Relaxation Studies of Enzymes: Concentration and pH Dependence of Isomerization Phenomena†

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ABSTRACT: Equations have been developed for the relaxation times for a variety of mechanisms involving enzyme isomerization coupled to proton transfers. The concentration and pH dependences of the relaxation time have been calculated and graphed for a number of representative mechanisms. We find that for most of the mechanisms examined, the relaxation time is not only pH but also strongly concentration dependent. The concentration dependence results from finite perturbations of the hydrogen ion concentration. For the systems tested, the relaxation time shows a clear concentration dependence at enzyme concentrations below 200 μM.

Temperature-jump spectroscopy has become an important tool for the elucidation of enzyme mechanisms. Transient kinetic studies employing this technique have dealt with such rapid processes as proton transfers, association phenomena, substrate and cofactor binding, and configurational changes. Several laboratories have reported relaxation processes attributed to an enzyme isomerization. The first such study was that of French & Hammes (1965) in ribonuclease A. The mechanism used to interpret their results was mechanism 2 of Table I. French et al. (1974) reported for carboxypeptidase A not only an effect analogous to that in ribonuclease A but also a second relaxation which they found to be correlated with an isomerization of a deprotonated form of the enzyme (Scheme I) for which τ^{-1} is

$$\tau^{-1} = \left(\frac{K_a}{K_a + [H]}\right)k_r + k_f \qquad (1)$$

Aspartate aminotransferase (Giannini et al., 1975) exhibited an isomerization coupled to two cooperative proton transfers (mechanism 8 of Table I). An isomerization was also found by Jentoft et al. (1977) to occur in hexokinase. The mechanism used to fit their data was also that of French and Hammes.

Scheme I

$$E' \underset{k_r}{\overset{k_f}{\rightleftharpoons}} E + H \overset{K_a}{\rightleftharpoons} EH$$

In these studies the relaxation was detected by relating the change in the absorption of a pH indicator to the change in the hydrogen ion concentration for the process. All of these isomerizations were observed in the pH region of 5–8. Their relaxation times were independent of the enzyme concentration, indicator concentration, and identity of the indicator. Inherent in all of these treatments was the assumption, first proposed by French & Hammes (1965), that the perturbation of the hydrogen ion concentration (δ[H]) is approximately zero. This assumption causes the terms involving the enzyme concentration and indicator concentration to drop out of the expressions for the reciprocal relaxation times (see Table I, column 3). There seems to be no a priori reason for such an assumption to hold for all enzyme systems. We have therefore derived the exact expressions for τ^{-1} for several representative mechanisms involving conformational changes. The purpose of this paper is to present these mechanisms, to examine their predicted pH, enzyme, and indicator dependences, and to compare these predictions with the originally postulated mechanisms.

Results and Discussion

Examination of Table I shows that exact derivations can yield quite complex expressions for τ^{-1} as a function of pH or enzyme concentration. These derivations incorporate no assumptions concerning δ[H], include all enzyme species, and contain terms arising from the indicator coupling and the self-ionization of water. The last column shows the approximate equations which result from the condition that δ[H] is not large enough to contribute to the experimental results, i.e., δ[H] = 0. The rate constants may be eliminated from these expressions by division by k_r and definition of the dimensionless parameter $\tau_r^{-1} = \tau^{-1}/k_r$. Values of log τ_r^{-1} for the exact expressions (column 2) have been plotted as a function of pH and [E°] for several representative mechanisms. The mechanisms have been divided into three groups based on the number of isomerizations and the number of protons coupled to the process. Each group will be discussed separately.

Single Isomerization Mechanisms. Three mechanisms have been derived for a single isomerization coupled to a single proton transfer. Mechanism 2, that of French & Hammes (1965), allows for ionization from only one of the conformational forms of the enzyme. Presumably, the ionizing group in the E'H form is buried in a hydrophobic pocket and cannot interact with the solvent. Mechanisms 1 and 3 are more general forms. The former allows for the rapid ionization of both conformational forms. This type of behavior would apply to an isomerization where the ionizing group remains on the surface of the protein in both conformational forms; however, a shift in the proximity of the various other charged groups in the neighborhood of the group in question will result in a shift of the apparent pK_a. When the shift is very large, i.e., $K_{a1} \ll K_{a2}$, the mechanism reduces to mechanism 2. If no change in the pK_a occurs, the enzyme-dependent terms cancel, and the process is concentration independent and τ^{-1} is found to be

$$\tau^{-1} = \left(\frac{[H]}{K_{a1} + [H]}\right)(k_f + k_r) \qquad (2)$$

Mechanism 3 was derived to take into account the formation of a strong hydrogen bond in the E'H conformation, which can reduce the rate of the ionization by several orders of magnitude so that it may be kinetically coupled to the

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Table I: Theoretical Relaxation Time Expressions for Enzyme Isomerizations

mechanism[a]	τ^{-1}_{exact}[d]	$\tau^{-1}_{approximate}$[b]
(A) single isomerization		
(1) $E' + H \overset{K_{a1}}{\rightleftharpoons} E'H \underset{k_r}{\overset{k_f}{\rightleftharpoons}}$ $EH \overset{K_{a2}}{\rightleftharpoons} E + H$	$\dfrac{k_f(\frac{[H]}{K_{a1}+\gamma})([E]^*+\gamma+K_{a2}) + k_r(\frac{[E]^*}{K_{a1}+\gamma}+1)}{[E]^*(\frac{[H]+K_{a1}}{K_{a1}+\gamma}) + K_{a2} + H}$ (where $\gamma = [E']^* + [H]$)	$k_r(\frac{[H]}{K_{a2}+[H]}) + k_f(\frac{[H]}{K_{a1}+[H]})$
(2) $E'H \underset{k_r}{\overset{k_f}{\rightleftharpoons}} EH = E + H$	$k_r(\frac{[E]^*+[H]}{[E]^*+[H]+K_a}) + k_f$	$k_r(\frac{[H]}{K_a+[H]}) + k_f$
(3) $E'H \underset{k_{1r}}{\overset{k_{1f}}{\rightleftharpoons}} EH$ $k_{2r} \diagdown\!\diagdown\, k_{2f}\ \ /\!/ K_a$ $E' + H$	$(k_{1r}+k_{2r}K_a)(\frac{[E]^*+[H]}{[E]^*+[H]+K_a}) + k_{1f} + k_{2f}$	$(k_{1r}+k_{2r}K_a)(\frac{[H]}{K_a+[H]}) + k_{1f} + k_{2f}$
(B) multiple isomerizations		
(4) $EH'' \overset{K}{=} E'H \underset{k_r}{\overset{k_f}{\rightleftharpoons}} EH \overset{K_a}{=} E + H$	$k_r(\frac{[H]+[E]^*}{[H]+[E]^*+K_a}) + k_f(\frac{K}{1+K})$	$k_r(\frac{[H]}{K_a+[H]}) + (\frac{K}{1+K})k_f$
(5) $E''H \underset{k_r}{\overset{k_f}{\rightleftharpoons}} E'H \overset{K}{=} EH \overset{K_a}{=} E + H$	$k_r(\frac{1}{K+1})[\dfrac{[H]+[E]^*}{[H]+[E]^*+K_a(\frac{K}{1+K})}] + k_f$	$k_r(\frac{1}{K+1})[\dfrac{[H]}{[H]+K_a(\frac{K}{1+K})}] + k_f$
(C) multiple proton transfers[c] noncooperative		
(6) $E'H_2 \underset{k_r}{\overset{k_f}{\rightleftharpoons}} EH_2 \overset{K_{a2}}{=} EH \overset{K_{a1}}{=} E$	$k_r\left[\dfrac{[H]^2+[H][EH]^*+4[H][E]^*}{[H]^2+[H][EH]^*+4[H][E]^*+K_{a2}([H]+[E]^*)+K_{a1}K_{a2}}\right] + k_f$	$k_r(\frac{[H]^2}{[H]^2+K_{a2}[H]+K_{a1}K_{a2}}) + k_f$
(7) $E'H_3 \underset{k_r}{\overset{k_f}{\rightleftharpoons}} EH_3 \overset{K_{a3}}{=} EH_2$ $\Vert K_{a2}$ EH $\Vert K_{a1}$ E	$k_r\left[\dfrac{[H]^3+[H]^2(9[E]^*+4[EH]^*+[EH_2]^*)}{[H]^3+[H]^2(9[E]^*+4[EH]^*+[EH_2]^*)+K_{a3}[[H]([EH]^*+4[E]^*)+K_{a3}[E]^*+[H]^2+K_{a2}[H]+K_{a1}K_{a2}}\right] + k_f$	$k_r\left[\dfrac{[H]^3}{[H]^3+K_{a3}([H]^2+K_{a2}[H]+K_{a1}K_{a2})}\right] + k_f$
cooperative		
(8) $E'H_2 \underset{k_r}{\overset{k_f}{\rightleftharpoons}} EH_2 \overset{K_a}{=} E + 2H$	$k_r(\frac{[H]^2+4[H][E]^*}{[H]^2+4[H][E]^*+K_a}) + k_f$	$k_r(\frac{[H]^2}{[H]^2+K_a}) + k_f$
(9) $E'H_3 \underset{k_r}{\overset{k_f}{\rightleftharpoons}} EH_3 \overset{K_a}{=} E + 3H$	$k_r(\frac{[H]^3+9[H]^2[E]^*}{[H]^3+9[H]^2[E]^*+K_a}) + k_f$	$k_r(\frac{[H]^3}{[H]^3+K_a}) + k_f$

[a] All mechanisms are coupled to the indicator equilibrium, $HIn \overset{K_{In}}{=} H + In$, and also to the self-ionization of water, $H_2O \overset{K_w}{=} H + OH$. [b] Reduction of the exact derivation for the assumption $\delta[H] \approx 0$ (or, what is numerically equivalent, $\alpha \to \infty$). [c] Protons released are not indicated, for simplicity. [d] The asterisk denotes that the expression is divided by $1 + \alpha + \beta$. The indicator buffering term $\alpha = [In]/(K_{In} + [H])$. The term $\beta = [OH]/(K_w + [H])$ results from the self-ionization of water.

isomerization. The concentration function for this mechanism is the same as that for mechanism 2. As a consequence, they are functionally indistinguishable. The observed rate constants are made up of contributions from both pathways: $k_r^{obsd} = k_{1r} + k_{2r}K_a$ and $k_f^{obsd} = k_{1f} + k_{2f}$.

The concentration and pH dependences of τ_r^{-1} for mechanism 1 are shown in Figure 1. At constant pK_a the shape of the pH curves is relatively independent of the value of K_e, where $K_e = k_f/k_r$. There are no values of K_e which produce a strong concentration dependence; the largest dependence is for $K_e = 1$ which yields only a 17% change in τ_r^{-1}. The effect of varying one of the pK_a values, the other parameters being constant, is much the same as that for K_e and is not shown. For example, with $K_{a1} = 10^{-7}$ and $K_e = 1$ there is little concentration dependence over a wide range of K_{a2} values. For $K_{a2} \geq 10^{-6}$ virtually complete concentration independence is found. Only when $K_{a2} < 10^{-8}$ does τ_r^{-1} vary significantly with concentration. Although the shapes of the pH profiles are quite dependent upon the value of K_{a2}, a large dependence of τ_r^{-1} upon pH is observed for any K_{a2}.

The pH curves for mechanisms 2 and 3 (Figure 2) are quite dependent upon the value of K_e. A significant concentration dependence is observed over a rather small range of K_e values ($10 > K_e > 10^4$). If $K_e \ll 1$, the process is independent of concentration; however, it retains its pH dependence. At values of $K_e \gg 1$ there is no pH or concentration dependence.

Compared to mechanisms 2 and 3, the additional ionization in mechanism 1 yields a smaller range of parameters which generate a concentration dependence. Where such a dependence is discernible, it is rather small (τ_r^{-1} increases only 10–20%). A strong concentration dependence occurs only when $K_{a2} \ll K_{a1}$, i.e., when the second ionization does not occur in the pH region under consideration. In this case mechanism 1 becomes indistinguishable from mechanisms 2 and 3.

Multiple isomerizations are exemplified by mechanisms 4 and 5. Introduction of a fast isomerization either preceding or following the slow step does not change the functional form of the expression for τ^{-1}. As a consequence, mechanisms 4 and 5 are indistinguishable from mechanisms 2 and 3. The measured rate constants are a combination of the true rate

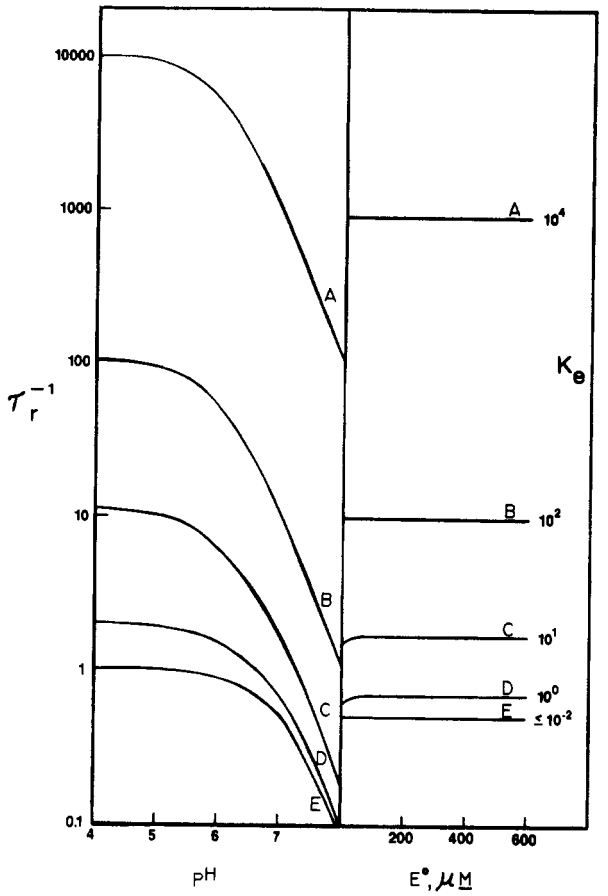


FIGURE 1: Calculated curves of $1/\tau_r$ as a function of pH ([E°] = 25 μM) and total enzyme concentration (at pH 7) for mechanism 1. For all curves $K_{a1} = 10^{-6}$, $K_{a2} = 10^{-7}$, $K_{In} = 10^{-6}$, and K_e ($=k_f/k_r$) is as indicated. Correspondingly lettered curves on the right and left are calculated with the same constants. The indicator concentration is 25 μM.

constant for the slow step and the equilibrium constant for the fast step. In addition, for mechanism 5 the observed K_a is a combination of the true K_a and the equilibrium constant for the fast step. These two mechanisms may be generalized to include any number of fast isomerizations in a linear reaction sequence. The observed values of the constants are given by the expressions

$$k_f^{obsd} = k_f\left(\frac{K}{1+K}\right),\ k_r^{obsd} = k_r,\ \text{and}\ K_a^{obsd} = K_a \qquad (3a)$$

and

$$k_f^{obsd} = k_f,\ k_r^{obsd} = k_r\left(\frac{K}{1+K}\right)\prod_{j=1}^{n}\frac{1}{K_j},\ \text{and}$$

$$K_a^{obsd} = K_a\left(\frac{K}{1+K}\right) \qquad (3b)$$

for mechanisms 4 and 5, respectively, where

$$\frac{1}{K} = \sum_{i=1}^{n}\left(\prod_{j=i}^{n}\frac{1}{K_j}\right)$$

n = total number of fast isomerizations, and K_j = equilibrium constant for the jth fast isomerization.

Multiple proton transfers may be divided into two classes, cooperative and noncooperative. The former does not allow for intermediate ionization states. Figure 3 shows the theoretical concentration dependences and pH dependences for the noncooperative mechanisms given in Table I. The shape of the pH curves is dependent upon the number of protons,

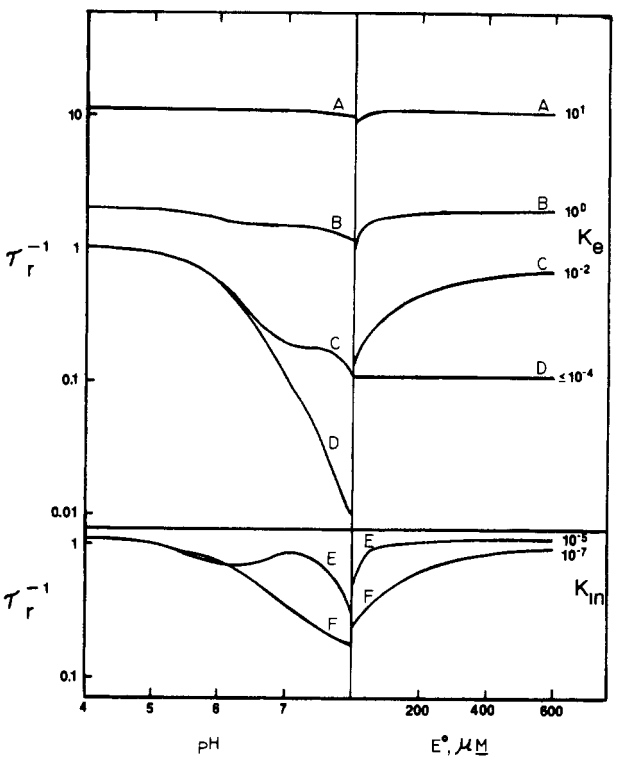


FIGURE 2: Calculated curves of $1/\tau_r$ as a function of pH ([E°] = 25 μM) and total enzyme concentration (at pH 7) for mechanisms 2–5. For curves A–D: $K_a = 10^{-6}$, $K_{In} = 10^{-6}$, and K_e is as indicated. For curves E and F: $K_a = 10^{-6}$, $K_e = 0.1$, and K_{In} is as indicated. The indicator concentration is 25 μM.

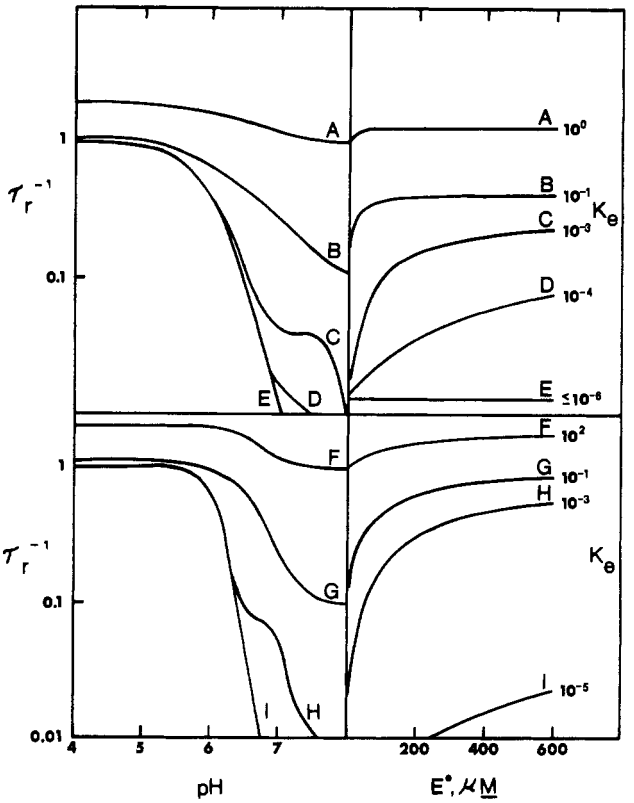
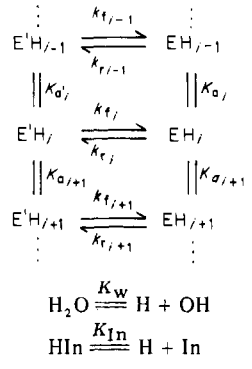


FIGURE 3: Calculated curves of $1/\tau_r$ as a function of pH ([E°] = 25 μM) and total enzyme concentration at pH 7 for mechanisms 6 (top) and 7 (bottom). For all curves $K_{ai} = 10^{-6}$, $K_{In} = 10^{-6}$, and K_e is as indicated. The indicator concentration is 25 μM.

with the overall steepness of the curve increasing with the number of proton transfers. For given values of the constants, the initial slope is steeper and the curves level off sooner with

Scheme II



concentration as the number of protons increases. Since the τ_r^{-1} profiles are almost identical for the cooperative and noncooperative cases, a graph of the former has not been included. For a specific set of the constants, however, the cooperative mechanisms generate a slightly steeper pH dependence and a stronger concentration dependence.

Indicator Dependence. Up to this point the effect of the indicator has not been considered. Since the indicator term α contains the K_{In} of the indicator, different indicators need not produce similar τ_r^{-1} values. Figure 2 shows the effect on the pH and concentration dependences of coupling the process to indicators of pK_{In} equal to 5 or 7 for mechanism 2. As the pK_{In} of the indicator increases, the steepness of the curve for $[E^0]$ decreases. The shapes of the pH profiles are quite dependent on the indicator. In one case the dependence is even reversed over a range of pH values. Plots of τ_r^{-1} vs. $[In^0]$ exhibit an *inverse* dependence on indicator since the indicator is coupled through only the proton balance.

General Treatment. So far, we have considered several specific mechanisms for enzyme isomerization coupled to proton transfer reactions. There are, of course, a large number of possible mechanisms other than those treated above. We have as a consequence developed a generalized mechanism (shown in Scheme II) in which each distinct ionizable form of the enzyme is permitted to undergo a conformational change with a unique rate constant. If the proton transfer reactions are considered to be rapidly established, a single (slow) relaxation time results. The kinetic equation is

$$\frac{-d}{dt}\left(\sum_{i=0}^{n}\delta[E'H_i]\right) = \sum_{i=0}^{n}\delta[E'H_i]k_{f_i} - \sum_{i=0}^{n}\delta[EH_i]k_{r_i} \quad (4)$$

Here δ signifies the difference between the instantaneous and equilibrium concentrations and n is the total number of protons. The species are related through mole balance equations

$$\sum_{i=0}^{n}\delta[E'H_i] + \sum_{i=0}^{n}\delta[EH_i] = 0 \quad (5)$$

$$\sum_{i=1}^{n} i\delta[E'H_i] + \sum_{i=1}^{n} i\delta[EH_i] + \delta[H] + \delta[HIn] + \delta[H_2O] = 0 \quad (6)$$

$$\delta[HIn] + \delta[In] = 0 \quad (7)$$

$$\delta[H_2O] + \delta[OH] = 0 \quad (8)$$

and ionization equilibria

$$K_{a_i} = \frac{[EH_{i-1}][H]}{[EH_i]} \qquad K_w = \frac{[H][OH]}{[H_2O]} \quad (9)$$

By means of these relationships, the number of species in the rate equation may be reduced to a single concentration variable. The resulting relaxation time is given by

$$\tau^{-1} = \{\sum[(A_i - [E'](FC1)B_i)k_{f_i} + ([E](FC1)D_i - (FC2)C_i)k_{r_i}] + k_{f_0} - (FC2)k_{r_0}\}/\{1 + \sum A_i - [E'](FC1)\sum B_i\} \quad (10)$$

where

$$A_i = [H]^i \prod_{j=1}^{i}\frac{1}{K'_{a\,j}} \qquad B_i = \frac{i}{[H]}A_i$$

$$C_i = [H^i]\prod_{j=1}^{i}\frac{1}{K_{a_j}} \qquad D_i = \frac{i}{[H]}C_i$$

and

$$FC1 = \{\sum iA_i + \sum iC_i(FC2)\}/\{\sum iB_i[E'] + \sum iD_i[E] + 1 + \alpha + \beta\}$$

$$FC2 = \{([E']\sum B_i + [E]\sum D_i)\sum iA_i - ([E']\sum iB_i + [E]\sum iD_i + 1 + \alpha + \beta)(1 + \sum A_i)\}/\{(\sum iB_i[E'] + \sum iD_i[E] + 1 + \alpha + \beta)(1 + \sum C_i) - ([E']\sum B_i + [E]\sum D_i)(\sum iC_i)\}$$

All summations are from $i = 1$ to $i = n$. The relaxation time expression (eq 10) may be used to calculate τ^{-1} for most of the mechanisms given in Table I by inserting appropriate values for the various rate and equilibrium constants. For example, one can generate the τ^{-1} expression for mechanism 2 by the following procedure: set $n = 1$ (1 proton ionization), and $K'_{a1} << K_{a1}$ and all k_{f_i} and $k_{r_i} = 0$ except for $i = 1$. By these operations eq 10 reduces algebraically to that shown in Table I for mechanism 2. It is clear that τ^{-1} expressions for a large number of mechanisms not shown in Table I may be generated by appropriate identification of the constants in eq 10.

Conclusions

We have demonstrated that relaxation times for enzyme isomerizations are not in principle independent of the enzyme and indicator concentrations. The experimental observations to date of concentration independence may be due to several factors. These are (1) the indicator term α dividing E (see Table I)[1] may have been large enough to mask any dependence, (2) any pH buffer present will generate a term analogous to α in the rate expression, (3) the values of the parameters may be such that no dependence is expected, (4) the experimental conditions may have been such as to not produce a noticeable dependence upon enzyme or indicator, and (5) $\delta[H]$ may indeed have been equal to zero.

Finally, a note of caution with regard to enzyme isomerization processes. Unambiguous interpretation of rate and equilibrium constants depends upon the ability to choose one particular mechanism over all others. Since mechanisms can be functionally equivalent, this often cannot be done without independent information concerning the process. One should therefore be very careful about assigning fundamental significance to these constants.

In the following paper (Feltch & Stuehr, 1979) we present detailed experimental data for the enzyme deoxyribonuclease I. This enzyme exhibits an isomerization process which has pH, enzyme concentration, and indicator concentration dependences as predicted by the theoretical considerations outlined in this paper.

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[1] E refers to any enzyme species appearing in the expressions for τ^{-1}.

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Relaxation Studies of Enzymes: Rapid Isomerization in Deoxyribonuclease I†

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ABSTRACT: Temperature-jump relaxation studies in deoxyribonuclease I were carried out at 10 °C and [I] = 0.1 M. The single observed relaxation time, which varied from 10^{-4} to 10^{-5} s, was characterized as a function of enzyme concentration, pH, and indicator concentration. The concentration and pH dependences of the relaxation time are in quantitative agreement with a mechanism involving an isomerization of the enzyme coupled to a rapid proton ionization process. The best fit forward and reverse isomerization rate constants are 6.5×10^3 and 7.2×10^4 s^{-1}, respectively; the apparent pK is 5.7. The addition of urea brought about reductions in both the amplitude of the relaxation effect and the enzyme activity.

Deoxyribonuclease I is an enzyme of molecular weight 31 000 that catalyzes the hydrolysis of native DNA to 5′-deoxyribonucleotides. Early work on the steady-state kinetics has done much to define the overall aspects of the reaction. The enzyme was found to be an endonuclease (Williams et al., 1961) having an absolute requirement for a divalent metal ion such as Mg^{2+} or Mn^{2+} (Wiberg, 1958). At least two phases of the reaction with native DNA have been recognized. The initial phase is characterized by both specificity (Weis et al., 1968) and the production of several single-stranded nicks before cleavage of the double strand (Dekker & Schachman, 1954). During the terminal phase there is little specificity (Laskowski, 1967) and autoretardation is observed (Vanecko & Laskowski, 1961). Both competitive product inhibition (Vanecko & Kaskowski, 1961) and decreasing affinity toward newly formed substrates (Vanecko & Laskowski, 1961; Cavalieri & Hatch, 1953) have been shown to be necessary to account for the latter phenomenon. Chemical modification experiments have implicated tyrosine (Hugli & Stein, 1971), serine (Poulos & Price, 1974), carboxyl (Poulos & Price, 1974), and histidine (Price et al., 1969) residues as essential to DNase activity. If indeed all of these groups are involved at the active site, the mechanism must be quite complex. To date, however, no detailed mechanistic scheme has been proposed. The required time sequence of events at the molecular level has not been characterized. We have therefore initiated a broad investigation to characterize as many rapid elementary steps as possible to help in the elucidation of the detailed mechanism. The principal investigative tool will be temperature-jump spectroscopy. The suitability of this technique for the study of such enzymatically important rapid processes as ionizations, configurational changes (isomerizations), association phenomena, enzyme–substrate binding, and enzyme–metal ion interactions is well documented (del Rosario & Hammes, 1970). This paper describes a rapid process involving the enzyme in the absence of substrates or cofactors.

Experimental Procedures

Reagents and Chemicals. Phosphocellulose was purchased from Accurate Chemical and Scientific Corp. Lima bean trypsin inhibitor and CNBr-activated Sepharose 4B were from Sigma Chemical Co. Bovine pancreatic deoxyribonuclease I was purchased from Sigma or Miles Laboratories. All other reagents were standard analytical grade. The indicators methyl red, bromthymol blue, and chlorophenol red were checked for purity by thin-layer chromatography, titration, and visible absorption spectra and found to be pure by these criteria. Urea was recrystallized from 70% ethanol.

Enzyme Purification. The crude enzyme was treated with phenylmethanesulfonyl fluoride to inactivate proteases. Typically, 1.0 g of protein was dissolved in 100 mL of 0.05 M Tris buffer, 5×10^{-3} M Ca^{2+}, pH 7.0, to which 10 mL of 0.02 M phenylmethanesulfonyl fluoride (in 95% ethanol) had been previously added. It was usually necessary to readjust the pH to 7.0 with 1.0 M base. The solution was allowed to stand at room temperature for 1 h, dialyzed vs. distilled water at 4 °C, and lyophilized. Some precipitation occurred after 1 h at room temperature and also after dialysis. This was removed by filtration through Whatman high-porosity fluted paper. Recovery of activity was >90%. The treated protein lost no activity after 24 h at room temperature, pH 7.0, in 0.05 M EDTA.

The phenylmethanesulfonyl fluoride treated enzyme was purified over a 2.5 × 70 cm column of phosphocellulose according to the procedure of Salnikow et al. (1970). The fractions containing enzyme activity were pooled, dialyzed vs. distilled water at 4 °C, and lyophilized. This material was further purified by affinity chromatography over a 2 × 25 cm lima bean trypsin inhibitor–agarose column according to the procedure of Otsuka & Price (1974). The purified DNase I was stored frozen in the buffer from the final column (0.02 M Tris and 5×10^{-3} M Ca^{2+}, pH 8.0) for up to several months without loss of activity.

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