WATER EXCHANGE AT THE ACTIVE SITE OF CARBONIC ANHYDRASE

A Synthesis of the OH- and H_2O-Models

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ABSTRACT We have measured the paramagnetic contribution to the magnetic relaxation rate of solvent protons in highly purified, buffer- and salt-free solutions of Co^{2+}-substituted human carbonic anhydrase B (HCAB), as a function of pH in the range 5.5–10 and as a function of magnetic field. We have also measured the optical absorption at 640 nm to characterize the enzyme. The relaxation rates vary with pH much as does the CO_2 hydration activity, increasing with increasing pH. We find that the relaxation rates at all intermediate values of pH can be described as linear combinations of the rates obtained at the extremes of pH used, indicating the existence of low- and high-pH forms of the enzyme with pH-dependent concentrations. The optical data can be similarly represented. The fraction of high-pH form present, determined from either the relaxation or optical data, has a pK_a of ~7.6 when approximated by a single ionization. The data are very similar to that for HCAB in the presence of buffer, in contrast to the bovine enzyme for which the pK_a is affected substantially by the presence of sulfate. Previous analysis of the high relaxation rates at high pH indicated rapid exchange of Co^{2+}-liganded protons, possible only if these exchanging protons were conveyed by water molecules. On the other hand, the present demonstration of the existence of two forms of HCAB in highly purified solutions, coupled with other data, argues strongly for ionization of a water molecule ligand of the metal ion at the active site, with OH^- as the solvent-donated ligand at high pH. We propose a mechanism of ligand exchange at high pH that reconciles these ostensibly conflicting requirements by invoking a pentacoordinate intermediate having both OH^- and H_2O as ligands. Proton exchange can be rapid between these ligands because charge transfer without net ionization can occur, so that the leaving water can carry away the initial OH^-. The low-pH form is a thermal mixture of tetra- and pentacoordinate species, the latter having low relaxation rates by analogy with inhibitor derivatives of the enzyme and model systems. The proposed associative ligand-exchange mechanism reconciles the distinctions between the OH- and H_2O-models of carbonic anhydrase by merging them, providing the first model is consistent with the observed pH dependence of hydration activity, optical absorption, and solvent magnetic relaxation.

INTRODUCTION

The commonly investigated mammalian isozymes of carbonic anhydrase, a ubiquitous class of enzymes that catalyze the reversible reaction $CO_2 + H_2O \rightleftarrows HCO_3^-$, are of two types: the erythrocyte bovine B and human C enzymes, which are the more active, and are sufficiently alike so that they are often regarded as identical,[1] and the less active erythrocyte human B enzyme (1). The native enzymes contain one Zn^{2+}-ion per carbonic anhydrase molecule of ~30,000 daltons essential for all catalytic activity. Replacement of Zn^{2+} by Co^{2+} leaves the enzymatic properties relatively unaltered[1] and, because of the unfilled d-shells of the Co^{2+}-ions, introduces paramagnetism and optical absorption bands in the visible spectrum that make the Co^{2+}-substituted enzymes particularly advantageous for investigation by a variety of physical methods (1–3).

The native human B enzyme (HCAB)[2] has an intrinsic pH-profile for hydration and esterase activity that increases with increasing pH and that, when approximated by a single ionization, gives a pK_a near 8 (4–6). A similar pK_a has been reported for Co^{2+}-substituted HCAB (4). The latter has a characteristic optical spectrum that varies

[1]Distinctions between the similar bovine B and human C enzymes of carbonic anhydrase and between the native and Co^{2+}-substituted enzymes will be made explicit when necessary.

[2]Abbreviations used: BCAB, HCAB, and HCAC for bovine carbonic anhydrase B, and the human B and C isozymes, respectively; NMRD for nuclear magnetic relaxation dispersion. In terms of a newer notation, which is becoming increasingly prevelant (1), HCAB belongs to class I, and both BCAB and HCAC to class II; the classification relates to the maximum enzymatic turnover rate.

with pH much as do CO_2 hydration and esterase activity, although none of these can be satisfactorily described by a single ionization (4–8).

For the bovine B (BCAB) and human C (HCAC) isozymes, the observed pK_a values for solvent relaxivity and the electronic spectrum are quite sensitive to the presence of low concentrations of anions, including sulfate. The binding properties of the former have only recently been characterized in some detail (9, 10); as little as 5 mM sulfate can increase the apparent pK_a for activity by 0.5 pH unit in carefully purified samples. A similar sensitivity of esterase activity to the presence of sulfate has been reported recently for BCAB (11). Based on a variety of measurements reported since it was first shown by extensive dialysis that the apparent pK_a of the bovine enzyme could be reduced substantially below an often quoted value near 7 (9, 12), as well as on early reports in the literature of experiments that used a minimal anion concentration in the buffered solutions (13), there can be little question that the intrinsic pK_a for some of the properties of the bovine enzyme is in the pH range 6.0–6.5 (8), and is perhaps lower (14) in the absence of deliberately added anions.

Prompted by this observation, Koenig et al. recently proposed a model for the interaction of monovalent anions with active carbonic anhydrase that was shown then (10) and subsequently (14) to explain a variety of disparate phenomena. In this model, anion binding is seen, in an equilibrium description, as a substitution of the anion for an OH^- moiety, the latter derived from an exchangeable water molecule with no net change of charge of the enzyme molecule. Thus, the pH-dependence of anion-binding arises from competition of the anion with OH^- and therefore depends on the pK_a of water, i.e., on an ionization in the solvent rather than on the enzyme. The kinetic picture of ligand exchange was that OH^- exchanged as part of an entire water molecule, whereas weakly interacting anions such as Cl^- and HCO_3^- exchanged in association with a proton; the latter exchange was generally mediated by a buffer. The proposed model only considered anion and solvent interactions with a single, active, high-pH form of the enzyme; no attempt was made at the time to describe an intrinsic low-pH form. In particular, the model did not include a description of the low activity, high-pK_a enzyme, HCAB.

About the same time, Bertini and collaborators published an extensive characterization of a variety of carbonic anhydrase-inhibitor complexes for several metal-substituted isozymes (3). In particular, Co^{2+}-substituted BCAB and its complexes with inhibitors were studied by optical spectroscopy (15), electron spin resonance (16), high-resolution NMR (17), and solvent nuclear magnetic relaxation measurements at relatively high fields (4–60 MHz) (18). More recently, small Co^{2+} and Zn^{2+} chelate complexes were synthesized that are reasonable models for the optical and catalytic properties of carbonic anhydrase (19, 20). Bertini et al. interpreted their results in terms of expanded coordination of the Co^{2+} ions in the substituted enzymes both upon anion binding and upon change of pH, and the proposed tetra- and pentacoordinated geometry was related in detail to anion binding, solvent proton relaxation, and enzymatic activity. Moreover, a comprehensive description of these extensive data indicated that the Co^{2+}-ligand derived from solution was OH^- (3, 19). These ideas have never been merged with those that derive from the strictures ostensibly set by the behavior of solvent proton relaxation, i.e,, that H_2O must be the exchanging molecule, even in the presence of buffer (6).

We now report measurements that compare the pH titrations of the optical and the solvent relaxation spectra of solutions of highly purified Co^{2+}-HCAB having no added ions or buffer. The relaxation data at high pH span the magnetic-field interval 0.01–300 MHz[3], a range of field quite useful in the present instance. Both the relaxation data and the optical data can be resolved into a superposition of contributions from two limiting spectra, attributed to high- and low-pH forms of HCAB. The relative concentrations found for each form as a function of pH, though not well-fit by a single titration, are the same for both the optical and relaxation data. Clearly, though it may not be necessary to invoke a low-pH form of carbonic anhydrase to explain anion binding (10), nonetheless, there are both low- and high-pH forms of HCAB in the absence of anions. The questions once again, are: which ionization on the enzyme distinguishes the high- and low-pH forms of HCAB, and of what relevance is this distinction to the other isozymes?

The problem at hand is to reconcile the evidence that H_2O exchanges with the active site of the enzyme, with the view that the solvent-donated ligand of the tetracoordinate metal at high pH is likely to be OH^-, not H_2O. To resolve this problem one must realize that the solvent-donated ligand is not the same as that which exchanges, i.e., that an OH^- on the metal ion does not mean that OH^- exchanges, and conversely, that if H_2O exchanges, it does not necessarily follow that H_2O is a ligand of the metal ion at the active site.

Accordingly, we present a model for the high- and low-pH forms of the several isozymes of carbonic anhydrase that is consistent with the earlier model of Koenig et al. (10), and incorporates the ideas of Bertini and collaborators regarding expanded coordination of the metal (3). The model also rationalizes the puzzling difference in the pK_a of the high and low activity isozymes in terms of metal stereochemistry. It stresses the importance of pentacoordination, which allows for associative (21) ligand exchange, thereby providing a means of proton transfer without net water ionization. This intermediate permits the rapid

[3]Magnetic field strength is given in units of the Larmor precession-frequency of protons in that field. The conversion is 4.26 KHz = 1 Oe = 1 G. The 300-MHz data were kindly taken for us by Dr. David Cowburn, The Rockefeller University.

proton transfer necessary for catalysis and explains relaxation. The present model resolves the controversy in the literature between the implications of the "OH-model" and the "H_2O-model" of the active site of carbonic anhydrase (1). In essence, the models merge.

MATERIALS AND METHODS

Protein Preparation

HCAB was purchased from Sigma Chemical Co. (St. Louis, MO) as a lyophilized powder of electrophoretically purified enzyme. All preparative procedures were done at room temperature. The commercial protein was demetallized by dialysis against a 100-fold greater volume of 0.05 M 2,6-dipicolinic acid in 0.1 M phosphate buffer, pH 6.9. The external solution was changed twice in a 24-h period. The apoenzyme solution was then dialyzed against deionized water, with 12 changes over a 48-h period, to remove the chelate and buffer ions. Co^{2+} was added by overnight dialysis against an unbuffered solution of 5×10^{-4} M spectroscopically pure $CoSO_4$, and excess metal salt was removed by dialysis against deionized water, with 6 changes in 12 h. The result was Co^{2+}-substituted HCAB at pH 5.51, close to 5.7, the value of the isoelectric point of the enzyme measured at 1°C without removing CO_2 (22).

The native Zn^{2+}-HCAB sample used as a blank was obtained by exhaustive dialysis of the material from Sigma Chemical Co. against deionized water, the final pH being 5.35. All samples were concentrated to ~3 mM protein using a Diaflo ultrafiltration membrane (Amicon Corp., Scientific Sys. Div., Lexington, MA).

The pH of the samples, typically ~0.5 ml volume, was increased from the isoelectric point by addition of microliter amounts of freshly prepared solutions of 1–2 M NaOH. All pH measurements were made using a microelectrode characterized by particularly low ion leakage to the solution.

Concentration Measurements

Concentration was measured from optical spectra obtained at room temperature by using a Cary 14 spectrophotometer (Varian Associates, Instrument Div., Palo Alto, CA) equipped with 0–0.1 and 0–1 optical-density slide wires. The protein concentration of the samples was obtained from the absorption of the 280-nm band, using a molar absorbance of 46,900 M^{-1} cm^{-1} (23). The cobalt concentration of the samples was obtained from the absorption of the 550-mm band, at pH 9.94, using 340 M^{-1} cm^{-1} for the high-pH limit of the molar absorbance (8, 24).

NMRD Measurements

Measurements of nuclear magnetic relaxation dispersion (NMRD), the magnetic-field dependence of the spin-lattic relaxation rate $1/T_1$ of solvent protons, were made using apparatus similar to, but considerably improved upon that used previously (25). In particular, the upper limit of the attainable magnetic field has been increased from 20 to 50 MHz so that measurements now routinely span the interval 0.01–50 MHz. This change, together with redesign that reduced amplifier noise and increased field stability, makes possible absolute reproducibilities of relaxation rates of about ±1% over the entire field range. A few measurements of relaxation rates were made at 80 and 300 MHz on IBM (IBM Instruments, Inc., Danbury, CT) and Nicolet (Nicolet Instrument Corp., Madison, WI) high resolution spectrometers, respectively.[3]

Data Reduction

The NMRD spectra for the Co^{2+}-substituted sample were decomposed at each pH into a paramagnetic contribution $NMRD_p$ and a diamagnetic contribution $NMRD_d$; the latter was obtained from a sample of native (Zn^{2+}) enzyme at essentially the same pH and concentration. Corrections for small differences in concentration were made by linearly scaling the data for the native protein, since it was demonstrated earlier that $NMRD_d$ is linear in carbonic anhydrase concentration to over 4 mM (26). These corrections must be made accurately for Co^{2+}-HCAB because, in contrast to, e.g., Mn^{2+}- and Cu^{2+}-protein complexes for which $NMRD_p \gg NMRD_d$ for all values of magnetic field, $NMRD_p$ is very similar to $NMRD_d$ for Co^{2+}-HCAB.

As was done previously (27), the diamagnetic data were fit by the Cole-Cole expression

$$1/T_{1d} = D + A \,\mathrm{Re}\{1/[1 + (i\nu/\nu_c)^{\beta/2}]\}, \qquad (1)$$

where Re stands for "the real part of," and ν is the magnetic field in frequency units. This function provides an excellent, though heuristic, description of $NMRD_d$ through a best fit determination of D, A, ν_c, and β, where D and A are the amplitudes of the constant and dispersive terms, and are proportional to the protein concentrations; ν_c is a measure of the inverse of the correlation time for the interaction that determines the relaxation; and β, which determines the shape of the variation of $1/T_{1d}$ with ν, is 2 for a Lorentzian dispersion and is <2 for the slower variation observed for all diamagnetic dispersions. Once this fit is obtained, it is straightfoward to generate $NMRD_d$ for any protein concentration by simply scaling D and A linearly (without altering the other two parameters, ν_c and β).

The theoretical description of $NMRD_p$ developed to explain relaxation of solvent protons by aquoions is inadequate for the description of $NMRD_p$ for solutions of metal-protein complexes (28). However, since our interest is in the characterization of the high- and low-pH forms of HCAB, any expression that provides a good heuristic description of the data would serve for the present purposes. It turns out, quite by chance, that in the case of Co^{2+}-HCAB, Eq. 1 also provides an excellent characterization of $NMRD_p$ within the relatively low experimental scatter, which made it particularly simple to resolve the paramagnetic effects, as a function of pH, into low- and high-pH contributions.

RESULTS AND ANALYSIS

Fig. 1 shows the NMRD spectra for native and Co^{2+}-substituted HCAB at the two extremes of the pH range

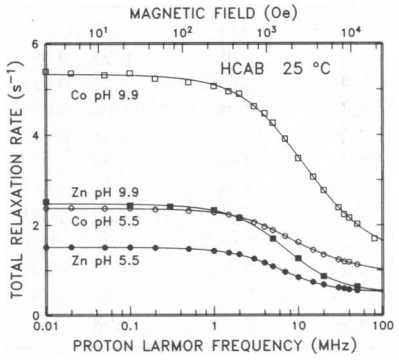


FIGURE 1 Magnetic-field dependence of the total relaxation rate (NMRD spectra) for samples of native (Zn^{2+}-containing) and Co^{2+}-substituted human carbonic anhydrase B at the extremes of the pH range covered, at 25°C. The samples were initially at low pH, with no added buffer, and brought to high pH by addition of NaOH. The native samples were 2.98 mM protein; the Co^{2+}-substituted samples were 2.87 mM Co^{2+}, 3.06 mM total protein. The curves through the data points result from a least-squares fit of Eq. 1 to the individual diamagnetic and paramagnetic contributions to the data.

used. The individual symbols show the data, and the curves through the points are derived from least-squares comparisons of the data with Eq. 1. The values derived for the parameters for the diamagnetic and paramagnetic contributions are listed in Table I. As discussed above, the paramagnetic dispersion was obtained by correcting the Co^{2+} data for the diamagnetic contribution at each pH. The pH dependence for the native enzyme is greater than that observed for other proteins (26, 27), amounting to an increase of almost a factor of two (after correcting for the solvent contribution of 0.4 s^{-1}) on going from pH 5.5 to 9.9. At a given pH, $NMRD_d$ is about that of apo-HCAB; these and related results from Hallenga and Koenig (27) are also in Table I.

It is apparent from Fig. 1 that $NMRD_p$, the difference between the NMRD spectra for the Co^{2+}-substituted and the native enzymes, is much less at low pH than it is at high pH, but by no means does it vanish. We have determined $NMRD_p$ for five intermediate values of pH, obtaining in each instance the complete NMRD spectra for the native and the Co^{2+}-substituted enzymes. The measurements in all cases were obtained by successively adding microliter quantities of 1M NaOH to the low-pH samples (Fig. 1). Note again that, other than this, the samples contained no deliberately added ions.

To represent the pH dependence of $NMRD_p$, noting that the data (Fig. 1) extend over four units of pH, we assumed that these data characterize the low- and high-pH limiting forms, and that the data at any intermediate pH could be resolved into contributions from each of these in proportion to the fractions of low- and high-pH forms present in solution. Accordingly, we smoothed the paramagnetic contribution to the low- and high-pH data (Fig. 1) by first fitting the two $NMRD_p$ spectra to Eq. 1 and then deriving the combination of these spectra that best fit the results at intermediate values of pH. These results are shown in Fig. 2. The symbols are the data, and the curves are linear combinations of those derived from the results (Fig. 1).

Note that the curves in Fig. 2, which agree very well with the data, are also purely heuristic. No commitment is made as yet to the mechanisms that determine $NMRD_p$; rather, the high- and low-pH data of Fig. 2, separated by an interval of four pH units centered near the pK_a for activity of HCAB, are taken as the respective $NMRD_p$ of the two limiting forms. F, the fraction of the high-pH form present at intermediate values of pH, is derived from these results (Fig. 2) and is plotted as a function of pH in Fig. 3. A least-squares fit of F to a single ionization is also shown; the derived pK_a is 7.6. The fit is reasonably good, though clearly, as with the enzymatic activity (4, 6), the data are not described well by a single ionization. Also shown are the results of optical measurements on the same sample, which are in excellent agreement with those previously reported (8). In this case, the fraction of high-pH form present is assumed to be linearly proportional to the height of the absorption peak at 640 nm. Clearly, the correlation between the pH dependences of the optical and relaxation spectra is high.

TABLE I

RESULTS OF A LEAST-SQUARES FIT OF THE COLE-COLE EXPRESSION (EQ. 1) TO THE DATA, AS INDICATED, FOR 25°C

Sample	pH	Concentration*	A	D‡	β	ν_c
		mM	s^{-1}	s^{-1}		*MHz*
Zn—HCAB	5.51	2.98	0.95	0.084	1.70	6.3
	5.92	2.98	0.96	0.088	1.70	6.3
	6.48	2.98	1.00	0.091	1.70	6.2
	7.05	2.98	1.04	0.094	1.69	6.2
	7.95	2.98	1.20	0.094	1.68	6.4
	9.17	2.98	1.53	0.085	1.68	6.8
	9.94	2.98	1.86	0.065	1.68	7.1
	~6	2.98§	0.90	0.056	1.68	6.4
Apo—HCAB	7.28	2.98‖	1.10	~0	1.48	5.3
Co—HCAB¶	5.51	2.87	0.46	0.40	1.53	32.2
	9.94	2.87	2.08	0.78	1.54	22.8

*The results for pH > 5.51 are all corrected, by linear interpolation, for the small dilution effects resulting from addition of NaOH.

‡The buffer contribution of 0.4 is not included in D.

§Computed from Hallenga and Koenig (27) for the concentration shown; original data were for 1.6 mM protein.

‖Sample concentration was 2.85 mM protein; the data were extrapolated linearly to this value.

¶The results are for the paramagnetic contribution to the relaxation rates. For the low-pH sample, data to 300 MHz were included.

Ancillary Results

The NMRD data for the pH 9.94 sample were extended to higher fields than the others, with one set of measurements

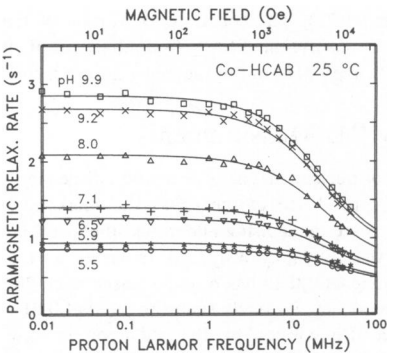


FIGURE 2 Paramagnetic contribution to the NMRD spectra for a range of pH values for the Co^{2+}-substituted samples of human carbonic anhydrase considered in Fig. 1 at 25°C. The data points are determined by subtracting the contribution of the native protein, determined from a smooth fit to the native dispersion, from the measured total rates for the Co^{2+}-protein. The curves through the data points of the uppermost and lowermost curves result from least-squares fits of Eq. 1. The remaining curves are weighted averages of these two curves, corresponding to a fraction F of the high-pH curve and a fraction 1-F of the low-pH curve.

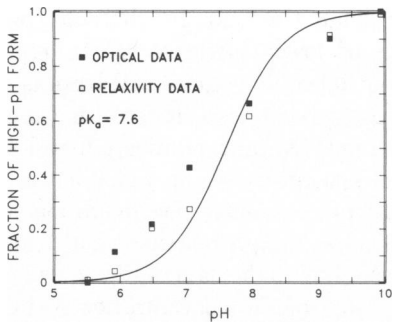


FIGURE 3 Fraction F of the high-pH form of the enzyme, as a function of pH, derived from the samples considered in Fig. 2. The open squares are from the NMRD data (Fig. 2) and the closed squares are from optical absorption measurements of the same samples.

at 80 MHz and another at 300 MHz, to check for any magnetic-field dependence of the correlation time that characterizes the Co^{2+}-proton interactions. Such behavior is invariably found, for example, in Mn^{2+}- and in Cu^{2+}-ions complexed with protein (25, 29). The fit by Eq. 1 (Figs. 1 and 2) includes these high-field data. The technical point, not relevant to the foregoing, but instructive for the general question of the shape of NMRD spectra in solutions of protein complexes and paramagnetic ions, is that $\beta = 1.53$ (rather than 2 if the dispersion were Lorentzian) and the limiting high-field value is 0.32 of the low-field value. This is close to the 0.3 expected on the most simplistic theory in the limit of rapid ligand exchange.

A MODEL FOR CARBONIC ANHYDRASE

Preliminary Considerations

Before presenting a model for the active site of carbonic anhydrase, it is useful to reexamine the distinctions between the OH-model and the H_2O-model discussed in the literature (1). These models refer to the solvent-derived ligand of the high-pH form of the enzyme. Consider the two reactions known to occur in aqueous solution

$$CO_2 + H_2O \rightleftarrows H^+ + HCO_3^- \qquad (2a)$$

$$CO_2 + OH^- \rightleftarrows HCO_3^-. \qquad (2b)$$

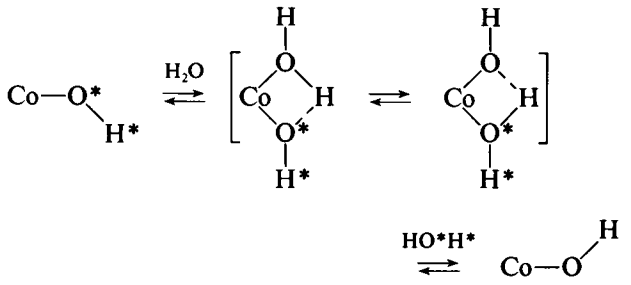
Eq. 2a represents the hydration of CO_2 and Eq. 2b the hydroxylation of CO_2 (30). The two mechanisms can be distinguished by their pH dependences. The rate of conversion of CO_2 into HCO_3^- for the mechanism of Eq. 2a is independent of pH, that described by Eq. 2b increases with pH, becoming competitive with the hydration reaction for pH ~ 10. Which of these reactions does carbonic anhydrase catalyze? There seems to be little argument in the literature that it is the hydration reaction, Eq. 2a. (However, Davis [31], among the first to consider the OH-model, concluded that this model is equivalent to saying that the OH^- ligand of the metal must also be the exchanging entity and, therefore, that the hydroxylation reaction was catalyzed by carbonic anhydrase. Subsequent authors, however, even when invoking the OH-model, state Eq. 2a as the reaction catalyzed [18]). Thus, H_2O must be considered a cosubstrate with CO_2 for the hydration reaction, which implies that solvent protons enter and leave the active site conveyed by water molecules (and not OH^- ions) during catalysis for both hydration and dehydration. By extension, solvent water molecules must (somehow) be in rapid exchange with the enzyme. To clarify the situation further, consider the thought-experiment in which an enzyme molecule is surrounded by a sphere that is permeable to the several substrates, but that prevents observation of the atomic configuration of the active site region; the enzyme is presumably encased in a "black box." Anything that monitors, for example, protons that have been relaxed by interactions with Co^{2+}-ions at the active site would see those protons leaving the enzyme (i.e., leaving the black box) as part of water molecules, never as part of hydroxide ions. Or, as another example, monitoring of the dehydration reaction product would show CO_2 and H_2O molecules leaving the protein. This thought-experiment makes no statement about the state of the substrate ions or molecules inside the black box, nor of the atomic details of the enzymatic mechanism, nor of the ligands of the metal ions, but shows that the exchanging entity that carries off relaxed protons, or isotopically labeled oxygen atoms (14), is a water molecule; a commitment to Eq. 2a as the reaction catalyzed ensures this.

The foregoing, we believe, settles an issue that has persisted for a decade, since the first proton relaxation data were interpreted as demanding that the exchanging entity be a water molecule (2, 32). It is apparent that one should expect the solvent-proton relaxation rates to be large in solutions of active enzyme, solely because Eq. 2a is catalyzed. One need not argue the converse—that from the observed rates water is what exchanges. The above discussion eliminates the controversy because it separates the question regarding the nature of the solvent-donated ligand of the metal-ion at the active site from that of the nature of the exchanging entities. Once this point is appreciated, it becomes possible to describe the low-pH and high-pH forms of HCAB on the basis of the present data, and extend the model to incorporate previous ideas regarding the bovine enzyme (9).

A Model for the Active Site of Co-HCAB

We propose that the high-pH form of Co-HCAB and, by extension, the high-pH forms of the several isozymes of mammalian erythrocyte carbonic anhydrase have a tetracoordinate Co^{2+}-ion with OH^- as a fourth ligand, in turn derived from a rapidly exchanging solvent water molecule. Specifically, we propose that both the oxygen and hydrogen of the OH^- ligand exchange in the solvent as with

Scheme I below



Scheme I

First, a water molecule from solvent associates with the metal ion to form a pentacoordinate intermediate; second, a rapid proton transfer takes place from this water to the OH^- ligand (perhaps going back and forth many times); and third, the original OH^- ligand leaves the metal ion only when protonated, i.e., as a water molecule. Although we will not discuss arguments from previous literature that support this idea until the Discussion section, we note now that the few precedents in the literature are convincing: that proton transfer can occur rapidly because no net dissociation of water occurs as a result of the transfer; and that the water off-rate can be sufficiently rapid to account for both proton relaxation ($\gg 10^5$ s^{-1} for proton off-rate [2]), and the loss of ^{18}O from substrate to solvent (33) ($\sim 10^6$ s^{-1} for oxygen off-rate [14]) for the native (Zn^{2+}) enzyme. We also note that the OH^- ligand of the metal-ion in the pentacoordinate intermediate acts as a proton acceptor, which we identify now as the proton acceptor that Koenig et al. (10) required as a fundamental part of their model. It now appears only in the transition state but, nonetheless, all the arguments that followed from their model remain valid.

Tetracoordinate Co^{2+} ions are characterized by (*a*) intense absorption bands in the visible region (3), (*b*) a relatively long electron spin relaxation time τ_S that results in a high solvent proton relaxivity associated with a relatively long correlation time that is dominated by τ_S (18), and (*c*) correlated with this, relatively narrow EPR signals at low temperatures (16). Hence, the relaxivity is high at high pH, now that we have a mechanism for rapid proton exchange, even with OH^- as the fourth ligand of the metal.

We propose that the low-pH form of pure HCAB, in the absence of anions, differs from the high-pH form in that the coordination spheres of the Co^{2+} ions can be expanded to pentacoordination at equilibrium, and may be a thermal mixture of tetra- and pentacoordination; the driving force is the addition of a proton to the region of the active site. This results in protonation of the OH^- ligand of the Co^{2+}-ions, the concomitant neutralization of the fourth ligand, and the increased bond length, which allows room for a possible fifth ligand, another water molecule.

Pentacoordinate Co^{2+} ions are characterized by absorption spectra of lower intensity (3), a relatively short electron spin relaxation time that results in a lower solvent-proton relaxivity associated with a relatively short correlation time (18), and relatively broad EPR signals (16). The tetracoordinate form, which has an exchanging H_2O ligand but is inactive for hydration and esterase activity, will have a high relaxivity and long correlation time and, according to the data of Fig. 2, will constitute no more than ~30% of the concentration of low-pH forms present in Co^{2+}-HCAB at equilibrium.

The anion-inhibited form of the enzyme, as in the model of Koenig et al. (10), results from a formal substitution in the active site of a monovalent anion with an OH^-. The result may be either tetra- or pentacoordinate, with a water molecule being the fifth ligand, or a thermal mixture of the two, depending upon the particular anion (3, 34) and isozyme. The tetracoordinate form, analogous to the high-pH form, has the anion as a fourth ligand of the Co^{2+} ions. Although the electron spin relaxation time is therefore long (18), water is excluded from the inner coordination sphere. The observed contribution to proton relaxation is small and due to outer sphere effects; the long correlation time is evidenced by the magnetic-field dependence of the relaxation dispersion (18). The pentacoordinate anion-inhibited form would have a water molecule added as a fifth ligand to the above, resulting, once again, in a form with a short electronic spin relaxation time. The result is an inner sphere contribution to the proton relaxation that is small because of the short correlation time, and a relaxation rate of comparable magnitude to that of the tetracoordinate, but distinguishable from it by its field dependence at relatively high fields (18).

In summary, the high-pH form of the enzyme is tetracoordinate with OH^- as a fourth ligand that exchanges as H_2O via a pentacoordinate intermediate. The electron spin relaxation time is relatively long, and the solvent proton relaxivity high. The enzyme at low pH, in the absence of anions, accepts a proton in the active site region (assumed here to protonate the metal-bound OH^-). Expansion of the coordinate sphere to include two water molecules, and the associated shortened electron spin relaxation time, can then occur. In the presence of anions, the enzyme can be inactivated by pH-dependent competition of the anions with OH^- for a ligand position, resulting in low proton relaxivity in the tetracoordinate form because of displacement of solvent from the inner sphere, and in the pentacoordinate form because of a shortened electron spin relaxation time.

The titration of the activity (4–6) and the spectrum (8) of carbonic anhydrase is not well described by a single ionization, as is well-known, and the (at least) two ionizations on the enzyme that are responsible may well contribute differently to relaxation, optical spectra, hydration activity, and esterase activity. That one ionization is on the

metal ion, if the two are treated independently, is clear enough; the other would most naturally be ascribed to the His 64 residue present in the active-site regions of both HCAB and BCAB. This complication has been ignored in the foregoing, mainly because there are insufficient data to allow resolution of the low-pH form into a greater variety of forms, which reflect the more than one variety of protonated species possible.

DISCUSSION

The analysis of the data in Fig. 2 shows quite clearly that one can reconstruct the paramagnetic component of the solvent proton relaxivity of Co-HCAB at all intermediate values of pH from a linear combination of the results at the two extremes of pH used, pH 5.51 and 9.94. This reconstruction holds for the entire NMRD spectrum, i.e., for the entire range of magnetic field, which indicates that at any pH, in the absence of anions, there is an equilibrium mixture of two forms of Co-HCAB, a low-pH and a high-pH form, each with its characteristic $NMRD_p$ spectra. (Or if there are two ionizations to be considered, either their pK_a values are very similar, or the relaxivity of one does not titrate significantly or is very small.)

The relaxivity values at low pH are about one-third the high-pH values at all fields, and the correlation time is ~50% shorter; the fits using Eq. 1 are in Table I. By contrast, the hydration activity at low pH is <5% of its high-pH value (5). Thus, whereas the hydration activity titrates essentially to zero at low pH, the relaxivity does not, indicating a substantive amount of tetrahedral material at low pH.

It is clear from Fig. 3 that a similar reconstruction can be made for the dependence of the optical spectra on pH; one can associate unique optical spectra with both the low- and high-pH forms of Co-HCAB and describe the spectra at intermediate values of pH as a linear combination of these two forms. The resulting decompositions of the relaxation and optical spectra are essentially the same, within the experimental scatter, with an equimolar mixture occurring at pH 7.6. We will refer to this value, rather loosely, as the pK_a. Note, however, that the extremes of pH (Fig. 3) are not good end points of the titration, and that a single ionization is but a first-order description of the pH-dependences observed. Thus, the uncertainty in the value of this approximation to a single pK_a is rather large.

The variations of both the relaxation and optical data are slower than can be attributed to a single ionization, and it can well be expected that additional ionizations in the active site region can affect the relaxation and optical spectra differently. Differences of this sort are not large for HCAB, as evidenced in Fig. 3. Both titrations do not differ significantly from the reported pK_a values of ~8.1 for esterase activities of Zn- and Co-HCAB (4) and the hydration activity of Zn-HCAB (5, 6). However, secondary ionizations complicate comparisons of the pH dependences of different properties of BCAB (8). The pH dependences of the relaxation and optical data of BCAB are quite different (8, 15), although the relaxation data correlate with part of the optical variation (10, 35, 36), and the correlation of either with hydration activity must await data from sulfate-free preparations. Moreover, for BCAB, it appears that catalysis of aldehyde hydrolysis has a markedly different pH dependence than does hydration of CO_2 (37). The situation for the bovine enzyme is more complex than was once thought.

The present model for the low- and high-pH forms of HCAB in the absence of anions, coupled with the earlier description of anion-enzyme complexes (10), can account for the greater part of the published data for all the mammalian carbonic anhydrase isozymes. The view of the low-pH form of the enzyme as a mixture of four and five coordinate species containing one or two coordinated water molecules is particularly unifying. The two major isoenzymes are described at low pH as predominantly tetrahedral (BCAB) or pentacoordinate (HCAB), which is consistent with the electronic and NMRD data. The difference in pK_a's is readily explained in terms of the well known decrease in pK_a of coordinated water with decreasing coordination number. The four coordinated high-pH forms are almost identical for any kind of spectroscopy. Moreover, and perhaps most significant, the incompatibility of the contending OH- and H_2O-models has been resolved; in some sense it has been an argument more semantic than substantive, but subtly so. Thus, the very statement that carbonic anhydrase catalyzes the hydration, and not the hydroxylation, of CO_2 implies that H_2O molecules are exchanged between solvent and the active site of the enzyme irrespective of the ligand of the metal ion. In the past, these two issues have not been carefully distinguished; the state of the ligand of the metal ion was often implicitly taken to be identical to the exchanging entity. The present model requires that it indeed is not.

The present view of the high-pH active enzyme differs only formally from the previous ideas (10) in that the postulated proton acceptor, needed to obtain proton transfer without a net dissociation of a water molecule, now appears as the OH^- ligand of the metal ions in the pentacoordinate transition state. Thus, the explanations of relaxation processes, the ^{13}C-NMR linewidths of substrates, the isotope-mixing of labeled substrates (4), the acceptor needed for anion-binding, and many other phenomena, can be shown to be unaltered (10). These added ideas regarding the low-pH form now allow for a unified description of the several mammalian erythrocyte isozymes, including the extensive physical data (optical, NMR, and EPR) for a great variety of metal-substituted enzymes and their complexes with anions. What remains is to find precedents for the postulated proton transfer in the pentacoordinate intermediate at high pH.

Kinetics of Proton Transfer

There are ample data to indicate that the rate of proton transfer to base in water solution from oxygen and nitrogen acids is limited by diffusion (38); i.e., the rate of proton transfer in the encounter complex is more rapid than the rate of complex formation, the latter given by a second-order rate constant of the order of 10^{10} $M^{-1}s^{-1}$. The reverse reaction, corresponding to a proton transfer against a pK_a gradient, proceeds more slowly; its rate, derived from equilibrium arguments, is the diffusion-limited rate, reduced by a factor equal to the antilog of the difference in pK_a's of the acid and base. If, for example, H_3O^+ and OH^- are taken as the acid and conjugate base, respectively, then the reverse of the diffusion-limited proton transfer, which requires dissociation of a water molecule, proceeds extremely slowly, $\sim 10^{-6}s^{-1}$. The actual dissociation of a given water in neat solution is acid-base catalyzed and much more rapid. It proceeds by proton transfer between H_2O and either OH^- or H_3O^+ (39) during a binary encounter, with diffusion-limited kinetics. Though proton transfer within the H_2O—OH^- binary complex is rapid, more rapid than the encounter rate, the latter is relatively slow near neutral pH, and the implied lower limit for the transfer rate is therefore relatively long. We would like to estimate how rapid this rate could be if the H_2O and OH^- were part of a termolecular complex with a protein-bound metal-ion.

There are data that indicate that proton transfer from acid to base in hydroxylic solvents, particularly water, often proceeds through a termolecular (or higher order) complex, with the hydroxyl group(s) acting as a proton shuttle. Grunwald and Eustace, in their review (40), point out that the near diffusion-limited second-order rate constants observed for these processes suggest that the participating solvent molecule is from the solvation shell of one of the reactants. Such solvent-aided proton transfer has been observed in aqueous solutions of Al^{3+} ions (41), and, somewhat more related to the present considerations, of $(NH_3)_5PtNH_2^{3+}$ (42) and $(H_2O)_5Cr\,OH^{2+}$ (43). Not only is proton exchange with solution rapid in the latter two cases, but proton transfer among the ligands of the metal-ion is rapid also, and ostensibly occurs via a bridging array of water molecules with concerted shuttling of protons along hydroxyl-water pairs and with no net dissociation of water molecules. Of particular note is that, for Cr^{3+}, the observed transfer of protons around the solvation shell precludes the particular interaction of liganded OH^- with a solvent H_2O in which a pair of protons interchange in a concerted manner, as a mechanism for proton exchange with solvent in this case.

Though there appears to be no example, other than Co^{2+} in carbonic anhydrase, in which expanded coordination and proton transfer in the resulting intermediate (transition) state allows for both rapid proton and oxygen exchange between solvation shell and solvent, the few examples of aquoions given above would appear to be ample and quantitative precedent for the mechanism proposed here for ligand exchange of the high-pH form of carbonic anhydrase. In particular, it describes a pathway for ligand exchange that proceeds independently of the existence of a low-pH form of the enzyme.

SUMMARY

We have presented a complete structural and kinetic description of the metal-ion coordination of the low- and high-pH forms of carbonic anhydrase in the absence of anions. Expanded (penta-) coordination plays an important role in the intermediate state for ligand exchange, and, indeed, the nature of the exchange is as important as the structure of the equilibrium forms. The metal ion of the high-pH form is tetracoordinate, with OH^- as the ligand supplied by solvent. However, the exchanging entity is a water molecule, which can transfer a proton to the OH^- in the pentacoordinate transition state. No reference need be made to a low-pH form in describing the catalytic mechanism. Both protons and oxygen can exchange rapidly, which thereby explains relaxation in the Co^{2+} enzyme (2) and isotope loss in the Zn^{2+} enzyme (14, 33), observations that previously argued for H_2O as the ligand.

The metal ion in the low-pH form has at least one water molecule as a ligand, and may be a thermal mixture of tetra- and pentacoordination (18). This view can acount for the loss of enzymatic activity at low pH with partial (HCAB) or almost complete (BCAB) retention of relaxivity. The difference in the relative amount of pentacoordinate low-pH forms can be responsible for the differences in the pK_a's between the low- and high-activity isozymes.

We believe that we have effected a synthesis of the OH- and H_2O-models that does violence to neither, yet accounts for the various observations that previously required aspects of one or the other model for their explanation. Finally, though we are well aware that there is more than one ionization that occurs in the active site (Fig. 3), we have considered one of them (presumably the ionization of His-63) to be secondary for the present considerations; it is not expected to have a qualitative impact on the foregoing considerations.

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