Relaxation Spectra of Aspartate Transcarbamylase. Interaction of the Catalytic Subunit with Carbamyl Phosphate, Succinate, and L-Malate*

Gordon G. Hammes,† Robert W. Porter,‡ and George R. Stark

ABSTRACT: A kinetic study of the binding of carbamyl phosphate, succinate, and L-malate to the catalytic subunit of aspartate transcarbamylase was performed using the temperature-jump method. A single relaxation process could be associated with the binding of carbamyl phosphate to the catalytic subunit. This process was quantitatively analyzed in terms of a bimolecular reaction mechanism. The rate parameters obtained are consistent with the equilibrium dissociation constant previously reported. In the presence of saturating carbamyl phosphate two relaxation processes, well separated on the time axis, could be associated with the binding of succinate, an unreactive analog of the substrate L-aspartate, to the catalytic subunit–carbamyl phosphate complex. The slower process could be quantitatively analyzed in terms of a relatively slow isomerization of the complex formed coupled to a rapid bimolecular reaction. The rate parameters obtained are consistent with overall equilibrium constants previously reported. A second, much faster relaxation process was observed at very low concentrations of succinate. The concentration dependence of the process could not be reliably established, but if it is attributed to the initial bimolecular reaction, a second-order rate constant of 2×10^6 M^{-1} sec^{-1} can be estimated. Similar studies with L-malate, a substrate analog which has a hydroxyl group in the position of the amino group of L-aspartate, revealed a single relaxation process that also could be quantitatively analyzed in terms of a two-step binding mechanism. The kinetic parameters for succinate and L-malate are similar (within a factor of two) except for the rate constant characterizing the conversion of the second enzyme–substrate complex to the first complex, which is almost ten times larger in the case of L-malate. This result suggests that the L-α substituent encounters the most significant steric interference in the isomerized complex, which is consistent with a mechanism in which rate enhancement occurs through a conformational change that forces the substrates together.

The aspartate transcarbamylase from *Escherichia coli* catalyzes the carbamylation of L-aspartate, the first step in the biosynthesis of CTP, and is subject to feedback inhibition by CTP (Gerhart and Pardee, 1962). The enzyme can be readily dissociated into two types of subunits (Gerhart and Schachman, 1965). The regulatory subunit (molecular weight 17,000; Weber, 1968; Hammes *et al.*, 1970) retains the ability to bind allosteric effectors, but is not a catalyst. The catalytic subunit (a trimer of molecular weight 100,000, with three active sites; Weber, 1968; Meighen *et al.*, 1970; Hammes *et al.*, 1970) retains full catalytic activity, but has no sensitivity to effectors such as CTP. An understanding of the regulatory mechanism requires investigation of the native enzyme. However, the catalytic mechanism is best probed by study of the isolated catalytic subunit, where the complications of homotropic and heterotropic allosteric effects are absent (Gerhart and Pardee, 1962). A detailed steady-state kinetic analysis of the carbamylation reaction catalyzed by the catalytic subunit indicates an ordered binding mechanism, in which carbamyl phosphate binds first, L-aspartate binds second, carbamyl-L-aspartate dissociates first, and inorganic phosphate dissociates last (Porter *et al.*, 1969). Equilibrium dialysis experiments (Changeux *et al.*, 1968) and ultraviolet difference spectroscopy (Collins and Stark, 1969) have shown that succinate and other unreactive aspartate analogs, which are competitive inhibitors of aspartate, bind more tightly to the enzyme in the presence of carbamyl phosphate. Moreover, sedimentation velocity (Gerhart and Schachman, 1968), immunological reactivity (von Fellenberg *et al.*, 1968), and ultraviolet difference spectroscopy (Collins and Stark, 1969) have suggested that a conformational change occurs upon the binding of aspartate analogs.

In this study the temperature-jump method has been used to study the kinetics of the binding of carbamyl phosphate to the catalytic subunit and the binding of succinate and L-malate to the catalytic subunit–carbamyl phosphate complex. The results indicate that the binding of carbamyl phosphate can be described by a simple bimolecular mechanism, while for both succinate and L-malate a relatively slow conformational change follows the initial bimolecular step. In the case of succinate, estimates of the rate constants for the initial bimolecular reaction have also been obtained. The rate and equilibrium constants obtained are consistent with a "compression" model for the catalysis previously proposed (Collins and Stark, 1969).

Experimental Section

Native aspartate transcarbamylase and its catalytic subunit were prepared according to the procedure of Gerhart and Holoubek (1967). The overproducing mutant strain of *E. coli* was grown by the New England Enzyme Center. Concentrated solutions of catalytic subunit were dialyzed extensively against unbuffered 0.02 M potassium acetate–5×10^{-4} M EDTA–1×10^{-3} M dithiothreitol, pH 8.0. Concen-

* From the Department of Chemistry, Cornell University, Ithaca, New York 14850 (G. G. H.), and the Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305 (R. W. P. and G. R. S.). *Received September 3, 1970.* This work was supported by grants from the National Institutes of Health (GM 13292, GM 11788).

† To whom correspondence should be addressed.

‡ National Institutes of Health Trainee in Biochemistry (GM 196-11).

trations were determined by absorbance at 280 nm, assuming an extinction coefficient of 0.70 cm^2/mg (Collins and Stark, 1969) and a molecular weight of 33,000 per site (Weber, 1968; Meighen *et al.*, 1970; Hammes *et al.*, 1970).

All chemicals were the best available commercial grade. Dilithium carbamyl phosphate (Sigma Chemical Company) was further purified by precipitation from aqueous solution by addition of cold ethanol (Gerhart and Pardee, 1962). All carbamyl phosphate solutions were freshly prepared, kept in ice, and used within 4 hr. Under these conditions, inorganic phosphate represents less than 10% of the total phosphate.

The temperature-jump apparatus used is described in detail elsewhere (Faeder, 1970). The reaction volume of this instrument is slightly less than 0.2 ml. A 10-kV discharge through the cell was used to obtain a temperature rise of 7.5° with a heating time constant of 8 μsec. The final temperature was 28° in all cases. The signal to noise ratio was typically about 1000. Solutions were prepared from freshly boiled, distilled, deionized water and contained 0.10 M potassium acetate. Fresh solution was flushed into the reaction cell for each temperature-jump. Enzyme concentrations were approximately 3 mg/ml. For each solution at least 6 oscilloscope traces were photographed. The relaxation times were calculated from a least-squares analysis of the logarithm of the signal amplitude *vs.* time. An IBM 1800 digital computer interfaced with a curve tracer was used to evaluate the photographed oscilloscope traces.

Results

Succinate. The existence of an ultraviolet difference spectrum, which accompanies the binding of succinate to the catalytic subunit–carbamyl phosphate complex (Collins and Stark, 1969), suggested utilizing the change in absorption of light at 289 nm (the wavelength at the maximum of the difference spectrum) to follow the course of the reaction. However, no change in absorption could be detected at this wavelength following a temperature jump, presumably because the absorption changes were too small. Attempts were made at pH 7.0, 0.02 M imidazole buffer, at pH 7.5, 0.01 M phosphate buffer, and at pH 8.0, 0.02 M glycylglycine buffer with concentrations of succinate corresponding to the equilibrium dissociation constants at the three pH values. Enzyme concentrations used were up to 10^{-4} M (3 mg/ml), which corresponds to an absorbancy of 1.4 at 289 nm.

However, in the presence of the pH indicator dye, phenol red (2×10^{-5} M), in an unbuffered solution containing 10^{-4} M catalytic subunit, saturating carbamyl phosphate (10^{-3} M), and succinate an absorbance change was observed at 560 nm in the time range 0.1–0.5 msec, which could be described by a single relaxation time. The observation of pH changes is consistent with the marked pH dependence of succinate binding to the enzyme (Porter *et al.*, 1969). No relaxation processes were observed when enzyme, carbamyl phosphate, or succinate was eliminated from the solution. The relaxation process is markedly pH dependent: at pH 7.0 the relaxation amplitude is smaller and the rate is slower than at pH 8.0. Unfortunately at pH 8.0, pH drift of the unbuffered solution is severe. A pH value of 7.4 ± 0.1 was finally selected as optimal: intermediate amplitudes and rates are observed, and the pH drift is controllable at this pH. Repurified carbamyl phosphate was used, since small amounts of inorganic phosphate can buffer out the small pH change being observed. A typical relaxation effect is shown in Figure 1.

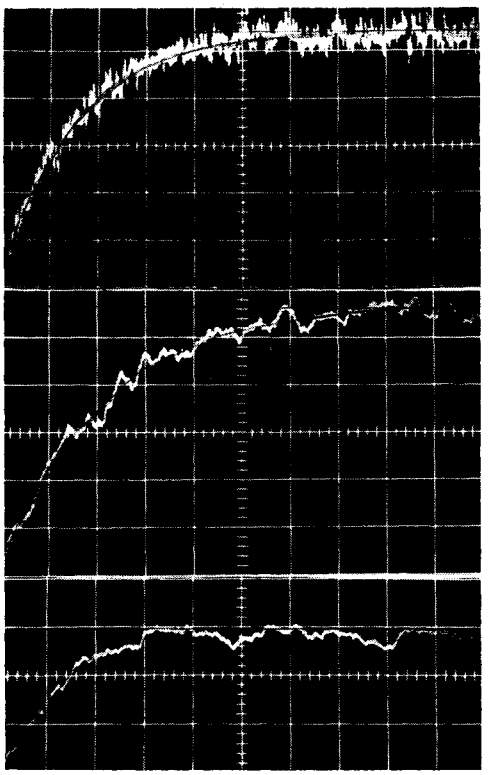

FIGURE 1: Temperature-jump relaxation effects of the catalytic subunit in 0.1 M potassium acetate and 2×10^{-5} M phenol red. The vertical scale is absorbancy at 560 nm (arbitrary scale) and the horizontal scale is time. Upper: succinate, 1.5×10^{-3} M; catalytic subunit, 0.9×10^{-4} M; and carbamyl phosphate, 10^{-3} M; pH 7.4. The horizontal scale is 0.5 msec/large division. Middle: L-malate, 3×10^{-2} M; catalytic subunit, 1.0×10^{-4} M; and carbamyl phosphate, 10^{-3} M; pH 7.5. The horizontal scale is 50 μsec/large division. Lower: carbamyl phosphate, 1.5×10^{-4} M; catalytic subunit, 1.4×10^{-4} M; pH 7.5. The horizontal scale is 20 μsec/large division.

The concentration dependence of the relaxation time for succinate at pH 7.4 is shown in Figure 2. For a simple bimolecular reaction mechanism such as eq 1, where E is enzyme, in

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \qquad (1)$$

this case the catalytic subunit–carbamyl phosphate complex, S is the substrate analog, and ES is the complex, the reciprocal relaxation time is

$$1/\tau_1 = k_{-1} + k_1[(\bar{E}) + (\bar{S})] \qquad (2)$$

where $(\bar{E})$ and $(\bar{S})$ are the equilibrium concentrations of enzyme and substrate analog. Equation 2 predicts that $1/\tau_1$ should be a linear function of $[(\bar{E}) + (\bar{S})]$; however, the reciprocal relaxation time in Figure 1 approaches a limiting value at large values of $[(\bar{E}) + (\bar{S})]$. A simple mechanism consistent with this concentration dependence is a bimolecular step followed by a relatively slow isomerization or conformational change of the enzyme–substrate complex.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ES' \qquad (3)$$

Here ES and ES′ represent two different forms of the enzyme–substrate complex. If the first step equilibrates very rapidly

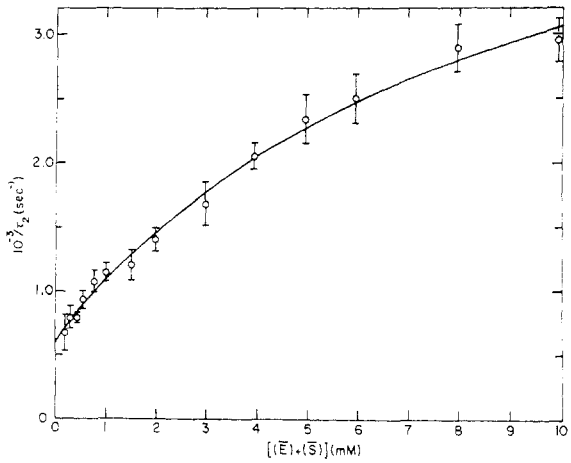

FIGURE 2: The reciprocal relaxation time for the interaction of succinate with the catalytic subunit–carbamyl phosphate complex, as a function of the sum of the equilibrium concentrations of the catalytic subunit and succinate. The initial concentration of catalytic subunit was 0.9×10^{-4} M. The initial concentration of succinate varied from 1.25×10^{-4} M to 1.0×10^{-2} M. The pH was 7.4 ± 0.1. The error bars represent the standard deviation for the traces analyzed. The solid line was calculated with eq 4 and the constants in Table I.

compared to the second step, the slow relaxation time is

$$1/\tau_2 = k_{-2} + \frac{k_2}{1 + \dfrac{k_{-1}}{k_1[(\bar{E}) + (\bar{S})]}} \qquad (4)$$

The data in Figure 2 were fit to eq 4 by a computer program using an iterative trial and error procedure. The best fit parameters obtained are summarized in Table I, and the curve in Figure 2 has been calculated with eq 4 and these parameters. The calculated curve is in excellent agreement with the experimental data. The parameters have an estimated uncertainty of about ±15%. The constants in Table I can be used to calculate the overall binding constant, defined as

$$K = \frac{(\bar{E})(\bar{S})}{(\bar{ES}) + (\bar{ES}')} = \frac{(k_{-1}/k_1)}{1 + k_2/k_{-2}} \qquad (5)$$

The calculated value of this constant is $K = 1.0 \times 10^{-3}$ M at pH 7.4. This value may be compared with the equilibrium dissociation constant obtained by ultraviolet difference spectroscopy, 0.7×10^{-3} M at pH 7.0 (Collins and Stark, 1969), and with the inhibition constant obtained by steady-state kinetics, 1.3×10^{-3} M, at pH 7.4 (Porter *et al.*, 1969).

A second, much faster relaxation process, in the time range 20–50 μsec, was observed at very low concentrations of succinate. This fast process has an amplitude in the direction of decreasing pH, as does the previously observed effect, whereas the heating curve has an amplitude in the opposite direction. However, the amplitude of this fast step was small, and at succinate concentrations lower than 1.25×10^{-4} M, it was too small to measure. On the other hand, at succinate concentrations higher than 5×10^{-4} M, the relaxation effect became so fast that it was obscured by the much larger amplitude of the heating curve. This concentration range was much too narrow to ascertain if in fact this fast step has the linear concentration dependence of a bimolecular reaction (eq 2). The observed reciprocal relaxation times for this concentration range were between 1.0×10^4 and 1.8×10^4 sec^{-1}. If the assumption is made that this relaxation process is, in fact, the bimolecular step, then approximate values for k_1 and k_{-1} may be calculated from the ratio k_{-1}/k_1 (8.7×10^{-3} M) and eq 2. These values of k_1 and k_{-1}, included in Table I, should be regarded as estimates only, with uncertainties of about ±50%. Moreover, the relaxation process observed could conceivably be related to other processes, *e.g.*, proton transfer reactions.

TABLE I: Summary of Rate and Equilibrium Constants at 28°.

	Succinate[a]	L-Malate[b]
k_1 (M^{-1} sec^{-1})	1.6×10^6	
k_{-1} (sec^{-1})	1.4×10^4	
k_{-1}/k_1 (mM)	8.7	15
k_2 (sec^{-1})	4600	3600
k_{-2} (sec^{-1})	620	5600
k_{-2}/k_2	0.13	1.6
K (mM)	1.0	10

[a] pH 7.4 ± 0.1. [b] pH 7.5 ± 0.1.

L-*Malate*. Similar temperature-jump studies were performed with another aspartate analog, L-malate, at pH 7.5 ± 0.1. A large relaxation amplitude was observed in the presence of saturating carbamyl phosphate (10^{-3} M) in the time range 50–100 μsec. A typical relaxation effect which can be described by a single relaxation time is shown in Figure 1. The concentration dependence of the reciprocal relaxation time is shown in Figure 3. These data were also fit to eq 4, which assumes the two-step mechanism of eq 3 to be valid. The calculated values for the kinetic parameters are given in Table I, and the curve in Figure 3 has been calculated with these parameters and eq 4. Because the reciprocal relaxation time is not markedly concentration dependent in the case of L-malate, the estimated uncertainties in the calculated parameters are slightly larger than for succinate.

A value of the overall equilibrium constant for the binding of L-malate to the catalytic subunit can be calculated from the kinetic parameters of Table I and eq 5. This value, 1.0×10^{-2} M at pH 7.5, may be compared with the equilibrium constant determined by ultraviolet difference spectroscopy, 1.0×10^{-2} M at pH 7.0 (Collins and Stark, 1969), and with the inhibition constant determined by steady-state kinetics, 3×10^{-2} M at pH 7.5 (Porter *et al.*, 1969).

A second relaxation process, which could be attributed to a bimolecular reaction, was not observed with L-malate. Such a process may have been obscured by the magnitude of the observed relaxation effect, which was considerably faster than the corresponding effect with succinate. Furthermore, the weaker binding of L-malate would lead to a very small relaxation amplitude at concentrations low enough to permit observation of a very fast bimolecular reaction.

Carbamyl Phosphate. The temperature-jump studies described above were performed in the presence of saturating carbamyl phosphate (10^{-3} M). If the concentration of carbamyl phosphate is reduced to the level of the molar concentration of enzyme, in the absence of analogs of L-aspartate, then another, very fast relaxation process can be observed, using the unbuffered system with a pH indicator, in the time range 20–50 μsec. The concentration dependence of the relaxation

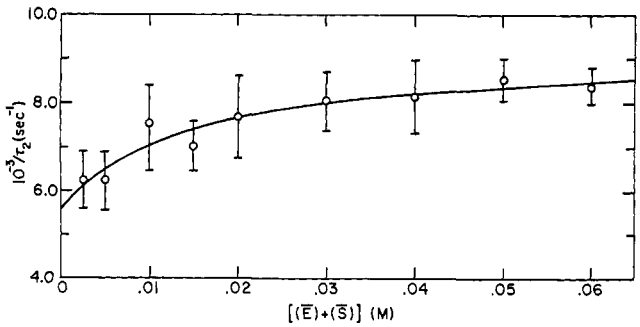

FIGURE 3: The reciprocal relaxation time for the interaction of L-malate with the catalytic subunit–carbamyl phosphate complex as a function of the sum of the equilibrium concentrations of the catalytic subunit and L-malate. The initial concentration of enzyme was 1.0×10^{-4} M. The initial concentration of L-malate varied from 2.5×10^{-3} M to 6.0×10^{-2} M. The pH was 7.5 ± 0.1. The error bars represent the standard deviation for the traces analyzed. The solid line was calculated from eq 4 and the constants given in Table I.

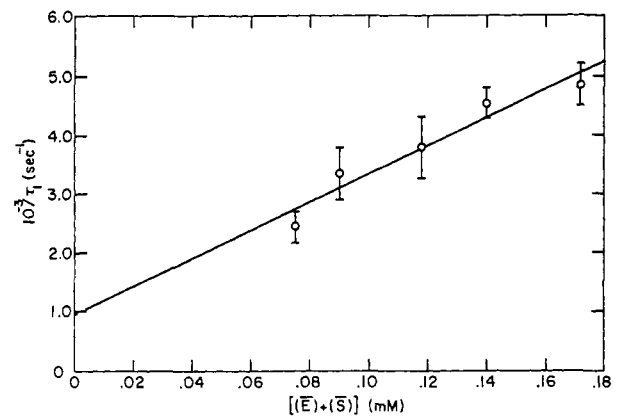

FIGURE 4: The reciprocal relaxation time for the interaction of carbamyl phosphate with the catalytic subunit as a function of the sum of the equilibrium concentrations of catalytic subunit and carbamyl phosphate. The initial concentration of enzyme varied from 0.7×10^{-4} M to 1.4×10^{-4} M. The initial concentration of carbamyl phosphate varied from 0.75×10^{-4} M to 2.5×10^{-4} M. The pH was 7.5 ± 0.1. The error bars represent the standard deviation for the traces analyzed. The straight line was obtained by least-squares analysis of the data.

time for this reaction was determined by varying the concentrations of both enzyme and carbamyl phosphate, in a range considerably higher than the equilibrium dissociation constant. Figure 4 shows the concentration dependence of the reciprocal relaxation time. The concentration range examined was rather narrow, because at lower concentrations of enzyme and carbamyl phosphate, the amplitude of the relaxation effect became too small to measure, and at higher concentrations the reaction became too fast to observe with the instrument described. The data in Figure 4 were quantitatively analyzed by a linear least-squares procedure. Values of $[(\bar{E}) + (\bar{S})]$ were calculated for different trial values of K, the equilibrium dissociation constant. Assuming the simple bimolecular mechanism of eq 1 and the linear concentration dependence of eq 2, the ratio of the intercept (k_{-1}) to the slope (k_1) gives an experimental value for the equilibrium constant, K. This value may be compared to the trial value of K used to calculate $[(\bar{E}) + (\bar{S})]$. This calculation was repeated for a series of trial values for K, until the experimental value for K agreed with the trial value. The best fit was obtained for a trial value of $K = 4.0 \times 10^{-5}$ M, which gave values of $k_1 = 2.4 \times 10^8$ M^{-1} sec^{-1} and $k_{-1} = 9.5 \times 10^3$ sec^{-1}. These values have estimated uncertainties of about ±25%. As shown in Figure 4, the straight line calculated by least-squares for this procedure passes within experimental error of all data points, confirming the linear concentration dependence and the existence of a bimolecular reaction mechanism. Furthermore, the value obtained for the equilibrium dissociation constant, 4.0×10^{-5} M at pH 7.5, is in reasonable agreement with the dissociation constant for carbamyl phosphate obtained by steady-state kinetics, 2.7×10^{-5} M, at pH 7.0, especially since this constant seems to increase with increasing pH (Porter *et al.*, 1969).

Discussion

In the derivation of the expressions for the relaxation times, the implicit assumption has been made that kinetic coupling of the reactions of interest with the proton transfer reactions of the indicator is negligible. This is valid so long as the rates of the proton transfer reactions are very rapid compared to the rates of the observed relaxation processes. This latter assumption is supported by the absence of observable relaxation processes in control systems lacking any one of the reactants and by the known rates of protolytic reactions (Eigen and Hammes, 1963).

The concentration dependence of the reciprocal relaxation times, shown in Figure 4, suggests a simple bimolecular reaction mechanism for the binding of carbamyl phosphate to the catalytic subunit, although other possible mechanisms cannot be excluded on the basis of the necessarily limited data obtained. The value calculated for the second-order rate constant is consistent with the lower limit estimated by steady-state kinetics, $k_1 \geq 0.5 \times 10^8$ M^{-1} sec^{-1}, at pH 7.8 (Porter *et al.*, 1969). On the basis of other experiments (Collins and Stark, 1969; Kirschner and Schachman, 1970), a conformational change has been associated with the binding of carbamyl phosphate. Although a one-step mechanism (eq 1) is sufficient to explain the results presented here, a conformational change may still be occurring which cannot be detected by the methods employed.

However, the concentration dependence of the reciprocal relaxation times shown in Figures 2 and 3 rules out a simple bimolecular reaction mechanism for the binding of succinate and L-malate to the catalytic subunit–carbamyl phosphate complex. Although the data shown in Figure 3 indicate that the observed reciprocal relaxation times are not markedly concentration dependent for L-malate, the observed values do seem to reach a limiting value at higher concentrations, as required by the mechanism of eq 3. The small increase in reciprocal relaxation time and the large uncertainties allow a straight line, which does not lie outside any of the error bars, to be drawn through the points in Figure 3. A straight line would be consistent with a simple bimolecular mechanism, but the equilibrium dissociation constant calculated from the ratio of the intercept to the slope is 0.2 M, which is inconsistent with equilibrium and steady-state values reported previously (Collins and Stark, 1969; Porter *et al.*, 1969).

The data are quantitatively consistent with the sequential mechanism of eq 3, where a rate-limiting isomerization follows a rapid bimolecular step. Recent magnetic resonance measurements have been interpreted in terms of a bimolecular reaction mechanism for the binding of succinate (Sykes *et al.*, 1970). If a sequential mechanism such as eq 3 was assumed,

it was estimated that less than 2% of the enzyme–substrate complex is in the form of the initial complex, ES. The results presented here indicate that about 12% of the saturated enzyme remains in the initial complex in the case of succinate, and about 60% in the case of L-malate. The minor differences in temperature, pH, and buffer seem insufficient to account for this discrepancy in mechanism. It is by no means certain that the kinetic process observed with the temperature-jump method is the same as that observed with nuclear magnetic resonance. However, if the two kinetic processes are the same, then a possible interpretation of the nuclear magnetic resonance results is that the motional freedom of succinate in the *initial* ternary complex with carbamyl phosphate (ES in eq 3) must be greater than its motional freedom in the ternary complex with inorganic phosphate (Sykes *et al.*, 1970). In the case of succinate, the observation of a second, much faster, rate process which may be the bimolecular step gives further support to the proposed mechanism: a two-step process with significant amounts of two enzyme–substrate complexes.

Another type of mechanism consistent with the kinetic data is one involving a preexisting equilibrium between two enzyme forms, both of which can bind the substrate analogs.

$$\begin{array}{ccc} E + S & \rightleftharpoons & ES \\ \uparrow\downarrow & & \uparrow\downarrow \\ E' + S & \rightleftharpoons & ES' \end{array} \qquad (6)$$

This mechanism would postulate that at low concentrations of S the rate of interconversion of E and E′ is being measured, whereas at high concentrations of S the rate of interconversion of ES and ES′ is being measured. If the rate of the latter process is faster than that of the former, the concentration dependence of the relaxation time would be similar to that observed. Although the observed concentration dependence of the relaxation time (τ_2) for succinate and L-malate is formally consistent with the mechanism of eq 6, this mechanism also predicts that the value of $1/\tau_2$ when $[(\bar{E}) + (\bar{S})] = 0$ should be the same for both substrate analogs. This is clearly not the case, so that if this mechanism is occurring, then the concentration of E′ must be much lower than the concentration of E. Since the data are fit very well by the mechanism of eq 3, and since the derived kinetic parameters are in good agreement with independently obtained equilibrium constants, assumption of a more complex mechanism is not warranted at this time. Similar studies with the native enzyme indicate that the binding of succinate and L-malate is more complex than the process described by eq 3 (Hammes and Wu, 1971); both the observed concentration dependence of the relaxation time and the magnitude of the rates are quite different from those reported here. Moreover, the cooperative nature of the binding process with the native enzyme also suggests a more complex process.

A comparison of the kinetic parameters derived for succinate and L-malate indicates that k_{-1}/k_1 differs by less than a factor of two, and that k_2 is quite similar for both analogs, whereas k_{-2} is almost a factor of ten greater for L-malate. Moreover, the value of k_2 is sufficiently large relative to the turnover number (390 sec^{-1} at pH 7.5; Porter *et al.*, 1969) to allow the isomerization process to be part of the catalytic mechanism. These findings are consistent with a proposed mechanism of action for the enzyme where a conformational change of the enzyme compresses the substrates toward the transition state (Collins and Stark, 1969) (*cf.* Jencks (1969) for a general discussion of the model). This mechanism predicts that the conformational change would be hindered by substituents in the L-α position which are forced toward the bound carbamyl group, and which must overcome the van der Waal's repulsive forces. When succinate binds, no repulsion can occur, so that the conformational change can occur to a greater extent, resulting in tighter binding. This is manifested in the present case by the fact that the ratio (ES)/(ES′) is 0.13 for succinate and 1.6 for L-malate, and that the difference in these ratios is the predominant factor in determining the difference in the free energy of binding for these two analogs. This is consistent with a mechanism in which the L-α substituent encounters steric interference only in the isomerized ternary complex (ES′).

The occurrence of a two-step binding mechanism has been observed for many other enzymes (Hammes and Schimmel, 1970), suggesting that this may be a general feature of enzymatic mechanisms. The mechanism for the binding of substrates to the native enzyme, where regulation as well as catalysis is of importance, is more complex, and is considered in an accompanying article (Hammes and Wu, 1971).

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