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Water in enzyme reactions: biophysical aspects of hydration-dehydration processes

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Abstract. Water has been recognized as one of the major structuring factors in biological macromolecules. Indeed, water clusters influence many aspects of biological function, and the water-protein interaction has long been recognized as a major determinant of chain folding, conformational stability, internal dynamics, binding specificity and catalysis. I discuss here several themes arising from recent progress in understanding structural aspects of 'direct' and 'indirect' ligands in terms of enzyme-substrate interactions, and the role of water bridges in enzyme catalysis. The review also attempts to illuminate issues relating to efficiency, through solvent interactions associated with enzymic specificity, and versatility. Over the years, carbonic anhydrase (CA; carbonate hydrolyase, EC 4.2.1.1) has played a significant role in the continuing delineation of principles underlying the role of water in enzyme reactions. As a result of its pronounced catalytic power and robust constitution CA was transformed into a veritable 'laboratory' in which active site mechanisms were rigorously tested and explored.

Key words. Water; enzymes; biophysical chemistry; hydration-dehydration; carbonic anhydrase.

Introduction

Water-protein interactions have long been recognized as major determinants of chain dynamics and binding specificity of globular enzymes [1–3]. Water molecules and buffers not only interact with the protein surface but also occupy internal cavities and deep clefts [3]. Aqueous solutions of electrolytes and nonelectrolytes are finding increasing applications as solvent media, and many reports deal with the properties of water as well as of mixtures of water with a variety of cosolvents [4–9].

Although aqueous media are of considerable biochemical and biophysical importance there do not exist at present satisfactory explanations for some of the rather unusual properties which characterize such systems [3, 10]. This is not surprising, since our knowledge of molecular interactions in liquid water is limited in spite of the considerable amount of experimental data. An incomplete understanding of the structure and dynamics of liquid water, in its normal bulk phase, is a large stumbling block when we look at relatively simple inorganic and organic reactions and more complex ones involving biological macromolecules. We have an inadequate understanding of water-water hydrogen bonds and are still unable to handle, conceptually and theoretically, major structural characteristics of the liquid state [1–3, 10, 11].

Advances in our structural knowledge of biomolecule hydration have come not only from X-ray crystallography but also from neutron diffraction data [12, 13]. With the recent availability of both high-intensity X-ray and neutron beams, and their unique signal-to-background ratios, the frontiers of future experiments are expected to move significantly in the next few years. Consequently, recent workshops have been dedicated to in-depth discussions on present limits to spatial and time resolutions. Furthermore, there have been major advances in our understanding of the dynamics of water close to a macromolecule. The picture has been clarified through nuclear magnetic resonance (NMR) dispersion measurements of the kind discussed by Denisov and Halle [14] and the multidimensional nuclear Overhauser enhancement techniques developed by Wüthrich's school that allow individual water molecules to be observed directly via their double couplings with protein protons [15–17]. Physicochemical characterization of

strong hydrogen bonds also includes infrared spectroscopy (IR and FTIR) [18, 19] and dielectric relaxation, as well as thermal analysis calorimetry [20]. Note the discussion of some interesting issues by Finney [21]. Interpretation of the information provided by recent investigators has been reviewed [22, 23]. Many workers have underlined the need to use as many relevant techniques as possible in trying to unravel problems relating to hydration in terms of water clusters and water bridges. Nevertheless, much remains to be understood about hydrogen bonding both in general and in aqueous systems in particular. Various issues that have been raised remain to be resolved to the general satisfaction of researchers in biophysical chemistry. Currently available evidence indicates that the possibility for strong hydrogen bonds to exist in aqueous media deserves serious consideration [24]. Indeed, while the exact nature of symmetrical hydrogen bonds in water is still debated, there is reason to believe that hydrogen bonds in water vary in strength and that some may be stronger than heretofore expected [24].

Enzyme catalysis

We may recall the elegant work done in the 1980s which showed that certain enzymes became active at relatively low hydration levels. For example, Rupley et al. showed that lysozyme activity commenced in a hydrated powder at ca. 0.2 wt% water [25]. On the basis of these and similar results, it has become generally accepted that we can usefully study enzyme and solvent dynamics at relatively low hydration levels and so gain relevant information [26–30]. Related studies allowed extension of these ideas to water in crystals [31], to docking dynamics [32] and to trajectories in lysozyme [33, 34]. A particularly exciting recent report dealt with water involvement in the structural basis of the conversion of T4 lysozyme into a transglycosidase [35].

Ordinarily, it is a simple matter to detect the formation of enzyme-substrate complexes by examining the influence of substrate concentration on the rate of product formation. Information concerning the affinities of hydrolases and hydrolyases for substrate water has been much more difficult to obtain because the concentration of substrate water cannot be treated as an isolated variable. The interpretation of results in solvent mixtures is clouded by the fact that a mixture of water with a cosolute cannot be easily changed without altering viscosity and polarity. In 1989, carbonic anhydrase was found to show a reversible activity loss in the presence of organic viscogenic cosolutes that could be better understood in terms of a decrease in solvent water content than in terms of changing viscosity [36]. The hydrated form of the enzyme containing at least two more water molecules than the normal partially dehydrated form appeared to be superactive in regard to k_{cat} in CO_2 hydration and HCO_3^- dehydration. An elegant study performed on the hydrolytic activity of calf intestinal adenosine deaminase showed that the decrease in k_{cat}/K_m appears to be related to a diminished water content in the presence of each cosolvent [37]. Only a hydrated form of adenosine deaminase, containing at least nine more water molecules than the dehydrated form, appeared to be capable of binding substrates or competitive inhibitors (as shown by effects upon K_m). Other important papers from Wolfenden's laboratory on the role of solvent water in biochemical recognition and equilibria should also be consulted [38–40].

Carbonic anhydrase

Ever since its discovery, more than 66 years ago, the zinc metalloenzyme carbonic anhydrase (CA; carbonate hydro-lyase, EC 4.2.1.1) has occupied a prominent position in biophysical chemistry. Its catalysis of the reversible hydration of CO_2 is among the most efficient reactions known: $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$. Many valuable summaries pertaining to structure, catalytic mechanism, inhibition and genetics have appeared [41–52]. Not only is water a substrate in this reaction, but it also participates in a network of H bonds that mediate the rate-limiting proton-transfer step [42–52].

CA is a ubiquitous enzyme found in all animals and photosynthesizing organisms as well as in some non-photosynthetic bacteria. There exist three evolutionary unrelated CA families, which have been designated α-, β- and γ-CA [49]. All known carbonic anhydrases from mammalian systems are of the type α [49]. The first β-CA's were identified in spinach and pea chloroplasts [49, 53–56]. β-CA's have been shown to be present in several higher plants, monocotyledon as well as dicotyledons and in plants exhibiting C_3, as well as C_4, metabolism [53]. Valuable kinetic data are available for β-CA from spinach [54–57]. An interesting γ-CA that has been purified and characterized is produced by the methanogenic archaebacterium *Methanosarcina thermophila* (MAMT) when grown on acetate [58].

Fourteen genetically distinct forms of α-CA (known as isozymes I–XIV) have been identified in mammalian systems [49, 49a]. These isozymes have different tissue distributions as well as intracellular locations, and each contains a catalytically obligatory zinc ion. Isozymes CA I, II, III and VII are cytosolic. Although catalyzing a deceptively simple physicochemical process, the enzymatic hydration mechanism has been illuminated in stages involving an array of complimentary methods encompassing enzymology, biophysical chemistry and structural biochemistry. CA II is the most studied form;

it has an exceptionally high CO_2 hydration turnover rate of about 10^6 s^{-1} at pH 9 and 25 °C [59–62].

This isozyme has a wide tissue distribution and is found in many different organs and cell types [63]. Its deficiency syndrome has been associated with osteopetrosis (a congenital disease that prevents formation of bone marrow), renal tubular acidosis and cerebral calcification [63]. It is traditionally purified from red blood cells which contain about 2 mg of CA II per gram of hemoglobin. CA I is the major nonhemoglobin protein in human red blood cells, with an estimated average concentration of 12 mg per gram of hemoglobin. It is found in several other tissues, but is not as widely distributed as CA II. It is less active than CA II, with a maximal CO_2 hydration turnover of about 2×10^5 s^{-1} at 25 °C [59–64]. It appears to be a backup for CA II. A human CA I deficiency has been noted, but does not appear to be associated with any clinical symptoms. CA III is a low-activity isozyme with a maximal CO_2 hydration turnover rate of about 8×10^3 s^{-1} at 25 °C [65]. It is much less sensitive to inhibition by sulfonamides than other CA isozymes; it occurs in slow-twitch red muscle fiber where it is the major soluble protein and may possibly be involved in facilitating diffusion of CO_2 to the tissue capillaries.

Most studies of metal ion binding in CA have been performed on human and bovine isozyme II. The zinc ion can be removed by dialysis against certain chelating agents [66–68]. In mammalian CA II the catalytic zinc ion is firmly bound to three rigid histidine side chains near the apex of a 15-Å deep conical cavity with a strongly polarized water molecule completing the tetrahedral coordination. The resulting apoenzymes are inactive. Several other metal ions can bind to the apoenzyme, but only Co^{2+} yields a product with nativelike catalytic activity. The spectroscopic properties of Co^{2+}-substituted enzyme have been very useful in studies of the metal ion center [46, 69]. At pH 7, the dissociation constant of the zinc ion is extremely low, ca. 4 pM. The water-facilitated zinc ion dissociation rate constant has been estimated as 1.4×10^{-6} s^{-1} at pH 7 [70]. The chelating agent, dipicolinate, is believed to accelerate zinc dissociation by forming an intermediate protein—zinc—chelator ternary complex. The role of water in the displacement process is of some interest [67, 68]. One factor of importance for the firm binding of zinc in CA is the rigidity of the metal ion center [48, 71]. Indeed, the structure of apo CA II is almost identical to that of the holoenzyme, except that H_2O or H_3O^+ occupies the space of the zinc ion. Indeed, all attempts to alter the direct zinc ligands so far have resulted in drastic reductions of zinc ion affinity and catalytic activity [72, 73]. Under the conditions leading to CO_2 hydration, the solvent-donated zinc ligand is essentially in the OH^- form, whereas for HCO_3^- dehydration the solvent-donated zinc ligand is an H_2O molecule. The crystal structure of CA II reveals a network of water molecules in the catalytic pocket [48, 71], some of which are illustrated schematically in figure 1. Of particular interest for the catalytic events are the water molecules which are displaced by the incoming substrate or inhibitor. In addition there are two 'shuttle waters' providing a water bridge which is thought to relay protons between the zinc-aquo complex and His64 or between the zinc-hydroxo complex and $His64H^+$ (cf. fig. 2).

Residence times of structural water molecules in proteins can also be determined by the ^{17}O magnetic relaxation dispersion technique, where the longitudinal relaxation rate, R_1, of water ^{17}O resonance is measured as a function of resonance frequency [74]. A water molecule contributes to the relaxation dispersion only if its residence time, τ_w, is longer than the rotational correlation time, τ_r, of the protein (ca. 10 ns) but shorter than the intrinsic ^{17}O relaxation time (ca. 2 μs). Within the catalytic pocket, five water molecules satisfy the criteria for long residence times, supporting the earlier studies of Pocker and Janjic [36].

Most monovalent anions inhibit CA, but the apparent dissociation constants vary considerably [75]. Studies of the pH dependence of the inhibition and the effects of the optical spectrum of Co^{2+}-substituted enzyme led to the conclusion that anionic inhibitors bind to the metal ion and prevent the catalytic hydration [46]. In addition to a hydrogen-bonded system involving Glu106, Thr 199 (OH), and the zinc-bound H_2O or OH^-, the latter aquo and hydroxo components also form a hydrogen bond with another water molecule (called the 'deep water'), located in a hydrophobic pocket, and which in turn is bonded to the peptide NH of Thr199. These hydrogen-bonding interactions appear to have a decisive influence on the binding of inhibitors [48]. Distances between zinc ion and the closest inhibitor atom vary between 1.9 and 3.4 Å. Most anions lacking a protonated ligand do not remove the zinc-bound solvent molecule. They bind close to the metal ion displacing the 'deep water'. Some inhibitors appear to overcome the 'doorkeeper' function of Glu106/Thr99 system which tends to select protonated ligand atoms as replacements of the zinc-bound solvent molecule [48]. The divalent anion, SO_4^{2-}, acts as a very weak inhibitor, so much so that the effect of ionic strength on reaction rates can be tested with regard to the extended the Debye-Hückel equation [76].

From the results of kinetic studies and pH rate profiles of CO_2 hydration and HCO_3^- dehydration by CA II in H_2O and D_2O, it appears that two ionizing groups of pK_a near 7 are involved in catalysis [47, 59, 60, 62]. One catalytic group has been identified as the zinc-bound H_2O ionizing to OH^-. The other group shuttles a

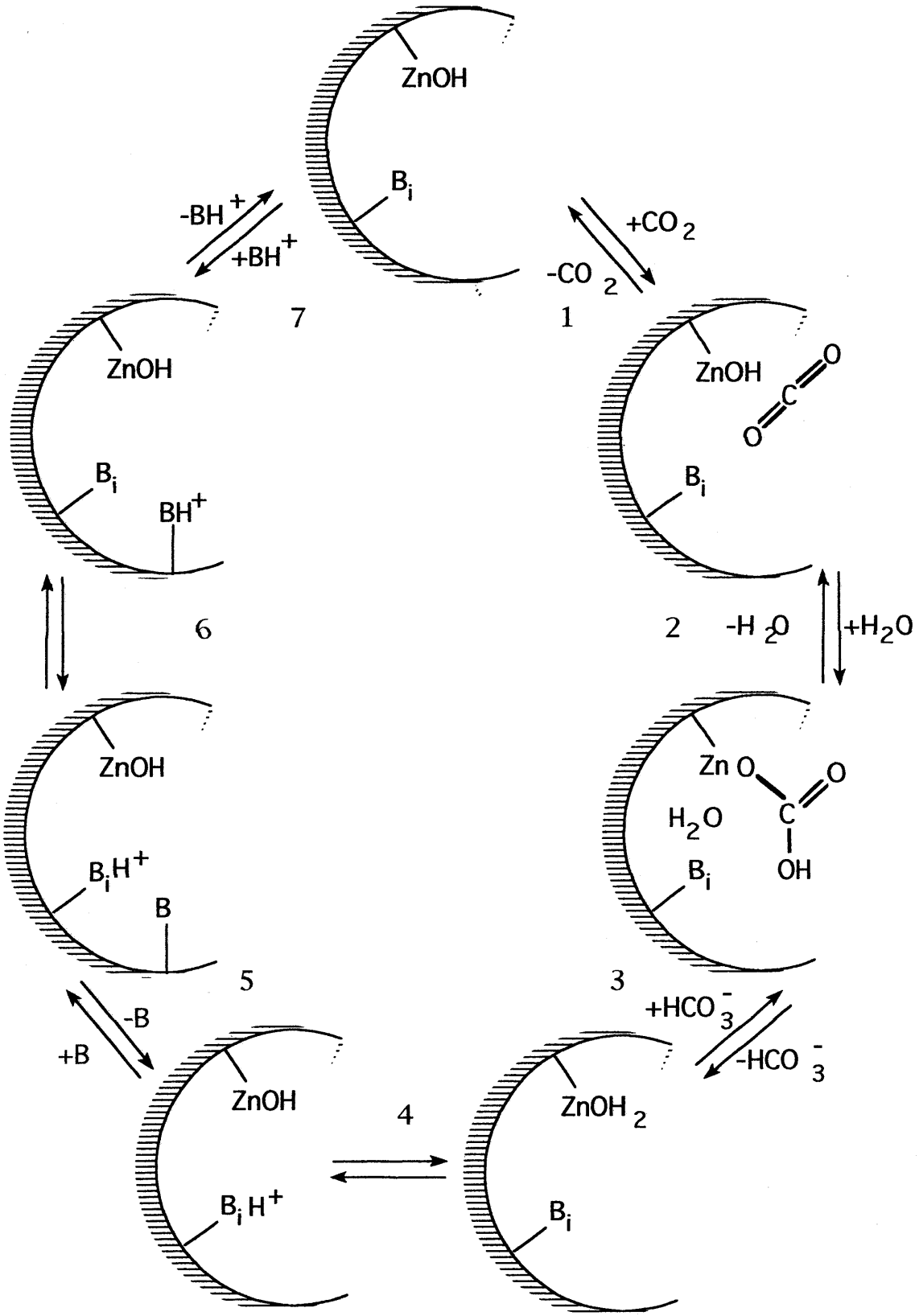

Figure 1. The catalytic cycle of carbonic anhydrase II. A schematic representation of the hydration of CO_2 (steps $1 \rightarrow 7$) and the dehydration of HCO_3^- (steps $7 \rightarrow 1$). B_i, internal proton shuttle group, identified as His-64; B, external buffer. Note: Two water molecules bridging the Zn-bound water and His-64, and several molecules bridging His-64 and external buffer, are omitted. Extra bonds between protein and catalytic zinc, and some H bonds involved in HCO_3^- and inhibitor (e.g. HSO_3^-) binding are also left out of this scheme. (Modified with permission from N., Janjic, Ph.D. thesis, University of Washington, 1988).

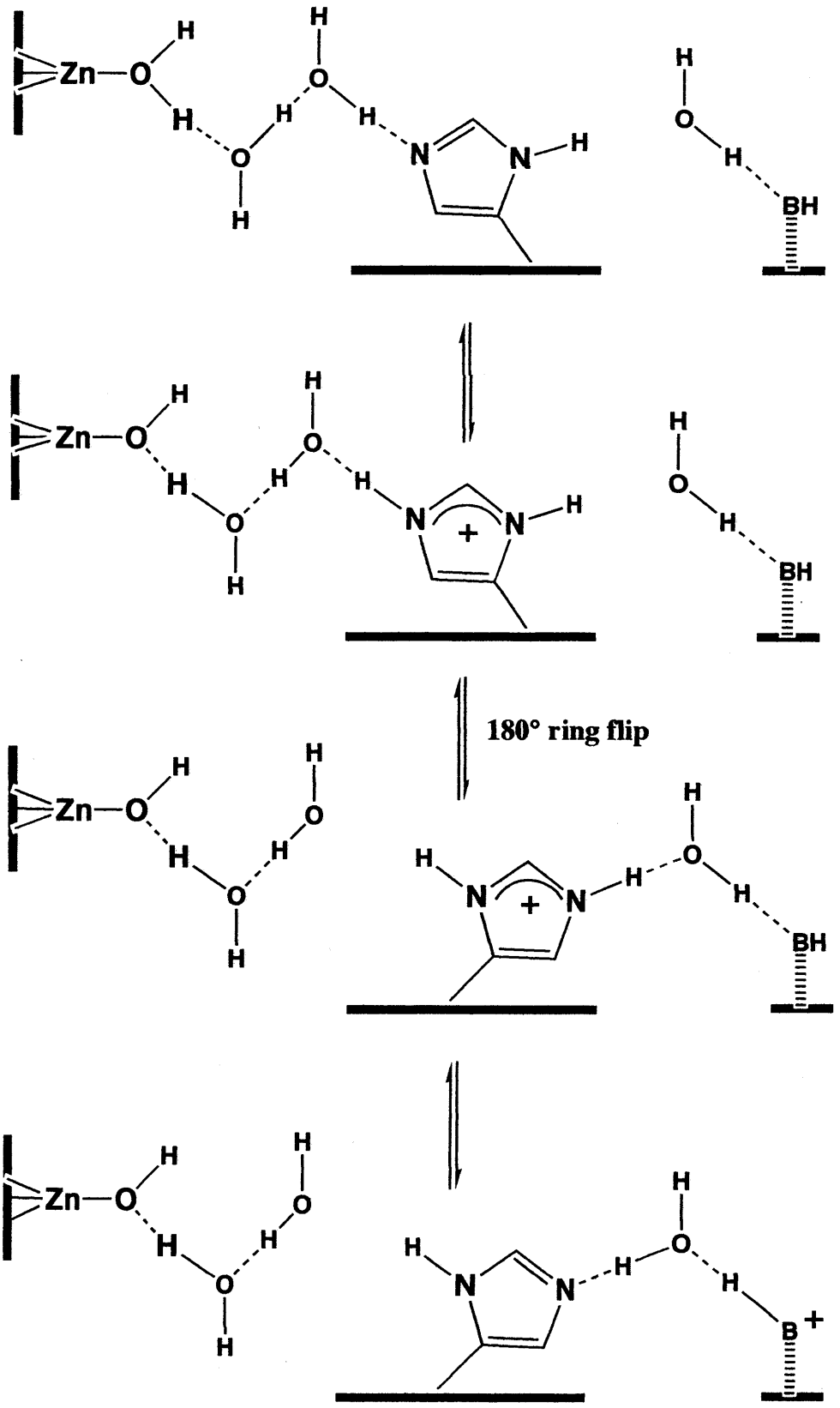

Figure 2. Protonation-deprotonation in the active site of carbonic anhydrase II: a proposed mechanism involving His-64 imidazole. Note: (i) Proton transfer between Zn-H_2O or Zn-OH^- and His-64-imid or His-64-imidH^+ may be rate-limiting; bridging water molecules are then of critical importance. (ii) At low buffer concentrations, H^+ transfer from His-64 to external buffer, BH (see fig. 1) becomes rate-limiting; other water molecules are then involved. (iii) In both cases deuterium isotope effects show that critical water bridges must be operative. (Modified with permission from Y. Pocker and N. Janjic N. (1989) J. Am. Chem. Soc. **111:** 731–733; copyright 1989 American Chemical Society).

proton between the catalytic site and buffer. This group was proposed to be His64. At low buffer concentration, the steady-state rates were found to depend on buffer concentration according to a pattern suggesting that buffer molecules abstract or donate a proton [77] via one or more water molecules acting as bridges with the proton shuttle group. Kinetic results also suggest that CO_2/HCO_3^- interconversion and proton transfer can be treated as two separate reactions (cf. fig. 1), as shown in equations 1 and 2.

$$EZnOH^- + CO_2 \leftrightarrow EZnOCO_2H^- \overset{+H_2O}{\underset{-H_2O}{\leftrightarrow}} EZnOH_2 + HCO_3^- \quad (1)$$

$$EZnOH_2 + B \leftrightarrow H^+EZnOH^- + B \leftrightarrow EZnOH^- + BH^+ \quad (2)$$

In the first stage (eq. 1) zinc-bound hydroxide participates in catalysis by converting CO_2 to HCO_3^-. In the second stage a proton transfer from enzyme to buffer B regenerates zinc-bound hydroxide while transforming B to BH^+. In CA II, stage (2) proceeds through an intramolecular proton transfer to an active-site residue His64, which subsequently releases the proton to the buffer (fig. 2). In the presence of sufficient buffer, the high rate of catalysis of CO_2 hydration ($\sim 10^6$ s^{-1} for isozyme II) appears to be determined by the intramolecular proton transfer between zinc-bound water and His64. In addition, zinc-bound water is also part of a hydrogen-bonded network involving additional water molecules which mediate proton shuttle to His64, prior to proton transfer to the buffer. Detailed kinetic studies of CA catalysis determined by stopped-flow spectrometry, in the CO_2 hydration direction, exhibit a maximal value of k_{cat}/K_m of 1.5×10^8 M^{-1} s^{-1} at high pH and a solvent isotope effect of 1.0 ± 0.1, indicating that in stage 1 there are no rate-contributing proton transfers and consistent with a direct nucleophilic attack of zinc-bound hydroxide on CO_2. When the buffer is 'ideal' and its pK_a exceeds 8, rate constants approaching 10^9 M^{-1} s^{-1} have been estimated [77, 78].

Chemical modification of CA II with acrolein is also consistent with the patterns observed in stage 1 of this enzymatic catalysis [79]. An important idea concerning the mechanism of action of CA is that the coordinated water is deprotonated prior to the reaction with CO_2. While theoretically attractive, this proposal has not yet been fully demonstrated. Such a demonstration would require, for example, a direct comparison of reaction efficiencies using a pair of structurally characterized aquo and hydroxo complexes of zinc with coordination environments that closely mimicked the active site of CA. Nevertheless, the pH dependence of certain macrocyclic zinc complexes suggests that reactions with CO_2 proceed via initial deprotonation of zinc-bound water [51]. The extraordinarily efficient catalysis of CO_2 hydration accomplished by CA II requires that the product, HCO_3^-, dissociate rapidly from the active site. Accordingly, the binding of HCO_3^- is rather weak, and consequently it has not so far been possible to obtain a crystal structure of the complex with unmodified enzyme. The conformational flexibility of His64 has implications for its function as a proton shuttle (fig. 2). Its capacity to shuttle protons efficiently is also associated with possession of a pK_a of 7, the ideal value for a dual function as proton donor and acceptor [77, 79, 80]. Our results have suggested that His64 has not only an optimal location but also an environment that permits water bridges appropriate for efficient general acid-base properties. The rate constant indeed depends upon a difference in pK_a between the buffer and the His64 residue. Brønsted plots corresponding to titration curves have been obtained for CO_2 hydration and for HCO_3^- dehydration. In wild-type CA II, the apparent rate constant for proton transfer from His64 to the buffer base (stage 2, eq. 2, and fig. 2) is not completely independent of the buffer structure. Interestingly, two buffer families that differ in their associated catalytic rates by a factor of 3, with the same pK_a, both for CO_2 hydration and for HCO_3^- dehydration, have been identified. The two families apparently differ in the number of water molecules that bridge between His64 and the buffer. The different rates may be associated with a difference of one H_2O in the relay of protons from $HisH^+64$ to buffer B and from BH^+ to His64. For the hydration of CO_2 catalyzed by CA II, it was shown that the solvent hydrogen isotope effect, $k_{cat(H_2O)}/k_{cat(D_2O)}$ was close to 3.8 for the high pH (alkaline) region of the hydration. However, the solvent isotope effect for the ratio $k_{cat}^{CO_2}/K_m^{CO_2}$ is near unity.

CA already had a particular fascination for investigators in biophysical chemistry in 1958 [44]. The early X-ray work gave a strong suggestion that a more or less organized network of up to nine water molecules was to be seen, but without further structural refinement, the possibility of artifacts forbade any definite conclusions concerning such a network. In almost all CA's, there are numerous water sites clustered over the protein surface, especially over its more polar regions. These water molecules, of course, are not static; they are undoubtedly undergoing rapid rotation, and exchanging places rapidly with neighboring water molecules. However, many sites occupied by water are commonly well defined in the most highly refined structures. The role of water in CA catalysis [48, 81–82a] and inhibition [48, 71] is so central that a

more complete knowledge of the state of water in the active site cavity would be of great interest. The essential zinc lies at the bottom of a cavity about 15 Å in depth; its ligands are three histidine residues, numbers 94, 96 and 119, and a water molecule. The active site cavity is lined with about 50 residues, 25 of which have side chains in the surface of the site. The amino acid side chains on one side of the cavity are predominantly hydrophilic and on the other side hydrophobic. It is attractive to assume that the CO_2 molecule enters the active site via the hydrophobic region and the HCO_3^- anion leaves the active site via the hydrophilic region. By the principle of microscopic reversibility, in dehydration the bicarbonate anion enters the active site via the hydrophilic region and CO_2 leaves via the hydrophobic region.

Catalytic versatility

Before 1964, CA was generally believed to catalyze only one process, namely CO_2 hydration and HCO_3^- dehydration. As originally discovered by Pocker and Meany [83], the enzyme also catalyzes the reversible hydration of acetaldehyde. In later work, Pocker and Meany demonstrated catalysis of the hydration of pyridine carboxaldehydes, and pyruvic acid and its esters [84–86]. Steric effects upon the hydration of aliphatic aldehydes were studied and characterized by Pocker and Dickerson [87]. A series of kinetic and mechanistic experiments upon the enzyme's esterase activities were conducted in an attempt to locate the major binding regions of CA II [88–94]. The binding sites were targeted with competitive inhibitors that often interacted with more than one site, thereby providing some specificity for the desired target [89]. Pocker and Sarkanen have reviewed the structure and catalytic properties of CA II and list eight different classes of compounds that are hydrated and/or hydrolyzed by this enzyme [42]. The importance of water in these processes is underlined when one realizes that the experimental binding of any substrate to the enzyme occurs in the presence of 55 M of a potentially competing ligand, namely, H_2O [42, 95–98]. Some interpretations of binding energies tend to ignore the presence of, and possible competition from, bound solvent. The study of catalytic versatility is, inter alia, an approach to both finding sites and optimizing ligands for these sites in CA. This approach not only recognizes hydrophobic regions but also the possible roles of bound solvent. It allows us to assess some of these roles from detailed kinetic and mechanistic studies [88–98].

Catalytic versatility can indeed be used to map qualitatively the major binding clefts of CA, and to find regions, including the active site cleft, that are sufficiently 'sticky' to provide a binding site. Enzyme-substrate and enzyme-inhibitor interactions are not random, but rather specific, usually involving the participation of specific residues from the enzyme interacting with specific regions of the substrate or inhibitor directly [99, 100]. Many direct interactions with substrates and inhibitors require that the water be displaced first [42, 88–98, 101]. Clearly, the nature of its interaction with the enzyme must be understood before any more detailed discussion is attempted.

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