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Carbon Dioxide Hydration Activity of Carbonic Anhydrase: Paradoxical Consequences of the Unusually Rapid Catalysis*

(enzymes/mechanism/acid-base catalysis/diffusion limit)

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ABSTRACT **The kinetic parameters for carbon dioxide hydration catalysis by carbonic anhydrase (EC 4.2.1.1) present an apparent paradox. The assumption of H_2CO_3 as the hydration product requires the rate of recombination of H_2CO_3 with enzyme to be faster than the diffusion limit. The alternative assumption of HCO_3^- as the product of hydration likewise requires active-site ionization rates to exceed the diffusion limit. We previously postulated the presence of special means for rapid active-site ionization. It is shown here that when proton transfer between enzyme and buffer species is taken into account, there is no need to invoke rates exceeding the diffusion limit. Bicarbonate ion thus appears as the most probable hydration product and dehydration substrate.**

The carbonic anhydrases (EC 4.2.1.1) of high and low specific activity from mammalian species are zinc metalloenzymes that catalyze the reversible hydration of carbon dioxide. They are remarkable in that they possess some of the highest measured turnover rates among all enzymes, but especially among those using acid–base catalysis (1–5). The interpretation of their kinetics has posed a dilemma, since rates exceeding the diffusion limit have invariably had to be postulated, as discussed below. In particular, the definite identification of the product of hydration (either H_2CO_3 or HCO_3^-, see *Scheme I*) has depended on the resolution of these kinetic

$$\begin{array}{ccccc} H^+ & + & HCO_3^- & \rightleftharpoons & H_2CO_3 \\ & & \searrow & & \swarrow \\ & & CO_2 & + & H_2O \end{array}$$

Scheme I

difficulties and, consequently, has remained a somewhat open question.

I wish to point out in this paper that the observed kinetics can be understood without having to invoke rates that exceed the diffusion limit, provided that the previously neglected contribution of the buffer to the ionization processes in the active site is taken into account. Such considerations may also apply to other enzymes of very high turnover rates for which the reaction mechanism involves similar acid–base catalysis.

H_2CO_3 AS PRODUCT

The very rapid rate of ionization of H_2CO_3 to HCO_3^- and the inability to extend the enzymatic studies to pH below the pK of the above ionization ($pK_{H_2CO_3} = 3.8$) make it difficult to directly ascertain whether H_2CO_3 or HCO_3^- is the product of CO_2 hydration. However, the observed enzyme kinetics and steady-state kinetic theory combine to shed some light on this problem.

Michaelis–Menten parameters of one-substrate, one-product reactions provide lower limits for all the rate constants of an enzymatic reaction, regardless of the number of intermediates involved (6). If we denote the substrate by S and the product (substrate for reverse reaction) by P, then the ratio k_{cat}/K_m determined in the forward direction (S to P) provides a lower limit for the second-order rate of recombination of S with enzyme. Similarly, the ratio of the Michaelis–Menten parameters determined for the reverse reaction (P to S), k'_{cat}/K'_m, constitutes a lower limit for the rate of recombination of P with enzyme.‡ The dehydration kinetic parameters (k'_{cat}/K'_m) are not yet available under the conditions of the hydration studies (5), but one can obtain the ratio k'_{cat}/K'_m by use of the Haldane relation that applies for carbonic anhydrase (2–4). Thus if we assume that H_2CO_3 is the hydration product, we can write,

$$\frac{k_{cat}/K_m}{k'_{cat}/K'_m} = \frac{[H_2CO_3]}{[CO_2]} = K_h \qquad [1]$$

where K_h is the equilibrium constant for the reaction: $CO_2 + H_2O \rightleftharpoons H_2CO_3$. The recent measurements on the high activity human C carbonic anhydrase (5) yield a maximal value for k_{cat}/K_m at high pH equal to $10^{8.13}\ M^{-1}\ sec^{-1}$ at 25° and 0.2 ionic strength. Under these conditions, K_h can be estimated to be $10^{-2.54}$ if we assume that the true first ionization of carbonic acid has the same ionic strength dependence as acetic acid (see ref. 7). Thus, the maximal value of k'_{cat}/K'_m at high pH is given by,

$$k'_{cat}/K'_m = (k_{cat}/K_m)/K_h = 10^{10.67} = 4.7 \times 10^{10}\ M^{-1}\ sec^{-1}$$

The rate of H_2CO_3 combination with carbonic anhydrase must then equal or exceed this limit. However, as first recog-

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‡ A more exact definition, of course, is that $(k_{cat} + k'_{cat})/K_m$ and $(k_{cat} + k'_{cat})/K_m'$ constitute lower rate limits for the recombination of S and P, respectively (6).

nized by DeVoe and Kistiakowsky (2), the diffusion limit for this reaction, as estimated by the model of Alberty and Hammes (8), is well below this value. Alberty and Hammes (8) considered a model in which a small substrate diffuses into a hemispherical cavity on the surface of an enzyme and gave the following expression for the diffusion limited rate of recombination of two molecules A and B.

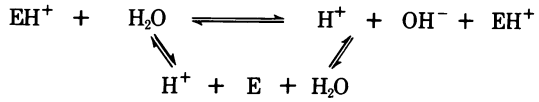
$$k_{AB} = \frac{2\pi N_A(D_A + D_B)\ (R_A + R_B)}{1000} \qquad [2]$$

where N_A is Avogardro's number, D_A and D_B are the diffusion coefficients of A and B in cm²/sec, and $R_A + R_B$ represents a "reaction distance" in cm that denotes the closest approach of A and B before they lose their identity through rapid chemical reaction and become products. Electrostatic interactions have been neglected in Eq. 2 for simplicity. Since "reaction distances" rarely exceed about 5 Å, the use of $D_{H_2CO_3} = 6 \times 10^{-6}$ cm²/sec for H_2CO_3 and $D_{CA} = 9 \times 10^{-7}$ cm²/sec for carbonic anhydrase results in an estimate of the diffusion-limited recombination rate of about 1.3×10^9 M^{-1} sec^{-1} (2), well below the above kinetic requirements. Consequently, HCO_3^- was considered the true dehydration substrate (2, 4).

One may inquire as to what hypothetical "reaction distance" is needed to account for the kinetic requirements. Inserting k_{AB} of 4.7×10^{10} M^{-1} sec^{-1} into Eq. 2 and using the above diffusion coefficients for H_2CO_3 and carbonic anhydrase, one calculates $R_A + R_B = 180$ Å. Recent attempts have been made to rationalize such large "reaction distances" in terms of a model where rapid diffusion of the substrate on the total surface of a spherical enzyme occurs (9). This does not seem realistic, since one must postulate for the kinetics of the human C isoenzyme a spherical enzyme exceeding the actual radius of carbonic anhydrase. More generally, however, such models are not in accord with what is known about the specificity and rates that govern the interactions of *small* molecules and substrates with proteins and enzymes (1, 8, 10, 11) that have well defined active-site cavities.

HCO_3^- AS PRODUCT

The alternative assumption that HCO_3^- is the hydration product removes the problem of rate of recombination with enzyme, since the maximum value of k'_{cat}/K'_m calculated for this product would not exceed 5×10^7 M^{-1} sec^{-1} for any carbonic anhydrase. However, since HCO_3^- (a base) would be the product of the enzymatic hydration of CO_2 (an acid), it follows that the enzyme must formally change its state of ionization during the catalysis. Since the pH–activity profile indicates that the basic form (denoted by E) is active in the hydration direction, the acid form (EH^+) must be active in the dehydration of HCO_3^- (2, 4, 5, 12, 13). Thus, during hydration, steady-state kinetic theory (6) requires that the enzyme return from its acid form to its basic form at a rate not less than the turnover number, approximately 5×10^5 sec^{-1} for the high-activity human C enzyme (5) at pH 7. We can describe the basic ionization processes as in *Scheme II* following Eigen (10),



Scheme II

where ionization occurs by protolysis or hydrolysis. Let us assume for simplicity that protolysis and hydrolysis make equal contributions at pH 7, as for imidazole (14) which has a similar pK, so that we will consider only the steps involving k_d and k_r in Eq. 3. The ionization constant K_a is

$$EH^+ \underset{k_r}{\overset{k_d}{\rightleftharpoons}} E + H^+ \qquad [3]$$

given by $K_a = k_d/k_r = 10^{-7}$ M for $pK_a = 7$. For k_d to exceed the turnover number of 5×10^5 sec^{-1}, k_r must exceed 5×10^{12} M^{-1} sec^{-1} to satisfy the kinetic requirements. This value greatly exceeds the expected diffusion limit for the recombination of a proton ($D_{H^+} \approx 8 \times 10^{-5}$ cm²/sec) with the enzyme. The limit can be estimated from Eq. 2 to be about 1.5×10^{10} M^{-1} sec^{-1} for a reaction radius of 5 Å. Thus, the assumption of HCO_3^- as dehydration substrate also leads to a paradox (1, 5, 15–17).

ACTIVE-SITE IONIZATION

The above difficulties have led to the assumption that the enzyme must have special means of facilitating the ionization of the essential active-site group controlling the activity (5). It was apparent that the ionization, if important, was *not* rate-limiting in the catalysis, since the concentration of non-inhibitory buffer species had no effect on the rate (5, 13). Furthermore, the pH–activity profiles observed with native and covalently modified carbonic anhydrases could be well described by kinetic mechanisms involving instantaneous proton equilibria (12, 13).

Different hypotheses have been advanced to account for very rapid active-site ionization. For example, the presence of an ordered, ice-like water structure in the active site of human carbonic anhydrase C (12) has naturally led to the suggestion (17) that this could facilitate the ionization rate. It is well known from the work of Eigen's group (10) that proton–hydroxyl recombination in ice is about two orders of magnitude greater than in solution. However, this suggestion is unlikely to apply in the carbonic anhydrase problem, since the greater reactivity of H^+ and OH^- in ice is due to their greater effective diffusion rates (10). The local ice-like water structure within the active site in carbonic anhydrase may greatly increase *intramolecular* proton transfer rates, but it cannot significantly increase the rate of reaction of *solvent* protons with the active site. To account for a recombination of H^+ with carbonic anhydrase with a rate constant exceeding 5×10^{12} M^{-1} sec^{-1}, one must postulate a "reaction distance" exceeding 1650 Å!

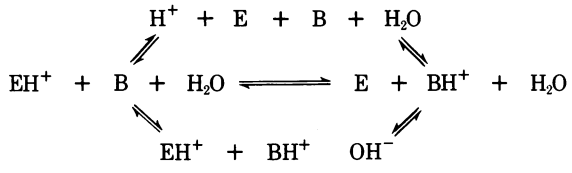
Eigen and Hammes (1) have pointed out that neighboring ionizing groups in an active site could facilitate the rate of ionization in acid–base catalysis. However, chemical modification studies do not provide evidence for the presence of *catalytically important* ionizing groups in the active site of carbonic anhydrase (5, 12, 13) that could function in such a role. In addition, the needed enhancement in the rate of ionization of carbonic anhydrase is somewhat near or above the limit that can be expected (1) from this type of mechanism.

BUFFER PARTICIPATION IN THE MECHANISM

The following alternative appears capable of resolving the difficulties discussed above. It has been suggested§ that a

§ Personal communication from Dr. W. P. Jencks to Dr. J. T. Edsall.

buffer-mediated deprotonation of the enzyme could be a kinetically significant step in the reaction. This seemed unlikely, since, as noted above, the buffer concentration had not been found to affect the catalysis. However, further analysis indicates that if the buffer-mediated deprotonation was *too fast* to be rate limiting in the catalysis, then there would not be an obvious buffer concentration dependence in the kinetics. *Scheme III*, following Eigen (10), illustrates the basic processes expected when enzyme (E) and buffer (B) are present together. There is now a new pathway for ionization of the enzyme involving direct proton exchange



Scheme III

with the buffer species with rate constants k'_d and k'_r for the second order dissociation and protonation of the enzyme (Eq. **4**).

$$EH^+ + B \underset{k'_r}{\overset{k'_d}{\rightleftharpoons}} E + BH^+ \qquad [4]$$

The *observed* rates of active-site ionization would then be the sum of the unassisted and buffer-assisted pathways. The upper limit on the former rate constants can be estimated from Eq. **2**, from which we get $k_r \leqslant 1.5 \times 10^{10}\ M^{-1}\ sec^{-1}$ and $k_d \leqslant 1.5 \times 10^{3}\ sec^{-1}$. The contribution of the latter pathway (Eq. **4**) can also be estimated, since the work of Eigen (10) has shown that the rate of proton transfer between a donor and an acceptor depends upon the difference of the pK values of the two conjugate bases, such that the transfer becomes diffusion limited (about $1 \times 10^9\ M^{-1}\ sec^{-1}$) if the acceptor has a much higher pK. At high pH, where maximal catalysis occurs, the buffers used in the kinetic studies have a greater pK value than carbonic anhydrase (pK is about 7), so that k'_d should be near the diffusion limit. Typically, buffer concentration may be about 5×10^{-2} M, so that at the buffer pK we can calculate a pseudo-first-order deprotonation rate: $k'_d[B] \approx {}^1/_2 \times 5 \times 10^{-2} \times 1 \times 10^9 \approx 2.5 \times 10^7\ sec^{-1}$. This easily satisfies the kinetic requirements of the enzymatic catalysis. At lower pH values, the difference between the pK values of the enzyme and buffer decrease, but so does the k_{cat} value for hydration, thus posing less stringent requirements for deprotonation rates. Thus, there is no need to postulate that certain protonation or deprotonation rates in the mechanism must possess unusually high values.

The above arguments provide theoretical justification for previous assumptions that ionization rates involving the enzyme can be considered instantaneous in comparison with the catalysis under the conditions of the kinetic studies (2, 4, 12, 13). It is not difficult to appreciate that under *in vivo* conditions, e.g., in erythrocytes, there is always a wealth of potential buffers from metabolites and small substrates, as well as from proteins such as hemoglobin. There would be no problem in facilitating active-site ionization of carbonic anhydrase under physiological conditions when needed. Similar arguments may also be extended to other "impossible enzymes" that use this type of acid–base catalysis and have unusually high turnover rates.

The above suggestion for analyzing and interpreting the kinetic data is, of course, subject to experimental verification. It would be necessary to decrease the buffer participation to the extent that it does become rate limiting. However, it should be noted that measurements in weakly buffered media present great difficulties, since the hydration of CO_2 produces both HCO_3^- and H^+ in the physiological pH range and since the rate of catalysis is itself pH dependent (12). Measurements could be done at very low substrate and buffer concentrations by use of isotope exchange techniques under equilibrium conditions.§ The problem can also be approached by direct attempts at measurement of the ionization rates in the active site by use of suitable spectroscopic techniques in the presence and absence of buffers.

In conclusion, the previously suggested kinetic schemes (2, 4, 12, 13) that assume rapid ionization of carbonic anhydrase and its substrate complexes are valid, provided that it is recognized that all the ionization rates are *apparent* rates reflecting contributions from both unassisted and buffer assisted pathways, the latter being the most important under physiological conditions and also under the conditions used for kinetic studies. The most probable product of hydration is HCO_3^-. This is hardly surprising in view of the numerous studies establishing the affinity of the active site of carbonic anhydrase to monoanions (12).

Similar conclusions to those reported here have been reached independently by Lindskog and Coleman (18).

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