Relaxation Spectra of Aspartate Transcarbamylase. Interaction of the Native Enzyme with Cytidine 5′-Triphosphate†

L. W. Harrison‡ and Gordon G. Hammes*

ABSTRACT: A kinetic study of the interaction between aspartate transcarbamylase and cytidine 5′-triphosphate in the presence and absence of carbamyl phosphate and succinate, an aspartate analog, has been carried out using the temperature-jump method. A single relaxation process was observed in the presence of carbamyl phosphate and succinate. The reciprocal relaxation time and amplitude of this process increase with increasing cytidine 5′-triphosphate concentration and reaches a limiting value at high concentrations. Two relaxation processes are observed in solutions containing enzyme and cytidine 5′-triphosphate in the absence of carbamyl phosphate and succinate. The faster process apparently reflects the interaction of cytidine 5′-triphosphate with the catalytic sites while the slower process is due to binding at the regulatory sites. The reciprocal relaxation time of the slower process has a concentration dependence that is similar, but not identical, to that observed in the presence of carbamyl phosphate and succinate. Only limited data could be obtained with the isolated regulatory subunit, but the concentration dependence of the relaxation time resembled that of the slower process observed with the native enzyme. These results are consistent with a mechanism involving a rate limiting conformational change for the binding of cytidine 5′-triphosphate to regulatory sites, although a mechanism involving coupled bimolecular reactions cannot be strictly excluded for the isolated regulatory subunit and the native enzyme in the absence of carbamyl phosphate and succinate. For the conformational change mechanism, carbamyl phosphate and succinate stabilize a different conformation than cytidine 5′-triphosphate. This may be of importance in the overall regulatory process.

The regulation of aspartate transcarbamylase from *Escherichia coli* by nucleotide effectors is a well-known example of allosteric control (Gerhart and Pardee, 1962). The enzyme is inhibited by CTP and activated by ATP, and is known to consist of regulatory and catalytic subunits (Gerhart and Schachman, 1965). Equilibrium binding studies have shown that CTP and ATP bind to the enzyme in a heterogeneous manner (Winlund and Chamberlin, 1970; Buckman, 1970; Matsumoto and Hammes, 1973). The data are consistent with the existence of two classes of binding sites, three having a high affinity for the nucleotide effectors and three having a relatively low affinity.

Kinetic investigations of the interaction of aspartate transcarbamylase with 5-bromocytidine 5′-triphosphate (BrCTP;[1] Eckfeldt *et al.*, 1970), carbamyl phosphate (Hammes and Wu, 1971a), and succinate and L-malate (Hammes and Wu, 1971b) have been carried out. The results suggest that at least three conformational changes are involved in the control process. In this work, the results of an investigation of the interaction of CTP with aspartate transcarbamylase are presented. This study was undertaken because of the detailed equilibrium binding data now available (Matsumoto and Hammes, 1973). By using sensitive pH detection and by carefully choosing experimental conditions, a considerably wider concentration range could be examined than in the study using BrCTP (Eckfeldt *et al.*, 1970). The results of this study show that a conformational change is rate limiting in the binding process in the presence of 1 mM carbamyl phosphate and 10 mM succinate, an aspartate analog, and also very probably in their absence. The results obtained have possible implications for the overall control mechanism.

Experimental Section

The CTP, dilithium carbamyl phosphate and succinate were obtained from Sigma Chemical Co. Carbamyl phosphate was further purified by precipitation from 50% ethanol (Gerhart and Pardee, 1962). All other chemicals were reagent grade or the best commercially available grades.

Aspartate transcarbamylase was prepared according to the procedure of Gerhart and Holoubek (1967). The mutant bacteria were obtained from the New England Enzyme Center. The subunits of the enzyme were dissociated by the method described by Nelbach *et al.* (1972). In this procedure, the mercury bound to the regulatory subunit is replaced with zinc by an excess of β-mercaptoethanol and zinc acetate. Solutions of native enzyme or subunit were transferred into 0.02 M potassium acetate, 5×10^{-4} M EDTA, and 1×10^{-3} M dithiothreitol (pH 8.0) by extensive dialysis. This treatment results in the removal of zinc from the isolated regulatory subunit. In some cases zinc was not removed by eliminating EDTA from the dialysis solution. Concentrations of native enzyme and catalytic subunit were determined by measurement of the absorbance at 280 nm, assuming extinction coefficients of 0.59 cm^2/mg (Gerhart and Holoubek, 1967) and 0.7 cm^2/mg (Collins and Stark, 1969), respectively. The concentration of the regulatory subunit was determined by the Lowry procedure (Lowry *et al.*, 1951) using native aspartate transcarbamylase as the protein standard.

The equipment and procedures used for temperature-jump relaxation measurements have been described elsewhere (*cf.* Faeder, 1970). When CTP binds to native aspartate trans-

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[1] Abbreviation used is: BrCTP, 5-bromocytidine 5′-triphosphate.

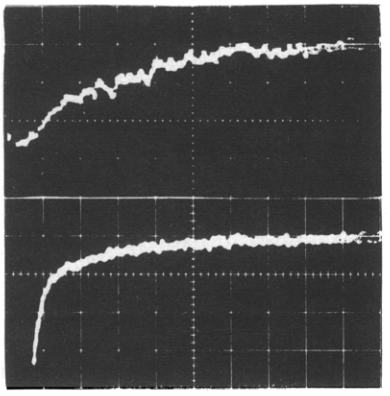

FIGURE 1: Oscilloscope traces of temperature-jump relaxation effects associated with the interaction of CTP and aspartate transcarbamylase in 0.1 M potassium acetate and 2×10^{-5} M Phenol Red, pH 7.5, 13°. The vertical scale is absorbancy (arbitrary units) and the horizontal scale is 100 μsec/large division. The upper trace is for CTP and enzyme in the presence of 1 mM carbamyl phosphate and 10 mM succinate. The concentrations of enzyme and CTP are 1.13×10^{-5} and 60.7×10^{-5} M, respectively. The lower trace is for CTP and enzyme alone. The concentrations of enzyme and CTP are 1.30×10^{-5} and 63.5×10^{-5} M. The sensitivity of the absorbancy scale in the upper trace is twice that of the lower trace. One relaxation process is present in the upper trace and two in the lower trace.

carbamylase, a difference spectrum is observed having absorbance maxima at 290 and 240 nm and a minimum at 267 (Eckfeldt *et al.*, 1970). Because of the large protein absorbance at these wavelengths, the kinetics of the CTP–enzyme interaction could not be followed directly spectrophotometrically. However, relaxation processes associated with the CTP–enzyme interaction could be observed in unbuffered solutions by following the pH changes occurring after a temperature jump. The pH and temperature were varied to optimize the sensitivity of detection, while at the same time remaining under conditions for which equilibrium binding constants could be estimated. A pH of 7.5 ± 0.05 at a final temperature of 13° was selected. The change in pH caused by the temperature jump was followed by measurement of the absorbancy changes at 555 nm of the pH indicator Phenol Red. Solutions were prepared from deionized distilled water and the pH was adjusted with potassium hydroxide or acetic acid. The temperature jump was 7.5° in all cases.

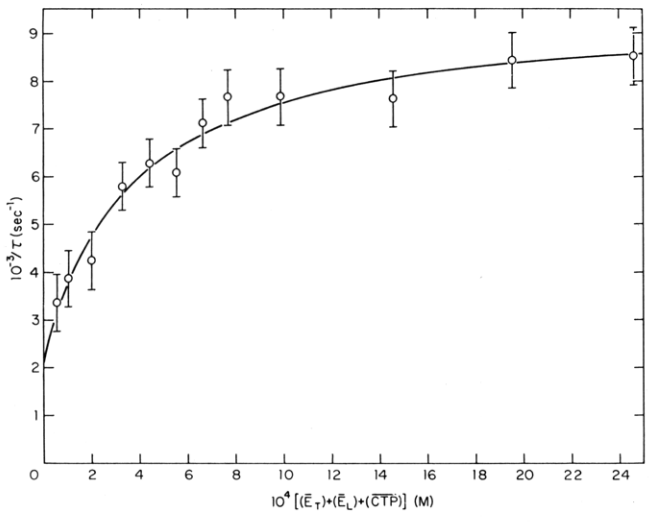

FIGURE 2: A plot of the reciprocal relaxation time, $1/\tau$, for the interaction of CTP with aspartate transcarbamylase as a function of the sum of the equilibrium concentrations of regulatory sites and CTP in the presence of 1 mM carbamyl phosphate and 10 mM succinate. The initial enzyme concentrations varied from 0.67 to 1.3×10^{-5} M and the initial concentrations of CTP varied from 0.7 to 25×10^{-4} M. The curve was calculated with eq 2 and the constants in Table I. The bars represent the estimated experimental uncertainty.

The kinetic information was transferred directly on-line to a PDP-11 digital computer by a Biomation Model 802 transient recorder. This system transfers 1024 data points at a maximum rate of 1 point/0.5 μsec with a precision of 8 bits for each point. The relaxation times and amplitudes were calculated by a least-squares analysis that analyzes the data in terms of one or two relaxation processes. The data of successive measurements on the same reaction mixture also were accumulated to enhance the signal to noise ratio. The data can be collected and stored on paper tape. A more complete description of these data collection and analysis systems is given elsewhere (Hilborn *et al.*, 1973; Hilborn, 1972). In some cases the oscilloscope traces were photographed and analyzed in the usual manner. The results were in good agreement with the computer analysis. The experimental uncertainty in the relaxation times was estimated to be about ±10%, with somewhat larger uncertainties in the relaxation amplitudes.

Results and Treatment of Data

Temperature jump measurements were carried out on the following systems: CTP and regulatory subunit; CTP and native enzyme in the presence and absence of 1 mM carbamyl phosphate and 10 mM succinate, an aspartate analog; and limited measurements of CTP and catalytic subunit in the presence and absence of carbamyl phosphate. In all cases except the last, measurements were made over as wide a concentration range of CTP as feasible. The range of CTP concentrations used was 2×10^{-5} to 2.5×10^{-3} M and that of native enzyme was 6.7×10^{-6} to 1.3×10^{-5} M based on an enzyme molecular weight of 310,000.

The simplest results were obtained for the interaction of CTP and native enzyme in the presence of 1 mM carbamyl phosphate and 10 mM succinate, and these results are considered first. No relaxation process was observed with solutions of enzyme, potassium acetate, and Phenol Red. When 1 mM carbamyl phosphate was added to the enzyme solution a relaxation process was observed; enzyme solutions containing both 1 mM carbamyl phosphate and 10 mM succinate (but no CTP) showed a relaxation process of very small amplitude with a relatively long relaxation time (>1 msec). This process could not be detected in the presence of CTP. Instead a single relaxation process was observed having a relaxation time in the range of 0.1–1 msec. A typical oscilloscope trace of the relaxation process is shown in Figure 1.

The dependence of the reciprocal relaxation time on the sum of the equilibrium concentration of free regulatory sites and CTP is shown in Figure 2. The equilibrium concentrations were calculated by using values of the binding constants determined by Matsumoto and Hammes (1973) extrapolated to 13°. No correction was made for the slight differences in pH. The constants used were 1.75×10^5 M^{-1} for the three "tight" regulatory sites and 7.1×10^3 M^{-1} for the three "loose" regulatory sites. The reciprocal relaxation time reaches a limiting value at high concentrations. The concentration dependence of the amplitude of the relaxation process parallels that of the reciprocal relaxation time, except that the relaxation amplitude decreases gradually at very high CTP concentra-

TABLE I: Kinetic Parameters for the Mechanism of Equation 1.[a]

Ligands	$10^{-3}k_{-2}$ (sec^{-1})	$10^{-3}k_2$ (sec^{-1})	$10^4(K_1 + K_2)$ (M)	k_2/k_{-2}
—	0.99	12.6	6.0	13
1 mM carbamyl phosphate–10 mM succinate	2.13	7.4	3.6	3.5

[a] pH 7.5, 13°, 0.1 M potassium acetate–2×10^{-5} M Phenol Red.

tions (>0.3 mM), probably due to the buffering effect of CTP. These results can be most easily explained by postulating that an isomerization of the enzyme–CTP complex is rate limiting in the binding process. This mechanism is complicated by the existence of two classes of regulatory sites. The simplest assumption to make is that while differences exist in the initial bimolecular reactions, the conformational change is identical at both classes of sites. This mechanism can be written as

$$\begin{array}{l} E_T + CTP \underset{}{\overset{K_1}{\rightleftharpoons}} \\ \qquad\qquad X_1 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} X_2 \\ E_L + CTP \underset{}{\overset{K_2}{\rightleftharpoons}} \end{array} \qquad (1)$$

Here E_L and E_T represent the loose and tight sites, respectively, X_1 and X_2 are different conformations of the enzyme–CTP complex, the K_i are equilibrium constants and the k_i are rate constants. If the steps prior to the formation of X_1 are assumed to be fast relative to the rate of interconversion of X_1 and X_2, the reciprocal relaxation time for the slowest relaxation process is

$$\frac{1}{\tau} = k_{-2} + \frac{k_2}{1 + \dfrac{K_1 + K_2}{[(\bar{E}_L) + (\bar{E}_T) + (\overline{CTP})]}} \qquad (2)$$

where the concentrations are the equilibrium concentrations. The data were fit to this equation by a nonlinear least-squares procedure. The parameters obtained are presented in Table I and the curve in Figure 2 has been calculated with these parameters and eq 2. The term $(K_1 + K_2)/(1 + k_2/k_{-2})$ is equal to 0.80×10^{-4} M, a value which agrees reasonably well with the sum of the dissociation constants for the two sites determined by equilibrium binding studies, 1.46×10^{-4} M (Matsumoto and Hammes, 1973).

In the absence of carbamyl phosphate and succinate, two distinct relaxation processes are associated with the CTP–native enzyme interaction. The faster process has relaxation times in the range of 10–50 μsec, while the slower process has relaxation times in the range 0.1–1 msec. A typical oscilloscope trace for these processes is included in Figure 1. For both processes, the reciprocal relaxation time increases with increasing CTP concentration and appears to approach a limiting value at sufficiently high concentrations, although the detailed concentration dependence of the relaxation times is not identical. The concentration dependence of the relaxation amplitudes parallels that of the reciprocal relaxation times in

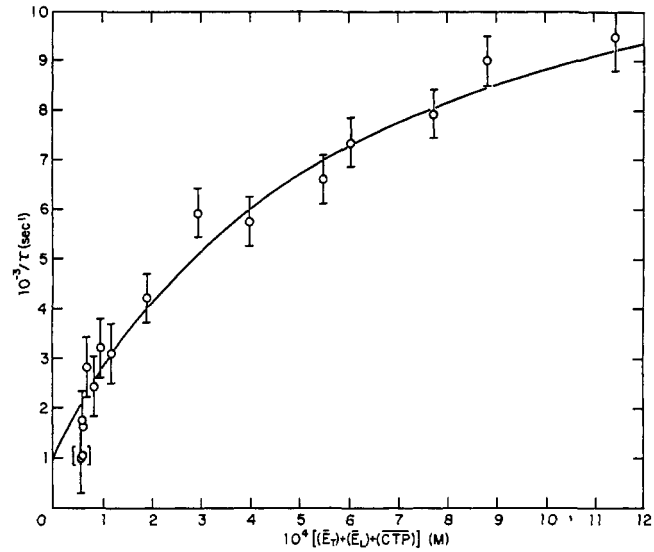

FIGURE 3: A plot of the reciprocal relaxation time, $1/\tau$, for the interaction of CTP with aspartate transcarbamylase as a function of the sum of the equilibrium concentrations of regulatory sites and CTP. The initial enzyme concentrations varied from 0.67 to 1.3×10^{-5} M and the initial concentrations of CTP varied from 0.25 to 12.4×10^{-4} M. The curve was calculated with eq 2 and the constants in Table I. The bars represent the estimated experimental uncertainty.

both cases, with a decrease again occurring at high concentrations. The simplest explanation of the faster process is that it is due to the interaction of CTP with the catalytic site since it is not observed in the presence of 1 mM carbamyl phosphate and 10 mM succinate. A relaxation process with similar relaxation times was observed in solutions of CTP and catalytic subunit. However, the addition of 1 mM carbamyl phosphate caused the appearance of a relaxation process with a larger amplitude which also is observed in the absence of CTP. This relaxation process is very similar to that observed with native enzyme in the presence of 1 mM carbamyl phosphate. This process had not been observed previously (Hammes *et al.*, 1971; Hammes and Wu, 1971a), probably because higher temperatures were used which decreased both the relaxation time and amplitude. A more thorough examination of the interaction of phosphate compounds with the catalytic site is currently in progress. Although the situation is not entirely unambiguous, all available evidence indicates that the faster relaxation process is due to the binding of CTP at the catalytic site; the concentration dependence of the relaxation time and amplitude suggests that a conformational change of the CTP–catalytic site complex is being observed.

The concentration dependence of the slower relaxation time is presented in Figure 3 as a function of the sum of the equilibrium concentrations of the free regulatory sites and CTP. In calculating the equilibrium concentrations, the binding constants associated with the regulatory sites were assumed to be identical to those in the presence of carbamyl phosphate and succinate, and the binding constant associated with the six catalytic sites was taken as 10^3 M^{-1} (Matsumoto and Hammes, 1973). The data are in almost quantitative agreement with those in Figure 2, except at high concentrations where the reciprocal relaxation time in the absence of carbamyl phosphate and succinate becomes distinctly larger than in their presence. Unfortunately relaxation times at concentrations greater than about 1 mM cannot be measured precisely because the two observed relaxation processes cannot be well

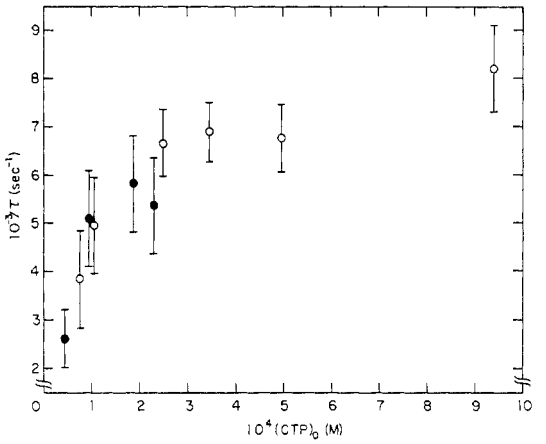

FIGURE 4: A plot of the reciprocal relaxation time, $1/\tau$, for the interaction of CTP with the isolated regulatory subunit of the enzyme as a function of the initial concentration of CTP. The initial protein concentration varied from 1.0 to 1.3 $\times$ 10^{-5} M. The solid circles represent data obtained with the zinc complex of the subunit while the open circles were obtained with the subunit which had been dialyzed against EDTA. The bars represent the estimated experimental uncertainty.

resolved: the two relaxation times differ only by about a factor of two at high CTP concentrations. By analogy with the earlier discussion, the data can be fit to eq 2, which corresponds to the mechanism of eq 1. The implicit assumption is made that the two observed relaxation processes are not appreciably coupled; this is probably valid since the CTP concentration is essentially buffered [(CTP) $\gg$ (E)] and binding at the catalytic and regulatory sites is essentially independent (Matsumoto and Hammes, 1973). The parameters obtained, assuming the mechanism of eq 1 to be valid, are included in Table I and the curve in Figure 3 has been calculated with these parameters and eq 2. The value of $(K_1 + K_2)/(1 + k_2/k_{-2})$ was calculated to be 0.44 $\times$ 10^{-4} M, in fair agreement with the equilibrium measurements. In carrying out the least-squares analysis the two points in brackets were found to deviate appreciably from the calculated curve, and therefore were not used in the final analysis. The significance of these excluded points is considered later.

Since precise values of the relaxation time could not be obtained at concentrations greater than about 1 mM, it cannot be determined if the reciprocal relaxation time in Figure 3 reaches a well-defined plateau. Consequently, another possible mechanism consistent with the data is that the relaxation time is determined by two consecutive bimolecular reactions. The first step involves CTP binding to the "tight" site and the second step involves binding to the "loose" site. This mechanism can be written as

$$\mathrm{E} + \mathrm{CTP} \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} \mathrm{ECTP}$$
$$\mathrm{ECTP} + \mathrm{CTP} \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} \mathrm{E(CTP)_2} \qquad (3)$$

where the relaxation times are

$$1/\tau = \frac{(a_{11} + a_{22}) \pm [(a_{11} + a_{22})^2 - 4(a_{11}a_{22} - a_{12}a_{21})]^{1/2}}{2} \qquad (4)$$

$$a_{11} = k_{12}[(\bar{\mathrm{E}}) + (\overline{\mathrm{CTP}})] + k_{21}$$

$$a_{22} = k_{23}[(\overline{\mathrm{CTP}}) + (\overline{\mathrm{ECTP}})] + k_{32}$$

$$a_{12} = k_{21} - k_{12}(\bar{\mathrm{E}})$$

$$a_{21} = k_{23}[(\overline{\mathrm{CTP}}) - (\overline{\mathrm{ECTP}})]$$

If it is assumed that only the slowest relaxation time is observed, which corresponds to the negative square root of eq 4, the data can be fit as well to this mechanism as to the isomerization mechanism. The parameters obtained are k_{12} = 3.9 $\times$ 10^7 M^{-1} sec^{-1}, k_{21} = 200 sec^{-1}, k_{23} = 5.5 $\times$ 10^6 M^{-1} sec^{-1}, and k_{32} = 3.4 $\times$ 10^3 sec^{-1}. In this calculation, the data obtained from concentrations above 2 $\times$ 10^{-4} M were used to obtain k_{23} and k_{32} since at these concentrations the contribution of the other rate constants is negligible. These values were then fixed and the other rate constants were obtained by a nonlinear least-squares analysis of the entire curve. The values of the equilibrium constants determined from the kinetic parameters are k_{12}/k_{21} = 1.9 $\times$ 10^5 M^{-1} and k_{23}/k_{32} = 1.6 $\times$ 10^3 M^{-1}, in reasonable agreement with the equilibrium binding constants obtained assuming independent sites, 1.8 $\times$ 10^5 and 7.1 $\times$ 10^3 M^{-1}.

The relaxation spectrum associated with the interaction of CTP with the isolated regulatory subunit also was examined. A single relaxation process of very small amplitude was observed. The subunit that was complexed with zinc was found to be associated with relaxation times that were very similar to those found with the subunit prepared by dialysis against EDTA. The data obtained are presented in Figure 4 where the reciprocal relaxation time is plotted versus the total concentration of CTP. The concentration of regulatory subunit was 1.1 $\times$ 10^{-4} M. A mechanistic interpretation of these data is not appropriate since the experimental error in the relaxation times is considerably greater than those in Figures 2 and 3, and reliable equilibrium binding constants are not available. The concentration dependence of the relaxation time obviously parallels that for the CTP–native enzyme interaction, although a distinction cannot be made between a mechanism of coupled bimolecular reactions and a mechanism involving a rate-limiting conformational change. The amplitude of this relaxation process decreases at much lower concentrations of CTP than for the native enzyme, suggesting the possibility of a different mechanism.

Discussion

The interaction between CTP and native aspartate transcarbamylase in the presence and absence of 1 mM carbamyl phosphate and 10 mM succinate can be described by the mechanism of eq 1, which postulates a rate-limiting conformational change in the binding process. This mechanism does not require interactions between the regulatory sites. In fact, the kinetic data do not provide evidence for homotropic interactions. However, a sequential binding mechanism with essentially the same conformational change occurring in each subunit (*cf.* Koshland *et al.*, 1966) or a concerted mechanism similar to that of Monod *et al.* (1965) cannot be excluded. If the complex binding isotherm (Matsumoto and Hammes, 1973) is due to homotropic interactions causing negative cooperativity, a concerted mechanism is not possible. Of course, a more complex mechanism involving both concerted and sequential subunit conformational changes also is possible.

In the absence of carbamyl phosphate and succinate, coupled bimolecular reactions also describe the data well. However, this mechanism seems less likely for several reasons.

First, at low CTP concentrations, the concentration dependence of the relaxation time is very similar in the presence and absence of carbamyl phosphate and succinate. Second, the relaxation amplitude approaches a limiting value which is consistent with the conformational change mechanisms, but not with the bimolecular reaction mechanism. Finally, it is difficult to accept the explanation that the bimolecular reaction can be observed in the absence of carbamyl phosphate and succinate, but not in their presence.

The question as to whether essentially the same conformational change occurs at both tight and loose sites cannot be answered unambiguously. At the lowest enzyme and CTP concentrations, very little of the loose site is occupied, while virtually all of the tight sites are. The deviations of the points at low concentrations from the calculated curve (Figure 3) may be due to the fact that the conformational changes are not exactly the same at both the "tight" and "loose" sites. Unfortunately the sensitivity of the temperature-jump method is not sufficient to make measurements under conditions where the tight sites are not appreciably occupied. Because of this, a mechanism assuming the conformational change occurs only at the "loose" sites also fits the data reasonably well. This mechanism can be represented as

$$E_L + CTP \overset{K_2}{\rightleftharpoons} X_1 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} X_2 \qquad (5)$$

Again assuming rapid equilibration of the first step, the reciprocal relaxation time for the slowest process is

$$\frac{1}{\tau} = k_{-2} + \frac{K_2}{1 + \dfrac{k_2}{[(\bar{E}_L) + (\bar{CTP})]}} \qquad (6)$$

This equation is similar in form to eq 2, and in fact the parameters obtained by a least-squares fitting of the data are essentially identical because $(\bar{E}_T) \ll (\bar{E}_L) + (\bar{CTP})$ under almost all accessible conditions.

The relaxation spectrum observed for the interaction between CTP and the isolated regulatory subunit also is ambiguous. In this case, it is not possible to ascertain whether coupled bimolecular reactions or a rate-limiting conformational change describes the data best.

The simplicity of the relaxation spectrum for such a potentially complex system is surprising. The bimolecular steps associated with ligand binding are not observed over the accessible concentration range. This can be attributed either to the associated relaxation amplitude being too small or to the associated relaxation time being too short. In any event, the assumption made in interpreting the data, namely that the initial binding process is fast compared to the conformational change, requires that the relaxation times associated with the bimolecular reactions are approximately an order of magnitude smaller than the observed relaxation times. This in turn requires that the second order rate constants be greater than about 10^8 M^{-1} sec^{-1}. Second-order rate constants of this order of magnitude are commonly associated with protein–ligand reactions (*cf.* Hammes and Schimmel, 1970).

The results obtained in this work should be compared with an earlier study (Eckfeldt *et al.*, 1970) of the interaction between BrCTP and the enzyme, where the reaction progress was monitored by following the change in the difference spectra accompanying the BrCTP–enzyme interaction. In the latter case, the range of concentrations utilized was necessarily smaller and the heterogeneity of effector binding to the regulatory site (Winlund and Chamberlin, 1970; Buckman, 1970; Matsumoto and Hammes, 1973) was not known at the time of the study. Nevertheless, the experimental results of the two studies are essentially the same. In the presence of carbamyl phosphate and succinate, the reciprocal relaxation time approaches a limiting value at high concentrations in both cases. In the absence of these ligands, the reciprocal relaxation time is a linear function of the appropriate concentration functions over essentially the same concentration range. However, in the case of CTP, lower concentrations could be studied where the linear relationship is no longer valid. A similar comment applies to the results obtained with the isolated regulatory subunit. Thus the same mechanism undoubtedly occurs for the interaction of both CTP and BrCTP with aspartate transcarbamylase, but more extensive data were obtained for the former case.

In terms of the mechanism of eq 1, the most significant change in parameters caused by the presence of carbamyl phosphate and succinate is a decrease in the ratio k_2/k_{-2}. This has significant implications for the allosteric control mechanism. If X_2 is assumed to be the conformation used as the "off" switch and X_1 that used as the "on" switch, then CTP should stabilize the X_2 conformation, while the substrates should stabilize the X_1 conformation, exactly as observed. In an accompanying paper (Wu and Hammes, 1973), evidence is presented that ATP stabilizes the X_1 conformation, again consistent with the proposed control mechanism. Thus the observed conformational change accompanying the binding of CTP to aspartate transcarbamylase probably plays a significant role in the overall allosteric control mechanism.

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Relaxation Spectra of Aspartate Transcarbamylase. Interaction of the Native Enzyme with an Adenosine 5′-Triphosphate Analog†

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ABSTRACT: The interaction of aspartate transcarbamylase from *Escherichia coli* with the activator 6-mercapto-9-β-D-ribofuranosylpurine 5′-triphosphate (sRTP) has been investigated at pH 7.0, 25°, in 0.15 M potassium acetate–0.04 M imidazole acetate using difference spectroscopy and the temperature-jump method. The sRTP does not serve as an affinity label for the regulatory or catalytic sites, but a difference spectrum is observed when sRTP binds to the catalytic subunit, the regulatory subunit and the native enzyme. A spectral titration of the catalytic subunit indicates three binding sites are present per catalytic subunit molecule with a dissociation constant of 2.5×10^{-4} M. Although a difference spectrum also accompanies binding to the regulatory subunit, the binding is too weak for quantitative characterization. In the absence of substrates, the difference spectrum of the sRTP–native enzyme interaction is very similar to that found for the catalytic subunit, but the addition of 2 mM carbamyl phosphate shifts the maximum in the difference spectrum from 332 to 325 nm. Further addition of the aspartate analog, succinate, decreases the absolute magnitude of the difference spectrum, but does not shift the wavelength maximum. The binding of sRTP to the isolated catalytic subunit gives rise to a temperature-jump relaxation process which is too fast and of too low an amplitude for detailed study. With native enzyme, two relaxation processes are seen. The faster one has a time constant similar to that found with the isolated catalytic subunit and disappears in the presence of 2 mM carbamyl phosphate so that it probably reflects the interaction of sRTP with the catalytic site. The reciprocal relaxation time for the slower process increases and approaches a constant value as the sRTP concentration is raised. This behavior is observed in the presence or absence of 2 mM carbamyl phosphate and 10 mM succinate, although the limiting value reached varies. The simplest mechanism consistent with the data is a rapid combination of sRTP and enzyme followed by a rate-limiting conformational change, a mechanism similar to that proposed for the interaction of cytidine 5′-triphosphate with the native enzyme. When both sRTP and 5-bromocytidine 5′-triphosphate are added to the enzyme, only a single relaxation process is observed suggesting that the same two conformational states occur with both activator and inhibitor complexes. Carbamyl phosphate and succinate and sRTP tend to stabilize one conformational state, while cytidine 5′-triphosphate stabilizes the other. A different conformational transition accompanies the binding of succinate to the native enzyme; sRTP decreases the reciprocal relaxation time of this process as its concentration is raised, while 5-bromocytidine 5′-triphosphate has the opposite effect. A multiconformational model involving both concerted and sequential conformational transitions is proposed for the overall regulatory mechanism.

The regulatory properties of aspartate transcarbamylase from *Escherichia coli* (EC 2.1.3.2) have been extensively studied. The feedback inhibition of enzyme activity by CTP controls the production of pyrimidine nucleotides (Gerhart and Pardee, 1962). In addition the purine nucleotide ATP is an activator of the enzyme suggesting that the relative amounts of purine and pyrimidine nucleotides also are regulated by aspartate transcarbamylase (Gerhart and Pardee, 1962). The effect of ATP and CTP on the enzyme activity is manifested by a change in the apparent affinity of the enzyme for aspartic acid. The binding of aspartic acid to the enzyme in the presence of a saturating concentration of carbamyl phosphate follows a sigmoidal binding isotherm, and the sigmoidicity of the isotherm is increased by CTP and decreased by ATP. The allosteric nature of the control mechanism has been convincingly demonstrated by the resolution of the enzyme into a catalytically active subunit which is not subject to nucleotide control and a catalytically inactive subunit which binds CTP strongly (Gerhart and Schachman, 1965). Native aspartate transcarbamylase consists of six catalytic subunits and six regulatory subunits (Weber, 1968; Hammes *et al.*, 1970; Meighen *et al.*, 1970; Winlund and Chamberlin, 1970; Rosenbusch and Weber, 1971; Matsumoto and Hammes, 1973). The binding of ATP and CTP to the enzyme is quite complex; at least two classes of regulatory binding sites on the native enzyme can be distinguished, and the binding isotherm indicates negative cooperativity in the binding process (Winlund and Chamberlin, 1970; Buckman, 1970; Matsumoto and Hammes, 1973).

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