Relaxation Spectra of Aspartate Transcarbamylase. Interaction of the Native Enzyme with Carbamyl Phosphate*

Gordon G. Hammes† and Cheng-Wen Wu

ABSTRACT: Temperature-jump studies were made of the interaction of carbamyl phosphate with aspartate transcarbamylase of *Escherichia coli* by observing the pH changes accompanying binding with a pH indicator. In the absence of aspartate analogs a single relaxation process (τ = 0.1–0.5 msec) could be associated with the bimolecular binding of carbamyl phosphate to native aspartate transcarbamylase. In the presence of 10 mM succinate, two relaxation processes were observed to accompany the binding of carbamyl phosphate to the native enzyme. The faster process ($\tau \sim$ 0.2–1 msec) could be quantitatively analyzed in terms of an ordered bimolecular binding of carbamyl phosphate and succinate, followed by an isomerization of the enzyme–carbamyl phosphate–succinate complex. The rate constants characterizing the isomerization are similar to those found for the succinate-induced conformational change of the carbamyl phosphate–catalytic subunit complex reported previously. These two conformational changes are probably identical in nature and of importance in the catalytic mechanism. The slower relaxation process ($\tau \sim$ 25–50 msec) has a concentration dependence which is consistent with the allosteric control mechanism of Monod *et al.* When BrCTP, an allosteric inhibitor, was added three relaxation processes were observed: one associated with the conformational transition induced by BrCTP in the presence of substrates, and two associated with the processes induced by carbamyl phosphate with 10 mM succinate in the absence of BrCTP. The BrCTP has no effect on the faster relaxation process, but reduced the rate of the slower conformational transition.

This confirms the interpretation suggested above, namely that the faster relaxation process is associated with the catalytic mechanism and the slower process with the control process. On the basis of these and other results a multiconformational model for regulation is proposed.

Most of the regulatory enzymes catalyze reactions involving two or more substrates which introduces a great number of complications in kinetic analyses. Whatever the kinetic mechanism, however, it is often only one of the two or more substrates which gives a sigmoidal velocity response. In fact, all the molecular models for the mode of action of allosteric enzymes have been treated as if only one substrate were involved (Monod *et al.*, 1965; Koshland *et al.*, 1966). Aspartate transcarbamylase of *Escherichia coli:* (ATCase,[1] EC 2.1.3.2) is the first enzyme in the pathway of pyrimidine biosynthesis and catalyzes the formation of carbamyl L-aspartate from L-aspartate and carbamyl phosphate. The final product of this pathway, CTP, is an allosteric inhibitor of this enzyme. The sigmoidal dependence of reaction velocity on the aspartate concentration (Gerhart and Pardee, 1962) and the cooperative binding of a L-aspartate analog (succinate) in the presence of saturating carbamyl phosphate (Changeux *et al.*, 1968) have been well established. Bethell *et al.* (1968) have also found a sigmoidal saturation curve of the initial velocity for carbamyl phosphate at a high constant concentration of L-aspartate. This finding suggests, but does not prove, cooperative binding of carbamyl phosphate. Direct studies of the binding of carbamyl phosphate to ATCase in the absence of L-aspartate or its analog have not been reported.

On the basis of temperature-jump studies we previously reported that BrCTP and L-aspartate analogs (succinate and L-malate) cause two distinct conformational transitions of native ATCase which are probably involved in the allosteric control mechanism (Eckfeldt *et al.*, 1970; Hammes and Wu, 1971). These experiments were carried out in the presence of saturating carbamyl phosphate. The present studies show that when the succinate concentration is high (10 mM), another conformational change related to the allosteric regulation can be detected by addition of carbamyl phosphate, as well as a conformational change probably related only to the catalytic mechanism. The heterotropic effect of BrCTP on the carbamyl phosphate binding to ATCase in the presence of 10 mM succinate is also considered.

Experimental Section

Aspartate transcarbamylase was 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. The enzyme was transferred from phosphate buffer into 0.02 M potassium acetate, 5×10^{-4} M EDTA, and 1×10^{-3} M dithiothreitol (pH 8.0) by extensive dialysis. Concentrations were determined by absorbance at 280 nm, assuming an extinction coefficient of 0.59 cm²/mg (Gerhart and Holoubek, 1967) and a molecular weight of 52,000 per site (Weber, 1968; Hammes *et al.*, 1970).

The dilithium carbamyl phosphate (Sigma Chemical Co.) 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. Under these conditions, inorganic phosphate represents less than 10% of the total phosphate. Aqueous solutions of succinic acid (Sigma Chemical Co.) 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).

* From the Department of Chemistry, Cornell University, Ithaca, New York 14850. *Received November 3, 1970.* This work was supported by a grant from the National Institutes of Health (GM 13292).

† To whom to address correspondence.

[1] Abbreviations used in this work are: ATCase, aspartate transcarbamylase; BrCTP, 5-bromocytidine triphosphate.

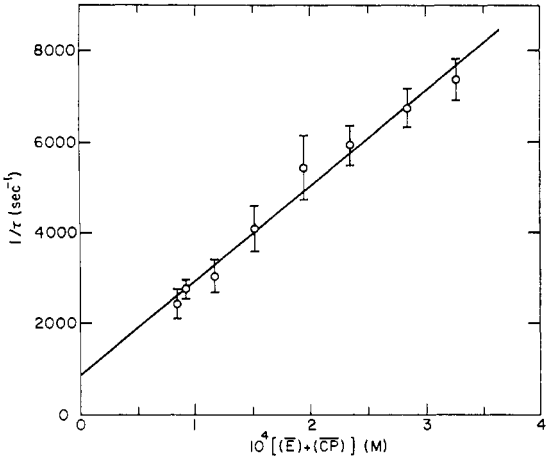

FIGURE 1: The reciprocal relaxation time, $1/\tau$, for the interaction of carbamyl phosphate with native aspartate transcarbamylase as a function of the sum of the equilibrium concentration of the enzyme, ($\bar{E}$), and carbamyl phosphate, ($\overline{CP}$). The initial enzyme concentration was 0.89×10^{-4} M. The initial concentration of carbamyl phosphate varied from 0.75×10^{-4} to 4.0×10^{-4} M and the pH was 7.4 ± 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 as described in the text.

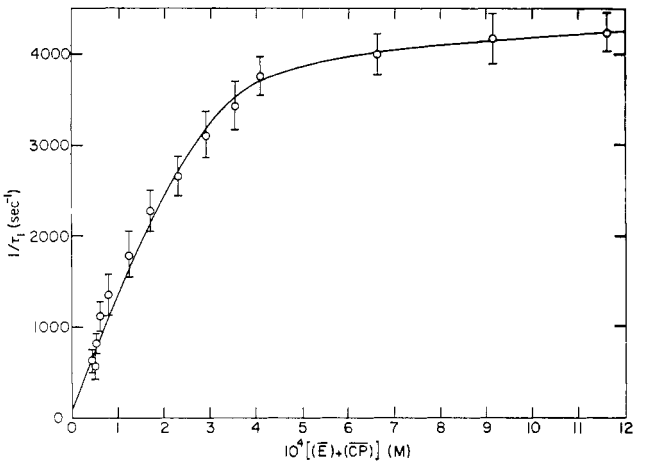

FIGURE 2: The reciprocal relaxation time, $1/\tau_1$, *vs.* the sum of the equilibrium concentrations of the enzyme, ($\bar{E}$), and carbamyl phosphate, ($\overline{CP}$), for the faster relaxation process associated with carbamyl phosphate binding to aspartate transcarbamylase in the presence of 10 mM succinate. The initial concentration of the enzyme was 0.3–0.9 $\times 10^{-4}$ M, and the pH was 7.4. The initial concentration of carbamyl phosphate varied from 1.25×10^{-4} to 1.25×10^{-3} M. The error bars represent the standard deviation for the traces analyzed. The solid line was calculated from eq 5 and the constants given in the text.

Phenol red (Eastman Organic Chemicals) was recrystallized before use.

The temperature-jump apparatus used is described in detail elsewhere (Faeder, 1970). The temperature jump was 7.5° and 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 pH 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 2×10^{-5} M pH indicator (phenol red). Fresh solution was flushed into the reaction cell for each temperature jump. At least six oscilloscope traces were photographed and analyzed for each solution. 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%. A PDP-9 digital computer interfaced with a curve tracer was used to evaluate the relaxation time from the photographed oscilloscope traces. Concentration-jump experiments were performed with a Durrum-Gibson stopped-flow apparatus as described previously (Hammes and Wu, 1971).

Results and Treatment of Data

Carbamyl Phosphate Binding to Aspartate Transcarbamylase in the Absence of Succinate. The temperature-jump studies of the interaction of native aspartate transcarbamylase with aspartate analogs reported previously (Hammes and Wu, 1971) were performed in the presence of saturating concentrations of 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 aspartate analogs, a fast relaxation process not seen previously can be observed. This process was detected by monitoring spectrophotometrically with a pH indicator (phenol red) and was characterized by a relaxation time in the range 0.1–0.5 msec.

The concentration dependence of the relaxation time for carbamyl phosphate at pH 7.4 is shown in Figure 1. The linear relationship between the reciprocal relaxation time, $1/\tau$, and the sum of the equilibrium concentration of the enzyme, ($\bar{E}$), and carbamyl phosphate, ($\overline{CP}$), as shown in Figure 1 suggests a simple bimolecular-binding mechanism

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

where ECP is the enzyme–carbamyl phosphate complex. The reciprocal relaxation time for this mechanism is

$$1/\tau = k_1[(\bar{E}) + (\overline{CP})] + k_{-1} \qquad (2)$$

The data were analyzed by assuming a value of the equilibrium dissociation constant and constructing a plot of $1/\tau$ *vs.* $[(\bar{E}) + (\overline{CP})]$; a least-squares analysis of this plot yielded a new value of the equilibrium constant which was then used to construct a new plot. This process was repeated until the initial and final values of the equilibrium constant were the same. Figure 1 represents the final plot of the data. The rate and equilibrium constants obtained are $k_1 = 2.1 \times 10^7$ M^{-1} sec^{-1}, $k_{-1} = 860$ sec^{-1}, and $k_{-1}/k_1 = 4.1 \times 10^{-5}$ M.

Carbamyl Phosphate Binding to Aspartate Transcarbamylase in the Presence of Succinate. Addition of carbamyl phosphate to a solution of native ATCase and a high, constant concentration of succinate (10 mM) causes the appearance of two well-defined relaxation processes, a faster process in the time range 0.2–1 msec (τ_1) and a slower process in the time range 25–50 msec (τ_2).

The reciprocal relaxation time associated with the faster process ($1/\tau_1$) increases with increasing concentration of carbamyl phosphate up to about 5×10^{-4} M; above this concentration the relaxation time is essentially independent of carbamyl phosphate concentration, as shown in Figure 2. This suggests that an isomerization or conformational change of the enzyme becomes rate limiting in the binding process

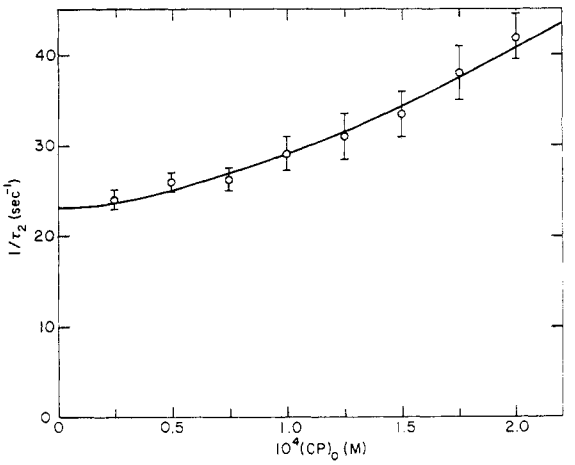

FIGURE 3: The reciprocal relaxation time, $1/\tau_2$, *vs.* the initial carbamyl phosphate concentration, $(CP)_0$, for the slower relaxation process associated with carbamyl phosphate binding to aspartate transcarbamylase in the presence of 10 mM succinate. The initial concentration of the enzyme was 0.2–0.4 $\times$ 10^{-4} M and the pH was 7.4. The initial concentration of carbamyl phosphate varied from 0.25 $\times$ 10^{-4} to 2.0 $\times$ 10^{-4} M. The error bars represent the standard deviations for the traces analyzed. The solid line is the computer fitted curve according to eq 7 and the parameters given in the text.

at high concentrations of enzyme and carbamyl phosphate. A simple mechanism of this type is

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

where ECP′ is the isomerized enzyme–carbamyl phosphate complex. If the first step is assumed to equilibrate much faster than the second step, the slowest reciprocal relaxation time is

$$1/\tau_1 = k_{-2} + \frac{k_2}{1 + k_{-1}/\{k_1[(\bar{E}) + (\overline{CP})]\}} \qquad (4)$$

A PDP-9 computer was programmed to fit the experimental data to eq 4 by searching for the "best-fit" value (least-squares criterion) of the variables k_{-1}/k_1, k_2, k_{-2}, and the equilibrium binding constant $K = k_{-1}/[k_1(1 + k_2/k_{-2})]$. By an iterative procedure, a best-fit equilibrium binding constant of 1.0 $\times$ 10^{-6} M was obtained. Since this value is far too low compared with the $K_{m,app}$ for carbamyl phosphate (5 $\times$ 10^{-5} M, Kleppe, 1966; 2 $\times$ 10^{-4} M, Bethell *et al.*, 1968) this fit of the data is not acceptable.

An alternative interpretation of the data is to assume that both steps in eq 3 equilibrate at comparable rates, and hence the two relaxation processes are coupled. The analytical expressions for the relaxation times can be expressed as

$$1/\tau_1 = (1/2)[(a_{11} + a_{22}) \pm \sqrt{(a_{11} + a_{22})^2 + 4(a_{11}a_{22} + a_{12}a_{21})}] \qquad (5)$$

where $a_{11} = k_1[(\bar{E}) + (\overline{CP}) + k_{-1}$, $a_{12} = k_{-1}$, $a_{21} = k_2$, $a_{22} = k_2 + k_{-2}$. The concentration dependence of the relaxation time corresponding to the negative square root is consistent with the data in Figure 2. With this interpretation, at low concentrations the relaxation time is essentially associated with the bimolecular reaction coupled to a relatively fast isomerization step. However, the bimolecular reaction becomes faster as $[(\bar{E}) + (\overline{CP})]$ becomes larger until the relaxation time essentially characterizes the isomerization step coupled to a relatively fast bimolecular reaction. Again, by means of an iterative computer analysis, the best-fit value of the overall equilibrium binding constant was found to be 0.84 $\times$ 10^{-5} M; and the rate constants obtained are $k_1 = 1.3 \times 10^7$ M^{-1} sec^{-1}, $k_{-1} = 380$ sec^{-1}, $k_2 = 3030$ sec^{-1}, and $k_{-2} = 1230$ sec^{-1}. The reciprocal relaxation time calculated from these parameters according to eq 5 is given as a solid line in Figure 2. As can be seen in the figure, the calculated curve fits the experimental data very well. Moreover, the standard deviation between the calculated and experimental relaxation times was considerably less than for the analysis using eq 4. A relaxation process having a relaxation time similar to the one expected for that associated with the positive square root could be seen, but the relaxation amplitude was too small to permit a quantitative analysis.

The slower relaxation process associated with τ_2 has the concentration dependence, shown in Figure 3, which rules out either a simple bimolecular mechanism (eq 1) or a two-step isomerization mechanism (eq 3). However, a concerted mechanism such as that proposed by Monod *et al.* (1965) is consistent with the experimental data. For aspartate transcarbamylase with six carbamyl phosphate sites (Hammes *et al.*, 1970), this mechanism can be described by eq 6, where R and

$$\begin{array}{ccc}
6CP + R_0 & \overset{k_0}{\rightleftharpoons} & T_0 + 6CP \\
\downarrow\uparrow & & \downarrow\uparrow \\
5CP + R_1 & \overset{k_1}{\rightleftharpoons} & T_1 + 5CP \\
\downarrow\uparrow & & \downarrow\uparrow \\
4CP + R_2 & \overset{k_2}{\rightleftharpoons} & T_2 + 4CP \\
\downarrow\uparrow & & \downarrow\uparrow \\
3CP + R_3 & \overset{k_3}{\rightleftharpoons} & T_3 + 3CP \\
\downarrow\uparrow & & \downarrow\uparrow \\
2CP + R_4 & \overset{k_4}{\rightleftharpoons} & T_4 + 2CP \\
\downarrow\uparrow & & \downarrow\uparrow \\
CP + R_5 & \overset{k_5}{\rightleftharpoons} & T_5 + CP \\
\downarrow\uparrow & & \downarrow\uparrow \\
R_6 & \overset{k_6}{\rightleftharpoons} & T_6
\end{array} \qquad (6)$$

T are different conformational states of the enzyme, K_R and K_T are the intrinsic dissociation constants for the binding of carbamyl phosphate 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. If the simplifying assumptions are made that (1) the vertical steps equilibrate rapidly relative to the horizontal steps, (2) the concentration changes of the free ligand during equilibration are negligible, and (3) all transitions from the R to T states are characterized by identical rate constants, namely, $k_0 = k_1 = k_2 = \cdots = k_6$ (for a discussion of the validity of these assumptions, see Hammes and Wu, 1971), the reciprocal relaxation time associated with the

conformational transitions can be expressed as (*cf.* Eckfeldt *et al.*, 1970)

$$1/\tau_2 = k_0 + k_{-0}\left(\frac{1 + (\overline{CP})/K_R}{1 + (\overline{CP})/K_T}\right)^6 \qquad (7)$$

By varying the four unknown parameters in the above equation, a best-fit curve to the experimental data (τ_2) was obtained with a computer analysis based on a least-squares criterion. This is shown as a solid line in Figure 3, and the best-fit kinetic parameters are $k_0 = 22$ sec^{-1}, $k_{-0} = 0.58$ sec^{-1}, $K_R = 0.89 \times 10^{-4}$ M, $K_R/K_T = 0.37$.

Effect of BrCTP on the Carbamyl Phosphate–Aspartate Transcarbamylase Interaction. A comparison of the relaxation times for ATCase–carbamyl phosphate–BrCTP solutions (with saturating succinate) in the absence and presence of 2.5×10^{-5} M BrCTP is given in Table I. Because the triphosphate tends to buffer the pH changes used to monitor the carbamyl phosphate–aspartate transcarbamylase interaction, a relatively low concentration of BrCTP was used for the study.

In the presence of BrCTP, three relaxation processes were observed. All of these 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 (Eckfeldt *et al.*, 1970). As shown in Table I, under these experimental conditions, BrCTP has no significant influence on the relaxation time τ_1. On the other hand, it decreases the value of $1/\tau_2$. Since convection effects in the temperature-jump apparatus can sometimes obscure relatively slow relaxation processes, values of τ_2 were also determined by concentration-jump experiments using a stopped-flow apparatus. As shown in Table I, these values are essentially the same as obtained with the temperature-jump apparatus.

A third relaxation process, τ_3, was detected when BrCTP was added to solutions containing carbamyl phosphate, enzyme and saturating succinate. This effect is undoubtedly the same as that observed previously (Eckfeldt *et al.*, 1970), and is associated with a conformational transition induced by BrCTP in the presence of both succinate and carbamyl phosphate. These two effects have similar relaxation times and disappear in the absence of BrCTP. Alternatively this relaxation process could conceivably be associated with the positive square root of eq 5. Although the lack of concentration dependence of the observed relaxation times and the absence of the process in the absence of BrCTP make this seem unlikely, this possibility cannot be rigorously excluded.

TABLE I: Relaxation Times for ATCase–Carbamyl Phosphate–BrCTP Solutions at 28.[a]

10^4 $(CP)_0$ (M)	No BrCTP		2.5×10^{-4} M BrCTP		
	$1/\tau_1$ (sec^{-1})	$1/\tau_2$ (sec^{-1})	$1/\tau_1$ (sec^{-1})	$1/\tau_2$ (sec^{-1})	$1/\tau_3$ (sec^{-1})
1.25	1230	31 (35)[c]	1260 (1310)[b]	19 (21)[b] (18)[c]	4500 (4700)[b]
1.5	1390	33	1350 (1370)[b]	20 (21)[b]	4310 (4710)[b]
2.0	2090	40	2280	22	4370

[a] Temperature jump. Reaction mixture: 0.3–0.9 $\times 10^{-4}$ M ATCase, 0.1 M potassium acetate (pH 7.4), 2×10^{-5} M phenol red, and 10 mM succinate. Concentrations of carbamyl phosphate and BrCTP as indicated above, monitored at 560 nm. [b] Temperature jump; monitored at 308 nm. [c] Concentration jump; monitored at 560 nm.

Discussion

The concentration dependence of the reciprocal relaxation time shown in Figure 1 suggests a simple bimolecular reaction for the binding of carbamyl phosphate to native ATCase in the absence of L-aspartate analogs. Carbamyl phosphate binding to the catalytic subunit was found to follow the same mechanism (Hammes *et al.*, 1971). The values obtained for the equilibrium dissociation constant (4×10^{-5} M) are identical for both the native enzyme and its catalytic subunit. No evidence of any irregularity due to sigmoidal binding can be found, although the concentration range studied was by necessity rather small.

In the presence of 10 mM succinate, the situation becomes much more complex. Two relaxation processes have been detected. The faster process (τ_1) occurs in a time range similar to that found in the absence of succinate. However, the concentration dependence of the reciprocal relaxation time shown in Figure 3 indicates that the carbamyl phosphate binding is not a simple bimolecular reaction under these conditions. The data are quantitatively consistent with a coupled two-step mechanism (eq 3): a bimolecular reaction followed by an isomerization of the complex formed. Again no evidence of unusual behavior, such as sigmoidal saturation, can be detected.

A question might be raised here that if the binding of carbamyl phosphate induces the isomerization of the complex formed, why is such a conformational change not observed in the absence of succinate? Actually, the isomerization requires the participation of succinate in the manner described by eq 8.

$$E + CP \rightleftharpoons ECP \underset{\text{fast}}{\overset{S}{\rightleftharpoons}} ECPS \rightleftharpoons ECPS' \qquad (8)$$

Here ECPS and ECPS′ are two isomerized forms of the enzyme–carbamyl phosphate–succinate complex. In the absence of succinate (S), the isomerization does not take place (or at least it cannot be detected). On the other hand, since the second step equilibrates rapidly with 10 mM succinate (Hammes and Wu, 1971), the overall effect observed will be the coupling of steps 1 and 3, which is kinetically indistinguishable from the mechanism given in eq 3. In fact, increasing the fraction of enzyme bound to carbamyl phosphate also increases the fraction of enzyme bound to succinate, so that it cannot be ascertained which substrate actually causes the observed behavior of the relaxation time. The interpretation presented here assumes that the concentration dependence is due to the binding of carbamyl phosphate, but that the conformational change of the enzyme occurs after the binding of succinate. This ordered binding mechanism proposed for carbamyl phosphate and L-aspartate analogs (succinate) is consistent with the following results: a steady-state kinetic study of the reaction catalyzed by the catalytic subunit indicates that carbamyl phosphate binds first and L-aspartate binds second (Porter *et al.*, 1969); by use of equilibrium dialysis Changeux *et al.* (1968) have demonstrated that the binding of succinate to both native ATCase and its catalytic subunit requires the presence of carbamyl phosphate for tight binding; and finally

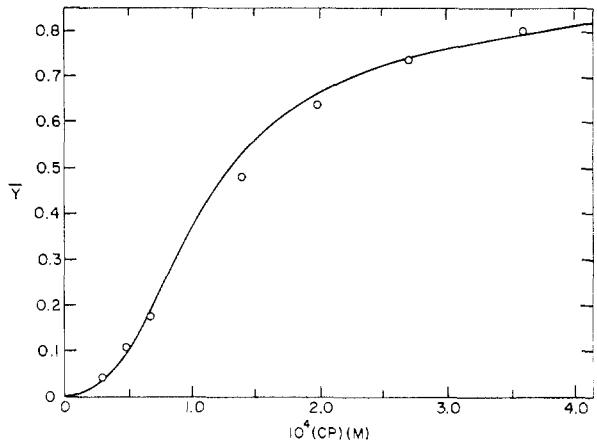

FIGURE 4: A plot of the fraction of enzyme saturated by carbamyl phosphate, $\bar{Y}$, *vs.* the concentration of carbamyl phosphate in the presence of high concentrations of aspartate or its analog. The curve represents the theoretical binding isotherm predicted for carbamyl phosphate in the presence of 10 mM succinate according to the concerted mechanism of eq 6 and the parameters given in the text. The circles are calculated from the enzyme activity measurements by Bethell *et al.* (1968) at high concentrations of L-aspartate (varying the carbamyl phosphate concentration). In this case, $\bar{Y}$ is expressed as v/V_m, where v is the initial velocity and V_m is the maximal velocity.

the relaxation processes associated with the conformational transitions of succinate binding to native ATCase vanish if no carbamyl phosphate is present (Hammes and Wu, 1971).

The rate constants characterizing the conformational transitions (k_2 and k_{-2}) are similar to those associated with the conformational transitions of the catalytic subunit induced by succinate in the presence of saturating carbamyl phosphate ($k_2 = 4600$ sec^{-1}, $k_{-2} = 620$ sec^{-1}, Hammes *et al.*, 1971). The latter conformational change has been postulated to be of importance in the catalytic mechanism. Very likely, these two conformational changes are identical in nature, but are observed by coupling with the binding of different ligands (the former with carbamyl phosphate binding in the presence of 10 mM succinate, and the latter with succinate binding in the presence of saturating carbamyl phosphate). This assumption is further supported by the fact that BrCTP, an allosteric inhibitor for the native enzyme but not for the catalytic subunit, has no effect on values of τ_1 (Table I). Furthermore, the equilibrium binding constant (k_{-1}/k_1) calculated according to eq 3 agrees well with that for carbamyl phosphate binding in the absence of succinate, and the rate constants are also quite similar.

Finally, it should be mentioned that an attempt was made to fit the data in Figure 2 to the allosteric model of Monod *et al.* (1965), using the restricted analysis presented in this and previous work (Eckfeldt *et al.*, 1970; Hammes and Wu, 1971). A satisfactory fit of the data could not be obtained, but this may not be the case if no restrictions are made on the relationship between the rate constants.

An interpretation of the slow relaxation time (τ_2) is not easy. At a saturating concentration of carbamyl phosphate and nonsaturating concentrations of succinate, a slow relaxation process is also seen ($1/\tau \geq \sim 50$ sec^{-1}; Hammes and Wu, 1971). This may be related to the relaxation process observed at high succinate concentrations and nonsaturating concentrations of carbamyl phosphate since $1/\tau_2 \leq \sim 40$ sec^{-1}. At saturating concentrations of both carbamyl phosphate and succinate this process cannot be seen, so that it cannot be ascertained whether one or two processes are actually occurring. A number of differences exist in the behavior of the relaxation times observed under these two different sets of conditions: the reciprocal relaxation time at a saturating carbamyl phosphate concentration and varying succinate concentration decreases with increasing succinate concentration, whereas $1/\tau_2$ increases with the increasing carbamyl phosphate concentration (Figure 3); the addition of BrCTP increases the reciprocal relaxation time in the former case and decreases it in the latter case; and the relaxation time varies from 2 to 20 msec in the range of 2–25 $\times$ 10^{-4} M succinate (10^{-3} M carbamyl phosphate), whereas it varies from 25 to 50 msec in the range of 0.25–2 $\times$ 10^{-4} M carbamyl phosphate (10 mM succinate). (However, in the latter case, the concentration dependence of the relaxation time could be attributed to increased saturation of the enzyme with succinate as the concentration of carbamyl phosphate is increased as discussed above.) Therefore, these two effects may be quite distinct, and in fact this is the basis for the analysis given in the results section.

Nevertheless, the analysis given only shows that a concerted mechanism is consistent with the data; it does not definitely rule out a complex sequential mechanism. However, if such a mechanism were occurring it would involve an extraordinary slow bimolecular reaction, which seems unlikely. The interpretation of the data given in the Results section involves some quite restrictive assumptions which have been discussed in detail elsewhere (Hammes and Wu, 1971). Obviously a unique fit of such a small amount of data to the mechanism of eq 6 is not possible because of the large number of adjustable parameters. Therefore, the derived parameters should only be given semiquantitative significance. Also the assumption that the cooperative unit contains six binding sites is by no means certain. Nevertheless, the predicted binding isotherm for carbamyl phosphate in the presence of 10 mM succinate is remarkably similar to the sigmoidal dependence of the initial velocity of the enzymatic reaction (expressed as v/V_m, where v is the initial velocity and V_m is the maximal velocity) on the concentration of carbamyl phosphate at high concentrations of L-aspartate (Bethell *et al.*, 1968). This is shown in Figure 4. This suggests that when L-aspartate or its analog is at high concentrations and carbamyl phosphate concentration is nonsaturating, the conformational transition associated with τ_2 is a rate-limiting step involved in the regulation of enzyme activity.

An important piece of evidence in accord with the interpretation given above is the effect of BrCTP on the relaxation processes. When the enzyme is saturated with carbamyl phosphate, BrCTP increases (or leaves unchanged) the reciprocal relaxation time associated with the conformational change induced by succinate (Hammes and Wu, 1971). On the other hand, at a succinate concentration of 10 mM, BrCTP decreases the reciprocal relaxation time, $1/\tau_2$, associated with the conformational transition induced by carbamyl phosphate. In both cases BrCTP antagonizes the effect of succinate or carbamyl phosphate, and alters the rate of the conformational changes. In accordance with these findings, Bethell *et al.* (1968) observed that CTP decreases the affinity of ATCase for carbamyl phosphate when the aspartate concentration is high, and decreases the affinity for aspartate when the carbamyl phosphate concentration is high. Furthermore, these effects could be attributed to binding of the nucleotides at the regulatory subunit, rather than to competitive inhibition of the carbamyl phosphate binding. In an analogous fashion, carbamyl phos-

TABLE II: Elementary Steps Detected with Relaxation Techniques.

Reactant	Mechanism	Effectors[a]
	Native Enzyme	
BrCTP	Bimolecular association–dissociation	
Carbamyl phosphate	Bimolecular association–dissociation	
BrCTP (10 mM succinate–1 mM carbamyl phosphate)	Conformational change (concerted or stepwise)	Carbamyl phosphate, succinate
Carbamyl phosphate (10 mM succinate)	Conformational change (stepwise)	
Carbamyl phosphate (10 mM succinate)	Conformational change (concerted)	BrCTP
Succinate (1 mM carbamyl phosphate)	Conformational change (concerted)	BrCTP
L-Malate (1 mM carbamyl phosphate)	Conformational change (concerted)	BrCTP
	Regulatory Subunit	
BrCTP	Bimolecular association–dissociation	
	Catalytic Subunit	
Carbamyl phosphate	Bimolecular association–dissociation	
Succinate (1 mM carbamyl phosphate)	Conformational change (stepwise)	
L-Malate (1 mM carbamyl phosphate)	Conformational change (stepwise)	

[a] Substances which influence the rate process in a manner not due to direct competition for the binding site.

phate decreases the rate of the conformational transition induced by BrCTP (at high concentrations of succinate), with an apparent binding constant for carbamyl phosphate of 1.4×10^{-4} M (Eckfeldt *et al.*, 1970).

A number of discrete steps have been observed to accompany the binding of substrates and effectors to aspartate transcarbamylase and its subunits (Eckfeldt *et al.*, 1970; Hammes *et al.*, 1970; Hammes and Wu, 1971). The results that have been observed are summarized in Table II. The conclusions which can be derived from these results can be briefly summarized as follows. (1) The bimolecular reactions of BrCTP and of carbamyl phosphate with the enzyme are quite similar for the native enzyme and its subunits, and antagonistic effects between effectors and substrates are not observed. (2) One of the conformational changes induced by succinate and carbamyl phosphate is essentially the same for the native enzyme and catalytic subunit. Since BrCTP has no effect on this process, it is presumably part of the catalytic mechanism. (3) Three distinct conformational changes are seen only with the native enzyme in the presence of various combinations of carbamyl phosphate, succinate, and BrCTP. Moreover, the rates of the conformational changes display an interactive relationship between BrCTP and the other two reactants. This implies that these conformational changes are involved in the control mechanism.

The conformational changes associated with succinate and carbamyl phosphate binding appear to be concerted in nature and that associated with BrCTP may be concerted or may involve a stepwise mechanism. In any event, the overall control mechanism is apparently a combination of all these molecular changes and must be considerably more complex than the limiting models presented by Monod *et al.* (1965) and Koshland *et al.* (1966). The multiplicity of conformational changes involved in the regulatory mechanism is clearly a desirable and reasonable feature: it provides versatility and sensitivity of the control mechanism to a variety of different molecules.

Buckman (1970) found that CTP and ATP perturbed the electron spin resonance spectrum of a spin label, whereas succinate and carbamyl phosphate did not; however, the spectral changes observed with CTP could be reversed by succinate and CTP. These results also suggest a complex pattern of conformational changes. The measurements of the sulfhydryl reactivity of the enzymes to *p*-chloromercuribenzoate and the peptide digestivity by proteolytic enzymes provide additional evidence that a multiplicity of conformational changes exists (Changeux *et al.*, 1968; von Fellenberg *et al.*, 1968; McClintock and Markus, 1968, 1969). However, in both cases the measurements cannot distinguish between the many conformational changes apparently occurring, nor can they readily distinguish between the conformational changes involved in catalysis and those involved in the control mechanism. Therefore, definitive conclusions about the mechanism of the allosteric regulation cannot be obtained from such measurements.

Although questions remain to be answered, the mechanism of the control process for aspartate transcarbamylase has been broken down into a number of elementary steps. The complexity found indicates that construction of any simple model to describe the regulatory mechanism is unlikely to be successful.

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Kinetic and Equilibrium Studies on the Interaction of Ribonuclease A and 2′-Deoxyuridine 3′-Phosphate*

Frederick G. Walz, Jr.

ABSTRACT: The ionization constants for the secondary phosphate and ring nitrogen groups of 2′-deoxyuridine 3′-phosphate were determined. Kinetic and equilibrium binding studies of the interaction of ribonuclease with this nucleotide were performed using the temperature-jump and difference spectra methods, respectively. Experiments were performed in the pH range 4.5–7.0 at 25°. Association constants and maximum difference extinction coefficients for 2′-deoxyuridine 3′-phosphate binding were determined at six pH values. Two relaxation processes were observed which can be attributed to an initial association–dissociation of the enzyme and deoxynucleotide followed by an isomerization of the enzyme–deoxynucleotide complex. The pH dependence of the individual rate constants was determined for this mechanism. There is no indication that 2′-deoxyuridine 3′-phosphate preferentially binds one of the isomeric states of ribonuclease. A detailed minimal mechanism for the pH dependence of the relaxation processes is presented and requires the participation of two acid-base groups of ribonuclease. Differences in these results with those previously reported for ribonuclease binding with uridine 3′-phosphate are discussed with reference to the participation of the nucleotide 2′-hydroxyl group on interaction with ribonuclease.

The RNase[1]-catalyzed degradation of RNA has been characterized as proceeding *via* a two-step process involving the formation and hydrolysis of pyrimidine 2′:3′-cyclic phosphate intermediates (Markham and Smith, 1952; Brown *et al.*, 1952). The 2′-hydroxyl group of phosphodiester substrates (*e.g.*, RNA and 3′-pyrimidine dinucleoside phosphates) has been postulated to interact with a basic group on RNase in a variety of reaction mechanisms proposed for the enzyme-catalyzed transesterification (Findley *et al.*, 1962; Ramsden and Laidler, 1966; Hammes, 1968; Usher, 1969; Roberts *et al.*, 1969). Likewise, the reverse of the hydrolytic step (*i.e.*, the formation of pyrimidine 2′:3′-cyclic phosphate from the 3′-nucleotide product) in these mechanisms is postulated, at least implicitly, to involve an interaction of the 2′-hydroxyl group of the 3′-nucleotide product with a basic group on the enzyme. The importance of the 2′-hydroxyl group in the binding of nucleosides with RNase was shown by inhibition studies of RNase activity (Ukita *et al.*, 1961), where cytidine was two times more effective as an inhibitor than deoxycytidine. Furthermore, binding studies of RNase with a variety of nucleotides including the phosphobenzyl ester of thymidine 3′-phosphate indicated the importance of a 2′-oxygen atom which apparently was required to give a significant difference optical rotatory dispersion curve for the RNase·nucleotide complex (Deavin *et al.*, 1966).

The present study concerning the pH dependence of the kinetic and equilibrium constants characterizing the interaction of 3′-dUMP with RNase were undertaken in an effort to elucidate the role of the 2′-hydroxyl group in nucleotide 3′-phosphate binding. The results of this study in conjunction with those from similar studies on RNase·3′-UMP binding (Hammes and Walz, 1969) provide, by comparison, an assessment of the participation of the 2′-hydroxyl group in the RNase·3′-nucleotide interaction.

Materials

Ribonuclease A. Phosphate-free, lyophilized ribonuclease A was obtained from Worthington Biochemical Corp. and was used without further purification. The concentration of ribonuclease A was determined as described previously (Hammes and Walz, 1969).

* From the Department of Biological Sciences, State University of New York at Albany, Albany, New York 12203. *Received October 26, 1970.* This work was supported by grants from the Research Foundation of the State University of New York and the National Science Foundation (GB-12750).

[1] The abbreviations used in this paper are RNase, bovine pancreatic ribonuclease A; 3′-dUMP, 2′-deoxyuridine 3′-phosphate; 3′-UMP, uridine 3′-phosphate; 3′-CMP, cytidine 3′-phosphate.