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Binding of Succinate to Aspartate Trancarbamylase Catalytic Subunit. pH and Temperature Dependence of Nuclear Magnetic Resonance Relaxation Times†

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ABSTRACT: The interaction of the inhibitor succinate with the catalytic subunit of *Escherichia coli* aspartate transcarbamylase has been studied by a transient nuclear magnetic resonance method. In the presence of carbamyl phosphate both the succinate proton relaxation rate, $1/T_2$, and the temperature dependence of relaxation change markedly over the pH range 7–10. The data are accounted for by a mechanism featuring two protonated groups on the enzyme affecting succinate binding and titratable over the pH range studied. Four distinct enzyme species are included, one of which does not interact with succinate and does not contribute to the relaxation, two others, one singly protonated and the other doubly protonated, both of which form fast-exchange complexes with succinate, and the fourth, a conformational isomer of the doubly protonated species in which succinate is bound more tightly. The concentration of these species is pH dependent, and pK_a values of 6.9 and 8.2 are calculated for the two titratable groups at the active site. A rotational correlation time of 7×10^{-8} sec is calculated for the fast exchange enzyme–inhibitor complexes suggesting that succinate is bound through both carboxyl groups. Hence the lower pK_a group on the enzyme is probably distinct from the succinate carboxyl group binding sites, its protonation instead catalyzing the conformational change which leads to tighter binding of succinate. The equilibrium constant for the conformational change of the doubly protonated species is 8×10^{-2} with rate constants of 3.2×10^3 sec^{-1} for the forward step and 2.5×10^2 sec^{-1} for the reverse. The higher pK_a group appears to interact directly with succinate, and its deprotonation at higher pH leads to only very weak, if any, binding of the inhibitor.

Allosteric control of the reaction catalyzed by aspartate transcarbamylase from *Escherichia coli* can be fully understood only when the catalytic mechanism itself is known in detail. Toward this end Stark and coworkers have employed a variety of physical techniques in studying the isolated catalytic subunit and have proposed a mechanism for the enzyme-catalyzed condensation of L-aspartate and carbamyl phosphate to form carbamyl aspartate (Collins and Stark, 1969, 1971; Porter *et al.*, 1969; Schmidt *et al.*, 1969; Davies and Stark, 1970; Davies *et al.*, 1970). In this mechanism carbamyl phosphate binds first and a conformational change following aspartate binding serves to force the substrates together and promote the reaction (Collins and Stark, 1969).

Since the time of this proposal several studies have elaborated on aspects of the mechanism. In particular, transient nuclear magnetic resonance (nmr) (Sykes *et al.*, 1970) and temperature-jump methods (Hammes *et al.*, 1971) have been used to study the kinetics of binding of the inhibitor succinate to the catalytic subunit. At pH 7.0 in the presence of saturating carbamyl phosphate the nmr results led to the conclusion that a conformational change takes place when succinate binds and that this change is largely concerted with the binding. The temperature-jump experiments of the binding of succinate to the catalytic subunit were done at pH 7.4 and from these

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it was concluded that approximately 12% of the bound succinate exists in an intermediate complex while the rest is bound in a conformationally isomerized enzyme–inhibitor complex.

The apparent conflict between the nmr and temperature-jump results in part prompted the present study. Since the two methods were used at somewhat different pH values and different temperatures, it seemed that an nmr study of the pH dependence of succinate binding to the catalytic subunit–carbamyl phosphate complex might be revealing. In fact, the nmr results are highly pH dependent. The temperature dependence of succinate nuclear relaxation times as a function of pH has led to the development of a model for succinate binding involving two ionizable groups at the active site.

Experimental Section

Native aspartate transcarbamylase was prepared according to the method of Gerhart and Holoubek (1967). The catalytic subunit was separated from the regulatory subunit by the method of Gerhart and Holoubek (1967) as modified by Kirschner (1971). Neohydrin was used as the mercurial to separate the subunits, and a DEAE-cellulose column was used to isolate the catalytic subunit. Catalytic subunit concentration was determined by absorption at 280 nm assuming an extinction coefficient of 0.72 cm^2/mg (Gerhart and Holoubek, 1967) and a mol wt of 33,000 per catalytic site (Weber, 1968; Meighen *et al.*, 1970; Hammes *et al.*, 1970), and by radioactive assays following the method of Porter *et al.* (1969).

Samples of aspartate transcarbamylase catalytic subunit and inhibitor used in relaxation measurements were prepared by mixing appropriate volumes of four stock solutions: (1) catalytic subunit dialyzed in imidazole–borate–glycylglycine buffer of appropriate pH; (2) imidazole–borate–glycylglycine buffers of varying pH; (3) unbuffered sodium succinate at pH 7.5; and (4) dilithium carbamyl phosphate (Sigma Chemical Co.) in imidazole–borate–glycylglycine buffers of varying pH prepared immediately prior to use. All solutions were made in D_2O, and pH values are reported as meter readings uncorrected for the effect of the deuterium isotope. The pH of the buffer solutions was adjusted by addition of acetic acid-d_4 or NaOD. 2-Mercaptoethanol (2×10^{-3} M) was used in all the buffers to stabilize the enzyme. Care was taken in preparing the solutions to avoid contamination by paramagnetic ions. EDTA of approximately the same concentration as the enzyme, 2×10^{-4} M, was present in all the buffers, and was apparently adequate for complexing any contaminants since the transverse relaxation times measured did not indicate consistently short relaxations characteristic of protons in the presence of paramagnetic ions, and the longitudinal relaxation time, T_1, was long in all cases. No special purification procedures were used for the chemicals required in the nmr samples.

The final samples were approximately 1.7×10^{-4} M in catalytic subunit, 0.004 M in carbamyl phosphate, 0.05 M in succinate, 0.015 M in imidazole, 0.015 M in borate, 0.005 M in glycylglycine, 2×10^{-3} M in 2-mercaptoethanol, and 2×10^{-4} M in EDTA. The pH values of the samples were measured before and after the experiment was run, with differences between the two measurements ranging from 0 to 0.25 pH unit, and the values reported are the average of the two readings. Relaxation measurements were made immediately after preparation of the samples, which were stored on ice between measurements so that the total time the samples were above 0° was less than 1 hr in all cases.

The buffer system was found to be particularly critical for this experiment. A buffer or buffers were required to cover a range of pH of 7.0–10.0. The system chosen had to maintain a pH independent of temperature over the range studied (17–40°), and a constant pH for the duration of the experiment. This latter was a problem because decomposition of carbamyl phosphate to yield cyanate and phosphate causes a lowering of the pH and decomposition is accelerated at higher temperatures. In order to minimize these effects, the samples were kept on ice between nmr measurements. The buffer used could not inhibit the binding of the substrates to the enzyme to any appreciable extent. Imidazole buffers adequately in the range of pH 7.0–8.0. Glycylglycine buffers at higher pH but could not be used because cyanate, a decomposition product of carbamyl phosphate, attacked the glycylglycine, thus lowering the buffer capacity and the pH. Borate buffers very well at higher pH, and the pH change over temperature is negligible, but preliminary investigations suggested that borate inhibited the enzyme, and radioactive assay confirmed the inhibition to be as great as 20% at the concentration required to maintain a constant pH with time. The final buffer system combined the advantages of all three individual buffer systems. By using imidazole, borate, and glycylglycine, (1) the full pH range was available in a single buffer system, (2) inhibition of the enzyme was small and constant over the whole pH range (about 10%), (3) the enzyme was protected by glycylglycine from attack by cyanate, and (4) the pH remained relatively constant throughout the course of the experiment. The highest pH samples did drop in pH about 0.2–0.3 unit during the course of the experiment, as a result of carbamyl phosphate decomposition. For the lower pH samples the change was less. There remained a small dependence of the pH on temperature; experiment indicated that a buffer measuring pH 7.0 at room temperature actually ranged from pH 7.10 to 6.90 over the temperature range studied, and that a pH 9.55 buffer ranged from 9.50 to 9.60.

All longitudinal and transverse relaxation times, T_1 and T_2, were measured on the Varian HR-220 spectrometer (London, 1973) and the Varian HA-100 instrument, using the adiabatic half-passage or $T_{1\rho}$ method as developed by Sykes (1969). A fast, exponential sweep from about 400 Hz off resonance into the center of the succinate peak was used for all the measurements. Temperatures of the probes at 220 and 100 MHz were controlled by the standard Varian temperature control unit. Probe temperatures were measured before and after each temperature change by a thermometer and ethylene glycol chemical shifts for the 220-MHz instrument and by a thermocouple and ethylene glycol chemical shifts for the 100-MHz instrument. These methods gave the same results within ±1°. Samples were equilibrated to probe temperature in a water bath outside the spectrometer just prior to use. This step helped shorten the time required for temperature equilibration in the probe and thus helped minimize decomposition of carbamyl phosphate.

Results

Temperature and pH Dependence of Relaxation. Succinate relaxation times of solutions of aspartate transcarbamylase catalytic subunit containing carbamyl phosphate and succinate were measured at five temperatures between 16.5 and 38.5°. Seven samples were used ranging in pH from 7.0 to 10.0. T_2 and T_1 values were calculated by least-squares determinations from the nmr exponential decays obtained by the $T_{1\rho}$ method of Sykes (1969). The results are plotted in Figure 1 as

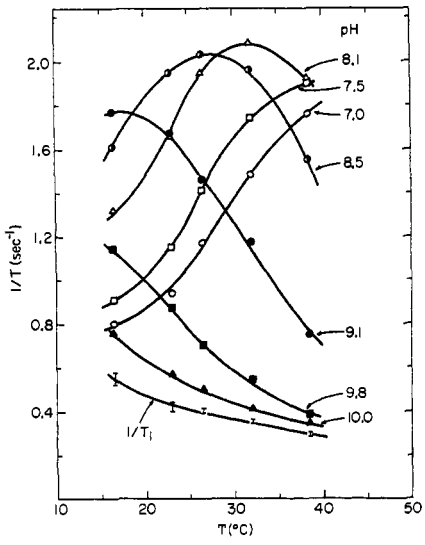

FIGURE 1: Reciprocal relaxation times $1/T_2$ and $1/T_1$ at 220 MHz for succinate in the presence of catalytic subunit and carbamyl phosphate as a function of temperature and pH. $1/T_2$ values: ○, pH 7.0; □, pH 7.5; △, pH 8.1; ◑, pH 8.5; ●, pH 9.1; ■, pH 9.8; ▲, pH 10.0. $1/T_1$ values are averaged over all the pH values and error bars indicate the average deviation. All samples are 1.7×10^{-4} M in catalytic subunit, 0.004 M in carbamyl phosphate, and 0.05 M in succinate.

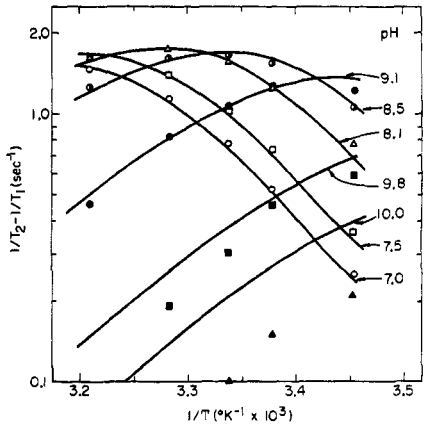

FIGURE 2: Logarithm of the difference in succinate relaxation rates $1/T_2 - 1/T_1$ at 220 MHz as a function of reciprocal temperature, experimental (points) and calculated (solid curves): ○, pH 7.0; □, pH 7.5; △, pH 8.1; ◑, pH 8.5; ●, pH 9.1; ■, pH 9.8; ▲, pH 10.0. Calculated curves are obtained from eq 9 and 13–17. Concentrations are as in Figure 1.

$1/T_2$ and $1/T_1$ *vs.* temperature. The values for $1/T_1$ were virtually unaffected by the addition of enzyme and were found to be independent of pH within the experimental error of $\pm 5\%$. Hence, $1/T_1$ values from all seven samples were averaged to give the single curve seen in Figure 1, with error bars indicating the average deviation from the value plotted. In addition, $1/T_1$ appears to change very little with temperature. $1/T_2$ on the other hand is markedly dependent on both pH and temperature. The samples from pH 7.0 to 8.1 show an increase in $1/T_2$ as temperature and pH increase, while the pH 9.1–10.0 samples show a decrease in $1/T_2$ with both temperature and pH. The pH 8.5 sample shows behavior intermediate between the low and high pH samples. In all cases, $1/T_2 > 1/T_1$, although at high pH and high temperature $1/T_2$ approaches closely the value of $1/T_1$.

In order to eliminate any effects on relaxation times due to changes in viscosity between samples at different temperatures, values for $1/T_1$ are subtracted from those for $1/T_2$ (Marshall *et al.*, 1972). The observed longitudinal relaxation rate $1/T_1 = 1/T_{1,I} + P_0/(T_{1,EI} + \tau)$ where $1/T_{1,I}$ and $1/T_{1,EI}$ are the relaxation rates for the inhibitor (succinate) free in solution (I) and bound to the enzyme (EI), respectively, τ is the mean lifetime of the enzyme–inhibitor complex, and P_0 is the fraction of inhibitor bound. For an enzyme as large as the catalytic subunit (mol wt 100,000) the rotational correlation time, τ_R, is sufficiently large that at $\omega_0 = 100$ or 220 MHz, the quantity $(\omega_0\tau_R)^2 \gg 1$ (Sykes *et al.*, 1970; Marshall *et al.*, 1972) and hence $1/T_{1,EI}$ is small. Since P_0 is also small, $1/T_1 \cong 1/T_{1,I} = 1/T_{2,I}$ where $1/T_{2,I}$ is the transverse relaxation rate of the free inhibitor. The value of interest in the following discussion, then, is $1/T_2 - 1/T_{2,I}$. A plot of $\ln(1/T_2 - 1/T_1)$ *vs.* inverse temperature is shown as the data *points* in Figure 2.

Analysis of Relaxation Data. Succinate is present in the samples free in solution or bound to the catalytic subunit, and the changes observed in the relaxation times of succinate as a function of temperature and pH are related to changes in the rates of exchange of succinate between these environments. The mathematical relationship developed by Swift and Connick (1962) for the exchange of protons between two environments, one (in this case succinate free in solution) much more highly populated than the other, is expressed as

$$\frac{1}{T_2} - \frac{1}{T_{2,I}} = \frac{P_0}{\tau} \times \frac{\dfrac{\tau}{T_{2,EI}}\left(1 + \dfrac{\tau}{T_{2,EI}}\right) + (\tau\Delta)^2}{\left(1 + \dfrac{\tau}{T_{2,EI}}\right)^2 + (\tau\Delta)^2} \tag{1}$$

assuming a simple bimolecular reaction

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \tag{2}$$

$P_0 = [EI]/[I]_0$, the fraction bound, where $[I]_0$ is the total concentration of inhibitor, $\tau = 1/k_{-1}$, Δ is the chemical-shift difference (in rads per second) between the bound and free species, and $T_{2,I}$ and $T_{2,EI}$ are the transverse relaxation times for free inhibitor and bound inhibitor, respectively.

A slow exchange limit arises when either

$$(\tau\Delta)^2 \gg \left[1 + \frac{\tau}{T_{2,EI}}\right]^2 \tag{3a}$$

or

$$\left[\frac{1}{T_{2,EI}}\right]^2 \gg \Delta^2, \left(\frac{1}{\tau}\right)^2 \tag{3b}$$

Under these conditions

$$\frac{1}{T_2} - \frac{1}{T_{2,I}} = \frac{P_0}{\tau} \tag{4}$$

If the inhibitor is exchanging rapidly with respect to the bound relaxation time, then

$$\frac{\tau}{T_{2,EI}} \ll 1 \tag{5}$$

and eq 1 becomes

$$\frac{1}{T_2} - \frac{1}{T_{2,I}} = \frac{P_0(\tau/T_{2,EI}) + (\tau\Delta)^2}{\tau \quad [1 + (\tau\Delta)^2]} \tag{6}$$

An additional simplification can be made to eq 6, provided that $(\tau\Delta)^2 \ll 1$. This leads to a fast exchange limit

$$\frac{1}{T_2} - \frac{1}{T_{2,I}} = P_0\left[\frac{1}{T_{2,EI}} + \tau\Delta^2\right] \tag{7}$$

As discussed by Sykes *et al.* (1970), the slow and fast exchange limits given in eq 4 and 7, respectively, may be distinguished by the temperature dependence of $1/T_2 - 1/T_{2,I}$. The difference decreases logarithmically with inverse temperature in the slow exchange limit as a result of the Arrhenius expression relating $1/\tau$ to the reciprocal of absolute temperature. An increase in $1/T_2 - 1/T_{2,I}$ with inverse temperature suggests the fast exchange limit, since both $1/T_{2,EI}$ and $\tau\Delta^2$ of eq 7 are directly proportional to $1/T$.

However, neither the fast nor the slow exchange limit of eq 1 is sufficient by itself to account for the marked pH dependence of relaxation.

The data of Figure 2 suggest that at pH 7.0 succinate relaxes according to the slow exchange limit, and, at higher pH, succinate follows a fast exchange limit. A possible interpretation for these observations is that there are two different forms for the succinate–catalytic subunit–carbamyl phosphate complex, in addition to the succinate free in solution. A simple relationship for $1/T_2 - 1/T_{2,I}$ is derived in Appendix A for a mechanism such as

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} EI \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} (EI)' \tag{8}$$

where there is a rapid initial equilibrium to form EI followed by a slow step yielding (EI)′. The result is

$$\frac{1}{T_2} - \frac{1}{T_{2,I}} = P_{EI}\left[\frac{1}{T_{2,EI}} + \tau_{EI}\Delta^2\right] + \frac{P_{(EI)'}}{\tau_{(EI)'} + T_{2,(EI)'}} \tag{9}$$

where $T_{2,EI}$ is the transverse relaxation time of succinate in the EI complex, $\tau_{EI} = 1/k_{-1}$, Δ is the difference in rads per second between the resonance frequency of free succinate and the effective frequency of the pure EI species, $\tau_{(EI)'} = 1/k_{-2}$, $T_{2,(EI)'}$ is the transverse relaxation time of the (EI)′ complex, and P_{EI} and $P_{EI'}$ are the fractional populations.

Mechanism of Succinate Binding. While eq 9 contains both the fast exchange and slow exchange terms required by the data of Figure 2, it does not explicitly provide for the observed pH dependence of relaxation. However, by making P_{EI} and $P_{(EI)'}$ functions of pH, the data may be fit. In the following section a mechanism is proposed which allows for the development of expressions for the fractional populations such that eq 9 fits both the pH and temperature dependence of succinate relaxation.

MODEL INVOLVING ONE TITRATABLE GROUP. A simple model which might account for the pH dependence of relaxation includes one group at the active site titratable over the range of pH studied

$$\begin{array}{ccc} E & \overset{H^+}{\rightleftarrows} & EH \\ {\scriptstyle k_{-1}}\uparrow\downarrow{\scriptstyle k_1 I} & & {\scriptstyle k_{-2}}\uparrow\downarrow{\scriptstyle k_2 I} \\ EI & \overset{H^+}{\rightleftarrows} & EHI \\ (EI)' & & \end{array} \tag{10}$$

where EHI corresponds to (EI)′ in eq 8.

At low pH and at saturating levels of succinate, a tight complex of succinate with the protonated enzyme, EHI, would predominate, exhibiting slow exchange behavior with τ_{EHI} as the lifetime of the EHI complex. At high pH, the enzyme would be unprotonated, the EI species would predominate, and the succinate would exchange off the enzyme more rapidly. Relaxation of succinate would be described by eq 9 with EI representing the complex of succinate and unprotonated enzyme, and (EI)′ the complex with the protonated species. The K_a of the ionizable group on the enzyme would determine the relative populations of these complexes as a function of pH.

Qualitatively this model completely fails to predict the drastic decrease in $1/T_2 - 1/T_1$ at high pH. Quantitatively the fit of experimental data to calculated curves based on eq 9 is exceedingly poor. Using the pK_a, $[(1/T_{2,EI}) + \tau_{EI}\Delta^2]$, and $[\tau_{(EHI)'} + T_{2,(EHI)'}]^{-1}$ as adjustable parameters no set of values could be found which even approximated the data.

MODEL INVOLVING TWO TITRATABLE GROUPS. The active site contains several positively charged groups (Collins and Stark, 1969), and we propose that, in the presence of succinate and carbamyl phosphate, two of these groups are titratable in the range of pH 7.0–10.0 resulting in the possibility for three different enzyme species, EH_2, EH, and E.[1] Succinate binds strongly to the EH_2 species forming a slow exchange complex; the relaxation of the inhibitor is then dominated by the lifetime of the complex according to the second term in eq 9. Interaction between succinate and the singly protonated EH species is weaker, resulting in fast exchange, and relaxation of the EHI species is characterized by $[1/T_{2,EHI} + \tau_{EHI}\Delta^2]$. Succinate binds poorly if at all to the completely deprotonated species, E, and not to an extent observable by nmr.

The binding mechanism proposed is

$$\begin{array}{ccccc} E & \overset{H^+}{\rightleftarrows} & EH & \overset{H^+}{\rightleftarrows} & EH_2 \\ & & {\scriptstyle k_{-1}}\uparrow\downarrow{\scriptstyle k_1 I} & & \uparrow\downarrow{\scriptstyle I} \\ & & EHI & \overset{H^+}{\rightleftarrows} & EH_2I \end{array} \tag{11}$$

There is strong evidence from a number of studies which indicates that, in the presence of carbamyl phosphate and at a pH of about 7, a conformational change accompanies succinate binding (Jacobson and Stark, 1973). Therefore, a conformational isomer $(EH_2I)'$ is added as an important part of the mechanism proposed here, although its existence has not been determined directly in the present study. Equation 11 is then modified to give the complete model.

$$\begin{array}{ccccc} E & \overset{H^+}{\rightleftarrows} & EH & \overset{H^+}{\rightleftarrows} & EH_2 \\ & & {\scriptstyle k_{-1}}\uparrow\downarrow{\scriptstyle k_1 I} & & \uparrow\downarrow{\scriptstyle I} \\ & & EHI & \overset{H^+}{\rightleftarrows} & EH_2I \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} (EH_2I)' \end{array} \tag{12}$$

[1] Positively charged species which persist over the pH range or which do not influence succinate binding are not explicitly included in the notation.

A calculation of the fractional populations of the individual enzyme–substrate complexes (see below) indicates that the equilibrium constant for the conformational change strongly favors the $(EH_2I)'$ species. The slow exchange behavior observed in the nmr is attributed to this species. $(EH_2I)'$ then corresponds to $(EI)'$ of eq 8, and eq 9 predicts that the relaxation of succinate in this complex is proportional to $[\tau_{(EH_2I)'} + T_{2,(EH_2I)'})^{-1}$. In light of the results discussed later, it now seems apparent that the EH_2I species relaxes identically with the EHI species, according to the fast exchange limit. However, due to the relatively small fractional population of the former species, it has been neglected in the final calculations and the resulting error is estimated to be no more than 5%. The EHI species, then, corresponds to EI in eq 8.

Qualitatively, this model accounts for the features observed in Figure 2. The value of $1/T_2 - 1/T_1$ as a function of inverse temperature for the low pH samples exhibits typical slow exchange characteristics as a result of the predominance of a fully protonated species. The increase in $1/T_2 - 1/T_1$ with pH between 7.0 and 8.5 is explained by the larger contribution to the relaxation effect from the binding of succinate to the increasing amount of EH species. As pH increases above 8.5, the value of $1/T_2 - 1/T_1$ shows a decrease due to an increase in concentration of the unprotonated enzyme species, E, a decrease in concentration of the enzyme sites to which the inhibitor can bind, and hence a loss of the relaxation mechanism provided by the binding process. This second model, in contrast to the first, predicts the large decrease in relaxation effect at high pH.

In order to obtain quantitative results from the proposed mechanism given in eq 12, it is necessary to obtain equations for calculating fractional populations of the species observed by nmr relaxation, calculate values for $1/T_2 - 1/T_{2,I}$ according to eq 9 for different sets of adjustable parameters, and, by comparing with experimental values of $1/T_2 - 1/T_1$, choose the set of parameters which gives the best fit.

Values for $[\tau_{(EH_2I)'} + T_{2,(EH_2I)'}]^{-1}$ and $[1/T_{2,EHI} + \tau_{EHI}\Delta^2]$ as functions of temperature, required by eq 9 for the calculation of $1/T_2 - 1/T_{2,I}$, were chosen on the basis of the experimental curves in Figure 2 and the mechanism proposed. The lowest pH sample consists predominantly of the $(EH_2I)'$ species and the curve of $P_0[\tau_{(EH_2I)'} + T_{2,(EH_2I)'}]^{-1}$ *vs.* inverse temperature is similar to the data for $1/T_2 - 1/T_1$ at pH 7.0. The fast exchange relaxation curve was more difficult to estimate. However, the goodness of fit of the data is quite sensitive to its value, and it was varied by trial and error until a best fit with reasonable values for the other parameters was obtained. The values for $[1/T_{2,EHI} + \tau_{EHI}\Delta^2]$ yielding a best fit vary from 600 to 900 sec^{-1} over the range of inverse temperature studied.

Appendix B contains a derivation of the equations needed to calculate fractional populations of the relaxing enzyme species. The final equations used are

$$\mathcal{P}_E = \frac{K_{a1}K_{EH}K_{con}}{D} \quad (13)$$

$$\mathcal{P}_{EHI} = \frac{K_{a1}K_{con}[H^+]}{D} \quad (14)$$

$$\mathcal{P}_{EH_2I} = \frac{K_{con}[H^+]^2}{D} \quad (15)$$

and

$$\mathcal{P}_{(EH_2I)'} = \frac{[H^+]}{D} \quad (16)$$

where

$$D = K_{a1}K_{EH}K_{con} + K_{a1}K_{con}[H^+] + (K_{con} + 1)[H^+]^2 \quad (17)$$

In these equations, $\mathcal{P}_E = [E]/[E]_0$ and similarly for the other $\mathcal{P}$ terms, $K_{a1} = [EHI][H]/[EH_2I]$, $K_{con} = [EH_2I]/[(EH_2I)']$, and $K_{EH} = K_{a2}K_{EHI}/[I]$, where $K_{a2} = [E][H]/[EH]$ and $K_{EHI} = [EH][I]/[EHI]$. The three equilibrium constants of primary interest are the two ionization constants, K_{a1} and K_{a2}, and the equilibrium constant for the conformational change, K_{con}.

The calculations given in eq 13–17 require the optimization of several parameters. The three equilibrium constants K_{a1}, K_{EH}, and K_{con} (or equivalently, pK_{a1}, pK_{EH}, and pK_{con}) must be assigned for a given temperature, and three ΔH values corresponding to the three pK values must be assigned to allow for calculation of equilibrium constants at other temperatures. It was found that the calculated values for $1/T_2 - 1/T_{2,I}$ for the pH 9.1, 9.8, and 10.0 samples are independent of the parameters pK_{a1}, pK_{con}, and the corresponding ΔH_1 and ΔH_{con}. Consequently, only the lower pH samples were used to optimize these parameters. The optimization was accomplished by an iteration and error calculation computer program. Values of pK_{a1} were varied from about 6.4 to 7.4, pK_{con} was varied from 0.8 to 1.4, and the ΔH values ranged from about 6 to 18 kcal/mol. For each set of parameters, $1/T_2 - 1/T_{2,I}$ was calculated from eq 9 and 13–17 for six temperatures and four pH values. The differences between the calculated values and the experimental values were squared, divided by the experimental values, and summed over all temperatures and pH values. The set of parameters having the smallest summed error was chosen as giving the best fit. The best fit values for these two equilibrium constants and the two ΔH values were then used in optimizing pK_{EH} and ΔH_{EH} by the same method, this time using six pH values ranging from 7.0 to 9.8. The error program was insensitive to pK values to an extent of about ± 0.2 pH unit, while values for ΔH could be varied by about ± 3 kcal/mol without affecting the fit. The final set of parameters is given in Table I, for 28°.

The parameters giving the smallest error were used to calculate the values for the fractional populations of the different enzyme species as a function of pH and temperature. The variation of these species with pH at 28° is given in Figure 3a, and the variation with temperature at pH 7.0 is presented in Figure 3b. It is clear from this figure that the relative concentration of EH_2I is small over the whole pH and temperature range. Thus, neglecting its contribution to the relaxation is justified as mentioned above.

The set of six parameters listed in Table I and the fast and slow exchange relaxation curves were then used to calculate $1/T_2 - 1/T_{2,I}$ from eq 9 for different pH values as a function of temperature. The results are presented as the smooth curves in Figure 2, along with the experimental points. As can be seen from the figure, the agreement is very good for all but the pH 9.8 and 10.0 samples.[2]

[2] Possible reasons for the poor fit at very high pH include a further pH-dependent conformational change, mutual interaction of high pK titratable groups, or titration of the carbamyl phosphate binding site.

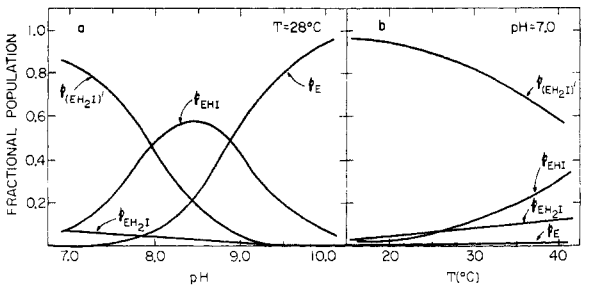

FIGURE 3: Fractional populations of enzyme species E, EHI, EH_2I, and $(EH_2I)'$: (A) as a function of pH at 28° and (B) as a function of temperature at pH 7.0. Values of $\mathcal{P}$ are given as the ratios of the concentrations of particular enzyme species to the total enzyme concentration and are calculated from eq 13–17 using the best fit parameters from Table I.

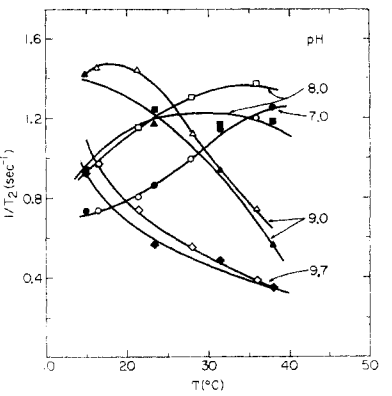

FIGURE 4: Frequency dependence of reciprocal relaxation time $1/T_2$ as a function of temperature for succinate in the presence of catalytic subunit and carbamyl phosphate: 220 MHz, ○, pH 7.0; □, pH 8.0; △, pH 9.0; ◇, pH 9.7; 100 MHz, ●, pH 7.0; ■, pH 8.0; ▲, pH 9.0; ◆, pH 9.7. Concentrations are approximately as in Figure 1.

It should be pointed out that an equally good fit to the data points can be obtained (with somewhat different parameters) assuming a mechanism like that of eq 11 where succinate is involved only in bimolecular interactions with the enzyme. However, as noted above, a variety of physical and chemical studies provide compelling evidence that a conformational change accompanies the binding of succinate to the catalytic subunit–carbamyl phosphate complex (Jacobson and Stark, 1973). Thus, the conformational isomer $(EH_2I)'$ has been explicitly included in the calculations.

Frequency Dependence of Relaxation. The relaxation rate (eq 9) may in general be dependent on the nmr frequency through Δ, $T_{2,EI}$ and $T_{2,(EI)'}$. Therefore, succinate relaxation times were measured at 100 as well as 220 MHz to determine any frequency dependence in the system under investigation. Because of the long correlation time for rotation of the complex, arguments similar to those given above for $T_{1,EI}$ lead to the conclusion that $T_{2,(EI)'}$ and $T_{2,EI}$ are not frequency dependent between 100 and 220 MHz (Marshall *et al.*, 1972). Any frequency dependence observed is thus due to the $\tau_{EI}\Delta^2$ term. Four different pH samples were measured by the $T_{1\rho}$ method at four temperatures. The results are presented in Figure 4, a plot of $1/T_2$ *vs.* temperature for the different samples at both frequencies. The $1/T_1$ values were found to be approximately the same as those in Figure 1, independent of pH as before, and independent of frequency within the experimental error of ±10%, so these values have not been included in the figure. The pH 7.0 sample shows no frequency dependence, in agreement with previous results (Sykes *et al.*, 1970) at 60 and 100 MHz. The $1/T_2$ values for the pH 8.0 samples at higher temperatures and for the pH 9.0 sample at lower temperatures show a relatively small frequency dependence. The apparent frequency dependence for the pH 9.7 sample is probably due to a difference in pH. This sample was observed to drop about 0.3 pH unit during the course of the experiment, in which the 100-MHz results were obtained first. Thus, the increase in $1/T_2$ values at 220 MHz is attributable to a lower pH for the sample.

Nuclear Relaxation Times of Bound Succinate. The fast exchanging complex between succinate and the enzyme (EHI) is described by $[1/T_{2,EHI} + \tau_{EHI}\Delta^2]$, which was determined for 220 MHz as a function of inverse temperature as discussed previously. The frequency dependence of relaxation (Figure 4) provides the data needed to approximate $\tau_{EHI}\Delta^2$ and hence $1/T_{2,EHI}$. Using the values calculated for the fractional population of EHI at pH 9.0 and 28° (Figure 3a), then

$$(1/T_2 - 1/T_1)_{220\ MHz} - (1/T_2 - 1/T_1)_{100\ MHz} = 0.08 = \mathcal{P}_{EHI}P_0(0.793\tau_{EHI}\Delta^2) \quad (18)$$

where Δ is the chemical-shift difference at 220 MHz.

From eq 18, at 28° $\tau_{EHI}\Delta^2 = 70$ sec^{-1}. This value must be regarded as approximate because of the small frequency dependence measured and because of some scatter in the data at 100 MHz. However, the result clearly indicates that exchange contributes only a small fraction to the total relaxation effect observed for EHI since at 28°, $[1/T_{2,EHI} + \tau_{EHI}\Delta^2] = 800$ sec^{-1}. Thus, $1/T_{2,EHI} \cong 730$ sec^{-1}.

While a small frequency dependence is measured for the fast exchange complex, none is found for the slow exchange complex, $(EH_2I)'$. However, the curvature of $[\tau_{(EH_2I)'} + T_{2,(EH_2I)'}]^{-1}$ as a function of inverse temperature suggests that $T_{2,(EH_2I)'}$ contributes to the relaxation at high temperatures. There is insufficient data for an exact calculation but limits can be placed on the value of $\tau_{(EH_2I)'}$. The upper limit is found if, at low temperature, the contribution from $T_{2,(EH_2I)'}$ is assumed negligible. Then, from a linear extrapolation to

TABLE I: Best Fit Parameters K_{al}, K_{EH}, and K_{con} at 28°, Corresponding ΔH Values for the Mechanism in Equation 11, and Rate Constants at 28° from Nmr and Temperature-Jump[a] Studies.

	Equilibrium Constant	ΔH (kcal/mol)
K_{al}	1.2×10^{-7}	12
K_{EH}	1.2×10^{-9}	12
K_{con}	7.9×10^{-2}	14
	Present Study[b]	**Temp-Jump Study[c]**
k_{-1} (sec^{-1})		1.4×10^4
k_1 (M^{-1} sec^{-1})		1.6×10^6
k_{-2} (sec^{-1})	2.5×10^{2} [d]	6.2×10^2
k_2 (sec^{-1})	3.2×10^{3} [e]	4.6×10^3

[a] Taken from Hammes *et al.* (1971). [b] Rate constants refer to those in eq 12. [c] Rate constants refer to those in eq 8. [d] Calculated from an average value of $\tau_{(EH_2I)'} = 4 \times 10^{-3}$ sec. [e] Determined from the relationship $K_{con} = k_{-2}/k_2$.

higher temperatures, $\tau_{(EH_2I)'} = 4.5 \times 10^{-3}$ sec at 28°. The lower limit is obtained by assuming that $T_{2,(EH_2I)'} \leq T_{2,EHI}$ in which case the lifetime is 3.7×10^{-3} sec. $T_{2,(EH_2I)'}$ should not be greater than $T_{2,EHI}$ because $(EH_2I)'$ represents a sterically restricted complex whose mobility, if anything, should be less than the EHI complex, and the transverse relaxation time is, in this case, directly proportional to the degree of motional freedom of the bound small molecule (Marshall *et al.*, 1972).

Discussion

Acid Dissociation Constants. The equilibrium constant for the first protonation can be related to another study with the catalytic subunit. Porter *et al.* (1969) found evidence for an ionizable group at the active site from the pH dependence of succinate inhibition in steady-state kinetic experiments. A pK_a of 7.1 was determined for the protonation of this group. Our value of $pK_{a1} = 6.9$ agrees closely with the results of Porter *et al.*; however, it must be pointed out that pK_{a1} involves protonation of a complex of enzyme and succinate, while the value from steady-state kinetics is for protonation of the uncomplexed catalytic subunit.

The presence of deuterium in the nmr studies must be considered in dealing with values for pK_a values. If all the calculations were done using the true pD rather than the meter reading, the pK values would increase by about 0.4. But Stark (1971) points out that the pK values of weak acids increase 0.5 to 0.7 pH unit in D_2O solution, and Sachs *et al.* (1971) have shown that the histidine imidazole ring pK is 0.4 pH unit higher in D_2O than in H_2O, almost exactly cancelling the glass electrode correction. Hence, the pK values and consequently the rate constants reported here are closely comparable to those in H_2O.

A pK_a of 7 suggests that a histidine residue may be involved in the binding of succinate (Porter *et al.*, 1969; Jacobson and Stark, 1973). As discussed below our nuclear relaxation data allow us to elaborate on the role this protonation plays in promoting succinate binding and an accompanying conformational change.

As noted in the Results section and Appendix B, the second equilibrium constant K_{EH} (1.2×10^{-9} M), which characterizes the $E \rightleftarrows EH \rightleftarrows EHI$ equilibrium, is actually the product of two dissociation constants divided by the succinate concentration (5×10^{-2} M), and hence $K_{a2}K_{EHI} = 6 \times 10^{-11}$ M. We cannot evaluate either of these individual dissociation constants solely on the basis of the experimental data presented here, but approximate values can be calculated if we use results from the temperature-jump study of Hammes *et al.* (1971). Their data are consistent with a mechanism like that of eq 8 and their calculated value of k_{-1}/k_1 is 8.7×10^{-3} M. As discussed below, the results of the present study are consistent in other respects with those of Hammes *et al.*, so it is probable that k_{-1}/k_1 corresponds to K_{EHI}.[3] Then K_{a2}, the equilibrium constant for protonation of E, is 7×10^{-9} or $pK_{a2} = 8.2$.[4] This value suggests the N-terminal amino group. However, the involvement of that residue in succinate (or aspartate) binding has not yet been demonstrated, and a pK_a in this range may be due to one of a number of amino acid groups in nonstandard environments.

Enthalpies. Several values of enthalpy changes were determined for the different steps in the mechanism proposed. The calculations of ΔH_{con} and ΔH_1 were coupled in such a way that, provided the sum of the two remained constant, each could vary by as much as about 4 kcal without significantly affecting the fit to the data. The value of ΔH_{con} given in Table I (14 kcal/mol) is reasonable considering that the step is a conformational change at the active site. ΔH_1 with a value of 12 kcal/mol seems too high for the ionization of an amino acid residue on a protein. Tanford (1962) suggests a value of 7–8 kcal/mol for the ΔH of ionization of imidazole rings in proteins, for example. However, the difference here may be explained by the fact that if the enzyme as a whole changes in net charge with temperature, at a constant pH, then the pK of an ionizable group will change due to electrostatic interactions, even if the ΔH for ionization is zero (Parsons and Raftery, 1972b). ΔH_{EH} (12 kcal/mol) is actually the sum of two ΔH values, so only broad limits can be determined from its value.

Comparison of Nmr and Temperature-Jump Results. It is instructive to compare the binding model and rate constants determined from the present study (eq 12) with those from the temperature-jump work of Hammes *et al.* (1971) (eq 8). The major difference is that while the protonation steps are explicitly included in the nmr model, they are only implicit in the temperature-jump model.

For a direct comparison of the nmr and temperature-jump results the intermediates of eq 12 must be identified with those of eq 8. $(EH_2I)'$ in eq 12 clearly corresponds to the isomerized state in the temperature-jump model, $(EI)'$. The combined intermediates EHI and EH_2I can be identified with the fast exchange, initial complex, EI, of the temperature-jump model. As discussed below, EHI and EH_2I probably represent the same binding configuration of succinate. The E species of eq 12 has no counterpart in the temperature-jump model.

The rate constants in eq 12 can also be related to those in eq 8, given the correspondence between the intermediates of the two mechanisms. Equating k_{-1}/k_1 to K_{EHI} as mentioned previously is now justified since EHI is identified with the initial complex EI. If k_2 and k_{-2} from eq 8 are called k_{2H} and k_{-2H} to distinguish them from those in eq 12, simple kinetics yields the relationships $k_{-2H} = k_{-2}$ and $k_{2H} = k_2[H^+]/K_{a1}$, assuming the protonation step is fast relative to the conformational change, and hence $k_{-2H}/k_{2H} = K_{con}K_{a1}/[H^+]$.

Comparison of rate constants from the present study and that of Hammes *et al.* is possible using the results in Table I. The two values for k_{-2} which correspond directly in the two mechanisms are in fair agreement. If k_{2H} is calculated from k_2, K_{a1}, and $[H^+]$ at pH 7.4, the result of 1.1×10^3 sec^{-1} is similarly fairly close to 4.6×10^3 sec^{-1} from the temperature-jump results. The ratios of k_{-2H}/k_{2H}, 0.23 from the present study and 0.13 from the temperature-jump work, compare reasonably well between the two studies.

Several fundamental differences between nmr and temperature-jump relaxation measurements make it somewhat hazardous to compare the methods quantitatively. In particular, in the work of Hammes *et al.* only steps coupled to rapid protonations of the enzyme were observed following a temperature jump. The nmr method, on the other hand, is sensitive to any significant change in the chemical environment of nuclei of the inhibitor whether hydrogen ions are released or not. Therefore, only qualitative correspondence between results of

[3] In this case, $K_{EHI}/I = 0.17$, which implies that about 85% of the singly protonated species is complexed with succinate. Thus a small error may be introduced into the calculations of Appendix B in which it was assumed that the concentration of EH was negligible compared to EHI.

[4] It might be expected that, with another residue at the active site having a pK_a just 1.2 units less, the two ionizing groups would interact yielding quite different results (Parsons and Raftery, 1972a). However, the two groups could well be sufficiently distant that little interaction would take place.

the two methods might be expected. Considering this, the agreement seen is quite satisfactory.

A previous nmr study of succinate binding to the catalytic subunit as a function of temperature, at pH 7.0 only, yielded a result of $k_{-2} = 1.7 \times 10^2$ sec^{-1} at 33° (Sykes *et al.*, 1970), a value somewhat smaller than that found here. The data were analyzed only in terms of slow exchange since the Arrhenius plot of ln $(1/T_2 - 1/T_1)$ was very nearly linear. The linearity of the Arrhenius plot at low temperature also prompted Sykes *et al.* to conclude that at pH 7.0 less that 2% of the bound succinate is in an intermediate fast exchange complex. This conclusion is borne out in the present study but only for low temperatures at pH 7.0 (Figure 3b). At a temperature of 28° and at pH 7.0, on the other hand, about 16% of bound succinate is in the intermediate complexes of EHI or EH_2I.

Hammes *et al.* in their temperature-jump study at pH 7.4 found that about 12% of the bound succinate consisted of a fast exchange intermediate EI while 88% was in the isomerized state (EI)′. From Figure 3a approximately 75% of the bound succinate is in the $(EH_2I)'$ complex at pH 7.4 and 28°. Considering that the concentration of intermediates is highly pH dependent and in the temperature-jump studies the pH was somewhat uncertain because no buffer could be used the agreement between the two studies is good.

Chemical Description of Intermediates. A unique characteristic of nuclear relaxation binding studies is that they can provide information about the chemical environment of bound molecules as well as exchange rates and equilibrium constants. Succinate protons in the EHI complex have a nuclear relaxation rate $1/T_{2,EHI} = 730$ sec^{-1}. Using the expression for intramolecular dipole–dipole dominated relaxation we can calculate an approximate rotational correlation time (τ_R) of the complex and compare it with that expected for the whole enzyme (Marshall *et al.*, 1972) (eq 19)

$$\frac{1}{T_{2,EHI}} = \frac{3}{40}\sum_{i,j}\frac{\hbar^2\gamma^4}{r_{ij}^6}\left[6\tau_R + \frac{10\tau_R}{1+(\omega_0\tau_R)^2} + \frac{4\tau_R}{1+4(\omega_0\tau_R)^2}\right] \tag{19}$$

Here γ is the magnetogyric ratio for protons, ω_0 is the nmr frequency in rads per second, and r_{ij} is the internuclear distance between protons on succinate with its carboxyl groups cis (Porter *et al.*, 1969). The result is $\tau_R = 7 \times 10^{-8}$ sec which is close (certainly within a factor of 2) to what is expected for the overall reorientation correlation time of a molecule the size of the catalytic subunit. Succinate is therefore held quite tightly in the EHI complex. If succinate were bound through only *one* of its carboxyl groups in the EHI complex, the relaxation $(1/T_{2,EHI})$ effect would be much smaller because of the several degrees of rotational freedom available. It therefore seems likely that succinate is bound through both its carboxyl groups in the EHI complex.

If succinate is already bound quite firmly in the EHI complex, then EH_2I is probably not significantly different structurally from EHI. We suggest that the addition of another proton to the enzyme takes place at a site not directly involved in electrostatic interaction with succinate, and is probably responsible for promoting the conformational change to $(EH_2I)'$. An attractive candidate for the conformational change promoter is a group which interacts with the carbonyl group of carbamyl phosphate (Collins and Stark, 1969). With inorganic phosphate in place of carbamyl phosphate, succinate exchanges rapidly from the catalytic subunit at pH 7.0 (Sykes *et al.*, 1970), and the temperature effect is much like that seen with carbamyl phosphate around pH 9 in the present study. This observation and others from ultraviolet (uv) difference spectroscopy (Collins and Stark, 1969) and nmr studies (Schmidt *et al.*, 1969) suggest that carbamyl phosphate raises the pK of the titratable group.

Appendix A

An expression can be developed for the transverse relaxation rate of nuclei on an inhibitor for the case

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} EI \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} (EI)' \tag{A1}$$

in which the frequency dependence of the relaxation rate is preserved. In order to simplify the notation, let I ≡ a, EI ≡ b, (EI)′ ≡ c, and $1/(k_1[E]) = \tau_{ab}$, $1/k_{-1} = \tau_{ba}$, $1/k_2 = \tau_{bc}$, and $1/k_{-2} = \tau_{cb}$.

Equation 17 from Swift and Connick (1962) is expressed as

$$\frac{1}{T_2} = \frac{1}{T_{2,a}} + \frac{1}{\tau_{ab}} - \frac{1}{\tau_{ab}\tau_{ba}} \times \frac{\frac{1}{T_{2,b}} + \frac{1}{\tau_{ba}} + \frac{1}{\tau_{bc}}\frac{1/T_{2,c}^2 + 1/(T_{2,c}\tau_{cb}) + \Delta\omega_c^2}{(1/T_{2,c} + 1/\tau_{cb})^2 + \Delta\omega_c^2}}{\left\{\frac{1}{T_{2,b}} + \frac{1}{\tau_{ba}} + \frac{1}{\tau_{bc}}\frac{1/T_{2,c}^2 + 1/(T_{2,c}\tau_{cb}) + \Delta\omega_c^2}{(1/T_{2,c} + 1/\tau_{cb})^2 + \Delta\omega_c^2}\right\}^2 + \left\{\Delta\omega_b + \frac{\Delta\omega_c}{\tau_{bc}\tau_{cb}[(1/T_{2,c} + 1/\tau_{cb})^2 + \Delta\omega_c^2]}\right\}^2} \tag{A2}$$

where $1/T_{2,a}$, $1/T_{2,b}$, and $1/T_{2,c}$ are the transverse relaxation times of I in the a, b, and c species, respectively, and $\Delta\omega_b$ and $\Delta\omega_c$ are the differences between the observed frequency and the frequencies of I in sites b and c, respectively.

If the substitution is made that

$$\Delta\omega = \Delta\omega_b + \frac{\Delta\omega_c}{\tau_{bc}\tau_{cb}[(1/T_{2,c} + 1/\tau_{cb})^2 + \Delta\omega_c^2]} \tag{A3}$$

and it is assumed that[5]

$$[1/T_{2,c}^2 + 1/(T_{2,c}\tau_{cb})] \gg \Delta\omega_c^2 \tag{A4}$$

then eq A2 simplifies to

$$\frac{1}{T_2} = \frac{1}{T_{2,a}} + \frac{\frac{1}{T_{2,b}} + \frac{\tau_{cb}}{\tau_{bc}(T_{2,c} + \tau_{cb})} + \frac{\Delta\omega^2}{1/T_{2,b} + 1/\tau_{ba} + \tau_{cb}/[\tau_{bc}(T_{2,c} + \tau_{cb})]}}{\frac{\tau_{ba}}{\tau_{ab}}\left[\frac{\tau_{ba}}{T_{2,b}} + 1 + \frac{\tau_{ba}\tau_{cb}}{\tau_{bc}(T_{2,c} + \tau_{cb})} + \frac{\tau_{ba}\Delta\omega^2}{1/T_{2,b} + 1/\tau_{ba} + \tau_{cb}/[\tau_{bc}(T_{2,c} + \tau_{cb})]}\right]} \tag{A5}$$

[5] This assumption can be shown to be valid in the present study since there is no observed frequency dependence for the low pH, slow exchange samples, where the c species predominates.

Based on the assumption that the mechanism in eq A1 involves an initial fast equilibrium followed by a slow isomerization, the following limits may be applied to eq A5

$$\frac{1}{T_{2,b}} \ll \frac{1}{\tau_{ba}} \tag{A6}$$

and

$$\frac{1}{\tau_{ba}} \gg \frac{1}{\tau_{bc}} \tag{A7}$$

Furthermore, since

$$\frac{\tau_{cb}}{T_{2,c} + \tau_{cb}} \leq 1 \tag{A8}$$

then

$$\frac{1}{\tau_{ba}} \gg \frac{1}{\tau_{bc}}\left(\frac{\tau_{cb}}{T_{2,c} + \tau_{cb}}\right) \tag{A9}$$

Equation A5 can then be written as

$$\frac{1}{T_2} = \frac{1}{T_{2,a}} + \frac{\dfrac{1}{T_{2,b}} + \dfrac{\tau_{cb}}{\tau_{bc}(T_{2,c} + \tau_{cb})} + \tau_{ba}\Delta\omega^2}{\tau_{ab}\left[1 + (\tau_{ba}\Delta\omega)^2\right]} \tag{A10}$$

If the final assumption is made that $(\tau_{ba}\Delta\omega)^2 \ll 1$ and if the τ values are replaced by fractional populations, then the resulting equation is

$$\frac{1}{T_2} - \frac{1}{T_{2,a}} = P_b\left(\frac{1}{T_{2,b}} + \tau_{ba}\Delta\omega^2\right) + P_c\left(\frac{1}{T_{2,c} + \tau_{cb}}\right) \tag{A11}$$

The $\Delta\omega$ term as defined in eq A3 can be simplified by applying the limit given in eq A4. If values for $1/T_{2,c}$, $1/\tau_{bc}$, and $1/\tau_{cb}$ from the text are substituted into the simplified expression, it can be seen that the dominant term for $\Delta\omega$ is $\Delta\omega_b$.

Appendix B

Given a mechanism such as that of eq 12, the fractional populations of any enzyme species of interest may be expressed in terms of a set of equilibrium constants, $[H^+]$ and [I]. The total enzyme concentration is

$$E_0 = E + EH + EH_2 + EHI + EH_2I + (EH_2I)' \tag{B1}$$

A set of equilibrium constants is defined as follows

$$K_{a2} = \frac{[E][H^+]}{[EH]} \tag{B2}$$

$$K_{EHI} = \frac{[EH][I]}{[EHI]} \tag{B3}$$

$$K_{EH_2I} = \frac{[EH_2][I]}{[EH_2I]} \tag{B4}$$

$$K_{a1} = \frac{[EHI][H^+]}{[EH_2I]} \tag{B5}$$

$$K_{con} = \frac{[EH_2I]}{[(EH_2I)']} \tag{B6}$$

$$K_{EH} = \frac{K_{a2}K_{EHI}}{[I]} \tag{B7}$$

If the concentration of I is sufficiently high, and if both K_{EHI} and K_{EH_2I} are sufficiently small, then the concentrations of EH and EH_2 can be neglected. These two conditions are assumed to hold in the system under investigation since [I] is 0.05 M, and the K_i values for succinate inhibition measured by Porter *et al.* (1969) suggest small K_{EHI} and K_{EH_2I}. Equation B1 can then be written as

$$E_0 = E + EHI + EH_2I + (EH_2I)' \tag{B8}$$

Using eq B2–B7, eq B8 can be rewritten as

$$E_0 = [E]\left(1 + \frac{[H^+]}{K_{EH}} + \frac{[H^+]^2}{K_{EH}K_{a1}} + \frac{[H^+]^2}{K_{EH}K_{a1}K_{con}}\right) \tag{B9}$$

or

$$\mathcal{P}_E = \frac{[E]}{E_0} = \left(1 + \frac{[H^+]}{K_{EH}} + \frac{[H^+]^2}{K_{EH}K_{a1}} + \frac{[H^+]^2}{K_{EH}K_{a1}K_{con}}\right)^{-1} \tag{B10}$$

Similar equations can be determined for the other species of interest

$$\mathcal{P}_{EHI} = \frac{[EHI]}{E_0} = \left(\frac{K_{EH}}{[H^+]} + 1 + \frac{[H^+]}{K_{a1}} + \frac{[H^+]}{K_{a1}K_{con}}\right)^{-1} \tag{B11}$$

$$\mathcal{P}_{EH_2I} = \frac{[EH_2I]}{E_0} = \left(\frac{K_{EH}K_{a1}}{[H^+]^2} + \frac{K_{a1}}{[H^+]} + 1 + \frac{1}{K_{con}}\right)^{-1} \tag{B12}$$

$$\mathcal{P}_{(EH_2I)'} = \frac{[(EH_2I)']}{E_0} = \left(\frac{K_{EH}K_{a1}K_{con}}{[H^+]^2} + \frac{K_{a1}K_{con}}{[H^+]} + K_{con} + 1\right)^{-1} \tag{B13}$$

When the terms on the right of these equations are combined and the reciprocals are taken, the results obtained are

$$\mathcal{P}_E = \frac{K_{a1}K_{EH}K_{con}}{D} \tag{B14}$$

$$\mathcal{P}_{EHI} = \frac{K_{a1}K_{con}[H^+]}{D} \tag{B15}$$

$$\mathcal{P}_{EH_2I} = \frac{K_{con}[H^+]^2}{D} \tag{B16}$$

$$\mathcal{P}_{(EH_2I)'} = \frac{[H^+]^2}{D} \tag{B17}$$

where

$$D = K_{a1}K_{EH}K_{con} + K_{a1}K_{con}[H^+] + (K_{con} + 1)[H^+]^2 \tag{B18}$$

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Purification and Characterization of Aspartic β-Semialdehyde Dehydrogenase from Yeast and Purification of an Isozyme of Glyceraldehyde-3-Phosphate Dehydrogenase†

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ABSTRACT: Aspartic β-semialdehyde dehydrogenase has been isolated from commercial baker's yeast in high yield. Purified aspartic β-semialdehyde dehydrogenase was judged homogeneous by sedimentation equilibrium and by polyacrylamide gel electrophoresis in the presence and absence of sodium dodecyl sulfate. Optimum conditions for purification include the use of deionized water, 5–10 mM EDTA, reducing agents, and low metal content ammonium sulfate, with all operations at 20–25°. The molecular weight of the enzyme is 156,000, and it appears to have four identical subunits of 41,000 ± 4,000. The 280:260 nm absorbance ratio is 1.9 without charcoal treatment. The copurification of aspartic β-semialdehyde dehydrogenase and yeast glyceraldehyde-3-phosphate dehydrogenase through six major purification procedures, as well as methods for the separation of five major isozymes of glyceraldehyde-3-phosphate dehydrogenase by hydroxylapatite chromatography, are also described.

During the past several years a number of laboratories have been engaged in comparative studies of dehydrogenases in an effort to establish general principles of enzyme catalysis and structure (*e.g.*, Sund, 1970). Among other things these studies have shown that the subunit molecular weights of dehydrogenases are quite variable and, more importantly, that there do not seem to be common critical residues at the active sites of these different dehydrogenases.

Recent analysis of the primary structures of alcohol dehydrogenase (Jörnvall and Harris, 1970; Jörnvall, 1970a,b), glutamate dehydrogenase (Smith *et al.*, 1970), and glyceraldehyde-3-phosphate dehydrogenase (Davidson *et al.*, 1967; Harris and Perham, 1968) have produced more puzzling results since the limited amount of sequence homology among these three enzymes does not provide any easily interpreted clues to their evolutionary history or to a generalized mechanism of action of these dehydrogenases. It appears that if these enzymes were in fact evolved from a common precursor, then the degree of divergence demanded by their respective functions was quite substantial. Since the reaction catalyzed by aspartic β-semialdehyde dehydrogenase is so similar to that catalyzed by glyceraldehyde-3-phosphate dehydrogenase, we decided that an investigation of its structure and properties would be valuable. The complexity of these two reactions is

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