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The Catalytic Mechanism of Carbonic Anhydrase

(metalloenzymes/enzyme mechanism/hydration of CO_2)

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ABSTRACT **It is shown that an "inverse" relationship between the pH dependencies of the rates of hydration of CO_2 and dehydration of HCO_3^- by carbonic anhydrase (EC 4.2.1.1) is a direct consequence of the thermodynamic equilibrium between CO_2 and HCO_3^- and independent of any assumptions about the catalytic mechanism. It is further shown that proposed mechanisms for carbonic anhydrase involving HCO_3^- as the substrate in the dehydration reaction and a proton transfer reaction, $EH^+ \rightleftharpoons E + H^+$, as an obligatory step during catalysis obey the rule of microscopic reversibility. This includes mechanisms in which the proton dissociation is from a zinc-coordinated water molecule. Such mechanisms can be in accord with the observed rapid turnover rates of the enzyme, since rapid proton exchange can occur with the buffer components, $EH^+ + B \rightleftharpoons E + BH^+$. Mechanisms in which H_2CO_3 is the substrate in dehydration avoid the proton-transfer step, but require that H_2CO_3 combines with enzyme more rapidly than in a diffusion-controlled reaction. Physicochemical evidence for and against a zinc-hydroxide mechanism is discussed.**

The zinc metalloenzyme carbonic anhydrase (EC 4.2.1.1) has been investigated by most techniques available for investigation of structure–function relationships (1–4). The crystal structure of the human C isoenzyme has been completed to a resolution of 2 Å (2, 3). Yet there is little direct evidence identifying the catalytic and substrate binding groups in the enzyme. It is known that an ionizing group on the enzyme with a pK_a near 7 is involved in catalysis, and that the titration of this group results in changes in the immediate environment of the metal ion. As to the identity of this group, the two major proposals are that its basic form represents (*a*) a zinc-coordinated hydroxide ion and (*b*) a basic amino-acid side chain, e.g., imidazole, directly or indirectly linked to the metal ion (1, 2, 4). Recently, Koenig and Brown (5) stated that proposed mechanisms involving a zinc-hydroxide as the active group violate the rule of microscopic reversibility, and they postulated that H_2CO_3 must be the appropriate substrate.

As shown in the present paper, the zinc-hydroxide mechanism does not violate microscopic reversibility and, in fact, cannot be ruled out on kinetic or physicochemical grounds. Present data from physicochemical techniques in solution, chemical modification of the enzyme, and x-ray diffraction are analyzed as they pertain to the various proposals for the mechanism of action of carbonic anhydrase.

Reversible hydration of CO_2

Eq. 1 summarizes the interconversions between the molecular species of the carbonic acid system (neglecting CO_3^{2-} and the nonenzymic reaction of CO_2 with OH^-),

$$\begin{array}{ccc} CO_2 + H_2O & \overset{K_1}{\rightleftharpoons} & H^+ + HCO_3^-, \\ K_h \backslash\backslash & & //K_{H_2CO_3} \\ & H_2CO_3 & \end{array} \qquad [1]$$

where the equilibrium constants have the following values at 25° (6): $K_h = 0.0026$; $K_1 = 10^{-6.35}$ M; $K_{H_2CO_3} = 10^{-3.77}$ M. The reversible hydration of CO_2 is a relatively slow reaction in the absence of a catalyst; the rate constant for hydration equals about 3.5×10^{-2} sec^{-1}.

The enzyme-catalyzed reaction

The most active carbonic anhydrases, the human C and the bovine enzymes, catalyze both hydration of CO_2 and dehydration in the neutral pH range with rate constants between 10^5 and 10^6 sec^{-1}. This rapid turnover has posed some problems, since any of the proposed mechanisms for the enzyme appears to require that second-order steps involving either H^+ or H_2CO_3 have to be faster than diffusion-controlled rates in simple model systems.

The pH dependence of the carbonic anhydrase-catalyzed reactions can be summarized by the statement that the activity depends on a group in the enzyme with a pK_a of 7, the basic form, E, being required for hydration of CO_2 while the acid form, EH^+, appears to be required for dehydration of HCO_3^- (1, 6, 7). Such an "inverse" relation between the pH-rate profiles for hydration and dehydration (Fig. 1) is a direct consequence of the pH dependence of the equilibria in Eq. 1, and the statement does not imply that hydration and dehydration follow different pathways, a violation of the principle of microscopic reversibility.

If the neutral H_2CO_3 molecule is the substrate in dehydration it must combine with E, and formally the pH-rate profile would be expected to be the same as that for hydration. However, since the only experimentally obtainable solution is one in which HCO_3^- is the dominating species, and the H_2CO_3 concentration varies inversely with pH, the pH-rate profile of dehydration will be observed to be the inverse of that for hydration regardless of which form, HCO_3^- or H_2CO_3, is the true substrate. All proposed mechanisms involving HCO_3^- as substrate are compatible with the principle of microscopic reversibility, which requires that the neutral substrate in the hydration reaction combines with E, while in dehydration the anionic form of the substrate combines with EH^+. E may be E–Zn–$^-$OH or it may involve the basic form of any other

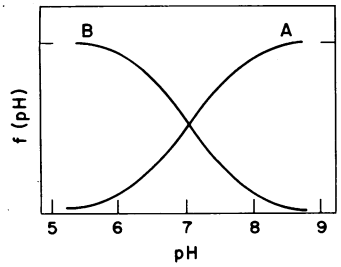

FIG. 1. (*A*) Functions following curve *A*: (*1*) pH-rate profile for CO_2 hydration (k_{cat}); (*2*) pH-rate profile for dehydration of H_2CO_3 (k_{cat}/K_m); (*3*) pH-rate profile for esterase activity ($k_{enz} = k_{cat}/K_m$); (*4*) pH dependence of relaxation enhancement of solvent protons by the Co(II) and Mn(II) enzymes; (*5*) pH dependence for OH^- release (or H^+ uptake) by the enzyme on anion or sulfonamide binding; (*6*) pH dependence for formation of basic forms of absorption or circular dichroism spectra of Co(II) enzyme. (A_2) Functions following sigmoid curve to high pH but displaced from *A* toward more alkaline pH: (*1*) pH-rate profile for hydration in the presence of anions; (*2*) pH rate profile for hydration by chemically modified enzyme in which histidyl residues near active site have been modified; (*3*) pH dependence for change of absorption spectrum of Co(II) enzyme in the presence of anions; magnitude of shift depends on binding affinity of anion. (*B*) Functions following curve *B*: (*1*) pH-rate profile for dehydration of HCO_3^- (k_{cat}/K_m); (*2*) pH dependence of anion binding affinity ($1/K_i$); (*3*) pH dependency of line broadening of ^{35}Cl resonance by Zn(II) enzyme (17).

ionizable group. The question of whether the required proton transfers in such mechanisms could be rapid enough to be compatible with the experimentally observed rate constants is a separate question not related to microscopic reversibility.

Steady-state kinetics of carbonic anhydrase

The enzyme-catalyzed hydration of CO_2 has been studied in detail by Kernohan (6) and by Khalifah (8), while the reverse reaction studied by Kernohan (6) and by Magid (9) is less fully documented. In these studies Michaelis–Menten kinetics have been consistently obtained, and the parameters obey the Haldane relation, Eq. **2**. An equivalent Haldane relation may be written

$$(k_{cat}^{CO_2}/K_m^{CO_2})(K_m^{HCO_3^-}/k_{cat}^{HCO_3^-}) = K_1/[H^+], \qquad [2]$$

considering H_2CO_3 as substrate.

While $K_m^{CO_2}$ is independent of pH, $k_{cat}^{CO_2}$ varies in a sigmoidal fashion, reaching a maximal value at alkaline pH. The pH-$k_{cat}^{CO_2}$ profiles for the bovine and human C enzymes correspond to simple titration curves having pK_a near 7 (curve *A* in Fig. 1). In the dehydration reaction, $k_{cat}^{HCO_3^-}/K_m^{HCO_3^-}$ varies as curve *B* in Fig. 1, reaching a maximal value at acid pH with the same apparent pK_a as the hydration reaction. The work of Magid (9) suggests that $k_{cat}^{HCO_3^-}$ is independent of pH while $K_m^{HCO_3^-}$ varies with pH. The experimental observation that the Michaelis–Menten parameters obey the Haldane relation of Eq. **2** is independent of any assumptions about the rate of proton transfers that may be involved in the mechanism or, indeed, any other assumptions about the mechanism.

Since the substrates have no pK_a values in the pH range covered by the kinetic studies there must be a titratable group on the enzyme participating in activity. A kinetic mechanism in accordance with all observations is summarized in Eq. **3**, where H_2O has been omitted for convenience.

$$\begin{array}{ccccc} & \text{Step 1} & & \text{Step 2} & \text{Step 3} \\ E + CO_2 & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & E{-}CO_2 & \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} & EH^+{-}HCO_3^- \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EH^+ \\ k_4 \downarrow\uparrow k_{-4}[H^+] & & k'_4 \downarrow\uparrow k'_{-4}[H^+] & & \\ EH^+ + CO_2 & \underset{k'_{-1}}{\overset{k'_1}{\rightleftharpoons}} & EH^+{-}CO_2 & & \end{array}$$

$$\text{Step 4}$$

$$+ HCO_3^- \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E + H^+ + HCO_3^-. \qquad [3]$$

In Eq. **3**, E and EH^+ denote the basic and acidic forms of the enzyme, respectively. This scheme, originally proposed by DeVoe and Kistiakowsky (10) and recently applied by Khalifah and Edsall (7) to summarize various pH effects on the kinetics of native and chemically modified carbonic anhydrases, constitutes a complete and reversible, catalytic cycle. If we let the cycle begin with Step 1, EH^+ is produced as an intermediate, but E is regenerated in Step 4. The pH-independent rate constants are related by Eq. **4**.

$$(k_1k_2k_3k_4)/(k_{-1}k_{-2}k_{-3}k_{-4}) = [(k_1k_2k_3)/(k_{-1}k_{-2}k_{-3})]K_a = K_1 \qquad [4]$$

where K_a is the acid dissociation constant of the activity-linked group on the enzyme. It is misleading to state (5) that K_1 depends on K_a. Eq. **4** *does* imply that a variation in K_a (for example from one isoenzyme to another) must be coupled to corresponding changes in $k_1k_2k_3/k_{-1}k_{-2}k_{-3}$. Eq. **4** is independent of any assumptions as to the rates of proton transfer.

Since $k_{cat}^{CO_2}$ approaches 10^6 sec^{-1} at neutral pH, it is required that k_4 has at least this formal value. With $K_a = 10^{-7}$ M, this implies that $k_{-4} \geqslant 10^{13}$ M^{-1} sec^{-1}, exceeding values for diffusion-controlled reactions with H_3O^+ by a factor of 10^2–10^3 (11). Before discussing alternative mechanisms involving H_2CO_3 as substrate, we shall consider the role of the buffer in proton exchange reactions.

Buffer-mediated proton transfer

If H_2O or OH^- are the proton acceptors in Step 4 of the hydration reaction (Eq. **3**) and H_2O or H_3O^+ are the proton donors in the reverse reaction, the pseudo first-order rate constants for the proton transfers between enzyme and medium would not be expected to be greater than 10^3–10^4 sec^{-1} at neutral pH (1, 8, 11). However, if a better donor–acceptor system than water is present, much more rapid proton exchange can take place at neutral pH. In all real experimental situations (and *in vivo*) the enzyme solution is buffered by a low-molecular-weight acid–base system (BH^+/B) or by groups on protein molecules. The proton exchange step in Eq. **3** may actually be written

$$EH^+ + B \rightleftharpoons E + BH^+. \qquad [5]$$

At neutral pH the most rapid pathway for proton transfer between two acid–base pairs is through a proton exchange between the acid of one pair and the base of the other pair, perhaps mediated by one or more intermediate water molecules. The proton exchange between imidazole (ImH^+/Im) and *p*-nitrophenol (NP–OH/NP–O^-) may serve as a model system,

$$Im + NP{-}OH \rightleftharpoons ImH^+ + NP{-}O^-. \qquad [6]$$

Both pairs have pK_a values near 7, and the rate constants in both directions have values near 5×10^8 M^{-1} sec^{-1} (see

Table 5 of ref. 11). Similar values may apply to the rate constants of Eq. **5** when a buffer system having a pK_a near 7 is used. If we assume a relatively low buffer concentration, [B] = [BH^+] = 20 mM, and second-order rate constants in Eq. **5** of $5 \times 10^8\ M^{-1}\ sec^{-1}$, the apparent first-order rate constants for the deprotonation of EH^+ and the protonation of E will both be $10^7\ sec^{-1}$ at neutral pH.

It can be argued that the catalytic group of the enzyme, being situated near the bottom of a deep cavity in the molecule (3), may not exchange protons with the medium as rapidly as in the model systems. On the other hand, it can also be argued that the function of the ice-like cluster of water molecules filling the active-site pocket (3) may be to facilitate such a proton exchange. The transfer of a proton (to an acceptor) through such a system of hydrogen-bonded water molecules could occur within less than 10^{-7} sec (11). The conclusion from these considerations must be that the mechanism of Eq. **3** may adequately describe the kinetics of carbonic anhydrase. Furthermore, it should be possible to test experimentally whether proton transfers between enzyme and medium can limit the rate of the catalyzed reaction under certain conditions.

H_2CO_3 as substrate in the dehydration reaction

Let us examine the alternative scheme where H_2CO_3 rather than HCO_3^- is the substrate in the dehydration reaction. Steps 2, 3, and 4 of Eq. **3** are replaced by

$$\underset{\text{Step 2'}}{E\text{–}CO_2 \underset{k'_{-2}}{\overset{k'_2}{\rightleftharpoons}} E\text{–}H_2CO_3} \underset{\text{Step 3'}}{\overset{k'_3}{\rightleftharpoons} E + H_2CO_3}, \quad E + H^+ \rightleftharpoons EH^+. \qquad [7]$$

Such a scheme was most recently proposed by Koenig and Brown (5), and it is compatible with the observed kinetics provided that the various steps can proceed at the required rates. The rate of proton exchange between E and EH^+ is no problem, since this step is not directly on the catalytic pathway. However, above pH 5, the concentration of H_2CO_3 must be small compared to that of HCO_3^- and the scheme has generally been rejected on the grounds that the formal value of k'_{-3} would have to be greater than $10^{10}\ M^{-1}\ sec^{-1}$ to comply with the observed kinetic parameters (1, 2, 10). This value is greater by at least a factor of 10 than rate constants for diffusion-controlled reactions between small molecules (other than H^+ and OH^-) and proteins (11). Koenig and Brown (5) recognized that the formation of H_2CO_3 by protonation of bulk HCO_3^- may lead to an increase of the apparent rate of diffusion of H_2CO_3 to the active site even at neutral pH. However, they calculated that this effect is small, and they had to postulate that the whole surface of the enzyme molecule acts as a sink for H_2CO_3, which is transported intact to the active site. While an analogous model probably applies to the rate of crystal growth, such a "surface diffusion" would not appear to operate in the reaction of a substrate with a specific site on an enzyme. Strictly speaking, the above discussion applies to a rate-limiting, bimolecular step in the second-order reaction between enzyme and substrate.

There are other arguments against E–H_2CO_3 being a significant intermediate in catalysis (12). The enzyme-substrate complex with the H_2CO_3 molecule would have to be much more stabilized by interacting with the enzyme than the complex with HCO_3^-, or an EH^+–HCO_3^- complex would be rapidly formed by intramolecular proton transfer. However, a characteristic property of the active site is its specific interaction with monovalent anions (1, 2). The esterase reaction of carbonic anhydrase is inhibited by HCO_3^- with a K_i of the same magnitude as obtained for other anions, e.g., Br^- and NO_3^- (13). The spectral effects of HCO_3^- on the cobalt enzyme are not very different from those of Cl^- (4, 14). Thus it seems likely that the substrate is present as a bound HCO_3^- ion in the enzymic active site.

The molecular mechanism of carbonic anhydrase

The simplest molecular mechanism proposed for carbonic anhydrase suggests that the activity-linked pK_a represents the dissociation of a Zn(II)-coordinated H_2O molecule (1, 2, 7, 14).

$$E\text{–}Zn(II)\text{–}OH_2 \rightleftharpoons E\text{–}Zn(II)\text{–}OH^- + H^+. \qquad [8]$$

Alternatively, the pK_a may be assigned to an ionizable amino-acid side chain in the active site, e.g., an imidazole group (2, 13). The mass of structural and functional information available on the enzyme can be used to support one or the other mechanism. However, no available evidence allows a definite choice, and we review briefly the information and its possible interpretation.

The x-ray data at pH 8.5 show the Zn(II) to be liganded to the side chains of three amino-acid residues (3). At least two of these, and perhaps the third, are histidyl residues (2, 3, 15). There is an open coordination site occupied by solvent (3). Thus the observed Zn(II) coordination in the enzyme is compatible with mechanisms involving coordinated OH^- or H_2O in the reaction. No additional, ionizing residue has been observed in the active site close enough to the Zn(II) to suggest that it participates in the reaction. Aside from the metal ligands, the only histidine in the active site retained in both the human B and C enzymes is 6 Å from the Zn(II) in the C structure (histidine 63). Carboxyketoethylation of histidine 63 in the human C enzyme with bromopyruvate (2, 16) yields a modified enzyme that has a maximal $k_{cat}^{CO_2}$ of about $3 \times 10^5\ sec^{-1}$ compared to $1.4 \times 10^6\ sec^{-1}$ for the native enzyme (7, 8). The product still shows a sigmoid pH-rate profile. Thus, unless this modified histidyl has free rotation such that the unmodified imidazole nitrogen can participate in the reaction, this would not seem to be the enzyme group participating directly in the reaction.

The solution data that appear to reflect the ionization of the group on the enzyme involved in activity are summarized at the bottom of Fig. 1 in terms of the pH dependencies of the various measurements. The visible absorption spectrum of the active Co(II) enzyme shows two distinctive forms of the Co(II), one at low pH and one at high pH (1, 4). Spectrophotometric titrations show that only two species are involved, and the pH function describing the transformations of the acid into the alkaline form follows curve *A* in Fig. 1. A reasonable explanation for this finding is that the group undergoing ionization is directly coordinated to the Co(II) ion and is the same as that involved in activity, compatible with a $Co(II)\text{–}OH_2 \rightleftharpoons Co(II)\text{–}OH^- + H^+$ equilibrium. Alternatively, the ionization of an adjacent group involved in activity might be influencing the coordination sphere of the metal ion.

Mounting evidence (x-ray, optical spectra, and electron spin resonance spectra) shows that the anionic inhibitors and sulfonamides react with carbonic anhydrase by direct coordination to the metal ion (1–3, 15). Measurements of proton equilibria accompanying anion and sulfonamide bind-

ing show that binding to the alkaline form of the Zn(II) or Co(II) enzymes is accompanied by OH^- release (or its equivalent, H^+ uptake) by the enzyme and that the pH function controlling this release is identical to that influencing activity (1, 14). A straightforward explanation of this finding is that the metal-coordinated group has an ionization curve corresponding to this pH function. The metal ion-hydroxide model implies that all monovalent anions *including* OH^- have unusually high affinities for the active site. If the alternative, H^+ uptake by a protein group, is chosen, one must postulate that all monovalent anions *except* OH^- have high affinities. In addition one must postulate that upon anion binding an adjacent group shifts its pK_a upward by several pH units, or that anion coordination involves the neutral species (e.g., HCl), which seems highly unlikely. Alternatively, it could be postulated that the anion displaces a protein ligand that takes up a proton. There is no evidence from the crystal structure of the inhibitor complexes or from electron spin resonance data that the latter occurs (2, 3, 15). These apparent similarities in the binding of OH^- and other monovalent anions would appear to be some of the strongest evidence supporting the OH^- hypothesis.

One finding not predicted by the Zn(II)–OH^- model is the pH dependence of solvent ^1H relaxation in the presence of the Co(II) or Mn(II) enzymes. It follows curve *A* in Fig. 1, sigmoid to high pH, with the acid form of both the Co(II) and Mn(II) enzymes producing little or no relaxation of solvent protons (5, 18). Koenig and Brown (5) and Lanir *et al.* (18) have used this finding to argue that a coordinated H_2O molecule cannot be present at low pH. Although this might be considered the most straightforward explanation of the nuclear magnetic resonance data, the mechanisms responsible for the presence of a solvent-occupied coordination site at high pH but its absence at low pH even when a monodentate site for weakly binding anions is present at low pH are not clear from present data available on the enzyme. The nuclear magnetic resonance calculations presume simple ligand exchange mechanisms based on model systems that may not be applicable to the enzyme. For example, the solvent relaxation at high pH might depend on rapid proton exchange with Co(II)–OH^- and not involve ligand exchange at all, while the low pH form may be limited to slow proton exchange with Co(II)–OH_2 or ligand exchange [Co(II)–O bond breaking]. Additional experiments with $H_2{}^{17}O$ might shed more light on this problem.

Conclusions

We conclude that the mechanism shown in Eq. **3** is an adequate model for the catalytic action of carbonic anhydrase. We have shown that this mechanism obeys the rule of microscopic reversibility and that the proton exchange between the active site and the medium can readily occur at the required rates when the buffer acts as a donor–acceptor system. Most evidence seems to favor the assignment of the pK_a associated with activity to the ionization of a Zn(II)-coordinated water molecule, although alternative assignments cannot be ruled out by present data.

One essential step in the hydration of CO_2 must be the rapid splitting of a H_2O molecule into H^+ and OH^- in conjunction with the donation of OH^- to the carbon atom of CO_2. This step (Step 2 in Eq. **3** or Step 2′ in Eq. **7**) poses some interesting kinetic problems (1, 8, 11) not discussed in this paper. It would seem that, regardless of the assignment of the activity-linked pK_a, the metal ion must play an important role in this process, since the metal is required for activity and the ionization on the enzyme controlling activity appears closely coupled to the metal ion.

Our interpretation of the role of the buffer, in catalysis by carbonic anhydrase, has been proposed independently by Khalifah (19).

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