'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

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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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1 D. N. Silverman, C. K. Tu, S. Lindskog and G. C. Wynns, *J. Am. Chem. Soc., 101,* 6734 (1979).
2 S. Lindskog, *Adv. Inorg. Biochem., 4,* 115 (1982).

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Kinetic Pathways and Carbonic Anhydrase Mechanisms

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Carbonic anhydrase (CA) exists in three forms: the low pH form (L), the high pH form (H) and the anion-inhibited 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.

1 S. M. Koenig, R. D. Brown and S. G. Jacob, 'Biophysics and Physiology of Carbon Dioxide', C. Bauer, G. Gros and H. Bartels (eds.), Springer, Berlin, 1980, p. 238.