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On the Mechanism of Action of Carbonic Anhydrase

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The catalytic mechanism of the enzyme carbonic anhydrase has recently been the subject of renewed debate. The generally accepted picture of the mechanism of the enzyme's action has been thrown into doubt by new evidence derived from spectroscopic techniques. In this paper some of the points of conflict are examined and the degree is assessed to which new evidence is incompatible with the previously held picture of CA action. Reinterpretations are put forward of the mechanisms of proton transfer between enzyme and solution and of the detailed mechanism of ligand displacement at the zinc ion. These show that the generally accepted picture of the mechanism of CA action is by no means disproved and remains plausible.

INTRODUCTION

Carbonic anhydrase (CA) (EC 4.2.1.1) (*1*, *2*) catalyses the hydration of carbon dioxide and aldehydes, the hydrolysis of esters, and other reactions whose common factor is attack by nucleophilic oxygen. Its enzymic activity depends on the ionization of an acidic group with a pK_a of approximately 7; only the alkaline, i.e., ionized form of the enzyme is active. The acidic group has until recently been taken to be a water molecule bound to zinc at the active site and considerable evidence in support of this has been adduced. However, there are some difficulties associated with this view and these have been stressed in a recent paper (*3*) which presents as an alternative a model in which an undissociated water molecule is bound to the zinc in the alkaline form and protonation of a protein group leads to loss of the zinc-bound water molecule. These arguments rest on ^{1}H- and ^{35}Cl-NMR relaxation data and kinetic calculations.

The arguments against the hypothesis of an ionizing metal-bound water are threefold (*3*):

(*i*) *Proton Relaxation*

In the analogous enzyme made by replacing Zn^{2+} with Co^{2+}, the paramagnetic metal ion causes pH-dependent proton relaxation (*4*). The sigmoid relaxivity/pH curve is interpreted as showing that the alkaline form of the enzyme exchanges protons with the solvent while the acidic form does not; thus the metal-bound water, if any, is inert in the acidic form while the metal-bound water or hydroxide in the alkaline form is labile. In fact if the curve were truly sigmoid—although the high-pH end is

not sufficiently well-defined to ascertain this—then a more rigorous interpretation would be that the transition complex in the relaxation process has the formula $[E^- \cdot H^+ \cdot OH^-]$, i.e., $EH + OH^-$ or $E^- + H_2O$.

From the line width, Koenig and Brown calculated a maximum proton residence time of 15 μsec per exchanging proton. The observed rate of exchange, R, is therefore given at alkaline pH (in M sec^{-1}) by

$$R = \frac{10^6}{30} \text{ sec}^{-1} \text{ [Enz]}$$

Here [Enz] is the total enzyme concentration in mole $liter^{-1}$, [EH] and $[E^-]$ the co-centrations of the acid and alkaline forms, respectively, and K_a the active-site acid dissociation constant of CA. It is argued in Reference 3 that exchange cannot be occurring between E^- and OH^- because this would necessitate a bimolecular reaction rate exceeding the diffusion-controlled limit. However, as shown above, the kinetics do not require exchange between E^- and OH^-. For a transition state $[E^- \cdot H^+ \cdot OH^-]$, the reaction rate can be analysed in two ways:

$$R = \frac{10^6}{30} \text{ [Enz] sec}^{-1} \text{ in alkali}$$

Therefore in general

$$R = \frac{10^6}{30} [E^-] \text{ sec}^{-1}$$

$$= \frac{10^6}{30[H_2O]} [E^-] [H_2O] \text{ sec}^{-1}$$

or

$$R = \frac{10^6}{30} \times \frac{[EH]}{[H^+]} \times K_a \text{ sec}^{-1}$$

$$= \frac{10^6 \times K_a}{30[OH^-][H^+]} [EH] [OH^-] \text{ sec}^{-1}$$

$$= \frac{10^6 \times 10^{-7}}{30 \times 10^{-14}} [EH] [OH^-] \text{ M}^{-1} \text{ sec}^{-1}$$

(In these equations K_a and concentrations have the dimensions mole $liter^{-1}$.) In either case the bimolecular rate constant is calculable, and the values are, respectively, $(10^6/(30 \times 55) = 600)$ and $(10^{13}/30 = 3 \times 10^{11})$ M^{-1} sec^{-1}. While the latter exceeds the diffusion-controlled limit, the former does not. Consequently an exchange mechanism involving the alkaline form of CA and an incoming H_2O molecule is feasible, and this does not necessarily involve ligand exchange at the metal. However, the fact that the incoming molecule in the exchange process must be water does not lead to the conclusion of Ref. 3 that "the metal ligand at high pH must be a water molecule." Indeed, one cannot even on this basis rule out the possibility that the incoming ligand is OH^-, since diffusion-control arguments may be misleading (see below).

(ii) Chloride Ion Relaxation

The metal ion, Zn^{2+} or Co^{2+}, also provides quadrupolar relaxation of $^{35}Cl^-$ ions in solution (*5*) and here an opposite pH-dependence is seen: the acid form of the

enzyme relaxes the chloride by means of inner sphere association and exchange, while the alkaline form does not.

The association rate between Cl^- and acidic CA, calculated from the residence time, is 6.6×10^7 mole^{-1} liter sec^{-1}. This represents a fairly high ligand exchange rate. The lability of metal-bound chloride at low pH, as contrasted with the low rate of ^1H–exchange and the inferred inertness of metal-bound water, has been considered (*3*) to show that the metal-bound water does not exist and that its supposed coordination site is in fact occupied, by default, by a protein group, which must therefore be vulnerable to displacement by chloride but not by water. This argument stands on the assumption that if a chloride were to enter the inner sphere of the ion it would do so by the Eigen-Wilkins mechanism (*6*) common in metal-ion aqueous systems, entailing the formation of a loose outer-sphere complex between the metal and the chloride followed by a dissociation of water leaving a coordination site vacant, into which the chloride falls. On this assumption the rate of chloride exchange is limited by the rate of water exchange. However, the Eigen-Wilkins mechanism is by no means universal in transition-metal substitutions (*6*) and it has not been established in this case. If the assumption that the Eigen-Wilkins mechanism operates here is not made, then two further possibilities arise.

(a) The displacement of water by chloride involves expansion of the coordination sphere of the metal ion, and coordination of Cl^- is therefore not limited in rate by dissociation of the water ligand.

(b) H_2O is not displaced at all by chloride, but the Cl^- ion merely adds itself to the zinc ion. This implies the existence of an optional fifth coordination site, open to Cl^- but not to H_2O.

There is a certain amount of evidence in support of these two options, although they cannot at present be distinguished (one way in which this might be done would be to look for proton relaxation in the acid form of CA induced by anions such as chloride; this would indicate an associative displacement). Cobaltous and zinc ions are noted for their flexibility of coordination number and geometry (*7*). ESR spectroscopy of the cobalt-containing enzyme has led to the suggestion that the metal ion can bind two cyanide groups (*8*), and expansion of the metal's coordination sphere in a normally four-coordinate metalloenzyme has also been observed in manganese carboxypeptidase (*9*), although of course manganese (II), a larger ion, would be more liable than zinc to expand its coordination sphere.

The rate of binding of carboxylate inhibitors has been found (*10*) to be very high—$10^{8.3}$ mole^{-1} liter sec^{-1} for cobalt carbonic anhydrase. The rates of water exchange in the free aquo complexes are for Zn^{2+}, $10^{7.5}$ sec^{-1} and for Co^{2+}, $10^{5.5}$ sec^{-1} (*6*); here each is coordinated by six waters, all of which are exchanging, whereas in carbonic anhydrase the smaller coordination number and the smaller number of bound waters would tend to reduce these figures unless an associative mechanism operates. A similar lability has been observed (*11*) in four-coordinate cobaltous complexes, but the mechanism in this case is not known. The X-ray determined structure of carbonic anhydrase in the alkaline form (*1*) shows a large part of the solid angle of the metal's coordination sphere left vacant by the three groups of the protein which coordinate the metal. This would in general be expected to increase *a priori* the likelihood of displacement by an associative in preference to a dissociative mechanism. Thus, as

argued in reference 10, it is very likely that the Eigen-Wilkins mechanism does not operate in carbonic anhydrase.

(iii) Rates of Proton Transfer

The simple kinetic scheme involving zinc-bound hydroxide as a nucleophile in the hydration reaction $HO^- + CO_2 \rightarrow HCO_3^-$ has been criticized on the grounds that excessively rapid dissociation would be entailed (*12*, *13*), and also because in the dehydration step an excessively fast supply of protons would be required (*3*). In the dehydration reaction the kinetics reveal the stoichiometry of the transition complex to be $(E^- \cdot H^+ \cdot HCO_3^-)$, which could correspond to a bimolecular reaction between E^- and H_2CO_3 or between EH and HCO_3^-. One is therefore faced with choosing between a pre-equilibrium protonation of E^- (mechanism A) and a pre-equilibrium protonation of HCO_3^- (mechanism B), either of which must keep pace with the overall

$$E^- + H^+ \underset{}{\overset{k_1}{\rightleftharpoons}} EH \xrightarrow{HCO_3^- k_2} E^- + H_2O + CO_2 \qquad (A)$$

$$HCO_3^- + H^+ \overset{k_1'}{\rightleftharpoons} H_2CO_3 \xrightarrow{E^- k_2'} E^- + H_2O + CO_2 \qquad (B)$$

dehydration rate given empirically by

$$\frac{d[CO_2]}{dt} = 8.9 \times 10^6 \text{ M}^{-1} \cdot \text{sec}^{-1} [EH][HCO_3^-] \ (14).$$

In A, the rate of step 1 is

$$R_1 = k_1[E^-][H^+] > \frac{d[CO_2]}{dt}$$

$$\therefore k_1[E^-][H^+] = k_1 \frac{K_a}{K_a + [H^+]}[Enz][H^+]$$

$$> 8.9 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1} [HCO_3^-][Enz] \frac{[H^+]}{K_a + [H^+]}$$

$$\therefore k_1 K_a > 8.9 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1} [HCO_3^-]$$

At $[HCO_3^-] = 20$ mM, the highest concentration used in CA kinetics (*14*),

$$k_1 > 8.9 \times 0.02 \times 10^{13} \text{ M}^{-1} \text{ sec}^{-1} \text{ (since } K_a = 10^{-7} \text{ M)}$$

$$= 1.8 \times 10^{12} \text{ M}^{-1} \text{ sec}^{-1}.$$

So k_1 exceeds the diffusion limit for protonation.

In B, k_1' is not excessive but k_2' is too high: if the pK_a of carbonic acid is 3.7 (*3*) then

$$R_2 = k_2'[E^-][H_2CO_3] = 8.9 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1} [EH][HCO_3^-]$$

$$k_2' = \frac{8.9 \times 10^6 [HCO_3^-][Enz][H^+]/(K_a + [H^+])}{[H_2CO_3][Enz] K_a/(K_a + [H^+])} \text{ M}^{-1} \text{ sec}^{-1}$$

$$= 8.9 \times 10^6 \times 10^{-3.7}/10^{-7} \text{ M}^{-1} \text{ sec}^{-1}$$

$$= 1.5 \times 10^{10} \text{ M}^{-1} \text{ sec}^{-1}.$$

This again exceeds the diffusion limit for collision between two substrates, one of which is neutral.

The overhigh value for k_2' has been rationalized (*3*) by the assumption that *all* H_2CO_3 molecules which collide with any part of the enzyme's surface are conducted without ionization to the active site by means of a novel mechanism of surface diffusion. However, if mechanism A is accepted, no such postulate is necessary. Instead, the difficulty is to explain the apparently impossibly high rate of protonation of the enzyme, and this is easily surmounted by considering that the enzyme may be protonated not only by direct reaction with $H^+_{(aq)}$ from the bulk phase but also by proton transfer from the protonated buffer species, which will always be present and in much higher concentration than 10^{-7} mole liter^{-1}. Thus mechanism A might be modified, introducing the buffer HA/A^-:

$$E^- + HA \underset{k''_{-1}}{\overset{k_1''}{\rightleftharpoons}} EHA^- \overset{k_2''}{\rightleftharpoons} EH + A^- \xrightarrow{HCO_3^- k_3''} E^- + H_2O + CO_2 + A^-$$

In order to justify such a scheme it is necessary to show that both $k_1''[E^-][HA]$ and $k_2''[EHA^-]$ exceed the rate of dehydration but do not exceed the diffusion limit, and to attempt this for all conceivable buffer systems would be impracticable. However, to take one example, consider the case of a 10 mM acetate buffer, $pK_a = 5$, with $[HCO_3^-] = 20$ mM. This corresponds to the highest concentration of bicarbonate used in a stopped-flow experiment (*14*) and to an unusually low buffer concentration. Then, proceeding as before,

$$R_1'' > \frac{d[CO_2]}{dt} = 8.9 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1} [HCO_3^-][EH]$$

and

$$R_1'' = k_1''[HA][Enz]\frac{K_a}{K_a + [H^+]}$$

$$> 8.9 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1} [HCO_3^-][Enz]\frac{[H^+]}{K_a + [H^+]}$$

$$\therefore k_1'' > 8.9 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1} \frac{[HCO_3^-][H^+]}{K_a[HA]}$$

$$= 8.9 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1} \frac{[HCO_3^-]}{K_a} \times \frac{K_b}{[A^-]} \text{ where } K_b = 10^{-5} \text{ M}$$

and $[A^-] = 10^{-2}$ M

$$= 8.9 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1} \times \frac{20 \times 10^{-3}}{10^{-7}} \times \frac{10^{-5}}{10^{-2}}$$

$$= 1.8 \times 10^9 \text{ M}^{-1} \text{ sec}^{-1}.$$

This is a much more reasonable value. Step 2 is less easily accessible. However, assuming step 1 to be a pre-equilibrium, step 2 is not rate-limiting as long as $k_2'' > k''_{-1}$, which in the case of acetate is very plausible. In most situations the concentration of buffers in solution will be much higher and a larger proportion of HA will be present, rendering this mechanism for proton supply still easier.

This also has a bearing on the problem of the diffusion of protons away from the active site in the hydration reaction. We suggested above (*2*) that proton transfer could be facilitated, as it is in ice (*15*), by the ordered water structure (*1*) present in the cavity containing the active site in alkaline CA.[1] However, this facilitation could not operate outside the cavity unless a sufficient flux of protons away from the enzyme could be maintained; perhaps such a flux could be brought about by proton transfer to the conjugate base A^-. It should be underlined that enhancement of enzyme protonation or deprotonation rates through the mediation of buffer species need not involve coordination of the buffer to the zinc, or even, if protons in the ordered region are exceptionally mobile, its entry into the active site cavity.

Unfortunately it is not possible to check that protons are being transferred by means of the buffer species, since, as the above example shows, only very small amounts of buffer are required to effect the necessary rate of proton transfer, and it is not possible to conduct an enzymic kinetic experiment in the total absence of any kind of buffer, particularly as the bicarbonate substrate will itself act as a buffer. However the acceleration of protonation and deprotonation equilibria by buffer components is a noted feature of many organic reactions (*16*).

SUMMARY

The traditional picture of the active site in carbonic anhydrase involves a zinc-bound water molecule. This becomes a hydroxide in alkaline carbonic anhydrase and this hydroxide is supposed to be the nucleophile in the hydration reaction, after which it is lost; the product leaves the active site and is replaced by a water molecule which ionizes, thereby reactivating the enzyme. Microscopic reversibility requires that the dehydration of bicarbonate should follow the reverse path, involving hydroxide protonation (mechanism A above). The alternative viewpoint is that the zinc-bound entity in the alkaline enzyme is H_2O and that in the acid form of the enzyme the zinc ion is coordinated exclusively by protein; the catalysed dehydration consequently involves H_2CO_3 rather than HCO_3^- (mechanism B above). Following the former mechanism one is obliged to postulate that the zinc in the acidic form has a coordination number varying between four and five according to the nature of the ligands (a postulate not in itself unreasonable (*12*)) and that the water molecule bound to zinc is surprisingly nonlabile, its rate of water-exchange being only 5×10^3 sec^{-1} or less. Delabilization of water in the inner sphere by a chelating ligand is not unprecedented; it has been observed in the case of a certain metal–ATP complex (*22*) in which the adenosine group, remote from the metal, forms a hydrogen bond with one of the coordinated water molecules, thereby selectively reducing its exchange rate.

If the latter mechanism operates it is necessary to explain three surprising facts. (i) The zinc-bound water present in the alkaline form does not ionize, even at pH 9,

[1] We note at this point that to regard the process of proton exchange as taking place between a zinc-bound water and OH^- is an oversimplification because of the existence in the active site of the ice-like arrangement of water molecules. Such a structure can facilitate H^+ transfer; even one or two intervening water molecules can have a dramatic effect on the transfer rate, e.g., as in the systems Me_3NH^+/NH_3 (24), imidazolium/imidazole (25) [cf. also (26)].

whereas studies on simple zinc complexes with one or more ligand waters have shown a range of pK_a's down to below 8 (*17*); furthermore, the lower the overall coordination number the smaller this pK_a should be (*18*). Formation of a characteristically strong zinc-oxygen bond in the transition complex is also a feature of several zinc-catalysed inorganic reactions involving hydroxide abstraction (*19*). (ii) The nucleophilicity of metal-bound hydroxide is not in doubt (*2*), but metal-bound water will be an extremely poor nucleophile. (iii) There is a surface diffusion process by which all H_2CO_3 molecules colliding with the enzyme at any point find themselves at the active site. This would be fundamental to the mechanism and, if confirmed, would have wide-ranging implications in enzyme chemistry. In addition to these facts, the activity-determining ionizing group in carbonic anhydrase would have to be identified.

It thus appears that at present the "traditional" mechanism for CA activity is the more economical of the two outlined above. A synthesis of the two mechanisms might to some extent be achieved. For example, there is no evidence to contradict the hypothesis that the zinc's ligands in the acid form of the enzyme could be protein groups (the nearby threonine hydroxyl group (*1*) might be a contender for the fourth coordination site), with or without the "optional" fifth site referred to above. The fourth protein group could then be displaced by hydroxide, bicarbonate, chloride, etc. This would not affect any of the kinetic arguments, but it would explain the absence of exchanging water in the acidic enzyme. In this connection an x-ray structure of the acidic active site would be vitally informative.

Three points must be made regarding assumptions made in the above discussion:

(i) It has been assumed throughout that the coordination of cobalt and zinc in acid and alkaline CA's are identical. This is reasonable in view of the ion's similar size and chemical properties and of the nearly identical behaviour of native and cobalt-substituted carbonic anhydrases. This means that the coordination number of cobalt in alkaline Co-carbonic anhydrase has been taken as four, viz., three protein and one solvent. Pending an x-ray structure determination this must be regarded as an assumption, particularly in view of the suggestion, based on magnetic circular dichroism, of a coordination number of five (*20*).

(ii) If the coordination number of the zinc is flexible, then the binding of hydroxide to the metal is not incompatible with the simultaneous binding of CO_2 to the metal, either in the ground-state Michaelis complex or in the transition complex in which the C—O bond is half formed. However there is as yet no positive evidence for such binding, although the validity of the evidence against it (*21*) has recently been disputed (*27*).

(iii) The ionization of water bound to zinc or cobalt does not imply that the metals in less active metal-substituted forms of CA are capable of inducing the ionization of a water molecule; for example, studies of manganese-substituted CA (*23*) show that the polarizing power of Mn^{2+} in this enzyme, at least with respect to sulphonamide inhibitors, is lower than that of Zn^{2+} or Co^{2+}.

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