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Chemical Relaxation Studies of Metal Ion Activated Enzymes. I. Opposing Bicipital Relaxation Phenomena in the Bovine Carbonic Anhydrase System. Theory and Observation

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Abstract: Temperature-jump investigations of bovine carbonic anhydrase have been carried out in solutions containing the enzyme, carbon dioxide, bicarbonate ion, and pH indicator in 0.15 *M* potassium fluoride over the pH range 6.0–8.0. A single relaxation has been observed, the amplitude of which is not only dependent on solution conditions but can actually be made to reverse its direction by appropriate adjustment of these conditions. The amplitude goes through a null point where, for a particular set of solution conditions, the amplitude of the relaxation is zero. This behavior, termed an opposing bicipital relaxation, is attributed to a change in the sign of the normal enthalpy for the overall hydration reaction which results from a coupling of the hydration reaction *via* proton to other proton ionization reactions in the system. An approach is presented for calculating the conditions necessary for a relaxation amplitude null in a general reaction system in which the observed reaction is coupled to several more rapid reactions. Agreement between the calculated and the experimental conditions required for a relaxation null may be used to further substantiate a reaction scheme proposed from relaxation time measurements.

The various roles that metal ions play in enzymatic reactions range from weak ionic interactions to specific associations with involvement of the metal ion in the catalytic mechanism.[1] Although numerous studies of metalloenzymes have been reported,[1,2] relatively little is known about the role of metal ions in the detailed catalytic mechanisms. We have initiated in our laboratories a series of investigations employing fast reaction methods for elucidating the kinetic and mechanistic role of metal ions in enzyme-catalyzed reactions. The application of fast reaction techniques permits kinetic experiments to be conducted under conditions which often result in direct observation of enzymatic intermediates.[3,4] Under such conditions, the role of the metal ion in the interconversion of the intermediates can be directly assessed. In this paper we describe the application of the temperature-jump relaxation method to the carbonic anhydrase system and the associated opposing bicipital relaxation phenomenon.

Carbonic anhydrase (carbonate hydro-lyase, E.C. 4.2.1.1) from bovine erythrocytes is a zinc-containing enzyme of the approximate mol wt 30,000. The enzyme catalyzes the reversible hydration of carbon dioxide.

$$2H_2O + CO_2 \rightleftharpoons H_3O^+ + HCO_3^-$$

The enzyme is monomeric and binds zinc in a 1:1 mole ratio.[5] The zinc(II) ion can be replaced by cobalt(II) ion with only minor loss of activity. The incorporation of other first row transition metals results in significant loss of activity.[5] The metal ion is coordinated at the active site and is known to influence the K_a of a catalytically active ionizable moiety. The exact nature of this ionizable group is still a matter of some controversy, but it is generally believed to be either a water molecule coordinated to the metal ion or an uncoordinated vicinal histidyl residue.[5-7]

The temperature-jump investigations of carbonic anhydrase were carried out in solutions containing the enzyme, carbon dioxide, bicarbonate ion, and pH indicator in 0.15 *M* potassium fluoride at a pH near neutrality. In these experiments a single relaxation has been observed, the amplitude of which is not only dependent on solution conditions but can actually be made to reverse its direction by appropriate adjustment of these conditions. The amplitude goes through a null point where, for a particular set of solution conditions, the amplitude of the relaxation is zero. Taylor and Rorabacher have observed a similar phenomenon in solutions of Co(II) and NH_3 and they have called this type of relaxation process an *opposing bicipital relaxation,*[8] a term which we shall adopt in this report. We have found no other examples of this phenomenon reported in the literature. In the following, thermodynamic considerations are used to explain the opposing bicipital relaxation phenomenon.

Experimental Section

Materials. Carbonic anhydrase from bovine erythrocytes was obtained from Worthington Biochemical Corp. and further purified by preparative polyacrylamide gel disk electrophoresis in a Buchler Poly-Prep apparatus.[9] The least anionic of the electrophoretically pure multiple forms designated BCA-B (bovine carbonic anhydrase B in the literature[6]) was used in these experiments. The specific activity of the enzyme was consistently 4000 ± 500 Wilbur-Anderson units/mg of protein. Enzyme activity was assayed electrometrically according to a modified Wilbur and Anderson procedure[10] at 0° with a Heath recording pH meter equipped with a multi-speed chart drive and a combination electrode. The reaction mixture contained 10.0 ml of 0.02 *M* Veronal buffer, pH 8.0, and 0.2 ml of enzyme solution at 0°. CO_2 solution (5 ml) saturated at 0° was injected by means of a syringe into the mixture and the time required to reduce the pH from 8.0 to 6.2 was recorded (*ca.* 3–10 sec). A blank determination was also performed substituting 0.2 ml of buffer for the enzyme solution. Carbonic anhydrase activity for the hydration reaction was calculated from the following formula: Wilbur–Anderson units = $(t_0 - t)/t$, where t_0 is the time of the uncatalyzed reaction and t is the time of the enzyme-catalyzed reaction. Protein was determined according to Lowry, *et al.*[11] The metal ion content of the enzyme was determined with a Perkin-Elmer Model 303 atomic absorption spectrophotometer. Protein samples frozen in liquid nitrogen were lyophilized in a Model USM-15 freeze-dryer from VirTis Co. To remove the Zn(II) ion from the native carbonic anhydrase, the enzyme was suspended (5 mg of protein/ml) in 0.1 *M* acetate buffer, pH 5.0, and dialyzed against 10 vol of 10^{-2} *M* 1,10-phenanthroline in the same buffer for approximately 7 days. When the demetallated enzyme was dialyzed against 10^{-2} *M* $CoCl_2$ in 0.1 *M* acetate buffer, pH 5.0, for 48 hr, about 80% of the originally present Zn(II) ion could be replaced by Co(II) ion with 75% retention of the original specific activity in the zinc enzyme.

All other chemicals used were of reagent grade. Solutions were prepared with deionized distilled water which had been degassed by boiling for 1 hr and stored *in vacuo* until used. Standard buffer solutions used for calibration of all pH equipment were obtained from Beckman.

Instrumentation. Relaxation measurements were made with a stopped-flow/temperature-jump relaxation spectrometer of the Hammes and Erman design[12] as modified by Faeder[13] and Tallman. In the temperature-jump mode, a temperature increase of 7.5° is achieved with a heating time constant of 8 μsec for a solution having 0.15 *M* ionic strength. The relaxation spectrometer is interfaced to a PDP-8/I minicomputer for on-line evaluation of the relaxation times.[14] The exponential relaxation curve is stored on a Tektronix Type 549 storage oscilloscope and is simultaneously digitized and recorded by a Biomation Model 802 transient recorder. The digital data from the transient recorder are read into the computer where they are optionally smoothed and are subjected to exponential least-squares analysis. The computer evaluates the relaxation time (sec), the reciprocal relaxation time (sec^{-1}), the correlation coefficient, and the number of data points used in the evaluation. Typically, 200–400 data points are used in each evaluation.

An Orion Model 701 digital pH meter with a Radiometer combination electrode was used for measurements and adjustments of pH for the relaxation experiments.

Procedure. Solutions were prepared for equilibrium temperature-jump measurement by mixing together in a volumetric flask appropriate aliquots of carbonic anhydrase, potassium bicarbonate, potassium fluoride, and pH indicator stock solutions and diluting to the mark. The pH was adjusted by addition of small volumes of 3 *N* HCl or 3 *N* NaOH (freshly prepared from triply washed NaOH pellets).

All stock solutions and run solutions were prepared in a glove box under nitrogen. Relaxation measurements were carried out under nitrogen by construction of a continuously purged nitrogen tent surrounding the cell and syringe compartments of the relaxation spectrometer. Such precautions were found essential to eliminate interferences caused by atmospheric CO_2. Relaxations were monitored spectrophotometrically by coupling the enzyme system with suitable pH indicators. The measurements were performed at 25° in an ionic medium of 0.15 *M* KF.

Results and Discussion

The relaxation spectrum of bovine carbonic anhydrase has been studied over the range of 10^{-4}–10^{-7} *M* enzyme concentration, 2×10^{-2}–1×10^{-4} *M* total carbonate concentration, and pH 6.0–8.0. Under each of these conditions, no more than a single relaxation process has been observed. The pH dependence of the observed *relaxation time* is shown in Figure 1. Figure 2 shows the pH dependence of the *relaxation amplitude.* The dependence of the relaxation time on pH and on enzyme, carbon dioxide, and bicarbonate concentrations is consistent with a mechanism of the type[7]

$$\begin{array}{ccc} CO_2 + E_b & \overset{\text{fast}}{\rightleftharpoons} & E_b \cdot CO_2 \\ \text{fast}\ \Big\updownarrow\ -H^+ \ +H_2O & & \Big\updownarrow\ \text{slow} \\ HCO_3^- + E_a & \overset{\text{fast}}{\rightleftharpoons} & E_a \cdot HCO_3 \end{array}$$

where E_b and E_a represent the base and acid forms of the enzyme, respectively. The relaxation we observe appears to be due to the slower hydration step with all other steps readjusting in rapid equilibrium.

The pH dependence of the relaxation time (Figure 1) may be interpreted qualitatively in the following way. At low pH, most of the carbonate in solution is in the form of CO_2, whereas the enzyme is predominantly in its acid form, apparently incapable of hydrating CO_2. Conversely, at high pH, most of the carbonate is present as HCO_3^-, and the enzyme is predominantly in its base form and is incapable of dehydrating HCO_3^-. Hence, the relaxation rate is lowest at the extremes of pH and reaches a maximum at an intermediate pH where enzyme and carbonate are present in compatibly reactive forms. The shift in the maximum for the cobalt enzyme to higher pH relative to the zinc enzyme reflects a slightly higher pK_a of the activity-linked ionizable group of the cobalt enzyme.[15,16] A quantitative interpretation of our relaxation time data in terms of the reaction mechanism of carbonic anhydrase is the subject of a forthcoming paper.[17]

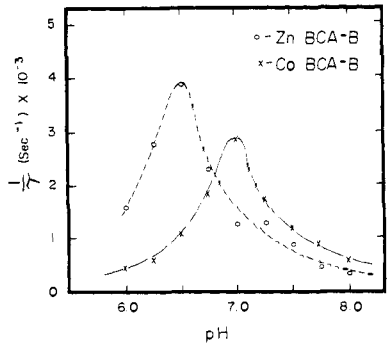

Figure 1. pH dependence of the reciprocal relaxation time for the carbonic anhydrase catalyzed CO_2-HCO_3^- system. Total concentrations: $C^0_{CO_3} = 1.00 \times 10^{-2}$ *M*, $C^0_{BCA\text{-}B} = 1.0 \times 10^{-5}$ *M*, $C^0_{IND} = 4.0 \times 10^{-5}$ *M*, 0.15 *M* KF. Indicators: Bromcresol Purple, pH 6.0–6.75; Bromthymol Blue, pH 7.0–7.75; Phenol Red, pH 8.0. The dashed lines drawn through the points have no theoretical significance.

Of particular interest in the present report is the behavior of the amplitude of the singly exponential relaxation process observed in the carbonic anhydrase system. As the pH is increased through the range 6.0–8.0, the amplitude undergoes a monotonic decrease, passes through a null point where no chemical relaxation is observed, and then increases in the direction opposite to that observed prior to the null point. This behavior is illustrated in Figure 2 where it is observed that the inversion of amplitude occurs over a narrow range of pH. The relaxation times in the *immediate vicinity* of the null are essentially the same within experimental error. The pH at which the null is observed is essentially independent of the nature of the metal ion activator and of the enzyme and total carbonate concentrations and does not appear to bear any specific relationship to the pH dependence of $1/\tau$.

Theory of Opposing Bicipital Relaxation. The above results suggest that the relaxation curves of opposite amplitudes represent the same relaxation process, the differing amplitudes resulting from a change in the direction of the equilibrium shift following a temperature-jump perturbation. The minimum requirement for this type of behavior is satisfied in a system of two parallel coupled reactions

$$A \overset{\text{fast}}{\rightleftharpoons} B + C \qquad \Delta H_{1a} \tag{1a}$$

$$R \overset{\text{slow}}{\rightleftharpoons} P + C \qquad \Delta H_{1b} \tag{1b}$$

the enthalpies of which have the same sign. If the relaxation rate of reaction 1a is fast compared to that of reaction 1b, a temperature-jump perturbation will cause a rapid change in the concentration of the coupling species C, resulting from the rapid relaxation of reaction 1a. The relaxation amplitude of the slower reaction 1b will then be governed by the combined effect of two perturbing forces: (1) the changing state function (temperature) and (2) the changing concentration of the coupling species, C. Since the enthalpies of reactions 1a and 1b have the same sign, these two perturbing forces oppose one another, and the direction in which reaction 1b shifts depends upon which of these two perturbing forces dominates. By judicious choice of conditions, the two opposing perturbing forces can be made to exactly cancel each other in which case there is no observed relaxation of reaction 1b

$$\delta[R] = \delta[P] = 0$$

Such a condition is called a null relaxation point:[8] the vanishing of a relaxation under conditions for which, in the absence of coupling, a relaxation would otherwise occur.

The enthalpies of the coupled reactions can be related to one another and to the solution conditions required for a

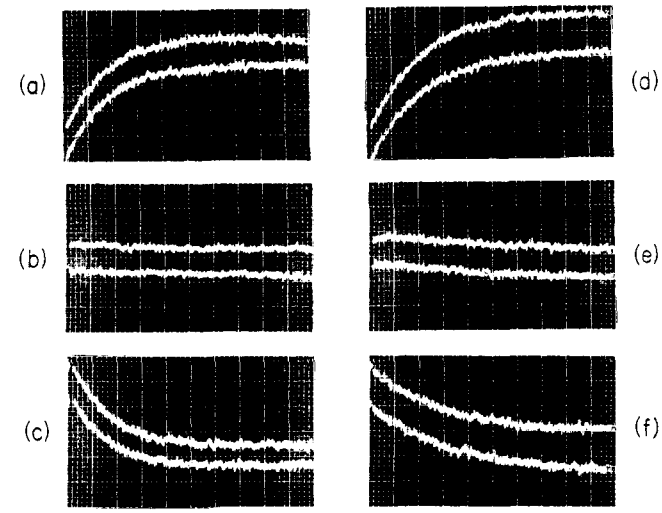

Figure 2. Opposing bicipital relaxation effect in the carbonic anhydrase catalyzed CO_2-HCO_3^- system. Zinc carbonic anhydrase: (a) pH 6.79, (b) pH 7.18, (c) pH 7.40. Cobalt carbonic anhydrase: (d) pH 6.94, (e) pH 7.32, (f) pH 7.56. Total concentrations: $C^0_{CO_3} = 1.00 \times 10^{-2}$ *M*, $C^0_{BCA\text{-}B} = 1.0 \times 10^{-5}$ *M*, $C^0_{IND} = 4.0 \times 10^{-5}$ *M*, 0.15 *M* KF. The Bromthymol Blue indicator was monitored at 615 nm. The horizontal scale is 0.5 msec per large division. The vertical scale is in arbitrary units of transmittance.

null relaxation. To illustrate the derivation of this relationship, consider the following general reaction scheme

$$\begin{aligned} A_1 &\rightleftharpoons B_1 + C \qquad K_1 \qquad \Delta H_1 \\ A_2 &\rightleftharpoons B_2 + C \qquad K_2 \qquad \Delta H_2 \\ &\vdots \qquad\qquad \vdots \\ A_m &\rightleftharpoons B_m + C \qquad K_m \qquad \Delta H_m \end{aligned} \tag{2a}$$

$$R \overset{\text{slow}}{\rightleftharpoons} P + C \qquad K \qquad \Delta H \tag{2b}$$

where the n dissociation reactions involving A_i and B_i (all different chemical species) are considered to be rapid and are coupled to the slower reaction between reactant, R, and product, P, by the coupling species, C. For each of the coupled reactions of eq 2 the following relationships can be written.[18]

$$\delta \ln K_i = \frac{\delta[B_i]}{[B_i]} + \frac{\delta[C]}{[C]} - \frac{\delta[A_i]}{[A_i]} = \frac{\Delta H_i}{RT^2}\delta T \tag{3}$$

$$\delta \ln K = \frac{\delta[P]}{[P]} + \frac{\delta[C]}{[C]} - \frac{\delta[R]}{[R]} = \frac{\Delta H}{RT^2}\delta T \tag{4}$$

Considering only the very fast change in the concentration of C which results from the rapid equilibrium of the n (2a) reactions, mass balance requires that

$$\delta[C] = \sum_i \delta[B_i] \tag{5}$$

and

$$\delta[A_i] = -\delta[B_i] \tag{6}$$

Equations 3 and 6 can be solved for $\delta[B_i]$ yielding

$$\delta[B_i] = \frac{(\Delta H_i/RT^2)\delta T - \delta[C]/[C]}{1/[A_i] + 1/[B_i]} \tag{7}$$

Substitution of eq 7 into eq 5 and solving for $\delta[C]$ gives

$$\delta[C] = \frac{\dfrac{\delta T}{RT^2}\displaystyle\sum_i \frac{\Delta H_i}{1/[A_i] + 1/[B_i]}}{1 + \displaystyle\sum_i \frac{1}{K_i/[B_i] + C/[B_i]}} \tag{8}$$

where $\delta[C]$ is the total change in the concentration of C which occurs rapidly after the temperature-jump perturbation but prior to significant relaxation of the slower step. If a null relaxation is observed

$$\delta[P] = \delta[R] = 0 \tag{9}$$

and the magnitude of $\delta[C]$ required for such a null, denoted $\delta[C]_N$, is obtained from eq 4.

$$\delta[C]_N = [C](\Delta H/RT^2)\delta T \tag{10}$$

Therefore, at the null relaxation point

$$\delta[C] = \delta[C]_N$$

and the right-hand sides of eq 8 and 10 are equated to yield

$$\Delta H = \frac{\sum_i \frac{\Delta H_i}{1/[A_i] + 1/[B_i]}}{[C] + \sum_i \frac{1}{1/[A_i] + 1/[B_i]}} \tag{11}$$

The extension of this derivation to other reaction systems is straightforward.

For the two coupled dissociation reactions of eq 1, eq 11 condenses to

$$\Delta H_{1a} = \Delta H_{1b}(1 + [C]/[A] + [C]/[B]) \tag{12}$$

This result implies an additional requirement for a relaxation null in the two reaction system. Not only must the enthalpies have the same sign, but the absolute value of the enthalpy change for the rapidly equilibrating reaction 1a must be greater than that for the more slowly equilibrating reaction 1b.

$$|\Delta H_{1a}| > |\Delta H_{1b}|$$

This conclusion does not agree with a previous statement[8] that, providing the two reactions are of the same molecularity and are not coupled to any other reactions, a complete cancellation of the equilibrium shift occurs only under the specific condition where $\Delta H_{1a} = \Delta H_{1b}$.

The relaxation null point corresponds to a condition for which the normal-mode enthalpy change[19] for the slower relaxation process is zero. An expression for this normal-mode enthalpy change, ΔH_N, is readily obtained from the null point enthalpy relationship. For reaction 2b the normal-mode enthalpy expression is obtained from eq 11 as

$$\Delta H_N = \Delta H - \frac{\sum_i \frac{\Delta H_i}{1/[A_i] + 1/[B_i]}}{[C] + \sum_i \frac{1}{1/[A_i] + 1/[B_i]}} \tag{13}$$

For the two coupled reactions of eq 1, eq 13 reduces to

$$\Delta H_N = \Delta H_{1b} - \frac{\Delta H_{1a}}{1 + [C]/[A] + [C]/[B]} \tag{14}$$

Equation 14 is equivalent to eq II.3.39 of Eigen and DeMaeyer[20] and to eq 54 of Thusius.[21] The sign of ΔH_N dictates the direction and magnitude of the equilibrium shift and, hence, the amplitude of the relaxation process. The equilibrium will shift to the right, to the left, or will not shift at all corresponding to $\Delta H_N > 0$, $\Delta H_N < 0$, or $\Delta H_N = 0$, respectively.

The CO_2–HCO_3^- System. Under the conditions of our experiments the relaxation amplitude does not appear to be significantly dependent upon enzyme concentration or on the nature of metal ion associated with the enzyme. Consequently, the essential features of the amplitudinal behavior in fluoride media can be understood by considering the coupled reaction system shown in Table I where the ionization

Table I

	ΔH, kcal/mol	pK	Ref
$HF \rightleftharpoons H^+ + F^-$	−3.0	3.20	22, 23
$HIn \rightleftharpoons H^+ + In^-$	2.1	7.10	24
$H_2O \rightleftharpoons H^+ + OH^-$	13.3	14.00	25
$CO_2 + H_2O \rightleftharpoons H^+ + HCO_3^-$	2.24	6.35	26

reactions of HF, HIn (protonated Bromthymol Blue indicator), and H_2O are assumed to equilibrate rapidly compared to the CO_2 hydration reaction.[27] Using literature values for the thermodynamic parameters, the normal enthalpy, ΔH_N, for the CO_2 hydration reaction in this coupled system was calculated as a function of pH from eq 13, and the result is shown by the dashed curve of Figure 3. Below pH 7.4, ΔH_N is positive and a temperature increase should produce an equilibrium shift to the right, resulting in a decrease in pH. Above pH 7.4, ΔH_N is negative and an equilibrium shift to the left is expected, resulting in a pH increase. A null relaxation point is predicted to occur at pH 7.4 where $\Delta H_N = 0$. These results are in good agreement with the experimental observations shown in Figure 2 and summarized in Table II.

Table II. Null Point pH Values[a]

	0.15 M KCl	0.15 M KF
Enzymatic[b]	6.3	7.2 (7.3)[c]
Nonenzymatic[d]	6.2	7.3
Calculated[e]	6.1	7.4

[a] Uncertainty ±0.1 pH unit. [b] Enzyme concentration 1.0×10^{-5} M, total carbonate concentration 1.0×10^{-2} M, Bromthymol Blue concentration 4.0×10^{-5} M. [c] Data for cobalt enzyme in parentheses. [d] Same as b without enzyme. [e] Values obtained from Figure 3.

In the vicinity of the null point (pH 7.4), the summation terms of eq 13 are dominated by the term reflecting the contribution from the HF ionization reaction. This is due to the fact that F^- is a relatively good base (pK_a = 3.2) and is present at high concentration (0.15 M). Consequently, the null point pH is expected to shift in going from a fluoride medium to a chloride medium since Cl^- is a much weaker base than F^-. In a chloride medium only the indicator and water ionization reactions are considered since the concentration of undissociated HCl is negligible. The solid line of Figure 3 indicates the pH dependence of ΔH_N under these conditions and predicts a shift in the null point pH from 7.4 to 6.1 in going from F^- to Cl^- supporting electrolyte. This result is in agreement with the experimentally estimated null point pH of 6.0 in 0.15 M KCl[28] (Table II). In the chloride medium the influence of the pH indicator on ΔH_N, which is masked in the fluoride medium, shows up as a broad plateau of negative ΔH_N in the vicinity of pH 7.0. At high pH, the water ionization equilibrium dominates the summation terms of eq 13 and drives the normal mode enthalpy to a limiting value of −11 kcal/mol in both Cl^- and F^- media. At pH 6.5, Figure 3 predicts the relaxation effect should exhibit an amplitude in the F^- medium which is opposite in direction to that observed in the Cl^- medium. Indeed, this behavior is observed experimentally. For purposes of comparison, Figure 3 also displays the behavior of ΔH_N for the CO_2 hydration reaction in pure water where it is seen that ΔH_N passes through zero at pH 6.6.

Since equilibria involving the enzyme do not appear to contribute significantly to the normal enthalpy of the over-

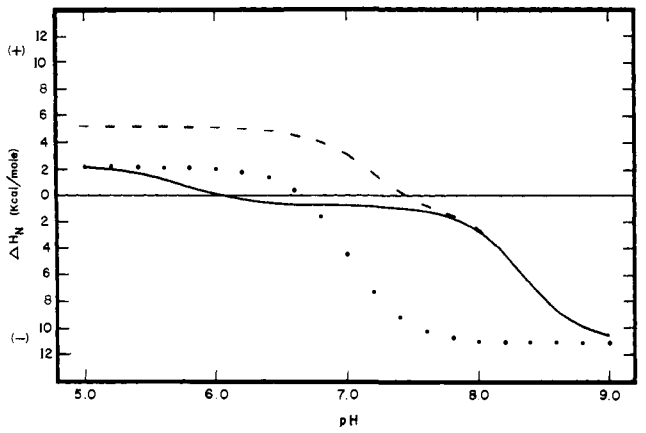

Figure 3. pH dependence of the normal enthalpy for the CO_2 hydration reaction. Reactions coupled to the hydration reaction: (• • •) water ionization; (——) indicator (4.0×10^{-5} *M* Bromthymol Blue) and water ionizations; (- - -) HF (0.15 *M* KF), indicator (4.0×10^{-5} *M* Bromthymol Blue), and water ionizations.

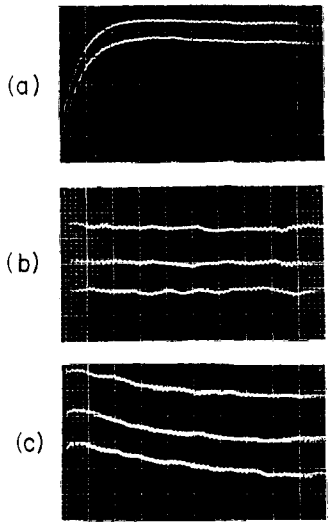

Figure 4. Opposing bicipital relaxation effect in the nonenzymatic CO_2-HCO_3^- system: (a) pH 6.05 (Bromcresol Purple indicator), (b) pH 6.25 (Bromthymol Blue indicator), (c) pH 6.60 (Bromthymol Blue indicator). Total concentrations: $C^0_{CO_3} = 1.00 \times 10^{-2}$ *M*, $C^0_{IND} = 4.0 \times 10^{-5}$ *M*, 0.15 *M* KCl. The horizontal scale is 100 msec per large division. The vertical scale is in arbitrary units of transmittance.

all hydration reaction in the pH range 6-7, the relaxation amplitude of the nonenzymatic CO_2-HCO_3^- reaction is expected to behave similarly to that observed for the enzyme system. Careful examination of the nonenzymatic system did indeed reveal an opposing bicipital relaxation effect (Figure 4) with a null pH of 6.2 in a Cl^- medium and 7.3 in a F^- medium. The nonenzymatic system relaxes too slowly for our instrument to obtain good quality data.[29] Table II summarizes the null relaxation conditions.

Conclusions

The relaxation effects of opposite amplitude observed in the carbonic anhydrase, CO_2, HCO_3^- system in 0.15 *M* KF appear to be due to the same relaxational process, the opposite amplitudes resulting from a change in the direction of equilibrium shift following a temperature-jump perturbation. This behavior is attributed to a change in the sign of the normal mode enthalpy for the overall hydration reaction which results from a coupling of the hydration reaction *via* proton to other proton ionization reactions in the system. Experimental observation of this behavior appears to be rare since to our knowledge only one other similar phenomenon has been reported in the literature. Under the conditions of our study, the enzyme does not appear to be involved in any equilibrium which significantly influences the conditions necessary for a relaxation null. At higher or lower pH, enzyme ionization reactions may contribute to the normal mode enthalpy expression and, hence, may influence the amplitude of the relaxation effect.

The approach outlined in this paper permits the calculation of the conditions for which a relaxation amplitude inversion should be observed, if indeed the conditions for such an inversion exist, in a reaction system in which the observed reaction is coupled to several more rapid reactions. Agreement between observed and calculated conditions for a relaxation null further substantiate a reaction scheme proposed from relaxation time measurements.

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References and Notes

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(18) Volume changes are neglected and ΔH is assumed to be independent of temperature over the range of interest. Also, we have neglected considerations of activity coefficients. All concentrations are equilibrium concentrations in moles per liter.
(19) The normal-mode enthalpies for a coupled reaction system are linear combinations of the enthalpies of the individual reaction steps. The observed amplitude of a relaxation process monitored spectrophotometrically may be expressed in terms of its normal-mode enthalpy, the normal-mode extinction coefficients, and the appropriate Γ function (see ref 20 and 21).
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(27) Under the conditions of our experiments, contributions to this reaction scheme by ionization reactions involving H_2CO_3 and CO_3^{2-} are considered negligible. Also, the formation of HF_2^- is not considered in this treatment. These approximations do not alter the qualitative results which follow.
(28) Precise measurement of the relaxation null pH was difficult in the KCl medium since the null occurs at such a low pH that the indicator exhibits only very low sensitivity to pH changes. The use of an indicator with a lower pK_a does not alleviate this problem since the null point then shifts to even lower pH as predicted by eq 13.
(29) We have found that, for identical conditions of concentrations and pH, the nonenzymatic relaxation effect in a F^- medium is slower than that observed in a Cl^- medium. This difference is not observed in the enzymatic system: T. J. McNeese and D. E. Tallman, paper in preparation.