the molecular frame of reference are sensitive to the nature of chemical bonding to the phosphorus atom, the ^{31}P shielding anisotropies of phosphate and phosphonate esters are considerably different.[58] These differences lead to the spectra shown in Figure 9. Unlike the overlapping spectra shown in Figure 8, Figure 9 indicates a significant downfield shift of the phosphonate spectrum relative to that of the phosphate. Deconvolution of the spectra was achieved by saturation of the subspectrum associated with the phospholipid.[58] The separated ^{31}P spectra could then be analyzed and assigned to a bilayer arrangement of the lipids. The ability to change the chemical shielding at the phosphorus nucleus without affecting other physical properties of the lipid molecule in the membrane suggests a method for selectively monitoring a single lipid class in the presence of other phospholipids[58] as has been proposed by other workers.[61]

(61) I. Vasilenko, B. De Kruijff, and A. I. Verkleij, *Biochim. Biophys. Acta*, 685, 144 (1982).

^{31}P NMR spectra of membranes are relatively easy to obtain. However, care must be paid to their interpretation since chemical shielding tensor components must be known, and since the angle made by the director with the principal axis of the shielding tensor can alter the spectrum dramatically.[51] Order parameters and correlation times for motions can only be obtained by spectral simulation, and these are often ambiguous. ^{31}P spectra provide a facile first look at a system to see if changes have occurred. Quantitative or speculative conclusions should be confirmed by other methods.

Overview

Magnetic resonance of membranes has reached a high level of sophistication in detection sensitivity and interpretation. Many of the theoretical and technical problems have been solved, and the methods are ready for application to complex biological systems. A high yield of biologically significant conclusions can be expected.

Cobalt(II) as a Probe of the Structure and Function of Carbonic Anhydrase

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Received July 30, 1982 (Revised Manuscript Received March 7, 1983)

Carbonic anhydrase, the premier zinc enzyme, has fascinating biochemical, chemical, and physicochemical properties. Its natural function is that of catalyzing the reversible hydration of carbon dioxide, which it does with one of the highest known turnover numbers.[1] Other catalytic activities are displayed in vitro; in particular, the enzyme is active towards hydration of aldehydes and hydrolysis of esters. The enormous amount of data obtained under different experimental conditions and with the aid of many techniques has often appeared inconsistent within the framework of the proposed models. Striking examples are provided by buffered and unbuffered carbonic anhydrase solutions that may exhibit completely different chemical properties, because small anionic molecules, like sulfate,[2] phosphate,[3] or acetate[4] are capable of interacting at the metal active site. Our purpose here is not to review the field comprehensively, as others[5-7] have but rather to elaborate a structural model of the enzyme that is capable of accounting for its very many physical and biochemical properties.

Ivano Bertini was born in Pisa, Italy, in 1940. He received a Doctor degree at the University of Florence in 1964. After spending a year as research associate, he joined the faculty of the University of Florence and rose to the rank of Professor in 1975. He was research associate at Princeton University, in 1968–1969. His research on the electronic properties of metal ions in systems of biological interest involves spectroscopic investigation of metalloproteins containing paramagnetic metal ions, as well as metal-substituted zinc proteins.

Claudio Luchinat was born in Florence, Italy, in 1952. He received a Doctor degree at the University of Florence in 1976, where he continues to work as research associate.

The physicochemical investigation of the cobalt(II)-substituted enzyme (CoCA) has recently been pursued extensively with gratifying results. High-spin cobalt(II) is being recognized as a powerful spectroscopic probe in biological systems. The cobalt(II) enzyme displays an activity sometimes lower and sometimes larger than the native enzyme, depending on the isoenzyme and on the substrate.[6] The catalytic properties and their pH dependence have been shown to be similar to those of the native enzyme,[8] although the available data on the kinetic studies overwhelmingly are for the native enzyme.

We attempt here to provide a unifying picture of the structure and function of carbonic anhydrase on the basis of recent results obtained on its cobalt derivative. The model rests on the following assumptions: (i) that the kinetic properties of native carbonic anhydrase can be transferred to its cobalt derivative and that (ii) the structural properties obtained from spectroscopical investigations of CoCA can be transferred to the native

(1) T. H. Maren, *Physiol. Rev.*, 47, 595 (1967).
(2) I. Bertini, G. Canti, C. Luchinat, and A. Scozzafava, *J. Am. Chem. Soc.*, 100, 4873 (1978).
(3) I. Bertini, C. Luchinat, and A. Scozzafava, *FEBS Lett.*, 93, 251 (1978).
(4) I. Bertini, C. Luchinat, and A. Scozzafava, *J. Chem. Soc., Dalton Trans.*, 1962 (1977).
(5) Y. Pocker and S. Sarkanen, *Adv. Enzymol.*, 47, 149 (1978).
(6) S. Lindskog, *Adv. Inorg. Biochem.*, 4, 115 (1982).
(7) I. Bertini, C. Luchinat, and A. Scozzafava, *Struct. Bonding*, 48, 45 (1982).
(8) S. Lindskog, *Biochemistry*, 5, 2641 (1966); J. E. Coleman, *J. Biol. Chem.*, 242, 5212 (1967).

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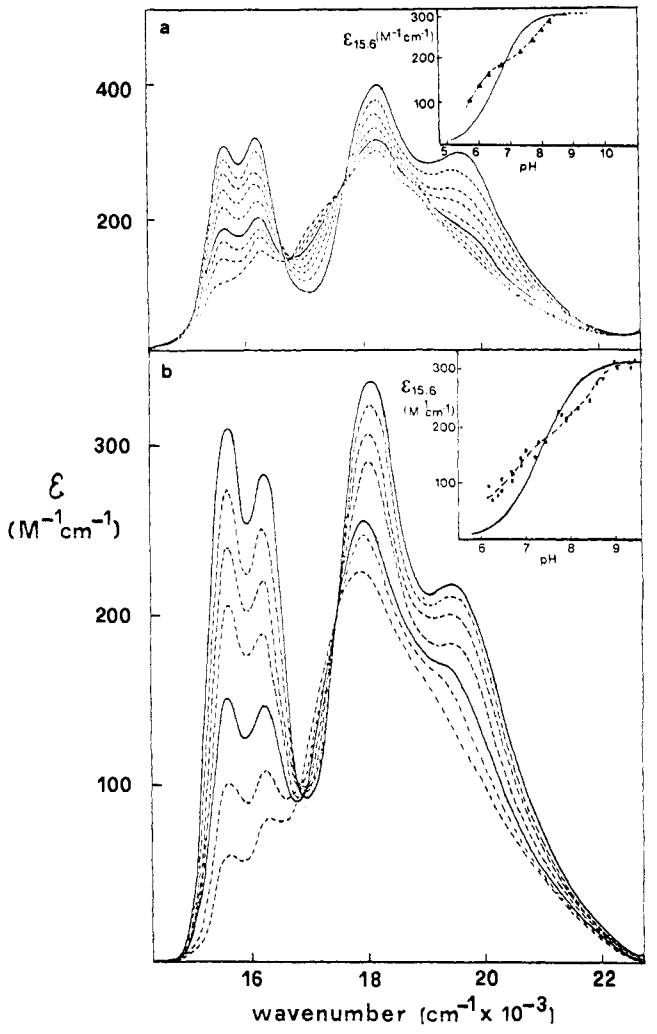


Figure 1. Electronic absorption spectra of CoBCAB (a) and CoHCAB (b) a function of pH: (a) pH 5.8, 6.0, 6.3, 6.7, 7.3, 7.7, 7.9, 8.2, 8.8, in order of increasing $\epsilon_{15.6}$; (b) pH 6.1, 6.6, 7.1, 7.8, 8.3, 8.6, 9.5, in order of increasing $\epsilon_{15.6}$. The insets represent the intensity of the 15.6 $cm^{-1} \times 10^{-3}$ d–d transition as a function of pH. The solid lines are calculated assuming a single pK_a value of 6.6 (a) and of 7.35 (b), respectively.

enzyme. The simplicity of the model and its capacity to answer the hitherto open questions are the chief indicators we have that attest to the validity of these assumptions.

Selected Structural Properties from X-ray Data

The protein as a whole has a rugby ball shape. The metal ion has been shown through X-ray studies[9] to be bound to three histidyl nitrogens at the bottom of the active cavity, which is 1.5 nm deep. On the surface of the cavity several hundreds of picometers away from the metal there is a threonine residue and farther away a histidine residue. Hydrogen bonded to the threonine there is also a glutamic residue that is buried into the protein. Although this residue has been suggested to be a possible candidate as the acidic group determining the pH dependent properties of the enzyme,[10] it will not be considered here.

(9) K. K. Kannan, in "Biophysics and Physiology of Carbon Dioxide", C. Bauer, G. Gros, and H. Bartels, Eds., Springer Verlag, Berlin, 1980, p 184, and references therein.
(10) K. K. Kannan, M. Petef, K. Fridborg, H. Cid-Dresdner, and S. Lövgren, *FEBS Lett.*, **73**, 115 (1977).

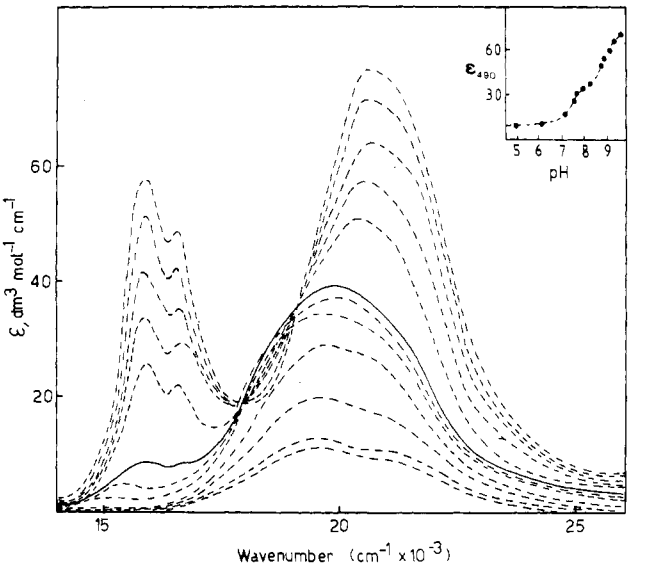


Figure 2. Electronic absorption spectra of the complex [tris(3,5-dimethyl-1-pyrazolylmethyl)amine]cobalt(2+) hydrate as a function of pH. The inset represents the intensity of the absorption at 20.4 × 10^{-3} cm^{-1}. The low-pH inflection corresponds to ligand protonation; the high-pH inflection to the deprotonation of the coordinated water.

These properties are common to both the low- and high-activity isoenzymes, for which X-ray studies are available. Another common feature is that the cavity can be divided into two halves, one mainly hydrophobic and the other hydrophilic. The shape and topology of the cavity for the two human isoenzymes (HCAB and HCAC) are slightly different, thus accounting for slight structural differences in the coordination polyhedra around the metal in both pure and inhibited systems and for marked differences in catalytic activity. The bovine enzyme (BCAB) is similar to the high-activity HCAC.

Cobalt(II), manganese(II), and copper(II) substitute at the zinc(II) site.[11] In addition to the three hystidyl nitrogens, solvent is believed to complete the coordination sphere; however, the spectroscopic studies shed more light on this kind of interaction than the X-ray data. We note at this stage that Clementi et al. have shown through Montecarlo calculations that two water molecules can be accommodated in the cavity at reasonable bond distances.[12]

A Structural Model of Cobalt Carbonic Anhydrase

The electronic absorption spectrum of CoCA is dramatically pH dependent. These spectra have been studied under a variety of experimental conditions and have been matters of elaborate speculation concerning the coordination geometry of the metal ion. The spectra of unbuffered solutions at different pH values for both CoHCAB and CoBCAB are shown in Figure 1.[13] The only statement on which there is general

(11) K. K. Kannan, in "Proceedings on Biomolecular Structure, Conformation, Function and Evolution", R. Srinivasan, Ed.; Pergamon Press, Oxford, 1979.
(12) E. Clementi, G. Corongiu, B. Jönsson, and S. Romano, *FEBS Lett.*, **100**, 319 (1979).
(13) I. Bertini, C. Luchinat, and A. Scozzafava, *Inorg. Chim. Acta*, **46**, 85 (1980).
(14) I. Bertini, G. Canti, C. Luchinat, and F. Mani, *Inorg. Chem.*, **20**, 1670 (1981).

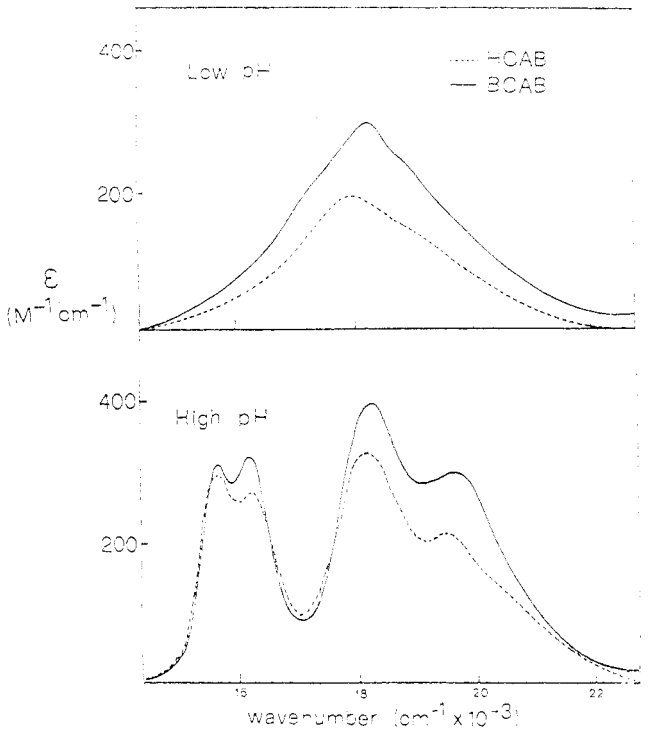
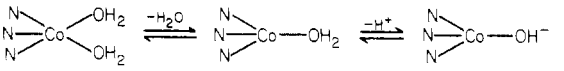


Figure 3. Low-pH and high-pH limit spectra of CoHCAB (---) and CoBCAB (–) in unbuffered solutions.

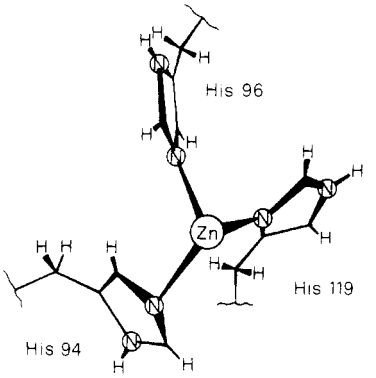
agreement is that these spectra are indicative of low-symmetry chromophores.

The spectra of Figure 1 indicate that at least two acidic groups are capable of affecting the electronic spectra of the metal ion, because the pH dependence of the absorption at 640 nm does not fit a simple sigmoidal curve (see insets, Figure 1). However, one group with a pK_a value of ~6 for the bovine isoenzyme and ~7.5 for the human isoenzyme seems responsible for the most dramatic changes. This presumably is a group attached to the metal. In the literature a simple cobalt(II) complex is reported that shows a pH dependence of the spectra similar to that of CoCA (Figure 2). The complex is five-coordinate including a water molecule[14] that is deprotonated (with a pK_a value of ~9.0). Such finding supports the early hypothesis of Lindskog and Coleman that a water molecule in the coordination sphere of carbonic anhydrase is the ionizing group.[15] The low pK_a values in the natural system can be accounted for by the partially hydrophobic nature of the cavity.

In Figure 3 the spectra of the acidic and alkaline forms of CoHCAB and CoBCA are shown. According to an early criterion proposed by Gray et al.,[16] which provides a satisfactory interpretation of all the CoCA inhibitor derivatives[2,7] and which is consistent with an independent set of measurements to be discussed in this Account, we suggest that the intensity of the d–d spectrum in a homogeneous series of cobalt(II) derivatives is capable of discriminating between tetra- or pentacoordination. For example, we assume that the high-pH spectra of CoCA with ϵ_{max} of 350–400 M^{-1} cm^{-1} are indicative of tetracoordination. Of course, not all the pseudotetrahedral species have the same molar absorbance; however, if the maximum intensity is larger than 300 M^{-1} cm^{-1}, we may tentatively assign the spectrum as pseudotetrahedral. With this criterion, the acidic form of CoHCAB with an ϵ value of 180 M^{-1} cm^{-1} probably contains a large percentage of pentacoordinate species, whereas the acidic form of CoBCAB with an ϵ value of 290 M^{-1} cm^{-1} is largely tetrahedral. If the tetrahedral species contains as the fourth ligand OH^- or H_2O (depending on pH), the five-coordinate species presumably contains two bound water molecules. The overall equilibrium is therefore



The ^1H NMR spectra of CoBCAB[17] exhibit one β-CH signal and three NH signals in accordance with the type of hystidyl coordination that was found in the X-ray analysis. The isotropic shifts of these protons are pH



dependent with about the same pK_a obtained from the electronic spectra. The presence of the NH throughout the pH range 5.8–9.5 shows that the histidine residues do not undergo deprotonation.

The water ^1H T_1^{-1} NMR measurements on CoBCAB have shown[2] that exchangeable protons are attached to the donor atoms from pH 5.6 to pH 10.5. Variable external magnetic field measurements have shown[18,19] that the T_1^{-1} values substantially obey the simple Solomon–Bloembergen theory for dipolar coupling between nuclei and unpaired electrons. The correlation time τ_c for this coupling is[19] ~10^{-1} s, which is related to the electron spin relaxation times. These, in turn, are related to the orbital degeneracy of the ground state and to the availability of low-lying excited states. In other words, *the correlation time can be related to the overall geometry of the chromophore.* Values of the order of 10^{-11} s have been taken as indicative of tetracoordination.[20] Both T_1^{-1} and the correlation time remain constant when the pH of CoBCAB is changed. This can be accounted for by the equivalence of two protons of coordinated H_2O and one proton of a coordinated OH^- that is closer to the metal. Such an effect has also been demonstrated in model complexes.[14,21] The proton-exchange mechanism from the coordinated hydroxide ion is discussed later.

(15) S. Lindskog and J. E. Coleman, *Proc. Natl. Acad. Sci. U.S.A.*, **70**, 2505 (1973).
(16) R. C. Rosenberg, C. A. Root, R.-H. Wang, M. Cerdonio, and H. B. Gray, *Proc. Natl. Acad. Sci. U.S.A.*, **70**, 161 (1973).
(17) I. Bertini, G. Canti, C. Luchinat, and F. Mani, *J. Am. Chem. Soc.*, **103**, 7784 (1981).
(18) M. E. Fabry, S. H .Koenig, and W. E. Schillinger, *J. Biol. Chem.*, **245**, 4256 (1970).
(19) I. Bertini, G. Canti, and C. Luchinat, *Inorg. Chim. Acta*, **56**, 99 (1981).
(20) I. Bertini, G. Lanini, and C. Luchinat, *Inorg. Chim. Acta*, in press.
(21) I. Bertini, G. Canti, C. Luchinat, and L. Messori, *Inorg. Chem.*, **21**, 3426 (1982).

The water ^1H T_1^{-1} NMR data on CoHCAB decrase at low pH. A thorough analysis at magnetic fields between 0.01 and 300 MHz has shown that the correlation time dependence on pH is responsible for this finding.[22] In particular, the low pH species has a τ_c of the order of 10^{-12} s, whereas the high pH species has $\tau_c \sim 10^{-11}$ s. The former value is consistent with the presence of a large percentage (>70%) of five-coordinate species. [*Note added in proof*: ^1H NMR studies on CoHCAB as a function of pH have shown that the T_1 values of the His-119 4H proton reflect the same variations in τ_c observed from the water ^1H investigations.[57]]

Predominance of five-coordinate species in the low-pH form of CoHCAB as compared with the predominance of tetrahedral species in CoBCAB (>80%) is also consistent with their pK_a values. The pK_a of coordinated water depends, among other factors, on the effective charge on the metal ion. Such charge depends on the nature and number of donor groups. In this specific case the isoenzyme that has a higher percentage of five-coordinate sites has a pK_a larger than the isoenzyme that is largely tetracoordinate.

A final comment relates to the acidic group that is capable of modulating the main pH dependence of the electronic spectra. This probably is a group not coordinate to the metal but close enough to interact with the donor groups, e.g., via the hydrogen-bond network. A reasonable candidate for this group is the histidine that dangles in the cavity.

Comments on the Formalism of the Catalyzed Reaction

CO_2 may be transformed into HCO_3^- through a hydration reaction

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

or a hydroxylation reaction

$$CO_2 + OH^- \rightleftharpoons HCO_3^- \quad (2)$$

If the catalyzed reaction is the reversible hydration, H^+ ions must be supplied rapidly to and from the active site: the calculated rate around neutral pH exceeds the limits set by diffusion. If the reaction is the reversible hydroxylation, the same problem is encountered in connection with the OH^- ion supply. Hydrogen ions, at variance with hydroxide ions, can be carried to and from the enzyme by buffer. Indeed, buffer effects on k_{cat}, have been measured.[23,24] Therefore, the catalyzed reaction is reaction 1.

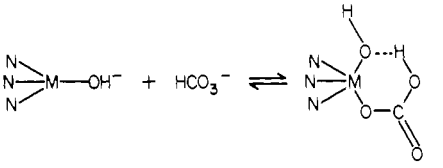
A comment is due on the active form of the enzyme. Within the above frame the pH dependence of the kinetic parameters[25-30] requires that the active species of the enzyme is the high pH form.

Isotope effect data[29-31] have been taken as indicative of the new rate-limiting step that becomes operative in the presence of buffer; this would be an intramolecular proton transfer within two groups in the enzyme having similar pK_a values:

$$H\text{-}E \rightleftharpoons E\text{-}H$$

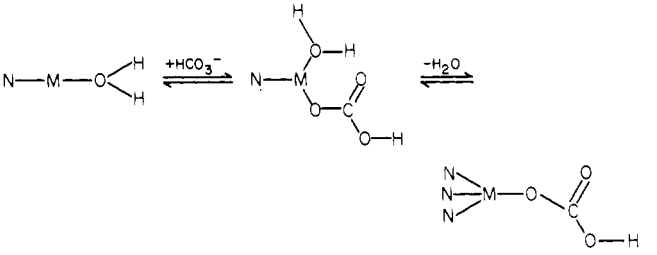
Mechanism of the Catalytic Process

There is now convincing evidence that the group responsible for the acid–base equilibrium in the enzyme is the metal-bound water molecule (see also next section). From the inhibitory effect of HCO_3^- on the hydration reaction, it has been proposed that bicarbonate may also bind to the basic form, giving rise to an adduct of the type[28]



This might also represent the first step of the dehydration reaction (and, according to the principle of microscopic reversibility, the last one in the hydration process). The second step would be the addition of a proton (the second substrate), which is brought to the enzyme by a buffer BH^+ molecule. Since there is no spectroscopic evidence that HCO_3^- binds to CoCA in its alkaline form,[32] it is probably preferable, as proposed by Lindskog,[6,30,33] that the first step is the addition of a proton, which allows the subsequent binding of HCO_3^-. Whichever the pathway, it is unlikely that buffer molecules like dimethylimidazolium or 2,4-lutidinium may penetrate deeply into the active cavity, so that we may postulate the existence of a proton-transfer group capable of shuttling a proton to the inner site of the cavity. Such a group has been proposed to be a histidine residue present in the cavity.

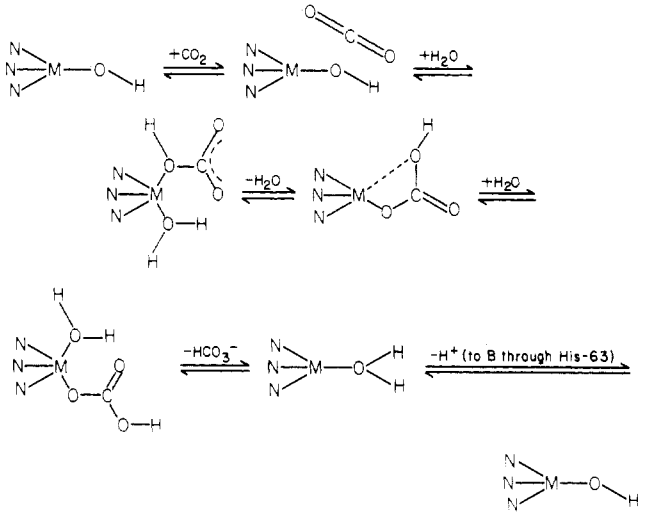
As it will be discussed later, the adducts of anions with the M – OH_2 form may be in general described as giving rise to equilibria between four- or five-coordinate chromophores. The binding of bicarbonate can therefore proceed through an associative mechanism of the type



CO_2 may be then released, with the possible aid of a water molecule. The above considerations are summarized in Scheme I for the hydration of CO_2; the initial step is the binding of CO_2 to a site (or at least a region)

(22) I. Bertini, R. D. Brown, III, S. H. Koenig, and C. Luchinat, *Biophys. J.*, **41**, 179 (1983).
(23) B.-H. Jonsson, H. Steiner, and S. Lindskog, *FEBS Lett.*, **64**, 310 (1976).
(24) H. Steiner, B.-H. Jonsson, and S. Lindskog, *FEBS Lett.*, **62**, 16 (1976).
(25) S. Lindskog in "Biophysics and Physiology of Carbon Dioxide", C. Bauer, G. Gros, and H. Bartels, Eds., Springer Verlag, Berlin, 1980, p 230, and references therein.
(26) R. G. Khalifah, *J. Biol. Chem.*, **246**, 2561 (1971).
(27) R. G. Khalifah in "Biophysics and Physiology of Carbon Dioxide", C. Bauer, G. Gros, and H. Bartels, Eds., Springer Verlag, Berlin, 1980, p 206, and references therein.
(28) Y. Pocker, T. L. Deits, and N. Tanaka in "Advances in Solution Chemistry", I. Bertini, L. Lunazzi, and A. Dei, Eds., Plenum Press, New York, 1981, p 253, and references therein.
(29) Y. Pocker and D. W. Bjorkquist, *Biochemistry*, **16**, 5698 (1977).
(30) H. Steiner, B.-H. Jonsson, and S. Lindskog, *Eur. J. Biochem.*, **59**, 253 (1975).
(31) K. S. Venkatasubban and D. N. Silverman, *Biochemistry*, **19**, 4984 (1980).
(32) Unpublished results from this laboratory.
(33) I. Simonsson and S. Lindskog, *Eur. J. Biochem.*, **123**, 29 (1982).

Scheme I

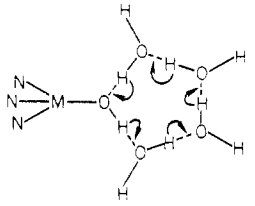


in the hydrophobic side of the cavity.[34]

This sequence is consistent with all the available information on the enzyme: (1) it acknowledges the role of buffers in the proton transfer step, (2) includes the invariant histidine residue as the proton-transfer group to account for the isotope effect, (3) takes advantage of the well-documented existence of a fifth coordination site to allow (i) the initial attack of CO_2 to the coordinated hydroxide, which presumably experiences an increased basicity upon coordination of water as a fifth ligand, and (ii) the dissociation of bicarbonate (or its binding in the reverse reaction).

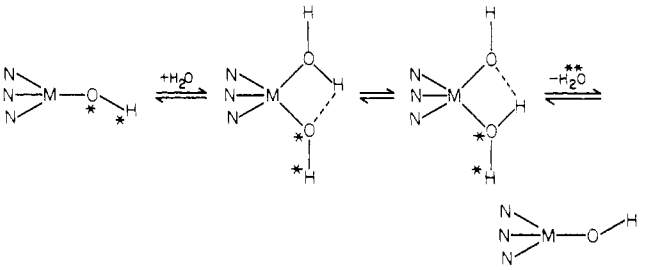
Water and ^{17}O Exchange

The exchange of solvent-related chemical species has been the subject of a lively debate for carbonic anhydrase because of its implications for the catalytic mechanism.[15,18,35,36] It is well-established that, if the catalytic process involved direct exchange of H^+ or OH^- species, their required rates would exceed the diffusion limit. For instance, the exchange rate of OH^- at pH 8 cannot exceed $10^{10} \times 10^{-6} = 10^4$ s^{-1}, whereas from water relaxation at high pH the lower limit for the proton exchange rate is close to 10^6 s^{-1}. Mechanisms such as



allow the proton to exchange, while the oxygen is retained in the metal coordination sphere. However, from isotope exchange experiments it has been possible to measure the dissociation rate of ^{18}O-containing species from the active site[37-40] at high pH, which is larger than 10^5 s^{-1}. Again, these values exceed the limit imposed by diffusion around neutral pH if the exchanging species were a metal-coordinated hydroxide. ^{17}O NMR experiments have independently shown that in the copper derivative the exchange of ^{17}O-containing species is an extremely fast process ($\gtrsim 10^7$ s^{-1}) even at high pH.[41]

All of these data can be rationalized if a mechanism is found for the exchange of a coordinated hydroxide as a part of a water molecule. The availability of a fifth coordination site in the metal coordination sphere has allowed us to propose[22] a mechanism analogous to that observed for the exchange of the hydroxide ion from $Cr(OH_2)_5OH^{2+}$:[42] the mechanism involves a proton transfer to the coordinated hydroxide from a transiently coordinated water molecule in the fifth coordination position:

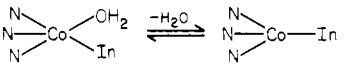


The latter proposal reconciles the presence of a coordinated hydroxide in the active enzyme with the extensive ^1H relaxation data and ^{17}O NMR and ^{18}O exchange data, by overcoming the limits imposed by diffusion. It should be noted, however, that in this mechanism no charge transport is involved; the presence of buffers is still required to explain the high turnover number of the enzyme, because a proton transport is involved for each catalytic cycle.

Inhibition

Sulfonamides, monoanionic species (halides, N_3^-, NCO^-, NCS^-, CN^-, CH_3COO^-, etc.), oxalate, aniline, imidazole, and triazoles bind the cobalt ion in CoCA in the stoichiometric ratio 1:1.[7] Some of them have been tested to be inhibitors of the catalytic activity of the native enzyme.[5,6] Upon binding, the electronic spectra of the metal ion are altered, and the NMR parameters of the inhibitor nuclei are affected by the paramagnetic center just like coordinated species.

^1H NMR spectra of the cobalt protein prove that the histidines remain coordinated upon binding of inhibitors.[17] The major effect is that in some cases the isotropic shifts are larger and the lines sharper than in the noninhibited enzyme. This is consistent with the shorter electronic relaxation times experienced by five-coordinate species. Inhibitors in general can give rise to tetrahedral species, to five-coordinate species, or to equilibria between the two.



The intensity of the overall absorption spectrum, including the presence of a weak band often observed in the range 13–15 $\times 10^3$ cm^{-1} for the five-coordinate complexes,[2,43] indicates whether the equilibrium is

(34) I. Bertini, E. Borghi, and C. Luchinat, *J. Am. Chem. Soc.*, **101**, 7069 (1979); I. Bertini, E. Borghi, G. Canti, and C. Luchinat, *J. Inorg. Biochem.*, in press.
(35) S. H. Koenig and R. D. Brown, *Proc. Natl. Acad. Sci. U.S.A.*, **69**, 2422 (1972).
(36) I. Bertini, G. Canti, C. Luchinat, and A. Scozzafava, *Biochem. Biophys. Res. Commun.*, **78**, 158 (1977).
(37) D. N. Silverman and C. K. Tu, *J. Am. Chem. Soc.*, **97**, 2263 (1975).
(38) D. N. Silverman and C. K. Tu, *J. Am. Chem. Soc.*, **98**, 978 (1976).
(39) C. K. Tu and D. N. Silverman, *J. Biol. Chem.*, **252**, 3332 (1977).
(40) D. N. Silverman, C. K. Tu, S. Lindskog, and G. C. Wynns, *J. Am. Chem. Soc.*, **101**, 6734 (1979).
(41) I. Bertini, G. Canti, and C. Luchinat, *Inorg. Chim. Acta*, **56**, 1 (1981).
(42) B. F. Melton and V. L. Pollack, *J. Phys. Chem.*, **73**, 3669 (1969).

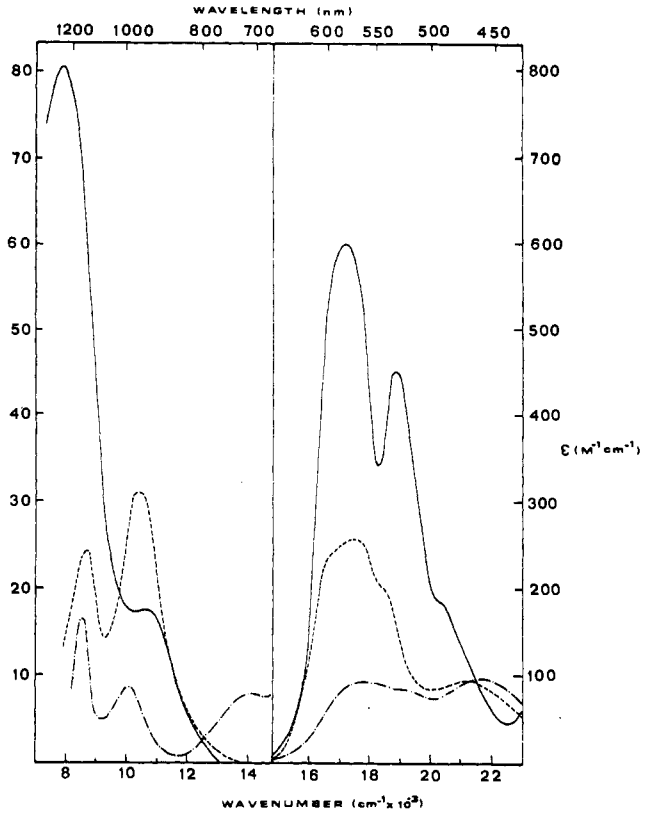


Figure 4. Electronic absorption spectra of CoBCAB adducts with cyanate (–), azide (- - -), and thiocyanate (–·–).

shifted toward four- or five-coordination or lies in between (Figure 4). The shape of the ESR spectrum also has been related to the coordination number.[44] Finally, water ^1H T_1^{-1} NMR measurements at various magnetic fields[19] have established that species assigned as pseudotetrahedral do not have coordinated water and display τ_c values of 10^{-11} s, whereas five-coordinate species have a water molecule in the coordination sphere and τ_c values of 10^{-12} s. This should be convincing proof of the kind of coordination in the presence of inhibitors as well as in the pure cobalt protein.

Ligands like sulfonamides, CN^-, NCO^-, etc. (Table I) give rise to full pseudotetrahedral species, whereas NCS^-, acetate, $Au(CN)_2^-$, etc. give rise to five-coordinate species. Possibly the interactions with the surface of the cavity play a role in determining the type of coordination, otherwise it would be difficult to understand the different behavior of NCO^- and NCS^-.

The affinity constants of inhibitors are pH dependent. To a first degree of approximation they are governed by the main active site ionization, i.e., the deprotonation of the coordinated water, and by possible acid–base equilibria of the inhibitor itself. Other ionizations in the enzyme may further modulate the affinity of inhibitors.[45] The experimental pH dependences of the apparent affinity constants, K_{app}, are grouped into three classes as shown in Figure 5; again the data have been obtained on either the zinc or cobalt enzymes. In some cases data are available on both derivatives.

(43) I. Bertini, G. Canti, C. Luchinat, and P. Romanelli, *Inorg. Chim. Acta*, **46**, 211 (1980).
(44) A. Bencini, I. Bertini, G. Canti, D. Gatteschi, and C. Luchinat, *J. Inorg. Biochem.*, **14**, 81 (1981).
(45) I. Bertini, C. Luchinat, and A. Scozzafava, *Bioinorg. Chem.*, **9**, 93 (1978).

Table I
Classification of CoCA Derivatives According to the Intensities of Their Absorption Spectra[a]

four-coordinate (ϵ_{max} > 300 M^{-1} cm^{-1})	four-to-five coordinate (ϵ_{max} = 200–300 M^{-1} cm^{-1})	five-coordinate (ϵ_{max} < 200 M^{-1} cm^{-1})
CoBCAB, high pH	CoBCAB, low pH	CoHCAB, low pH
CoHCAB, high pH	HCO_3^-	SCN^-
CN^-	F^-	HSO_3^-
NCO^-	Cl^-	I^-
SH^-	Br^-	$Au(CN)_2^-$
aniline	N_3^-	$Ag(CN)_2^-$
sulfonamides	phosphate	formate
anthranilate	benzoate	acetate
trichloroacetaldehyde	imidazole, low pH	bromoacetate
thiadiazole		oxalate
imidazole, high pH		malonate
1,2,4-triazole		succinate
tetrazole		glutarate
		glycine
		L(+)-alanine
		D(–)-alanine
		2,4-pentanedione
		1,2,3-triazole

[a] The inhibitor adducts refer to the bovine isoenzyme, with the exception of imidazole.

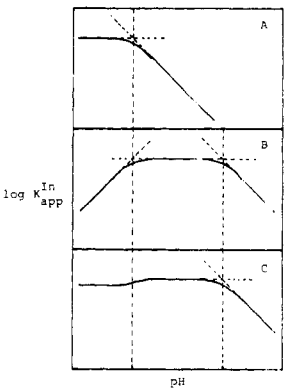
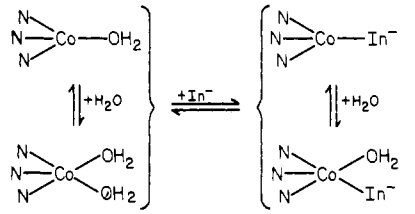


Figure 5. Experimental pH dependence of the apparent affinity constants of class A, B, and C inhibitors for carbonic anhydrase. The dashed lines represent the enzyme pK_a (left) and the inhibitor pK_a (right).

Class A

Mononegative anions, which are conjugated bases of strong acids, formally bind the low-pH form of the enzyme, the decrease in affinity at high pH (Figure 5A) resulting from formal competition with the hydroxide ion. The negative slope in the high-pH region is larger than 1 for dinegative ions,[33,45] suggesting that protonation of a second group is required for binding. The same behavior depicted in Figure 5A is shown by aniline[46] and *N*-methylimidazole,[47] which bind the low-pH



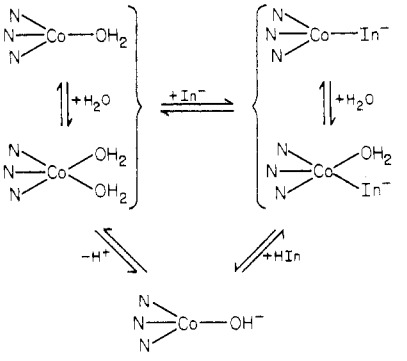
(46) I. Bertini, C. Luchinat, and A. Scozzafava, *J. Am. Chem. Soc.*, **99**, 581 (1977).

form as neutral species. The bicarbonate ion also belongs to this class[32] despite that K_M is pH independent;[25-30] apparently K_M measured at nonequilibrium conditions in kinetic experiments does not correspond to the thermodynamic dissociation constant.

Class B

The pH dependence shown in Figure 5B is observed for sulfonamides,[48,49] hydrated trichloroacetaldehyde,[50] HCN,[51] H_2S,[51,52] and α-amino acids[53] in their zwitterionic form.

Such behavior is accounted for on the basis of either the binding between the low-pH form of the enzyme and the anionic form of the inhibitor or the binding between the high-pH form of the enzyme and the neutral form of the inhibitor. There is convincing evidence that the latter pathway is followed by sulfonamides.[54] In any case the resulting adducts contain the anionic form of the inhibitor.



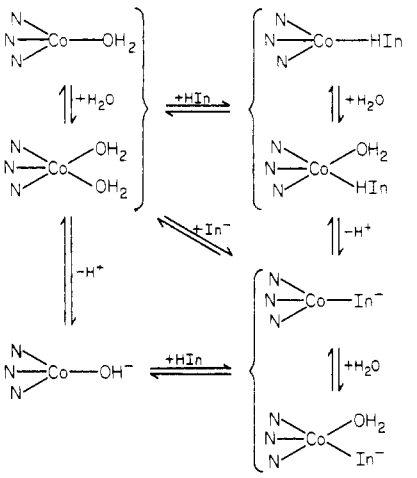
It should be noted that even for class A inhibitors a concerted reaction of In^- and H^+ with the high-pH form cannot be a priori ruled out as a possible kinetic pathway.

Class C

All the inhibitors discussed so far are usually regarded as noncompetitive for the hydration reaction and competitive for the dehydration,[6] although recently mixed inhibition patterns and evidence for noncompetitivity have been reported.[27,55]

Imidazole is the only ligand that is reported to be an inhibitor that is competitive with carbon dioxide in the native human enzyme.[26] The existence of a specific binding site of CO_2 in the hydrophobic side of the cavity has been proposed but never definitively proved. It seems now that there is at least a region of the cavity in which CO_2 interacts.[34] Imidazole evidently competes for the CO_2 binding site. Indeed, X-ray studies have shown that it binds to a different place in the cavity with respect to other inhibitors.[10] The affinity constant

Scheme II

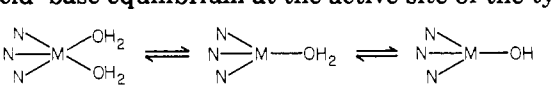


of imidazole is substantially independent on the enzyme ionization.[25,56] Recently, other triazoles with nitrogens in the 1-3-positions of the pentaatomic ring have been found to tightly bind CoCA, both human and bovine, also in an essentially pH-independent fashion.[47] Apparently, the presence of a second nitrogen in position 3 is a requirement to stabilize the molecule in the binding region of CO_2. Both imidazole and triazoles bind the metal ions, as shown by the electronic spectra of the cobalt derivative and by ^1H NMR data.[47] Owing to their peculiar acid–base behavior they may in principle be able to act as class A inhibitors like aniline and *N*-methylimidazole and class B inhibitors, since they have a weakly acidic proton. The binding scheme shown in Scheme II may therefore account for the pH dependence of K_{app} (Figure 5C).

As a matter of fact, *N*-methylimidazole (which cannot ionize) is a class A inhibitor, as well as tetrazole, which is an acid with pK_a value of 5 and therefore behaves as a regular anion. In Scheme II there are two different types of adducts, one with the neutral ligand and one with the anionic ligand, both of which may either be four- or five-coordinated. Following spectroscopic criteria (the intensity of the absorption), we assign the adducts with 1,2,4-triazole as tetrahedral and those with 1,2,3-triazole as five-coordinate. The adduct with imidazole displays a major pH dependence in its electronic spectra:[47,56] the low-pH spectrum is consistent with five-coordination and the high-pH spectrum is consistent with tetracoordination.

Concluding Remarks

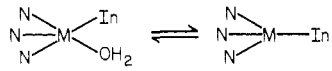
The entire body of physicochemical data on all the isoenzymes of carbonic anhydrase are consistent with an acid–base equilibrium at the active site of the type



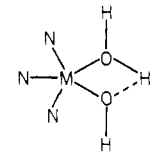
The relative share of five-coordinate species determines the shape of the electronic spectra, the electronic spin relaxation rate, and ultimately the pK_a of the acid–base equilibrium. Bovine carbonic anhydrase is largely tetrahedral at low pH, whereas the human isoenzyme is largely five-coordinate. The analysis of the kinetic

(47) G. Alberti, I. Bertini, C. Luchinat, and A. Scozzafava, *Biochim. Biophys. Acta*, **16**, 688 (1981).
(48) J. C. Kernohan, *Biochim. Biophys. Acta*, **118**, 405 (1966).
(49) S. Lindskog and A. Thorslund, *Eur. J. Biochem.*, **3**, 453 (1968); P. W. Taylor, R. W. King, and A. S. V. Burgen, *Biochemistry*, **9**, 3894 (1970).
(50) I. Bertini, E. Borghi, G. Canti, and C. Luchinat, *J. Inorg. Biochem.*, **11**, 49 (1979).
(51) A. Thorslund and S. Lindskog, *Eur. J. Biochem.*, **3**, 117 (1967).
(52) Y. Pocker and J. T. Stone, *Biochemistry*, **7**, 2936 (1968).
(53) I. Bertini, C. Luchinat, and A. Scozzafava, *Bioinorg. Chem.*, **7**, 225 (1977).
(54) R. W. King and A. S .V. Burgen, *Proc. R. Soc. London, Ser. B*, **193**, 107 (1976).
(55) Y. Pocker and T. L. Deits, *J. Am. Chem. Soc.*, **103**, 3949 (1981).
(56) R. Bauer, P. Limkilde, and J. T. Johansen, *Carlsberg Res. Commun.*, **42**, 325 (1977).
(57) I. Bertini, G. Lanini, and C. Luchinat, *J. Am. Chem. Soc.*, in press.

data points out that the hydroxo species is the active one. Inhibitors of the enzyme that act as coordination ligands give rise to the general equilibrium



The importance of five-coordination is reflected on the catalytic pathway: indeed, five-coordinate intermediate species with bicarbonate bound, which have been detected, allow the detachment of bicarbonate through an equilibrium of the type of that shown above. Finally, the rapid OH^- exchange is accounted for by assuming the existence of an intermediate of the type



We acknowledge with deepest thanks the contributions of P. Vanni of the University of Florence, who ably assisted us in our initial investigations of carbonic anhydrase. Enlightening discussions during recent years with H. B. Gray, S. H. Koenig, S. Lindskog, B. G. Malmström, Y. Pocker, and D. N. Silverman have allowed us to capture what we believe are the key properties of carbonic anhydrase.

Registry No. Carbonic anhydrase, 9001-03-0.

Electron-Deficient Carbocations

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Received May 20, 1982 (Revised Manuscript Received December 3, 1982)

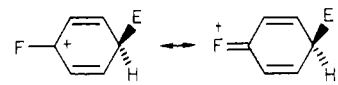
To refer to a carbocation as "electron deficient" may appear superfluous. Carbocations did not even gain respectability as organic intermediates until 1932,[1] and it was only with the advent of the direct observation of carbocations, particularly by the use of NMR in the early 1960's,[2] that these species became commonly studied as discrete entities in solution. However, they are now quite routinely examined and it is appropriate to consider gradations of stability.

What we consider as electron-deficient carbocations are those species, 1, in which the substituent R is less

1

electron donating than hydrogen. The classification of substituents as to their electron-donating ability relative to hydrogen is well-known to organic chemists from their influence on electrophilic aromatic substitution. These are categorized in a qualitative fashion in all introductory textbooks in organic chemistry; most usually as ortho,para directing and activating (e.g., alkyl, NR_2, OH, OR, O_2CR); ortho,para directing and deactivating (F, Cl, Br, I); and meta directing and deactivating (NO_2, NR_3^+, SO_3H, CO_2R, COR, CF_3, and CN).[3]

The class of deactivators, but ortho,para directors, clearly reflects the operation of two effects, namely, inductive electron withdrawal and resonance electron donation. In fact, for fluorine, even though the inductive electron withdrawal due to electronegativity ought to be the greatest of any element, the compensating resonance donation (eq 1) is so strong that this



(1)

group is frequently a net activator. Thus, the partial rate factors for electrophilic aromatic substitution in fluorobenzene range from 0.68 to 2.98,[4a] and the σ_p^+ constant for fluorine derived from cumyl chloride solvolysis is −0.07.[4b] The activating effect of fluorine is also manifested in aliphatic reactivity; for example, the rate ratio k(2-fluoropropene)/k(propene) toward addition of trifluoroacetic acid via an intermediate carbocation is 71 (eq 2).[5]

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(1) Whitmore, F. C. *J. Am. Chem. Soc.* **1932**, *54*, 3274–3283.

(2) (a) Olah, G. A.; Tolgyesi, W. S.; Kuhn, S. J.; Moffatt, M. E.; Bastier, I. J.; Baker, E. B. *J. Am. Chem. Soc.* **1963**, *85*, 1329–1334. (b) Deno, N. C.; Richey, H. G., Jr.; Hodge, J. D.; Wisotsky, M. J. *Ibid.* **1962**, *84*, 1498–1499.

(3) (a) Streitwieser, A., Jr.; Heathcock, C. H. "Introduction to Organic Chemistry", 2nd ed.; Macmillan: New York, 1981. (b) Pine, S. H.; Hendrickson, J. B.; Cram, D. J.; Hammond, G. S. "Organic Chemistry", 4th ed.; McGraw-Hill: New York, 1980. (c) Roberts, J. D.; Caserio, M. C. "Basic Principles of Organic Chemistry", 2nd ed.; W. A. Benjamin, Menlo Park, CA: 1977.

(4) (a) Stock, L. M.; Brown, H. C. *Adv. Phys. Org. Chem.* **1963**, *1*, 35–154. (b) Brown, H. C.; Okamoto, Y. *J. Am. Chem. Soc.* **1958**, *80*, 4979–4987.

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