Kinetics of Carbonic Anhydrase–Inhibitor Complex Formation. A Comparison of Anion- and Sulfonamide-Binding Mechanisms*

Palmer W. Taylor† and A. S. V. Burgen

ABSTRACT: The relationship between the mechanism of formation of anion and sulfonamide complexes with human carbonic anhydrase has been investigated by stopped-flow kinetics. The results are consistent with a mechanism involving a pH-dependent equilibrium between two coordination forms of the enzyme where anions selectively combine with the coordination form of the enzyme predominating at low pH, while sulfonamides combine with the high-pH form. The effect of pH on the anion affinity correlated with the pH dependence of the spectral change for the Co(II) human isozymes. Formation of the anion complex appears to be rate limited by prior dissociation of the sulfonamide; however, it has not been possible to demonstrate a rate limitation on sulfonamide association by dissociation of the anion. Carboxymethylation of carbonic anhydrase B alters the apparent affinity and pH dependence of anion binding to the enzyme. This is due largely to an alteration in the relative populations of combining and noncombining forms of the enzyme and to a lesser extent a reduction in the intrinsic affinity of anions for the chemically modified enzyme. This contrasts with the binding of sulfonamides where carboxymethylation causes a substantial reduction in binding affinity. Comparison of a ring-substituted *p*-carboxybenzenesulfonamide to its isosteric uncharged nitro congener shows a 2.9-kcal difference in the respective binding affinities which is not observed in the unmodified enzyme. This difference, which presumably is a consequence of electrostatic repulsive forces between carboxyl groups on the inhibitor and carboxymethylated enzyme, is reflected in a reduced association rate with little change in the dissociation rate. The resulting kinetic asymmetry permits a description of the effect of charge repulsion on the reaction energy profile.

Monovalent anions and aromatic sulfonamides are inhibitory ligands to the metalloenzyme, carbonic anhydrase, although the sulfonamide complexes usually possess considerably higher affinities (Maren, 1967; Verpoorte *et al.*, 1967; Pocker and Stone, 1967; Thorslund and Lindskog, 1968). The native metal, Zn, in the enzyme is required for both the binding of inhibitors and catalytic activity, however selective replacement by Co(II) leads to the retention of very similar catalytic and ligand binding characteristics (Lindskog, 1963; Coleman, 1967). Changes in the Co(II) d–d transitions resulting with the addition of either sulfonamides or inhibitory anions suggest that each type of inhibitor binds within or close to the coordination sphere of the metal (Lindskog, 1963, 1966; Coleman, 1968). The X-ray crystallographic structure of the sulfonamide complex substantiates this as the sulfonamido group has been found to reside within coordination distance to the Zn (Fridborg *et al.*, 1967). Studies employing infrared spectroscopy (Riepe and Wang, 1968) and ^{35}Cl nuclear magnetic resonance (Ward, 1969) have indicated direct metal coordination for enzyme-bound azide and chloride; these inhibitors are displaced from the coordination site with addition of sulfonamides. Recent studies on proton relaxation of H_2O have demonstrated that both anions and sulfonamides will displace H_2O presumed to be coordinated to the metal (Fabry *et al.*, 1970).

Although both anions and sulfonamides appear to occupy a common coordination site on the enzyme, a number of findings have indicated differences in the mechanism of stabilization of these two types of inhibitor complexes. The aromatic ring of the sulfonamide appears to bind within a hydrophobic cleft on the protein (Fridborg *et al.*, 1967; Chen and Kernohan, 1967). Thus stabilization of the complex may be conferred through both a ligand–metal bond and hydrophobic forces involving the essential aromatic ring. Ultraviolet difference spectroscopy has indicated that a change in enzyme conformation accompanies sulfonamide and anion binding (King and Burgen, 1970), however, the magnitude of the sulfonamide perturbation is about five times greater. Moreover, the environment and titration behavior of individual histidine residues for the sulfonamide and anion–enzyme complexes differ substantially (King and Roberts, 1971), indicating dissimilarities in the conformation of the respective complexes. Anions are noncompetitive inhibitors of the enzyme (Verpoorte *et al.*, 1967; Kernohan, 1965), while sulfonamides have more recently been shown to inhibit CO_2 hydration competitively (Kernohan, 1966b; Lindskog and Thorslund, 1968).

Anions of strong acids exhibit a maximal inhibitory capacity for the enzyme at low pH and appear to bind with the coordination form of the Co(II) enzyme which predominates at low pH (Lindskog, 1966; Thorslund and Lindskog, 1968; Ward, 1969). However, we have recently shown that improbably high association rate constants would be required if the low-pH coordination form of carbonic anhydrase and sulfonamide anion were the attacking species (Taylor *et al.*, 1970b). Therefore, it is likely that combination occurs between the neutral sulfonamide species and the alkaline coordination form of the enzyme. It is this coordination form which is catalytically active for CO_2 hydration (Kernohan, 1965). Ultraviolet difference spectroscopy, however, has revealed that the bound sulfonamide resembles its anionic species (King and Burgen, 1970) so that the probable mechanism of sulfonamide complex formation involves initial ligand combination as a neutral species with subsequent loss of the sulfonamido proton.

We have further examined the relationship between anion

* From the Medical Research Council, Molecular Pharmacology Unit, Medical School, Cambridge, England. *Received June 2, 1971.*

† Present address: Division of Pharmacology, Department of Medicine, University of California, San Diego, La Jolla, Calif. 92037; to whom correspondence should be addressed.

and sulfonamide complex formation mechanisms by following the kinetics of these reactions by stopped-flow measurements of fluorescence quenching. In contrast to sulfonamide binding, anion association is too rapid to directly examine the kinetics of this reaction by the stopped-flow method. However, equilibrium affinities and certain limitations on the rate constants may be determined by competition with the sulfonamide interaction. In the accompanying paper we show that kinetics of the anion interaction may be directly investigated by nuclear magnetic resonance spectroscopy (Taylor *et al.*, 1971).

Carboxymethylation of human carbonic anhydrase B places a negatively charged carboxymethyl group in the vicinity of the ligand binding site (Bradbury, 1969a,b). The effect of this protein modification on the comparative binding kinetics and energetics for both types of ligands has also been examined.

Methods

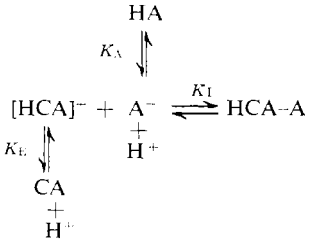
Materials. The isozymes, carbonic anhydrases B and C, were purified from human erythrocytes according to the method of Armstrong *et al.* (1966) and stored as slurries in 60% ammonium sulfate. Carboxymethylated isozyme B was prepared by reaction with 10 mM bromoacetate for 6 hr at pH 7.4 (Whitney *et al.*, 1967); the unreacted bromoacetate and reaction products were separated as previously described. *p*-Nitrobenzenesulfonamide (*p*-NBS),[1] *p*-carboxybenzenesulfonamide (*p*-CBS), bromoacetic acid, and bromopyruvic acid were obtained from commercial sources and recrystallized twice from ethanol or petroleum ether (bp 30–60°). Salicylazobenzenesulfonamide (SABS) was synthesized in the laboratory (Taylor *et al.*, 1970a).

Kinetic Studies. The stopped-flow instrumentation and methods for measuring complex formation kinetics and affinities have been described in detail (Taylor *et al.*, 1970a). Briefly, these studies were conducted under pseudo-first-order conditions where 0.50–1.25 μM enzyme was reacted with at least a 10-fold molar excess of *p*-NBS. Rate constants were determined from slopes of semilogarithmic plots of the voltage deflection *vs.* time and represent the mean value from at least three separate reactions. To examine the effect of anions on the association kinetics, equivalent concentrations of anion were added to both the sulfonamide and enzyme reactant solutions. Loss of HCN was minimized by adding CN^- as a 0.1 or 0.01 M NaCN solution to the reactants immediately before they were withdrawn into fitted syringes which served as the reactant reservoirs of the stopped-flow instrument. Over the pH range 5.5–10.8, the following 20 mM buffers were used: pH 5.5–7.0, imidazole or phosphate;[2] pH 7.0–9.0, Tris-SO_4; pH 9.0–10.2, 2-amino-2-methyl-1,3-propanediol sulfate; pH 10.2–10.8, triethylamine hydrochloride. A final measurement of pH was also made at the completion of the reaction by removing a sample from the collection syringe of the stopped-flow apparatus. All reactions were carried out at 25.0 ± 0.2°.

Results

Anion Inhibition of Sulfonamide Association Rates. Examined in the presence of increasing concentrations of monovalent anions, the association rate between isoenzyme C and *p*-NBS is inhibited as shown by the decrease in the apparent rate constant, k_i. When the data are plotted as $\log [(k_0/k_i) - 1]$ *vs.* log (anion) (Johnson *et al.*, 1942), where k_i and k_0 are the observed rate constants for sulfonamide association in the presence and absence of inhibitory anion, the slopes fall in the range of 1.0 ± 0.1 predicted for a 1:1 complex between the anion and carbonic anhydrase (Figure 1). The apparent affinity of the anions may be determined from the concentration corresponding to a zero ordinate, *i.e.*, where $k_0 = 2k_i$. Affinities have been expressed subsequently as association constants, K_I, which are reciprocals of the above concentration values. The order of inhibition for the four anions parallels that previously observed for the inhibition of esterase activity (Verpoorte *et al.*, 1967).

Determination of K_I for anions such as SCN^- from plots similar to Figure 1 over a pH range yields the relationship shown in Figure 2a. At low pH inhibition becomes pH independent and maximal, while at higher pH the apparent affinity is directly proportional to the H^+ concentration. Since appreciable protonation of SCN^- does not occur over this pH range, an ionization on the enzyme with a pK of 6.5 governs the affinity of SCN^- for isozyme C. For cyanide, a more effective inhibitor of the enzyme, a different pH dependence is observed (Figure 2b). The pK for dissociation of HCN at 25° is 9.21 (Izatt *et al.*, 1962), so that both CN^- and HCN must be taken into account in assessing binding. Assuming that it is the same form of the enzyme which combines with cyanide as with thiocyanate, the pH profile of cyanide inhibition can be accounted for by considering only CN^- as the reactive species. This may be represented in.



(1)

Then $K_{I,app} = [K_I K_A(H^-)/((H^+) + K_E)((H^+) + K_A)]$, where $K_{I,app}$ and K_I denote the apparent and intrinsic affinity of the ligand and K_A and K_E are H^+ dissociation constants of the anion and enzyme, respectively. With SCN^-, where $K_A \gg H^+$, the above equation reduces to $K_{I,app} = K_I(H^+)/((H^+) + K_E)$; thus, at low pH, $K_{I,app} \rightarrow K_I$. Studies employing inhibition of esterase activity (Thorslund and Lindskog, 1968), ^{35}Cl nuclear magnetic resonance (Ward, 1969), and Co(II) spectral changes (Lindskog, 1966) have also demonstrated preferential binding of anions of strong acids to the low-pH form of bovine carbonic anhydrase B. For CN^-, since $K_A < K_E$, $K_{I,app}$ will be considerably smaller than K_I over the complete pH range. Taking pK_E = 6.5 and pK_A = 9.2, a value of 7.45 is calculated for pK_I (Figure 2b). Therefore, when considering only CN^- as the reactive species, the intrinsic affinity is found to be 500 times greater than the maximal apparent affinity. This high affinity will allow us in subsequent kinetic studies to place additional constraints on CN^--binding kinetics and mechanism.

Relationship between the pH Dependence of Anion Binding and Co(II) Carbonic Anhydrase Spectra. The spectra arising from d–d transitions of Co(II) carbonic anhydrase demonstrate a pH-dependent equilibrium between two coordination forms of the enzyme (Lindskog, 1963; Lindskog and Nyman,

[1] Abbreviations used are: *p*-NBS, *p*-nitrobenzenesulfonamide; *p*-CBS, *p*-carboxybenzenesulfonamide; SABS, *p*-(salicyl-5-azo)benzenesulfonamide.

[2] High concentrations of both imidazole base and $H_2PO_4^-$ will inhibit sulfonamide association rates with carbonic anhydrase B, however the concentrations of inhibitory species employed here are less than their apparent dissociation constant.

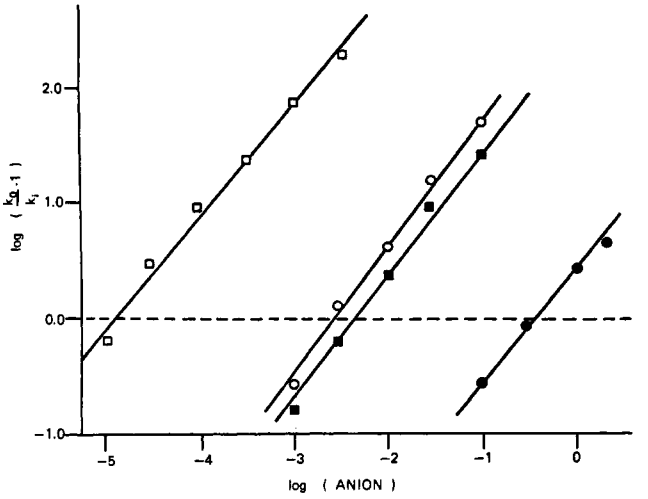


FIGURE 1: Inhibition of the association rate between *p*-nitrobenzenesulfonamide and human carbonic anhydrase C by various anions: (□) CN, (○) SCN^-, (■) ClO_4^-, (●) Cl^- (pH 7.6), 0.625 μM enzyme. k_0 and k_i are the association rate constants in the absence and presence of the respective amount of anion. A slope of 1.0 is compatible with a 1:1 complex between enzyme and anion, and the intersection with the horizontal dotted line gives a measure of the apparent affinity of the anion, $K_{I,app}$; CN, $K_{I,app} = 7.8 \times 10^{+4}$; SCN^-, 3.51×10^2; ClO_4^-, 2.13×10^2; Cl, 2.86.

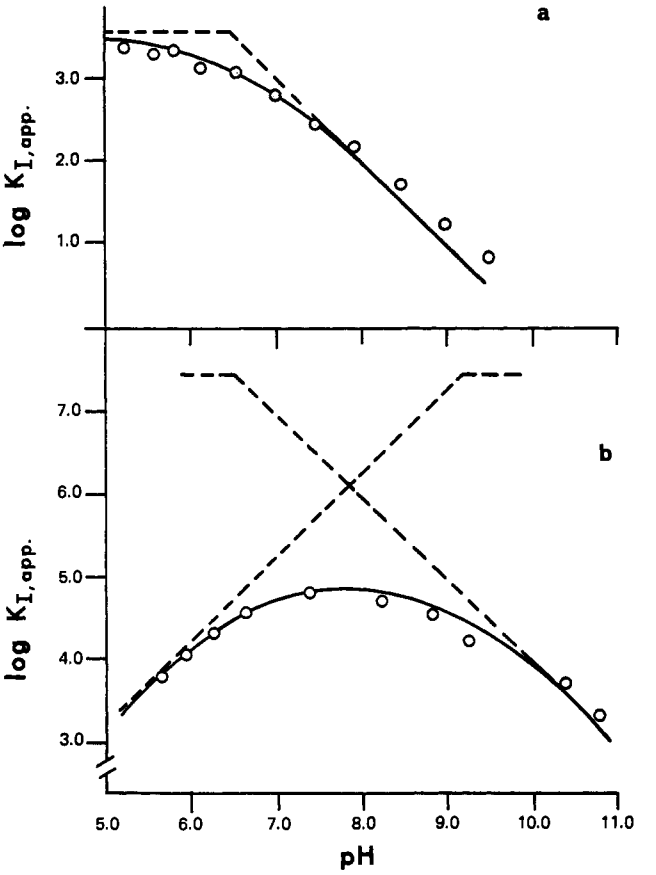


FIGURE 2: pH dependence of the apparent affinity of anions for human carbonic anhydrase C: (a) SCN^- and (b) CN^-. The solid lines are theoretical, calculated from eq 1 in the Results section assuming $K_E = 3.18 \times 10^{-7}$ and $K_A = 6.2 \times 10^{-10}$ for CN^- and $K_A < 10^{-3}$ for SCN^-. Intersection of the dotted lines of slopes -1.0 and 0.0 gives pK_E, and of the lines of slope $+1.0$ and 0.0 gives pK_A.

1964). The H^+ dependence of this equilibrium varies with the particular isozyme; the transition pH is 6.5 for human C and 7.3 for human B. Carboxymethylation of human B shifts this pH to 9.1 (Taylor *et al.*, 1970b). A similar pH dependence of sulfonamide complex formation is observed for the Zn and Co(II) enzymes, and the H^+ dependence of sulfonamide association rates correlates closely with the spectral pH dependence. Thus, the metal coordination structure in the "nonliganded" enzyme governs the specificity of the macromolecule toward association of the sulfonamide ligand.

A unique Co(II) spectrum is generated for the carbonic anhydrase–sulfonamide complex which is pH independent, thus indicating that with ligand binding the coordination state of the enzyme becomes fixed. Correspondingly, dissociation rates of sulfonamide complexes are also pH insensitive (Taylor *et al.*, 1970b). Since anion–carbonic anhydrase complexes also give rise to pH-independent spectra (Lindskog, 1966), similar behavior might be expected to apply for anionic ligands.

The intrinsic affinity of SCN^- for isozyme B is found to be very similar to that of isozyme C, however the ionization controlling anion binding has a pK of 7.5 (Figure 3). Carboxymethylation results in an alkaline shift of this group which now exhibits a pK of ~9.2. Thus, with each isozyme the pH dependence of anion affinities also correlates well with the respective Co(II) spectral transitions, and anion binding may be adequately described by eq 1.[3]

The higher apparent affinity of SCN^- for the carboxymethylated enzyme at high pH simply reflects the presence of a greater fraction of the coordination form of the enzyme that binds anions. Carboxymethylation, in fact, causes a three-fold reduction in K_I, the intrinsic affinity for anion association; while K_E, the pH-dependent equilibrium between coordination forms of the enzyme, is shifted by a factor of 50. The latter shift would be positive as denoted by eq 1; *i.e.*, an en-

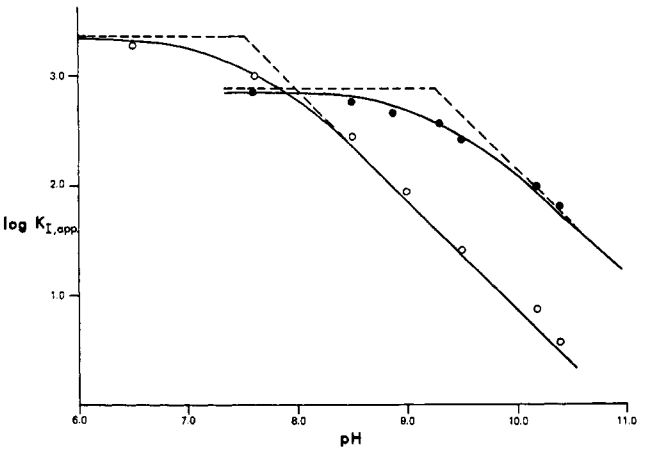


FIGURE 3: pH dependence of the observed affinity of SCN^- for human carbonic anhydrase B (○) and its carboxymethylated derivative (●). Solid lines are calculated from eq 1 in the Results section assuming $K_E = 3.18 \times 10^{-8}$ for the B isozyme and 6.32×10^{-10} for its carboxymethylated derivative.

[3] The dissociation rate of the carbonic anhydrase C–formate complex is nearly pH independent over the range 6.3–9.5 (Taylor *et al.*, 1971). Thus, parallels between the H^+ dependence of the Co(II) spectral changes and the kinetics of the respective association and dissociation processes may be drawn for the formate anion as well as the sulfonamides.

TABLE I: A Comparison of Affinities and Rate Constants for Various Inhibitor Complexes of Native and Carboxymethylated Carbonic Anhydrase B.

	Carbonic Anhydrase B				Carboxymethyl Carbonic Anhydrase B			
Inhibitor	K_I (M^{-1})[a]	$-\Delta F^\circ$ (kcal/mole)	k_a (M^{-1} sec^{-1})[b]	k_d (sec^{-1})[c]	K_I (M^{-1})[a]	$-\Delta F^\circ$ (kcal/mole)	k_a (M^{-1} sec^{-1})[b]	k_d (sec^{-1})[c]
SCN^-	2.24×10^3	4.6			7.24×10^2	3.9		
$O_2NC_6H_4SO_2NH_2$-*p*	6.41×10^7	10.6	3.5×10^6	0.049	1.46×10^6	8.4	3.0×10^6	2.18
$^-O_2CC_6H_4SO_2NH_2$-*p*	1.30×10^7	9.7	2.7×10^6	0.210	2.2×10^3	4.6	1.08×10^4	4.9

[a] Intrinsic affinity calculated from the apparent affinity using eq 1 in text for SCN^- and the formula, $K_I = K_{I,app}[((H^+) + K_S) \times (K_E + (H^+))/K_E(H^+)]$ for the sulfonamides; $K_E = 3.18 \times 10^{-8}$, carbonic anhydrase B; $K_S = 5.02 \times 10^{-10}$, *p*-NBS; 1.26×10^{-10}, *p*-CBS. [b] Determined by direct measurements of association rates over the pH range, 7.6–10.2, and calculated from the formula analogous to that in footnote *a* for *p*-NBS. For *p*-CBS, this value was calculated from $K_I k_d$. [c] k_d was determined by directly following dissociation of the complex as previously described.

hanced affinity for H^+; however, if the equilibrium, K_E, is considered to be OH^- dependent, carboxymethylation results in a decrease in OH^- affinity qualitatively analogous to the decrease in SCN^- affinity.

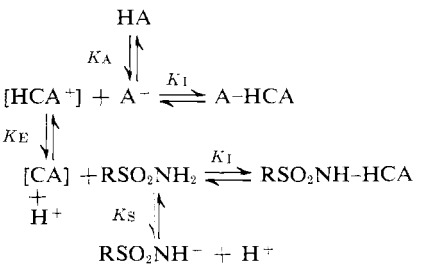
An equilibrium between metal-coordinated H_2O and OH^- has been proposed as the ionization responsible for generation of the catalytically active form of the enzyme and the spectroscopic change observed for the Co(II) enzyme (Lindskog, 1963; Coleman, 1968; Bradbury, 1969a). Substitution of either OH^- or SCN^- for metal-coordinated H_2O would yield an identical charge difference associated with ligand coordination. Carboxymethylation exerts a quantitatively different influence on the equilibria for OH^- and SCN^- association which illustrates that a Coulombic repulsive force between the carboxymethyl group and the coordinated OH^- could not, by itself, account for the large shift in K_E. This can not rule out the possibility of an equilibrium dependent on OH^-–metal coordination but it indicates an essential role for the involvement of other interaction forces in this equilibrium.

With anion association at both pH 6.0 and 9.0 ultraviolet difference spectra arising from perturbation in the environment of aromatic amino acid residues are evident (King and Burgen, 1970).[4] This indicates some difference in protein conformation between the anion-liganded enzyme and the coordination form predominating at high pH for which OH^-–metal coordination has been suggested. Moreover, the iodide complex of carbonic anhydrase and the alkaline coordination form of the enzyme exhibit strikingly different histidine titration behavior (King and Roberts, 1971). The altered environment of two histidine residues and appearance of an additional titratable histidine for the iodide complex also suggest conformational differences between these two forms.

The carboxymethyl group in the chemically modified enzyme is linked to the 3′-N of essentially a single histidine residue (Bradbury, 1969a,b). The high specificity of the reaction appears to be dependent on initial reversible binding of the haloacetate to the anionic binding site thereby directing the alkylation. Thus, the covalently linked carboxymethyl group residue is in very close vicinity to the active site. Provided that the conformational position of the alkylated histidine remains unchanged, the position of the carboxylate anion would be constrained by orientations allowed through rotation around the C–N bond.

The small difference in free energy of stabilization between SCN^- complexes of isoenzyme B and its carboxymethylated derivative indicates minimal charge repulsion between the carboxymethyl group and the bound ligand. It seems likely that upon binding the formal charge on the anion is lost requiring a net positive charge on the coordination site to become neutralized. In this case it can be similarly argued that an electrostatic interaction between the carboxymethyl group and the coordination site of the anion, which would serve to stabilize the unliganded form of the enzyme, must be equally small.

Co(II) spectra of the carboxymethylated enzyme qualitatively resemble those of the native enzyme and differ markedly from spectra generated when carboxylate anions reversibly bind to the enzyme (Taylor *et al.*, 1970b). Thus, both the spectroscopic observations and comparative anion-binding affinities show no evidence for substantial interaction between the carboxymethyl group and the metal, and hence, are compatible with an orientation of the covalently linked group which is directed away from the metal. In contrast to the small loss in intrinsic anion affinity resulting from carboxymethylation, this protein modification has a striking effect on the sulfonamide interaction (Table I). The neutral sulfonamide ligand and the alkaline coordination form of the enzyme are the likely combining species (Taylor *et al.*, 1970b) which differs from the anion-binding case as shown in eq 2. With the *p*-NBS complex, carboxymethylation is responsible for a 45-fold reduction in affinity, K_I, while with *p*-CBS the difference is 2000-fold (Table I).[5]



(2)

[4] Difference spectra equivalent to those reported by King and Burgen (1970) are also observed at pH 6.0 and 9.0 with various monovalent anions which are inhibitory to the enzyme.

[5] Despite the appreciable reduction in binding energy, the sulfonamide still appears bound with the sulfonamido group coordinated to the

The difference in free energy of stabilization between the *p*-NBS- and *p*-CBS–carboxymethyl enzyme complexes is 3.8 kcal which is 2.9 kcal in excess of that observed for the unmodified enzyme. These congeneric inhibitors are essentially isosteric, therefore the 2.9-kcal differential can be expected to arise from Coulombic repulsive forces between juxtapositioned carboxyl groups on the enzyme and sulfonamide. In contrast to complexes with the anionic ligands, the charge residues ~8 Å from the metal, and there is no opportunity for delocalization or neutralization of charge through coordination of the charged center of the bound ligand.

Comparisons in binding energy between charged and uncharged analogs have been employed to estimate interaction distances between a quaternary nitrogen and an anionic site on the protein for cholinesterase (Adams and Whittaker, 1950) and certain hapten–antibody systems (Grossberg and Pressman, 1960; Marlow *et al.*, 1969). Similar criteria could be applied to the Coulombic repulsive forces between charged centers here in estimating the intercarboxylate distance as the native B enzyme complexes provide a satisfactory control for eliminating differences in binding energy arising from nonelectrostatic interactions. However, the presumed charge repulsion affects primarily the association process (compare k_a and k_{-d} in Table I) presenting an obvious kinetic asymmetry; thus, consideration of relative reaction kinetics may give a more detailed description of reaction mechanism.

The simplest representation of the influence of charge on the energetic course of the reaction is shown in Figure 4a. However, this reaction profile seems unlikely. In a single-step reaction, repulsive forces destabilizing the complex, which should affect the equilibrium distance of the bound ligand, would also be expected to lower the transition state barrier for the dissociation process. In the *p*-NBS- and *p*-CBS–carboxymethyl enzyme complexes the difference in free energy of stabilization of the complex, $\Delta(\Delta F°)$, is not paralleled by a reduction in the free energy of activation of the dissociation process, $\Delta(\Delta F_{-1}^{\pm})$. According to transition state theory, $\Delta F^{\pm}$ contains an electrostatic term (Laidler, 1963; Jones, 1968) which has the usual Coulombic dependence on the inverse of the distance between charged centers. $\Delta F_{-1}^{\pm}$, in contrast to $\Delta F_1^{\pm}$, appears insensitive to charge repulsion in the carboxymethyl enzyme implying that the distance between charged groups is unaltered when going from the complex to the reaction transition state.

This leads to a consideration of a more detailed reaction profile where the enhanced energy barrier, arising from charge repulsion, clearly must precede the rate-limiting transition-state barrier for complex formation as shown in Figure 4b. Kinetic constants would then be related to activation energies by

$$k_a = \left(\frac{RT}{nh}\right) e^{\frac{-(\Delta F_1 + \Delta F_2^{\pm})}{RT}}$$

$$k_d = \left(\frac{RT}{nh}\right) e^{\frac{-\Delta F_{-2}^{\pm}}{RT}}$$

Repulsion between like charges in the combining species would affect ΔF_1, and, consequently, exert a selective influence

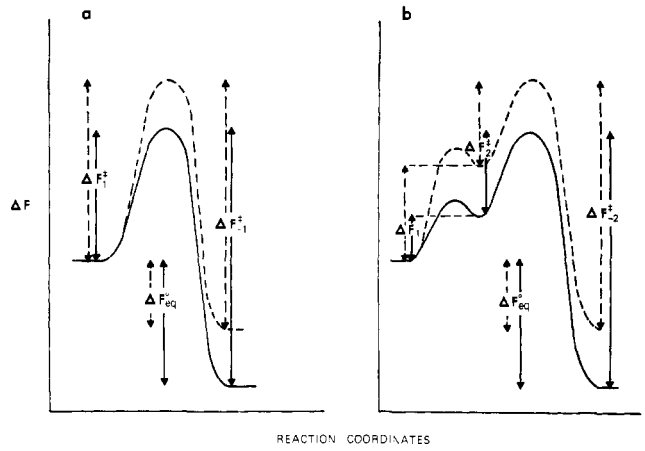
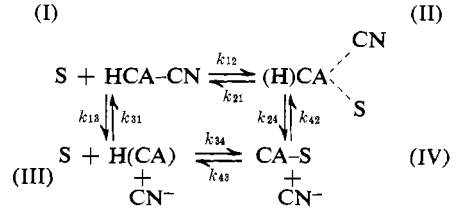


FIGURE 4: Reaction energy profiles for sulfonamide association with carboxymethyl carbonic anhydrase B. The solid line represents the profile for *p*-nitrobenzenesulfonamide association while the dotted line represents *p*-carboxybenzenesulfonamide. Free-energy values have been calculated from the kinetics of the forward and reverse rates. The relative magnitudes of ΔF_1 and $\Delta F_2^{\pm}$ are arbitrary.

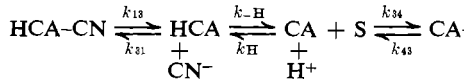
on k_a. This two-step reaction scheme would preclude an interpretation relating the reduction in energy of stabilization to the equilibrium distance between interacting groups as can be done for a simple binding isotherm with a dominant electrostatic force. However, this scheme will accommodate a mechanism involving a change in enzyme conformation arising from ligand association. The reaction profile of Figure 4b allows a discrete energy state to become populated although a concerted mechanism is also applicable to these considerations. In this case the energy profile would simply have an asymmetric transition state rather than the two energy barriers shown in Figure 4b.

Kinetic Relationships between Anion and Sulfonamide Binding. Considering the equilibrium data alone, eq 2 satisfactorily describes the overall reaction path for inhibitor binding to the enzyme. Moreover, the influence of structural modification of either the ligand or enzyme can be analyzed in terms of individual rate or equilibrium constants. Stopped-flow studies only permit a direct measure of sulfonamide binding kinetics, as anion ligand exchange is considerably more rapid and yields smaller spectroscopic changes. Further rate studies are therefore limited to examining the kinetic relationship between association of the two types of ligands. Interconversion between the CN^- and sulfonamide complexes can be represented by eq 3.



(3)

The reaction path, I → III → IV, is equivalent to eq 2 and may be rewritten to include the proton-dependent equilibrium on the enzyme



(4)

metal as the Co(II) spectrum of the *p*-CBS complex is virtually identical with the complex with the unmodified enzyme. Furthermore, we have found that ultraviolet difference spectra equivalent to those observed with the native enzyme–sulfonamide complex are present with both the *p*-NBS and *p*-CBS complexes with the carboxymethyl enzyme.

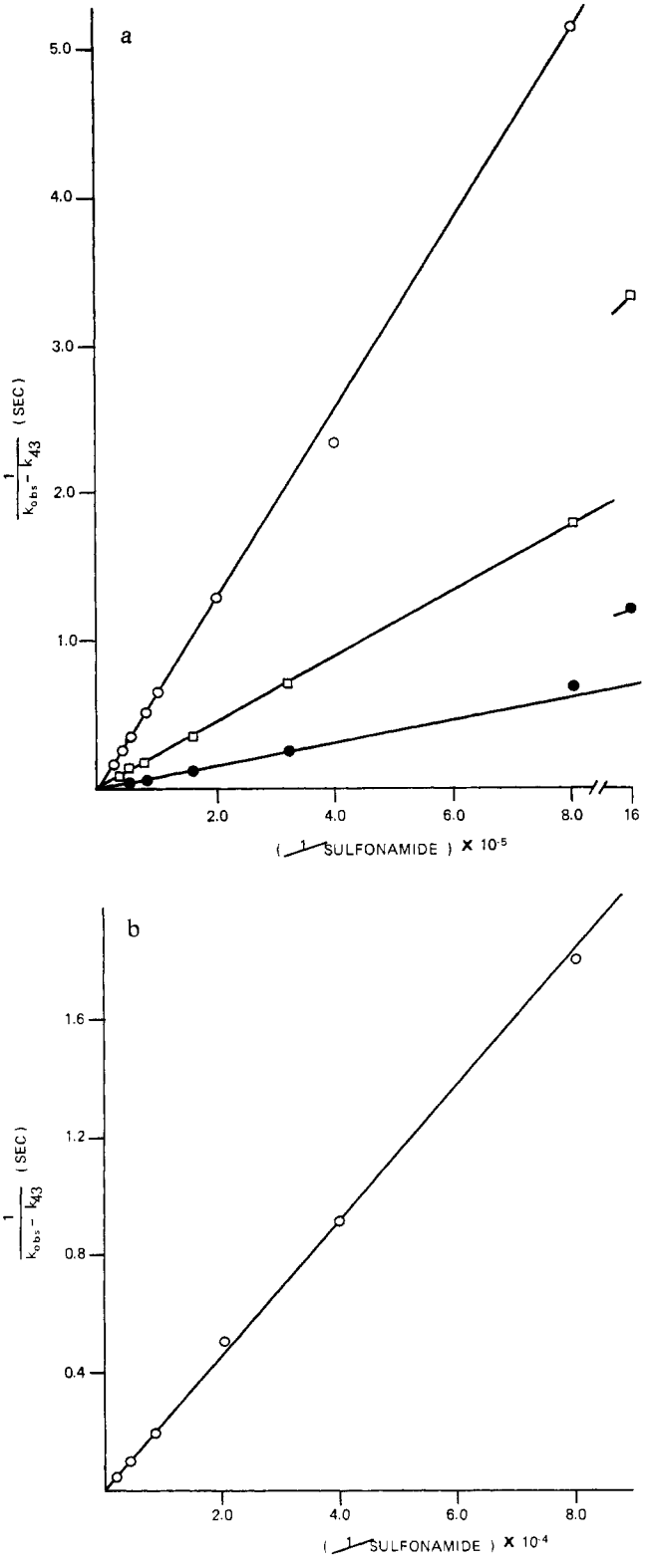


FIGURE 5: (a) Observed rate of formation of sulfonamide complexes in the presence of cyanide as a function of sulfonamide concentration. Data are plotted according to eq 4 in the text where k_{obsd} is the observed pseudo-first-order rate constant for the overall reaction. (○) 10^{-3} M cyanide and *p*-NBS; (●) 10^{-3} M cyanide and SABS; (□) 3×10^{-3} M cyanide and SABS. All reactions were carried out with the C isozyme in 20 mM of Tris-sulfate, pH 7.6. (b) Observed rate of formation of *p*-carboxybenzenesulfonamide complex in the presence of 15 mM bromopyruvate as a function of sulfonamide concentration. The reactions were carried out within 2 min after adding the bromopyruvate to the enzyme in order to minimize irreversible alkylation by bromopyruvate. Human C isozyme, 20 mM phosphate, pH 6.5.

The steady-state rate constant for formation of CA–S from HCA–CN becomes

$$k_{obsd}\ (sec^{-1}) = \frac{k_{13}k_{34}k_{-H}(S) + k_{31}(CN)k_H(H^+)k_{43}}{k_{31}(CN)[k_H(H^+) + k_{34}(S)] + k_{34}k_{-H}(S)}$$

which may be rewritten to give

$$\frac{1}{k_{obsd} - k_{43}} = \frac{1}{k_{13} - k_{43}}\left[1 + \frac{k_{31}(CN)}{k_{-H}}\right] + \frac{k_{31}(CN)k_H(H^+)}{(k_{13} - k_{43})k_{34}k_{-H}(S)}$$

Plots of $1/k_{obsd} - k_{43}$ *vs.* 1/(S) yield $[1/(k_{13} - k_{43})][1 + (k_{31} \times (CN)/k_{-H})]$ as the ordinate intercept. Since $[1 + (k_{31}(CN)/k_{-H})] \geq 1$, and k_{43}, the sulfonamide dissociation rate, is known to be 5×10^{-2} and 3×10^{-2}, respectively, for *p*-NBS and SABS complexes (Taylor *et al.*, 1970a), a minimal value for k_{13} can be obtained from the ordinate intercept.

The observed rate of reaction between the HCA–CN complex and sulfonamides is linearly proportional to sulfonamide concentration (Figure 5a), so that plots of $1/(k_{obsd} - k_{43})$ *vs.* 1/(S) appear to intersect at the origin. The precision of the results at high sulfonamide concentration simply allows us to say this intercept is $\leq 10^{-2}$ sec. Therefore, the above reaction path for conversion of the cyanide into the sulfonamide complex requires the CN^- dissociation rate, k_{13}, to exceed 10^2 sec^{-1}. The intrinsic affinity of CN^- was 2.8×10^7 M^{-1} (Figure 2b), therefore k_{31} would be greater than 2.8×10^9 M^{-1} sec^{-1}.

This interpretation yields a surprisingly high association rate for CN^-, a value which exceeds that measured for carboxylate anion association by an order of magnitude (Taylor *et al.*, 1971). A rate constant of this magnitude indicates no activation energy other than diffusion for association (Alberty and Hammes, 1958) and can be questioned as exceeding the rate limitation for ligand–macromolecule association. Very few unidentate ligand-substitution processes exhibit rate constants of 10^9 or higher (Eigen and Wilkens, 1965), and geometric limitations on access to the combining site on the enzyme (Fridborg *et al.*, 1967) might further limit a diffusion-controlled association.

Two alternative possibilities to this reaction scheme should, therefore, be considered. If HCN were an associating species, its intrinsic affinity would be lower than CN^- by the ratio, $K_A : K_E$, (~500-fold) and parallel reductions in the association rate would be possible. The pH profile requires that HCN combine only with the alkaline coordination form of the enzyme. The findings that acetonitrile (CH_3CN) is an ineffective inhibitor of the enzyme (Pocker and Stone, 1967, 1968), and that cyanide probably does not coordinate to the metal through the nitrogen (E. Grell, personal communication) argue against HCN as a liganded species. We are left with the possibility of a mechanism involving initial combination as HCN with subsequent loss of the proton. This has a parallel to the sulfonamide–enzyme association scheme (Taylor *et al.*, 1970b).

A second possibility is that prior dissociation of the CN^- ligand is not an obligatory preliminary step for sulfonamide association. In this case the reaction would proceed through formation of a ternary intermediate complex (es) as shown by I → II → IV. Initial sulfonamide association forming the intermediate, II, may not involve the coordination sphere of the metal; but, nevertheless, could assist the subsequent dissociation step of the leaving group, CN^-. Fluorescence quenching, being dependent on association of the sulfonamide, would re-

sult through population of either the intermediate state or the final complex, and the rate would be expected to be linearly proportional to sulfonamide concentration.

Anions show noncompetitive inhibition with respect to esterase and CO_2 hydration activity (Verpoorte *et al.*, 1967; Pocker and Stone, 1968; Kernohan, 1965) even though association of the substrate and anion in the vicinity of the metal coordination site is thought to be required for catalytic activity and inhibition, respectively. This associative scheme, I → II → IV, allowing for ligand binding without prior displacement of the anion, would appear to be more compatible with noncompetitive inhibition kinetics. It seems likely that the aromatic portions of the ester substrates and the sulfonamides would occupy a common site on the enzyme.

Formation of a reversible bromopyruvate–isozyme C complex results in ~16% quenching of the protein fluorescence which may be restored upon addition of a nonquenching sulfonamide; the apparent rate of this reaction, conversion from the bromopyruvate to the sulfonamide complex, is also to directly proportional to sulfonamide concentration. As shown in Figure 5b, kinetic behavior identical to that of the CN^- complex is found for the case when the fluorescence change is a consequence of dissociation of the leaving anion. This provides no evidence for population of an intermediate state to an appreciable extent during the course of reaction; however, the presumed rapid dissociation rate of the bromopyruvate complex, if analogous to other carboxylate anions (Taylor *et al.*, 1971), also does not allow us to distinguish between alternative reaction paths in eq 3 for this inhibitor of comparatively low affinity.

If a sulfonamide–enzyme complex is reacted with an anion, *i.e.*, carrying out the reaction in reverse order to that used to obtain the data of Figure 5, the apparent rate is limited by sulfonamide dissociation and independent of anion concentration (Table II). Irrespective of the attacking species, the *p*-NBS dissociation rate is essentially constant. In a mechanism where dissociation of the initial complex is the rate-limiting step, the observed rate should be independent of the nature and concentration of the attacking ligand provided that the reaction can be driven to near completion. Whereas, if a ternary complex were formed the dissociation rate could be accelerated by an increasing concentration of the replacing ligand.

TABLE II: Observed Dissociation Rates for Human Carbon Anhydrase C–*p*-Nitrobenzenesulfonamide Complex; 20 mM Tris-Sulfate Buffer (pH 7.6).[a]

Nonquenching Ligand	Concn (M)	k_{obsd} (sec^{-1})
p-Carboxybenzene-sulfonamide	5×10^{-5}	0.049
	1.5×10^{-4}	0.049
	5.0×10^{-4}	0.050
	1.5×10^{-3}	0.047
Cyanide (as HCN + CN^-)	3×10^{-4}	0.068
	1×10^{-3}	0.071
	3×10^{-3}	0.063
	1×10^{-2}	0.058
	3×10^{-2}	0.057

[a] A 1:1 complex of 1.25 μM *p*-nitrobenzenesulfonamide and human carbonic anhydrase C was reacted with the above concentration of nonquenching ligand and the rate of change in fluorescence measured.

It is unlikely that the reaction path, IV → II → I, makes an appreciable contribution to the dissociation reaction, and it, therefore, appears that the step corresponding to k_{43} constitutes the rate-limiting step for sulfonamide dissociation. Microscopic reversibility requires that under the same concentration conditions intermediate II, likewise, would not be involved in the cyanide to sulfonamide direction. However, rates in this direction are measured at different reactant concentrations. When alternative reaction paths are considered, it must be kept in mind that the reaction energy profile and corresponding transition states are concentration dependent (Jencks, 1969). Thus conversion of the cyanide into sulfonamide complex through intermediate II cannot be ruled out under conditions of high sulfonamide concentration.

References

Adams, D. H., and Whittaker, V. P. (1950), *Biochim. Biophys. Acta 4*, 543.

Alberty, R. A., and Hammes, G. G. (1958), *J. Phys. Chem. 62*, 154.

Armstrong, J. McD., Myers, D. V., Verpoorte, J. A., and Edsall, J. T. (1966), *J. Biol. Chem. 241*, 5137.

Bradbury, S. L. (1969a), *J. Biol. Chem. 244*, 2002.

Bradbury, S. L. (1969b), *J. Biol. Chem. 244*, 2010.

Chen, R. F., and Kernohan, J. C. (1967), *J. Biol. Chem. 242*, 5813.

Coleman, J. E. (1967), *J. Biol. Chem. 242*, 5212.

Coleman, J. E. (1968), *J. Biol. Chem. 243*, 4574.

Eigen, M., and Wilkens, R. G. (1965), *Advan. Chem. Ser. No. 49*, 55.

Fabry, M. E., Koenig, S. H., and Schillinger, W. E. (1970), *J. Biol. Chem. 245*, 4256.

Fridborg, K., Kannan, K., Liljas, A., Lundin, J., Strandberg, B., Strandberg, R., Tilander, B., and Wiren, G. (1967), *J. Mol. Biol. 25*, 505.

Grell, E., and Bray, R. C. (1971), *Biochim. Biophys. Acta 236*, 503.

Grossberg, A., and Pressman, D. (1960), *J. Amer. Chem. Soc. 82*, 5478.

Izatt, R. M., Christersen, J. J., Pack, R. T., and Bench, R. (1962), *Inorg. Chem. 1*, 828.

Jencks, W. (1969), Catalysis in Chemistry and Enzymology, New York, N. Y., McGraw-Hill, p 583.

Johnson, F. H., Eyring, H., and Williams, R. W. (1942), *J. Cell Comp. Physiol. 20*, 247.

Jones, M. (1968), Ligand Reactivity and Catalysis, New York, N. Y., Academic Press, p 146.

Kernohan, J. C. (1965), *Biochim. Biophys. Acta 96*, 304.

Kernohan, J. C. (1966a), *Biochim. Biophys. Acta 118*, 405.

Kernohan, J. C. (1966b), *Biochem. J.* 98, 31P.

King, R. W., and Burgen, A. S. V. (1970), *Biochim. Biophys. Acta 207*, 278.

King, R. W., and Roberts, G. C. K. (1971), *Biochemistry 10*, 558.

Laidler, K. J. (1963), Reaction Kinetics, Vol. 2, New York, N. Y., Macmillan, p 6.

Lindskog, S. (1963), *J. Biol. Chem. 238*, 945.

Lindskog, S. (1966), *Biochemistry 5*, 2641.

Lindskog, S., and Nyman, P. O. (1964), *Biochim. Biophys. Acta 85*, 462.

Lindskog, S., and Thorslund, A. (1968), *Eur. J. Biochem. 3*, 453.
Maren, T. (1967), *Physiol. Rev. 47*, 595.
Marlow, H. F., Metcalfe, J. C., and Burgen, A. S. V. (1969), *Mol. Pharmacol. 5*, 166.
Pocker, Y., and Stone, J. T. (1967), *Biochemistry 6*, 668.
Pocker, Y., and Stone, J. T. (1968), *Biochemistry 7*, 2936.
Riepe, M. E., and Wang, J. H. (1968), *J. Biol. Chem. 243*, 2779.
Taylor, P. W., Feeney, J. and Burgen, A. S. V. (1971), *Biochemistry 10*, 3866.
Taylor, P. W., King, R. W., and Burgen, A. S. V. (1970a), *Biochemistry 9*, 2638.
Taylor, P. W., King, R. W., and Burgen, A. S. V. (1970b), *Biochemistry 9*, 3894.
Thorslund, A. and Lindskog, S. (1968), *Eur. J. Biochem. 3*, 117.
Verpoorte, J. A., Mehta, S. and Edsall, J. T. (1967), *J. Biol. Chem. 242*, 4221.
Ward, R. L. (1969), *Biochemistry 8*, 1879.
Whitney, P. L., Nyman, P. O., and Malmstrom, B. G. (1967), *J. Biol. Chem. 242*, 4112.

Investigation of the Mechanism of Ligand Binding with Cobalt(II) Human Carbonic Anhydrase by ^{1}H and ^{19}F Nuclear Magnetic Resonance Spectroscopy*

Palmer W. Taylor,† J. Feeney, and A. S. V. Burgen

ABSTRACT: ^{1}H and ^{19}F nuclear magnetic resonance spectroscopy has been used to investigate interaction between various carboxylate anion inhibitors and cobalt(II) human carbonic anhydrase C. Analysis of the frequency and temperature dependence of the transverse relaxation time, T_2, has shown four situations where relaxation of ligand nuclei is controlled by the chemical-exchange rate. With mono- and difluoroacetates the paramagnetic contribution to relaxation for protons is dependent on the chemical shift difference between the free and bound states, while for ^{19}F relaxation, a chemical-exchange dependence can be demonstrated at low temperatures. Rates of association and dissociation of the various carboxylate ligands with the enzyme have been determined from the relaxation and equilibrium affinity measurements and have been found to be in the range of 2×10^{8} M^{-1} sec^{-1} and 10^{5} sec^{-1}, respectively. Anion association is thus two to three orders of magnitude greater than rates of similar substitution processes on aquocobalt(II). If anion binding displaces a H_2O molecule from the coordination sphere of the metal, an extremely labile H_2O is coordinated to the metal in the enzyme. This capacity for enhanced ligand substitution rates would be potentially advantageous for metalloenzyme catalysis. Dissociation rates for these carboxylate inhibitors are four to six orders of magnitude greater than for the sulfonamide inhibitors. The dissociation step for anionic ligands is nearly pH independent, a finding consistent with the scheme for anion binding presented in the previous paper.

The interaction of the various anions with the metalloenzyme, carbonic anhydrase, has been widely studied since the initial finding of Roughton and Booth (1946) that Cl^- acts as an inhibitor of the enzyme-catalyzed CO_2–hydration reaction. Anions appear to be noncompetitive inhibitors of the enzyme (Kernohan, 1965; Pocker and Stone, 1967; Verpoorte *et al.*, 1967) and preferentially bind to the coordination form of the enzyme which predominates at low pH (Lindskog, 1966; Thorslund and Lindskog, 1968; Taylor and Burgen, 1971). Ward (1969, 1970) has recently examined the interaction of Cl^- with carbonic anhydrase by nmr, and, on the basis of the magnitude of the quadrupolar relaxation, concluded that Cl^- binds directly to the metal coordination site in the enzyme. This is in agreement with the ir spectroscopic results of Riepe and Wang (1968) on enzyme-bound azide which are consistent with direct ligand–metal coordination. More recently Fabry *et al.* (1970) have observed that sulfonamide and monovalent anion ligands upon binding to the cobalt(II) enzyme will displace a water molecule which apparently exists within the coordination sphere of the metal. X-Ray crystallographic studies on the human C isozyme–sulfonamide complex also demonstrate that the sulfonamido group resides within coordination distance of the metal.

In contrast to other d-transition metals, cobalt(II) may be substituted for the single zinc atom in the enzyme with retention of similar catalytic and inhibitor binding properties (Lindskog, 1963; Coleman, 1967a). This has greatly aided the study of ligand–carbonic anhydrase interactions since characteristic visible (Lindskog, 1963, 1966) and electron spin resonance (Grell and Bray, 1971; Taylor *et al.*, 1970a) spectral differences occur with ligand binding to the enzyme.

We have further examined anion binding by ^{1}H and ^{19}F nmr employing various carboxylate ligands and cobalt(II) human carbonic anhydrase C. Exchange rates determined by

* From the Medical Research Council, Molecular Pharmacology Unit, Medical School, Cambridge, England.
† Present address: Division of Pharmacology, Department of Medicine, University of California, San Diego, La Jolla, Calif. 92037; to whom correspondence should be addressed.