Shyy, Y.-J., Tian, G., and Tsai, M.-D. (1987) *Biochemistry 26*, 6411-6415.

Smith, G. M., & Mildvan, A. S. (1982) *Biochemistry 21*, 6119-6123.

Sternlicht, H. (1965) *J. Chem. Phys. 42*, 2250-2251.

Tagaya, M., Yagami, T., & Fukui, T. (1987) *J. Biol. Chem. 262*, 8257-8261.

Tomasselli, A. G., & Noda, L. H. (1980) *Eur. J. Biochem. 103*, 481-491.

Tomasselli, A. G., & Noda, L. H. (1983) *Eur. J. Biochem. 132*, 109-115.

Tomasselli, A. G., Marquetant, R., Noda, L. H., & Goody, R. S. (1984) *Eur. J. Biochem. 142*, 287-289.

Tomasselli, A. G., Mast, E., Janes, W., & Schiltz, E. (1986) *Eur. J. Biochem. 155*, 111-119.

Vasavada, K. V., & Nageswara Rao, B. D. (1988) *J. Magn. Reson.* (in press).

Vasavada, K. V., Kaplan, J. I., & Nageswara Rao, B. D. (1984) *Biochemistry 23*, 961-968.

Villafranca, J. J. (1984) *Phosphorus-31 NMR: Principles and Applications* (Gorenstein, D. G., Ed.) pp 155-174, Academic, New York.

Hydration of CO_2 by Carbonic Anhydrase: Intramolecular Proton Transfer between Zn^{2+}-Bound H_2O and Histidine 64 in Human Carbonic Anhydrase II[†]

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ABSTRACT: The energy barrier for the intramolecular proton transfer between zinc-bound water and His 64 in the active site of human carbonic anhydrase II (HCA II) has been studied at the partial retention of diatomic differential overlap (PRDDO) level. The most important stabilizing factor for the intramolecular proton transfer is the zinc ion, which lowers the pK_a of zinc-bound water and electrostatically repels the proton. The energy barrier of 127.5 kcal/mol for proton transfer between a water dimer is completely removed in the presence of the zinc ion. The zinc ligands, which donate electrons to the zinc ion, raise the barrier slightly to 34 kcal/mol for a 4-coordinated zinc complex including three imidazole ligands from His 94, His 96, and His 119 and to 54 kcal/mol for the 5-coordinated zinc complex including the fifth water ligand. A few model calculations indicate that these energy barriers are expected to be reduced to within experimental range (~10 kcal/mol) when large basis set, correlation energies, and molecular dynamics are considered. The proton-transfer group, which functions as proton receiver in the intramolecular proton transfer, helps to attract the proton; and the partially ordered active site water molecules are important for proton relay function.

Carbonic anhydrase is a zinc metalloenzyme that catalyzes the reversible hydration of CO_2 to bicarbonate ion and a proton. In human carbonic anhydrase II (HCA II) the maximal turnover number is $10^6\ s^{-1}$ at 25 °C. It is now widely accepted that the initial nucleophilic attack occurs by a Zn^{2+}-bound hydroxide ion and that the subsequent proton transfer are catalyzed by a non-Zn^{2+}-liganded histidine and by buffer in HCA II (Lindskog, 1983; Lindskog et al., 1984; Lipscomb, 1983; Pocker & Sarkanen, 1978; Prince, 1979; Coleman, 1980). A plausible sequence (Figure 1) for the hydration reaction consists of (1) binding of CO_2 near Zn^{2+}; (2) conversion of CO_2 to HCO_3^- by nucleophilic attack of Zn^{2+}-bound OH^- on C of CO_2; (3) internal proton transfer within Zn^{2+}-bound HCO_3^-; (4) binding of H_2O to Zn^{2+} and ionization of this Zn^{2+}-bound H_2O (probably assisted by the negatively charged Glu 106–Thr 199 proton network) to facilitate release of HCO_3^-; and (5) the coordinated transfer of H^+ from Zn^{2+}-bound H_2O to a proton-transfer group (His 64 in HCA II), then to buffer, and finally to solvent. In this mechanism, the mechanistic role of a 5-coordinated Zn^{2+} species is unclear, although such intermediates could be accommodated in several steps depicted in Figure 1. In step 2, for instance, one oxygen of CO_2 could bind to Zn^{2+} at the fifth coordination site. Also, in step 4, both an OH and terminal O of HCO_3^- could bind to Zn^{2+} before the product release.

It is known that the active site of human carbonic anhydrase II is a conical cavity, which is about 15 Å wide at the entrance and 16 Å deep reaching toward the center of the enzyme (Eriksson et al., 1986). The zinc ion is located near the apex, and His 64, the proton-transfer group in HCA II, is about 7 Å away from Zn^{2+}. Active site water molecules between the zinc ion and His 64 (Figure 2) are partially ordered (Eriksson et al., 1986). In the X-ray structure, these water molecules are not in contact with solvent further out in the cavity. His 64 bridges these two solvent areas (Eriksson et al., 1986). Thus, for the deprotonation step (step 5, Figure 1) of the enzyme-catalyzed CO_2 hydration, two independent proton transfers are required: (1) an intramolecular proton transfer from the zinc-bound H_2O to His 64 and (2) a proton transfer from the protonated His 64 to buffer and then to solvent. The buffer-assisted proton transfer is the rate-limiting step at low buffer concentrations (Jönsson et al., 1976), while the intramolecular proton transfer is rate limiting at high buffer concentrations (Steiner et al., 1975; Pocker & Bjorkquist, 1977). It is possible that the intramolecular proton transfer may involve more than two protons: the deuterium isotope effects

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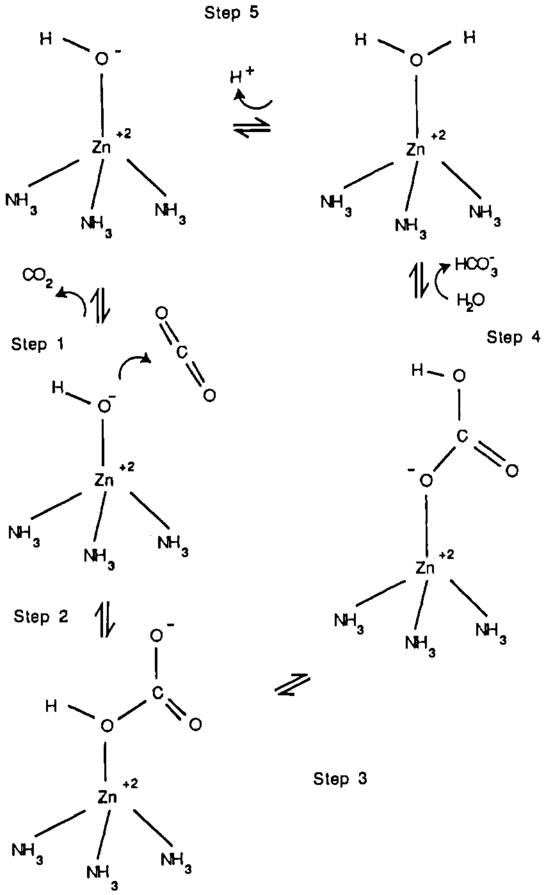

FIGURE 1: Carbonic anhydrase catalyzed CO_2 hydration consists of the following steps: (1) binding of CO_2 near Zn^{2+}; (2) conversion of CO_2 to HCO_3^- by nucleophilic attack of Zn^{2+}-bound OH^- on C of CO_2; (3) internal proton transfer of Zn^{2+}-bound HCO_3^-; (4) binding of H_2O to Zn^{2+} [ionization of this Zn^{2+}-bound H_2O (probably by the Glu 106–Thr 199 proton network) in an associative coordination may facilitate release of HCO_3^-]; and (5) the coordinated transfer of H^+ from Zn^{2+}-bound H_2O to a proton-transfer group (His 64 in HCA II), then to buffer, and then to solvent (see the text).

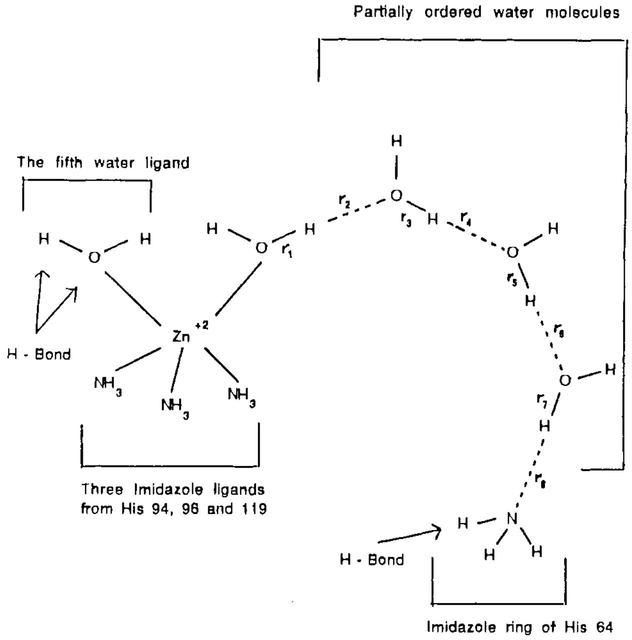

FIGURE 2: Schematic drawing for intramolecular proton transfer from the Zn^{2+}-bound H_2O to His 64 in the active site of HCA II. The system includes (1) the zinc ion and its three imidazole ligands from His 94, His 96, and His 119, denoted as $Zn^{2+}(NH_3)_3$ in the text; (2) the possible fifth water ligand of the zinc ion; (3) three partially ordered water molecules, which serve as the proton relay; and (4) the NH_3 molecule, which simulates the imidazole ring of His 64 (the proton–transfer group). Possible solvent water molecules that may form hydrogen bonds to the fifth water ligand and to His 64 are indicated by arrows.

are 3–4 for the $k_{cat}^{CO_2}$ and $K_M^{CO_2}$ values (Steiner et al., 1975; Pocker & Bjorkquist, 1977; Venkatasubban & Silverman, 1980). The recently refined X-ray structure of HCA II has identified two or three partially ordered water molecules, in addition to the zinc-bound H_2O, between the zinc-bound H_2O and His 64 (Eriksson et al., 1986).[1] These water molecules may function as the proton relay during the intramolecular proton transfer.

This paper investigates the energetic aspects of the intramolecular proton transfer, putting emphasis on the catalytic effects of the possible catalytic elements in the active site.

METHODS

PRDDO. Partial retention of diatomic differential overlap (PRDDO) (Halgren & Lipscomb, 1973; Marynick & Lipscomb, 1982), which uses an orthogonalized basis set, is a close approximation to SCF MO calculations at the minimum basis set level. Treatment of third-row atoms is included in the extended version of PRDDO (Marynick & Lipscomb, 1982), in which a double-ζ basis for d orbitals of the transition metals is applied. In the calculation of two-electron integrals, one-, two-, and three-center integrals of the form $(\chi_{ia}\chi_{jb}|\chi_{kc}^2)$ are retained, where χ_{ia} is a symmetrically orthogonalized AO mainly centered on the atom a. Also retained are one- and two-center exchange integrals of the form $(\chi_{ia}\chi_{ja}|\chi_{ia}\chi_{ja})$ and $(\chi_{ia}\chi_{jb}|\chi_{ia}\chi_{jb})$. Problems of rotational invariance are avoided by choice of local axes which are unique in anisotropic environments. Retention of $\sim N^3$ integrals in PRDDO is shown to simulate efficiently the STO-3G SCF results (Halgren et al., 1978). Here, N is the number of orbitals.

Molecular Geometries. Three ammonia molecules are used to simulate the three imidazole ligands of Zn^{2+} from His 94, His 96, and His 119 in the active site of carbonic anhydrase. The substitution of NH_3 for imidazole is justified by the finding that NH_3 and imidazole transfer a similar amount of charge to the zinc ion (Pullman & Demoulin, 1979). The Zn^{2+}–NH_3 distance is held fixed at 2.102 Å based on X-ray structures of small model compounds (Mak & Huang, 1987), and a C_3 symmetry is imposed on the $Zn^{2+}(NH_3)_3$ complex. In the tetracoordinated zinc complex, we have Zn^{2+}–O = 1.9269 Å, H–O–Zn = 127.6°, and H–O–H = 105.1° for the water ligand and Zn^{2+}–O = 1.9093 Å and H–O–Zn = 126.5° for the hydroxide ligand. In the pentacoordinated zinc complex, the water ligand has Zn^{2+}–O = 1.93 Å, O–H = 1.0 Å, H–O–H = 105.0°, O–Zn^{2+}–O = 100°, and N–Zn^{2+}–O = 59.47°. The proton-transfer group, His 64, is simulated by an ammonia molecule, with N–H = 1.0 Å and H–N–H = 109.47°. Finally, the geometry of the water chain between Zn^{2+}-bound H_2O and His 64 is O–H = 1.0 Å, H–O–H = 109.47°, O···H = 1.8 Å, O–H···O = 180.0°, and H···O–H = 109.47°. The water molecules hydrogen bonded to the fifth water ligand and to the terminal NH_3 are assumed to have the same geometry as the water molecules in the water chain.

RESULTS

To examine the catalytic effects of various catalytic elements in the active site of carbonic anhydrase on the intramolecular

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[1] According to our recent correspondence with Eriksson, there are actually two water molecules between the zinc-bound water molecule and His 64 in their refined X-ray structure (with permission from Eriksson).

Table I: Barriers of Proton Transfers (Reaction Energies)

no.	reaction species[a]	barriers (kcal/mol)[b] first	second	third	fourth	ΔE^{+}
1	$(H_2O)_2$	127.5	104.0[c]	95.0[d]		127.5
2	$(H_2O)_4$	119.0	37.3 (156.3)	26.5 (182.8)		182.8
3a	$H_2O–NH_3$	124.7				124.7
3b	$(H_2O)_2–NH_3$	111.5	40.4 (151.9)			151.9
3c	$(H_2O)_3–NH_3$	112.4	30.1 (142.5)	16.4 (158.9)		158.9
3d	$(H_2O)_4–NH_3$	117.2	32.2 (149.4)	5.0 (154.4)	5.9 (160.3)	160.3
4a	$Zn^{2+}–(H_2O)_2$	–0.4				–0.4
4b	$Zn^{2+}–(H_2O)_3$	–6.5	3.9 (–2.6)			–2.6
4c	$Zn^{2+}–(H_2O)_4$	–9.8	–12.2 (–22.0)	13.8 (–8.2)		–8.2
5a	$Zn^{2+}–(H_2O)_2–NH_3$	–14.7	–15.9 (–30.6)			–30.6
5b	$Zn^{2+}–(H_2O)_3–NH_3$	–12.0	–20.2 (–32.2)	–8.6 (–40.8)		–40.8
5c	$Zn^{2+}–(H_2O)_4–NH_3$	–12.1	–17.8 (–29.9)	–9.9 (–39.8)	–4.1 (–43.9)	–43.9
6a	$Zn^{2+}(NH_3)_3–(H_2O)_2$	55.3 (37.1)[e]				55.3
6b	$Zn^{2+}(NH_3)_3–(H_2O)_3$	40.4	19.6 (60.0)			60.0
6c	$Zn^{2+}(NH_3)_3–(H_2O)_4$	36.7	4.5 (41.2)	19.4 (60.6)		60.6
7a	$(H_2O)–Zn^{2+}–(H_2O)_2$	31.8				31.8
7b	$(H_2O)–Zn^{2+}–(H_2O)_3$	15.7	10.6 (26.3)			26.3
7c	$(H_2O)–Zn^{2+}–(H_2O)_4$	12.4	–5.1 (7.3)	16.2 (23.5)		23.5
8a	$(H_2O)–Zn^{2+}–H_2O–NH_3$	10.3				10.3
8b	$(H_2O)–Zn^{2+}–(H_2O)_2–NH_3$	7.5	–10.8 (–3.3)			7.5
8c	$(H_2O)–Zn^{2+}–(H_2O)_3–NH_3$	10.1	–13.2 (–3.1)	–5.5 (–8.6)		10.1
8d	$(H_2O)–Zn^{2+}–(H_2O)_4–NH_3$	9.7	–10.7 (–1.0)	–7.0 (–8.0)	–2.3 (–10.3)	9.7
9a	$Zn^{2+}(NH_3)_3–H_2O–NH_3$	34.7				34.7
9b	$Zn^{2+}(NH_3)_3–(H_2O)_2–NH_3$	30.2	–1.6 (28.6)			30.2
9c	$Zn^{2+}(NH_3)_3–(H_2O)_3–NH_3$	34.6	–3.5 (30.9)	–2.3 (28.6)		34.6
9c	$Zn^{2+}(NH_3)_3–(H_2O)_4–NH_3$	34.2	–0.9 (33.3)	–4.3 (29.0)	–0.8 (28.2)	34.2
10a	$(H_2O)–Zn^{2+}(NH_3)_3–(H_2O)_4–NH_3$	53.9	–12.3 (41.6)	–8.0 (33.6)	–2.3 (31.3)	53.9
10b[f]	$10a–H_2O$	52.9	–13.7 (39.2)	–13.7 (25.5)	–12.2 (13.3)	52.9
10c[g]	$H_2O–10a$	56.5	–10.4 (46.1)	–7.5 (38.6)	–2.1 (36.5)	56.5
10d[g]	$H_2O–10a$	52.9	–12.0 (50.9)	–6.4 (44.5)	–2.1 (41.4)	52.9

[a] Reaction species such as $Zn^{2+}–(H_2O)_n–NH_3$ of reaction 5 represent the molecular arrangements with Zn^{2+} coordinated to the first water molecule of the hydrogen-bonded water chain, $(H_2O)_n$, and NH_3 hydrogen bonded to the last water molecule (Figure 2). The direction of proton transfer is from the first water molecule to the last water molecule. In reactions 6, 9, and 10, the $(NH_3)_3$ group represents the three histidine ligands of zinc, His 92, His 94, and His 119. In reactions 7, 8, and 10, the H_2O molecule appearing to the left of Zn^{2+} is the fifth water ligand on zinc. Also see Figure 2. [b] For each reaction step, two barriers are listed: (1) the barrier of the single-step proton transfer and (2) the corresponding barrier (included in parentheses) relative to the initial state of individual reactions. The reaction barrier for the entire proton transfer, ΔE^{+}, is listed in the last column. For reactions without energy barriers, the ΔE^{+} values are the overall reaction energies. [c] Inclusion of one water molecule on either end of the water dimer. [d] Addition of one NH_3 molecule to the H_3O^+ end and two water molecules to the OH^- end. [e] r_1 = 1.0 Å and r_2 = 1.6 Å are used. [f] In reaction 10b, one water molecule is hydrogen bonded to the terminal NH_3 as a proton acceptor. [g] In reactions 10c and 10d, one water molecule is hydrogen bonded to the fifth water ligand as a proton acceptor and a proton donor, respectively.

proton transfer, we divided the active site into separate units (Figure 2): the Zn^{2+} ion, the three imidazole ligands, the fifth water ligand, the imidazole ring of histidine 64, and the water chain connecting between zinc-bound H_2O and His 64. Table I contains the calculated reaction barriers for the studied intramolecular proton transfers.

Reaction 1. Proton Transfer in a Water Dimer. A direct proton transfer in a water dimer involves deprotonation of the first water molecule and protonation of the second water molecule. Our calculated barrier is 127.5 kcal/mol. This barrier is reduced to 104 kcal/mol when a water molecule is hydrogen bonded to either side of the water dimer and to 95 kcal/mol when one NH_3 molecule is hydrogen bonded to H_3O^+ and two water molecules to OH^-.

Reaction 2. Proton Transfers along a Water Tetramer. For proton transfer in a linear water tetramer a large barrier of 119 kcal/mol is obtained for the first proton transfer, reflecting the unfavorable deprotonation of the first water molecule. The energy barriers for the second and the third proton transfers between H_3O^+ and H_2O are 37.3 and 26.5 kcal/mol, respectively. For the first, second, and third proton transfers, the energy barriers relative to the initial four-water state are 119.0, 156.3, and 182.8 kcal/mol, respectively. The increasing energy barrier is due to the unfavorable separation of H_3O^+ from OH^-.

Reaction 3. Proton Transfers along a Water Chain with an NH_3 Molecule Hydrogen Bonded to the Water Molecule at the Proton-Receiving End. The ammonia molecule at the proton-receiving end of the water chain simulates the imidazole group of His 64 in HCA II. The barriers for the first, second, third, and fourth proton transfers are 112–125, 30–40, 5–16, and 6 kcal/mol, respectively, depending on the number of water molecules involved in the proton transfer of reactions 3a–d (Table I). The ΔE^{+} values are 124.7, 151.9, 158.9, and 160.3 kcal/mol for the one- (reaction 3a), two- (reaction 3b), three- (reaction 3c), and four-water (reaction 3d) systems, respectively. The increase of energy barrier with the length of the water chain, as in reaction 2, reflects the unfavorable separation of OH^- and H_3O^+. The presence of NH_3 is shown to lower the barriers of reaction 3c slightly as compared with the barriers of reaction 2.

Reaction 4. Proton Transfers along a Water Chain with the Zinc Ion Coordinated to the First Proton-Donating Water Molecule. The Zn^{2+} ion not only lowers the pK_a value of its coordinated water molecule but also "repels" a proton of this water molecule. There are basically no energy barriers for this proton-transfer process, except for the last proton transfer in which the number of solvating water molecules around the transferring proton is reduced from two to one. The ΔE^{+} values for the two- (reaction 4a), three- (reaction 4b), and four-water systems (reaction 4c) are –0.4, –2.6, and –8.2 kcal/mol, respectively. Unlike reaction 3, proton transfers along longer water chains are favored, due to smaller repulsions between the positive zinc ion and the proton in a longer water chain.

Reaction 5. Proton Transfers along a Water Chain with *the Zinc Ion Coordinated to the First Water Molecule (on the Proton-Donating End) and an NH_3 Molecule Hydrogen Bonded to the Last Water Molecule (on the Proton-Receiving End).* No energy barriers are found for proton transfers from Zn^{2+}-bound H_2O to NH_3 through one (reaction 5a), two (reaction 5b), or three (reaction 5c) intermediate water molecules. The reaction energies are −30.6, −40.8, and −43.9 kcal/mol, respectively. These negative reaction energies are much larger in magnitude than those of reaction 4, primarily due to additional stabilization from the NH_3 molecule. [The proton affinity of NH_3 (205.7 kcal/mol) is larger than that of H_2O (169.7 kcal/mol) (Scheiner, 1982a).]

Reaction 6. Proton Transfers along a Water Chain with the Zinc Ion and Its Three Ammonia Ligands, $Zn^{2+}(NH_3)_3$, Coordinated to the First Water Molecule. Coordination of three ammonia ligands, $(NH_3)_3$, to Zn^{2+} is shown to donate ~0.9 electrons to the zinc ion. This electron donation raises the pK_a value of the zinc-bound H_2O, thus increasing the barrier of the first proton transfer from −9.8 kcal/mol of reaction 4c to 36.7 kcal/mol of reaction 6c. The energy barriers of reactions 6a–c are larger than those of reactions 4 and 5 but much smaller than that of reaction 1. This result implies that although the stabilizing effect of Zn^{2+} is reduced in the presence of three NH_3 ligands, it remains indispensable for facilitating the proton transfer.

Reaction 7. Proton Transfers along a Water Chain with the Zinc Ion and Its One Water Ligand, (H_2O)-Zn^{2+}, Coordinated to the First Water Molecule. Like the three ammonia ligands in reaction 6, the water ligand in reaction 7 donates ~0.3 electron to the zinc ion. This donation also destabilizes the proton transfers. For the two- (reaction 7a), three- (reaction 7b), and four-water (reaction 7c) systems, the energy barriers of the first proton transfer are 31.8, 15.7, and 12.4 kcal/mol, respectively; and the ΔE^+ values are 31.8, 26.3, and 23.5 kcal/mol, respectively. The destabilizing effect of the water ligand, judging from the calculated ΔE^+ values, is about 25 kcal/mol smaller than that of three ammonia ligands in reaction 6.

Reaction 8. Proton Transfers along a Water Chain with the Zinc Ion and Its One Water Ligand, (H_2O)-Zn^{2+}, Coordinated to the First Water Molecule, and an NH_3 Molecule Hydrogen Bonded to the Last Water Molecule. For the one- (reaction 8a), two- (reaction 8b), three- (reaction 8c), and four-water (reaction 8d) systems, the calculated ΔE^+ values are 10.3, 7.5, 10.1, and 9.7 kcal/mol, respectively. The reaction barriers arise primarily from the first proton transfer. The values are smaller than those of reaction 7 due to the presence of a terminal NH_3 molecule.

Reaction 9. Proton Transfers along a Water Chain with the Zinc Ion and Its Three Ammonia Ligands, $Zn^{2+}(NH_3)_3$, Coordinated to the First Water Molecule, and an NH_3 Molecule Hydrogen Bonded to the Last Water Molecule. From our previous calculations above, we see that the presence of Zn^{2+} and terminal NH_3 stabilizes the proton transfer, while inclusion of three ammonia ligands to zinc destabilizes the reaction. Combination of these two opposing effects in reactions 9a–d yields a barrier of ~34 kcal/mol for the first proton transfer and no barriers for the subsequent proton transfers.[2]

Table II: Mulliken Charges on the Zinc Ion

reaction no.	Q(Zn)	ΔE	reaction no.	Q(Zn)	ΔE
4c	1.542	−9.8	7c	1.289	12.4
5c	1.536	−12.1	8c	1.289	10.1
6a	0.872	55.3	8d	1.290	9.7
6b	0.824	40.4	9a	0.817	34.7
7b	1.291	15.7	9b	0.825	30.2

[a] Q(Zn) values, in electrons, are the atomic charges on the zinc ion. ΔE values, in kcal/mol, are the energy barriers of the first proton transfer.

Reaction 10. Proton Transfers along a Water Chain with the Zinc Ion and Its One Water and Three Ammonia Ligands, (H_2O)-$Zn^{2+}(NH_3)_3$, Coordinated to the First Water Molecule, and an NH_3 Molecule Hydrogen Bonded to the Last Water Molecule. In reaction 10a, the energy barrier of the first proton transfer is 53.9 kcal/mol, and there is no energy barrier for subsequent proton transfer to the terminal NH_3. When the Zn–O distance is increased from 1.93 to 2.0 Å, the barrier is reduced to 45.6 kcal/mol. In reaction 10b, one water molecule is hydrogen bonded to the terminal NH_3, the energy barrier of the first proton transfer is slightly lowered by ~1.0 kcal/mol, and the ensuing reaction is more exothermic. In reactions 10c and 10d, one water molecule is hydrogen bonded to the fifth water ligand as proton acceptor and proton donor, and the reaction barriers are 56.5 and 52.9 kcal/mol, respectively. These barriers are similar to the barrier of reaction 10a, which implies that solvent hydrogen bonds to the terminal NH_3 and to the fifth water ligand have negligible effects on the energy barrier of the intramolecular proton transfer.

DISCUSSION

Three individual proton transfers are involved in the intramolecular proton transfer between Zn^{2+}-bound H_2O and His 64: (1) The deprotonation of a H_2O molecule with the simultaneous protonation of a second H_2O molecule is accompanied by a large energy barrier (119 kcal/mol in reaction 2). (2) Proton transfer between H_3O^+ and H_2O has no energy barrier, provided that the geometry of the water dimer is fully optimized (Scheiner, 1981). The barriers are 1.4, 7.5, and 16.8 kcal/moi when the O···O distance is held fixed at 2.55, 2.75, and 2.95 Å, respectively (Scheiner & Harding, 1981a, 1983). In reaction 2, the presence of a terminal OH^- after the first proton transfer raises the barriers of the second and the third proton transfer between H_3O^+ and H_2O to 37 and 27 kcal/mol, respectively. (3) Proton transfer from H_3O^+ to the terminal NH_3 has no energy barrier (Scheiner & Harding, 1981a,b, 1983; Scheiner, 1982a,b). In reactions 3b–d, the presence of an OH^- ion destabilizes the proton transfer from H_3O^+ to NH_3. Positive barriers are obtained for these reactions.

The energy barriers for the three types of proton transfers discussed above are significantly lowered or completely removed when the proton donor is coordinated to zinc and the ammonia molecule serves as the ultimate proton receiver. The presence of the zinc ion lowers the pK_a value of the Zn^{2+}-bound H_2O and repels the proton electrostatically. The imidazole group of His 64, simulated by the ammonia molecule here, "attracts" the proton (because proton affinity of the ammonia molecule is greater than that of the water molecule). Inclusion of zinc ligands (one water and three imidazole molecules) in the model systems is shown to reduce the Mulliken charges (Mulliken, 1955) on the zinc ion (Table II), thus destabilizing the proton transfer. The partially ordered active site water molecules are important for the proton relay function. The number of water molecules involved in the proton relay does

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[2] In the most recently refined X-ray structure (Eriksson et al., 1986), the Zn^{2+}–N distance is 2.0 Å, for which the energy barrier is calculated to be 37 kcal/mol. When the Zn^{2+}–N distance is increased from 2.0 Å to 2.2 and 2.4 Å, the energy barrier is decreased from 37 kcal/mol to 31 and 29.8 kcal/mol, respectively. Coordination of three ammonia ligands at large Zn^{2+}–N distances thus favors fast intramolecular proton transfer.

Table III: Energy Corrections for Proton Transfer in the 4-Coordinated $Be^{2+}(NH_3)_3$–H_2O–NH_3 Complex[a]

method	barrier (kcal/mol)	method	barrier (kcal/mol)
PRDDO	13.2	4-31G*	3.7
4-31G	6.4	4-31G/MP2	3.9

[a] The 4-coordinated Be^{2+} complex simulates the zinc complex in reaction 9a with Be^{2+} substituting Zn^{2+}. The geometries used in the energy calculations of 4-31G, 4-31G*, and 4-31G/MP2 are optimized at the 4-31G level.

not seem to affect the overall reaction barrier of the intramolecular proton transfer. Finally, hydrogen bonds from water molecules to the fifth water ligand, and to the terminal NH_3, have negligible effects on the reaction barrier.

We have obtained for the intramolecular proton transfer a PRDDO barrier of 34 kcal/mol in a 4-coordinated Zn^{2+} complex and a barrier of 54 kcal/mol for the 5-coordinated Zn^{2+} complex. These values are larger than the estimated barrier of 10 kcal/mol for the HCA II catalyzed reaction of CO_2 hydration (Liang & Lipscomb, 1986). Further lowering of our PRDDO barriers may be derived from: (1) usage of extended basis sets, and (2) inclusion of correlation energies. In Table III, we examine the effects of these corrections in reducing the barriers of proton transfers in the tetracoordinated Be^{2+} complex (Be^{2+} substitutes Zn^{2+} of reaction 9a). We find that (1) the barrier is lowered from 13.2 kcal/mol of PRDDO calculations to 6.4 kcal/mol when the 4-31G basis is applied; (2) addition of the polarization functions to the heavy atoms, thus forming the 4-31G* basis, further reduces the energy barrier to 3.7 kcal/mol; and (3) correlation correction to the 4-31G wave function at the MP2 level yields a barrier of 3.9 kcal/mol. Further barrier lowering is expected, if stabilizations due to inclusion of both polarization functions and correlation energies are combined. Earlier calculations have shown that PRDDO barriers are ~20 kcal/mol higher than the corresponding 4-31G barriers (Scheiner & Kern, 1979) for multiple proton transfers between formic acid dimer and DNA base pairs. It is also known that when polarization functions and electron correlations are considered, the energy barriers are lowered, relative to the 4-31G results, by 5–10 kcal/mol for proton transfer between NH_4^+ and H_2O (Scheiner & Harding, 1983), and by ~15 kcal/mol (Rodwell et al., 1980) for internal proton transfer in formic acid, vinyl alcohol, and propene. These results suggest that our PRDDO barriers of proton transfer may be lowered up to ~35 kcal/mol when large basis set and correlation corrections are considered. Protein dynamics may also contribute in reducing the energy barriers, for which further investigation is required. It is documented that the barrier of 200 kcal/mol for the ring rotation of Tyr 35 in rigid BPTI can be reduced to 23 kcal/mol when relaxation of the protein structure is allowed during the rotation, and to 10 kcal/mol when protein dynamics is incorporated (McCammon & Karplus, 1979, 1980; Northrup et al., 1982). Besides barrier corrections for individual proton-transfer reactions, the relative differences in PRDDO barriers among processes that are compared here also need to be scaled down. In our previous studies of internal proton transfer of Zn^{2+}-bound HCO_3^- (Liang & Lipscomb, 1987), differences in energy barriers between water-assisted proton transfer and the direct proton transfer are 30 kcal/mol in PRDDO, 25 kcal/mol in 4-31G** SCF MO calculations, and 11 kcal/mol when Moller–Plesset perturbation (Moller & Plesset, 1934) correction of the 4-31G** wave functions to the second order (MP2) is considered.

Scheiner and colleagues have studied the multiple proton transfers in the three- and five-water systems and found that the sequential and concerted proton transfers yield the same energy barriers (Scheiner, 1981) (10 kcal/mol for the three-water system, and 16 kcal/mol for the five-water system, with the O···O distances set at 2.95 Å). A concerted multiple proton transfer usually gives a smaller energy barrier than does the sequential proton transfer, provided that distances between heavy atoms are held constant. Here, we have optimized the molecular geometries and studied the concerted proton transfers for a few proton-transfer reactions (Table IV). It is noted that the conclusions obtained from Table IV regarding the catalytic effects of various catalytic elements are in agreement with those obtained from the rigid molecule study of Table I. The optimized O···O distance between Zn^{2+}-bound H_2O and the first solvent water molecule, 2.4 Å, is shorter than the 2.8 Å used in the rigid molecule study. The most favorable position for the first hydrogen atom in the Zn^{2+}–$(H_2O)_3$–NH_3 complex is close to halfway between the two oxygen atoms. A complete transfer of the first proton is achieved in concert with subsequent proton transfers. For the intramolecular proton transfer in the 4-coordinated Zn^{2+} complex, the energy barrier is 20.0 kcal/mol at O···O ($r_3 + r_4$, Table IV) = 2.6 Å and 37.2 kcal/mol at O···O = 2.8 Å.

Immediately following the intramolecular proton transfer from Zn^{2+}-bound H_2O to His 64, the proton can be transferred either directly from the protonated His 64 to a water molecule or first to a buffer molecule and then to solvent (the buffer-assisted proton transfer).[3] A barrier of 20–40 kcal/mol is obtained for the direct proton transfer from the protonated His 64 to a water molecule, according to the simulation of proton transfer between NH_4^+ and H_2O at O···N of 2.7–3.1 Å (Scheiner & Harding, 1981a,b, 1983; Scheiner, 1981, 1982a,b). For the buffer-assisted proton transfer, if we take the imidazole molecules as the buffer molecules, then the energy barrier of proton transfer between NH_4^+ (the proton-transfer group) and NH_3 (the buffer molecule) is 1.7–14.7 kcal/mol at N···N of 2.73–3.15 Å (Scheiner & Harding, 1981c). These results indicate that it is important for the enzyme to include the buffer-assisted proton transfer to ensure a fast proton turnover. The final proton transfer from the protonated buffer molecule to solvent is probably entropy driven, since there is a vast number of configurations corresponding to a single proton in the bulk water region. Following each proton turnover, it is necessary for the imidazole ring to flip over to enable the successive proton transfers from Zn^{2+}-bound H_2O to His 64. No steric hindrance for this rotation of His 64 is indicated in the X-ray structure (Eriksson et al., 1986).

The proton-transfer role of His 64 is seriously challenged by the recent site-specific mutagenesis studies on His 64 of CA II (Forsman et al., 1988). The results show that mutations from His 64 to lysine, glutamine, glutamic acid, and alanine lead to changes of k_{cat} values of CO_2 hydration by 1.5–3.5-fold as compared to the native enzyme at pH 8.8 and 25 °C. Gothe and Nyman (1972) also reported the presence of a 30% enzyme activity in His 64 carboxyketoethylated CA II. Similar residue activities were observed in CA I when His 200 is chemically modified by bromoacetate and bromopyruvate (Whitney, 1970; Khalifah & Edsall, 1972; Khalifah, 1980). These results indicate that His 64 is not an obligatory proton-transfer group as previously hypothesized. This, however,

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[3] Khalifah (1973), Prince and Woolley (1973), and Lindskog and Coleman (1973) have proposed the buffer-assisted proton transfer to account for the discrepancy between the theoretically predicted turnover number of 10^3–10^4 s^{-1} and the experimentally measured turnover number of 10^6 s^{-1} in carbonic anhydrase catalyzed CO_2 hydration.

Table IV: Concerted Proton-Transfer Reactions[a]

Zn^{2+}–$(H_2O)(H_2O)$			(H_2O)–Zn^{2+}–(H_2O)–(NH_3)		
r_1	r_2	ΔE	r_1	r_2	ΔE
1.0	1.2535	0.0	1.0	1.4946	0.0
1.2	1.1414	−2.5[b]	1.2	1.2768	−6.0[b]
1.4	1.1324	6.1	1.4	1.0354	3.5
1.6	1.0194	13.2	1.6	1.1149	8.2
1.8	1.0188	22.2	1.8	1.0456	1.0

	Zn^{2+}–(H_2O)–(NH_3)		Zn^{2+}–$(H_2O)_2$–(NH_3)				
r_1	r_2	ΔE	r_1	r_2	r_3	r_4	ΔE
1.0	1.3000	0.0	1.0	1.2361	1.0	1.5956	0.0
1.2	1.2286	−10.0[b]	1.3259	1.1459	1.2	1.2713	−19.2[b]
1.4	1.1903	−7.9	1.3946	1.0374	1.4	1.2093	−16.6
1.6	1.0773	−6.4	1.5120	1.0065	1.6	1.1001	−11.0
1.8	1.0717	−0.8	1.5116	1.0056	1.8	1.0904	−3.4

Zn^{2+}–$(H_2O)_3$–(NH_3)						
r_1	r_2	r_3	r_4	r_5	r_6	ΔE
1.0	1.2139	1.0153	1.4051	1.0064	1.6501	0.0
1.1993	1.1741	1.0473	1.3656	1.0052	1.6288	−12.3[b]
1.2	1.1786	1.0474	1.3693	1.0038	1.6388	−11.7
1.4174	1.0140	1.2	1.1201	1.0888	1.3567	−28.4
1.4220	1.0131	1.2320	1.1138	1.2	1.3407	−25.0
1.4432	1.0136	1.2515	1.1034	1.4	1.1392	−17.7
1.6052	1.0545	1.5733	0.9983	1.6	1.0665	−9.5
1.6196	1.0504	1.5587	0.9962	1.8	1.0682	−0.6

$Zn^{2+}(NH_3)_3$–(H_2O)–(NH_3)			$Zn^{2+}(NH_3)_3$–$(H_2O)_2$–(NH_3)				
r_1	r_2	ΔE	r_1	r_2	r_3	r_4	ΔE
1.0	1.3630	0.0	1.0	1.3567	1.0064	1.5941	0.0
1.2	1.3010	1.5	1.2	1.2379	1.0212	1.5309	2.8
1.4	1.1810	11.5	1.2665	1.1093	1.2	1.2858	3.8
1.6	1.0928	20.0	1.2760	1.0587	1.4	1.1376	12.1
1.8	1.0924	37.2	1.3405	1.0512	1.6	1.0738	25.1
			1.3775	1.0420	1.8	1.0479	38.3

[a] r_1, r_2, r_3, r_4, r_5, and r_6 are O···H or N···H distances as depicted in Figure 2. Geometry optimizations are carried out at the PRDDO level. The $Zn^{2+}(NH_3)_3$ complex, as described under Methods, is held fixed during the optimizations. See footnotes of Table I and Figure 2. [b] The most favorable positions for the first proton.

does not affect our conclusion, because the NH_3 molecule used here to simulate His 64 can also represent an alternative proton-transfer group or a properly located buffer molecule.

According to the X-ray structure of HCA II at high pH (Eriksson et al., 1986; Kannan, 1980), one solvent water molecule occupies the fourth coordination site of zinc, forming a distorted tetrahedral complex with the three histidine ligands of zinc. Near the hydrophobic pocket, a second water molecule, the "deep" water, is found, which is ~3.2 Å away from Zn^{2+}. This deep water is thought to be displaced by CO_2 during substrate binding. The presence of a 5-coordinate zinc complex at high pH was suggested by absorption spectra of Co^{2+}-substituted carbonic anhydrase (Bauer et al., 1977; Kindskog et al., 1971) and X-ray structures of enzyme–inhibitor complexes: imidazole was found to bind to Zn^{2+} at 2.8 Å as a fifth ligand (Kannan et al., 1977), and sulfonamide binds at both the fourth and the fifth coordination sites of zinc (Kannan et al., 1977). Eriksson's recent refinement of the sulfonamide complex, however, supports the 4-coordinated structure for the metal ion (Eriksson et al., 1986; E. A. Eriksson, T. A. Jones, and A. Liljas, unpublished results). Also, spectroscopic investigation of imidazole binding indicates a possible 4-coordinated binding structure at high pH, with the deprotonated imidazolate ion occupying the fourth coordination site (Khalifah et al., 1987; Bertini & Luchinat, 1983). In addition, NMR measurements of solvent proton relaxation rates have been reinterpreted in terms of a 4-coordinated structure at high pH, with OH^- as a fourth ligand that exchanges as H_2O via a pentacoordinate intermediate (Koenig et al., 1983; Wells et al., 1979; Koenig & Brown, 1973). Thus, most of the recently obtained experimental results support the presence of the 4-coordinated zinc complex at high pH, which is energetically more favorable for the intramolecular proton transfer than the 5-coordinated structure.

It is noted that the experimental pK_a value of 7 for the Zn^{2+}-bound H_2O is similar to that of His 64. Assuming that the pK_a value of His 64 is larger than that of the Zn^{2+}-bound H_2O, a smaller concentration of unprotonated His 64 would be available to receive a proton from Zn^{2+}-bound H_2O—this would hinder the intramolecular proton transfer. On the other hand, if the pK_a value of His 64 is smaller than that of the Zn^{2+}-bound H_2O, a larger energy barrier for the intramolecular proton transfer would be expected. Thus, it may be important for the enzyme to modulate the pK_a values of both Zn^{2+}-bound H_2O and His 64 near the value of physiological pH 7.4 in order to ensure efficient and synchronous intramolecular proton transfer.

REFERENCES

Bauer, R., Limkilde, P., & Johanssen, J. T. (1977) *Carlsberg Res. Commun. 42*, 325.

Bertini, I., & Luchinat, C. (1983) *Acc. Chem. Res. 16*, 272.

Coleman, J. E., et al. (1980) in *Biophysics and Physiology of Carbon Dioxide* (Bauer, C., Gros, G., & Bartels, H., Eds.) pp 133–285, Springer-Verlag, Berlin.

Eriksson, E. A., Jones, T. A., & Liljas, A. (1986) in *Zinc Enzymes* (Bertini, I., Luchinat, C., Maret, W., & Zeppezauer, M., Ed.) pp 317–328, Birkhäuser, Boston.

Forsman, C., Behravan, G., Jönsson, B.-H., Liang Z.-W., Lindskog, S., Ren, X., Sandstrom, J., & Wallgren, K. (1988) *FEBS Lett. 229*, 360.

Gothe, P. O., & Nyman P. O. (1972) *FEBS Lett. 21*, 159.

Halgen, T. A., & Lipscomb, W. N. (1973) *J. Chem. Phys. 58*, 1569.

Halgren, T. A., Kleier, D. A., Hall J. H., Jr., Brown, L. D., & Lipscomb, W. N (1978) *J. Am. Chem. Soc. 100*, 6595.

Jönsson, B.-H., Steiner, H., & Lindskog, S. (1976) *FEBS Lett. 64*, 310.

Kannan, K. K. (1980) in *Biophysics and Physiology of Carbon Dioxide* (Bauer, C., Gros, G., & Bartels, H., Ed.) p 184, Springer-Verlag, New York.

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

Khalifah, R. G. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 1986.

Khalifah, R. G. (1980) in *Biophysics and Physiology of Carbonic Dioxide* (Bauer, C., Gros, G., & Bartels, H., Eds.) p 206, Springer-Verlag, Berlin.

Khalifah, R. G., & Edsall J. T. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 172.

Khalifah, R. G., Rogers, J. I., & Mukherjee, J. (1987) *Biochemistry 26*, 7057.

Koenig, S. H., & Brown, R. D. (1973) *Ann. N.Y. Acad. Sci. 222*, 752.

Koenig, S. H., Brown, R. D., Bertini, I., & Luchinat, C. (1983) *Biophys. J. 41*, 179.

Liang, J.-Y., & Lipscomb, W. N. (1986) *J. Am. Chem. Soc. 108*, 5051.

Liang, J.-Y., & Lipscomb, W. M. (1987) *Biochemistry 26*, 5293.

Lindskog, S. (1983) in *Zinc Enzymes* (Spiro, T. G., Ed.) p 77, Wiley, New York.

Lindskog, S., & Coleman, J. E. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2505.

Lindskog, S., Henderson, H. E., Kannan, K. K., Liljas, A., Nyman, P. O., & Strandberg, B. (1971) *Enzymes (3rd Ed.) 5*, 587.

Lindskog, S., Engberg, P., Forsman, C., Ibrahim, S. A., Jönsson, B.-H., Simonsson, I., & Tibell, L. (1984) in *Ann. N.Y. Acad. Sci. 429*, 61.

Lipscomb, W. N. (1983) *Annu. Rev. Biochem. 52*, 17.

Mak, T. C. W., & Huang, S.-H. (1987) *Polyhedron 6*, 1111.

Marynick, D. S., & Lipscomb, W. N. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 1341.

McCammon, J. A., & Karplus, M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 79*, 3585.

McCammon, J. A., & Karplus, M. (1980) *Biopolymers 19*, 1375.

Moller, C., & Plesset, M. S. (1934) *Phys. Rev. 46*, 618.

Mulliken, R. S. (1955) *J. Chem. Phys. 23*, 1833.

Northrup, S. H., Pear, M. R., Lee, C. Y., McCammon, J. A., & Karplus, M. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4035.

Pocker, Y., & Bjorkquist, D. W. (1977) *Biochemistry 16*, 5698.

Pocker, Y., & Sarkanen, S. (1978) *Adv. Enzymol. Relat. Areas Mol. Boil. 47*, 149.

Prince, R. H. (1979) *Adv. Inorg. Chem. Radiochem. 22*, 349.

Prince, R. H., & Woolley, P. R. (1973) *Bioorg. Chem. 2*, 337.

Pullman, A., & Demoulin, D. (1979) *Int. J. Quant. Chem. 16*, 641.

Rodwell, W. R., Bouma, W. J. & Radom, L. (1980) *Int. J. Quantum Chem. 18*, 107.

Scheiner, S. (1981) *J. Am. Chem. Soc. 103*, 315.

Scheiner, S. (1982a) *Chem. Phys. Lett. 93*, 540.

Scheiner, S. (1982b) *J. Chem. Phys. 77*, 4039.

Scheiner, S., & Kern, C. W. (1979) *J. Am. Chem. Soc. 101*, 4081.

Scheiner, S., & Harding, L. B. (1981a) *J. Am. Chem. Soc. 103*, 2169.

Scheiner, S., & Harding, L. B. (1981b) *Chem. Phys. Lett. 79*, 39.

Scheiner, S., & Harding, L. B. (1981c) *J. Am. Chem. Soc. 103*, 2169.

Scheiner, S., & Harding, L. B. (1983) *J. Phys. Chem. 87*, 1145.

Scheiner, S., Lipscomb, W. N., & Kleier, D. A. (1976) *J. Am. Chem. Soc. 98*, 4770.

Steiner, F., Jönsson, B.-H., & Lindskog, S. (1975) *Eur. J. Biochem. 59*, 253.

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

Wells, J. W., Kandel, S. I., & Koenig, S. H. (1979) *Biochemistry 16*, 3883.

Whitney, P. L. (1970) *Eur. J. Biochem. 16*, 126.