Nuclear magnetic relaxation methods were used to investigate the interaction of the inhibitor succinate with aspartate transcarbamylase from Escherichia coli. Over the pH range 7 to 9, the dissociation constant for succinate remains less than the inhibitor concentration used for most of this work (0.05 M). As a result, the enzyme predominantly exists in a single "gloss" conformational state. Succinate binding to this enzyme state (generally known as the R form) parallels the behavior seen previously with the isolated catalytic subunit (Beard, C. B., and Schmidt, P. G. (1973) Biochemistry 12, 2255-2264). The pH and temperature dependence of succinate proton relaxation rates, \(1/T_1 = 1/T_r\), in the presence of carbamyl phosphate, is interpreted in terms of a binding mechanism involving three forms of the enzyme, differing by their states of protonation. The least protonated form of the enzyme does not interact with succinate, the singly protonated species binds succinate to form a rapidly dissociating complex, and the doubly protonated species undergoes a conformational isomerization upon succinate binding, yielding a slow exchange complex. Relaxation data provide sufficient information to determine \(pK_a\) values of 7.2 and 8.9 for two ionizing groups, as well as the dissociation constant for succinate in the fast exchange complex, \(K_d = 1.6 \times 10^{-2}\) M. Rate constants for the forward and reverse steps of the isomerization, \(1.3 \times 10^6 \text{s}^{-1}\) and \(23 \text{s}^{-1}\), respectively, indicate a significantly slower reverse rate from that obtained in the earlier NMR study of the isolated catalytic subunit. In experiments where the succinate concentration was varied, the relaxation rates showed sigmoidal binding of that ligand to the fast exchange complex above pH 9.1, (a) indicating cooperative binding of succinate, and (b) suggesting that above pH 9.1, the system cannot be characterized by a single dissociation constant, ionization constant, or relaxation effect. CTP and ATP were tested for their ability to affect succinate binding to the fast exchange complex. Heterotropic interactions were observed for CTP but not for ATP. Addition of low concentrations of the transition state analog N-(phosphonomethyl)-L-aspartate to the enzyme-carbamyl phosphate-succinate complex sharply decreased the relaxation rate, indicating that the measurement are sensitive only to succinate bound specifically to the active site.

NMR spectra of succinate methylene protons exhibit a single resonance, and nuclear magnetic relaxation rates, \(1/T_1\) and \(1/T_r\), of this resonance have provided an abundance of information about interaction of the inhibitor with aspartate transcarbamylase from Escherichia coli. In particular, the \(pK_a\), temperature, and succinate concentration dependence of relaxation rates in the presence of aspartate transcarbamylase catalytic subunit \(C_a\) and bound carbamyl phosphate led us to propose a binding sequence for succinate. The results were accounted for by a mechanism involving two protonation groups on the enzyme affecting succinate binding and titratable over the pH range 7 to 10. A fit of the data to the model provided estimates of several parameters including acid dissociation constants, rate and equilibrium constants for a conformational isomerization, and the rotational correlation time of bound succinate (1). The succinate binding scheme for \(C_a\) is consistent with and elaborates on the mechanism proposed by Hammes et al. (2) from temperature-jump relaxation measurements.

Having characterized the catalytic subunit-succinate interaction and determined the usefulness of NMR for such a system, we have now extended our previous investigation to include the native enzyme \(C_aR_a\). Of particular importance are differences arising from constraints imposed by the presence of the regulatory subunits. Because of the relatively high concentrations of succinate used (0.01 to 0.10 M), the enzyme sites are generally saturated and allosteric effects are not operative below about pH 9. The results resemble those seen for \(C_a\) but with important quantitative differences. Above pH 9, succinate binding becomes progressively weaker, and cooperative binding is found, in contrast to results with \(C_a\) at high pH.

Central to the interest in aspartate transcarbamylase as a regulatory enzyme are the effects of the nucleotides CTP and ATP on the modes of substrate binding and catalytic efficiency, regardless of whatever model or combination of models of allosteric behavior obtain. We have measured succinate
relaxation as a function of concentration of ATP and CTP in order to probe changes in inhibitor affinity induced by binding of nucleotide effectors to the regulatory sites.

It is important to determine whether the NMR measurements are sensitive to more than specific interactions at the active site. The transition state analog N-(phosphonacetyl)-L-aspartate (PALA) is known to bind specifically and tightly to the active site (3). Titration of a C₅₆₄/succinate/carbamyl phosphate solution with PALA indicates that low concentrations of PALA effectively displace all of the bound succinate leaving no significant residual relaxation effect.

**EXPERIMENTAL PROCEDURES**

Aspartate transcarbamylase was purified from *Escherichia coli* according to the method of Gerhart and Holoubek (4), with the exception that nucleic acids were degraded by DNase and RNase as suggested by Piget (5), rather than separated by DEAE-Sephadex chromatography. Concentrations of the purified enzyme were determined by an assay involving radioactive L-aspartate (6).

Buffered samples of aspartate transcarbamylase native enzyme and succinate used in NMR relaxation measurements were prepared by mixing appropriate volumes of four stock solutions: (a) borate/imidazole/glycylglycine buffers of varying pH, (b) native enzyme dialyzed into the same buffer of appropriate pH, (c) dithiolum carbamyl phosphate (Sigma Chemical Co. or Calbiochem) in the same buffers of varying pH prepared immediately prior to use, and (d) unbufferehd sodium succinate at pH 7.0 to 9.1. All solutions were made in D₂O and pH values are reported as meter readings uncorrected for the effect of the deuterium isotope. Acetic acid-d₄, or NaOD was used to adjust the pH of the buffer solutions. Samples contained 2-mercaptoethanol to stabilize the enzyme and EDTA to complex any contaminant metal ions which would interfere with the relaxation measurements.

The final samples were generally 1.5 x 10⁻⁴ to 3.5 x 10⁻¹ M in catalytic sites (based on six succinate binding sites per native enzyme molecule of M₀ = 310,000), and contained 0.004 M carbamyl phosphate, 0.01 to 0.05 M succinate, 0.015 M imidazole, 0.015 M borate, 0.005 M glycylglycine, 2 mM 2-mercaptoethanol, and 0.2 mM EDTA. Because of the instability of carbamyl phosphate, relaxation measurements were made immediately after preparation of the samples, which were stored on ice between measurements. The total time these were above 0°C was never more than 80 min. In spite of these precautions, and the use of the borate/imidazole/glycylglycine buffer formulated to maintain a constant pH throughout the experiments, the pH samples show a trend of increasing relaxation rate with decreasing temperature as found with the studies of C₅₆₄.

Several of the experiments described below involve serial additions of succinate, transition state analog N-(phosphonacetyl)-L-aspartate (PALA, a gift from Professor George Stark, Stanford University), or nucleotides ATP and CTP (Sigma). For these studies, stock solutions of inhibitor or regulatory effector were prepared in borate/imidazole/glycylglycine buffer, and the pH was adjusted to that of the sample to which each solution would be added. The stock concentrations were chosen on the basis of the expected potency of the ligand.

Following a method described by London and Schmidt (7), for each effector, successive amounts (0.5 to 30 μl) of stock solution were added to the NMR tube by means of a Hamilton microsyringe, and the sample was mixed by a small plunger moved vigorously through the solution several times. Relaxation rates were measured for the initial solution and after each of 5 to 10 additions. For each effector, a control was run in which buffer was used in place of enzyme. Measurements were made for each control without effector and after effector had been added to reach the maximum concentration obtained in the experimental samples.

Longitudinal and transverse relaxation rates, 1/ T₁ and 1/ T₂, were measured by the adiabatic half-passage or 1/ T₁ₙ method as developed by Sykes (8), using the Varian HR-220 spectrometer (7). A Varian temperature control unit was used to control the probe temperature, which was determined before and after each temperature change by measurement of ethylene glycol chemical shifts.

**RESULTS**

**pH and Temperature Dependence of Relaxation — Longitudinal (1/ T₁) and transverse (1/ T₂) relaxation rates for succinate in the presence of carbamyl phosphate and native enzyme were determined as a function of pH ranging from 7.1 to 9.6 and at 5 temperatures between 16 and 38°. For all samples, 1/ T₁ is greater than 1/ T₂, but in contrast to the results obtained for a similar study of the catalytic subunit (1), the values for 1/ T₂ show a small but systematic pH dependence. The greatest range is found at 16° where the value of 1/ T₁ varies from 0.59 s⁻¹ at pH 7.1 to 0.72 s⁻¹ at pH 9.1, and back to 0.52 s⁻¹ at pH 9.6. At all pH values, 1/ T₁ shows a monotonic decrease with increasing temperature as found with the studies of C₅₆₄.

For an accurate analysis, the experimental data must be independent of possible viscosity changes as the temperature and pH change (1,9); hence values for 1/ T₁ are subtracted from corresponding values for 1/ T₂. The logarithm of these differences versus reciprocal temperature are shown as the points in Fig. 1.

Fig. 1 reveals a complex pattern for the succinate proton relaxation rate as a function of pH and temperature changes in the presence of C₅₆₄. As might be expected, a simple mechanism where, for example, succinate exchanges with a single type of binding site is totally inadequate to account for the data. Fortunately, a similar pattern of relaxation rates was previously found for experiments with the catalytic subunit, C₅₆₄. Except at the very highest pH values, the succinate binding mechanism developed for C₅₆₄ works for C₅₆₄. This scheme is illustrated in Equation 1.

\[
E \xrightarrow{k_1} EH \xrightarrow{k_2} EHI \xrightarrow{k_{12}} (EHI)_n
\]

Two titratable groups on the enzyme affect succinate binding in the pH range of interest. Of the three resulting enzyme species, E, EH, and EHI, only the latter two are capable of binding succinate. It is suggested from other studies (10) as well as the NMR data that the enzyme-succinate complex undergoes a slow conformational rearrangement after oue site binding. This is included as the (EHI) = (EHI)_n step. EH and EHI represent a similar binding environment for succinate and a detailed analysis shows that EHI is always present in much lower concentration than EH. Therefore, in addition to succinate free in solution, two major enzyme-succinate complexes, (EHI)_n and EH, whose concentrations are pH-dependent, are observable by NMR.

Referring to Fig. 1, it is apparent that each of the two complexes has a different type of relaxation effect. The lower pH samples show a trend of increasing relaxation rate with increasing temperature, characteristic of the slow exchange limit of NMR relaxation. In this limit, \(|(EHI)_n| > |EH| |H| and the relaxation rate is dominated by the reciprocal of the exchange lifetime \(k_e = \tau(EHI)_n\)⁻¹. At higher pH, a decrease in relaxation with increasing temperature is observed, which is interpreted as a fast exchange limit. In this case, \(|EH| > |(EHI)_n| and the bound relaxation rate 1/ T₂(EHI) dominates the measured relaxation. Swift and Connick (11) developed the modified Bloch equations for exchange from more than one site. Their Equation 11 can be simplified for the present situation to yield Equation 2 (1,12):

\[
NMR Relaxation of Succinate-ATCase Complexes
\]

2263
$T(EH,I)'$ turns out to be about 1 order of magnitude smaller than the forms EH, or EH$. At low pH (7.1), the doubly protonated fraction of succinate bound in each of the two environments can be qualitatively accounted for by the temperature data can be qualitatively accounted for by the actual numbers vary in each form as a function of pH.

Above pH 9.1, there is a dramatic increase in relaxation falls. Fig. 2a shows plots of the fraction of enzyme in the concentration of the unprotonated enzyme form unable to bind succinate detectably, and the measured NMR shift to the left and $P(\text{C}E\text{H}_{	ext{IIr}})$ increases at the expense of $P(\text{C}E\text{H}_{	ext{I}})$. Since at 28°C $l/\tau$, (2) complexes, respectively, and $l/T_2 - l/T_1$ is the measured relaxation rate difference (dominated by $l/T_1$).

The right hand side of Equation 2 contains four terms governing the observed value of succinate relaxation. Two of these, $\tau(\text{E}H_{\text{II}},)$ and $T_1(\text{E}H_{\text{II}})$, are independent of pH. $P_{\text{E}H_{\text{II}}}$ and $P_{\text{E}H_{\text{I}}}$ on the other hand are strongly dependent on pH. The fraction of succinate bound in each of the two environments naturally depends on the relative concentrations of enzymes in the forms $E$ or $EH$. At low pH (7.1), the doubly protonated species predominates and $P_{\text{E}H_{\text{II}}}$ > $P_{\text{E}H_{\text{I}}}$. Since at 28°C $l/\tau(\text{E}H_{\text{II}})$ turns out to be about 1 order of magnitude smaller than $1/T_2(\text{E}H_{\text{I}})$, the net effect (Equation 2) is a small value for the observed relaxation rate.

As the pH is raised above 7.1, the equilibrium of Equation 1 shift to the left and $P_{\text{E}H_{\text{I}}}$ increases at the expense of $P_{\text{E}H_{\text{II}}}$.

This leads to an increase in $T_1$, because of the relatively large value of $1/T_2(\text{E}H_{\text{II}})$. Above pH 9.1, there is a dramatic increase in the concentration of the unprotonated enzyme form (E) unable to bind succinate detectably, and the measured NMR relaxation falls. Fig. 2a shows plots of the fraction of enzyme in each form as a function of pH. The trend of $1/T_1$ versus pH described for 28°C generally holds for the other values of temperature. The actual numbers vary substantially because the terms of Equation 2 are temperature-dependent, as discussed in the following section.

Temperature Dependence—For a fixed value of pH, the temperature dependence of relaxation arises from changes in all four terms on the right hand side of Equation 2. The fractions of succinate in each form, $P_{\text{E}H_{\text{I}}}$ and $P_{\text{E}H_{\text{II}}}$, depend on several equilibrium constants whose values are temperature-dependent through the enthalpy change, $\Delta H$. $T_1(\text{E}H_{\text{II}})$ depends on the rotational correlation time, $\tau_R$, of the enzyme-succinate complex, and this value is moderately temperature-dependent through an activation energy for rotational diffusion. Of course, the exchange lifetime, $\tau(EH_{\text{II}})$, depends on temperature with a rather large activation energy.

At pH 7.1, most of the enzyme is fully protonated and saturated with succinate. Here the temperature dependence reflects slow exchange of succinate from the $(\text{E}H_{\text{II}})$ complex; $1/T_1 - 1/T_1 = P_{\text{E}H_{\text{II}}}/\tau(EH_{\text{II}})$ and this function increases as $\tau(EH_{\text{II}})$ decreases, with increasing temperature. At higher pH values, the temperature dependence of relaxation is not dominated by any one term in Equation 2. Increasing temperature shifts the equilibria of Equation 1 up and to the left. At pH 9.1 for example, this leads to a dome shape for $1/T_1$ versus temperature. At the highest pH values, 9.3 and 9.6, the relaxation rate decreases steeply with increasing temperature due mainly to a drastic decrease in the fraction of succinate bound to any form of the enzyme, at the highest temperatures.

Quantitative Fit to Succinate Binding Mechanism—A previous study (1) described the method by which Equations 1 and 2 can be used to fit the experimental data, by optimizing several parameters. These include: (a) the acid dissociation constants $K_{\text{E}H_{\text{I}}}$ = $[\text{EH}]$/$[\text{E}][\text{H}][\text{I}]$ and $K_{\text{E}H_{\text{II}}}$ = $[\text{E}H][\text{I}]/[\text{E}][\text{H}]$, the succinate dissociation constant $K_{\text{E}H_{\text{I}}}$ = $[\text{E}][\text{H}][\text{I}]/[\text{EH}]$, and the equilibrium constant describing the conformational change, $K_{\text{eq}}$ = $[\text{E}H_{\text{II}}]/([\text{E}H_{\text{I}}][\text{E}])$. The doubly protonated species $E$ predominates at low pH where other work has shown that the dissociation constant for succinate is at least an order of magnitude smaller than the concentration of inhibitor used here (6, 13); thus succinate saturates the $E$ site; (b) the $\Delta H$ values for each of the equilibrium constants, from which the temperature dependence of concentrations of the various enzyme species can be determined; and (c) the temperature dependence of the relaxation effects, $1/T_1(\text{E}H_{\text{I}})$ and $1/\tau(EH_{\text{II}})$.

Values for $K_{\text{E}H_{\text{I}}}$, $K_{\text{E}H_{\text{II}}}$, $\Delta H_{\text{I}}$, $\Delta H_{\text{II}}$, and $(\Delta H_{\text{E}H} + \Delta H_{\text{II}})$ could be determined with some degree of certainty from the data in Fig. 1. However, these data are insufficient by
themselves to determine uniquely the higher pH equilibrium constants and enthalpy changes individually. Therefore, values of \( K_{\text{BHH}} \) and \( K_{\text{D}} \) determined from a succinate titration study described below were used to refine the fit of the pH and temperature dependence of succinate relaxation rates. Best fit values of \( \Delta H_{\text{BHH}} \) and \( \Delta H_{\text{D}} \) were then determined using the values of \( K_{\text{BHH}} \) and \( K_{\text{D}} \) from the succinate titration experiment and the equilibrium constants and enthalpies determined from the low pH data.

The values for the equilibrium constants and enthalpies determined as best fit parameters are given in Table I. These parameters were used to calculate the fractional populations of each of the five enzyme species as a function of pH and temperature. Some of the results are plotted in Fig. 2: as a function of pH at 28° in Fig. 2a, and as a function of temperature at pH 8.5 in Fig. 2b.

The fitting procedure yielded a temperature-dependent \( 1/\tau (EHI)_i \) with a value of 33 s\(^{-1}\) at 28°. This corresponds to the rate constant \( k_3 \) in Equation 1. From the best fit value for \( K_{\text{BHH}} = 2.5 \times 10^{-2} \), the forward rate constant for isomerization is calculated to be \( k_3 = 1.3 \times 10^{-1} \) s\(^{-1}\).

The values for \( 1/T_1(EHI) \) which best fit the data of Fig. 1 vary from 1400 to 850 s\(^{-1}\) over the temperature range studied. From these results an effective rotational correlation time \( \tau_R \) can be estimated using Equation 3:

\[
\frac{1}{T_1(EHI)} = \frac{3}{40} \frac{\Delta T g^2}{r_{ij}^6} \left[ 6\tau_R + \frac{10\tau_R}{1 + (\omega_0 T)^2} + \frac{4\tau_R}{1 + 4(\omega_0 T)^2} \right]
\]

where \( g \) is the magnetogyric ratio for protons, \( \omega_0 \) is the NMR frequency in rad s\(^{-1}\), and \( r_{ij} \) is the internuclear distance between protons on succinate with its carbonyl groups (cis). This equation is strictly valid only for isotropic reorientation of the internuclear vectors coupling the dipole. Rapid internal reorientation can decrease the relaxation rate observed (see "Discussion"). Using the value 1000 s\(^{-1}\) for \( 1/T_1(EHI) \) at 28°, \( \tau_R \) decreases in binding.

The fitting procedure yielded a temperature-dependent \( 1/\tau (EHI)_i \) with a value of 33 s\(^{-1}\) at 28°. This corresponds to the rate constant \( k_3 \) in Equation 1. From the best fit value for \( K_{\text{BHH}} = 2.5 \times 10^{-2} \), the forward rate constant for isomerization is calculated to be \( k_3 = 1.3 \times 10^{-1} \) s\(^{-1}\).

The values for \( 1/T_1(EHI) \) which best fit the data of Fig. 1 vary from 1400 to 850 s\(^{-1}\) over the temperature range studied. From these results an effective rotational correlation time \( \tau_R \) can be estimated using Equation 3:

\[
\frac{1}{T_1(EHI)} = \frac{3}{40} \frac{\Delta T g^2}{r_{ij}^6} \left[ 6\tau_R + \frac{10\tau_R}{1 + (\omega_0 T)^2} + \frac{4\tau_R}{1 + 4(\omega_0 T)^2} \right]
\]

where \( g \) is the magnetogyric ratio for protons, \( \omega_0 \) is the NMR frequency in rad s\(^{-1}\), and \( r_{ij} \) is the internuclear distance between protons on succinate with its carbonyl groups (cis). This equation is strictly valid only for isotropic reorientation of the internuclear vectors coupling the dipole. Rapid internal reorientation can decrease the relaxation rate observed (see "Discussion"). Using the value 1000 s\(^{-1}\) for \( 1/T_1(EHI) \) at 28°, \( \tau_R \) decreases in binding.

### Table I

**Best fit values of equilibrium constants, enthalpies, rate constants, and rotational correlation time for succinate binding mechanism in Equation 1**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value (kcal/mol)</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( K_{\text{BHH}} )</td>
<td>6 ( \pm 2 ) \times 10^{-3}</td>
<td>28</td>
</tr>
<tr>
<td>( K_{\text{D}} )</td>
<td>2.5 ( \pm 0.8 ) \times 10^{-1}</td>
<td>12</td>
</tr>
<tr>
<td>( k_3 )</td>
<td>1.4 ( \pm 0.3 ) \times 10^{-3}</td>
<td>2</td>
</tr>
<tr>
<td>( K_{\text{HHI}} )</td>
<td>1.6 ( \pm 0.4 ) \times 10^{-3}</td>
<td>8</td>
</tr>
</tbody>
</table>

\( k_{-3} \) is a very good approximation to the succinate titration data. The dominant equilibrium being observed is a simple one-step binding of succinate to the \( E \) species to form \( EHI \).
Comparison of Fig. 3 with Fig. 4, the succinate titration results for catalytic subunit, indicates a very marked deviation from the native enzyme results from the standard saturation curves seen for the catalytic subunit. It is expected from Equation 4 that the dependence of $1/T_s$ on $P_{EHI}$ should allow for the calculation of the succinate dissociation constant $K_{EHI}$. Such is the case for the catalytic subunit data, but, while the downward trend of the relaxation rates at low succinate concentration observed in Fig. 3 makes it impossible to treat the native enzyme data using a simple $EH = EHI$ equilibrium.

The experimental data in Fig. 3 were recast in a more familiar form, the extent of saturation as a function of succinate concentration observed in Fig. 4 it can be seen that the relaxation rate observed depends upon two quantities: the fraction of succinate bound in $EHI$ and the relaxation effect of that complex, $1/T_s(EHI)$. The latter quantity should be independent of succinate concentration and independent of pH.

From the value for $1/T_s(EHI)$, $[EHI]/[EHI]$, can be calculated as a function of [I]. However, $1/T_s(EHI)$ could not be determined directly from the experimental data. Therefore, Equation 4 can be rewritten as

$$
\frac{1}{[I]} = \frac{1}{T_s} + \frac{[EHI]}{E_a} \cdot \frac{P_{EH}}{P_{EHI}} + \frac{K_{EHI}}{[I]} \cdot \frac{P_{EH}}{P_{EHI}}
$$

where $[EHI] = [EH] + [EHI]$ and $P_{EH}$ are the concentration of $EHI$ and the fraction of enzyme in $EHI$, respectively, at saturating succinate levels. At saturating succinate, $[EH] = 0$ and $P_{EH} = 1$ (that is, $[(EH)] + [EHI] = E_a$), and a plot of $(1/T_s - 1/1) E_a$ versus $1/ [I]$ can be extrapolated to $1/ [I] = 0$ to yield a maximum value equal to the constant (at a given pH) $P_{EHI}/T_s(EHI)$. Only the lowest pH data could be extrapolated to yield a value accurate to an estimated 10%. (At higher pH, the concentration of succinate was not close enough to the saturation level to yield reliable extrapolated results.) However, $P_{EHI}$ can be calculated as a function of $pH$ from Equation 6

$$
P_{EHI} = \frac{K_{EHI} K_{con}}{K_{EHI} [H^+]}
$$

using values for $K_{EHI}$ and $K_{con}$ from Table 1. The low pH data yielded $1/T_s(EHI) = 800 \text{s}^{-1}$, which was used as a constant to determine $P_{EHI}/T_s(EHI)$ for the higher pH samples.

The values of $[EHI]/[EHI]$, were computed from the experimental data in Fig. 3 using Equation 5, and are plotted versus succinate concentration in Fig. 5.

The peculiar behavior at low succinate concentration shown in Fig. 3 can be explained by the shape of the saturation curves seen in Fig. 4. The sigmoidicity in the saturation curves is characteristic of positive cooperativity observed for succinate binding to the $C_{R_1}$ carbamyl phosphate complex (10).

Values for the succinate dissociation constant $K_d$ (apparent) can be determined from hyperbolic saturation curves using Equation 7:

$$
P_{EHI} = K_d(\\text{apparent}) + [I]
$$

A least squares fit to the data for pH 8.9 which is hyperbolic yields $K_d(\text{apparent}) = 2.3 \times 10^{-2} \text{ M}$. Although the curves of fraction bound for the pH 9.1 and 9.4 samples are not exactly hyperbolic, it is possible to use Equation 7 to estimate values for $K_d(\text{apparent})$ from the higher succinate concentration points. The results are approximately $3.6 \times 10^{-2} \text{ M}$ and $5.2 \times 10^{-2} \text{ M}$ at pH 9.1 and 9.4, respectively. The maximum succinate concentration measured at the highest pH did not even saturate half the enzyme sites, and hence the estimated value of $K_d(\text{apparent})$ is not reliable.

Porter et al. (6) noted that the observation of pH-dependent dissociation constants may be interpreted as the coupling of an acid dissociation step to an inhibitor binding step, such as that given by Equation 1 (E $\Leftrightarrow$ EH $\Leftrightarrow$ EHI). Under these conditions,

$$
K_d(\text{apparent}) = \frac{K_{EHI}[H^+] + K_{EH}}{K_{EHI}K_{EH} + [H^+]} \quad (8)
$$

where $K_{EH} = [E][I]/[EHI]$, and the other equilibrium constants are defined above. Under the conditions that $K_{EH}$ is very large,
and \( K_{eq} = [H^+] \), then Equation 8 simplifies to

\[
K_{eq}(\text{apparent}) = K_{eq} \left( \frac{1}{[H^+]} \right) + K_{em}
\]

From Equation 9 it is evident that a straight line plot of \( 1/[H^+] \) versus \( K_{eq}(\text{apparent}) \) will yield values for both \( K_{em} \) and \( K_{eq} \). Such a plot using the approximate values of \( K_{eq}(\text{apparent}) \) at the three lower pH values given above results in \( K_{em} = 0.012 \) M and \( pK_{eq} = 8.8 \). These are the values used in the fit to the pH- and temperature-dependent succinate relaxation data. (Note that the values given here apply to a different temperature (24°) than those in Table I (28°).)

Graphing the saturation data given in Fig. 5 in the form of a Hill plot yields slopes equal to the Hill coefficient, \( n \), which characterizes the degree of cooperativity in succinate binding. The values of \( n \) are 0.94, 1.1, 1.5, and 1.4 for pH 8.9, 9.1, 9.4, and 9.7, respectively, as determined from a linear least squares fit to the data.

PALA Titration of Native Enzyme – Values for \( 1/T_1 \) and \( 1/T_2 \) were determined for succinate in the presence of CTP and carbamyl phosphate at varying concentrations of the transition state analog PALA. The temperature was maintained at 28° and the pH of the sample was 8.5. The experimental data for \( 1/T_2 - 1/T_1 \) are plotted versus PALA concentration in Fig. 6. A control experiment run in the absence of enzyme yielded no effect of PALA on the succinate relaxation rate.

It is evident from Fig. 6 that addition of PALA sharply decreases the relaxation effect of the succinate interaction with the enzyme. The fact that \( 1/T_2 \) approaches \( 1/T_1 \) above 3 mM PALA indicates that the relaxation measurements are sensitive only to succinate which binds specifically to the active site.

**CTP and ATP Titration of Native Enzyme** – Succinate relaxation rates were measured as a function of CTP or ATP concentration in the presence of native enzyme and carbamyl phosphate in order to determine the effect of these nucleotides on succinate binding. For the CTP experiment, four samples of pH 8.6 to 9.4 were measured at 21° over a CTP concentration range of 0 to 3 mM. The ATP experiment was run at 28° over an ATP concentration range of 0 to 3.6 mM with three samples ranging in pH from 8.9 to 9.6. In an attempt to magnify the effect of ATP on succinate relaxation rates, the succinate concentration was made about half that in the CTP samples, and the enzyme concentration was doubled. The values for \( 1/T_1 \) were virtually independent of CTP concentration, and showed a small increase as ATP concentration increased. However, both \( 1/T_1 \) and \( 1/T_2 \) for succinate were unchanged in the presence of either ATP or CTP in the absence of enzyme, indicating no paramagnetic contaminants.

Most of the samples decreased somewhat in pH, generally 0.05 to 0.15 pH units, over the course of the experiments. Because the effects being measured are quite small, and the relaxation rates are very dramatically dependent on pH above 9.1, it is imperative that the data be normalized to a single pH in order to avoid false interpretations of the changes observed. A case in point is the highest pH sample for the ATP experiment, for which a 50% increase in relaxation rate was measured. Essentially the entire effect was attributable to a decrease in pH. The data in Fig. 1 were used to correct the relaxation rates observed in this experiment. Because of the need to make these corrections, the results for this experiment can be interpreted only qualitatively; there is insufficient data in Fig. 1 to obtain an accurate correction curve at high pH.

The corrected experimental results for the CTP and ATP...
concentration effects are plotted as $1/T_2 - 1/T_1$, versus nucleotide concentration in Figs. 7 and 8, respectively. Note that the total relaxation effect for the CTP samples is less than that for the ATP samples, as a result of the lower enzyme concentration, higher succinate concentration, and lower temperature. The change in succinate relaxation rate induced by CTP, however, is much greater than that produced by ATP.

**DISCUSSION**

**Comparison of Succinate Binding to $C_R$ and $C_3$**

The behavior of succinate proton relaxation with $C_R$ is qualitatively similar to that seen with $C_3$ (1). At low pH, relaxation is dominated by slow exchange of succinate from the $(EHJY)$ complex. Above pH 8, the singly protonated species $EH$ dominates the measurement; exchange of succinate is sufficiently rapid that nuclear relaxation of the inhibitor is largely determined by a weighted average of relaxation rates for succinate free in solution and bound in $EH$.

The relatively high concentration of succinate used in the pH-temperature work (0.05 mM) is sufficient to saturate $C_R$, catalytic sites below pH 8 (Fig. 2). This conclusion is supported by other data on succinate binding at pH 7.0 (13) and pH 7.4 (14). Furthermore the "gross" conformational transitions known to occur for $C_R$ with addition of carbamyl phosphate and succinate precede saturation of the catalytic sites (15, 16).

In fact, even though 0.05 mM succinate does not saturate at pH 8.9 and pH 9.1, the binding isotherms are essentially hyperbolic (Fig. 5), suggesting that a limiting form of the active site predominates. Thus, over the range of pH 7 to 9, the enzyme is predominantly in what is often called the R form (17), its most active state, and the NMR data reflect chemical exchange of succinate from both the $EH$ and $(EHJY)$ complexes in their R form. Under the conditions of 0.05 mM succinate concentration and below pH 9, no effects due to homotropic interactions are expected, although at much lower concentrations ($10^{-4}$ M to $10^{-5}$ M) allosteric effects are clearly evident in succinate binding isotherms (13) and in relaxation kinetic data (14).

In the absence of overall conformational changes the $C_R$, catalytic sites might well bind succinate in much the same way as $C_3$. In fact the NMR data below pH 9.1 are adequately fit to the model of Equation 1 which derives from the previously studied binding of succinate to $C_3$ (1). With $C_R$ unique values of $K_{on}$ and $K_{off}$ were obtained directly from the pH-temperature data, and the values of $K_{on}$ and $K_{off}$ (determined from succinate saturation experiments) were used to complete the fitting procedures. Several quantitative comparisons can be made between the results for the native enzyme and those for the catalytic subunit.

**Rate and Equilibrium Constants** — Values for $pK_a$, 6.9 and 7.2 for $C_3$ and $C_R$, respectively, are within the estimated uncertainty in their measurement. The value for $K_{on}$ = 2.5 x $10^{-4}$, describing the isomerization between $EH_1$ and $(EHJY)$, is about a factor of 3 smaller than that for the catalytic subunit. Thus, the conformational isomer, $(EHJY)'$, is favored even more for $C_R$ than for $C_3$. The slow exchange limit for the low pH samples allows determination of the rate constants associated with the isomerization. At 28°C, a value of $k_{-1} = 33$ s$^{-1}$ was obtained (refer to Equation 1), and hence, $k_0 = 1.8 	imes 10^9$ s$^{-1}$. The forward rate constant ($k_2$) is a factor of 2.5 smaller for the native enzyme and the reverse rate constant ($k_{-2}$) a
factor of about 8 smaller. The Arrhenius activation energies for the isomerization of \((\text{EHI})'\) back to EHI can be calculated from the slopes of the curves for \(\log \left( \frac{1}{T} \right) \) versus reciprocal temperature. The values for Cs, \(E_a = 21\) kcal/mol, and C,R,6, \(E_a = 18\) kcal/mol, differ very little.

The dissociation constant for succinate binding to the singly protonated complex, \(K_{\text{EHI}} = 1.6 \times 10^{-2}\) M (at 28°C), is 2 to 3 times that for the catalytic subunit, although the difference may not be significant. The value for \(pK_a = 8.9\) agrees well with that of 8.7 obtained from the succinate titration of Cs.

**Rotational Correlation Time** - A nuclear relaxation rate of \(1/T_2 (\text{EHI}) = 1000\) s\(^{-1}\) was found from the pH-temperature study for succinate bound to C,R,6. This value is a function of the overall rotational correlation time of the protein and also depends strongly on the degree of internal reorientational freedom of the bound inhibitor. An estimate of the correlation time for overall reorientation of C,R,6 can be made based on measurements of solutions of several proteins including hemoglobin, \(t_\chi = 4 \times 10^{-9}\) s (18, 19); chymotrypsin, \(t_\chi = 1.2 \times 10^{-9}\) s (20); ribonuclease (concentrated solution), \(t_\chi = 3 \times 10^{-9}\) s (21). Since the correlation time is proportional to the molecular volume (and the degree of hydration) we expect \(t_\chi\) to be roughly proportional to protein molecular weight. While variations in shape, solution viscosity, and temperature will subject an extrapolation to some uncertainty, we estimate that the overall correlation time of C,R,6 is \(t_\chi = 2 \pm 1 \times 10^{-7}\) s under conditions of the NMR experiment.

If succinate were rigidly bound in the EHI complex the relaxation rate \(1/T_2 (\text{EHI})\) would then be 2000 s\(^{-1}\) (Equation 3). On the other hand, if succinate were bound loosely, for example by only one carboxyl group, rapid internal motion would serve to decrease the relaxation rate by at least a factor of 4 (9). Thus we conclude that succinate is probably bound through both carboxyl groups in the EHI site. On the other hand if the modes of succinate binding were precisely the same in EHI of C, and C,R,6 we would have expected \(1/T_2 (\text{EHI})\) for C,R,6 to be 3 times that of C for which \(1/T_2 (\text{EHI}) = 700\) s\(^{-1}\) (1). The difference is actually a factor of 1.4 suggesting that the EHI complex in C,R,6 is subject to slightly less steric restraint than in C.

While differences do appear in the parameters characterizing succinate chemical exchange with C, and with fully liganded C,R, (R form), these differences are not large and do not suggest a major perturbation of succinate binding due to the presence of regulatory chains. Probably the most important differences are the slower rate constants for the isomerization step \(\text{EHI} \rightleftharpoons (\text{EHI})'\) in C,R,6. The conformational change which this step represents (2) is thought to be important for promotion of the reaction of carbamyl phosphate and L-aspartate (10).

**Comparison of NMR and Temperature-Jump Kinetics**

It is of interest to compare our NMR results with the temperature-jump kinetic data of Hammes and Wu (14). For C,R,6 with succinate and saturating carbamyl phosphate at pH 7.4, a single relaxation time was observed after the temperature-jump, the value of which increased with increasing succinate concentration and plateaued above \(10^{-5}\) M succinate. This behavior was ascribed to an isomerization of C,R,6 where the rate constants were dependent on the degree of succinate binding.

We do not detect this overall isomerization step in the NMR because the concentration of succinate is so high as to drive the equilibrium overwhelmingly toward one isomer. On the other hand, we do monitor the higher pH bimolecular step and the active site conformational isomerization which had previously been observed in the temperature-jump for C (2) but which processes were not detected for succinate binding to C,R,6 (14). Presumably the amplitudes of relaxations associated with these steps were too low for C,R,6. Since the temperature-jump measurements employed pH indicator dyes, the assumption is that insufficient numbers of protons were taken up or released. This possibility is consistent with the present results inasmuch as the best fit parameters for the mechanism of Equation 1 predict that following a temperature jump to 20°C near pH 7.4, significantly fewer protons would be released per mol of active site for C,R,6 than for C.

**Succinate Binding and pH Dependence of C,R,6 Catalyzed Reaction Velocity**

With C, the pH dependence of the reaction velocity with L-aspartate as substrate is a skewed bell-shaped curve centered near pH 8.5 (6, 22), but for C,R,6 the profile is strikingly different. At 50 mM L-aspartate and with saturating carbamyl phosphate, the activity rises sharply between pH 6 and 7, plateaus between pH 7 and 9, then rises dramatically, peaking near pH 10, and finally falls precipitously above pH 10 (22). Weitzman and Wilson (23) found that the steep rise in activity at high pH was accompanied by loss of the sigmoidal behavior of the curve of velocity versus aspartate concentration.

Using the NMR data for succinate we looked for a possible source of the peculiar behavior of the velocity profile at high pH. However, the affinity of succinate for the C,R,6-carbamyl phosphate complex has a markedly different pH dependence than the reaction velocity. Succinate binds most tightly at low pH and the overall dissociation constant increases monotonically from pH 7 to 9.6.

The fall off in succinate binding to C,R,6 at high pH probably relates to the steep drop in enzyme activity above pH 10, but there is no clue in the succinate data to account for the activity rise between pH 9 and 10. Interactions involving the aspartate amino group are therefore most likely involved in this phenomenon.

**Effects of Nucleotides ATP and CTP on Succinate Relaxation Rates**

As noted with reference to the succinate saturation experiment, in the pH range that the nucleotide titrations were performed, the changes measured in succinate relaxation rates are attributed entirely to changes in the EHI complex, as a result of the magnitude of the relaxation effect from this species. The results in Fig. 7 show that CTP has a measurable effect on the relaxation rate of succinate above pH 8.9 at 21°C. However, in contrast to results with PALA, CTP addition leads to succinate relaxation reaching a plateau for the pH 8.9 and 9.1 samples. This trend is reminiscent of inhibition of enzyme activity by the nucleotide and antagonism by 5-bromocytidine triphosphate of the succinate-induced enhanced reactivity of C,R,6 toward sulfhydryl reagents (24). The decrease in relaxation rate is not large (from 1.67 to 1.2 s\(^{-1}\) for pH 9.1) and the effect diminishes as the pH decreases to pH 8.6. A diminished effect of CTP is expected when the concentration of succinate substantially exceeds the dissociation constant of the dicarboxylic acid and indeed decreasing pH strengthens the succinate interaction. Recent findings of Wedler and Cao (25) are important for interpreting the CTP and ATP effects on succinate relaxation. These workers determined...
from equilibrium isotopic exchange kinetic studies that CTP and ATP promote changes in the aspartate reaction velocity by different mechanisms. CTP appears to interfere (indirectly) with L-aspartate binding while ATP promotes the rates of the catalytic events. Since in the NMR we are simply monitoring succinate binding, the lack of an ATP effect and the presence of a CTP effect are compatible with the isotope exchange results.

Comparison of Results in D₂O and H₂O – For technical reasons, D₂O is substituted for ordinary water in the NMR relaxation experiments. We recently repeated the temperature-pH studies on succinate plus C₅ and C₆R₆ with buffers made in H₂O (28). Measurements of 1/T₂ were made from linewidths of the succinate resonance. The results were qualitatively similar to those reported here for C₅R₅ and earlier for C₃ (1) in D₂O. The main difference lies in the weak acid dissociation constants pKₐ₁ and pKₐ₂. For C₅R₅, pKₐ₁ = 6.8 and pKₐ₂ = 8.3 in H₂O, compared to values of 7.2 and 8.8 for these parameters in D₂O determined in the present work.

The D₂O pKₐ values are based on uncorrected pH meter readings in order to more closely reflect the expected isotope effect. In fact, when 0.4 pH unit is added to correct the D₂O pKₐ values for the glass electrode behavior, the values in D₂O are 0.8 to 0.9 pH unit higher than in H₂O. A similar difference was found by Stark (27) in comparing the reaction velocity for C₅ as a function of pH (or pD) in H₂O and D₂O. The curves were the same shape but shifted 0.8 pH unit higher in D₂O. The fact that pKₐ₁ and pKₐ₂ from our NMR work are both shifted by approximately the same amount in D₂O allows us to conclude that there is probably no major effect of the isotope on the enzyme mechanism.

REFERENCES
Proton magnetic relaxation of aspartate transcarbamylase - succinate complexes.
C B Ireland and P G Schmidt


Access the most updated version of this article at http://www.jbc.org/content/252/7/2262

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/252/7/2262.full.html#ref-list-1