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Kinetic Pathways and Carbonic Anhydrase Mechanisms

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*Carbonic anhydrase (CA) exists in three forms: the low-pH form (L); the high-pH form (H); and the anion-inhibited from (A). The latter includes the bicarbonate complex. All three forms have been demonstrated in CA I and, when sulfate is removed, in CA II. The L-form of CA III has not yet been seen, even at pH 5. Equilibrium among the three forms in a sample of CA can be established, in principle, by kinetic pathways connecting any two forms; which pathway dominates is as yet an open question. By invoking the usual ping-pong mechanism of CA, during which hydration of CO_2 causes the enzyme to go from H to L, the kinetic pathway connecting A and H is ignored, essentially by definition. Rarely has the A–H pathway been considered (*cf. Koenig et al., *1980). Though there are few data to demonstrate the relative kinetics of the A–L and A–H pathways, it can be argued that the latter is buffer-mediated, which could distinguish the two. In this case, the lifetime of a bound anion would be buffer-dependent. We have investigated this point by measuring the nuclear relaxation rates of fluorine of trifluoroacetate in Co^{2+}–CA II solutions. The fluorine linewidth, and thus the anion exchange rate, is independent of buffer concentration up to ~50 mM, which argues for the A–L pathway predominating.*

Introduction

Carbonic anhydrase catalyzes the reversible interconversion

$$CO_2 + H_2O \rightleftharpoons H^+ + HCO_3^- \quad (1)$$

This is a two-substrate, two-product reaction, a point that will become more pertinent as we examine the enzymatic pathways involved. An increasingly popular way to express the enzymatics is by the two half reactions:

$$CO_2 + E \rightleftharpoons ECO_2 \rightleftharpoons EH^+HCO_3^- \rightleftharpoons EH^+ + HCO_3^- \quad (2)$$

$$EH^+ + B \rightleftharpoons E + BH^+ \quad (3)$$

B in eqn. 3 indicates the role of buffer in the transfer of protons between enzyme and buffered solution. It should be noted that buffer can influence the observed kinetics under steady-state conditions, but only when the turnover number of eqn. 2, which increases as $[CO_2]$ (for hydration) or $[HCO_3^-]$ (for dehydration) increase, is comparable to the residual rate of the deprotonation step in eqn. 3 in the absence of buffer.

Writing the reactions as eqns. 2, 3 is an explicit commitment to an ordering of the interaction of H^+ and HCO_3^- with enzyme: during hydration of CO_2, HCO_3^- is released before product H^+; and conversely, during dehydration of HCO_3^-, H^+ as substrate binds to enzyme before HCO_3^-. (The protonated enzyme complex is often equated with a low-pH form of the enzyme. This is strictly incorrect, though often convenient and not very wrong. However, at low pH, other proton acceptor sites become protonated and can alter the properties of the binding site of the proton of EH^+. Suffice it for now to note that the pH-dependence of the activity of carbonic anhydrase is not described very well by a single ionization). In addition to this explicit ordering, eqns. 2, 3 contain an implicit statement regarding the kinetic pathways for the binding of monovalent anions other than HCO_3^-; *e.g.* acetate. These anions, too, might interact preferentially with EH^+, rather than displace an OH^- from E, to form EH^+A^-. That the kinetic pathways for anion–enzyme interactions are important to consider becomes clearer when the equilibria among three forms of the enzyme (E, EH^+, and EH^+A^-) are considered, as in Scheme I.

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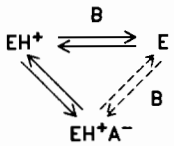


Scheme 1.

Here the dashed equilibria represent the processes which, by inference from eqns. 2, 3, are slower than the longer pathway via EH^+ that couples E and EH^+A^-. Can this be so?; is it so? If one takes the liberty of equating EH^+ with the low-pH form of the enzyme, and if one thinks of processes taking place at, say, pH ~ 8, well above the pK_a for activity of enzymes II and III (~6.3 and <5 respectively (*cf.* Lindskog, 1982)) then $[EH^+]$ will be so low that anions, including HCO_3^-, might be expected to interact with E (and buffer) directly. This is analogous to H^+ and HCO_3^- interacting with enzyme and buffer simultaneously, rather than consecutively, obviating the need for eqn. 3.

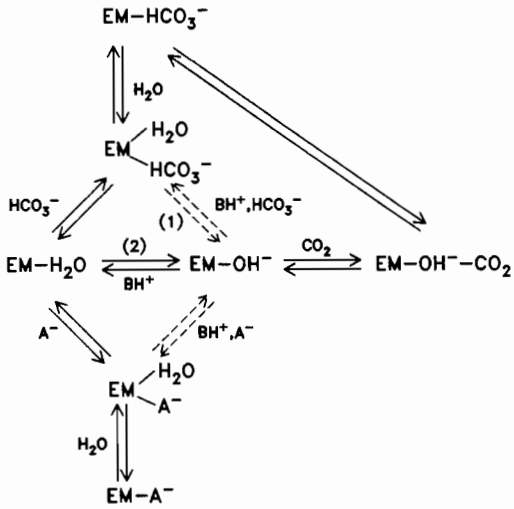
There are but few data that relate to the foregoing question. These include: (a), new results, presented here, on the off-rate of a particular anion (trifluoroacetate) from EH^+A^- as a function of buffer concentration; (b), a quantitative analysis, and reinterpretation, of the recent results of Pocker and Deits (1981, 1982) on inhibition of hydration activity at high pH by anions; and (c), a reconsideration of the ^{13}C NMR linewidth data for CO_2 and HCO_3^- in the presence of carbonic anhydrase (Koenig *et al.*, 1974; Simonsson *et al.*, 1979, 1982). All results indicate the release of HCO_3^-, followed by H^+, as the pathway of the hydration reaction.

To pose the problem still more effectively requires extending Scheme I to include enzymatic pathways. In so doing, we elaborate the Scheme to include transition state intermediates.

Ligands, Intermediates, and Pathways

Scheme II is a summary of much of current thinking regarding carbonic anhydrase, a scheme that we believe is close to a consensus view. Pentacoordinate intermediates are indicated.

Here M represents the metal ion at the active site, with its three protein histidyl ligands. E of eqn. 1 becomes $EM-OH^-$, in this notation, and EH^+ becomes $EM-H_2O$, or a thermal mixture of this and a pentacoordinate form with two H_2O ligands (Bertini *et al.*, 1981). $EM-H_2O$ itself, however, may have to be regarded as a mixture of states with a proton of the water shifted to a nearby proton acceptor (Bertini *et al.*, 1981; Koenig *et al.*, 1983). EH^+A^- is a thermal mixture of $EM-A^-$ and the pentacoordinate form with an H_2O ligand added (Bertini *et al.*, 1981). Two possible catalytic pathways are indicated by (1) and (2). In pathway (1), which does not include the protonated enzyme (loosely, the low-pH



Scheme II

form), and which corresponds to simultaneous release of H^+ and HCO_3^- during hydration, the mandatory requirement for buffer suggests that the dashed equilibria, Schemes I and II, must be buffer catalyzed. The equilibrium $EM-H_2O-A^- \rightleftarrows EM-H_2O$ presumably is not. The foregoing suggests that the lifetime of A^- on $EM-A^-$ will be buffer dependent if the dashed pathway, involving no change of charge, predominates at equilibrium over the pathway that connects the anionic form with the low-pH form. We have examined this possibility using trifluoroacetate anion, by redoing the experiments of Taylor *et al.* (1971) for several values of buffer concentration.

Experimental and Results

Under appropriate conditions, the linewidths of NMR resonances of anionic inhibitors of carbonic anhydrase are determined by the off-rates of these anions from the enzyme. The fluorine signal from trifluoroacetate (among others) in equilibrium with Co^{2+}–substituted solutions of human C (II) carbonic anhydrase has been examined in some detail by Taylor *et al.* (1971), for a range of temperature, but at only one buffer concentration.

We have performed similar experiments, for a range of buffer concentration and temperature, on the almost identical Co^{2+}–substituted bovine enzyme (II). Experimental protocol followed that of Taylor *et al.* (1971). All our data are at pH 8, well above the pK_a for activity. Conditions were chosen such that the linewidths broaden as temperature is lowered, due to a large chemical shift when the anion is on the paramagnetic Co^{2+}–ion; the longer it remains, the greater the precessional phase loss and the greater the linewidth.

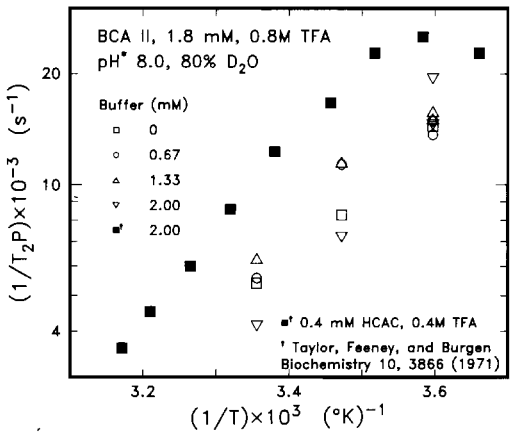


Fig. 1. Temperature dependence of the transverse relaxation rate of ^{19}F in trifluoroacetate at 94.1 MHz, 1.8 mm Co^{2+}–BCA (II), 0.8 *M* TFA, pH* 8.0, 80% D_2O and tris-sulfate concentrations of (□) 0; (○) 0.67; (△) 1.33; and (▽) 2.0 m*M*. The measured linewidths are corrected by values for the apo enzyme. (■) indicates data of Taylor *et al.* (1971) for 0.4 m*M* Co^{2+}–HCAC (II), 0.4 *M* TFA, pH* 7.6, 100% D_2O and 2 m*M* tris-sulfate. P is the ratio of enzyme to TFA concentrations. pH* indicates uncorrected meter readings.

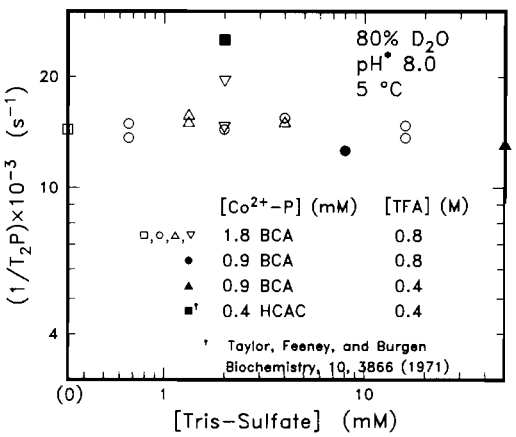


Fig. 2. Buffer dependence of the transverse relaxation rates of ^{19}F in trifluoroacetate at 94.1 MHz in the presence of Co^{2+}–BCA (II) at 5 °C, pH* 8.0, 80% D_2O and the following concentrations of enzyme and TFA: (□, ○, △,▽) 1.8 m*M* BCA, 0.8 *M* TFA (●); 0.9 m*M* BCA; 0.8 *M* TFA; (▲) 0.9 m*M* BCA, 0.4 *M* TFA. (■) indicates the data of Taylor *et al.* (1971). P is the ratio of enzyme to TFA concentrations. pH* indicates uncorrected meter readings.

Our results are compared with those of Taylor *et al.* (1971) in Fig. 1. The observed variation of linewidth with temperature confirms that we are in the appropriate range of conditions, in which the linewidths should be inversely proportional to buffer concentration if buffer is the major determinant of anion lifetime. As seen in Fig. 2, which shows line broadening at 5 °C as a function of buffer concentration, we find no systematic buffer effects, within experimental error. From this we infer that the main pathway for anionic exchange between enzyme and solution at equilibrium is $EH^+A^- \rightleftarrows EH^+ + A^-$, even at high pH where the equilibrium concentration of EH^+ is but a very small fraction of the total anion-free enzyme. Thus, at equilibrium at high pH, dissociation of EH^+A^- is into A^- and EH^+, the latter catalyzed by buffer to $E + H^+$; association is the reverse of this pathway.

Note that the nature of the experiment is such that the lifetime of the anion–enzyme complex is measured independently of the form of the dissociated state of the enzyme; it is the absence of a buffer effect that implies the dissociated state, and thereby the pathway. By analogy, HCO_3^- would behave similarly, which is one argument for the validity of the sequence in eqns. 2, 3.

Discussion

Anion Off-Rates

That the dissociation pathway for the anion–enzyme complex leaves the enzyme protonated, *i.e.*, in its low pH form, is surprising for two reasons. First, this pathway, in contrast with one in which the anion is replaced directly by OH^-, involves the Coulomb barrier required to separate the dissociation products. Second, there is an argument that follows by analogy with eqn. 3 which, to first approximation, represents interchange of a proton by collision between two proton acceptors, E and B. If an anion is added to EH^+, to form EH^+A^-, subsequent transfer of the proton to buffer and separation of the reactants could occur as before, but with concerted release of the anion as well. This reaction might be thought to be at least as rapid as proton release in eqn. 3, since involvement of the anion would reduce the Coulomb barrier substantially. This process is the dashed pathway, Scheme I, and has been postulated previously (Koenig *et al.*, 1980). Why isn't it observed? We suggest the reason is not that it is intrinsically slow, but that the observed sequential release of anion, then proton, is unusually rapid in carbonic anhydrase, and may relate to the existence of a protein-contributed proton acceptor in the active site, presumably His 64 (*cf.* Lindskog *et al.* (1983)).

Lindskog (1983) has suggested, referring to eqns. 2, 3, that the location of the proton after the catalytic step in the hydration reaction is not the same as that from which it is removed by encounter with buffer. Rather, there is an internal proton transfer, ostensibly from an H_2O ligand of the metal–ion (where it is deposited enzymatically) to His 64 (where it can be removed by buffer). This trans-

fer is needed to explain the proton-deuteron isotope effect in hydration (Ventkatasubban and Silverman, 1980; Lindskog, 1983). We suggest here that release of HCO_3^- may be concerted with this transfer; in essence, shift of the proton to His 64 leaves the metal–ion too negative to hold the anion. Buffer subsequently removes the proton from His 64. That this process contributes to the isotope effect means that it is more or less rate limiting and therefore comparable in rate to k_{cat}, or $\sim 10^5$ s^{-1}. If concerted with anion release, the anion lifetime would be $\sim 10^{-5}$ s, and rather insensitive to the particular type of anion. This agrees quantitatively with the results of Taylor *et al.* (1971) who find anion lifetimes in the range of 10–20 μs for various anions.

^{13}C-Linewidths

Koenig *et al.* (1974) were the first to use the linewidth of ^{13}C in CO_2 and HCO_3^- for a quantitative study of the rate of enzymatic interconversion at chemical equilibrium. This experiment measures the kinetics relating to eqn. 2 only, since it measures the lifetimes in solution of CO_2 and HCO_3^- without regard to either the state or rate of protonation of the enzyme. Their results for human carbonic anhydrase I (Koenig *et al.* 1974), and subsequent work by Simonsson *et al.* on both the human I (1982) and II (1979) enzymes, are in agreement that the fundamental catalytic rates are more rapid than those measured kinetically (which include the step in eqn. 3), and that they are uninfluenced by buffer. This is a second argument for the validity of the sequential reactions of eqns. 2, 3, a point stressed recently by Lindskog also (1983).

Inhibition by Anions at High pH

Pocker and Deits (1981, 1982) recently reported on the inhibition of carbonic anhydrase hydration activity by anions at high pH, under conditions of saturating concentrations of CO_2. They interpreted their results by invoking a novel ternary complex of CO_2, anion, and E, the high-pH form of the enzyme. They used only one buffer concentration in their experiments, apparently not considering that this concentration would make eqn. 3 rate limiting under the circumstances for which they had to invoke a novel ternary complex. We can readily explain their data by taking the buffer limitation into account.

If we compare the binding of anions to the protonated form of the enzyme,

$$EH^+ + A^- \rightleftharpoons EH^+A^-, \tag{4}$$

with the interaction of buffer with this form, eqn. 3, we see that under steady state conditions there will be competition between buffer and anion for the EH^+ that is generated. The effects of the competition on the turnover velocity V can be quantitated if eqns. 3, 4 are taken into account in deriving the usual expression for V under steady station conditions of high substrate concentration ($[CO_2] > K_M$) and high pH:

$$V \cong \frac{k_{cat}[E_T]}{1 + \frac{k_{cat}}{K_B}\left(1 + \frac{[A^-]}{K_I}\right)} \tag{5}$$

K_B is defined here as the off-velocity of protons in eqn. 3, and is directly proportional to buffer concentration, giving an explicit dependence of V on buffer concentration. Equation 5 is, moreover, formally identical to the term derived by Pocker and Deits, who assumed a novel ternary complex, no buffer involvement, and the Michaelis–Menton formalism. At low buffer concentration, anions will bind to the protonated form (as they do at low pH under equilibrium conditions) resulting in the inhibition observed by Pocker and Deits (1981, 1982). For the one anion that we have considered, Cl^-, we can account quantitatively for the results of Pocker and Deits, using the known value of K_I. Their experiments should be repeated for a range of buffer concentrations.

Lindskog *et al.* (1983) have also addressed the issue of inhibition of hydration activity by anions at high pH. They used 50 m*M* buffer, ostensibly a large enough concentration to minimize the buffer effects, eqn. 3. Their interest was to investigate the internal proton transfer step, presumed rate limiting for their conditions. They used SCN^-, an anion with a relatively high affinity for carbonic anhydrase, and observed 50% inhibition at about 1 m*M* anion, even at 50 m*M* buffer. Pocker and Deits, who used 20 m*M* buffer, ought to have seen (in our view) 50% inhibition at a lower anion concentration; however, they report 5 m*M* as the observed value. Thus, there is lack of agreement in the data obtained at the two laboratories, which may stem from the use of the human II enzyme by Lindskog *et al.* (1983) and the bovine II enzyme by Pocker and Deits (1981, 1982). Nonetheless, Lindskog *et al.* (1983) also suggest another explanation, different from ours, for the observations of Picker and Deits; one that, like ours, obviates the need for a novel ternary complex of enzyme, anion, and CO_2, and depends on a buildup of intermediates under steady state kinetic conditions.

Summary

The point that we would emphasize here is that the sequence in eqns. 2, 3 implies something special about the pathways for exchange of anions with carbonic anhydrase. Contrary to what one might expect, which is that direct (but buffer-aided) interchange of anions with the OH^- ligand of the enzyme

at high pH ought to be the most rapid step, the dominant pathway is *via* the protonated form. The reasons, we suggest, relate to the particular structure of the active site of carbonic anhydrase that makes turnover so rapid, and are unique to the enzyme and not the system of ligands of the metal-ions.

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