Tautomerization of Histidine 64 Associated with Proton Transfer in Catalysis of Carbonic Anhydrase

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The imidazole $^{15}$N signals of histidine 64 (His$^{64}$), involved in the catalytic function of human carbonic anhydrase II (hCAII), were assigned unambiguously. This was accomplished by incorporating the labeled histidine as probes for solution NMR analysis, with $^{15}$N at ring-$^{60}$ and N-$^{62}$, $^{13}$C at ring-$^{61}$, $^{13}$C and $^{15}$N at all carbon and nitrogen, or $^{15}$N at the amide nitrogen and the labeled glycine with $^{13}$C at the carboxyl carbon. Using the pH dependence of ring-$^{15}$N signals and a comparison between experimental and simulated curves, we determined that the tautomer equilibrium constant ($K_T$) of His$^{64}$ is 1.0, which differs from that of other histidine residues. This unique value characterizes the imidazole nitrogen atoms of His$^{64}$ as both a general acid (a) and base (b): its e2-nitrogen as (a) releases one proton into the bulk, whereas its d1-nitrogen as (b) extracts another proton from a water molecule within the water bridge coupling to the zinc-bound water inside the cavity. This accelerates the generation of zinc-bound hydroxide to react with the carbon dioxide. Releasing the productive bicarbonate ion from the inside separates the water bridge pathway, in which the next water molecules move into beside zinc ion. A new water molecule is supplied from the bulk to near the d1-nitrogen of His$^{64}$. These reconstitute the water bridge. Based on these features, we suggest here a catalytic mechanism for hCAII: the tautomerization of His$^{64}$ can mediate the transfers of both protons and water molecules at a neutral pH with high efficiency, requiring no time- or energy-consuming processes.

Carbonic anhydrase (CA$^2$) (EC 4.2.1.1) is a ubiquitous enzyme that catalyzes the reversible hydration of carbon dioxide (1). Isozymes of carbonic anhydrase regulate or function in such diverse physiological processes as pH regulation, ion transport, water-electrolyte balance, bicarbonate secretion-absorption, bone resorption, maintenance of intraocular pressure, renal acidification, and brain development (2). Nonfunctioning CA is implicated in such diseases as osteopetrosis syndrome, glaucoma, respiratory acidosis, epilepsy, and Ménière syndrome. Diseases due to CA deficiency include those affecting bones, the brain, and the kidneys. Consequently determining the detailed structure/function relationships or mechanisms responsible for its catalytic properties is mandatory for developing inhibitors or replacement therapies.

CA is present in at least three gene families ($\alpha$, $\beta$, and $\gamma$), which has made it a popular model for the study of the evolution of gene families and protein folding, and for transgenic and gene target studies (2). Among the three families, the $\alpha$ family is the best characterized, with 11 known isozymes identified in mammals. Earnhardt and co-workers have summarized maximal $k_{cat}$ and $k_{cat}/K_m$ values for CO$_2$ hydration by isozyme I–VII (3). The human isozyme II (hCAII) has a remarkably high turnover rate or catalytic efficiency ($k_{cat}/K_m = 1.5 \times 10^8$ M$^{-1}$ s$^{-1}$) that is very close to the frequency with which with which the enzyme and substrate molecules collide with each other in solution.

It is widely accepted that the hydration of CO$_2$ catalyzed by hCAII proceeds through several chemical steps as shown in Scheme 1 (1, 4, 5): the direct nucleophilic attack of the zinc-bound hydroxide ion on the carbonyl carbon of substrate CO$_2$ (structures 1–2), the formation of a zinc-bound bicarbonate intermediate (structures 2–3), the isomerization of the bicarbonate ion (structures 3–4), the exchange of the product bicarbonate ion with a H$_2$O (structures 4–5), and the regeneration of the zinc-bound hydroxide ion by the transfer of a proton to bulk solvent (structures 5–1). The proton transfer step (structures 1–5) consists of two substeps: 1) an intra-molecular transfer of protons to another residue in the enzyme and 2) a release of protons to the outside of the enzyme with the aid of a base. The intra-molecular proton transfer is the rate-limiting step of the maximal turnover rate ($10^6$ s$^{-1}$) at high concentrations of a base, whereas the proton release into the medium is rate-limiting at low buffer concentrations.

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‡ The abbreviations used are: CA, carbonic anhydrase; hCAII, human carbonic anhydrase II (EC 4.2.1.1), developing inhibitors or replacement therapies.

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In this reaction mechanism, His$^{64}$ is thought to play an important role in shuttling protons between the inside and outside of the active site cleft (6–9). As depicted in Fig. 1, the “in” (a) and “out” (b) conformations, representing the direction of the imidazole ring toward and away from the active site, were observed in pH-dependent x-ray crystallographic studies of hCAII (4, 5, 10–12). The side chain imidazole ring takes the in conformation at pH 7.8, where His$^{64}$ should be electrically neutral because of the pK$_a$ value of 7 as determined by $^1$H NMR (13). In this conformation, the δ1-nitrogen of His$^{64}$ appears to be involved in a water bridge or solvent network connected to the zinc-bound hydroxide ion through a hydrogen bond (12, 14, 15). In contrast, the T200S mutant of this enzyme was found to have His$^{64}$ in the out conformation at pH 8.0, retaining the full enzymatic activity (16). Because the out conformation of the imidazole ring was also observed at pH 5.7 (10), a swinging movement between the in and out conformations was assumed in connection with the proton transfer between a water molecule near a zinc ion and a bulk water molecule (5); the productive proton, which is transferred to the δ1-nitrogen via the water bridge, is released from its nitrogen to the bulk solution after swinging of the imidazole ring. This model is attractive because it appears to be able to account for the flow of water molecules in terms of space shared with the imidazole ring. However, there is no evidence supporting the notion that the two conformers are in the kinetically stable state at a given pH. In addition, molecular dynamics simulations show that His$^{64}$ vibrates rather than swings; it could be flexible enough to find the optimum geometry between active site solvent molecules and the bulk solvent (17–19).

Despite much effort, the proton-transfer mechanism involving the dynamic behavior of His$^{64}$ still remains controversial: the specific or reasonable manner in which His$^{64}$ participates in the proton-transfer needs to be explored. To address these issues, we labeled His$^{64}$ with $^{15}$N nucleus to identify the tautomeric forms of the imidazole ring in connection with the chemical mechanism of proton transfer in hCAII. The goal of our study is to detail the mechanisms responsible for the catalytic properties of carbonic anhydrase.

**MATERIALS AND METHODS**

*Isotope Labeling of hCAII—*To detect imidazole $^{15}$N signals and assign one of them to His$^{64}$, selectively labeled enzymes were obtained from a double-auxotroph requiring glycine and histidine of bacterial cell Escherichia coli BL21(DE3) containing the pET-hCAII gene and pLys-S, grown in the presence of labeled histidines and/or glycine. The double auxotroph was prepared using two distinct procedures. First is the generalized transduction method using phage P1 vir (20). In this experiment, the glyA gene encoding the serine-glycine hydroxymethyl transferase in the chromosome of *E. coli* BL21(DE3) (21) was replaced with the deficient gene glyA6 in the chromosome of a glycine auxotroph *E. coli* IQ417 (22) via the P1 phage particle. The second procedure is the ampicillin treatment method for the isolation of histidine auxotrophic mutants (23). The cells treated with 0–4 μg/ml acridine mutagen ICRI91 (6-chloro-9-[3-(2-chloroethylamino)-proplpylamino]-2-methoxy-acridine dihydrochloride, Sigma) were grown in an M9 medium containing 50 μg/ml ampicillin and enriched histidine auxotroph. The isolated double auxotroph requiring histidine and glycine, designated HS004, was cultured in an M9 medium containing 20 μg/ml histidine and 80 μg/ml glycine at 37 °C. By using this auxotroph transformed by the pET-hCAII-gene plasmid, four types of selectively labeled enzymes ([ring-$^{15}$N]His-hCAII, [ring-$^{13}$C-$^{15}$N]His-hCAII, [U-$^{15}$C/$^{13}$N]His-hCAII, and [α-$^{15}$N]His/[U-$^{13}$C]Gly-hCAII) were prepared. A uniformly $^{15}$N-labeled enzyme ([U-$^{15}$N]hCAII) was obtained from a bacterial cell *E. coli* BL21(DE3) containing pET-hCAII gene and pLys-S plasmids grown in an enriched M9 medium with $^{15}$NH$_4$Cl. The pET-hCAII gene (24) was a generous gift from Prof. Sly (St. Louis University School of Medicine). All the isotopically labeled chemicals were purchased from Cambridge Isotope Laboratories.

*Expression and Purification of Enzyme—*The gene expression was induced by the addition of 1.2 mM isopropyl β-D-galactopyranoside and 1.2 mM ZnSO$_4$ upon reaching the log...
Catalytic Mechanism of Carbonic Anhydrase

TABLE 1
Parameters for NMR measurements and solution conditions

| Experiment | Sample | Spectral widths (Hz) | Points | Phase delay or mixing time | pH | Ref. for pulse sequence |
|------------|--------|----------------------|--------|---------------------------|----|------------------------|
| \(^{15}\text{N}/\text{H} \text{HSQC}^a\) | \([\alpha\text{-}^{15}\text{N}]\text{-His}[1\text{-}^{13}\text{C}]\text{Gly}\text{-hCAII} \) | \(^{15}\text{N}\) 800 \(\text{H} 1\) 6250 | 1024 36 32 | 2-H 2.25\(^a\) | 90:10 | 5.2 31 |
| \(\text{HNCO} \) | \([\text{U}\text{-}^{13}\text{C}/\text{N}]\text{His}\text{-hCAII} \) | \(^{15}\text{N}\) 800 \(\text{H} 1\) 6250 | 30 1024 | N-H 2.25\(^a\) | 90:10 | 5.2 32 |
| \(\text{HNCA} \) | \([\text{U}\text{-}^{13}\text{C}/\text{N}]\text{His}\text{-hCAII} \) | \(\text{H} 1\) 6250 \(\text{C} 1250 \) \(^{15}\text{N}\) 800 | 1024 36 32 | N-H 2.25\(^a\) | 90:10 | 5.2 33 |
| \(\text{HCCH} \) | \([\text{U}\text{-}^{13}\text{C}/\text{N}]\text{His}\text{-hCAII} \) | \(^{13}\text{C}\) 2500 \(\text{H} 1\) 6250 | 64 1024 | C-H 1.80 \(^a\) | 90:10 | 5.2 34 |
| \((\beta\text{-}\beta\text{-}\beta\text{-}\beta\text{-}\beta\text{-}\beta)\text{hH}) \) | \([\text{U}\text{-}^{13}\text{C}/\text{N}]\text{His}\text{-hCAII} \) | \(^{13}\text{C}\) 2500 \(\text{H} 1\) 6250 | 32 1024 | C-H 1.80 \(^a\) | 90:10 | 5.2 35 |

**Parameter for NMR measurements and solution conditions**

- **Experiment**
- **Sample**
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- **Points**
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- **pH**
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* For the detection of amide N-H correlation cross-peaks.
* For the detection of imidazole N\(^{\text{15}}\)-H\(^{\text{15}}\), N\(^{\text{15}}\)-H\(^{\text{14}}\), and N\(^{\text{15}}\)-H\(^{\text{14}}\) correlation peaks.
* For the detection of imidazole N\(^{\text{14}}\)-H\(^{\text{14}}\) or N\(^{\text{14}}\)-H\(^{\text{14}}\) correlation peaks.

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phase (A\(_{600} = 0.6\)) in the growth curve. The cells were collected 16 h later and the harvest was extracted in 50 mM Tris sulfate, 0.1% Triton X-100, pH 8.0, after sonication. The enzyme was purified by affinity column chromatography as described by Osborne and Tashian (25), followed by gel filtration with Sephadex G-75. The purified sample was stored as a lyophilized powder at \(-20°C\). The zinc-free apoenzyme was prepared by treating the purified sample with pyridine-2,6-dicarboxylic acid (dipicolinic acid), according to Hunt et al. (26). Protein concentrations were determined by using the extinction coefficient \(e = 54800 \text{ m}^{-1} \text{ cm}^{-1}\) at 280 nm for hCAII (27). The purity was confirmed by reverse-phase high performance liquid chromatography on a C4-column (YMC Co.). The molecular weight (29,000) of native enzyme was confirmed by the sedimentation equilibrium method with an Optima XL-A (28). The enzyme activity was confirmed by the hydrolysis rate of 1 mM 4-nitrophenyl acetate (29, 30).

**NMR Measurements**—The lyophilized powder was dissolved in 20 mM acetate buffer with 200 mM Na\(_2\)SO\(_4\), pH 5.2, to prepare 1.5 mM of the selectively labeled enzyme samples and 3.0 mM of the uniformly labeled enzyme sample. All NMR experiments were determined by using the extinction coefficient \((29,000)\) of native enzyme was confirmed by the sedimentation equilibrium method with an Optima XL-A (28). The enzyme activity was confirmed by the hydrolysis rate of 1 mM 4-nitrophenyl acetate (29, 30).

Forms of imidazole, the N\(^{\text{15}}\)-H tautomer and the N\(^{\text{14}}\)-H tautomer, exist in tautomeric equilibrium. The acid-base equilibrium constants \(K_1\) and \(K_2\) are given by

\[
K_1 = \frac{[\text{imidazolium cation}]}{[\text{imidazolium cation}]} \quad \text{and} \quad K_2 = \frac{[\text{imidazolium cation}]}{[\text{imidazolium cation}]} \quad \text{coefficients}.
\]

The experimentally determined value \(K_2\) is given by \(K_2 = K_1 + K_2\). The \(K_1\) and \(K_2\) values of \(\text{1-H histidine}\) are 0.25 and 6.2 in aqueous solution, respectively (40–42). These values were derived from small model compounds (43). The tautomeric equilibrium constant \(K_T\) is given by the following equation.

\[
\delta_{\text{obs}} = \delta_{\text{basic}} \cdot \frac{1}{1 + 10^{(pK_a - pF)}} + \delta_{\text{acidic}} \cdot \frac{1}{1 + 10^{(pH - pF)}}
\]

where \(\delta_{\text{obs}}\) is the observed 1\(^{\text{5}}\)N chemical shift. The limiting chemical shifts at basic and acidic pH are represented by \(\delta_{\text{basic}}\) and \(\delta_{\text{acidic}}\), respectively. Parameters \(\delta_{\text{basic}}\) and \(\delta_{\text{acidic}}\) were determined by fitting Equation 1 to the experimental data using Kaleida Graph software (Synergy Software Co.). \(\delta_{\text{basic}}\) is the population-weighted average value of the 1\(^{\text{5}}\)N chemical shifts of pyrrole-like (>\(\text{N}\)-H) and pyridine-like (>\(\text{N}\)-N) types; the proportion of N\(^{\text{15}}\)-H or N\(^{\text{14}}\)-H type nitrogen to the entire nitrogen, P(N\(^{\text{15}}\)-H or N\(^{\text{14}}\)-H), is approximated expressed as a function of \(\delta_{\text{basic}}\) as,

\[
P = \frac{\delta_{\text{obs}} - \delta_{\text{basic}}}{\delta_{\text{basic}} - \delta_{\text{obs}}}
\]

where \(\delta_{\text{obs}}\) and \(\delta_{\text{basic}}\) are 1\(^{\text{5}}\)N chemical shifts of pyrrole-like (>\(\text{N}\)-H) and pyridine-like (>\(\text{N}\)-N) nitrogen types, respectively. These values were derived from small model compounds (43). The tautomeric equilibrium constant \(K_T\) is given by the following equation.
The procedure cannot be verified as being highly accurate, but certainly it is more accurate than any other determination for solutions, including the use of C-N coupling constants (44).

**RESULTS**

**Assignment of $^1$H, $^{13}$C, $^{15}$N Signals of His$^{64}$—**There is no strategy for the simple direct assignment of the imidazole ring within histidine residues. By combining a unique method of amide assignment and the following techniques of intra-residual assignment, we carried out the unambiguous imidazole assignment of His$^{64}$ in hCAII. The double-labeling method (45) was applied to the amide assignment of His$^{64}$, which was performed by using a selectively labeled enzyme, $[^{15}$N]Hist/[1-$^{13}$C]Gly-hCAII. Among 12 histidine residues in hCAII, only His$^{64}$ is linked to Gly; the peptidyl bond between Gly$^{63}$ and His$^{64}$ is labeled by both $^{15}$N and $^{13}$C. Twelve singlets of histidine resonances in the decoupling spectrum shown in Fig. 2A change into 11 singlets and one doublet in the non-decoupled spectrum shown in Fig. 2B. This spectral change clearly demonstrates that the doublet is due to His$^{64}$. This amide assignment was further confirmed in the two-dimensional HNCA spectrum of [U-$^{13}$C/$^{15}$N]Hist-hCAII at $^{15}$N = 117.2 ppm. The cross-peak between the amide proton and the Cα carbon of His$^{64}$ was observed in the spectrum where Cα = 55.5 ppm. Fig. 3C shows the two-dimensional HCCH spectrum in which the amide assignment was further confirmed in the two-dimensional HCCH spectrum shown in Fig. 4. As a result, this weakening signal allows us to assign three other observable resonances, N$^{61}$-H$^{61}$, N$^{62}$-H$^{62}$, and N$^{62}$-H$^{62}$. Consequently, the H$^{61}$, N$^{61}$, and N$^{62}$ nuclei of His$^{64}$ were assigned to the $^1$H and $^{15}$N chemical shifts of 8.03, 177.8, and 175.4 ppm, respectively. The identification of N$^{61}$ and N$^{62}$ nuclei can be confirmed at basic pH regions. By gradually changing the pH from 5.2 to basic, one of their signal intensities change characteristically; the N$^{61}$-H$^{62}$ resonance weakens in intensity where the 3$^N_{N^{61},H^{62}}$ coupling is too small (2–3 ppm uncertainty in the denominator) can cause a significant error, especially when the numerator in Equation 3 will allow quite reasonable estimates even a 2–3 ppm uncertainty in the limiting shift values of the denominator.

The amide assignment of His$^{64}$ in hCAII clearly demonstrates that the doublet is due to His$^{64}$. This decoupled spectrum shown in Fig. 2 of histidine resonances in the decoupling spectrum shown in Fig. 2. The amide assignment of His$^{64}$ in hCAII by using the double labeling method. $^{15}$N/$^1$H HSQC spectra of [α-$^{15}$N]Hist/[1-$^{13}$C]Gly-hCAII were obtained by the measurements (A) with decoupling (B) without decoupling the $^{13}$C-carbonyl region during $t_1$ at pH 5.2 and 25 °C. A doublet ($^{15}$n = 117.2 ppm and $^1$H = 8.41 ppm) in B was assigned to the His$^{64}$ amide.

$K_T = \frac{P_{N\text{H}-1}}{P_{N\text{H}+2}}$ (Eq. 3)

The amide assignment of His$^{64}$ in hCAII by using the double labeling method.
of the imidazole ring. To determine the $K_T$ value, it is essential to observe the $^{15}$N signals of $N^{61}$ and $N^{62}$ simultaneously. The $^{15}$N signals of the imidazole nitrogen nuclei characteristically reflect the charged states of the imidazole ring (43). In the cationic imidazolium form, both nuclei attached to protons showed a chemical shift at around 176.5 ppm. In the neutral form, $N^{61}$ and $N^{62}$ $^{15}$N exhibit signals at 167.5 ppm when these nitrogen atoms are protonated and at 249.5 ppm when they are not protonated, allowing us to distinguish between two tautomeric forms involving these nitrogen atoms. In favorable cases, these $^{15}$N signals of $N^{61}$ and $N^{62}$ are observed in a “fast exchange” regime, where their signals are averaged to give a single resonance. Note that the rate of proton-exchange between these two nitrogen atoms is more than $1.6 \times 10^8 \text{s}^{-1}$. When the proton prefers one of the nitrogen nuclei, the weight averaging of the chemical shifts occurs in the $N^{61}$ and $N^{62}$ signals.

For the observation of imidazole $^{15}$N signals of His$^{64}$ in hCAII, two-dimensional $^{15}$N/$^1$H-correlation spectroscopy was used, which detects the $N^{61}$-$H^1$, $N^{61}$-$H^2$, $N^{62}$-$H^1$, and $N^{62}$-$H^2$ resonances described above. As shown in Fig. 4, the signals of His$^{64}$ were observed to be regarded as “fast” at pH 7.9. In this measurement, all other imidazole $^{15}$N signals except the signal number 6 were also observed as the fast exchange. For signal number 6, considering the signals to be observed in the region of pH 5.2–6.7, one of the exchange rates may change from fast to “intermediate” with increasing pH. However, for this signal disappearance, this could not be concluded easily because the signal intensity is related not only to the exchange, but also to some other factors such as $J$-coupling constants dependent on pH.

The pH Dependence and Tautomeric Proportion of Histidine Residues—$^{15}$N chemical shifts were monitored as a function of pH to investigate the profile of acid–base and tautomeric equilibrium of the histidine residue. The pH-titration curves of $N^{61}$ and $N^{62}$ for all 12 histidine residues are shown in Fig. 5. To simply illustrate the pH dependence of the $^{15}$N chemical shift, the variation of the $^{15}$N chemical shifts with pH are simulated by substituting the $pK_a$ value of $L$-histidine (6.2), the chemical shift value of the $+N-H$ type, and the variable weight average of $>N-H$ and $>N$: chemical shifts for Equations 1–3, as shown in Fig. 6. This figure allows us to facilitate the investigation of the tautomeric proportion in histidine residues under the fast exchange situation. Comparing Figs. 5 and 6, the approximate $K_T$ values of the histidine residues are quite obvious. In the case of pH-independent $^{15}$N chemical shifts, their titration curves need not be compared with that of Fig. 6. In both cases, the $K_T$ values were calculated by Equations 2 and 3 using the basic $^{15}$N-limiting shift. Table 2 summarizes the acid-base and tautomeric equilibrium constants of the histidine residues.

According to their titration profiles, histidine residues of hCAII were classified into three groups, A, B, and C, as summarized in Table 2. Group A consists of seven histidine residues sensitive to the tested pH changes (Group A: the change between acid and base limiting shift values is $>30$ ppm for either $N^{60}$ or...
N\textsuperscript{c2}). These histidine residues would be distributed on the surface or in a solvent-accessible position in the molecule. For this study, one of them was unambiguously assigned to His\textsuperscript{N64} as described above. His\textsuperscript{N64} occurs in the equivalent proportion of the tautomer: $K_T = 1.0$. To our knowledge, no behavior similar to that of His\textsuperscript{N64} has been found in any other protein. The six other histidine signals are designated as 1–6. The $K_T$ constants are found to be in the range from 0.01 to 0.4. Curves 1 and 2 show that the hydrogen atoms are localized on N\textsuperscript{c2} of their histidine residues, whereas curves 3–6 show normal tautomeric profiles, similar to that of L-histidine amino acid in aqueous solution. These histidine residues are thought to be on the surface of the molecule. Group B consists of two pH-insensitive histidines, designated as 7 and 8 (Group B: the change between acid and base limiting shift values is <0.1 ppm for both N\textsuperscript{c1} and N\textsuperscript{c2}). The N\textsuperscript{c1} signals of 7 and 8 appeared as >N-H type, and the N\textsuperscript{c2} signal as the <N-H type, thus indicating that these histidine residues exist as N\textsuperscript{c1}-H tautomers in all pH values tested. Group C consists of three slightly pH-sensitive histidines designated as curves 9–11 (Group C: the change between acid and base limiting shift values is between 0.5 and 5 ppm for either N\textsuperscript{c1} or N\textsuperscript{c2}). The N\textsuperscript{c1} of 9 and 10, and N\textsuperscript{c2} of 11 appear as >N-H types. This result shows that 9 and 10 histidines occur as N\textsuperscript{c1}-H tautomers; N\textsuperscript{c2} of 9 experiences a 7 ppm low field chemical shift change compared with typical pyrrole-like (>N-H) nitrogen and N\textsuperscript{c2} of 10 at 12.5 ppm. Number 11 of the histidine residue behaves like a N\textsuperscript{c2}-H tautomer; N\textsuperscript{c2} is a 9.5-ppm low field chemical shift change.

**Identifications and Assignments of Zinc-bound and Buried Histidine Residues**—Crystal structure shows two kinds of interior or not exposed histidine residues: zinc-bound histidines, His\textsuperscript{94}, His\textsuperscript{96}, and His\textsuperscript{119}, and buried histidines, His\textsuperscript{107} and His\textsuperscript{122} (12). These residues except for His\textsuperscript{122} are illustrated in Fig. 1. First, we distinguished the zinc-bound histidines from the buried histidines by comparing the C\textsuperscript{13}-H\textsuperscript{13} correlation signal of the holoenzyme with that of the apoenzymes. The pH titration experiment was carried out on [ring-C\textsuperscript{13}-C\textsuperscript{13}]His-hCAII using 13C/1H HSQC experiments. The H\textsuperscript{13} titration profiles are consistent with those from the 15N/H experiments described above; the p$K_a$ values and chemical shift values of H\textsuperscript{13} were confirmed. Fig. 7, A and B, shows the spectra of holo- and apoenzymes labeled with [ring-C\textsuperscript{13}-C\textsuperscript{13}]His at pH 7.0. Comparing them, the His\textsuperscript{94} signal and three other signals (numbers 9–11) disappear from the spectrum of the apoenzyme. Instead of these signals, several other signals appear. This observation shows that signals 9–11 were from three zinc-bound imidazoles of the histidine residues. This result is consistent with that of the above described 15N experiment in which either a N\textsuperscript{c1} or N\textsuperscript{c2} signal is observed in the region between 205 and 215 ppm, which is of the zinc-bound nitrogen type (48). Subsequently, we tentatively assigned signals 9–11 to the zinc-bound histidine residues by using the crystal structure of enzyme. Among the three His residues coordinated with the zinc ion, His\textsuperscript{119} is

**TABLE 2**

| Group | Residual or signal number | $K_T$ (±5%) | Nucleus | $pK_a$ (±0.1) | Limiting shift (ppm) | $\gamma_{NH}$ |
|-------|--------------------------|-------------|---------|-------------|---------------------|-------------|
| A     | 1                        | <0.05       | N\textsuperscript{c1} | 5.8         | 179.0–243.5        |             |
|       | 2                        | 0.1         | N\textsuperscript{c1} | 5.9         | 174.6–168.2        |             |
|       | 3                        | 0.4         | N\textsuperscript{c1} | 6.1         | 177.2–238.1        |             |
|       | 4                        | 0.4         | N\textsuperscript{c2} | ND$^a$      | 174.6–168.2        |             |
|       | 5                        | 0.4         | N\textsuperscript{c2} | 6.6         | 177.8–222.9        |             |
|       | 6                        | 0.3         | N\textsuperscript{c2} | 6.6         | 173.8–190.3        |             |
|       | His\textsuperscript{64}  | 1.0         | N\textsuperscript{c2} | 6.6         | 179.4–230.6        |             |
| B     | 7 (His\textsuperscript{122}) | N\textsuperscript{c1} | 5.0         | 174.9–185.3        |             |
|       | 8 (His\textsuperscript{107}) | N\textsuperscript{c1} | 5.0         | 174.2–208.3        |             |
| C     | 9 (His\textsuperscript{94/96}) | N\textsuperscript{c1} | 5.0         | 175.8–207.7        |             |
|       | 10 (His\textsuperscript{94/96}) | N\textsuperscript{c1} | 5.0         | 167.8–167.8        |             |
|       | 11 (His\textsuperscript{119}) | N\textsuperscript{c1} | 5.0         | 249.3–249.3        |             |

$^a$ Numbers 1–6 histidine residues are located on the surface of molecule, which includes histidines 3, 4, 10, 15, 17, and 36. The number 1 or 2 may be from His\textsuperscript{13} (see also “Discussion”). His\textsuperscript{64} is assigned using unique NMR techniques. The residues in parentheses are tentatively assigned using crystal structure.

$^b$ ND, not determined.
Catalytic Mechanism of Carbonic Anhydrase

![Diagram of Carbonic Anhydrase](image)

His122. For H\(_1\) of His122, the NOE cross-peak was confirmed by using \(^{15}\)N/\(^1\)H HMQC-NOESY as shown in Fig. 8C. The H\(_N\) chemical shifts are added to Table 2. Scalar spin-spin coupling constants (\(J_{NH}\)) of the N-H bonds of the imidazole ring are summarized in Table 2. The values of \(J_{NH}\) provides a direct measure of covalent bond character; the observed values of 92–97 Hz indicate that these imino protons are fixed covalently about 90–100% (50).

The H\(_N\) chemical shifts were monitored as a function of pH to calculate \(pK_a\) values. In the \(^{15}\)N-labeled enzyme, all five H\(_N\) signals were observed in the region of pH 5.7–8.8. All H\(_N\) chemical shifts were slightly sensitive to pH change, as shown in Fig. 8D. For Group B, the H\(_N\) signal of His\(^{94/96}\) (H\(_1\)) shifts to a slightly lower field as the pH increases, which is different from the pH dependence of zinc-bound histidine residues in the direction of shift. The titration curve does not exhibit sigmoid behaviors and the difference between chemical shifts at acidic and basic is very small, 0.06 ppm. The H\(_N\) signal of His122 (H\(81\)) shifts to a slightly higher field. In the H\(_N\) of His94/96 and H\(_N\) of His119 of Group C, the titration curves exhibited the clearly sigmoid behaviors dependent on pH required to calculate \(pK_a\) values and limiting shifts using \(\delta_{obs}\) of the proton instead of \(\delta_{obs}\) in Equation 1. The \(pK_a\) values of H\(_N\) of His94/96 (number 9), H\(_N\) of His94/96 (number 10), and H\(_N\) of His119 are 7.3 ± 0.04, 7.2 ± 0.02, and 7.2 ± 0.03, respectively. The \(pK_a\) values of His94, His96, and His119 probably reflect the titration behavior of other residues or groups because these residues are unattached to water molecules. Importantly, these \(pK_a\) values are in good agreement with that of His64 determined in our measurements. The coincidence implies that the titration behavior of His64 is reflected on those of zinc-bound histidine residues. However, the possibility that the observed effect is due to the ionization of zinc-bound water could not be ruled out.

**DISCUSSION**

Implication of Tautomeric Equilibrium Constant of Histidine Residues—We determined the tautomeric equilibrium constant (\(K_T\)) of the imidazole ring of His64 to be 1.0, according to the unambiguous assignment of \(^{15}\)N signals, the analysis of their pH dependences, and a comparison of experimental and simulated titration curves. This value was different from those of other histidine residues in this enzyme, whereas its \(pK_a\) value of 7.2–7.3 was indistinguishable from those of the others (Table 2). The \(K_T\) value of 1.0 indicates that two imidazole nitrogen atoms (N\(_{61}\) and N\(_{62}\)) can be equally involved in the catalytic reaction. It is therefore reasonable to assume that one of the imidazole nitrogen atoms acts as a general acid, whereas the other acts as a general base, as shown in Equation 4.

\[
\text{H}^+ + \text{His}^+ \rightarrow \text{His}^2^+ + \text{H}^+ \quad (\text{Eq. 4})
\]

Because the tautomeric equilibrium of an imidazole group is dominated by hydrogen bond interactions with the \(\delta_1\)-nitrogen where an acid or base interacts strongly, the usual equilibrium condition gives a large deviation of the \(K_T\) values from 1 (51).
For example, the N\(^{61}\)-H tautomer dominates in the imidazole group of cis-uronic acid, as indicated by \(K_T = 5.2\) (Equation 5), in which the intramolecular hydrogen bond can be formed, whereas the N\(^{62}\)-H tautomer is favorable in Equation 6 with the \textit{trans}-configuration preventing the hydrogen bond though a carboxylate anion (\(K_T = 0.37\)).

These \(K_T\) values suggest that the imidazole group intrinsically tends to be the N\(^{62}\)-H tautomer, unless a hydrogen bond interacts with the \(\delta_1\)-nitrogen of the imidazole ring. In fact, the \(K_T\) values for 6 histidine residues exposed to the solvent (Group A in Table 2) were shown to be less than 0.4, indicating the prevalence of the N\(^{62}\)-H tautomer.

As shown in Equation 7, the conformational flexibility along the C\(\beta\)-C\(\gamma\) bond of 3-(imidazol-4-yl)propionic acid permits the partial formation of a hydrogen bond. In this case, the N\(^{62}\)-H tautomer still dominates, as in Equation 6, but the equilibrium shifts in favor of the N\(^{61}\)-H tautomer (\(K_T = 0.61\)). Based on this analogy, His\(^{64}\) should have a structure-specific determinant to promote the partial formation of a hydrogen bond. As illustrated in Equation 8, we consider that a negative charge of the zinc-bound hydroxide ion is responsible for increasing the population of the N\(^{61}\)-H tautomer, and a network of water molecules is responsible for attenuating the hydrogen bonding effect to a level comparable with that of the counterpart.

Using this equation, we could consider that the tautomerization of His\(^{64}\) would be coupled to the ionization of the zinc-bound solvent.

To our knowledge, no real compound model has been reported to explain the \(\epsilon_2\)-nitrogen of an imidazole group in hydrogen bond interactions. In this case, we assumed that a hydrogen bond partner in close proximity to the \(\epsilon_2\)-nitrogen affects the change in the \(K_T\) value, in contrast to the \(\delta_1\)-nitrogen case as described above, is expected to decrease to much less than 0.4. This implies that one of the \(K_T\) values in Group A, <0.05 of signal number 1 or 0.1 of number 2, is from His\(^{18}\) because the \(\epsilon_2\)-nitrogen of His\(^{15}\) can form a hydrogen bond with oxygen of Lys-9 as an acceptor (distance: 3.19 Å), which may stabilize the N\(^{62}\)-H tautomeric form. For His\(^{107}\) and His\(^{122}\) in Group B, two hydrogen bond interactions are seen in the imidazole group, as shown in Fig. 8E, a and b, respectively. The conditions of His\(^{107}\) and His\(^{122}\) existing in a hydrogen bond network are apparently similar. Based on the structures, both histidine residues should take only the N\(^{61}\)-H tautomer. This is also supported by our measurement for \(J\) values, in which the \(J\) value of His\(^{107}\) is close to that of His\(^{122}\), indicating that hydrogen localization on the imidazole nitrogen of His\(^{107}\) is essentially equal to that of His\(^{122}\). However, the apparent \(K_T\) values, 7.6 for His\(^{107}\) and >20 for His\(^{122}\), were calculated by Equations 2 and 3, although a small error contained in the difference \(\delta_{\text{N-H}} - \delta_{\text{NH}}\) in Equation 2 could make the comparison between their \(K_T\) values difficult. For the difference of these histidine residues, it is possible to argue the difference of their strengths of hydrogen bonds in terms of chemical shift values. Comparing Fig. 8E, a and b, we note that the distance of hydrogen bond between N\(^{61}\) of His\(^{122}\) and the carbonyl oxygen of Ala\(^{142}\) (3.12 Å) is appreciably longer than that between \(\delta_1\)-nitrogen of His\(^{107}\) and the carboxyl oxygen of Glu\(^{117}\) (2.84 Å). Similarly, there is a slight increase in distance between the \(\delta_1\)-nitrogen of His\(^{122}\) and the hydroxyl oxygen of Tyr\(^{51}\) (2.78 Å) compared with that between the \(\delta_1\)-nitrogen of His\(^{107}\) and the hydroxyl oxygen of Tyr\(^{194}\) (2.66 Å). Such an increase in distances could lead to a weakening of hydrogen bond. In the N\(^{61}\)-H tautomer illustrated in Fig. 8E, a, the chemical shift values of N\(^{61}\) (177.5 ppm) and N\(^{62}\) (240.5 ppm) for His\(^{107}\) agree well with the expected limiting shifts due to donation (+10 ppm) and acceptance (~−10 ppm) of hydrogen bonds, respectively (43, 52). Using these limiting shifts, the \(K_T\) value for His\(^{107}\), >20, is determined by the calculation using Equations 2 and 3, taking only N\(^{61}\)-H tautomer. In contrast, the corresponding values of N\(^{61}\) (167.8 ppm) and N\(^{62}\) (249.3 ppm) for His\(^{122}\) do not accord with the above empirical rule, but appear to be independent of the hydrogen bonds with Tyr\(^{51}\) and Ala\(^{142}\). That is, assume that neither \(\delta_1\)-nor \(\epsilon_2\)-nitrogen atoms of His\(^{122}\) is firmly involved in the hydrogen bond interactions but the \(\epsilon_2\)-nitrogen is rather involved in hydrogen bond interaction with the hydroxyl oxygen of Tyr\(^{51}\) because the slight or partial negative charge of the carbonyl oxygen of Ala\(^{142}\) can likely balance with the amide of His\(^{122}\). For His\(^{122}\), thus, the limiting shifts without the hydrogen bond, 167.5 and 249.5 ppm, are used to determine its \(K_T\) value, >20, taking only the N\(^{61}\)-H tautomer. For Group C, because of zinc coordination and hydrogen bonding, His\(^{94}\) and His\(^{96}\) would exist only in one tautomeric form. Although their N\(_{\text{tyr}}\) chemical shifts are ~10 ppm lower than a typical chemical shift, 167.5 ppm, the imidazole N-H spin-coupling constants range from 90 to 98 Hz. Therefore the N\(^{61}\) protons of His\(^{94}\) and His\(^{96}\) and the H\(^{\epsilon_2}\) of His\(^{119}\) are essentially 100% localized on these nitrogen atoms, based on their one-bond coupling constants.

\textbf{Catalytic Mechanism of Carbonic Anhydrase II—}It has been accepted that protonation of the N\(^{61}\) of His\(^{64}\) results from the ionization of the water molecule to generate the hydroxide ion near the zinc ion, as shown in Equation 9 (5).
The protonation of δ1-nitrogen is confirmed to be appropriate because transfer of the proton was achieved by a concerted process in a dynamics study (53). Using this process, the intramolecular proton transfer step could be said to occur in the active site. However, this equation is limited for explaining the release of the proton into the bulk solution in the catalytic reaction, because the proton travels only inside the water bridge between His64 and the zinc ion like a shuttle, and it cannot jump from the water bridge to bulk solvent. For the proton release, a crystallographic study has proposed the swinging mechanism of His64, as depicted in Fig. 1: that the productive proton transferred to the δ1-nitrogen of His64 is released from its nitrogen to the bulk solution after swinging (16). Although this mechanism is plausible, note that the swinging rate of the imidazole ring is considered to be the same as the rotation rate of the ring, such as the side chain of Phe or Tyr, to explain why the rate is comparable with the effective turnover (10⁶ s⁻¹) of this enzyme. This analogy cannot be appropriate because the imidazole hydrogen bond ability of the ring and rotational symmetry are quite different from those of phenyl or hydroxyl-phenyl rings. Therefore, using the out conformation resulting from the imidazolium ion in the next reaction step was a problem. This problem has made it exceedingly difficult to reveal a reasonable pathway to transfer the productive proton via His64 in the proposed proton release mechanism, in view of the vague or indistinguishable tautomerization.

Here we clearly demonstrate the relation between His64 structures and the proton release in the catalytic reaction of hCAII. Using two neutral tautomers, the imidazole ring of His64 need not swing to transfer the productive proton in the reaction because the imidazolium cation is thought to be a transient intermediate in mediating the tautomerization, assuming that this intermediate is different from the out conformation of imidazolium in the character of its structure. Instead of swinging, we notice a variety of water molecule locations in the active site in crystal structures of hCAII. The relationship between the variation of water molecule locations and molecular proton transfer step could be said to occur in the active site.
the reaction (Scheme 1) makes it reasonable that the water bridge (Fig. 1) can split in a process such as isomerization of a zinc-bound bicarbonate ion (54, 55) or the exchange of the product bicarbonate ion with a water molecule in the reaction. This indicates that a flow of the water molecules should occur in the active site to continue the reaction. We consider that behavior of water molecules such as the split and flow would occur within the N/H-N/H tautomer without hydrogen bond interaction, as shown in Fig. 9. In this scheme, the CO₂ hydration reaction proceeds in the following steps. 1) The zinc-bound hydroxide makes a direct nucleophilic attack on the carbonyl carbon of substrate CO₂ (Fig. 9, A and B). 2) This attack forms a zinc-bound bicarbonate intermediate in the active site (Fig. 9, B and C). 3) The bicarbonate intermediate isomerizes into the productive complex to be replaced with the solvent molecule shown as B, resulting in a split of the water bridge between His⁶⁴ and the zinc ion. This split changes the N/H-N/H tautomer of His⁶⁴ into the N/H-N/H tautomer via the transient imidazolium intermediate, which triggers the release of the product proton (shown in light blue), resulting in proton transfer among His⁶⁴, H₂O, H₂O, and H₂O in that order (Fig. 9, C and D). In this step, we adopted the Lipscomb model for isomerization of the bicarbonate ion on the zinc ion according to the recent papers (54, 55). However, this does not necessarily give it any preference to the Linskog model; our scheme might not depend on the isomerization mechanism of the bicarbonate ion. 4) Releasing the product bicarbonate from the active site center, the water molecules remaining in the cave move into beside the zinc ion to reconstitute the water bridge, to which a brand new water molecule, shown as H₂O, is supplied from the bulk solution (Fig. 9, D and E). 5) Immediately, the zinc electric repulsion causes rapid ionization into the hydroxyl ion. This ionization would be coupled to the tautomerization of His⁶⁴ (Fig. 9, E and F). Through the reconstituted water bridge, the protons transfer from the zinc-bound site to His⁶⁴ where transferring protons would be achieved by a concerted process (53). 6) The regeneration of the initial mode is achieved by proton release from the e²-nitrogen of His⁶⁴ to the bulk solvent (Fig. 9, F and A), leading to the subsequent cycle of the catalytic reaction. Thus, this scheme explains the effective proton release following the intra-molecular proton transfer step in the catalytic reaction of hCAII. This scheme can be also used to explain the unique pH-dependent activity (9, 56, 57) of this enzyme, which has its maximum activity in pH 7. First, lowering pH accelerates that His⁶⁴ would not participate in the hydrogen-bonded pathway because this residue takes the out conformation at low pH regions as shown in Fig. 1. This implies that the productive protons are transferred by another hydrogen-bonded pathway without His⁶⁴. Using this alternative pathway would decrease the proton transfer ability. Second, increasing pH would inhibit the addition of the proton (shown in pink) to the e²-nitrogen of His⁶⁴ in the C–D step in Fig. 9, or it might accelerate the
replacement of some water molecules between the zinc ion and His$^{64}$ with hydroxyl ions. The loss of their protons may decrease the effective transfer of the productive proton by tau-rometerization of His$^{64}$. In this study, our heteronuclear NMR approach to His$^{64}$ shows that both the N$_{\text{im}}$-H and N$_{\text{imid}}$-H tautomeric forms in equilibrium with an imidazolium ion are in the same popula-
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