capability of the chromophore [5], while T_1 measurements of the 4H signal of the coordinated His 119 show that the lower nuclear relaxing efficiency is due to a shorter electronic relaxation time [6]. All of these data are taken as evidence for a large percentage of five coordinate cobalt(II) in the low pH form of the human isoenzyme. The metal donor set would then be constituted by three histidine nitrogens and two water oxygens.

There are convincing experimental results and arguments to indicate that the main activity-linked acid base group is a coordinated water molecule. The existence of five coordinated chromophore in the low pH form of CoHCAB, as opposed to the mainly four-coordinate CoBCAB, for the first time satisfactorily accounts for the difference in pK_a of the coordinated water.

CoBCAB and CoHCAB are further differentiated by the higher affinity for imidazole and related ligands of the human isoenzyme [7]. The pH dependence of the affinity of such ligands for the enzyme is accounted for.

At low pH the behavior of the two isoenzymes towards anionic inhibitors appears to be different. While the spectra of the adducts with CoBCAB are pH insensitive in the range of existence of the complexes, the human isoenzyme shows an increase in molar absorbance at low pH. Such behavior is shown particularly by thiocyanate. Whereas the affinity of the inhibitor for the isoenzyme is governed by a pK_a of ~7.5, that of the change in molar absorbance is governed by a pK_a of 6.5.

Such differences are reminiscent of the different behavior of the copper derivatives with respect to sulfonamides [8].

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Mechanistic Aspects of Coordination, Catalysis and Control in Carbonic Anhydrase

Y. POCKER*, THOMAS L. DEITS, CONRAD T. O. FONG and CAROL HSING MIAO

Department of Chemistry, University of Washington, Seattle, Wash. 98195, U.S.A.

Among the most significant developments in the bioinorganic chemistry of carbonic anhydrase during the past few years are those associated with the role of coordinated zinc(II) ion in the activation of H_2O and bicarbonate. Illustrative of the catalytic processes encompassed by these developments are many hydration–dehydration processes and a variety of hydrolysis reactions. Contributing to the intensive interest and research that this field has generated is the unusual catalytic efficiency connected with the physiologically important process, eqn. 1:

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

The problems of elucidating the details of the enzymatic pathway are compounded not only by its multistep character but also by the fact that each of the intermediates in the proposed catalytic cycle coexists in several forms related not only through proton transfers but also via ligand addition and dissociation processes.

Substitution of an alkyl group (methyl through n-pentyl) for the proton of bicarbonate dramatically alters the properties of the resultant alkyl carbonate esters, $ROCO_2^-$, toward the enzyme. While bicarbonate is the natural substrate of various carbonic anhydrases with turnover numbers of $\sim 10^6$ s^{-1}, the alkyl carbonates show no detectable activity as substrates. The alkyl carbonates, however, bind efficiently to the various carbonic anhydrases and act as typical anionic inhibitors of enzyme catalyzed CO_2 hydration and HCO_3^- dehydration, with K_i values comparable to those of the corresponding RCO_2^- anions. It appears that the substitution of an alkyl group inhibits a proton transfer essential in the enzyme-catalyzed dehydration of HCO_3^-, and further that the bicarbonate proton permits a unique binding interaction with carbonic anhydrase.

Examination of the kinetic features (temperature-jump, stopped-flow and NMR) of the catalytic system emphasizes a number of additional points, notably: (a) The requirement of several binding sites. (b) Non-protein ligand lability during the interconversion of ES, EP and ESP. (c) Accessibility of two different coordination numbers for Zn(II)-, Co(II)- and Mn(II)-carbonic anhydrases. (d) Accessibility of two or more protonation states in the active site. (e) The potentially important role of the

'spectator' ligands (*i.e.* the three histidyl residues) in regulating catalytic activity and selectivity.

The results we present and the mechanism we propose provide an appealing structural model for carbonic anhydrase catalysis. The implications of these labile structures lead to fruitful mechanistic insights into a number of kinetic observations on this fascinating catalyst and provide some guidance in the search for the catalytic mechanism of carbonic anhydrase.

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The Rate of Exchange of Water Between the Active Site of Carbonic Anhydrase and Solvent

D. N. SILVERMAN* and C. K. TU

Department of Pharmacology, University of Florida, College of Medicine, Gainesville, Fla. 32610, U.S.A.

In the dehydration of HCO_3^- to form CO_2, carbonic anhydrase catalyzes the removal of an oxygen from substrate. Using an ^{18}O labeling technique, we have determined the rate at which this oxygen is released from the enzyme and appears in solvent as $H_2^{18}O$ [1]. In this technique, the isotopic content of CO_2 in solution is measured with a mass spectrometer. From the rate of depletion of ^{18}O in CO_2 and from the rate of exchange of ^{18}O between ^{12}C- and ^{13}C-containing species of CO_2, the rate of release from the enzyme of substrate oxygen is calculated. This is described by the rate constant k_{H_2O}: rate of release = k_{H_2O} $[E_{tot}]$. Although there is no proof from this technique that the site from which oxygen is released is the metal, such an interpretation is the most consistent with current ideas of the mechanism and the metal as the binding site of HCO_3^- [2].

We have observed k_{H_2O} in the absence of buffer for human carbonic anhydrases I and II obtained from red cells and from cat carbonic anhydrase III obtained from the skeletal muscle of the hindlimb. In the range of pH from 5.7 to 8.2, $k_{H_2O} = 1.5 \times 10^3$ sec^{-1} for isozyme III and is independent of pH. For isozyme I, $k_{H_2O} = 3 \times 10^4$ sec^{-1}, and is independent of pH from 5.7 to 7.5; data at higher pH are too uncertain to evaluate. In both of these cases the solvent hydrogen isotope effect, k_{H_2O}/k_{D_2O}, is 2.5 ± 20%. In contrast, we have observed a strong pH dependence of k_{H_2O} for isozyme II which reaches a maximum plateau of 5×10^5 sec^{-1} at pH 6.6 and decreases as pH is increased. The solvent hydrogen isotope effect at this maximum plateau is $k_{H_2O}/k_{D_2O} = 8.0 \pm 10\%$.

We interpret the data for isozyme II to be consistent with an intramolecular proton transfer as the step which determines the rate of oxygen release from the active site. This proton transfer occurs with a rate constant of 10^6 sec^{-1} and is likely the same step that determines the steady-state turnover rate. On the other hand, the pH independence of k_{H_2O} for isozyme III indicates no rate-limiting proton transfer for oxygen release with k_{H_2O}/k_{D_2O} determined by secondary isotope effects or solvation changes. The case for isozyme I is less certain because of the smaller pH range of observation; but isozyme I clearly behaves in a manner more similar to isozyme III than to isozyme II.

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Kinetic Pathways and Carbonic Anhydrase Mechanisms

SEYMOUR H. KOENIG and RODNEY D. BROWN, III*

Thomas J. Watson Research Center, Yorktown Heights, N.Y., U.S.A.

Carbonic anhydrase (CA) exists in three forms: the low pH form (L), the high pH form (H) and the anion-inhibited form (A). The latter includes the bicarbonate complex. All three forms have been demonstrated in CA I and, when anions are removed, CA II. The L-form of CA III has not yet been seen, even at pH 5. Equilibrium among the three forms in a sample of CA can be established, in principle, by kinetic pathways connecting any two forms and which one dominates is as yet an open question. By invoking the usual ping–pong mechanism of CA, during which hydration of CO_2 causes the enzyme to go from H to L, the kinetic pathway connecting H and A is ignored, essentially by definition. Rarely has the H–A pathway been considered [1]. Though there are few data to demonstrate the relative kinetics of the L–A and H–A pathways, it can be agreed that the latter is buffer-mediated, which could distinguish the two. In particular the lifetime of a bound anion could be buffer-dependent. We have investigated this point by measuring the relaxation rates of fluorine of trifluoroacetate in CO–CA II sections. The implications of the findings for the fundamental enzyme mechanism of CA will be discussed.

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