# Relaxation Spectra of Aspartate Transcarbamylase. Interaction of the Native Enzyme with Aspartate Analogs*

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ABSTRACT: Kinetic studies of the interaction of native aspartate transcarbamylase with two aspartate analogs, succinate and L-malate, were carried out using the temperature-jump method. In the presence of saturating carbamyl phosphate, a single relaxation process was observed for the binding of succinate to the enzyme–carbamyl phosphate complex. The reciprocal relaxation time decreases with increasing succinate concentration, and can be quantitatively analyzed in terms of a concerted mechanism analogous to the model of Monod *et al.* (Monod J., Wyman, J., and Changeux, J. P. (1965), *J. Mol. Biol. 12*, 88). Similar studies with L-malate revealed a relaxation process that also could be quantitatively analyzed in terms of the concerted mechanism. When both BrCTP (an allosteric inhibitor) and succinate were present with saturating carbamyl phosphate, two relaxation processes were observed, which were associated with two distinct conformational transitions of the enzyme induced by BrCTP and succinate, respectively. This result suggests that more than two conformational states of the enzyme are of importance in the allosteric regulation. A mechanism involving four conformational states is postulated to account for both the homotropic and heterotropic effects observed experimentally.

Aspartate transcarbamylase of *Escherichia coli* is a well-characterized allosteric regulatory enzyme. Native aspartate transcarbamylase exhibits two classes of allosteric interactions (Monod *et al.*, 1963) among the specific ligands involved in the regulation of its activity: homotropic effects as evidenced by the sigmoidal dependence of reaction velocity on the concentration of aspartate (Gerhart and Pardee, 1962) and carbamyl phosphate (Bethell *et al.*, 1968), and the cooperative binding of aspartate analogs to the enzyme in the presence of carbamyl phosphate (Changeux *et al.*, 1968); and heterotropic effects between the substrate (aspartate) and the feedback inhibitor, CTP, or the activator, ATP (Gerhart and Pardee, 1962). More conclusively, the allosterism of aspartate transcarbamylase is demonstrated by the fact that the enzyme can be separated into two types of subunits (Gerhart and Schachman, 1965). The regulatory subunit binds allosteric effectors but is catalytically inert, while the catalytic subunit is enzymatically active but is insensitive to allosteric effectors. Native aspartate transcarbamylase consists of six regulatory subunits (mol wt 17,000) and six catalytic subunits (mol wt 33,000) (Weber, 1968; Meighen *et al.*, 1970; Hammes *et al.*, 1970).

Two limiting models have been proposed to account for the allosteric behavior of regulatory enzymes, the model of Monod *et al.* (1965) and the model of Koshland *et al.* (1966). Both models deal with the allosteric effect in terms of indirect interactions between topographically distinct binding sites mediated by the protein molecule through a conformational transition. A critical assumption in the model of Monod *et al.* is that the conformational change is concerted for all the subunits, while in the model of Koshland *et al.* a sequential change of subunit conformations as a function of ligand binding is assumed. Although a decision between a concerted and a sequential mechanism cannot be readily derived from equilibrium studies, kinetic studies of elementary steps involved in substrate or effector binding to aspartate transcarbamylase can in principle distinguish between these mechanisms.

A temperature-jump study of the interaction of the inhibitor analog BrCTP with native aspartate transcarbamylase and its regulatory subunit has been presented previously (Eckfeldt *et al.*, 1970). In the presence of saturating carbamyl phosphate and succinate, a conformational change of the native enzyme is rate limiting in the binding process; a corresponding change in conformation is not observed with the regulatory subunit. This implies that this conformational transition may be involved in the allosteric control mechanism. The kinetic data obtained are consistent with both a concerted mechanism similar to the model of Monod *et al.* (1965) and a mechanism involving a bimolecular reaction followed by a conformational change, although the former mechanism appears to provide the best explanation for all of the data. A general sequential mechanism such as the scheme of Koshland *et al.* (1966), which predicts a spectrum of relaxation times, is not compatible with the experimental observations.

This work presents the results of a temperature-jump study of the interaction of native aspartate transcarbamylase with two aspartate analogs, succinate and L-malate, in the presence of saturating carbamyl phosphate. Under these conditions, the binding of both succinate and L-malate is accompanied by a conformational transition of native aspartate transcarbamylase which does not correspond to any processes observed with the isolated catalytic subunit (Hammes *et al.*, 1971). When both BrCTP and succinate are present (with saturating carbamyl phosphate), two relaxation processes are observed, which indicates that two distinct conformational transitions of native aspartate transcarbamylase are induced by BrCTP and succinate. A mechanism involving four conformational states is proposed to account for both the homotropic and heterotropic interactions observed experimentally.

## Experimental Section

*Materials.* Aspartate transcarbamylase of *Escherichia coli* was prepared according to the method of Gerhart and

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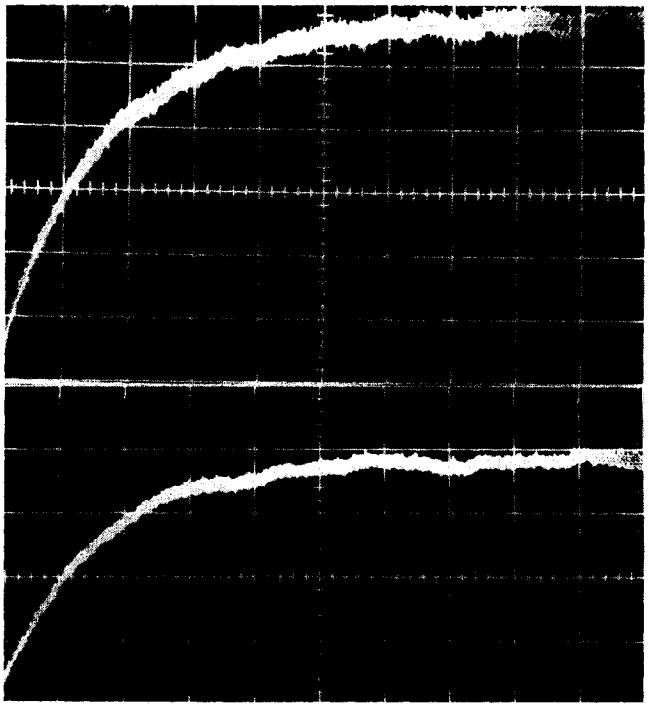


FIGURE 1: Temperature-jump relaxation effects of native aspartate transcarbamylase in 0.1 M potassium acetate, pH 7.4. The vertical scale is absorbance at 560 nm (arbitrary units) and the horizontal scale is time. The final temperature for all experiments was 28°. Top: succinate binding to aspartate transcarbamylase; succinate, $1 \times 10^{-3}$ M; ATCase, $0.94 \times 10^{-4}$ M; carbamyl phosphate, $1 \times 10^{-3}$ M; and phenol red, $2 \times 10^{-5}$ M. The time scale is 10 msec/large division. Bottom: L-malate binding to aspartate transcarbamylase. The reaction mixture was the same as above except the enzyme concentration was $0.89 \times 10^{-4}$ M and succinate was replaced by $2.5 \times 10^{-2}$ M L-malate. The time scale is 5 msec/large division.

Holoubek (1967). The necessary bacteria were obtained from the New England Enzyme Center. The purity of the enzyme obtained has been described previously (Eckfeldt *et al.*, 1970). The enzyme was transferred from phosphate buffer into 0.02 M potassium acetate–$5 \times 10^{-4}$ M EDTA–$1 \times 10^{-3}$ M dithiothreitol, pH 8.0, by extensive dialysis. Concentrations were determined by the absorbance at 280 nm, assuming an extinction coefficient of 0.59 $cm^2$/mg (Gerhart and Holoubek, 1967) and a molecular weight of 52,000 per site (Weber, 1968; Hammes *et al.*, 1970). Aspartate transcarbamylase activity was determined by the production of carbamyl aspartate according to the method of Gerhart and Pardee (1962).

Dilithium carbamyl phosphate (Sigma Chemical Company) was further purified by precipitation from aqueous solution with cold ethanol (Gerhart and Pardee, 1962). All carbamyl phosphate solutions were freshly prepared, kept in ice, and used within 4 hr. Aqueous solutions of succinic acid (Sigma Chemical Company) were neutralized with KOH to pH 7.5. The 5-bromocytidine triphosphate was synthesized by bromination of CTP (P-L Biochemicals) in formamide as described previously (Eckfeldt *et al.*, 1970). Phenol red (Fischer Scientific Company) and neutral red (Eastman Organic Chemicals) were recrystallized before use.

*Methods.* The temperature-jump relaxation spectrum was measured by use of equipment and procedures reported previously (Faeder, 1970; Eckfeldt *et al.*, 1970). The final temperature was 28° in all cases. Solutions were prepared from freshly boiled, deionized, distilled water and contained 0.1 M potassium acetate. The pH of the solutions was adjusted with KOH or acetic acid to 7.4 using a Radiometer PHM-26 pH meter. The uncertainty in the pH is estimated to be ±0.05 pH unit. The pH change accompanying the relaxation process was monitored spectrophotometrically at 560 nm with $2 \times 10^{-5}$ M pH indicator, either phenol red or neutral red. Because of a slight pH drift after each temperature jump, fresh solution was flushed into the reaction cell for each temperature jump. For measurements related to BrCTP interactions with the enzyme, the concentration changes were also monitored at 308 nm (Eckfeldt *et al.*, 1970). For each solution at least 6 oscilloscope traces were photographed. The photographed oscilloscope traces were analyzed by means of a curve tracer interfaced with a PDP-9 digital computer. The relaxation time was calculated from a least-squares analysis of the logarithm of the signal amplitude *vs.* time, and has an uncertainty of about ±10%.

Concentration-jump experiments were performed with a Durrum-Gibson stopped-flow apparatus equipped with a Kel-F syringe block and mixing chambers. Equal volumes of a solution containing aspartate transcarbamylase, substrates, pH indicator, and salt, and a similar solution without enzyme and substrates were mixed in about 4 msec. The change in transmittance was then monitored at 560 nm on a storage oscilloscope, where the trace was photographed and analyzed as described for temperature-jump experiments.

TABLE I: Reciprocal Relaxation Time for the Interaction of Succinate with the Aspartate Transcarbamylase–Carbamyl Phosphate Complex at 28°.[a]

| $10^4 \times$ (Succinate)$_0$ (M) | Temperature Jump | | Concentration Jump |
|---|---|---|---|
| | Phenol Red | Neutral Red | Phenol Red |
| 2.5 | 234 | 182 | |
| 5 | 104 | 94 | 93 |
| 10 | 62 | 56 | 42 |

[a] Final concentrations of reaction mixture were: $9.37 \times 10^{-5}$ M ATCase, $1 \times 10^{-3}$ M carbamyl phosphate, $2 \times 10^{-5}$ M phenol red or neutral red, 0.1 M potassium acetate, pH 7.4.

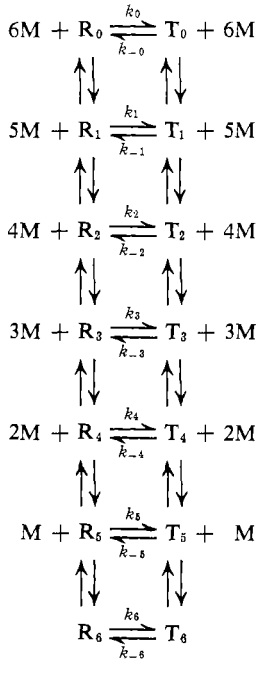
## Results and Treatment of Data

*Succinate Binding.* The difference spectra produced by the binding of substrates or substrate analogs to native aspartate transcarbamylase are too small to be useful for kinetic measurements with the temperature-jump method. However, when a pH indicator, phenol red, was added to solutions of succinate and enzyme in the presence of saturating carbamyl phosphate at pH 7.4, a relaxation process could be detected at 560 nm in the time range 2–20 msec. A typical oscilloscope trace of the relaxation process following a temperature jump is shown in Figure 1. Elimination of either the enzyme or succinate or carbamyl phosphate abolished the relaxation effect. The process also vanished in solutions buffered at pH 7.4 with 0.01 M imidazole-HCl or phosphate buffer. When neutral red, a dye with a p$K$ similar to phenol red but quite different structurally, was used as an indicator, very similar relaxation times were observed. This is shown in Table I at several different succinate concentrations. The

amplitude of the relaxation effect is opposite in direction because neutral red has an acid–base color change which is the reverse of that of phenol red. These results indicate that the relaxation process is related to the interaction of succinate with the aspartate transcarbamylase–carbamyl phosphate complex and is not due to dye–protein interactions. In addition, phenol red or neutral red at the concentrations used in the above experiments showed no effect on the enzyme activity. Essentially identical relaxation times were obtained with concentration-jump experiments in a stopped-flow apparatus; the results obtained are included in Table I.

At constant succinate concentrations which were much greater than the enzyme concentrations, the relaxation time associated with the succinate–aspartate transcarbamylase interaction in the presence of saturating carbamyl phosphate is independent of the enzyme concentration. This is illustrated in Figure 2.

Figure 3 shows the reciprocal relaxation time ($1/\tau$) as a function of the initial succinate concentration. Since the concentrations of succinate used in all experiments were much higher than that of the enzyme, the initial concentration is to a good approximation equal to the concentration of free succinate. As can be seen in Figure 3, the reciprocal relaxation time decreases rapidly as the concentration of succinate increases and approaches a constant level at higher succinate concentrations. This rather unusual concentration dependence is characteristic of a mechanism involving an isomerization of the enzyme, with associated rate constants that are dependent on the degree of saturation of the enzyme with succinate. The mechanism of Monod *et al.* (1965) is precisely of this type and can be described by the following scheme




(1)

where M is the substrate or the effector, R and T are different conformational states of the enzyme, $K_R$ and $K_T$ are the intrinsic dissociation constants for the binding of M to the R and T states, and the $k_i$'s are the rate constants associated with the interconversions between the R and T states. The assumption has been made that the cooperative unit consists of six subunits; this is suggested by the known number of substrate binding sites (Hammes *et al.*, 1970) and polypeptide chains (Meighen *et al.*, 1970) in the native enzyme.

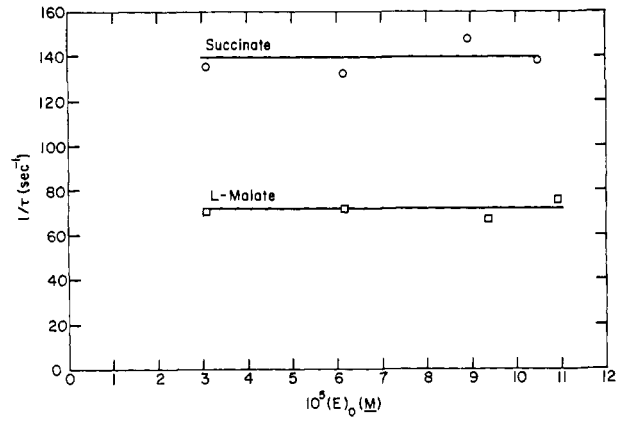



FIGURE 2: Plot of the reciprocal relaxation time *vs.* the enzyme concentration. Solutions contain $1 \times 10^{-3}$ M succinate (○—○) or $2.5 \times 10^{-2}$ M L-malate (□—□), $1 \times 10^{-3}$ M carbamyl phosphate, 0.1 M potassium acetate, $2 \times 10^{-5}$ M phenol red, and aspartate transcarbamylase, concentrations as indicated.

An analysis of the data is now presented based on the following simplifying assumptions. (1) The vertical steps equilibrate rapidly relative to the horizontal steps; this is a reasonable assumption since the relaxation time is much longer than that usually observed with bimolecular enzyme–substrate reactions (Hammes, 1968a,b). (2) The concentration changes of free ligand during equilibration are negligible; this is justified by the use of ligand concentrations that are much greater than the enzyme concentrations. (3) All transitions from T to R states are characterized by identical rate constants ($k_{-0} = k_{-1} = k_{-2} = \cdots = k_{-6}$); this is a quite arbitrary assumption and is discussed further below. With these assumptions, the reciprocal relaxation time associated with the conformational transitions can be expressed as (Eckfeldt *et al.*, 1970)

$$\frac{1}{\tau} = k_{-0} + k_0\left(\frac{1 + (M)/K_T}{1 + (M)/K_R}\right) \qquad (2)$$

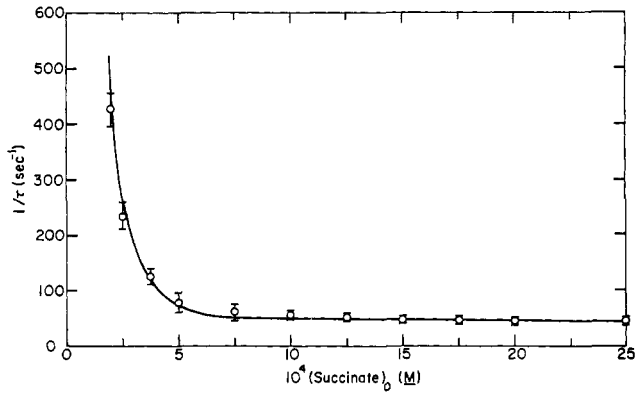



FIGURE 3: The reciprocal relaxation time for the interaction of succinate with the aspartate transcarbamylase–carbamyl phosphate complex as a function of the initial succinate concentration. The initial concentration of enzyme was $0.94 \times 10^{-4}$ M; carbamyl phosphate, $1 \times 10^{-3}$ M; potassium acetate, 0.1 M; and phenol red, $2 \times 10^{-5}$ M; pH 7.4. The initial concentration of succinate varied from $2 \times 10^{-4}$ M to $2.5 \times 10^{-3}$ M. The error bars represent the standard deviations for the traces analyzed. The solid line is the computer fitted curve according to eq 2 and the parameters in Table II.

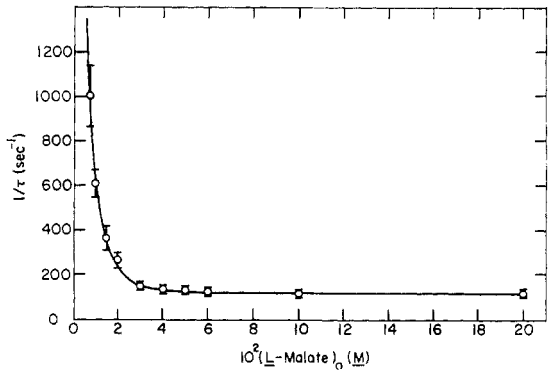



FIGURE 4: The reciprocal relaxation time for the interaction of L-malate with the aspartate transcarbamylase–carbamyl phosphate complex as a function of the initial concentration of L-malate. The initial concentration of enzyme was $0.89 \times 10^{-4}$ M; carbamyl phosphate, $1 \times 10^{-3}$ M; potassium acetate, 0.1 M; and phenol red, $2 \times 10^{-5}$ M; pH 7.4. The initial concentration of L-malate varied from $0.75 \times 10^{-2}$ M to $2.0 \times 10^{-1}$ M. The error bars represent the standard deviations for the traces analyzed. The solid line is the computer fitted curve according to eq 2 and the parameters in Table II.

There are four unknown parameters in this equation: $k_{-0}$, $k_0$, $K_T$, and $K_R$. A computer program was written to fit the experimental data to eq 2. If $K_R$ is much smaller than $K_T$, as in the case of succinate binding, $k_{-0}$ can be estimated by the value of $1/\tau$ when the succinate concentrations are very high. As can be seen in Figure 3, $1/\tau$ approaches a constant value at high concentrations of succinate. By varying the other three parameters ($k_0$, $K_T$, and $K_R$), the computer gave the best-fit curve (based on a least-squares criterion) shown as a solid line in Figure 3; the calculated curve is in good agreement with the experimental data. The best-fit kinetic parameters are listed in Table II.

A very fast relaxation process ($\tau \sim$ 20–50 μsec) of low amplitude was also observed for solutions containing aspartate transcarbamylase, phenol red, and salt. This relaxation process disappeared when succinate was added with a saturating amount of carbamyl phosphate. Variations of both enzyme and dye concentrations have been tested and have not produced significant changes in the relaxation time. Because of its overlap with the absorption changes associated with the heating of the solution, this fast relaxation process cannot be reliably analyzed, and the nature of the relaxation process cannot be ascertained.

TABLE II: Kinetic Parameters for the Concerted Mechanism of Eq 1.

| | Succinate | L-Malate |
|---|---|---|
| $k_{-0}$ (sec$^{-1}$) | 46 | 117 |
| $k_0$ (sec$^{-1}$) | 6900 | 6860 |
| $L_0$ ($=k_0/k_{-0}$) | 150 | 57 |
| $K_R$ (M) | $3.1 \times 10^{-4}$ | $1.5 \times 10^{-2}$ |
| $K_T$ (M) | $1.1 \times 10^{-2}$ | $1.2 \times 10^{-1}$ |
| $c$ ($=K_R/K_T$) | 0.028 | 0.13 |
| $K_{0.5}$[a] (M) | $5.7 \times 10^{-4}$ | $2.4 \times 10^{-2}$ |

[a] Concentration of substrate analog for half-saturation of the enzyme.

TABLE III: Relaxation Times for ATCase–Succinate–BrCTP Solutions at 28°.[a]

| $10^4 \times$ (Succinate)$_0$ (M) | No BrCTP $1/\tau_1$ (sec$^{-1}$) | $2.5 \times 10^{-4}$ M BrCTP | |
|---|---|---|---|
| | | $1/\tau_1$ (sec$^{-1}$) | $1/\tau_2$ (sec$^{-1}$) |
| 3.75 | 126 | 1640 | 3960 |
| 5 | 104 | 210 (159)[b] | 3870 (4110)[b] |
| 7.5 | 76 | 66 | 4220 |
| 10 | 62 | 58 (55)[b] | 3640 (3360)[b] |
| 100 | | | 4020 (4200)[b] |

[a] Reaction mixture: $0.92 \times 10^{-4}$ M ATCase–1 mM carbamyl phosphate–0.1 M potassium acetate–$2 \times 10^{-5}$ M phenol red, pH 7.4. Concentrations of succinate and BrCTP as indicated above. Monitored at 560 nm. [b] Monitored at 308 nm.

*L-Malate Binding.* Similar temperature-jump studies were performed with another aspartate analog, L-malate. Two relaxation processes were observed in the presence of saturating carbamyl phosphate. A very fast relaxation process could be detected when the L-malate concentrations were low (<10 mM). However, this process had a very small amplitude and was too fast to be studied quantitatively.

Another relaxation process with a large amplitude (see Figure 1) was seen in the time range 1–10 msec. This was not observed in the absence of carbamyl phosphate and was independent of the enzyme concentration at constant L-malate concentrations as shown in Figure 2. The concentration dependence of the reciprocal relaxation time associated with this process is shown in Figure 4. The curve obtained is similar to that associated with succinate binding (Figure 3), and can be analyzed in terms of the mechanism of eq 1. Applying the same procedures described above, the best-fit curve for L-malate binding is shown as a solid line in Figure 4, and the kinetic parameters derived are presented in Table II.

*Effect of BrCTP on the Succinate–Aspartate Transcarbamylase Interaction.* Thus far, only the homotropic effects of aspartate analogs have been considered. However an examination of the heterotropic effects between substrate (or its analogs) and inhibitor (or activator) is crucial for an understanding of the regulatory mechanism.

Relatively low concentrations of BrCTP must be used to study its effect on the succinate–aspartate transcarbamylase interaction because the triphosphate tends to buffer the pH changes used to monitor the interaction. Two relaxation processes were observed when both BrCTP and succinate were present with saturating carbamyl phosphate. These two effects could be detected by monitoring either at 560 nm using phenol red as a pH indicator or at 308 nm using the difference spectra produced by BrCTP and the enzyme. The relaxation times measured at 0 and $2.5 \times 10^{-4}$ M BrCTP for various succinate concentrations are given in Table III. The slower relaxation process (associated with $\tau_1$) obviously corresponds to the conformational transition of the enzyme–carbamyl phosphate complex induced by succinate since at high concentrations of succinate the relaxation time approaches the same limiting values found for the interaction of succinate with the aspartate transcarbamylase–carbamyl phosphate complex in the absence of BrCTP. Furthermore, the relaxation process also vanishes at concentrations where

the enzyme–carbamyl phosphate complex is saturated with succinate. At low concentrations of succinate, however, $\tau_1$ was decreased by the addition of BrCTP. The faster relaxation process ($\tau_2$) can be associated with the conformational transition of aspartate transcarbamylase induced by BrCTP in the presence of saturating succinate and carbamyl phosphate, since it is not observed in the absence of BrCTP and the relaxation time is the same as that found for the enzyme–BrCTP interaction (in the presence of saturating succinate and carbamyl phosphate) studied previously (Eckfeldt *et al.*, 1970). Variation of the succinate concentration did not change the $\tau_2$ values significantly. This is exactly as expected; the relaxation time for the BrCTP–aspartate transcarbamylase interaction is approximately the same in the absence and presence of succinate and carbamyl phosphate at a BrCTP concentration of $2.5 \times 10^{-4}$ M (Eckfeldt *et al.*, 1970).

## Discussion

The binding of both succinate and L-malate to aspartate transcarbamylase saturated with carbamyl phosphate is consistent with the concerted mechanism of eq 1. A sequential mechanism (Koshland *et al.*, 1966) does not seem probable since a spectrum of relaxation times related to conformational transitions is not observed. However, a sequential mechanism could, in principle, give rise to a relaxation time with a concentration dependence similar to that shown in Figures 3 and 4. This could occur if a conformational transition associated with the binding of one of the substrate molecules (other than the last one) is slow relative to all other binding steps and conformational transitions in the sequential mechanism.[1] However, it seems unlikely that in a situation where binding is observed continuously over a rather narrow concentration range, a conformational change associated with the binding of one particular molecule would be so different from that associated with the binding of all others that only its rate would fall in the accessible time range. Alternatively, a combination of a sequential and a concerted mechanism could be operative. Although alternatives to the mechanism of eq 1 exist, it is the simplest and most credible that is consistent with the data.

In principle, three relaxation times should be observed for the mechanism of eq 1. However, the two relaxation times related to the bimolecular binding steps could not be identified, although a relatively fast relaxation time was associated with L-malate binding. Either the relaxation times are too fast, no pH changes accompanying binding, or the amplitudes are too small for detection. If the former is the case, the bimolecular rate constant is greater than about $10^8$ $M^{-1}$ $sec^{-1}$, which is not unreasonable relative to known rates of enzyme–substrate reactions (Hammes, 1968a,b).

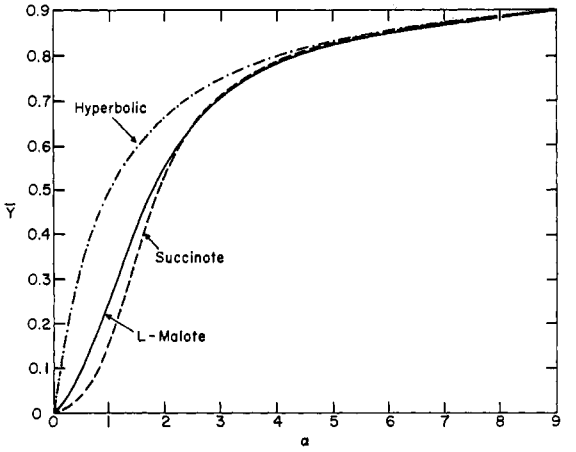



FIGURE 5: The binding isotherms of succinate and L-malate to the aspartate transcarbamylase–carbamyl phosphate complex calculated according to the concerted mechanism (eq 1) with the parameters given in Table II. Simple hyperbolic binding isotherm, –·–·–; succinate binding isotherm, ---; L-malate binding isotherm, —. $\bar{Y}$ is the fraction of enzyme saturated, and $\alpha$ is $(M)/K_R$.

The quantitative analysis of the data in terms of eq 2 is quite restrictive because of the arbitrary assumption that the $k_{-i}$'s are equal. A unique fit of the data to such a complex mechanism cannot be obtained, and the parameters in Table II should only be given qualitative significance. For example, $L_0$ should be the same for L-malate and succinate; this is only approximately true, but the difference is well within the precision of the analysis. However, regardless of the details of the analysis, the reciprocal relaxation time should extrapolate to the same value at zero concentration for all substrate analogs since at this limit $1/\tau = k_0 + k_{-0}$, which is a property of only the enzyme. This in fact is true, well within the experimental uncertainty. A reasonable agreement can be seen between the values of $K_R$ and the corresponding binding constants with the carbamyl phosphate–catalytic subunit complex. In the case of succinate, $K_R$ is $3.1 \times 10^{-4}$ M, while the binding constant with carbamyl phosphate–catalytic subunit complex is $5.5\text{–}7.4 \times 10^{-4}$ M (Changeux *et al.*, 1968; Collins and Stark, 1969). In the case of L-malate, the corresponding values are $1.3 \times 10^{-2}$ M and $1\text{–}3 \times 10^{-2}$ M (Collins and Stark, 1969; Porter *et al.*, 1969). The concentration required for half-saturation of the binding sites on native aspartate transcarbamylase was calculated to be $5.7 \times 10^{-4}$ M for succinate and $2.4 \times 10^{-2}$ M for L-malate. A value of $6.3 \times 10^{-4}$ M was reported for succinate by Changeux *et al.* (1968) based on equilibrium dialysis experiments.

The value of $c$ is significantly different for succinate and L-malate. This difference indicates that the binding should be more cooperative in the case of succinate. The predicted binding isotherms of succinate and L-malate calculated from the kinetic parameters in Table II are shown in Figure 5, along with that for simple hyperbolic binding. Unfortunately, reliable equilibrium data are not available for comparison, although only qualitative agreement would be expected.

The relaxation processes associated with succinate and L-malate bindings were measured with phenol red, a pH indicator. These processes are interpreted in terms of confor-

[1] For example, if the slow conformational change is represented as $EM_j \underset{k_r}{\overset{k_f}{\rightleftharpoons}} EM_j'$ and only $EM_j'$ combines with additional modifier or substrate molecules, the reciprocal relaxation time is

$$1/\tau = \frac{k_f}{1 + \sum_{l=1}^{j}\left(\prod_{i=1}^{l} K_{j+1-i}\right)\Big/(M)^l} + \frac{k_r}{1 + \sum_{l=1}^{n-j}(M)^l\Big/\left(\prod_{i=1}^{l} K_{j+i}\right)}$$

where the $K_i$'s represent the dissociation constants for formation of the $EM_i$ species and $n$ substrate molecules are bound per enzyme molecule. The assumption also has been made that the concentration of M is much greater than that of enzyme. Rapid conformational changes can be accounted for by appropriate definitions of the dissociation constants. If $k_f \ll k_r$, the reciprocal relaxation time could decrease inversely proportional to some power ($n - j$ or less) of S.

mational transitions of the native enzyme involved in the allosteric regulation. If this interpretation is valid, the allosteric transition must be pH dependent. In fact, high pH (10.2) has the effect of desensitizing native aspartate transcarbamylase from homotropic and heterotropic effects without causing dissociation (Weitzman and Wilson, 1966). Conceivably, the T and R states differ in the ionization constants of their protein side chains, and protons behave as allosteric effectors which shift the R–T equilibrium and alter the cooperativity of the substrate binding (*cf.* the Bohr effect for $O_2$ binding to hemoglobin).

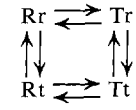
A temperature-jump study of succinate and L-malate binding to the catalytic subunit of aspartate transcarbamylase is reported in the preceding article (Hammes *et al.*, 1971). The observed concentration dependence of the relaxation times is quite different, and the magnitudes of the rates are much faster than reported here. A two-step binding mechanism, with a rate-limiting isomerization preceded by a relatively rapid bimolecular reaction, was proposed for succinate and L-malate binding to the catalytic subunit, while the binding of these two aspartate analogs to the native enzyme requires a more complex mechanism similar to the model of Monod *et al.* (1965). Since this mechanism is observed only with the native enzyme, it is probably mediated by some subunit–subunit interactions, and is also probably involved in the allosteric regulation. On the other hand, the simple two-step binding observed with the catalytic subunit reflects the catalytic mechanism where allosteric effects are absent. Such effects presumably also occur with the native enzyme, but they cannot be detected.

Obviously in the case of the catalytic mechanism, the reciprocal relaxation times associated with any relevant elementary steps must be greater than or equal to the turnover number. In contrast, relaxation processes associated with conformational changes involved in allosteric control mechanisms can be quite slow. This, in fact, is the case for yeast glyceraldehyde phosphate dehydrogenase (Kirschner *et al.*, 1966) and threonine-sensitive homoserine dehydrogenase and aspartokinase (Janin and Iwatsubo, 1969). The reciprocal relaxation time associated with succinate and L-malate binding to aspartate transcarbamylase at high concentrations of substrate analogs is also slow compared to the turnover number, although this may not be the case for the actual substrate, aspartate.

The kinetic data obtained for the homotropic effects of BrCTP (Eckfeldt *et al.*, 1970) and aspartate analogs are both consistent with a concerted model of Monod *et al.* (1965). However, for BrCTP $1/\tau$ increases with increasing BrCTP concentration, whereas for aspartate analogs $1/\tau$ decreases with increasing succinate or L-malate concentration. The concentration dependence observed with BrCTP is mechanistically ambiguous; a mechanism involving a rapid bimolecular reaction followed by a relatively slow isomerization of the complex formed also fits the data (Eckfeldt *et al.*, 1970). However, for succinate or L-malate binding in the presence of saturating carbamyl phosphate, this mechanism is not consistent with the data.

Since the binding of succinate and L-malate to native aspartate transcarbamylase can be quantitatively analyzed by the concerted model of Monod *et al.* (1965), the binding of the natural substrate, aspartate, is probably also subjected to a similar allosteric control mechanism. However, based on the tryptic digestivity of peptide chains and the rate of pCMB reactions, McClintock and Markus (1969) suggested that aspartate binding to native aspartate transcarbamylase is in accordance with a sequential mechanism. Nevertheless, since the second substrate, carbamyl phosphate, was absent in their experiments, the conclusion that the allosteric control mechanism for aspartate transcarbamylase is not compatible with a concerted model is premature. A detailed kinetic study of the reaction catalyzed by the catalytic subunit indicates an ordered binding mechanism, in which carbamyl phosphate binds first and L-aspartate binds second (Porter *et al.*, 1969). The requirement of carbamyl phosphate for relatively tight binding of succinate to both native aspartate transcarbamylase and its catalytic subunit has been demonstrated by Changeux *et al.* (1968) by use of equilibrium dialysis. In the present investigation, the relaxation processes associated with the conformational transitions of the native enzyme induced by succinate or L-malate vanished if no carbamyl phosphate was present.

Probably the most surprising phenomenon observed is the simultaneous existence of two distinct conformational transitions induced by BrCTP and by succinate. The kinetic data associated with the binding of both BrCTP (Eckfeldt *et al.*, 1970) and aspartate analogs are consistent with a mechanism of the type proposed by Monod *et al.* (1965). This model has generally been discussed in terms of two possible conformational states of the enzyme. The allosteric effectors are postulated to bind one or the other of the two possible forms, and either activate or inhibit the enzyme by shifting the equilibrium between the two conformational states. This model is not sufficient for aspartate transcarbamylase, since if this model were correct only the faster relaxation process would be observed in the presence of both substrates and effector. In other words, if only two conformations exist, but the mechanism of interconversion is different when BrCTP and succinate are bound, to a good approximation only the fastest mechanism would occur when BrCTP or succinate is present. The fact that two distinct conformational transitions are seen when both the substrates and inhibitor are present indicates that more than two conformational states exist. Moreover, since the addition of BrCTP enhances the rate of the conformational transition induced by succinate, this conformational transition and that induced by BrCTP are coupled with each other. A simple model consistent with the experimental observations can be written as



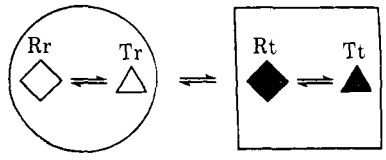

(3)

Four different conformation states, Rr, Rt, Tr, and Tt, are postulated as shown in the scheme. The horizontal T → R transitions are induced by aspartate analogs in the presence of saturating carbamyl phosphate, whereas the vertical r → t transitions are induced by BrCTP in the presence of substrates (succinate and carbamyl phosphate). Either the R–T or r–t transitions are in accord with the concerted mechanism of Monod *et al.* (1965), and Rr is the species binding substrate the best. Ligands which shift the conformational equilibria from R to T or from r to t inhibit the enzyme activity (allosteric inhibitor), and *vice versa*.

In the absence of all substrates and effectors, the enzyme is essentially in the Tt form, and the binding of BrCTP is a simple bimolecular process. When saturating amounts of carbamyl phosphate are added, the enzyme is converted primarily to the Tr state; therefore the effect observed with

varying succinate in the presence of saturating carbamyl phosphate is mainly due to the Rr ⇌ Tr transition. On the other hand, saturating amounts of carbamyl phosphate and succinate shift the enzyme to R forms so that mainly the Rr ⇌ Rt transition is observed when BrCTP is added at saturating concentrations of succinate and carbamyl phosphate. When both BrCTP and succinate are present (but not saturating the enzyme) in the presence of saturating concentrations of carbamyl phosphate, R ⇌ T and r ⇌ t transitions are seen simultaneously. The above predictions are consistent with all the experimental observations. However, again it should be pointed out that the regulation by BrCTP might also involve the alternative mechanism discussed earlier.

A more schematic model which can account for both homotropic and heterotropic effects can be written as




(4)

Equation 4 is a general case of eq 3 since Rr ⇌ Tt and Rt ⇌ Tr transitions are possible in the former case. However, these two models cannot be distinguished by the kinetic data.

The multiplicity of conformational changes observed and the cooperative effects of succinate and BrCTP on the conformational equilibria suggest that the control process is quite complex. Although the conformational change associated with succinate binding appears to be concerted in nature and that associated with BrCTP binding may be concerted, the overall control mechanism must still be considerably more complex than the simple model of Monod *et al.* (1965). At least two different molecular changes are capable of turning the enzyme on and off in the case of aspartate transcarbamylase. The fact that a number of different molecular changes can regulate enzyme activity would seem to be a reasonable and desirable feature of an overall control mechanism.

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