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*

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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

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nmr coupled with affinity measurements on the respective ligands have yielded new information on the kinetics and mechanism of the anion–carbonic anhydrase interaction.

Experimental Section

Carbonic anhydrase C was isolated from human erythrocytes according to the DEAE separation procedures of Armstrong *et al.* (1966), and enzyme activity was monitored as esterase activity against *p*-nitrophenyl acetate (Verpoorte *et al.*, 1967). The cobalt(II) derivative was prepared as described by Lindskog (1963). Prior to addition of Co^{2+}, various batches of apoenzyme had 4–11% of the activity that could be restored by addition of Zn. Concentration of cobalt(II) enzyme was determined from the OD taking ϵ_{280} to be 56.2 (Armstrong *et al.*, 1966). To ensure standardization between various preparations the extinction coefficient at 602 mμ, an isosbestic point for the cobalt(II) C isozyme, was also measured. The ratio $\epsilon_{280}:\epsilon_{602}$ was $3.2 \pm 0.3 \times 10^3$.

Samples were prepared for nmr relaxation measurements by dialysis against three changes of ten volumes of a solution of 67 mM K_2SO_4 in D_2O containing 2 mM of the following buffers: pH 6.3–7.0, phosphate; pH 7.0–9.0, Tris-sulfate; pH 9.0–9.5, 2-amino-2-methyl-1,3-propandiol sulfate. pH values have been recorded as the uncorrected glass electrode reading with no correction made for the deuterium isotope effect and will be designated as pH*. For relaxation measurements with the Zn enzyme a prior dialysis against 0.1 mM EDTA (pH 7.5) was required to remove trace metal contaminants. In most instances all measurements were completed within 20 hr after dialysis in D_2O buffer to minimize decomposition of the samples or leakage of Co^{2+} from its site in the enzyme. Titrations were carried out directly in sample tubes using an Agla micrometer syringe, appropriate corrections being made for volume dilution.

^{1}H and ^{19}F spectra were recorded on a Varian HA-100 spectrometer at 100 and 94.2 MHz. A smaller number of measurements were made at 60 and 56.4 MHz using a Varian 56/60 spectrometer. Samples contained 10–20 mM dioxane as an internal reference. There was no evidence from either line broadening or differences in the enzyme cobalt(II) spectra to indicate dioxane binding to the enzyme. Chemical shifts were measured relative to the dioxane resonance which also served as the tuning reference with typical line widths ranging between 0.27 and 0.31 Hz. In the ^{19}F experiments an external reference contained in coaxial capillary inserts was employed. The external reference was trifluoroacetic acid and to this was added ~30% hexafluorobenzene which served as a resolution standard.

Line widths were recorded as the width at half-maximal amplitude and represent the average from at least six spectra. Care was taken to avoid saturation. Variations in the instrumental broadening between samples were corrected from similar measurements on the reference peak. Line widths, $\Delta\nu$, were related to the transverse relaxation time by the expression $1/T_2 = \pi\Delta\nu$. Upon completion of a temperature series an amount of *p*-carboxybenzenesulfonamide (*p*-CBS)[1] sufficient to saturate at least 95% of the enzyme binding sites was added and the temperature series rerun. Thus, the reported values represent specific broadening which can be eliminated with addition of sulfonamide. Similarly, chemical shift changes represent the difference in shift with respect to the sample reference in the absence and presence of sulfonamide. With mono- and difluoroacetates, where multiple resonance peaks are observed, the same component of the multiplet was used for all measurements.

A Varian Model V-6040 temperature regulator was used to control temperature. Probe temperatures were calibrated from chemical shifts of methanol or measured directly with a thermocouple. Affinity measurements for the various ligands were determined as described in the previous paper (Taylor and Burgen, 1971).

Relaxation Theory

The theory for paramagnetic contribution to relaxation (Swift and Connick, 1962; Luz and Meiboom, 1964a,b) has been used for interpretation of the parameters controlling relaxation. For the case when the nuclei of interest can exist in two environments, *i.e.*, the coordination sphere of the paramagnetic ion and the bulk solvent, the paramagnetic contribution to relaxation may be expressed in eq 1 where P is the ratio

$$\frac{1}{T_2} = \frac{P}{\tau_M}\left[\frac{\dfrac{1}{T_{2M}^2} + \dfrac{1}{\tau_M T_{2M}} + \Delta w_M^2}{\left(\dfrac{1}{T_{2M}} + \dfrac{1}{\tau_M}\right)^2 + \Delta w_M^2}\right] \quad (1)$$

of ligand in the paramagnetic environment relative to that in bulk solvent and is much less than 1. τ_M is the residence time of the ligand in the paramagnetic environment. For the fast exchange condition this equation shows two general relaxation mechanisms: one involving T_{2M} which contains the dipolar and scalar terms (Bloembergen *et al.*, 1948), and the other involves the chemical shift difference in frequency between the free and bound states, Δw_M, which in itself, can constitute a mode of relaxation. By considering the relative magnitude of each term, three limiting cases may be derived. (a) If exchange is slow relative to either relaxation mechanism, *i.e.*, $\Delta w_M^2 \gg 1/T_{2M}^2, 1/\tau_M^2$ or $1/T_{2M}^2 \gg \Delta w_M^2, 1/\tau_M^2$; then $1/T_2P = 1/\tau_M$ and relaxation will be controlled by the rate of exchange of the ligand between the bulk phase and the paramagnetic environment. (b) If $1/\tau_M^2 \gg \Delta w_M^2 \gg 1/T_{2M}\tau_M$; then $1/T_2P = \tau_M\Delta w_M^2$ and relaxation is controlled by the difference in precessional frequency between the free and bound states. (c) If $1/T_{2M}\tau_M \gg 1/T_{2M}^2, \Delta w_M^2$; then $1/T_2P = 1/T_{2M}$ and the T_{2M} process controls relaxation. τ_M is related to temperature by

$$\frac{1}{\tau_M} = \frac{kT}{h}e^{-[(\Delta H^\pm/RT)-(\Delta S^\pm/R)]}$$

The relaxation rate for case a will, therefore, increase with increasing temperature, provided that a primary kinetic process controls τ_M, the lifetime in the paramagnetic environment.[2] The shift difference Δw_M is proportional to reciprocal temperature (Swift and Connick, 1962), and case b will show a decreased relaxation rate with increasing temperature. The temperature dependence of $1/T_{2M}$ is complex and will be dependent on whether the scalar or dipolar term is dominant (Peacocke *et al.*, 1969).

[1] Abbreviation used is: *p*-CBS, *p*-carboxybenzenesulfonamide.

[2] If exchange of a ligand between two magnetically distinct environments requires a prior isomerization step in the complex, it is conceivable that the opposite temperature dependence could be observed for exchange-dependent relaxation. Thus, for a complex ligand-binding scheme, a negative temperature dependence cannot always rule out exchange-controlled relaxation.

TABLE I: Affinities of Various Carboxylate Ligands for Zinc- and Cobalt(II)-Substituted Human Carbonic Anhydrase C; 20 mM Tris-SO_4 Buffer, pH 7.6.

Ligand	$K_{I,app}$[a] (M^{-1})	
	Zinc	Cobalt(II)
Formate	56	480
Acetate	8.4	130
Monofluoroacetate	7.8	81
Difluoroacetate	20.4	140
Trifluoroacetate	84	95

[a] Apparent affinity determined as described in the Experimental Section. Intrinsic affinities may be calculated according to eq 1 in the preceding paper, $K_{I,app} = K_I(H^+)/((H^+) + K_E)$ (Taylor and Burgen, 1971).

Results

Affinity Measurements. In Table I are given the affinities of various carboxylate ligands for zinc and cobalt(II) human carbonic anhydrase C. The relationship between pH and the apparent affinity of anionic ligands has been examined in detail (Taylor and Burgen, 1971), and the intrinsic affinity may be calculated from these experimental values according to eq 1 in that paper. Since 1.1 pH units separate the experimental pH and pK_E for the C isozyme, the intrinsic affinity will be 13.5-fold greater than the apparent affinity measured here at pH 7.6.

With the lone exception of trifluoroacetate, each ligand exhibits a considerably higher affinity for the cobalt(II) enzyme in preference to the zinc enzyme. Previous studies have shown the opposite to hold for CN^- and SH^- in that these ligands show a higher affinity with the zinc enzyme of bovine carbonic anhydrase B (Lindskog, 1963). There is thus some degree of ligand specificity toward the nature of the metal. The reason for the divergence with the trifluoro-substituted derivative is unknown, however additional substituents may distort the positioning of the bound ligand or lead to an additional means by which stabilization energy in the complex can be conferred. Pocker and Dickerson (1968) have also noted that *tert*-butyl alcohol and pivaldehyde do not bind to carbonic anhydrase which contrasts with the corresponding primary or secondary alcohols or aldehydes.

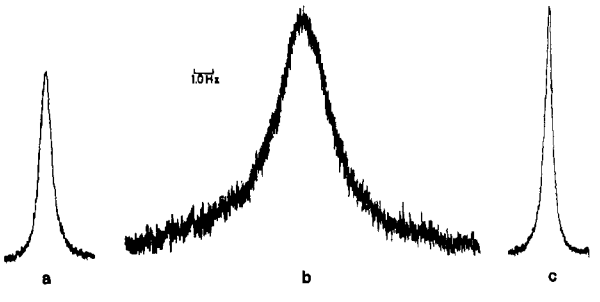

FIGURE 1: ^1H resonance spectrum for formate at 100 MHz, pH* 7.6, 27°. (a) 400 mM formate and 0.8 mM zinc human carbonic anhydrase C; (b) 400 mM formate and 0.1 mM cobalt(II) human carbonic anhydrase C; (c) 400 mM formate, 0.1 mM cobalt(II) human carbonic anhydrase C, 2.0 mM *p*-CBS.

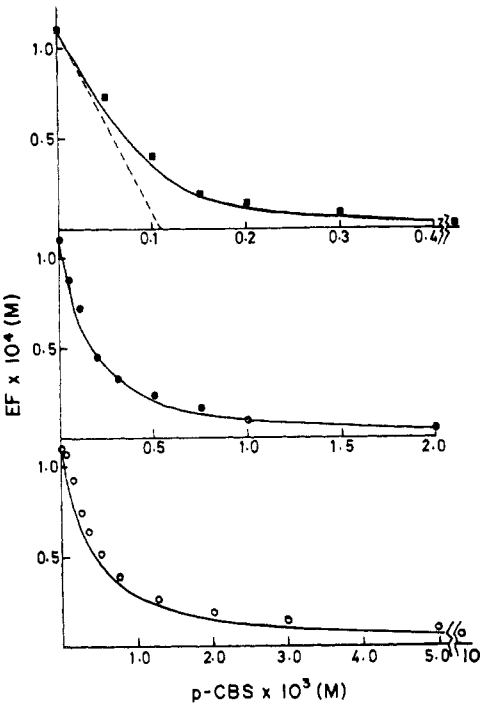

FIGURE 2: Titration of formate–cobalt(II) human carbonic anhydrase C complexes by *p*-carboxybenzenesulfonamide. Transverse relaxation rates, $1/T_2$, for 400 mM formate were measured in the presence of 0.11 mM enzyme and increasing concentrations of sulfonamide, 25°.

$$EF = \frac{\left[\left(\frac{1}{T_2}\right) - \left(\frac{1}{T_2}\right)_\infty\right]1.1 \times 10^{-4}}{\left(\frac{1}{T_2}\right)_0 - \left(\frac{1}{T_2}\right)_\infty}$$

which is equivalent to bound formate concentration, is plotted against the total sulfonamide concentration; $(1/T_2)_0$ and $(1/T_2)_\infty$ are the respective relaxation rates before addition of sulfonamide and at a saturating concentration of sulfonamide: ○, pH* 6.68; ●, pH* 7.24; ■, pH* 8.28. The solid lines are calculated according to

$$EF = \frac{E_T - S_T - KF_T + \sqrt{(S_T - E_T + KF_T)^2 - 4KF_TE_T}}{2}$$

where EF is the bound formate concentration; E_T, F_T, and S_T are the total enzyme, formate, and sulfonamide concentrations. K is ratio of the apparent affinities of the enzyme for formate and sulfonamide and was determined at each pH from the formulae given in the previous paper relating the apparent and intrinsic affinities. The intrinsic affinities are: K_I(formate) = 6.5×10^3 (Table IV) and K_I(*p*-CBS) = 5.2×10^6 (Taylor *et al.*, 1970b,c).

Nmr Titrations. Figure 1 shows the effect of the zinc enzyme on the line width of the formate protons and the additional line width that appears when zinc is replaced by cobalt(II) in the enzyme.

In order to determine whether the nmr experiment yields a quantitative correspondence with our previous fluorescence studies on ligand–carbonic anhydrase interactions (Taylor *et al.*, 1970b,c; Taylor and Burgen, 1971), displacement of a bound anion, formate, from the cobalt(II) enzyme was followed as a function of sulfonamide concentration at various pH values. In Figure 2, the solid line represents the expected titration curve using the affinity for formate and *p*-CBS previously determined by fluorescence titration and assuming each ligand forms a 1:1 complex with the enzyme. The experimental values of the affinities derived from the nmr are in close quan-

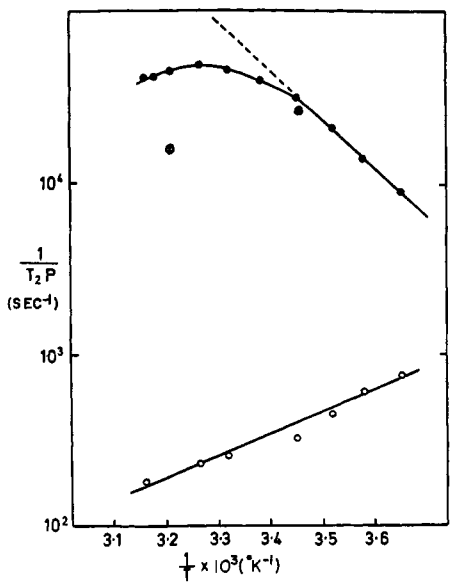

FIGURE 3: Influence of temperature on the transverse relaxation rate for formate and acetate complexes of cobalt(II) human carbonic anhydrase C, pH* 7.6. $1/T_2P$ is plotted as a function of reciprocal temperature, where P is the ratio of enzyme to inhibitor concentration and $1/T_2$ is the observed relaxation rate; (●) 400 mM formate in the presence of 0.1 mM enzyme at 100 MHz; ⊗ at 60 MHz; (○) 240 mM acetate in the presence of 1.5 mM enzyme at 100 MHz. At each temperature, measurements were made in the absence and presence of *p*-CBS, and plotted values are the difference between the two as described in the Experimental Section.

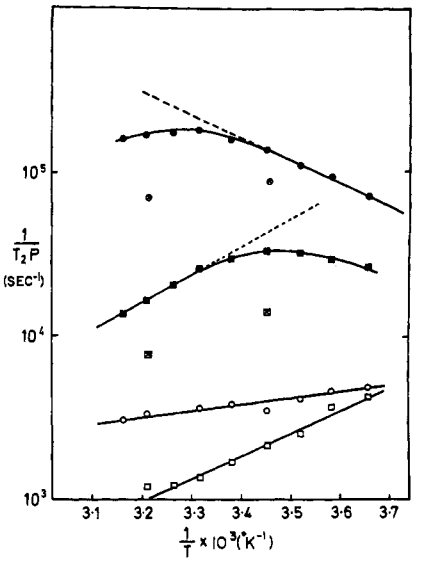

FIGURE 4: Influence of temperature on the transverse relaxation rate of ^{1}H and ^{19}F for mono- and difluoroacetate complexes of cobalt(II) carbonic anhydrase C, pH* 7.6. P is the ratio of enzyme to inhibitor concentration: (●) ^{19}F in CH_2FCOO^- at 94.2 MHz; (■) ^{19}F in CHF_2COO^- at 94.2 MHz; (○) ^{1}H in CH_2FCOO^- at 100 MHz; (□) ^{1}H in CHF_2COO^- at 100 MHz; (⊗) ^{19}F in CH_2FCOO^- at 56.4 MHz; (⊠) ^{19}F in CHF_2COO^- at 56.4 MHz. The relaxation rates represent the difference between measurements made in the absence and presence of *p*-CBS.

titative agreement with those obtained from the fluorescence titrations. This is reassuring in view of the fact that the nmr measurements were carried out in D_2O and the fluorescence titrations in H_2O solution. Thus the nmr experiment indicates a 1:1 ligand binding to a site from which aromatic sulfonamides can cause specific displacement of the anion. When the amount of sulfonamide is sufficient to saturate the binding site, the line width of the anion is similar to that observed in the absence of enzyme and is comparable with that found for the reference dioxane.[3]

Temperature Dependence of Relaxation. Figure 3 shows the temperature dependence of the transverse relaxation rate, $1/T_2$, for formate and acetate at ligand concentrations where the enzyme is, at least, 97% saturated; therefore, differences in P, the ratio of bound to free ligand, can be regarded as negligible in the analysis of the temperature dependence of relaxation. $1/T_2$ for formate in the low-temperature range, shows a positive temperature dependence suggesting that relaxation is dependent on chemical exchange. For acetate there is a negative temperature dependence and the observed value of $1/T_2$ is smaller despite acetate having a lower affinity than formate; it is thus very improbable that the observed relaxation rate is exchange limited. It would also seem unlikely that an exchange limitation on relaxation would exist for protons on substituted acetates.

If we turn to examination of a second nucleus, ^{19}F, faster relaxation rates may be anticipated owing to the greater degree of electronic polarization (Eaton and Phillips, 1965);

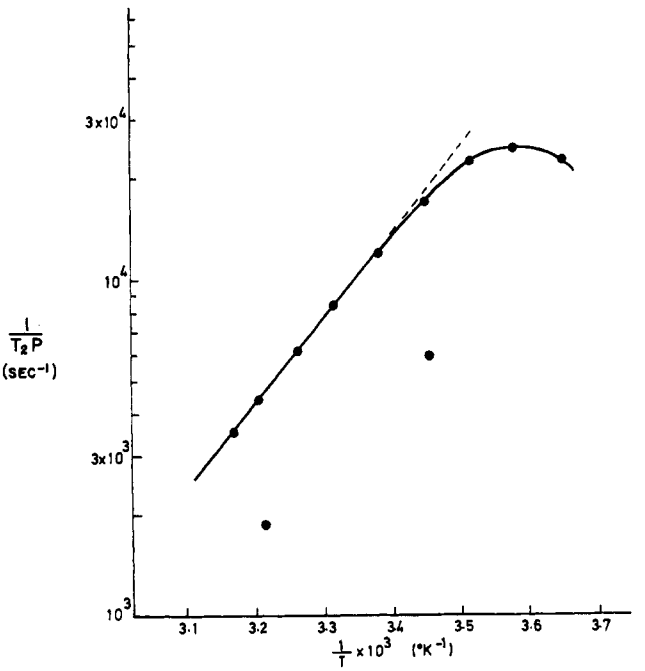

FIGURE 5: Influence of temperature on the transverse relaxation rate of ^{19}F for 400 mM trifluoroacetate with 0.40 mM cobalt(II) isozyme C, pH* 7.6. P is the ratio of enzyme to trifluoroacetate concentration; (○) 94.2 MHz; (⊗) 56.4 MHz. Relaxation rates represent the difference between measurements made in the absence and presence of *p*-CBS.

[3] In early experiments with cobalt(II) human carbonic anhydrase B, a complete return to natural line width for the anionic ligand resonance was not always observed on addition of sulfonamide. Subsequent addition of EDTA in quantities stoichiometric with the enzyme decreased $\Delta\nu$ to natural line widths. Since EDTA does not rapidly remove appreciable quantities of metal from the coordination site at neutral pH (Lindskog and Nyman, 1964), nor does it inhibit the enzyme at low concentrations (Davis, 1959), small amounts of cobalt(II) probably had previously dissociated from the coordination site. Stability of cobalt(II) in the enzyme appeared to be more of a problem with the human B than the C isozyme.

TABLE II: Frequency Dependence of the Transverse Relaxation Rate for Various Carboxylate Ligands with Cobalt(II) Carbonic Anhydrase C.

Ligand	Nucleus	T^{-1} (°K^{-1}) $\times 10^3$	A	B	A:B
Formate[a]	^{1}H	3.46	2.95×10^4	2.46×10^4	1.20
		3.22	4.7×10^4	1.76×10^4	2.67
Monofluoroacetate[b]	^{19}F	3.46	1.37×10^5	8.5×10^4	1.61
		3.22	1.75×10^5	7.2×10^4	2.45
Difluoroacetate[b]	^{19}F	3.46	3.3×10^4	1.39×10^4	2.37
		3.22	1.82×10^4	7.9×10^3	2.30
Trifluoroacetate[b]	^{19}F	3.46	1.7×10^4	5.9×10^3	2.89
		3.22	4.9×10^3	1.75×10^3	2.80

[a] A, $1/T_2P$ at 100 MHz; B, $1/T_2P$ at 60 MHz. [b] A, $1/T_2P$ at 94.2 MHz; B, $1/T_2P$ at 56.4 MHz.

hence, this increases the likelihood of observing exchange limited relaxation. Figures 4 and 5 show the temperature dependence of relaxation rates found for three fluoroacetates. The positive temperature dependence observed at low temperatures again is indicative of chemical exchange limited relaxation. In contrast to the ^{19}F results, the proton resonances for mono- and difluoroacetate show negative temperature dependences and, accordingly, considerably smaller values of $1/T_2$. As would be predicted for coordination of these ligands through the carboxylate group, relaxation rates for the mono- and difluoroacetate protons fall in the range between formate and acetate.

Frequency Dependence of Relaxation. Relaxation controlled by chemical exchange, case a in the theoretical section, should be completely independent of the Larmor frequency. When the residence time is smaller than the chemical-shift difference between the free and bound state, case b, the observed relaxation rate should be proportional to the square of the resonance frequency. The relationship between T_{2M} and frequency, case c, can be quite complex and will be dependent on whether the dipolar or scalar term is dominant (Fabry *et al.*, 1970; Navon *et al.*, 1970).[4]

Table II shows results from relaxation measurements at the lower frequency. The ratio of the two experimental frequencies is 1.67, thus relaxation controlled solely by case b will give a ratio of 2.78 between relaxation rates measured at 100 and 60 MHz, respectively, for protons and 94.2 and 56.4 MHz for ^{19}F, whereas a ratio $\leqslant 1$ would be predicted for the other two cases. A comparison of Table II and Figures 3, 4, and 5 clearly shows that when a negative temperature dependence of relaxation is observed the above ratio approximates the value predicted for case b. However, where a positive temperature dependence appears, which is consistent with exchange controlled relaxation, the ratio approaches 1.0. Thus cases a and b appear to satisfactorily describe T_2 relaxation processes for the nuclei in Table II, and eq 1 in the theoretical section may be approximated by

$$\frac{1}{T_2P} = \frac{\Delta w_M^2}{\frac{1}{\tau_M} + \tau_M \Delta w_M^2} \tag{2}$$

Correspondingly, the equation describing the ratio of relaxation rates at 100 and 60 MHz is

$$R = \frac{(2.78 + \tau_M^2 \Delta w_M^2)}{(1 + \tau_M^2 \Delta w_M^2)} \tag{3}$$

where Δw_M is the value measured at 100 MHz.

Chemical Shift Differences. For proton resonances, chemical shift differences in the presence and absence of sulfonamide over the complete temperature range have been measured with respect to an internal reference. For ^{19}F, this was not possible since the use of only an external reference diminishes the precision of the measured shift differences.

Swift and Connick (1962) have used the following relation to describe the observed chemical shift, Δw, with respect to the mode of relaxation

$$\Delta w = \frac{P \Delta w_M}{\left[\left(\frac{1}{T_{2M}} + \frac{1}{\tau_M}\right)^2 + \Delta w_M^2\right] \tau_M^2} \tag{4}$$

Examination of eq 4 shows that for case a $\Delta w \rightarrow 0$ whereas for case b the values of Δw are finite (*i.e.*, $\Delta w = P \Delta w_M$). An illustration of this behavior for the formate ligand is given in Figure 6 where it can be seen that as the temperature decreases the value of $\Delta w \rightarrow 0$ rapidly as one passes from case b to case a.

For the condition, $1/T_2P = \tau_M \Delta w_M^2$, *i.e.*, case b, an independent determination of Δw_M and τ_M should give the ob-

[4] An indication of the frequency dependence of T_{2M} may be obtained from the proton relaxation studies of water association with cobalt(II) carbonic anhydrase (Fabry *et al.*, 1970). $1/T_{1M}$ for water was found to decrease with increasing frequency. The difference in $1/T_{1M}$ between 60 and 100 MHz, however, is small.

Because the electron spin relaxation time for cobalt(II) is very short ($\tau_s \leqslant 10^{-11}$ sec), this will be the relevant correlation time for ligands coordinated to the cobalt(II) in the enzyme (Luz and Meiboom, 1964a,b). Therefore the Bloembergen equations for the longitudinal and transverse relaxation times, T_1 and T_2, reduce to

$$\frac{1}{T_{1M}} = \frac{1}{T_{2M}} = \frac{4}{3}\frac{S(S+1)\gamma_I^2 g^2 \beta^2 \tau_s}{r^6} + \frac{2}{3}\frac{S(S+1)A^2}{\hbar^2}\tau_s$$

On the basis of a nondetectable chemical shift for coordinated H_2O, Fabry *et al.* (1970) concluded that the first or dipolar term is dominant and have estimated a value of 2.2×10^3 sec^{-1} for the second term. Although the relative magnitude of the two terms will differ for the more distant formate or acetate protons, for any coordinated ligand the frequency dependence of $1/T_{2M}$ should be a function of τ_s which for the cobalt(II) enzyme has been shown to decrease with increasing frequency (Fabry *et al.*, 1970).

TABLE III: Correspondence between Observed $1/T_2P$ and Values Calculated from Lifetime and Chemical Shift Measurements.

Ligand	Nucleus	Temp (°C)	Δw (Hz)	P	Δw_M (Hz)	τ_M (sec)[a]	$4\pi^2\Delta w_M^2\tau_M$ (sec^{-1})[b]	$1/T_2P$[c] (sec^{-1})
Formate	^{1}H	41	2.6	4,000	1.1×10^4	6.3×10^{-6}	2.7×10^4	4.8×10^4
Monofluoroacetate	^{1}H	7	−0.9	3,000	2.7×10^3	1.1×10^{-5}	3.1×10^3	4.5×10^3
Difluoroacetate	^{1}H	7	−1.4	1,000	1.3×10^3	2.8×10^{-5}	2.2×10^3	3.3×10^3
Acetate	^{1}H	27	−0.7	240	1.7×10^2			2.2×10^2
Monofluoroacetate	^{19}F	41	−2.1	16,000	3.4×10^4	2.8×10^{-6}	1.28×10^5	2.0×10^5
Difluoroacetate	^{19}F	37	−1.5	4,000	6.0×10^3	6.0×10^{-6}	0.86×10^4	1.6×10^4
Trifluoroacetate	^{19}F	27	−5.5	1,000	5.5×10^3	5.8×10^{-6}	6.9×10^3	8.4×10^3

[a] Lifetimes are measured by extrapolation of the chemical-exchange-dependent relaxation rate to the appropriate temperature. [b] Chemical shift differences are measured in hertz, hence 2π is inserted to convert into radians; calculated values are from $1/T_2P = \tau\Delta w_M^2$ (*cf.* case b in text). [c] Observed $1/T_2P$.

served relaxation rate. For instance, with mono- and difluoroacetates the relaxation rate calculated from the product of τ_M, which is obtained from an extrapolation of the ^{19}F data, and the chemical shift for protons should be comparable with the observed proton relaxation rate for these inhibitors (Table III). Although calculated values are somewhat less than the observed relaxation rates, reasonable consistency is found throughout the series of ligands. Accuracy of these procedures is limited, however, by the relatively small observed chemical shift differences, extensive extrapolation to obtain τ_M and the large heat of ionization of the Tris buffer giving rise to a pH difference over the temperature range.

Nevertheless, the temperature dependence of formate chemical shifts (Figure 6) and the estimation of the relaxation rates from the chemical shifts (Table III) both reinforce the supposition that cases a and b control relaxation for the carboxylate ligands. A probable exception to this arises with acetate, which exhibits the lowest relaxation rate. Calculation of τ_M from case b yields 1.98×10^{-4} sec which is considerably greater than corresponding values for other ligands, yet acetate exhibits the lowest affinity for the enzyme (Table I).

Thus the temperature and frequency dependence of the relaxation rates for anions bound to cobalt(II) enzyme can be summarized in Table IV. A positive sign indicates an increasing relaxation rate with increasing temperature or frequency.

Zinc Enzyme. A few studies have also been carried out for the zinc enzyme primarily to establish that the observed relaxation of the cobalt(II) enzyme is essentially a consequence of paramagnetic contribution. For the ligand nuclei and the diamagnetic enzyme, $1/T_2P$ was found to be in the range of 50–250 sec^{-1}, and appeared to exhibit a negative temperature dependence. Thus, with the possible exception of acetate the diamagnetic contribution to relaxation is less than 10% of the total and may be neglected. Owing to the low relaxation rates observed for the zinc enzyme, quantitative relaxation studies require more C isozyme than is currently available and have not been pursued in detail.

TABLE IV

Relaxation Mode	Temp (°C)	Frequency
$1/\tau$ case a	+	No change
Δw^2 case b	−	+ (αw_0^2)
$1/T_{2M}$ case c	±	−

pH Dependence of Relaxation. The apparent affinity of anions for carbonic anhydrase shows a marked pH dependence which is consistent with the kinetic scheme presented in the previous paper (Taylor and Burgen, 1971).

$$[HCA]^+ + A^- \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} HCA\text{-}A \qquad (5)$$

$$K_E \Big\downarrow$$

$$CA + H^+$$

The pH dependence of the apparent affinity appears to be due to the ionization represented by K_E. This would be reflected only in the apparent association rate between ligand and enzyme, and the dissociation rate of the complex would be expected to be pH independent. Kinetic confirmation of this scheme can be obtained by following the pH dependence of relaxation in the exchange limitation region.

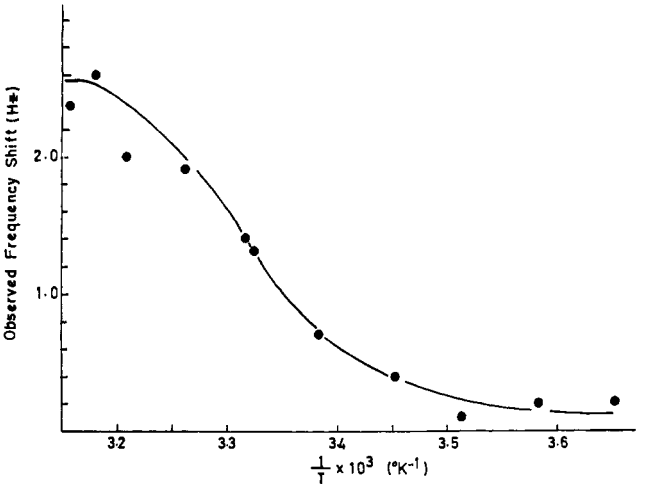

FIGURE 6: Frequency shift difference, Δw, of the formate proton for solution containing 400 mM formate and 0.10 mM cobalt(II) carbonic anhydrase C, pH* 7.6. Chemical shifts are measured relative to an internal reference, dioxane, and the recorded values are differences observed in the absence and presence of *p*-CBS.

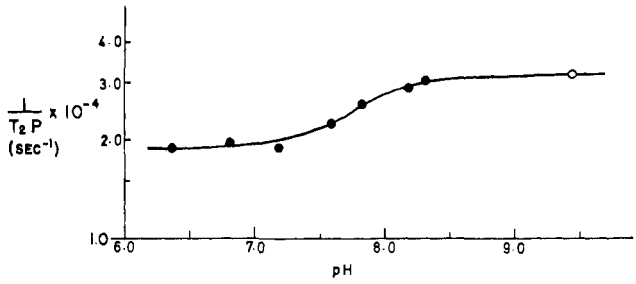

FIGURE 7: pH* dependence of the transverse relaxation rate of the formate proton for a solution containing 400 mM formate and 0.1 mM cobalt(II) carbonic anhydrase C. P is the ratio of enzyme to formate, however for the open circle at pH 9.42, eq 1 in the preceding paper has been used to correct for incomplete saturation of the enzyme.

TABLE V: Estimated Association and Dissociation Rate Constants for Various Carboxylate Anion Complexes of Cobalt(II) Carbonic Anhydrase C, 25°.

Ligand	K_I (M^{-1})[a]	k_1 (M^{-1} sec^{-1})[b]	k_{-1} (sec^{-1})[c]
Formate	6.5×10^3	3.9×10^8	6.0×10^4
Monofluoroacetate	1.1×10^3	2.2×10^8	2.0×10^5
Difluoroacetate	1.9×10^3	1.9×10^8	1.0×10^5
Trifluoroacetate	1.3×10^3	2.0×10^8	1.5×10^5

[a] Intrinsic affinity calculated from the apparent affinity at pH 7.6 (Table I) and eq 1 of (Taylor and Burgen, 1971). [b] Calculated from k_{-1} and K_I. [c] Determined from nmr measured lifetimes and extrapolation to 25°.

At 12° for formate, only a small variation in relaxation rate with pH is observed (Figure 7). Parallel measurements were also conducted at 27°, and the relaxation rate at this temperature was greater than the 12° value throughout the pH range. This makes it unlikely that the dominant process which controls relaxation at 12° changes with pH, thereby ensuring that relaxation of the formate ligand is governed by its dissociation from the paramagnetic environment.

Variation of the relaxation rate with pH, which presumably reflects the primary process represented by k_{-1}, varies by a factor of only 1.5. This may be compared to an apparent affinity difference of ~500 between pH 6.5 and 9.5 for anions of relatively strong acids (Taylor and Burgen, 1971), thus essentially confirming the above kinetic pathway. The origin of the transition in the vicinity of pH 7.8 is unknown, however it does not correspond with pK_E which is 6.5 for the C isozyme.

Kinetics of Anion Complex Formation. For the four anions showing evidence for exchange-dependent relaxation, kinetic constants for the overall association and dissociation processes may be estimated (Table V). In calculating association rate constant the scheme given by eq 5 has been used where the intrinsic affinity considers only ligand combination with the $[HCA]^-$ form of the enzyme. Thus, the product of the intrinsic affinity and the dissociation rate gives the rate constant for the actual association step, k_1. Since affinities were determined at 25°, corresponding dissociation rate constants are computed by extrapolation from low temperature. This can be accomplished best by extrapolation of the longer arm of the temperature curve and evaluating T_2P as the sum of two contributing terms (see eq 2).

The association rate constants for the ligands are similar and approach the limitation set by the rate of diffusional encounter between reactants (Alberty and Hammes, 1958; Eigen and Wilkens, 1965). Unfortunately, ligands in this series exhibit similar affinities which makes it difficult to obtain meaningful correlations between the affinity and the respective rate constants.

Concentration Dependence of Exchange. Examination of the concentration dependence of the apparent exchange rate can establish whether ligand substitution is affected by the following bimolecular interchange mechanism:

$$HCA\text{-}F + F^{-*} \rightleftharpoons HCA\text{-}F^* + F^-$$

If the attacking ligand can influence the dissociation rate of the corresponding leaving ligand, the measured exchange rate will be concentration dependent. As shown for formate in Figure 6, the exchange rate is concentration independent; thus, the above mechanistic pathway does not contribute to the nmr-determined formate exchange. Dissociation of the leaving group, as a unimolecular process, can be said to control turnover of the ligand on the enzyme; consequently, nmr measured lifetimes of the complex provide a valid measure of the dissociation process

$$HCA\text{-}F \xrightarrow{k_{-1}} [HCA]^+ + F^-$$

Discussion

Investigations employing nmr dispersion (Fabry *et al.*, 1970), nmr quadrupolar relaxation (Ward, 1969, 1970), visible spectroscopy (Lindskog, 1966) and ir spectroscopy (Riepe and Wang, 1968) all have indicated direct coordination of the inhibitor anions with the single zinc or cobalt(II) in the enzyme. It, therefore, becomes appropriate to compare kinetically ligand substitution behavior in the cobalt(II) enzyme with simple cobalt(II) coordination complexes.

Ligand coordination to fully aquated metals proceeds through diffusion-limited formation of an outer sphere (ion pair) complex followed by explusion of H_2O from the inner coordination sphere and replacement by the attacking ligand (Eigen, 1963; Eigen and Wilkens, 1965)

$$L + M(H_2O)_n \overset{K_0}{\rightleftharpoons} L \cdots M(H_2O)_n \underset{k_{-01}}{\overset{k_{01}}{\rightleftharpoons}} L - M(H_2O)_{n-1}$$

The first step of formation of the low-affinity outer sphere complex is in equilibrium with the rate-limiting ensuing step of expelling the leaving ligand so that $k_1 = K_0 k_{01}$. Correspondingly, ligand substitution rates are primarily sensitive to the nature of the metal rather than the attacking ligand. Observed rates for association of monovalent anions with octahedrally coordinated Co^{2+} are in the range of 10^5–10^6 M^{-1} sec^{-1} (Kustin and Pasternack, 1969; Davies *et al.*, 1969; Eigen and Wilkens, 1965). k_{01}, computed from the observed bimolecular rate constant and K_0, the ion-pair equilibrium constant for the single-charged anion (Eigen, 1954), is approximately 10^6 sec^{-1} and is in good agreement with the rate constant for water dissociation from aquocobalt(II) determined by nmr (Swift and Connick, 1962).

The spectral and magnetic properties of cobalt(II) in car-

bonic anhydrase, however, more closely resemble a tetrahedrally coordinated metal, although certain spectral characteristics are unique to the enzyme making the precise geometry uncertain (Lindskog and Ehrenberg, 1967; Dennard and Williams, 1966; Grell and Bray, 1971). Substitution rates are usually lower for tetrahedral complexes when compared with corresponding octahedral species, although the instability of aqueous tetrahedral species precludes making a systematic comparison for cobalt(II). Swift and Connick (1962), however, have obtained evidence from ^{17}O relaxation measurements and visible spectra on aqueous solutions of Co^{2+} for the existence of a tetrahedrally coordinated species at high temperature. From relaxation measurements they have calculated a 13-fold greater lifetime for bound H_2O on the tetrahedral species. This would yield a reduction of similar magnitude for the maximal rate of ligand substitution on tetrahedrally coordinated cobalt(II).

The cobalt(II) enzyme, on the other hand, exhibits ligand substitution rates which are two to three orders of magnitude greater than octahedral aquocobalt(II). Two possible explanations could be used to account for this difference. (1) In the ligand substitution mechanism discussed above for water displacement from aqueous metal coordination complexes (Eigen and Wilkens, 1965), the reaction proceeds through an intermediate having a decreased coordination number relative to the initial or final species (*cf.* dissociative mechanism of Langford and Gray, 1965). In this case, the unique metal coordination geometry in the enzyme probably confers an excessive degree of lability on the displaced ligand.[5] The difference in proton relaxation rates of H_2O with addition of inhibitory ligands to cobalt(II) carbonic anhydrase have implicated coordinated H_2O to be the displaced ligand (Fabry *et al.*, 1970). (2) Ligand substitution on carbonic anhydrase occurs *via* a mechanism involving an intermediate species of increased coordination number; that is, coordination of the attacking group occurs prior to dissociation of the leaving group (associative mechanism of Langford and Gray, 1965). With this alternative, the interconversion rate between coordination forms resulting in an intermediate of expanded coordination could rate limit the association process.

In either case, a potentially selective advantage for catalysis is rendered to the enzyme–metal coordination site, in that it possesses an inherently higher capacity for rapid ligand exchange and turnover rates.

Maximal ligand substitution rates for simple aqueous zinc coordination complexes are found to be greater for cobalt(II) complexes, H_2O exchange for zinc being $\sim 10^8$ sec^{-1} (Eigen and Wilkens, 1965; Eigen, 1963). It is unlikely that a parallel difference would exist between the zinc and cobalt(II) enzymes since (a) ligand association rates for the cobalt(II) enzyme are already close to the diffusion limitation, (b) the respective affinities of carboxylate ligands for the zinc relative to the cobalt(II) enzyme are lower (Table I). Higher affinities would correspond to a faster association rate.

Despite the rapid substitution rates observed for the carboxylate anions, certain problems are evident if OH^- is considered to be coordinated to the metal in a similar fashion to the inhibitory anions. An equilibrium between metal-coordinated OH^- and H_2O has been proposed as the ionization responsible for the pH-dependent conversion between catalytically active and inactive forms of the enzyme and the pH-dependent cobalt(II) spectral change (Lindskog, 1963; Coleman, 1967a,b; Wang, 1970). In this postulated scheme, the OH^- ligand would combine with the same coordination form as do the inhibitory anions; *i.e.*, in eq 5 the equilibrium, K_E, would be represented by

$$[HCA]^+ + OH^- \overset{K_E}{\rightleftharpoons} [HCA\text{-}OH]$$

Coordination with OH^-, in contrast to the carboxylate anions, would form a catalytically active species.

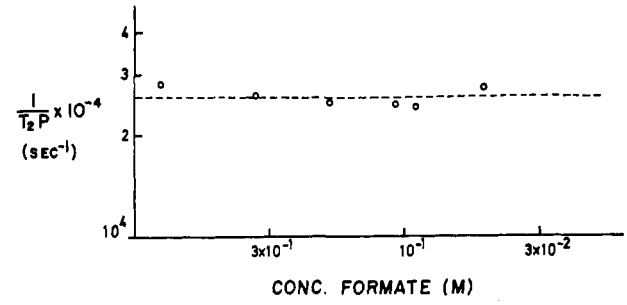

FIGURE 8: Concentration dependence of $1/T_2P$ for formate protons at 12°; pH 7.6. A sample of 800 mM formate with 0.2 mM cobalt(II) carbonic anhydrase C was sequentially diluted, and relaxation rates were measured at the respective formate concentrations.

Relaxation measurements for water coordinated to d-transition metals have shown essentially identical exchange rates for ^{17}O and ^1H (Swift and Connick, 1962, Pearson *et al.*, 1960; Pearson and Lanier, 1964) demonstrating the lack of an independent rapid dissociation step for H^+ from metal-coordinated H_2O. Thus, at least in aquometal complexes, coordination of OH^- involves the microscopic process of association of OH^- from the bulk solvent to the coordination sphere rather than dissociation of a proton from coordinated H_2O. The required high affinity for the presumed equilibrium of OH^- coordination (K_E for isoenzyme C = 3×10^{-8} M) coupled with the rapid ligand replacement rate necessary to satisfy a maximal CO_2 turnover rate of 6×10^5 sec^{-1} (Gibbons and Edsall, 1964) make it difficult to accept a metal-coordinated OH^- as the catalytically active species. Rates for exceeding the diffusion limitation would be required to replenish OH^- on the enzyme after its transfer to CO_2. Only H_2O would be present in sufficient concentration to satisfy this kinetic criterion. Recombination by a proximal protein ligand, which is effectively a unimolecular reaction, would also be kinetically compatible.

From a comparison of the kinetic constants for anion association and corresponding data for sulfonamide ligands (Taylor *et al.*, 1970b), we may draw some conclusions regarding their comparative interaction mechanisms. Association and dissociation are both slower processes in sulfonamide complex formation. Sulfonamide association rates have been found to be as high as 2×10^7 M^{-1} sec^{-1} and, thus, may differ from carboxylate anion association rates by as little as an order of magnitude. Sulfonamide dissociation rates range around 10^{-1} sec^{-1} giving a factor of $\sim 10^5$ separating the rates of dissociation for the two types of complexes. The complex between carboxymethyl human carbonic anhydrase B and *p*-

[5] The protein ligands which coordinate cobalt(II) in the enzyme have yet to be definitely established; however, on the basis of studies with metal coordination complexes, it seems unlikely that the existing protein ligands *per se* could alone be responsible for an increase of this magnitude in the lability of the remaining coordinated H_2O. For example, the overall charge on the complex (Margerum and Rosen, 1967) and the nucleophilicity of the existing ligands (Kustin and Pasternack, 1969) have a comparatively small influence on enhancing ligand substitution rates.

carboxybenzenesulfonamide has a similar affinity to the enzyme–formate complex (Taylor and Burgen, 1971). Despite the near equivalence in binding energy, ligand exchange rates for the formate ligand are four orders of magnitude greater. Thus longer residence times and a higher free energy of activation for the dissociation step characterize the sulfonamide complex.

This high activation energy for dissociation may be indicative of a change in enzyme conformation being responsible for the slow dissociation step. Ultraviolet difference (King and Burgen, 1970) and protein nmr spectroscopy (King and Roberts, 1971) have shown that some alteration in macromolecule conformation accompanies the binding of both sulfonamide and anion ligands. Ultraviolet difference spectra generated with sulfonamide and anion binding are qualitatively similar, although the magnitude of the sulfonamide-induced effect is fourfold greater. Recent nmr studies on the human B isozyme have shown distinct differences in histidine titration behavior between the anion and sulfonamide complexes; however, since assignment of the histidine residues is not complete, it is not possible to ascertain whether these differences are a consequence of a generalized conformational change or represent environmental differences localized to the vicinity of the binding ligand. Further work is therefore required to establish any correlation between the slow dissociation rate with the ligand-dependent conformational change.

A second explanation for the markedly different kinetics between the two types of ligands may reside in the intrinsic nature of the sulfonamide interaction: in addition to the sulfonamido–metal bond (Fridborg *et al.*, 1967), a hydrophobic interaction involving the aromatic ring is believed to contribute to the stabilization energy of complex formation (Chen and Kernohan, 1967; Coleman, 1968; Taylor *et al.*, 1970b). Thus, the interaction involves two distinct loci on the ligand and macromolecule which would not be the case for the anion complex. Consequently, the sulfonamide interaction may be expected to possess parallel kinetic behavior to reactions for bidentate ligands, and could be represented by sequential steps reflecting the two discrete interaction sites

$$E + S \underset{k_b}{\overset{k_a}{\rightleftharpoons}} (ES)_1 \underset{k_d}{\overset{k_c}{\rightleftharpoons}} (ES)_2$$

Formation of $(ES)_1$ results in the second step being effectively unimolecular thereby imparting a statistical advantage to bond formation; hence, the additional stability of the sulfonamide complex would have its origin in the ratio of the respective rates, k_c/k_d.

In chelates of coordination complexes the situation is analogous; that is, considerably slower dissociation rates and parallel increases in affinity are characteristic of the bidentate ligand chelate when compared to corresponding unidentate ligands (Melson and Wilkins, 1962; Eigen and Wilkens, 1965; Margerum *et al.*, 1967; Basolo and Pearson, 1967). As the initial combination step is equivalent to the unidentate ligand coordination, the ratio, k_c/k_d, would similarly be responsible for conferring excess stabilization energy to the bivalent chelate. k_d will exhibit a high activation barrier in that dissociation must additionally accommodate a degree of translation or rotation of the interacting group to prevent immediate bond reclosing (Margerum *et al.*, 1967). The same barrier must be traversed in the corresponding association step, k_c, but it can be energetically compensated by the higher probability for reaction relative to the unidentate ligand, which has the requirement of diffusion to a position suitable for combination.

Examination of complex formation with carboxylate anions has provided a useful comparison here since essentially a single locus of interaction presumably is involved. Thus for sulfonamides a multistep, "chelate effect" scheme is consonant with the observed kinetics, and amplification of the overall binding energy can be achieved when the proper geometric apposition of reacting groups is possible. Nevertheless, the ligand-generated conformational change and the proton donation characteristics of the ligand (Taylor *et al.*, 1970c) are also essential features of sulfonamide complex formation whose energetic contributions remain largely unknown. Further elucidation of the reaction mechanism will require transient methods where the corresponding physical process can be correlated with the component elementary step.

Acknowledgments

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References

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

Armstrong, J. McD., Meyers, D. V., and Verpoorte, J. A., and Edsall, J. T. (1966), *J. Biol. Chem. 242*, 5813.

Basolo, F., and Pearson, R. G. (1967), Mechanisms of Inorganic Reactions, New York, N. Y., Wiley.

Bloembergen, H., Purcell, E. M., and Pound, R. V. (1948), *Phys. Rev. 73*, 679.

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

Coleman, J. E. (1967a), *Nature (London) 214*, 193.

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

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

Davies, G., Kustin, K., and Pasternack, R. B. (1969), *Int. J. Chem. Kinet. 1*, 45.

Davis, R. P. (1959), *J. Amer. Chem. Soc. 81*, 5674.

Dennard, A. E., and Williams, R. J. P. (1967), *Advan. Coord. Chem. 2*, 115.

Eaton, D. R., and Phillips, W. D. (1965), *Advan. Magn. Res. 1*, 103.

Eigen, M. (1954), *Z. Physik. Chem. (Leipzig) 1*, 176.

Eigen, M. (1963), *Ber. Bunsenges. Phys. Chem. 67*, 753.

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

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

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

Gibbons, B. H., and Edsall, J. T. (1964), *J. Biol. Chem. 239*, 2539.

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

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

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.

Kustin, K., and Pasternack, R. F. (1969), *J. Phys. Chem. 73*, 1.

Langford, C. H., and Gray, H. B. (1965), Ligand Substitution Processes, New York, N. Y., Benjamin, pp 1–16.
Lindskog, S. (1963), *J. Biol. Chem. 238*, 945.
Lindskog, S. (1966), *Biochemistry 5*, 2641.
Lindskog, S., and Ehrenberg, A. (1967), *J. Mol. Biol. 24*, 133.
Lindskog, S., and Nyman, P. O. (1964), *Biochim. Biophys. Acta 85*, 462.
Luz, Z., and Meiboom, S. (1964a), *J. Chem. Phys. 40*, 1058.
Luz, Z., and Meiboom, S. (1964b), *J. Chem. Phys. 40*, 2686.
Margerum, D. W., Rohrabacher, D. B., and Clarke, J. F. G. (1967), *Inorg. Chem. 2*, 667.
Margerum, D. W., and Rosen, H. M. (1967), *J. Amer. Chem. Soc. 89*, 1088.
Melson, G. A., and Wilkens, R. G. (1962), *J. Chem. Soc.*, 4208.
Navon, G., Shulman, R. G., Wyluda, B. J., and Yamane, T. (1970), *J. Mol. Biol. 51*, 15.
Peacocke, A. R., Richards, R. E., and Sheard, B. (1969), *Mol. Phys. 16*, 177.
Pearson, R. G., and Lanier, R. D. (1964), *J. Amer. Chem. Soc. 86*, 765.
Pearson, R. G., Palmer, J., Anderson, M. M., Allred, A. L. (1960), *Z. Electrochem. 64*, 110.
Pocker, Y., and Dickerson, D. G. (1968), *Biochemistry 7*, 1995.
Pocker, Y., and Stone, J. T. (1967), *Biochemistry 6*, 668.
Riepe, M. E., and Wang, J. H. (1968), *J. Biol. Chem. 243*, 2779.
Roughton, F. J. W., and Booth, V. H. (1946), *Biochem. J. 40*, 319.
Swift, T. J., and Connick, R. E. (1962), *J. Chem. Phys. 37*, 307.
Taylor, J. S., Mushak, P., and Coleman, J. E. (1970a), *Proc. Nat. Acad. Sci. U. S. 67*, 1410.
Taylor, P. W., and Burgen, A. S. V. (1971), *Biochemistry 10*, 3859.
Taylor, P. W., King, R. W., and Burgen, A. S. V. (1970b), *Biochemistry 9*, 2638.
Taylor, P. W., King, R. W., and Burgen, A. S. V. (1970c), *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.
Wang, J. H. (1970), *Proc. Nat. Acad. Sci. U. S. 16*, 874.
Ward, R. L. (1969), *Biochemistry 8*, 1879.
Ward, R. L. (1970), *Biochemistry 9*, 2447.

Reaction of Aromatic Aldehydes with Glyceraldehyde 3-Phosphate Dehydrogenase*

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ABSTRACT: The reaction of glyceraldehyde 3-phosphate dehydrogenase with a series of aromatic aldehydes has been studied at 25° by following the appearance of NADH. A plot of log (V_{max}/K_m) *vs.* σ, the Hammett substituent constant, is reasonably linear with a slope of 1.24. A plot of log V_{max} *vs.* σ has a slope of 1.27. Thus, the rate of the reaction is facilitated by electron-withdrawing substituents. Trimethylacetyl phosphate is an inhibitor toward benzaldehyde. A plot of $1/V$ *vs.* (I) is linear in the presence or absence of arsenate. In the absence of arsenate, trimethylacetyl phosphate is also an inhibitor for reaction of cinnamaldehyde with the enzyme, but in that case a plot of $1/V$ *vs.* (I) is sigmoidal. In the presence of 0.013 M arsenate, trimethylacetyl phosphate is an inhibitor toward cinnamaldehyde only at concentrations greater than required for maximum inhibition in the absence of arsenate, and a plot of $1/V$ *vs.* (I) is linear.

D-Glyceraldehyde 3-phosphate dehydrogenase (D-glyceraldehyde 3-phosphate:NAD^+ oxidoreductase (phosphorylating), EC 1.2.1.12) is a key enzyme of carbohydrate metabolism, catalyzing several different reactions depending on the reaction conditions (Colowick *et al.*, 1966). The normal dehydrogenase reaction in the presence of inorganic phosphate involves the conversion of D-glyceraldehyde 3-phosphate into 3-phosphoglyceroyl phosphate. In the presence of arsenate the product is 3-phospho-D-glyceric acid. NAD^+ is required as a cofactor and in the reaction is converted into NADH. In addition, an acyl phosphatase activity has been noted in the presence of NAD^+ (Harting and Velick, 1954; Park and Koshland, 1958; Malhotra and Bernhard, 1968; Phillips and Fife, 1969), and esterase activity has been detected toward phenolic esters with an enzyme from which NAD^+ has been removed (Park *et al.*, 1961). The same thiol ester intermediate is apparently formed in reaction of the enzyme with acetyl phosphate and *p*-nitrophenyl acetate (Mathew *et al.*, 1967).

Trimethylacetyl phosphate, although not a substrate, is an excellent inhibitor for the acetyl phosphatase activity and the dehydrogenase reaction involving glyceraldehyde 3-phosphate (Phillips and Fife, 1969). In the latter reaction, plots of $1/V$ *vs.* (I) were sigmoidal, but when aliphatic aldehydes were employed as substrates such plots were linear even at relatively high concentrations of inhibitor (Fife and Rikihisa, 1970). To determine the factors of critical importance in acyl phosphate inhibition and to obtain further information

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