is calculated to be 11.3 kcal/mol. Experimentally it is thought[12] that the PT transfer step is the rate-determining step, while it appears that our calculations predict that the rate-determining step should be the hydration step. However, the difference in the E_a is small and certainly well within the limit of accuracy of the semiemprical AM1 method. However, to determine how large the error bar might be, we have done a series of AM1 calculations on a series of PTs between *neutral* and a *charged* species and compared our results to those of ab initio calculations. We have chosen this comparison because in the present situation a proton is being transferred between a "neutral" (a water molecule liganded to a zinc ion) and a charged species (the bicarbonate ion). The results are given in Table VI. The worst case is the $CH_3\cdots H\cdots CH_3$ case, where ab initio calculations give an E_a of 22.3 kcal/mol, while AM1 gives a value of zero. The best case is the last entry in Table VI, where we find that AM1 does predict an E_a, albeit the AM1 value is 4.1 kcal/mol lower than the ab initio result. However the trend in these calculations is clear: AM1 *underestimates* the E_a for proton transfers between a neutral and a charged species by, on average, 7.4 kcal/mol.

Thus, we expect that the calculated E_a for the intramolecular PT is too low in the case of our bicarbonate relay mechanism. A rough estimate of its magnitude is 7.4 kcal/mol, and with this correction added to the calculated E_a for the intramolecular PT

Table VI. AM1 E_a's Compared to ab Initio E_a's for Proton Transfers between Charged Species

| | E_a, kcal/mol | |
reaction	AM1	ab initio[a]
anions		
$CH_3^- + CH_4$ to $CH_4 + CH_3^-$	0.0	22.3[a]
$NH_2^- + NH_3$ to $NH_3 + NH_2^-$	0.0	7.1[a]
$HO^- + H_2O$ to $H_2O + HO^-$	0.0	0.8[a]
$HC_2^- + H_2C_2$ to $H_2C_2 + HC_2^-$	0.0	10.9[a]
$NC^- + HCN$ to $HCN + NC^-$	0.0	7.7[a]
cations		
$H_4N^+ + H_3N$ to $H_3N + H_4N^+$	0.0	4.2[a]
$H_3O^+ + H_2O$ to $H_2O + H_3O^+$	0.0	0.0[a]
$H_2CNH_2^+ + NH_3$ to $H_2CNH + NH_4^+$	1.8	5.9[b]

[a] Cao, H. A.; Allavena, M.; Tapia, O.; Evleth, E. M. *J. Phys. Chem.* **1985**, *89*, 1581. [b] Hillenbrand, E. A.; Scheiner, S. *J. Am. Chem. Soc.* **1985**, *107*, 7690.

Scheme V
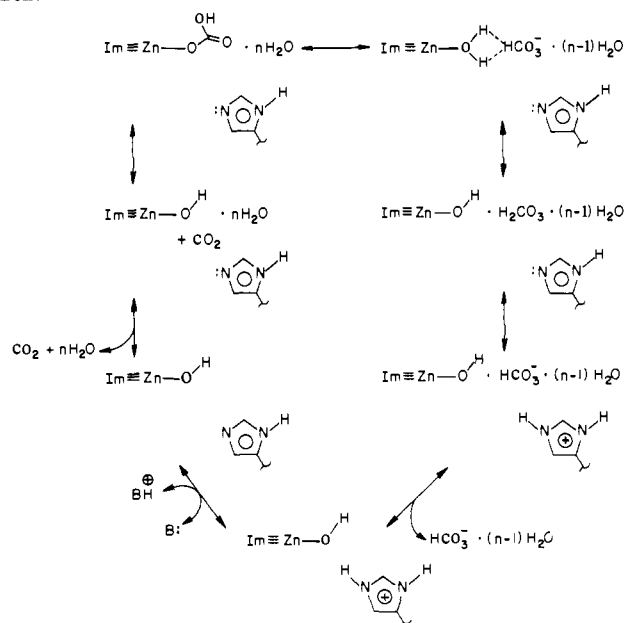
(**43** to **45**) we conclude that the "true" E_a for this reaction is probably closer to 18.7 kcal/mol. Thus, we predict that the intramolecular proton-transfer step should be the rate-determining step of our catalytic cycle. The catalytic cycle that one can draw where bicarbonate is the proton relay group is given in Scheme V.

The CA mechanism has been shown to follow ping pong kinetics,[6] which means in the case of CA that the catalytic cycle is separated into two half-reactions, one that involves hydration of CO_2 and the loss of bicarbonate (**3** to **1**; see Scheme III), and the other involving the removal of a proton from the active site (**1** to **3**, see Scheme III). The catalytic cycles given in Scheme V, however, cannot be separated into two half-reactions and, thus, does not follow ping pong kinetics. One way in which the bicarbonate ion might participate in the intramolecular proton transfer (while retaining ping pong kinetics) is if the bicarbonate ion that is *bound* to zinc is completely displaced from the active site and is subsequently replaced by another bicarbonate ion, which is not bound to the zinc, that then facilitates the PT. Thus, the catalytic cycle would involve two half-reactions (hydration and loss of bicarbonate followed by reintroduction of a bicarbonate ion or potentially a buffer molecule other than bicarbonate and PT) and, therefore, would follow ping pong kinetics. There is evidence that the presence of buffers does catalyze the intramolecular PT,[6,14] but it is as yet unknown whether the catalysis is due to the participation of the buffer molecule in the PT process or due to some as yet unspecified reason. We note that the buffers used in the experimental studies were substituted imidazoles or

Scheme VI

Scheme VII

Scheme VIII

Scheme IX
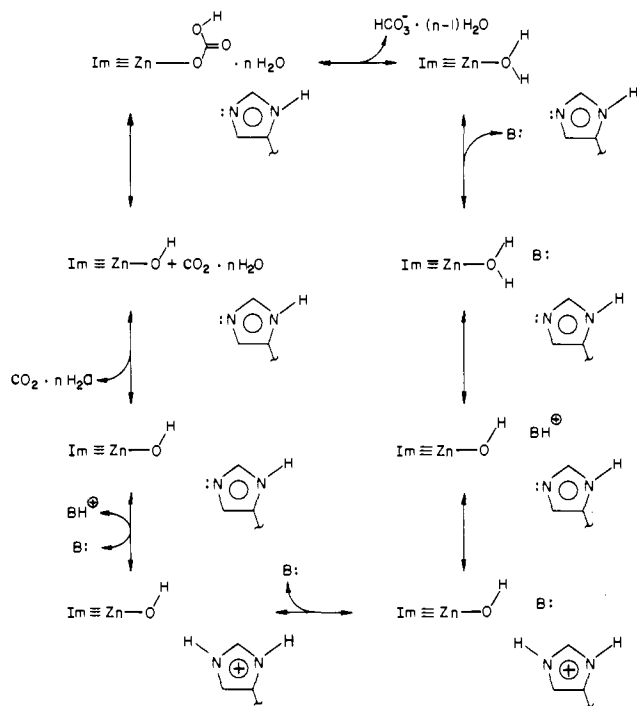
lutidines and not the bicarbonate ion itself. However, our proposed mechanism does not necessarily have to use bicarbonate as a proton shuttle but could equally as well be an imidazole or a lutidine. The generalized mechanism is given in Scheme VI.

Finally, recall that the E_a for the two-water-proton relay (**24** to **26**) is predicted to be about 18 kcal/mol, while the corrected E_a for the bicarbonate relay mechanism (**43** to **45**) is 18.7 kcal/mol. Thus, within the accuracy of our calculations we are unable to distinguish between these two mechanisms.

Extensions

The zinc–hydroxide mechanism is a recurrent mechanistic motif for zinc metalloenzymes,[42] and we would like to make some specific predictions concerning the pathway followed by two other zinc metalloenzymes; namely, carboxypeptidase A (CPA) and liver alcohol dehydrogenase (LADH). In the following we will only briefly discuss the course that the "hydration" step might take in the zinc–hydroxide mechanism for these two enzymes. It should be stressed that in both cases the zinc–hydroxide mechanism is not the only pathway that has been proposed, but given our present results we are able to comment only on this mechanistic type.

CPA catalyzes the hydrolysis of C-terminal amino acids and shows a strong preference for cleaving C-terminal residues that contain aromatic or branched chains.[43] Three realistic mechanisms have been proposed to explain this activity, namely, the zinc–carbonyl, zinc–hydroxide, and the anhydride intermediate.[43] Since we are able to comment only on the zinc–hydroxide mechanism, it is given in Scheme VII. For details on the other mechanisms see ref 43. In this mechanism first the substrate is introduced into the active site, and then the zinc–water form of the enzyme (**49**) transfers a proton to Glu-270 forming the zinc–hydroxide form of the enzyme (**50**). This latter form then attacks the carbonyl carbon, forming a zinc-bound tetrahedral intermediate (**51**), which is then subsequently cleaved by Glu-270. The hy-
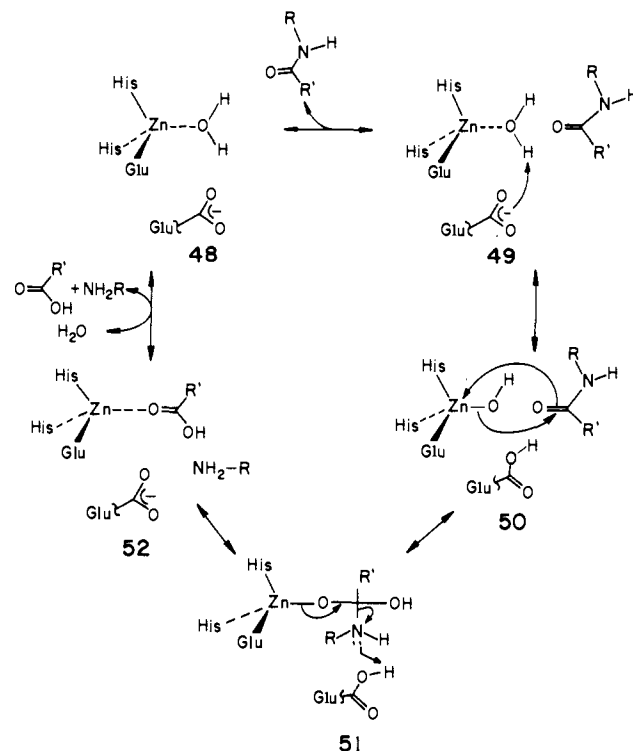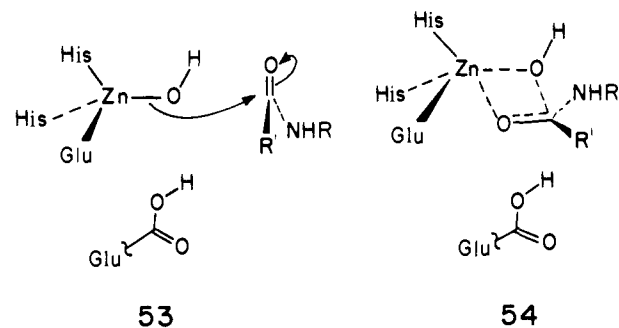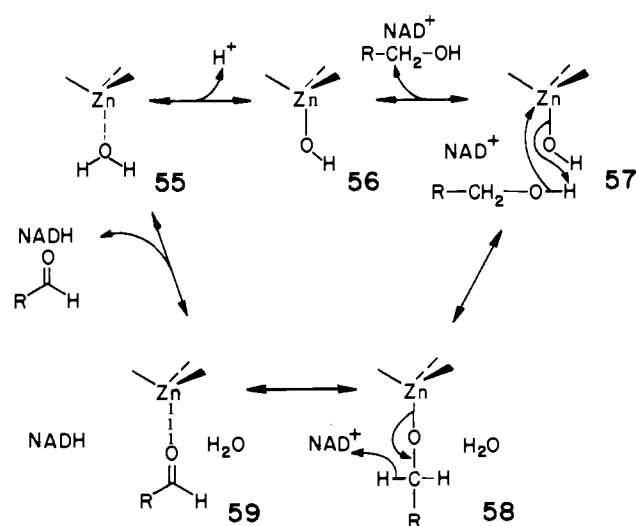
(42) For a concise recent description of the zinc–hydroxide mechanism and its role in zinc metalloenzyme chemistry see: Coleman, J. E. In *Zinc Enzymes*; Bertini, I., Luchinat, C., Maret, W., Zeppezauer, M., Eds.; Birkhäuser: Boston, 1986; p 317.

(43) Vallee, B. L.; Galdes, A.; Auld, D. S.; Riordan, J. F. In *Zinc Enzymes*; Spiro, T. G., Ed.; Wiley: New York, 1983; p 25. Also see: Gardell, S. J.; Craik, C. S.; Hilvert, D.; Urdea, M. S.; Rutter, W. J. *Nature* **1985**, *317*, 551. Rutter, W. J.; Gardell, S. J.; Roczniak, S.; Hilvert, D.; Sprang, S.; Fletterick, R. J.; Craik, C. S. *Protein Eng.* **1987**, 257.

drolyzed substrate then leaves the active site, and the catalytic cycle continues. The step we would like to comment on is the hydrolysis step that forms **51**.

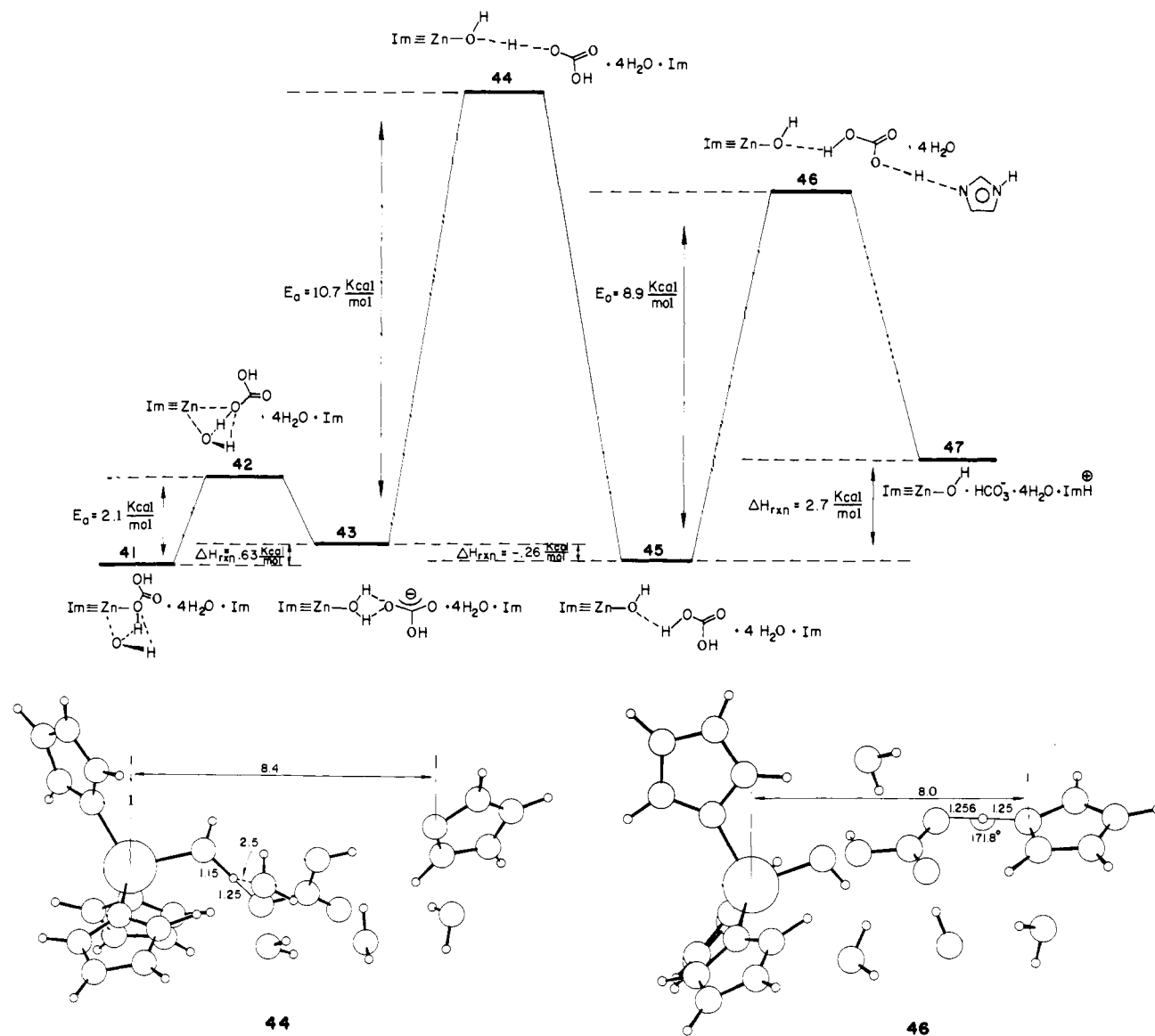Two TSs can be suggested for this step. The first involves an outer-sphere attack on the carbonyl carbon (with the TS **53**), while

Figure 10. Reaction profile for the bicarbonate relay mechanism and the geometries of **44** and **46** (distances in angstroms).

the other possibility involves an inner-sphere attack of the hydroxyl (with the TS **54**; see Scheme VIII) From our results on the hydration of CO_2 by CA, we feel that the inner-sphere mechanism is the most reasonable reaction path. Thus, the carbonyl carbon ligates with the zinc ion in a fifth coordination site and the hydroxyl oxygen then attacks the carbonyl carbon, forming the tetrahedral intermediate.

LADH converts a secondary or a primary alcohol into its corresponding ketone or aldehyde with the aid of the coenzyme NAD^+.[44] Again several mechanisms are possible, with one of them being classified as zinc–hydroxide mechanism.[44] For a discussion of the other mechanisms see ref 44. The zinc–hydroxide mechanism for LADH is given in Scheme IX. The zinc–water form (**55**) of this enzyme loses a proton to the surrounding medium, thus forming the zinc–hydroxide form of LADH (**56**). NAD^+ and an alcohol are then introduced into the active site of LADH, followed by the formation of the zinc–alkoxide form (**58**) of the enzyme and the release of a water molecule. A hydride transfer to the bound NAD^+ then ensues, giving the corresponding aldehyde or ketone and NADH (**59**). The loss of the carbonyl compound and NADH from LADH regenerates **55**. For LADH we would like to discuss the formation of the zinc–alkoxide form

(44) Elkund, H.; Bränden, C.-I. In *Zinc Enzymes*; Spiro, T. G., Ed.; Wiley: New York, 1983; p 123.
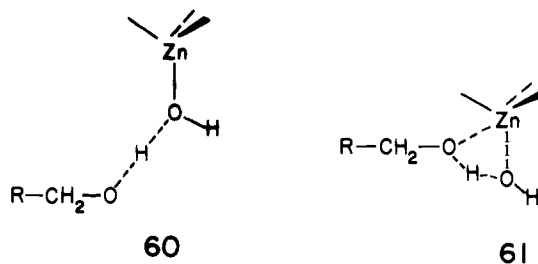
Scheme X

of the enzyme from the zinc–hydroxide intermediate (i.e., **57** → **58**).

Again the requisite proton transfer can occur via either an outer- (TS **60**) or inner-sphere (TS **61**; see Scheme X) mechanism, and again we feel that the inner-sphere mechanism is more likely. In the inner-sphere pathway the alcohol oxygen coordinates with the zinc and then transfers its proton to hydroxide forming water and the zinc–alkoxide form of the enzyme.

Conclusions

Two mechanisms for the activity of CA come out of our calculations. The specific AM1 predictions for the first mechanism, which can be considered the "classic" CA mechanism,[2] can be summarized as follows (see Scheme III): the hydration step

proceeds via the reaction of a zinc–bicarbonate intermediate with CO_2 through a trigonal bipyramidal TS, and our calculations predict that water, apparently, inhibits this reaction. The hydrolysis of the subsequently formed zinc–bicarbonate intermediate, however, requires the presence of several water molecules in the CA active site. Once water replaces bicarbonate, it transfers a proton to His-64 via the surrounding waters (two-water-proton relay) and regenerates the zinc–hydroxide intermediate. His-64 subsequently transfers the proton to buffer external to the enzyme and the catalytic cycle continues. The second mechanism (see Scheme VI), which we will call the AM1 mechanism, is identical with the above one except for the involvement of a buffer molecule in the intramolecular PT. Finally, we note that our calculations have not unambiguously been able to eliminate the mechanism of Kannan et al.,[4] and therefore it is still a distinct mechanistic possibility.

It is useful at this point to differentiate our computational results from those of other researchers. Previous workers have found that the hydration of CO_2 proceeds via a nearly activationless inner-sphere mechanism,[15] in which CO_2 initially coordinates with the zinc ion, while our calculations suggest an inner-sphere mechanism where CO_2 does not initially coordinate with the zinc ion. In both cases, though, the zinc ion is found to serve as a template to bring CO_2 and a hydroxide ion into contact. We attempted to locate an outer-sphere TS, which has been espoused by Lipscomb,[13] but were able to locate only a hilltop that led to an E_a that was greater than that for the inner-sphere mechanism.

The two-water-proton relay mechanism has been studied by Liang and Lipscomb,[24] and they have calculated an E_a for this process of 34 kcal/mol (from four-coordinated zinc) and 54 kcal/mol (from five-coordinated zinc), while we have predicted

the E_a to be 18 kcal/mol (from four-coordinated zinc). Both predictions are certainly too high, and the reason for the overestimation of this E_a is due in part to the theoretical model employed[24] and the neglect of the contributions from tunneling and the protein environment,[40] which tend to reduce the E_a of proton transfers.

Recently, the role of His-64 in the intramolecular PT has been studied with the aid of site-directed mutagenesis and has yielded the interesting conclusion that this group is *not* crucial to HCA activity.[45] These authors studied glutamine, glutamic acid, lysine, and alanine mutants and found that the k_{cat} was reduced only by a factor of 1.5–3.5 relative to the native enzyme. Our conclusions are consistent with these results in that they do suggest that other protein residues or molecules (e.g., bicarbonate) can serve as a proton-transfer group and thus keep k_{cat} close to that observed for the native enzyme.

(45) Forsman, C.; Behravan, G.; Jonsson, B.-H.; Liang, Z.; Lindskog, S.; Ren, X.; Sandström; J.; Wallgren, K. *FEBS Lett.* **1988**, *229*, 360.

Free Energy Perturbation Simulations of the Inhibition of Thermolysin: Prediction of the Free Energy of Binding of a New Inhibitor

Kenneth M. Merz, Jr., and Peter A. Kollman*

Contribution from the Department of Pharmaceutical Chemistry, University of California, San Francisco, San Francisco, California 94143. Received September 19, 1988

Abstract: We report free energy perturbation simulations on a series of thermolysin inhibitors. The three compounds that were examined are transition-state analogues and have the general structure carbobenzoxy-Gly^p(X)-L-Leu-L-Leu, where X = NH, O, and CH_2. While the NH and O compounds have had their crystal structure solved as well as having their K_i's determined, this is not true for the CH_2 compound. Our simulations predict the CH_2 compound to have a K_i similar to that of the NH compound ($\Delta\Delta G_{bind}$ = –0.3 kcal/mol favoring the NH compound), while both have K_i's greater than the O compound. The reason why the CH_2 compound is a potent inhibitor can be traced to two factors: (1) the solvation free energy for the CH_2 is less than that for the NH and O compounds (hydrophobic effect) and (2) the CH_2 compound does not have a repulsive interaction between the main chain carbonyl of Ala 113 and the oxygen. We have also redone the NH → O simulations reported earlier (*Science* **1987**, *235*, 574) using a new set of charges based on a more complete quantum mechanical geometry optimization of models of the inhibitors. These new charges led to calculated $\Delta\Delta G$ for NH → O of 5.9 kcal/mol, in contrast to the 4.2 kcal/mol calculated earlier. By mutating the new charges into the old, we were able to more closely reproduce ($\Delta\Delta G_{calcd}$ = 4.05 kcal/mol) the calculated results of Bash et al., albeit with different relative contributions of solvation and binding free energies. We have also carried out simulations in which we modified the force field parameter used for the neutral carboxylic acid X–C–O–H torsion. This was done because Glu 143 interacts with the perturbation site, and hence we expect that the smaller torsion barrier used previously (3.6 kcal/mol) might bias the calculation considering that the experimental value for this torsion is approximately 10 kcal/mol. In these simulations, which also used the new charges, we find that the free energy value for the CH_2 → NH perturbation changes very slightly (–0.3 vs 0.0 kcal/mol), while for the NH → O perturbation there is a much larger change (5.9 vs 3.3 kcal/mol). Concomitantly, a significant change in the active site geometry between the two parameter sets is observed, with the newer set matching the experimental structure more closely.

Thermolysin is a zinc-requiring endopeptidase that has a specificity for a peptide bond in which the adjacent side chains are

hydrophobic (e.g., leucine, isoleucine, and phenylalanine). The crystal structure has been solved to a resolution of 1.6 Å,[1] which