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Drug–Biomolecule Interactions: Mechanism of Ligand Interactions with Carbonic Anhydrase Studied by Magnetic Resonance Relaxation and Rapid Reaction Methods

PALMER TAYLOR

Abstract □ Kinetics of interaction between the metalloenzyme carbonic anhydrase and either monovalent anions or aromatic sulfonamides were examined by three distinct fast reaction techniques: stopped flow, equilibrium perturbation, and magnetic resonance relaxation. By correlating spectroscopic data on conformational and ionization equilibria of the complex and free species with the reaction kinetics, a relatively complete description of the mechanism can be presented. A proton-dependent equilibrium between two coordination forms of the free enzyme can be demonstrated spectroscopically. Anions selectively combine with the form predominating at low pH. For a series of carboxylate ligands, formate and substituted acetates, anion association is found to be three orders of magnitude greater than similar ligand substitution processes known in inorganic chemistry. For sulfonamide association, the attacking species are the form of carbonic anhydrase predominating at high pH and the neutral sulfonamide. Combination involving the neutral species is followed by loss of a proton to form the sulfonamido anion in the stabilized complex. This obligate proton transfer offers a probable explanation for the unique specificity of sulfonamides in inhibiting this enzyme. The anionic sulfonamido moiety can be shown to resemble closely a transition intermediate in the catalytic step of substrate hydration.

Keyphrases □ Ligand–carbonic anhydrase interactions—kinetics studied by magnetic resonance relaxation and rapid reaction methods □ Carbonic anhydrase–ligand interactions—kinetics studied by magnetic resonance relaxation and rapid reaction methods □ Drug–biomolecule interactions—mechanism of ligand interactions with carbonic anhydrase, magnetic resonance relaxation and rapid reaction methods □ Interactions—drugs with biomolecules, symposium

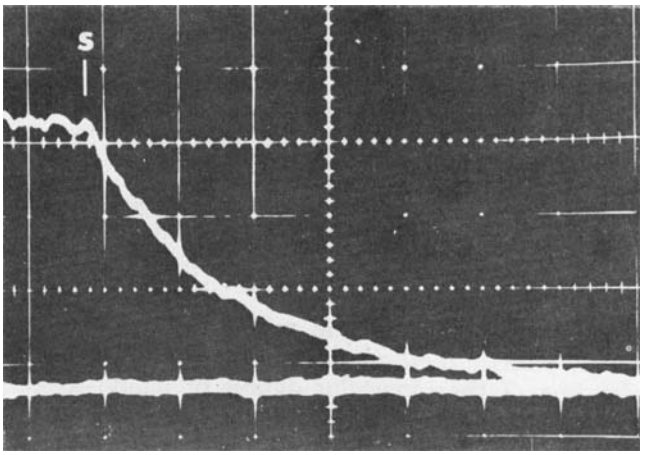


Figure 1—*Oscilloscope trace recording the change in carbonic anhydrase fluorescence for the reaction between 14 μM p-nitrobenzenesulfonamide and the human C isozyme. The oscilloscope was triggered with the initiation of flow so that the time dependence of fluorescence was measured during and after flow. Stoppage of flow occurs at s, and each large horizontal increment corresponds to 50 msec. The horizontal trace at the bottom of the screen was triggered ~10 sec later and corresponds with the fluorescence of the complex at equilibrium. Fluorescence was monitored at 345 nm; the excitation wavelength was 290 nm.*

The molecular basis of specificity in the formation of a ligand–macromolecule complex is of interest in many fields of the biological sciences, since complex formation represents the common initial event that leads to a divergence in response. The interaction of a macromolecule with a drug shows a capacity for selective recognition of ligand structure. In addition, receptors may exhibit specific capacity to translate an interaction with chemical mediators. The latter is generally considered to occur through ligand-sensitive conformational changes. Thus, in the study of specificity of drug–macromolecule interactions, both differential affinity and changes induced in the structure of the complex should be considered.

Complex formation between a drug and macromolecule usually involves an inherently rapid process(es). Examination of such interactions at a level where primary processes and transient species are detected requires methods applicable to following

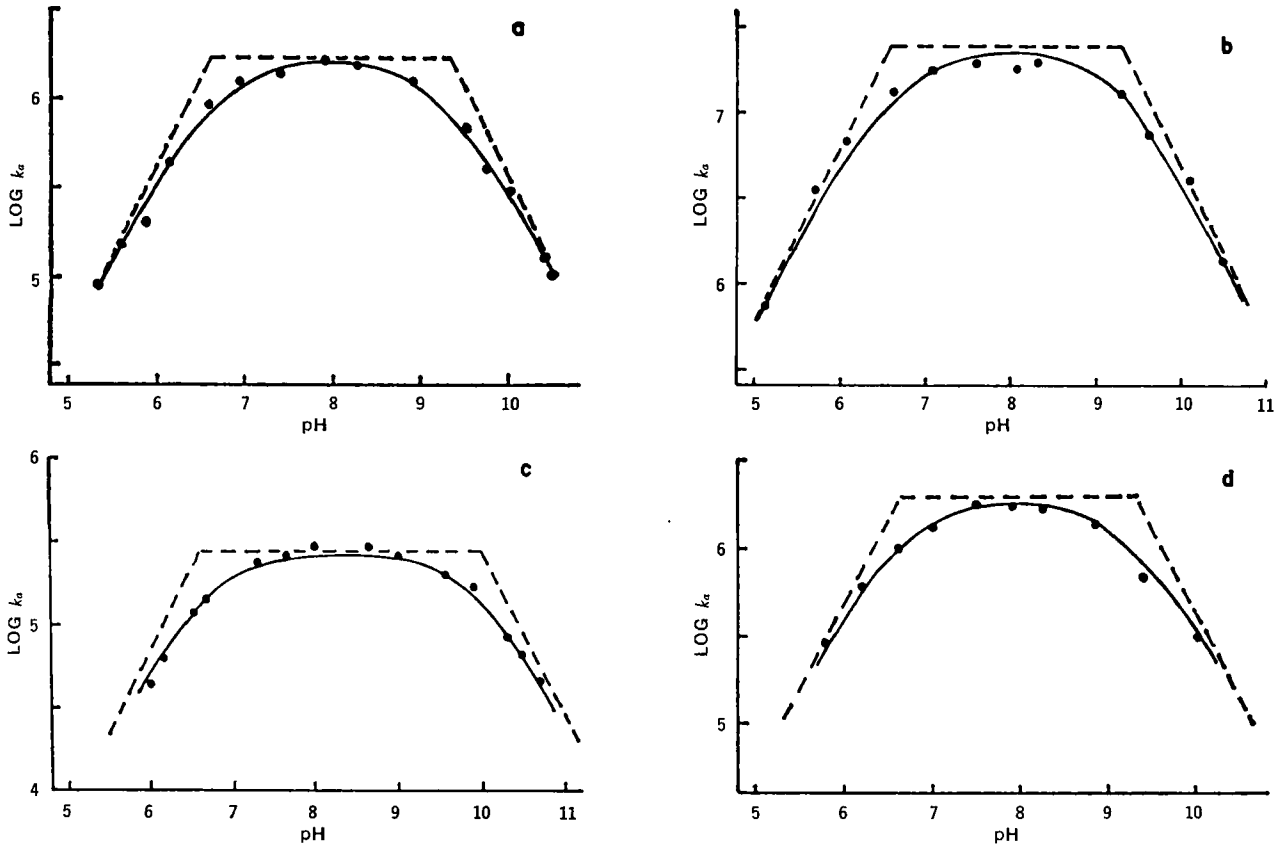


Figure 2—*The pH dependence of the bimolecular association rate constants,* k_a, *between human carbonic anhydrase C and various sulfonamides:* (a) p-*nitrobenzenesulfonamide and the Zn enzyme,* (b) *4-(salicyl-5-azo)benzenesulfonamide and the Zn enzyme,* (c) *5-dimethylaminonaphthalene-1-sulfonamide and the Zn enzyme, and* (d) p-*nitrobenzenesulfonamide and the Co (II) enzyme. The solid lines were calculated using Eq. 1, assuming* pK_E *to be 6.60 and* pK_S *to be 9.30 for* p-*nitrobenzenesulfonamide and salicylazobenzenesulfonamide and 10.00 for 5-dimethylaminonaphthalene-1-sulfonamide. The intersections of the dotted lines having slopes of 1.0 and 0.0 also yield the respective pK values. The kinetic constants were determined by stopped-flow measurements of fluorescence quenching associated with complex formation. (Reprinted, with permission, from Ref. 8.)*

reaction dynamics in the milli- and microsecond range. Kinetic methods useful in gaining an understanding of the molecular details of ligand–macromolecule interactions are discussed in this article.

Rapid mixing (stopped flow) and equilibrium perturbation techniques, when coupled to a suitable detection system, can monitor directly the course of the drug–macromolecule interaction. These two methods differ greatly in their applicable time range, the conditions under which kinetic constants are obtained, and the information that can be derived from the experiment. In addition, magnetic resonance relaxation times can be employed to estimate kinetic parameters. This situation arises when ligand exchange from two magnetically distinct environments, the binding site on the macromolecule and the bulk solvent, becomes the dominant relaxation process.

The level at which kinetic data can be interpreted is limited by the available structural information on the reacting species. Carbonic anhydrase is a metalloenzyme with a molecular weight of 29,500 and properties that are amenable to the use of spectroscopic probes. The crystal structure of this enzyme is now well characterized (1), and the active site exists at the base of a rather narrow cleft containing a single Zn atom. The Zn appears to be tetrahedrally coordinated with one H_2O and three residues from the protein core serving as ligands.

Catalysis takes place in the immediate vicinity of the Zn, and the enzyme inhibitors, monovalent anions and aromatic sulfonamides, appear to bind within the coordination sphere of the metal (1–4). The Zn may be replaced by Co (II) with the retention of very similar catalytic and inhibitor binding properties (2, 4). Substitution of this d^7-transition metal having unpaired electrons provides a valuable intrinsic probe of the enzyme coordination structure, since optical and magnetic resonance spectroscopy may be used to correlate conformational and ionization states with the reactive species.

In the selection of a system, it is also advantageous to keep ligand structure relatively simple. With the aromatic sulfonamide, only a single ionization equilibrium, the sulfonamido proton, must be considered. Conformation is also well defined with the two oxygens and the amino and aryl groups tetrahedrally disposed around the sulfur (6).

RESULTS AND DISCUSSION

Stopped-Flow Studies—The overall kinetics of association and dissociation may be followed with stopped-flow instrumentation by monitoring the quenching of the protein-tryptophan fluores-

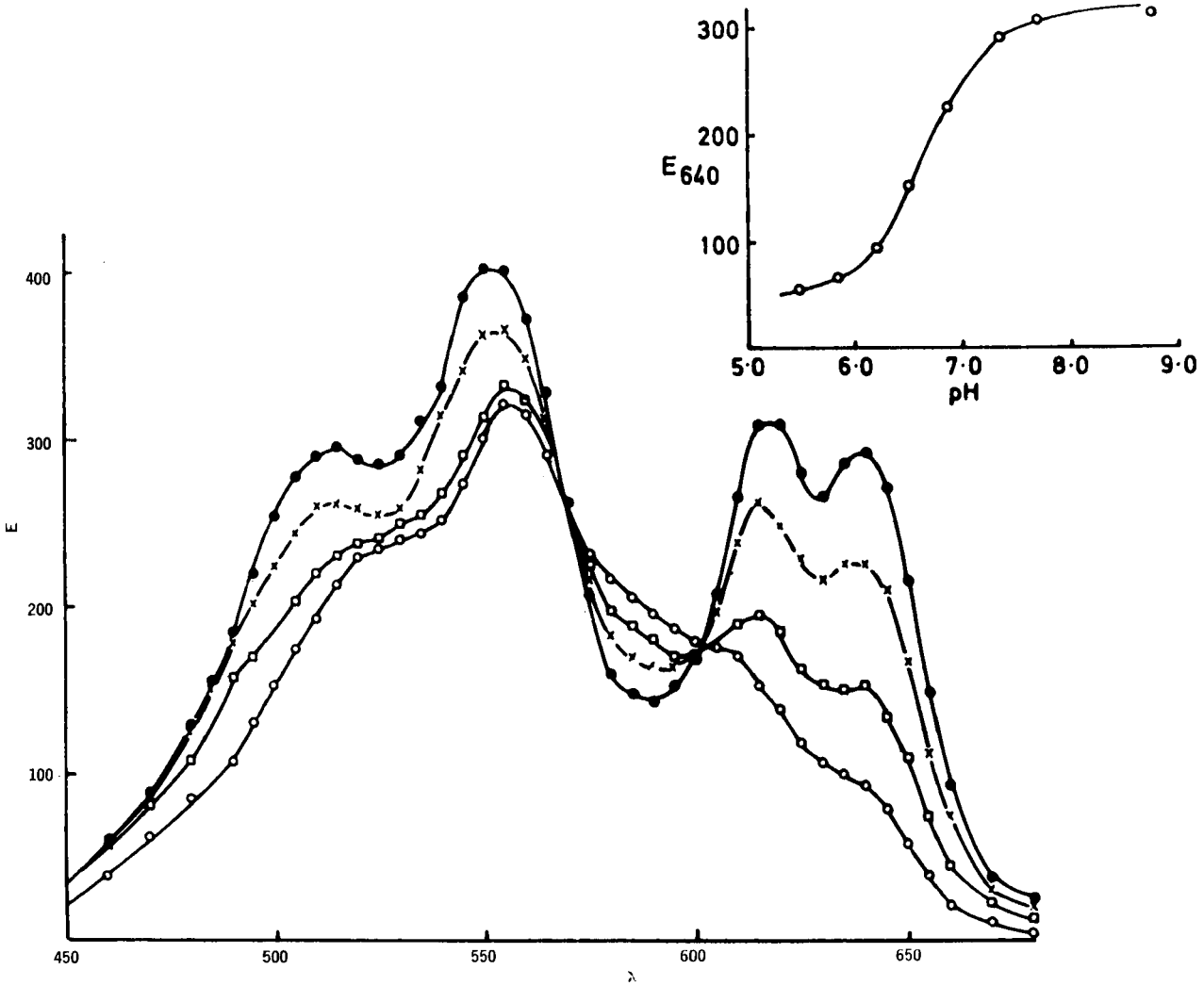


Figure 3—*The pH dependence of the Co (II) absorption spectrum of human carbonic anhydrase C; 0.32 mM enzyme in 20 mM K_2SO_4 was adjusted in pH by 0.05 N NaOH or 0.05 N H_2SO_4, and the spectra were recorded in 2.0-cm cells. Key: ○, pH 6.19; □, pH 6.50; ×, pH 6.86; and ●, pH 7.35. E is the molar absorptivity. The inset is a plot of the absorptivity for the peak at 640 nm as a function of pH. (Reprinted, with permission, from Ref. 8.)*

cence (7). If there is overlap between the tryptophan emission spectrum and the absorption spectrum of the sulfonamide, these species can serve as efficient donor–acceptor pairs of excited-state energy in the complex.

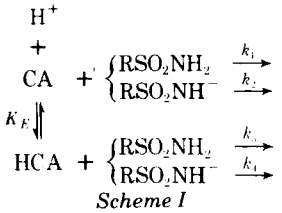
Figure 1 shows the time-dependent change in protein fluorescence associated with complexation of *p*-nitrobenzenesulfonamide and human carbonic anhydrase C. During the period where the trace is approximately horizontal, the transit time between the mixing chamber and observation tube is less than the reaction time, and fluorescence of the mixed, but unreacted, reactants is observed. At s, the flow is abruptly stopped and a first-order approach to the equilibrium fluorescence of the complex is observed.

Dissociation of the complex may also be followed by the stopped-flow procedure, which has been employed to obtain association and dissociation rate constants for a large series of sulfonamides (7). Correlation of individual kinetic parameters with structure provides an additional dimension to the usual structure–activity considerations. For example, substituent variation on the aromatic ring primarily affects the association rate process, and it is only with substitution *ortho* to the sulfonamido group that the dissociation rate becomes affected (7).

The pH dependence of the association rate between the sulfonamide and the enzyme shows two ionization equilibria which influence the association process (Fig. 2). The apparent pK at high pH corresponds with the titratable pKa of the sulfonamido proton on the various sulfonamides, while the apparent pK at 6.5 is independent of the combining ligand, suggesting that an ionization on the enzyme is responsible (8).

The Co (II) spectra of the human carbonic anhydrase C show clear isosbestic points, demonstrating a proton-dependent equilibrium between two discrete coordination forms of the enzyme (Fig. 3). The pK for this equilibrium is 6.6, which is in good accord with the pH dependence of the association rate. To confirm that the proton-dependent coordination change controls the low pH arm of the association rate, a pK of 7.3 can be assigned for both the spectral transition and the association rate in a different isozyme, carbonic anhydrase B.

Chemical modification of the β isozyme with bromoacetate forms a less active derivative in which a single histidine has been stoichiometrically carboxymethylated (9). The pH for the transition between coordination forms in the carboxymethylated enzyme shifts to 9.1, and there is a corresponding change in the ionization equilibrium affecting the sulfonamide association rate (8). Thus, Scheme I illustrates the sulfonamide association.



Scheme I

Reaction pathways represented by k_2 and k_3 may be ruled out

on the basis that they do not fit the observed pH dependence of the kinetics. Thus:

$$k_{obs} = \frac{k_1 + k_4(K_S/K_E)}{(1 + K_S/H^+)(1 + H^+/K_E)} \qquad \text{(Eq. 1)}$$

where K_E and K_S represent protonic dissociation constants on the enzyme and sulfonamide.

The pathways denoted by k_1 and k_4 exhibit identical pH dependencies, and a decision on the critical reaction pathway must be arrived at in a different way. Since k_{obs} is $2 \times 10^7\ M^{-1}\ sec^{-1}$, when $K_S/K_E = 10^{-3}$, k_4 must necessarily be greater than $10^{10}\ M^{-1}\ sec^{-1}$. A rate constant of that magnitude exceeds the diffusion limitation for a system of this nature. In other words, the low concentration product of the reactants for pathway k_4 requires that k_4 be improbably large to satisfy the observed rate. Thus, combination between the sulfonamido anion and the form of the enzyme represented by HCA can be ruled out and k_1 is left as the dominant reaction path for sulfonamide association.

Spectroscopic studies clearly show that the sulfonamido anion RSO_2NH^- is the stabilized species in the complex (10). Thus, complex formation involves initial combination between neutral species with subsequent transfer of a proton from the sulfonamide (Fig. 4). Although it is not known whether sulfonamide coordination occurs through the nitrogen or oxygen, striking similarities between sulfonamide coordination and CO_2 hydration are apparent. Both ligands combine with the coordination form of carbonic anhydrase predominating at high pH and subsequently involve a proton dissociation step.

The distances between the two oxygens in the linear CO_2 molecule and the tetrahedral sulfonamido group are similar, ~ 2.36–2.46 Å (6). As shown in Fig. 4, the sulfonamido anion in the complex rather closely resembles a transition state intermediate in CO_2 hydration. A requirement for combination by the neutral sulfonamide species followed by loss of the proton while in the bound state provides the likely explanation why aromatic sulfonic acids, although geometrically similar to the sulfonamides, exhibit a very low affinity for the enzyme.

The rate of dissociation of the complex is pH independent (7). Correspondingly, while the Co (II)·spectrum of the enzyme–sulfonamide complex differs from the free enzyme, it is unaffected by solution pH changes (7). Thus, the binding of an inhibitory ligand fixes the coordination state where no ionization equilibria affect either the coordination structure of the metal or the ligand dissociation rate.

Since the dissociation rate is pH independent, the dissociation of the sulfonamido proton does not take place in a rate-limiting step. Alternatively, an internal proton transfer between the ligand and a site on the enzyme would be consistent with the proposed mechanism.

Monovalent anions also bind to carbonic anhydrase but form

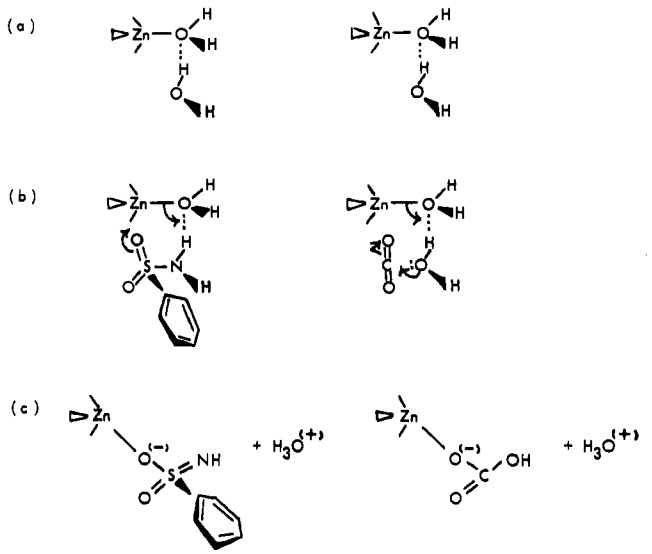


Figure 4—*Proposed scheme for sulfonamide and CO_2 interactions with the active site of carbonic anhydrase.*

Table I—Factors Controlling Relaxation of a Ligand in Association with a Paramagnetic Environment[a]

$$\frac{1}{T_2} = P\left[\frac{\frac{1}{T_{2M}^2} + \frac{1}{\tau_M T_{2M}} + \Delta\omega_M^2}{\left(\frac{1}{T_{2M}} + \frac{1}{\tau_M}\right)^2 + \Delta\omega_M^2}\right];\ P \ll 1$$

Case

a) If $\frac{1}{T_{2M}^2} > \frac{1}{\tau_M}$ or $\Delta\omega_M^2 > \frac{1}{\tau_M^2}$, then $\frac{1}{T_2} = \frac{P}{\tau_M}$

b) If $\frac{1}{\tau_M} > \Delta\omega_M^2 > \frac{1}{T_{2M}\tau_M}$, then $\frac{1}{T_2} = P\tau\Delta\omega_M^2$

c) If $\frac{1}{T_{2M}\tau_M} > \frac{1}{T_{2M}^2}, \Delta\omega_M^2$, then $\frac{1}{T_2} = \frac{P}{T_{2M}}$

Case	Relaxation Mode	Temperature Dependence	Frequency Dependence
a)	$1/\tau$	+	No change
b)	$\Delta\omega^2$	−	+ ($\alpha\omega_0^2$)
c)	$1/T_{2M}$	±	−

[a] From Swift and Connick (17). P is the ratio of ligand in the paramagnetic environment relative to the bulk solvent, τ_M is the residence time of the ligand in the paramagnetic environment, T_{2M} is the dipolar and scalar contribution to the relaxation time, and $\Delta\omega_M$ is the chemical shift difference in radians.

less affine complexes than the sulfonamides (11). The affinity of the anion–enzyme complex is pH independent and maximal at low pH, while at higher pH values the stability of the complex is inversely proportional to the hydrogen-ion concentration (Fig. 5). As in the case of the sulfonamides, ionization equilibria with pK_E's of 6.6, 7.3, and 9.1 determine the pH profile of anion binding to the C, B, and carboxymethylated B isozymes, respectively (12). Scheme II describes the specificity of ligand binding to carbonic anhydrase.

The coordination form predominating at low pH (HCA) selectively binds anions, while the high pH form (CA) is selective for sulfonamides. The pH dependence of association is dependent on the ionization equilibrium, K_E, and ionization equilibria on the ligand. Carboxymethylation of histidine$_{200}$ shifts ionization equilibrium K_E, thereby influencing the pH dependence of binding of both types of ligands.

NMR Relaxation Studies—The rapid rates of association and dissociation for the anion complex preclude measurement of the individual kinetic constants, k_a and k_{-a}, by flow methods. In an associating system, where the binding site and the solution provide magnetically distinct environments, NMR relaxation of ligand nuclei can be controlled by chemical exchange of the ligand between the free and bound states. This mode of relaxation only becomes dominant when the exchange rate is slow relative to other relaxa-

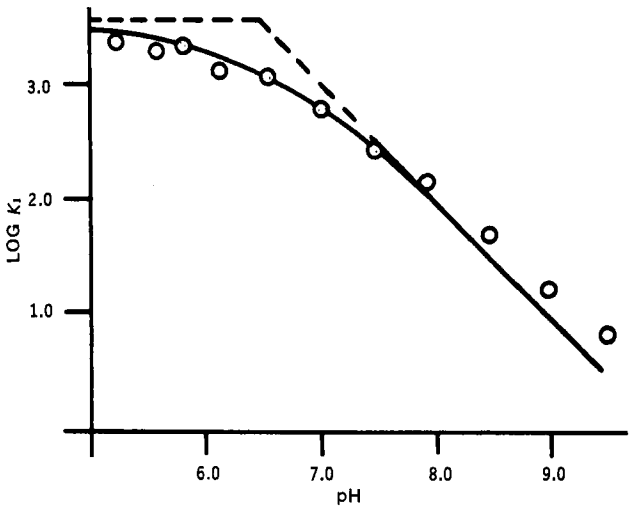


Figure 5—*The pH dependence of the equilibrium affinity of SCN^- for human carbonic anhydrase C. The solid line is calculated by assuming that $pK_E = 6.6$ and that the anions combine only with the coordination form of carbonic anhydrase predominating at low pH.*

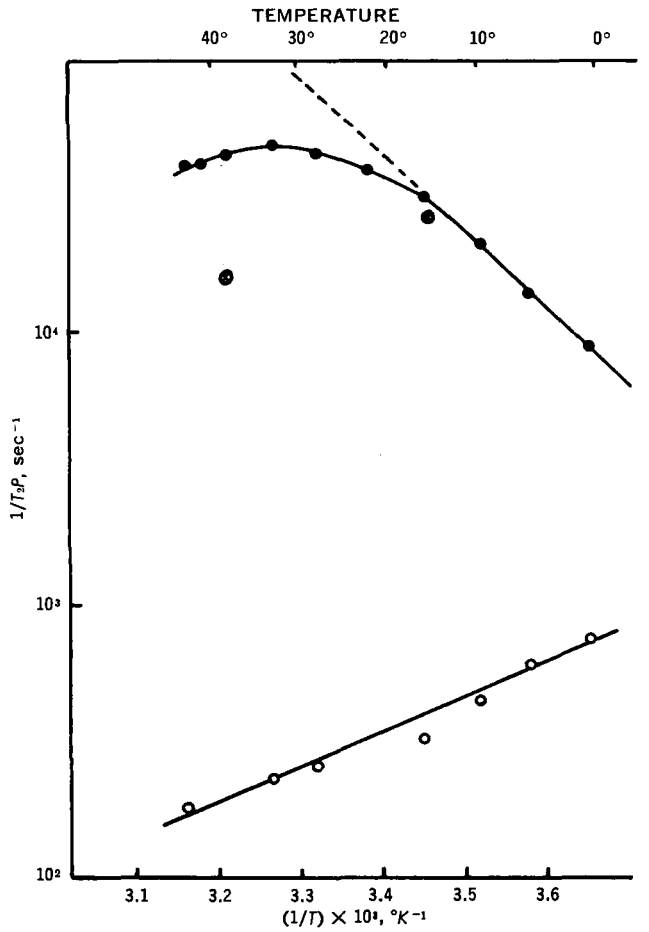
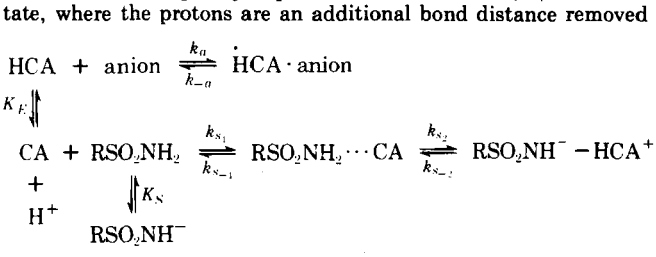


Figure 6—*Influence of temperature on the transverse relaxation rate ($1/T_2$) for formate and acetate complexes of Co (II) human carbonic anhydrase C, pH 7.6; $1/T_2P$ is plotted as a function of temperature where* P *is the ratio of bound to free ligand. Since the enzyme is at least 97% saturated at the ligand concentrations employed,* P *is equal to the ratio of added enzyme to ligand. Key: 400 mM formate in the presence of 0.1 mM enzyme at 100 MHz, ●, and at 60 MHz, ⊗; and 240 mM acetate in the presence of 1.5 mM enzyme at 100 MHz, ○. At each temperature, measurements were made in the presence and absence of* p-*carboxybenzenesulfonamide, and the plotted values represent the difference in the relaxation rates between the two conditions. The sulfonamide serves to dissociate all of the bound ligand.*

tion processes (Table I). With paramagnetic Co (II) enzymes, the contribution of electron–nuclei relaxation enhances the contribution of dipolar and scalar terms, $1/T_{2M}$, and the chemical shift difference, thereby increasing the likelihood of exchange-controlled relaxation (Case *a*).

The temperature dependence of relaxation of the single formate proton is consistent with exchange control in the low temperature range (Fig. 6) (Case *a*, Table I). Confirmation that the lifetime of the ligand in the paramagnetic environment, τ_M, controls and governs relaxation was obtained by measurements of chemical shift, $\Delta\omega_M$, and the frequency dependence of relaxation (13). For acetate, where the protons are an additional bond distance removed



Scheme II

Scheme III

from the metal, neither the magnitude of the relaxation rate nor its temperature dependence is indicative of exchange control.

Accordingly, a second nucleus must be examined to obtain additional direct kinetic information. Relaxation rate measurements with substituted fluoroacetates showed that ^{19}F relaxation at low temperatures also is governed by the exchange rate (13). On the other hand, protons on the same ligands, as would be anticipated from the acetate data, exhibit a different relaxation mode (Fig. 7). The kinetic constants, k_a and k_{-a}, may be estimated from the lifetime of the complex and equilibrium affinities for formate and the three fluoroacetates (Table II). Ligand association is a rapid process and approaches the diffusion limitation of a bimolecular reaction.

The crystal structure determination and NMR dispersion experiments provided good evidence that when inhibitory ligands bind to carbonic anhydrase a coordinated H_2O molecule is displaced (1, 14). When H_2O is the leaving group, ligand–metal association processes occur as shown in Scheme III (15).

The first step, which leads to an outersphere complex, is in rapid equilibrium with the slower ensuing step, which involves entry of the ligand into the metal coordination sphere. The second step, k_{01}, is rate limited by H_2O dissociation from the metal (15, 16); thus, ligand–metal substitution rates are sensitive to the nature of

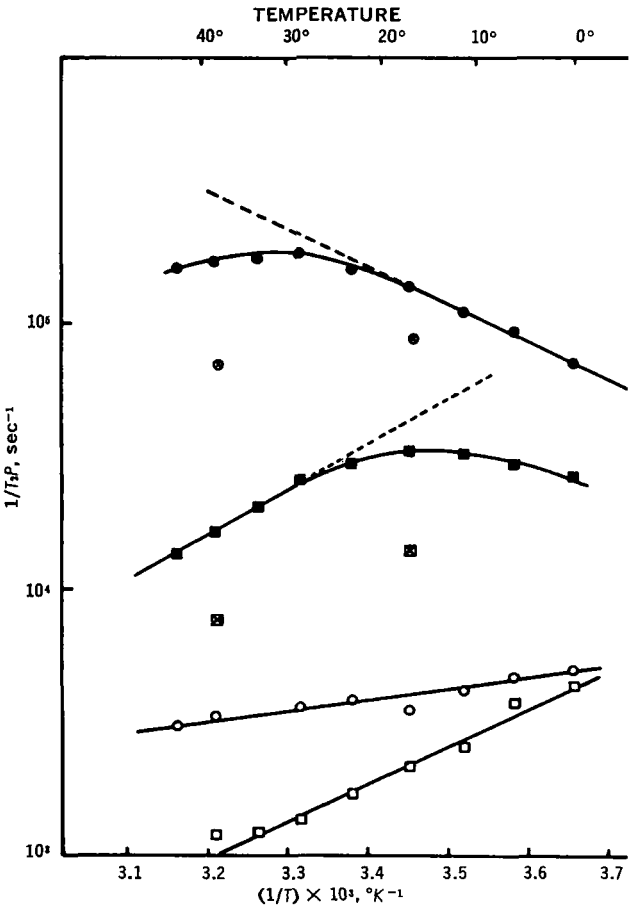


Figure 7—*Influence of temperature on the transverse relaxation rate ($1/T_2$) of ^1H and ^{19}F for mono- and difluoroacetate complexes of Co (II) carbonic anhydrase C, pH 7.6;* P *is the ratio of enzyme-to-inhibitor concentration: ●, ^{19}F in CH_2FCOO^- at 94.2 MHz; ■, ^{19}F in CHF_2COO^- at 94.2 MHz; ○, ^1H in CH_2FCOO^- at 100 MHz; □, ^1H in CHF_2COO^- at 100 MHz; ⊗, ^{19}F in CH_2FCOO^- at 56.4 MHz; and ⊠, ^{19}F in CHF_2COO^- at 56.4 MHz. The relaxation rates represent the difference in measurements made in the absence and presence of* p-*carboxybenzenesulfonamide.*

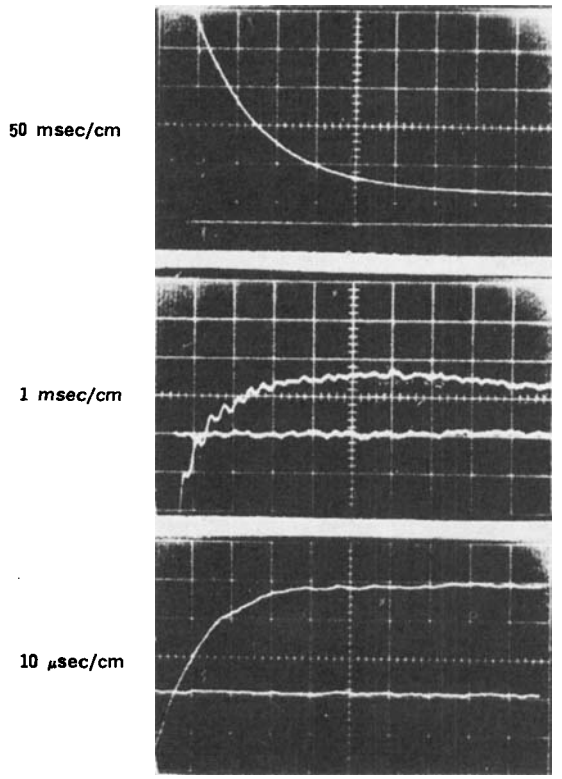


Figure 8—*Temperature-jump relaxation spectra for the association between 3-nitro-4-hydroxybenzenesulfonamide and human carbonic anhydrase B. A 30-kv pulse is discharged across a 0.7-cm path cell containing 4 × 10^{-4} M enzyme and sulfonamide in 0.2 M K_2SO_4 and 0.1 M bis(2-hydroxyethyl)-iminotris(hydroxymethyl)methane buffer, pH 6.0. Absorption at 425nm is monitored on a split-beam spectrophotometer. Key: top panel, 50-msec/cm sweeptime, τ_1 = 115 msec; middle panel, 1-msec/cm sweeptime, τ_2 = 1.1 msec; and bottom panel, 10-μsec/cm sweeptime, τ_3 = 13 μsec. The initial temperature is 18.5°.*

the metal rather than the ligand. For tetrahedral aquo–Co (II), k_{01} is estimated to be 10^5 sec^{-1}, which yields a bimolecular rate constant of the order of 10^5 M^{-1} sec^{-1} (13, 17). By contrast, the ligand association rates with the Co (II) enzyme exceed 10^8 M^{-1} sec^{-1}.

Enhanced rates of ligand substitution and turnover are obviously advantageous to the catalytic potential of an enzyme. The unusual spectra arising from *d–d** transitions, which have been observed for many metalloenzymes, indicate unique coordination geometry in the catalytically active form of the enzyme. However, it requires this kind of experiment where the dynamics of ligand exchange can be measured to establish that the metal in metalloenzymes plays a more crucial role than simply the orientation of the substrate.

Anion interactions with carbonic anhydrase can be characterized by far more rapid dissociation rates when compared with the aromatic sulfonamides. Although both monovalent anions and sulfonamides appear to coordinate with the metal, sulfonamide binding seems to involve two loci of interaction, the metal coordination sphere and a hydrophobic surface in the active site cleft to which the aromatic ring binds. The slower dissociation rate coupled with a second potential interaction site, confering stability to the complex, makes it seem probable that sulfonamide complex formation proceeds through a multistep mechanism (Scheme IV).

In a similar manner to chelation with bidentate ligands, formation of the initial complex makes the second step effectively unimolecular, imparting a statistical advantage toward complex formation. Thus, an apparent amplification of binding energy can be achieved through proper apposition of binding groups.

$$RSO_2NH_2 + CA \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} RSO_2NH_2 \cdots CA \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} RSO_2NH^- - HCA^+$$

Scheme IV

Table II—Estimated Association and Dissociation Rate Constants for Various Carboxylate Anion Complexes of Co (II) Carbonic Anhydrase C at 25°

Ligand	K_1, M [a]	k_1, M^{-1} sec^{-1} [b]	k_{-1}, sec^{-1} [c]
Formate	1.54 × 10^{-4}	3.9 × 10^8	6.0 × 10^4
Monofluoroacetate	9.1 × 10^{-4}	2.2 × 10^8	2.0 × 10^5
Difluoroacetate	5.3 × 10^{-4}	1.9 × 10^8	1.0 × 10^5
Trifluoroacetate	7.7 × 10^{-4}	2.0 × 10^8	1.5 × 10^5

[a] Intrinsic affinity: calculated from the apparent affinity at pH 7.6 and $K_1 = K_{1(app)}$ $[H^+/(H^+ + K_E)]$, where K_E is the protonic dissociation constant described in Scheme I. [b] Calculated from k_{-1} and K_1. [c] Determined from NMR measured lifetimes, τ_M, and extrapolation to 25°: $k_{-1} = 1/\tau_M$.

Equilibrium Perturbation Studies (Temperature Jump)—Low concentrations of reactants are necessary in the stopped-flow measurements; otherwise the time limit of resolution (~3 msec) is exceeded. Thus, in the stopped-flow experiment the bimolecular step, k_1, is rate limiting, and one cannot follow the reaction under conditions where a detectable concentration of intermediate would build up during the reaction. With high concentrations of reactants, a net change in reactant concentration must be achieved by methods other than mixing.

If an equilibrium between complex and free species is perturbed by the rapid adjustment of an external parameter controlling the equilibrium state, the transient approach to a new equilibrium (relaxation) can be monitored. The difference between initial and final equilibrium defines the amplitude of the relaxation step(s), and the time dependence of reequilibration can be related to the rate constants of the reaction (18). In contrast to the flow techniques, mixing is avoided and the concentration of reactants is close to the equilibrium concentration.

By discharging a high voltage pulse through a sample cell, a 5–10° temperature rise can be effected in a few microseconds. With the temperature-jump method, rate phenomena with relaxation times in the microsecond to second range are accessible to direct measurement.

The temperature-jump studies on complex formation between 3-nitro-4-hydroxybenzenesulfonamide and carbonic anhydrase B reveal as many as three relaxation processes (Fig. 8)[1], none of which is present if the reactants are examined individually. Assignment of relaxation steps to primary processes in the reaction pathway is achieved from the analysis of the concentration dependence of the relaxation rate and amplitude.

The slow step of the relaxation times, τ_1, at low concentrations is equivalent to the steady-state kinetic constants derived from the stopped-flow measurements. The relaxation step in the microsecond range, τ_2, is of low amplitude and precluded making a definitive assignment. The fast step, τ_3, shows a rate dependency on buffer concentration and appears to involve ionization in the bound state of the phenolic moiety on the sulfonamide.

A complete analysis of the relaxation profile will be presented elsewhere. It should be evident from these considerations that temperature-jump studies, with their wide temporal range of applicability, offer an essential means for examining the complete spectrum of primary processes associated with complex formation.

With ligand–macromolecule interactions where ionization and conformational changes can be anticipated to accompany complex formation, multiple kinetic and spectroscopic methods must be employed to provide a complete description of reaction mechanism. Through this approach, an understanding of the factors confering specificity in the recognition of ligand structure and the response to ligand binding can be developed.

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Drug–Biomolecule Interactions: Proton Magnetic Resonance Studies of Complex Formation between Bovine Neurophysins and Oxytocin at Molecular Level

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Abstract □ Proton magnetic resonance spectroscopy was used to monitor individual amino acid residues in bovine neurophysin, in the nonapeptide hormone oxytocin, and in the complex formed between them. For neurophysin I alone, a normal titration curve for the C-2 proton resonance of the lone histidine residue was obtained with an apparent ionization constant of 6.9. Addition of oxytocin to a solution of neurophysin I at pH 6.5 resulted in several changes in the spectrum. The effect on the histidine C-2 proton resonance signal indicated a slow exchange process between two states, probably representing a conformational change in the protein. The apparent pK of the histidine residue in the hormonal complex was shifted to 6.7, indicating a slightly more positive (less electron dense) environment for the histidine residue. Resonances of the single tyrosine residue of oxytocin were observed to broaden significantly, but not to shift appreciably, on the addition of neurophysin II. These observations may indicate involvement of the tyrosyl residue of oxytocin in the hormone–"carrier protein" interaction.

Keyphrases □ Neurophysins, bovine—complex formation with oxytocin, PMR □ Oxytocin—complex formation with bovine neurophysins, PMR □ Hormonal interactions—bovine neurophysins–oxytocin, PMR □ PMR—monitoring bovine neurophysins–oxytocin complex formation □ Drug–biomolecule interactions—PMR studies of complex formation between bovine neurophysins and oxytocin at the molecular level □ Interactions—drugs with biomolecules, symposium

High-resolution proton magnetic resonance (PMR) has proved useful in providing information about selected residues in proteins during denaturation (1–3) and inhibitor–enzyme interactions (4, 5). (For reviews, see Refs. 6 and 7.) A few studies of protein–polypeptide or protein–protein interactions using PMR techniques have been reported (8–10).

The neurohypophyseal nonapeptides oxytocin and 8-arginine (or 8-lysine) vasopressin are biologically important hormones known to associate noncovalently with the proteins neurophysin I and neurophysin II (11, 12). These proteins are found in the neurosecretory granules of the pituitary glands of several species associated with the hormonal peptides (13). The physical and chemical properties of the neurophysins, which have monomeric molecular weights near 10,000, have been investigated (14, 15), and some amino acid sequences for bovine neurophysins I and II have been proposed (16, 17). The study of the molecular interactions between neurophysins and peptide hormones has been approached using classical equilibrium methods (18–22) and spectroscopic techniques (23–26).

This article presents PMR observations on highly purified bovine neurophysins, the hormone oxytocin, and the molecular complex formed between them. In particular, high-resolution PMR methods allow the study of the possible involvement of the lone histidine residue of neurophysin I or the single tyrosyl residue of oxytocin in the hormonal complexes[1].

[1] A preliminary account of some of this work has appeared (27).