EXCHANGE OF LABELED NUCLEI IN THE CO_2—HCO_3^--SOLVENT SYSTEM CATALYZED BY CARBONIC ANHYDRASE

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ABSTRACT Silverman et al. (1979. *J. Am. Chem. Soc.* 101:6734–6740) have reported measurements of the loss of ^{18}O to solvent from the isotopically labeled CO_2—HCO_3^- system and of the mixing of ^{18}O and ^{13}C labels within the system, as catalyzed by human carbonic anhydrase C in the pH range 6–8. This work is an extension of earlier work (Silverman and Tu. 1976. *J. Am. Chem. Soc.* 98:978–984) on the very similar bovine enzyme. The more recent work is analyzed by its authors in terms of the "hydroxide" model for the apparent pH-dependence of enzymatic activity, a model in which the pH-dependence is associated with the presumed ionization of an H_2O ligand of the active-site metal ion to OH^-. From a comparison of their data with a solution of the coupled differential equations that describe the kinetics of isotope exchange in terms of the model, Silverman et al. derived a pH-dependent rate of exchange for the water molecule which is formed at the active site of the enzyme during dehydration. By contrast, using the same data and a model in which active enzyme has a water molecule on the metal ion at the active site, and similar differential equations, we derive a value for the rate of exchange of water that is pH-independent. This model has the attraction that it explains the magnetic relaxation rate of solvent water protons in the Co^{2+}-substituted enzyme, whereas the hydroxide mechanism cannot explain these data without the introduction of unfounded ad hoc assumptions; further, the presence of an OH^- ligand of the metal has never been demonstrated. We also include an analysis of analogous data for the bovine enzyme. One result of our analysis is that the pK_a for activity of the enzyme samples used is near 6.0, implying that the bulk of the data were taken when the enzyme was essentially all active. It is straightforward to account for the pH-dependence of the data near and below the pK_a by using an empirically-derived value for the pK_a. However, we have recently developed a model for the low pH (inactive) enzyme that has been successful in interpreting a wide range of data, and we show that this new view can explain the few points at low pH quite adequately. Additionally, we consider the recent kinetic results for the human C enzyme, obtained at chemical equilibrium by studies of the linewidths of nuclear magnetic resonances of ^{13}C in labeled substrate (Simonsson et al. 1979. *Eur. J. Biochem.* 93:409–417) and show that these experiments and those of Silverman et al. are all consistent with kinetic data from nonequilibrium stopped-flow experiments, viewed in terms of our model, in the limit of low substrate concentration. Results at higher concentrations indicate that the Michaelis constants and equilibrium constants differ somewhat.

INTRODUCTION

Carbonic anhydrase (EC 4.2.1.1) catalyzes the interconversion: $CO_2 + H_2O \rightleftharpoons H^+ + HCO_3^-$ (cf. Pocker and Sarkanen, 1978; and Lindskog, in press, for reviews). For a given concentration of reactants, the enzymatic activity is generally observed to be greatest at high pH, decreasing to a negligible value as the pH is lowered. However, the pK_a for activity

depends on the source of the enzyme (human erythrocyte, bovine, etc.); on whether the Zn^{2+}-ion at the active site has been replaced by, for example, Cd^{2+} or Co^{2+}; and upon the concentration of anions in the buffer. Values for pK_a in the range 7–8 are typical (Wells et al., 1979) of older measurements, whereas more recent results obtained on samples dialyzed extensively against distilled water indicate that the pK_a for the bovine enzyme can be reduced to 6 (Wells et al., 1979) and even lower (Bertini et al., 1977; Jacob et al., 1978; Koenig et al., 1980).

The nature of the ionization responsible for the pH-dependent activity has been the subject of many investigations and of much contention (Lindskog, in press; Koenig et al., 1980). Most investigators have searched for an ionizing group on the enzyme; a popular view has been that a water molecule ligand of the metal-ion at the active site ionizes at high pH, enabling a nucleophilic attack on the CO_2 by the resulting OH^- ligand of the metal-ion to produce HCO_3^-. Lindskog, who has expounded this view in some detail, nonetheless concludes his recent review (Lindskog, in press.) by noting that "as long as unequivocal evidence for the existence of Zn^{2+}-bound OH^- in the enzyme is lacking, this model must be continually questioned and tested against alternative models." By contrast, we had concluded (Koenig and Brown, 1972), on the basis of earlier investigations of the magnetic relaxation of solvent protons in solutions of both the Co^{2+}-substituted human B and bovine enzymes (Fabry et al. 1970), that a rapidly exchanging water molecule (off-rate $> 10^5\ s^{-1}$) is a ligand of the metal-ion when the enzyme is active. More recently we have shown that the pK_a for the titration of the magnetic relaxation effects and associated optical spectra in the visible, both of which correlate with enzymatic activity, decreases as the samples are purified by dialysis (Jacob et al., 1978). We argued at the time that, unless the exchangeable water was a fifth ligand and acting as a reporter group, the ionization responsible for the apparent pH-dependence of activity and correlated phenomenon could not be the ionization of a water ligand of the metal; there must be a water molecule rather than a hydroxyl on the metal-ion of the active enzyme and the explanation for the observed pK_a must lie elsewhere.

Concomitant with the catalyzed interconversion of carbon dioxide and bicarbonate by carbonic anhydrase, one finds an enzymatic enhancement of the interchange of label when these substrate molecules are prepared with labeled nuclei. Thus Koenig et al. (1973, 1974) studied the disappearance of label catalyzed by the human B enzyme, using high resolution magnetic resonance techniques: they labeled CO_2 and HCO_3^- enriched with ^{13}C by preparing the ^{13}C nuclei in identifiable spin states, and then measured the lifetime against enzymatic conversion of these labeled species. Similar experiments have since been performed with the human C enzyme by Simonsson et al. (1979).

Some isotopic labels may be detected more directly using mass spectrometry. Silverman and Tu (1976) have studied isotope exchange in some detail, extending the early work of Mills and Urey (1940). Their experiments were of two types: in one, ^{18}O-labeled HCO_3^- was added to a solution of bovine enzyme and, after waiting for chemical (but not isotopic) equilibrium, the fraction of ^{18}O remaining in a succession of samples of CO_2 drawn from the test solution was measured. They deduced the pH-dependence of a quantity θ, the observed rate constant for the catalyzed loss at equilibrium of ^{18}O from the CO_2—HCO_3^- system to the solvent water. In addition, Silverman and Tu (1976) measured the rate of catalyzed mixing of ^{18}O-labeled and ^{13}C-labeled HCO_3^- to give a doubly labeled species, which rate they

characterized by a rate constant ϕ. They also attempted to relate the data for θ to kinetic parameters of the enzyme obtained from earlier stopped-flow transient measurements (Kernohan, 1964, 1965), invoking the hydroxide mechanism, i.e., the model with an ionizing water ligand of the Zn^{2+} ion. They remarked that the catalyzed rate of ^{18}O depletion from species of CO_2 cannot be compared with results of nonequilibrium, initial catalyzed rates of hydration and dehydration, unless processes that contribute to ϕ are also considered. They suggested that after ϕ is considered, the data might be explained by invoking a pH-dependent off-rate for the water molecule produced at the active site of the enzyme during dehydration, but they did not quantitate this idea. It is important to note, however, that they identify this intermediate state with the form of the enzyme at low pH, which is presumed by them to have a water ligand on the metal-ion.

More recently, Silverman et al. (1979) have extended the measurements of θ and ϕ to the human C enzyme and also related their data to the model for enzyme action that assumes an ionizing water ligand of the Zn^{2+} ion at the active site to explain the pH-dependent effects. They deduced a set of five coupled differential equations, involving two kinetic parameters, for which they are able to find two solutions that they then relate (though not rigorously) to θ and ϕ. By these procedures, they are able to fit their data; they again conclude that the water off-rate is pH-dependent, as was conjectured by Silverman and Tu (1976) for the bovine enzyme.

Silverman et al. (1979) note that their model is inconsistent with the implications of the magnetic relaxation results (Jacob et al., 1978; Koenig and Brown, 1972; Fabry et al., 1970) which show high relaxation rates at high pH, and no relaxation at low pH. A model with OH^- on the metal at high pH and H_2O on the metal at low pH would be expected to behave just the reverse, unless some unique conditions prevail. Silverman et al. (1979) suggest several (in our view, unlikely) possibilities to resolve the apparent discrepancy: "(1) there is a difference in the exchange properties of water at the active sites of the human zinc and cobalt-substituted bovine enzymes, (2) the water containing the oxygen abstracted from the bicarbonate is not exchanging from the inner coordination sphere of the zinc in the human enzyme, (3) the proton exchange with solvent observed by proton relaxation in the presence of cobalt-substituted bovine carbonic anhydrase is not involved in the catalysis."

The purpose of the present paper is to demonstrate that the results of Silverman et al. (1979) for the human C enzyme and of Silverman and Tu (1976, and unpublished data) for the bovine enzyme can be accounted for in detail, within the experimental uncertainty, by a model for enzymatic activity that has a water molecule ligand, with a pH-independent off-rate, on the metal ion of the active enzyme; i.e., a model entirely consistent with the magnetic relaxation data. The question then arises as to how to regard the inactive enzyme; i.e., the enzyme at low pH. It turns out to be adequate for the present purposes to take the pK_a for activity as an empirically determined parameter, and then use a simple titration profile to describe the concentration of active enzyme at a given pH. Moreover, our analysis shows that the pK_a for the enzyme samples is low, near 6.0, so that the inactive form of the enzyme need be considered to explain only a few data points. Our analysis, following Silverman et al. (1979), is cast in a form that involves two kinetic parameters: the velocity of catalysis at equilibrium, and the lifetime of a water molecule on an active enzyme molecule. Again, five coupled differential equations are found that relate the kinetic scheme to the observables

measured in the experiments. We find all the solutions of these equations, i.e., the five roots and the five eigenfunctions that decay as single exponentials, and relate these rigorously to θ and ϕ. On the basis of these solutions, we show that all the results of Silverman and co-workers (1976, 1979, and unpublished data) over the entire range of pH considered are consistent with the results of stopped-flow kinetic measurements of enzymatic activity for both the human C and bovine enzymes, as well as with the measurements by magnetic resonance methods of catalyzed exchange of nuclear labels (Simonsson et al., 1979), and the results of magnetic relaxation studies. Thus we demonstrate that the data for catalyzed isotope exchange and isotope loss can be explained by a model that includes a water molecule as a ligand of the metal ion of the active enzyme.

We have recently presented a model for the active site of carbonic anhydrase at values of pH below the observed pK_a that is consistent with the relaxation data (Koenig et al., 1980). The model preserves, as it must, the water molecule as a ligand of the metal when the enzyme is catalytically active; the generally observed inactivity of enzyme solutions at low pH is ascribed to inhibition of activity by the presence of monovalent anions in the solution that bind, along with a proton, and displace the water ligands. These new views, coupled with the earlier model, allow a simple description of the activity of carbonic anhydrase over the entire range of pH. In more recent experiments, where the prescence of adventitious monovalent anions is assiduously minimized (Wells et al., 1979; Bertini et al., 1976; Jacob et al., 1978; Koenig et al., 1980), and divalent sulfate is used as the anion to maintain ionic strength, we pointed out (Koenig et al., 1980) that the relatively low concentration of (strongly binding) monovalent bisulfate ions in equilibrium with SO_4^{2-} is adequate to inhibit the enzyme at low pH. In the experiments of Silverman and his collaborators (1976, 1979) there are no deliberately added monovalent anions; however, the ionic strength is maintained throughout with SO_4^{2-}. This anion is present in sufficient concentration to account for the pK_a of the activity of the samples used in the isotope-exchange experiments. This conclusion is based on the shifts of pK_a upon addition of sulfate as observed by Koenig et al. (1980). Therefore, in analyzing the isotope-exchange data, we consider the enzyme to be inactivated at low pH by HSO_4^-. We reemphasize that, for the present arguments, there is no need to consider a particular mechanism for the inactivity of carbonic anhydrase at low pH; the existence of an empirically derived pK_a for activity is adequate. Moreover, these considerations only apply to the small fraction of the data taken below pH $\sim$ 6.5. However, certain details of the results, at present just outside experimental error, support our bisulfate arguments. Additionally, this model eliminates the pK_a for activity as an empirical parameter, and one of our aims is to correlate as much data for carbonic anhydrase with as few model parameters as possible.

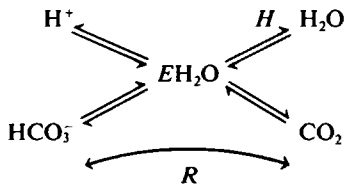
THEORY

The formal, rigorous solutions of the model equations relevant to the experiments of Silverman et al. (1976, 1979) are derived in the Appendix. However, the essence of the theoretical results can be derived rather simply by the following reasoning. First, we consider that the enzyme catalyzes the reaction

$$H^+ + HCO_3^- \rightleftharpoons CO_2 + H_2O,$$

and accordingly we treat the reaction as one with four reactants. Though the proton concentration is controlled and altered by variation of pH, we carefully distinguish between protons as reactant on the one hand, and alterations of the properties of the enzyme (if any) that may be pH-dependent, on the

other. Second, we consider that the conditions are saturating for H_2O as substrate, unless water is displaced (competitively) by HCO_3^- (as substrate) or another anion (as inhibitor). Within this context, we derive an expression for δ, the fraction of active enzyme with ^{18}O-labeled water when the CO_2—HCO_3^- system has been labelled with ^{18}O, by a simple kinetic argument. Expressions for θ and ϕ follow directly from that result.



Scheme I

With reference to Scheme I, R is the catalyzed velocity, in M s^{-1}, of interconversion of HCO_3^- and CO_2 at equilibrium. In the limit of low substrate concentration, it is the same as the initial velocity of catalysis obtained from traditional stopped-flow measurements. At equilibrium, of course, the velocities are equal for both directions of the catalyzed reaction, but the algebra is somewhat more straightforward when we express R in terms of $[CO_2]$ and the relevant Michaelis-Menten parameters:

$$R = k_{cat}^{CO_2}[CO_2][EH_2O]/K_M^{CO_2} \quad M\ s^{-1}. \tag{1}$$

Here $[EH_2O]$ is the concentration of active enzyme and is related to the concentration of total enzyme by the factors that account for binding of substrate and the pK_a for activity. H is the velocity of water exchange and is given in terms of a presumed pH-independent quantity τ, the lifetime of a water molecule on the enzyme, by

$$H = [EH_2O]/\tau \quad M\ s^{-1}. \tag{2}$$

Under the experimental conditions used by Silverman and co-workers (1976, 1979), the enzyme concentration is a few nanomolar whereas $[HCO_3^-] + [CO_2]$ is kept constant at several millimolar. Thus substrate labeled with ^{18}O can bring the EH_2O molecules into isotopic steady state almost instantaneously, and without significant loss of label from the CO_2—HCO_3^- system. Assuming, for the moment, that only a small fraction f (the general case is considered in the Appendix) of the CO_2—HCO_3^- system is labeled with ^{18}O, the velocity at which $EH_2{}^{18}O$ molecules are created is

$$fR/3 \quad M\ s^{-1}. \tag{3}$$

That is, an $EH_2{}^{18}O$ is created in one out of three conversions of HCO_3^- into CO_2; the remaining times, the ^{18}O finds itself in the CO_2 product.

By contrast, $EH_2{}^{18}O$ molecules lose label by two mechanisms: exchange of water with solution contributes a term δH, and catalyzed conversion of CO_2 to HCO_3^-, with the subsequent replacement of the labeled water on the enzyme molecule with unlabeled water from solution, contributes a term δR. Thus the velocity at which $EH_2{}^{18}O$ molecules are lost is

$$\delta(R + H) \quad M\ s^{-1}. \tag{4}$$

Equating the steady-state loss and creation rates gives the steady state value of δ:

$$\delta = \frac{fR}{3(R + H)}. \tag{5}$$

This steady state is established very rapidly compared with the rate at which f changes with time, as already indicated.

The rate constant θ, defined as the velocity of loss of ^{18}O label from the CO_2—HCO_3^- system to solution, per unit concentration of total ^{18}O content in the system, is then

$$\theta \simeq \frac{\delta H}{f[S]} = \frac{RH}{3(R + H)[S]} \text{ s}^{-1} \tag{6}$$

where $[S] = [HCO_3^-] + [CO_2]$. In the experimental protocol of Silverman and co-workers (1976, 1979), $[S]$ is kept constant as pH is varied.

The rate constant ϕ, defined as the velocity of creation of doubly-labeled substrate when ^{18}O-labeled and ^{13}C-labeled substrate are introduced into a solution of carbonic anhydrase, per unit of labeled substrate, is given by

$$\phi \simeq \frac{\delta R}{f[S]} = \frac{\theta R}{H} = \frac{R^2}{3(R + H)[S]} \text{ s}^{-1} \tag{7}$$

since each catalytic encounter of a ^{13}C-labeled CO_2 with an ^{18}O-labeled enzyme produces doubly labeled HCO_3^-.

Note that summing Eqs. 6 and 7 gives

$$\theta + \phi = \frac{R}{3[S]} \text{ s}^{-1} \tag{8}$$

so that from the sum of the experimentally derived values of θ and ϕ one can obtain R, a quantity directly comparable to stopped-flow data, independently of the value of H. Moreover, it should be noted that, since R is proportional to $[CO_2]$ and H is not, the relative contributions of θ and ϕ to R depend on $[CO_2]$ and will vary with pH for fixed $[S]$ and with $[S]$ for fixed pH. Appreciation of this point greatly simplifies the application of the theory to the interpretation of the experimental data.

The expressions derived for θ and ϕ are approximate in that the assumption $[HCO_3^-] \gg [CO_2]$ was implicit in the derivation: it was assumed that ^{18}O is lost during dehydration, which is correct, but any replacement of ^{18}O to the HCO_3^- reservoir from the lower concentration of ^{18}O-labeled CO_2 was ignored. The approximate results are quite correct above pH 7. The exact result for θ, derived in the Appendix, is

$$\theta = \frac{RH}{3(R + H)[S] - R[CO_2]}. \tag{9}$$

Eq. 8 remains unaltered, so that the exact expression for ϕ may be readily obtained by subtracting Eq. 9 from 8. (These results are "exact" in that they treat the kinetics properly; there is a subsequent mathematical approximation made in approximating a square root, however, that contributes an error of at most 3%). Our expressions that relate our R and H to θ and ϕ are identical to those that relate R_1 and R_{H_2O} of Silverman et al. (1979) to θ and ϕ. Thus the numerical values derived from the data for R and H are the same as those for R_1 and R_{H_2O}. However, the relation of these parameters to the kinetic parameters of the enzyme are, as might be expected, model dependent.

It still remains to write the expression that relates $[EH_2O]$, the concentration of active enzyme, to the known concentrations of total enzyme $[E_T]$, substrates, and anionic inhibitor. In our model, (Koenig et al., 1980), water, HCO_3^-, and HSO_4^- compete for the same ligand of the metal ion, whereas CO_2 would be expected to bind noncompetitively, with a value for the dissociation constant K_{CO_2} that is insensitive to the particular ligand of the metal ion. The anions bind in association with a proton, the latter at a proton acceptor in the active site region. It then follows that

$$[EH_2O] = \frac{[E_T]}{\left[1 + \frac{[CO_2]}{K_{CO_2}}\right]\left[1 + \frac{[H^+][HCO_3^-]}{K_{HCO_3^-}} + \frac{[H^+][HSO_4^-]}{K_{HSO_4^-}}\right]} \text{ M} \qquad (10a)$$

where $K_{HCO_3^-}$ and $K_{HSO_4^-}$ are pH-independent equilibrium dissociation constants, with units of M^2. This expression will be discussed in greater detail below.

We stress here that the form of Eq. 10a is more restrictive than is necessary for the present purposes; it would be adequate to assume a pK_a for activity and use

$$[EH_2O] = \frac{[E_T]}{\left[1 + \frac{[CO_2]}{K_{CO_2}}\right]\left[1 + \frac{[H^+][HCO_3^-]}{K_{HCO_3^-}}\right]\left[1 + \frac{[H^+]}{K_a}\right]}. \qquad (10b)$$

Our conclusions would remain unaltered. In particular, the concentrations of $[HSO_4^-]$ and $[HCO_3^-]$ never become high enough to verify the competition implicit in Eq. 10a, and the data at low pH are so few that the (average) ratio $[HSO_4^-]/K_{HSO_4^-}$ may be replaced by the constant K_a^{1-} without altering the theoretical description of the data. However, Eq. 10a derives from situations where this form is indicated (Koenig et al., 1980; Jacob et al., 1980), and we use it here for consistency with this larger body of data.

RESULTS

In this section we analyze the data obtained by Silverman and co-workers (1976, 1979, and unpublished) for θ and ϕ for both bovine and human C native carbonic anhydrase. Data for θ and ϕ are given for both enzymes in aromatic cationic buffers and for the unbuffered human C enzyme as a function of pH for a single value of total substrate concentration; and as a function of total substrate concentration for a single value of pH. For unbuffered solutions of the bovine enzyme, only $\theta + \phi$ is considered.[1] All measurements were at 25°C. In all cases the ionic strength was maintained at 0.2 by addition of Na_2SO_4. The quoted compositions of all the samples are given in Table I.

The points in Fig. 1 *A* are the results for *R* for buffered bovine carbonic anhydrase obtained using Eq. 8. The solid line through the points is from a least-squares comparison of the data with Eqs. 1 and 10a. $[E_T]$, $[S]$, and $[SO_4^-]$ are known quantities. An estimate of the value for K_{CO_2} of 0.015 M was taken from the result of Pocker and Bjorquist (1977) for $K_M^{CO_2}$, leaving the three quantities $K_{HCO_3^-}$, $K_{HSO_4^-}$, and $k_{cat}^{CO_2}/K_M^{CO_2}$, which were treated as adjustable parameters. (The data are insufficient to allow a four-parameter fit, so that a value for K_{CO_2} must be assumed.) The binding of HCO_3^- at the concentration used, given the choice for K_{CO_2}, is too weak to yield a value for $K_{HCO_3^-}$ in the present case. The derived values of the other parameters are in Table I.

The dashed curve, Fig. 1 *A*, is the intrinsic enzymatic activity as a function of pH in the limit of zero concentration of substrate. In terms of our model it is given explicitly by

[1]Silverman informs us that there is a fundamental difficulty in obtaining consistent data from unbuffered samples. It appears that *H* but not $R + H$ is very sensitive to metal-ion contamination and aging effects, and Silverman cautions that these aspects may not have been adequately controlled in the data considered here.

$$\frac{k_{cat}^{CO_2}}{K_M^{CO_2}} \times \frac{1}{\left[1 + \frac{[H^+][HSO_4^-]}{K_{HSO_4^-}}\right]} \quad (M\,s)^{-1} \tag{11a}$$

and is directly comparable with results of stopped-flow measurements of the initial velocity of CO_2 hydration. In terms of an empirical pK_a, Eq. 11a could be replaced by

$$\frac{k_{cat}^{CO_2}}{K_M^{CO_2}} \times \frac{1}{\left[1 + \frac{[H^+]}{K_a}\right]} \quad (M\,s)^{-1}, \tag{11b}$$

with no alteration of our present conclusions. The pK_a for activity, defined as the pH at which the activity of the solution is one-half its value at high pH, is 6.08, indicative of a relatively pure enzyme preparation.

Fig. 1 *B* shows the data for θ and ϕ, combined in Fig. 1 *A* to yield a value for R, plotted separately. The solid lines in Figure 1 *B* are the results of a least-squares comparison of Eqs. 6 and 7 with the data, with H, or equivalently τ, as the only unknown parameter. The values of the others are known from the fit, Fig. 1 *A*. The result is $\tau = 0.51$ μs.

The points in Fig. 1 *C* are for θ and ϕ as a function of $[S]$ at pH 6.8, for samples otherwise identical to those of Figs. 1 *A* and 1 *B*. In principle, we have sufficient information to predict

TABLE I
CHARACTERISTICS OF THE SAMPLES, AND RESULTS OF ANALYSES OF MEASUREMENTS ON THESE SAMPLES, AS SHOWN IN THE FIGURES

		Characteristics of samples					Values derived from the present analyses				
Sample	Figure(s)	Buffer	$[E_T]$	$[S]$	pH	K_{CO_2}	pK_a^*	$K_{HCO_3^-}$	$K_{HSO_4^-}$	$k_{cat}^{CO_2}/K_M^{CO_2}$	τ
		mM	*nM*	*mM*		*mM*		$(M)^2 \times 10^9$	$(M)^2 \times 10^{12}$	$(M\,s)^{-1} \times 10^{-8}$	μs
Bovine‡	1 *A,B*	25	1.9	10	*v*§	15‖	6.1	¶	4.6	2.7	0.51**
Bovine‡	1 *C*	25	1.9	*v*	6.8	15					0.29
Bovine‡	2	0	2.0	10	*v*	15	5.6	8.2	0.44‡‡	1.9	5.7**
Human C§§	3 *A,B*	50	1.6	15	*v*	8.3‖‖	6.1	2.6	3.5	2.0	0.37**
Human C§§	3 *C*	50	1.2	*v*	7.4	8.3					1.4
Human C§§	4 *A,B*	0	1.6	15	*v*	8.3	6.0	2.4	7.8	2.2	¶¶
Human C§§	4 *C*	0	1.2	*v*	7.4	8.3					6.3

Buffer, when present, is imidazole, and all solutions contain 0.066 M SO_4^{2-} · $[S] = [CO_2] + [HCO_3^-]$ is the concentration of total substrate used and $[E_T]$ is the concentration of enzyme. All results are for 25°C. Blank spaces indicate that results from the analysis of Figs. 1*A*, 2, 3*A*, or 4*A* were used in each case.

*From Figs. 1*A*, 2, 3*A*, or 4*A*.

‡Data for the bovine enzyme are largely unpublished, and were supplied by Silverman (cf. also Silverman and Tu, 1976).

§*v*, variable parameter.

‖From Pocker and Bjorquist (1977).

¶The value is too large to be obtained from the available data.

**From Figs. 1*B* or 3*B*, and for the unbuffered bovine enzyme, from data not illustrated.

‡‡The uncertainty of this value is very large because of the low value of pK_a.

§§Data for the human C enzyme are from Silverman *et al.* (1979).

‖‖From Steiner *et al.* (1975).

¶¶No analysis possible (see text).

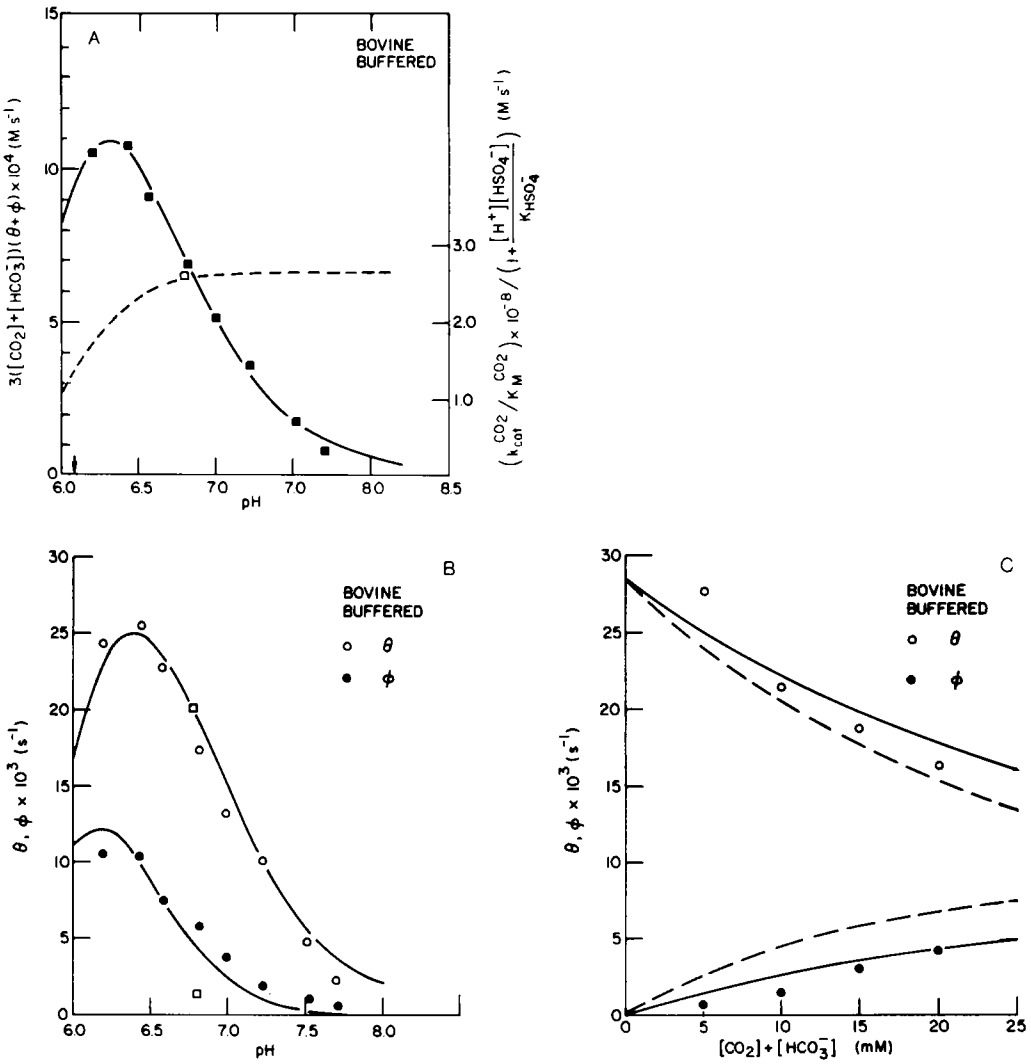


FIGURE 1 Results for buffered bovine carbonic anhydrase. The data are those of Silverman and Tu (1976 and unpublished). (*A*) The filled squares are results for $R = 3[S](\theta + \phi)$ obtained from the experimental data for θ and ϕ. The open square is from another run, Fig. 1*C*, corrected for differences in total enzyme concentration. The solid line is from a three-parameter least-squares comparison of the experimental results with Eqs. 1 and 10. The dashed curve is the predicted value of a quantity that should be directly comparable to $k_{cat}^{CO_2}/K_M^{CO_2}$ as obtained from initial catalytic velocities measured in stopped-flow experiments. The arrow at the base line near pH = 6 indicates the pK_a for the dashed curve. (*B*) The open and filled circles are the experimental results for θ and ϕ, respectively, as a function of pH; the squares are values from Fig. 1*C*, corrected as above. The solid lines result from a least-squares determination of the single parameter τ, the lifetime of a water molecule ligand of the metal ion of the enzyme, that best fits these data, using Eqs. 2, 6, 7, and values for other parameters obtained from the fit in Fig. 1 *A*. (*C*) Is analogous to Fig. 1 *B*, except that the results are a function of total substrate concentration rather than pH. Information regarding samples, and results of the comparisons of experiment and theory are all collected in Table I.

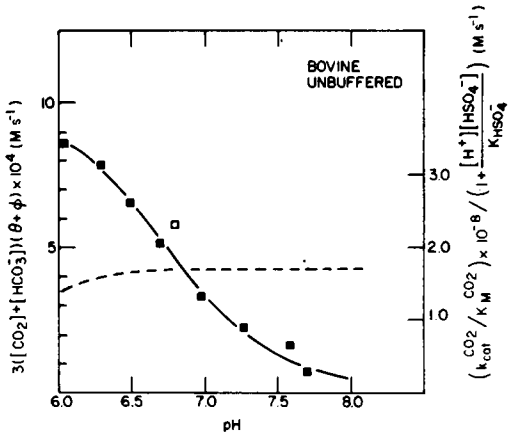


FIGURE 2 Results for R for unbuffered bovine carbonic anhydrase.[1] Other than for the absence of buffer, all comments relating to Figure 1 A apply. The pK_a of 5.6 for the dashed curve was too low to indicate.

these results; the predictions are the dashed curves that, although they represent the data qualitatively, do not agree all that well. The problem appears to be that the data for $\theta + \phi$, Fig. 1 A, has considerably less scatter than the data for θ and ϕ considered separately. This is seen when data from Fig. 1 C are replotted in Fig. 1 A and 1 B, as shown by the open squares. The values of ϕ are relatively small, and the fractional uncertainties are large. Therefore, we derived the value of τ that best fits the data of Fig. 1 C, again using for the other parameters the values derived from the data of Fig. 1 A. The result is $\tau = 0.29$ μs, and the fit is indicated by the solid curves.

Fig. 2 contains data for unbuffered bovine carbonic anhydrase,[1] but is otherwise analogous to Fig. 1 A. The main point to note is that enzymatic activity as indicated by $\theta + \phi$ is rather insensitive to the presence of buffer.

Fig. 3 A–C are results for buffered samples of human C carbonic anhydrase and are in all ways analogous to the results for the bovine enzyme in Fig. 1 A–C. The data are from the recent work of Silverman et al. (1979).

Fig. 4 A–C are analogous results for unbuffered samples of the human C enzyme[1] (Silverman et al., 1979). Though the data in Fig. 4 A and C are amenable to the same analysis used in the previous figures, those of Fig. 4 B are not. There appears to be a problem with the partitioning of $\theta + \phi$ for the data for pH < 6.8 where, to be consistent with the concentration-dependent data, Fig. 4 C, on almost any model, the results for θ and ϕ should have crossed. We have not included a fit to these data.[1]

Again, the compositions of the samples and the results of the comparisons of data and theory are in Table I. As was found for the bovine enzyme, the sum $\theta + \phi$, proportional to enzymatic activity, is essentially independent of buffer, whereas the ratio θ/ϕ, proportional to the product of enzymatic activity and water lifetime, depends very strongly on buffer concentration. Additionally, the parameters that characterize the two enzymes are quite close in numerical values.

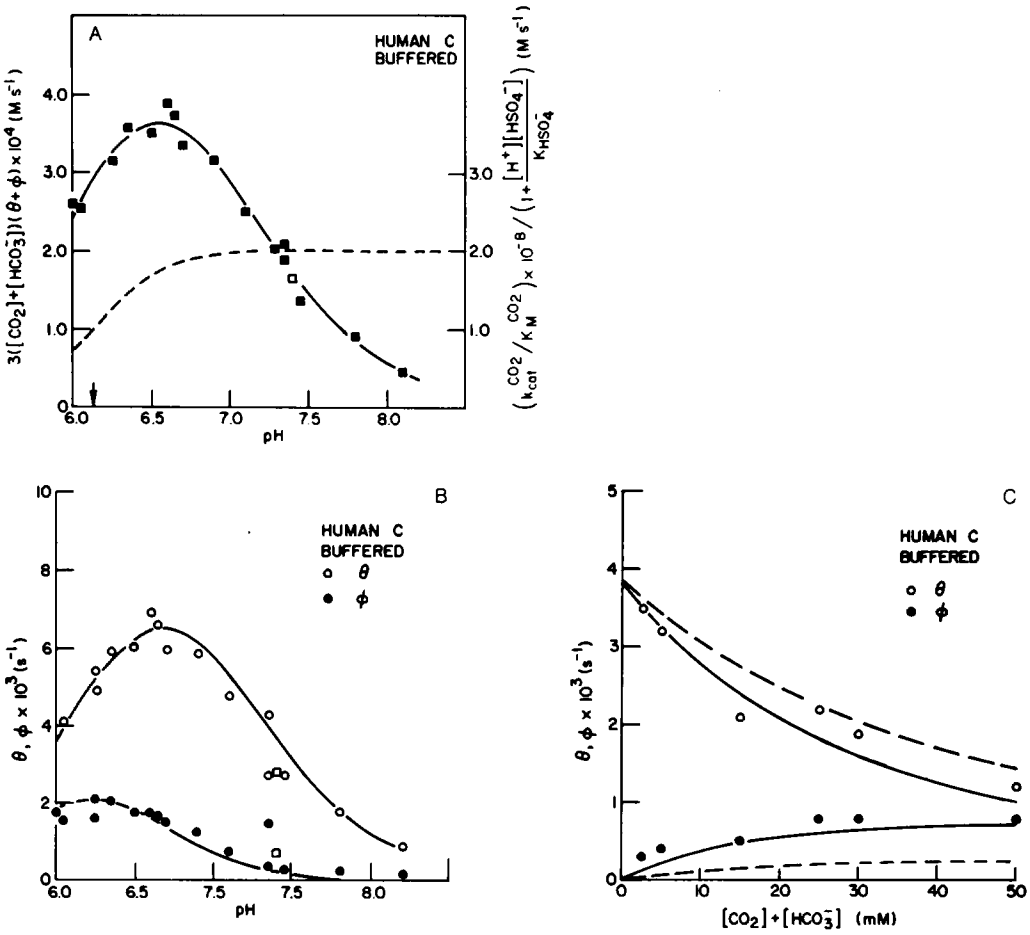


FIGURE 3 Results for buffered human C carbonic anhydrase. The data are those of Silverman et al. (1979). Other than the difference in the source of enzyme, all comments relating to Fig. 1 *A–C* apply.

DISCUSSION

General Considerations

The most significant conclusion from the foregoing is that Scheme I, the essence of which is that active enzyme (active equally for hydration and dehydration at a given pH) has a water molecule as ligand of the active-site metal ion, can account in a straightforward manner for the results of the isotope-mixing and isotope-loss experiments of Silverman and co-workers (1976, 1979, and unpublished data). As noted above and elsewhere (Jacob et al., 1978, 1980; Koenig et al., 1980), this Scheme is the only one that can explain the fairly extensive solvent proton magnetic relaxation data (Wells et al., 1979; Jacob et al., 1978; and Fabry et al., 1970) that now exist, without the introduction of *ad hoc* assumptions that have no experimental basis. Thus, it is clear that the experimental results analyzed here cannot be used as an

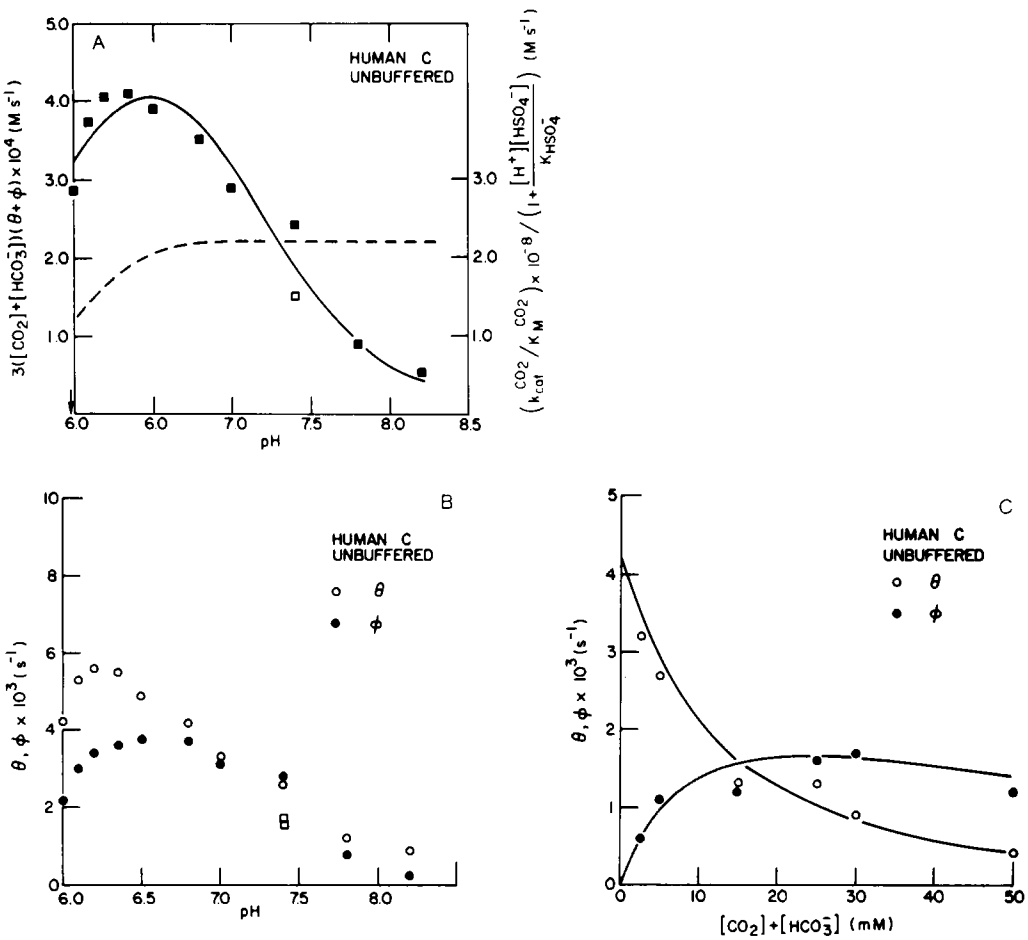


FIGURE 4 Results for the unbuffered human C enzyme.[1] The data are those of Silverman et al. (1979). Other than the difference in source of enzyme and absence of buffer, all comments relating to Figs. 1 *A–C* apply. Additionally, we believe there is a problem with the data in Fig. 4*B* (see text); though the sum $\theta + \phi$ behaves properly (Fig. 4*A*) as do the variations of θ and ϕ with concentration (Fig. 4*C*), almost any model consistent with the results of Figs. 4 *A* and 4 *C* would predict a crossing of θ and ϕ when the pH is lowered below ~7. Moreover, it is not simply that the data points are interchanged.

argument in support of the "hydroxide mechanism" of hydration activity (Silverman et al., 1979), nor of a pH-dependent off-rate for a water molecule on carbonic anhydrase; the data can be accounted for equally well, and indeed in greater detail, by the present considerations.

On the basis of our analysis we find that the pK_a for activity is in all instances no greater than 6.1. We have previously considered the factor that determines this pK_a (Koenig et al., 1980) and concluded that it relates to inhibition of activity due to binding of HSO_4^-. Our prediction would be that reduction of the concentration of Na_2SO_4 added to maintain ionic strength would lower this pK_a. In terms of the experimental observations themselves, without resorting to application of our model to derive this pK_a, such a shift would be observed as a shift in the peak of the data for $\theta + \phi$ to a lower value of pH.

An interesting aspect of the present analysis is the conclusion that addition of aromatic buffers (2,4-lutidine; 3,5-lutidine; and imidazole) drastically reduces the magnitude of τ. This is a quantity that is very difficult to measure, and indeed we are unaware of other experiments that can yield a value for τ. Magnetic relaxation experiments set an upper limit (Koenig and Brown, 1972) for τ of ~ 10 μs, a value quite consistent with the results of the present analysis (cf. Table I). Line-broadening experiments, such as that of Koenig et al. (1973, 1974) and Simonsson et al. (1979) in which the rate of interconversion of carbon dioxide and bicarbonate are measured independently of the rate of dissociation of the co-substrates H^+ and H_2O from the enzyme, give no information at all about τ. Traditional stopped-flow experiments also yield little information about τ unless conditions can be found for which τ is rate limiting. Thus, given the foregoing deductions regarding the influence of aromatic buffers on the pH-independent parameter τ, some speculations are in order.

We have recently concluded an investigation of the interaction of the small aromatic neutral molecules aniline and phenol with bovine carbonic anhydrase (Jacob et al., 1980). These results, as well as related results of other investigators, can all be understood if these aromatic molecules bind at the aromatic binding site to which sulfonamide inhibitors attach, even for apo-enzyme (King and Burgen, 1970). Once bound, they influence the properties of the enzyme because the polar substituents on the phenol ring of the small neutral molecules alter the relative affinities of the metal ion for water and anions. The dissociation constants are of order 0.01–0.02 M, not much different from the value of 0.005 M that Silverman et al. (1979) report for the influence of imidazole on ϕ (cf. their Fig. 4). It is thus tempting to ascribe the influence of the aromatic buffers on τ, not to their buffering properties, but to binding of the neutral (high-pH) form of the buffer molecules to the enzyme, at the aromatic binding site. Perhaps their hydrophobic character enhances the leaving rate of water molecules from the active site. In any event, it is now possible to interpret in detail, according to the present model, interactions that have been difficult to probe in the past. It remains to develop a corpus of data on the influence of these aromatic molecules on the catalyzed mixing of isotopically labeled substrates.

Quantitative Considerations

BOVINE ENZYME The experiments of Silverman and co-workers (1976, 1979) are done at chemical equilibrium (though not iostopic equilibrium) and as such the results are not complicated by questions regarding the rate of transport of protons to and from the enzyme, a problem in the interpretation of stopped-flow kinetic experiments (cf. Lindskog, in press) ever since the early work of DeVoe and Kistiakowsky (1961). Because of possible rate limitations set by proton transport, results for $k_{cat}^{CO_2}/K_M^{CO_2}$ from kinetic measurements represent a lower limit to the true value of this parameter. This situation appears to be confirmed by the present analysis. Thus, for the bovine enzyme, the average of the results in Table I give $k_{cat}^{CO_2}/K_M^{CO_2} = 2.3 \pm 0.4 \times 10^8\ (M\ s)^{-1}$, whereas comparable results from stopped-flow measurements are the (buffer-dependent) value of $5 \times 10^7\ (M\ s)^{-1}$ reported by Pocker and Bjorquist (1977), $4 \times 10^7\ (M\ s)^{-1}$ obtained using Magid's results (1973) and the Haldane relation, and $1.4 \times 10^8\ (M\ s)^{-1}$ reported by Khalifah (1971).

Values for the dissociation constants are a bit more complex to analyze, partly because the parameters that we have defined, $K_{HCO_3^-}$ and $K_{HSO_4^-}$, are pH-independent equilibrium

quantities that underlie the pH-dependent parameters that are usually considered. Additionally, though pH-independent, their values are buffer dependent (Koenig et al., 1980), a fact that explains the often observed buffer-dependence of the Michaelis constant for dehydration. In any event, the value for the equilibrium dissociation constant $K_{HCO_3^-}$ of 8.2×10^{-9} M^2 found for unbuffered bovine enzyme may be compared with the value 2.5×10^{-9} M^2 that can be inferred from the steady-state data of Magid (1973). The dissociation of HCO_3^- in the presence of buffer, on the other hand, is too great for a value to be obtained from the available equilibrium data; the binding of substrate, with the assumption that $K_{CO_2} \simeq K_M^{CO_2}$, is dominated by CO_2, a phenomena not encountered in stopped-flow dehydration experiments. The value for $K_{HCO_3^-}$ is similar to that obtained for Cl^- in the absence of buffer (Koenig et al., 1980), 7.2×10^{-9} M^2, consistent with the approximately similar positions they occupy in the Hofmeister lyotropic sequence (Koenig et al., 1980; Fridovich, 1963). For the data for both the buffered and unbuffered bovine enzyme, the substrate concentration is never great enough to verify the lack of competition between CO_2 and HCO_3^-, indicated in Eq. 10.

The values obtained here for $K_{HSO_4^-}$ of 0.44×10^{-12} and 4.6×10^{-12} M^2 for unbuffered and buffered bovine enzyme, respectively, may be compared with the (interpolated) value of 0.5×10^{-12} M^2 obtained for unbuffered bovine enzyme by Koenig et al. (1980). This last result was obtained from the pH-dependence of the optical spectrum of the Co^{2+}-substituted enzyme for various values of $[SO_4^{2-}]$. The excellent agreement of the two values for the unbuffered enzyme, derived from totally different experiments, is further support both for the role of HSO_4^- as the determinant of the pK_a for activity, and for the view that the titration behavior of enzymatic activity and optical spectra are correlated under all circumstances (Koenig et al., 1980; Jacob et al., 1980).

HUMAN C ENZYME For the human C enzyme, in addition to the comparison with stopped-flow results, it is possible to compare the values derived here for the kinetic parameters with results of other equilibrium experiments. Simonsson et al. (1979) have measured the kinetic parameters for dehydration of HCO_3^- at equilibrium using magnetic resonance methods. Indeed, Silverman et al. (1979) have used these results to predict values for R_1 (akin to our R, and related to k_{cat}/K_M) and have shown that the results of the two types of equilibrium experiments are mutually consistent. However, they never derived numerical values for the kinetic parameters θ and ϕ, and did not attempt to compare values for the dissociation constants of substrate. We include these comparisons here since they relate to the form of the denominator of Eq. 10a, and give some insight into how different the equilibrium constants may be from the analogous Michaelis constants.

From the results in Fig. 3 *A–C* and 4 *A–C*, we obtain the (average) value $k_{cat}^{CO_2}/K_M^{CO_2} = 2.1 \times 10^8$ $(M\ s)^{-1}$. Simonsson et al. (1979) measured the equivalent parameter for dehydration. When we take their value at pH = 7.1 (one pH-unit above the pK_a for the CO_2—HCO_3^- equilibrium) and apply the Haldane relation, the result for $k_{cat}^{CO_2}/K_M^{CO_2}$ is 1.1×10^8 $(M\ s)^{-1}$. Steiner et al. (1975) report the value 1.2×10^8 $(M\ s)^{-1}$ from stopped-flow hydration measurements, and Khalifah (1971) reports 1.3×10^8 $(M\ s)^{-1}$. These all agree reasonably well.

The basis for Eq. 10a is the assertion that CO_2 and HCO_3^- bind noncompetitively to carbonic anhydrase. That this is so may be inferred from the results of Khalifah (1971) who finds that $K_M^{CO_2}$ is the same above and below the pK_a for activity of the enzyme. In our terms,

this means that CO_2 binds equally well when either water, an inhibiting anion, or HCO_3^- is a ligand of the metal. Moreover, because binding of HCO_3^- is proportional to $[H^+]$, and the product $[H^+][HCO_3^-]$ is proportional to CO_2, the relative amount of bound CO_2 and HCO_3^- is independent of pH. The total that is bound in these experiments, of course, decreases as pH is increased, since $[CO_2] + [HCO_3^-]$ is held constant, and the pK_a for the CO_2—HCO_3^- equilibrium (Harned and Bonner, 1945) is 6.1. It was pointed out above that the binding of HCO_3^- to the buffered bovine enzyme is small compared with that of CO_2 for the conditions considered and, indeed, it is clearly small for all conditions. Thus the issue of competition does not arise. By contrast, for the buffered human C enzyme, $K_{HCO_3^-} \simeq 2.6 \times 10^{-9}$ M^2, so that at pH 6.1, the value at which $[CO_2] = [HCO_3^-]$, the bicarbonate dissociation constant is 3.3 mM, to be compared with the value 8.3 mM used for K_{CO_2}. (We have equated the Michaelis constant and the equilibrium dissociation constant for CO_2 in order to reduce the number of adjustable parameters, but the question arises as to whether this procedure will have a substantive effect on the arguments.) Thus, for the human C enzyme, HCO_3^- binding will dominate over CO_2 binding by a factor of ~2.5 under all conditions. Moreover, at pH 7.1, 33 mM HCO_3^- will half-saturate the enzyme. However, Simonsson et al. (1979) (cf. their Fig. 2) went as high as 0.2 M HCO_3^- at this pH, meaning that the enzyme molecules, on the basis of the above, should have been saturated with both HCO_3^- and CO_2, at least on the assumption of noncompetition between the two substrates. They found at most partial saturation at this concentration of substrate. The implication of this follows.

Stopped-flow data are not (usually) complicated by the presence of product, in this case CO_2, so that a comparison of the Michaelis constant $K_M^{HCO_3^-}$ with the dissociation constant computed from $K_{HCO_3^-}$ at a particular pH should be meaningful. The data of Steiner et al. (1975) at pH = 7.4, the only measurement at a pH well above the pK_a for activity, give $K_M^{HCO_3^-}$ = 0.032 M, and those of Magid (1971) give $K_M^{HCO_3^-}$ = 0.045 M. These agree very well, considering the differences in buffer composition and other variables. From Table I, for the buffered enzyme, $K_{HCO_3^-} = 2.6 \times 10^{-9}$ M^2, and dividing by $[H^+]$ gives 0.065 M for the equilibrium dissociation constant at pH = 7.4. This value for the equilibrium dissociation constant of HCO_3^-, derived from the equilibrium experiments of Silverman et al. (1979), and assuming noncompetitive binding of CO_2 and HCO_3^-, i.e., Eq. 10a, is in excellent agreement with the two Michaelis constants above. By contrast, the value derived by Simonsson et al. (1979) from the equilibrium experiments, assuming competitive binding of CO_2 and HCO_3^-, is 0.16 M at pH 7.4. The one way that we can see to reconcile these differences is if K_{CO_2}, which we took as equal to $K_M^{CO_2}$, is in fact greater by a small factor. However to investigate this point further will require more data, with greater concentrations of substrate at lower values of pH to enhance the conditions under which competition of CO_2 and HCO_3^- should be observable.

SUMMARY

We have shown that the extensive studies by Silverman and co-workers (1976, 1979) of the catalyzed mixing and loss of isotopic label within and from the CO_2—HCO_3^- system at chemical equilibrium can be interpreted in terms of a model for carbonic anhydrase that has a water molecule ligand at the active-site metal ion when the enzyme is active. The interpreta-

tion is straightforward and yields, for the first time, values for the residence lifetime of these water molecules. These lifetimes are pH-independent, in contrast to the conclusions of Silverman et al. (1979) who, however, never quoted a value for the lifetime itself. The lifetimes become shorter in the presence of aromatic buffers, and we have suggested that this effect is more related to the binding of the neutral form of the buffers to the known aromatic binding site of the enzyme than to the buffering properties of the buffers. The advantage of the model used here is that it also, in an equally straightforward manner, explains the solvent proton relaxation data for the Co^{2+}-substituted enzyme, as well as a wealth of other observations (Koenig et al., 1980; Jacob et al., 1980), whereas the much discussed alternate view, that the ligand is an OH^-, has difficulty explaining these data, and other data as well. Finally, we have addressed the interpretation of the magnetic resonance studies of ^{13}C linewidths of labeled substrates (Simonsson et al., 1979), experiments also performed at chemical equilibrium, and shown how the results may be included in our global view of the nature of the active site of carbonic anhydrase.

APPENDIX

In this section we derive expressions for the measured quantities θ and ϕ in terms of the kinetic properties of the enzymes. Though the equations appear quite analogous to those derived by Silverman et al. (1979), subtle distinctions lead to opposite conclusions regarding the off-rate of water molecules from carbonic anhydrase. Therefore we present the following in some detail, and rigorously connect defined quantities with measured quantities so that the source of differences between our conclusions and others (Silverman et al., 1979) is clearly indicated.

Following the notation of Silverman et al. (1979), and the earlier procedures of Mills and Urey (1940), we define the experimentally observable variables $^{12}\alpha$, $^{13}\alpha$, $^{12}\gamma$, $^{13}\gamma$, and δ respectively as the fraction of oxygen nuclei in all the ^{12}C-containing CO_2 molecules that are ^{18}O; the analogous fraction for ^{13}C-containing CO_2 molecules; the fraction of the oxygen nuclei in all the ^{12}C-containing HCO_3^- anions that are ^{18}O; the analogous fraction for ^{13}C-containing HCO_3 anions; and the fraction of active enzyme molecules that are $EH_2{}^{18}O$. Further, we let r be the constant fraction of HCO_3^- anions that contains ^{13}C. Using Scheme I, it is straightforward to derive the following rate equations that describe the time-dependence of these five variables:

$$\frac{d}{dt}{}^{12}\alpha = -\frac{R}{[CO_2]}({}^{12}\alpha - {}^{12}\gamma) \tag{A1}$$

$$\frac{d}{dt}{}^{13}\alpha = -\frac{R}{[CO_2]}({}^{13}\alpha - {}^{13}\gamma) \tag{A2}$$

$$\frac{d}{dt}{}^{12}\gamma = -\frac{R}{3[HCO_3^-]}(-2{}^{12}\alpha + 3{}^{12}\gamma - \delta) \tag{A3}$$

$$\frac{d}{dt}{}^{13}\gamma = -\frac{R}{3[HCO_3^-]}(-2{}^{13}\alpha + 3{}^{13}\gamma - \delta) \tag{A4}$$

$$\frac{d}{dt}\delta = \frac{R}{[EH_2O]}[{}^{12}\gamma(1 - r) + {}^{13}\gamma r] - \frac{(R + H)}{[EH_2O]}\delta. \tag{A5}$$

Here $[CO_2]$ is the sum of the concentrations of CO_2 molecules of all isotopic compositions; $[HCO_3^-]$ the analogous sum for HCO_3^-, and $[EH_2O]$ that for active enzyme. With the significant exception of the

appearance of $[EH_2O]$ rather than [EOH] in Eq. A5, these five simultaneous differential equations are identical in form to Eqs. 5–9 of Silverman et al. (1979). Writing $[EH_2O]$ in Eq. A5 is consistent with our Scheme I, in which active enzyme is active for both directions of catalysis and inactivated enzyme (in our view, HSO_4^--inhibited) is inactive for both directions.

The problem at hand is to solve these five equations: i.e., to find the five characteristic exponential decays and the combinations of the five variables that correspond to each; to describe the time-dependence of the five variables in terms of the five exponential solutions, in principle subject to the initial conditions of the experiments; to relate these to the measured quantities; and, finally, to identify which of the five decay constants to identify with θ and ϕ.

The key to the solution is to realize that $[EH_2O] \sim 10^{-9}$ M, whereas $[CO_2]$ and $[HCO_3^-]$ are of order 10^{-3} M. Using this fact, it is clear, from comparing Eq. A5 with Eqs. A1–A4, that $(d\delta/dt)$ is about 10^6-fold faster than the time derivatives of the other variables. Physically this means that the substrate, present in such large concentrations compared with enzyme, brings the enzyme molecules into isotopic steady state essentially instantaneously compared with the rates at which isotopic equilibrium is attained within the CO_2—HCO_3^- substrate system, and at which isotopic label is lost to solvent. Thus, on the time scale of changes in substrate isotopic composition, which is the time-dependence that we seek, the enzyme composition has settled and we can set $d\delta/dt = 0$, reducing Eq. A5 from a differential to an algebraic equation:

$$\delta = \rho({}^{12}\gamma(1 - r) + {}^{13}\gamma r) \tag{A6}$$

where for convenience we define $\rho \equiv R/(R + H)$. The initial rate at which δ changes can also be obtained from Eq. A5 by inserting the boundary condition that ${}^{13}\gamma_0 = 0$ initially and ${}^{12}\gamma_0 = s$, the fraction of ^{13}C added initially. The rate constant for this decay, λ_5, is clearly

$$\lambda_5 = \frac{s(1 - r)R - (R + H)}{[EH_2O]}. \tag{A7}$$

Eq. A6 can be substituted into Eqs. A1–A4. However, it is convenient to first define four new variables

$$\alpha_- = {}^{12}\alpha - {}^{13}\alpha \tag{A8}$$

$$\alpha_+ = (1 - r){}^{12}\alpha + r{}^{13}\alpha \tag{A9}$$

$$\gamma_- = {}^{12}\gamma - {}^{13}\gamma \tag{A10}$$

$$\gamma_+ = (1 - r){}^{12}\gamma + r{}^{13}\gamma \tag{A11}$$

α_+ and γ_+ are, by definition, the fractions of ^{18}O nuclei in all the isotopic forms of CO_2 and HCO_3^- respectively, when the ^{13}C is uniformly distributed between CO_2 and HCO_3^-. Once this state is achieved, one might expect (correctly) that it would decay as a single exponential with decay constant θ. Similarly, α_- and γ_- are the differences in ^{18}O composition between ^{12}C- and ^{13}C-containing CO_2 and HCO_3^- species, respectively, and one might expect that these differences would approach zero as a single exponential decay. This is indeed correct, and the decay constant will be shown to $\theta + \phi$; the difference will decrease from both isotopic mixing and ^{18}O loss.

After substituting Eq. A6 into Eqs. A1–A4, and these in turn into Eqs. A8–A11, one obtains

$$\frac{d}{dt}\alpha_+ = -\frac{R}{[CO_2]}(\alpha_+ - \gamma_+) \tag{A12}$$

$$\frac{d}{dt}\alpha_- = -\frac{R}{[CO_2]}(\alpha_- - \gamma_-) \tag{A13}$$

$$\frac{d}{dt}\gamma_+ = -\frac{R}{3[HCO_3^-]}[(3-\rho)\gamma_+ - 2\alpha_+] \tag{A14}$$

$$\frac{d}{dt}\gamma_- = -\frac{R}{3[HCO_3^-]}(3\gamma_- - 2\alpha_-). \tag{A15}$$

Since these four equations are uncoupled pairwise, their solution becomes trivial. We first consider the pair, Eqs. A12 and A14, that describe the variation of ^{18}O distribution independent of the fraction of ^{13}C. Imposing an exponential time-dependence on the solutions for α_+ and γ_+, with decay constant λ, gives the homogeneous algebraic equations

$$\left(\lambda - \frac{R}{[CO_2]}\right)\alpha_+ + \frac{R}{[CO_2]}\gamma_+ = 0 \tag{A16}$$

$$\frac{2R}{3[HCO_3^-]}\alpha_+ + \left(\lambda - \frac{(3-\rho)R}{3[HCO_3^-]}\right)\gamma_+ = 0. \tag{A17}$$

In the usual fashion, for a self-consistent solution of two such homogenous equations (i.e., with zeros on the right hand side), the determinant of the coefficients must be zero. Thus

$$\begin{vmatrix} \lambda - \dfrac{R}{[CO_2]} & \dfrac{R}{[CO_2]} \\ \dfrac{2R}{3[HCO_3^-]} & \lambda - \dfrac{(3-\rho)R}{3[HCO_3^-]} \end{vmatrix} = 0, \tag{A18}$$

which leads to the quadratic equation for λ

$$\left(\lambda - \frac{R}{[CO_2]}\right)\left(\lambda - \frac{(3-\rho)R}{3[HCO_3^-]}\right) - \frac{2R^2}{3[CO_2]\,[HCO_3^-]} = 0. \tag{A19}$$

The two solutions are

$$\lambda_\pm = \frac{3R(R+H)\,([CO_2] + [HCO_3^-]) - R^2[CO_2]}{6[CO_2]\,[HCO_3^-]\,(R+H)} \times [1 \mp (1-D)^{1/2}] \tag{A20}$$

where

$$D = \frac{12H(R+H)\,[CO_2]\,[HCO_3^-]}{\{3(R+H)\,([CO_2] + [HCO_3^-]) - R[CO_2]\}^2} \tag{A21}$$

As Silverman et al. (1979) pointed out (in connection with another solution) the term D is always sufficiently less than unity such that $(1-D)^{1/2}$ can be approximated by $1 - (D/2)$ with a maximum error of ~3%. Then

$$\lambda_- = \frac{3R(R+H)\,([CO_2] + [HCO_3^-]) - R^2[CO_2]}{2[CO_2]\,[HCO_3^-]\,(R+H)} \tag{A22}$$

$$\lambda_+ = \frac{RH}{3(R+H)\,([CO_2] + [HCO_3^-]) - R[CO_2]}. \tag{A23}$$

The latter is identical to Eq. 11 of Silverman et al. (1979), which they identify with θ, though this identification is not obvious from the foregoing as yet. However, it is not difficult to conclude, for the

conditions of the isotope mixing and loss experiments, that $\lambda_- \gtrsim 10\lambda_+$ so that after a time equal to a small multiple of $1/\lambda_-$, both α_+ and γ_+, the fractions of ^{18}O in all CO_2 and HCO_3^-, respectively, will both be observed to decay with the smaller rate constant λ_+. Silverman and co-workers measure α_+ (α in their notation) and define θ as its long term decay rate. Therefore, in agreement with the assertion of Silverman et al. (1979), we can make the identification $\theta = \lambda_+$. Similarly, the pair of variables α_- and γ_-, Eqs. A13 and A15, give the determinant

$$\begin{vmatrix} \lambda' - \dfrac{R}{[CO_2]} & \dfrac{R}{[CO_2]} \\ \dfrac{2R}{3[HCO_3^-]} & \lambda' - \dfrac{R}{[HCO_3^-]} \end{vmatrix} = 0, \tag{A24}$$

which only differs from Eq. A18 by the term containing ρ. The two roots that determine the time dependence of both α_- and γ_- are

$$\lambda_-' = R\left(\frac{1}{[CO_2]} + \frac{1}{[HCO_3^-]}\right) \tag{A25}$$

$$\lambda_+' = \frac{R}{3([CO_2] + [HCO_3^-])}. \tag{A26}$$

The result λ_+' is identical to Eq. 13 of Silverman et al. (1979) which they associate with $\theta + \phi$. Again this is not yet obvious from the foregoing. Nonetheless, we have that both α_e and γ_- decay with the same rate constants with $(\lambda_-'/\lambda_+') \geq 12$. The equal sign holds at pH 6.1 when $[CO_2] = [HCO_3^-]$; as the pH is increased, the ratio increases very rapidly so that measurements may readily be made in conditions where λ_+' predominates (which amounts to waiting for ^{18}O isotopic equilibrium to occur within the CO_2—HCO_3^- system, which is already at chemical equilibrium).

The experimentally determined quantities are $^{12}\alpha$, $^{13}\alpha$, and α_+ from which $(^{12}\alpha - {}^{13}\alpha) \equiv \alpha_-$, $(^{12}\alpha - \alpha_+)$, and $(\alpha_+ - {}^{13}\alpha)$ are computed. From Eqs. A24 and A25, α_- varies as the sum of two exponential decays; if sufficient time is allowed for the more rapid one to die off, then $^{12}\alpha - {}^{13}\alpha$ will decay as λ_+' which, as noted, is identical to what Silverman et al. (1976) call $\theta + \phi$ and define as the longer decay of $^{12}\alpha - {}^{13}\alpha$. This justifies equating λ_+' with the experimental values derived for $\theta + \phi$ from the variation of $^{12}\alpha - {}^{13}\alpha$.

Furthermore, from the definitions of α_- and α_+, in Eqs. A8 and A9, we can show that

$$^{12}\alpha - \alpha_+ = r\alpha_- \tag{A27}$$

and

$$\alpha_+ - {}^{13}\alpha = (1 - r)\,\alpha_-. \tag{A28}$$

Thus both these quantities, readily derived from experiment, vary with time as does α_- once the slower decay, λ_+', dominates, as noted by Silverman and Tu (1976). This rate, by definition of the experimental procedures, is $\theta + \phi$, so that

$$\theta + \phi \equiv \lambda_+' = \frac{R}{3([CO_2] + [HCO_3^-])}. \tag{A29}$$

Thus we have found all the five rate constants and shown rigorously how to associate the experimentally derived values for θ and $\theta + \phi$ to the parameters R and H of the model in Scheme I. We note in passing that ϕ itself is a bit artificial since no quantity ever varies in time with a decay constant equal to ϕ. It would seem more reasonable to define another quantity, say ϕ', equal to $\theta + \phi$; θ and ϕ' would be the two characteristic decay constants that describe the data most simply.

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