Relaxation Spectra of Aspartate Transcarbamylase. I. Interaction of 5-Bromocytidine Triphosphate with Native Enzyme and Regulatory Subunit*

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ABSTRACT: Equilibrium and kinetic studies of the interaction of the modifier analog 5-bromocytidine triphosphate (BrCTP) with native aspartate transcarbamylase and its regulatory subunit have been carried out utilizing difference spectroscopy and the temperature-jump method. Binding constants were determined under a variety of conditions. A single relaxation process was observed with relaxation times in the range of 0.1–1 msec for solutions containing BrCTP and native ATCase, both in the presence and absence of carbamyl phosphate and the aspartate analog, succinate, and for solutions containing BrCTP and the regulatory subunit. Rapid mixing experiments revealed that the entire binding process was complete in less than 5 msec. The concentration dependence of the relaxation times indicates a bimolecular reaction is rate determining in the case of the regulatory subunit, and probably for the native enzyme in the absence of substrates. In the presence of carbamyl phosphate and succinate, a conformational change of the native enzyme becomes rate limiting; a corresponding change in mechanism is not observed with the regulatory subunit. These data are consistent with two types of allosteric control mechanisms: a mechanism similar to that of Monod *et al.* involving two conformational states of the enzyme and one involving a rapid bimolecular reaction followed by a relatively slow conformational change. The concentration dependence of the relaxation time in the presence of phosphate is best explained by the two state mechanism. Therefore, this type of mechanism is most consistent with all of the data.

Aspartate transcarbamylase from *Escherichia coli* is one of the best-known and characterized regulatory enzymes. The first enzyme in the pathway of pyrimidine biosynthesis, it catalyzes formation of carbamyl-L-aspartate from L-aspartate and carbamyl phosphate. Cytidine triphosphate, the ultimate product of that pathway, strongly inhibits ATCase[1] in a characteristic "feedback" manner (Gerhart and Pardee, 1962). Because of the lack of chemical similarity between inhibitor and substrates, ATCase was early proposed to be an allosteric enzyme (Monod *et al.*, 1965) *i.e.*, one which possesses distinct, separate (but interacting) binding loci for substrates and inhibitors. This was demonstrated conclusively by showing that the inhibitor binding sites reside in regulatory subunits which can be physically separated from that part of the enzyme containing catalytic sites (Gerhart and Schachman, 1965). These isolated regulatory subunits can bind CTP but are catalytically inert; the catalytic subunits are enzymatically active but are not inhibited by CTP. Recombination of the separated subunits gives almost complete restoration of the original allosteric properties.

Two limiting models have been proposed to account for the allosteric behavior of regulatory enzymes, both based on the subunit structure of the enzyme and the change of conformational equilibria accompanying ligand binding. The concerted model of Monod *et al.* (1965) postulates the existence of reversible equilibria between two conformational states of the enzyme. Each conformational state has a different affinity for the ligand; therefore the conformational equilibria shift when ligand binds to the enzyme. An alternative mechanism, which is an extension of the Adair model for oxygen binding to hemoglobin (Adair, 1925a,b), postulates a sequential change of the enzyme conformation as each ligand is bound (Koshland *et al.*, 1966).

Equilibrium dialysis, sedimentation velocity experiments, and sulfhydryl reactivity (Changeux *et al.*, 1968; Gerhart and Schachman, 1968; Changeux and Rubin, 1968) have been quantitatively analyzed in terms of a concerted model. On the other hand, McClintock and Markus (1968, 1969) have found that the model best describing the tryptic digestivity and sulfhydryl reactivity depends on which ligands occupy the catalytic sites. At this time a detailed mechanistic explanation of the allosteric properties of ATCase is still not available.

In principle, kinetic studies of the elementary steps involved in substrate and modifier binding to ATCase can establish the mechanism of the allosterism. We report here a temperature-jump study of the interaction of the modifier analog 5-bromocytidine triphosphate (BrCTP) with native ATCase and its regulatory subunit. The results show that the binding mechanism is bimolecular for the regulatory subunit and probably for the native enzyme in the absence of substrates, but in the case of native ATCase in the presence of carbamyl phosphate and the aspartate analog, succinate, a conformational change becomes rate limiting. The conformational change, which requires the intact enzyme and substrates, can be quantitatively interpreted in terms of a concerted model for allosterism. A simple type of sequential model also fits the

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

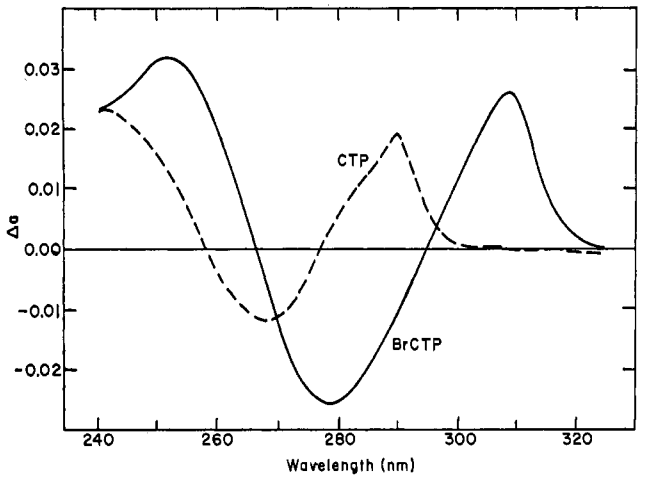

FIGURE 1: Ultraviolet difference spectra for the interaction of ATCase with CTP and BrCTP. Absorbance of the sample cell which contains the native ATCase and CTP (or BrCTP) in compartment a and the buffer in compartment b, minus the absorbance of the reference cell which contains enzyme alone in compartment a and CTP (or BrCTP) in compartment b. The path length of each compartment is 1 cm. Measurements were done at 25 ± 0.1° on a Zeiss PMQ II spectrophotometer. The buffer was 0.15 M KCl–0.04 M potassium phosphate, pH 7.0: (- - - -) 1.0×10^{-4} M CTP–1.03×10^{-5} M ATCase; (———) 8.6×10^{-5} M BrCTP–9.5×10^{-6} M ATCase.

TABLE I: Dissociation Constants and Difference Extinction Coefficients of BrCTP–ATCase and BrCTP–Regulatory Subunit Complexes at 25°.

System	Substrate	$10^5 K$ (M)	$\Delta\epsilon_{308}$[c] (M^{-1} cm^{-1})
ATCase–BrCTP[a]	None	5.2	750
ATCase–BrCTP[a]	2 mM Carbamyl phosphate		
	10 mM Succinate	10.2	1050
ATCase–BrCTP[b]	None	5.0	775
ATCase–BrCTP[b]	2 mM Carbamyl phosphate		
	10 mM Succinate	6.25	790
RSU–BrCTP[a]	None	6.7	850

[a] 0.15 M KCl and 0.02 M imidazole-HCl, pH 7.0. [b] 0.15 mM KCl and 0.02 M potassium phosphate, pH 7.0. [c] Assuming an equivalent protein molecular weight of 52,000 per site for the native enzyme and 17,000 per site for the regulatory subunit.

data. However, results obtained in the presence of phosphate suggest that the concerted mechanism provides the most consistent interpretation of all of the data.

Experimental Section

Enzyme. Aspartate transcarbamylase was prepared according to the procedure of Gerhart and Holoubek (1967) starting with frozen cells of an overproducing mutant strain of *E. coli*. We are indebted to Dr. K. Weber for supplying us with the bacterial strain, and to the New England Enzyme Center for growing some of the bacteria used. Approximately 330 g wet weight of cells was processed at a time, yielding about 2 g of purified enzyme. Disc electrophoresis (Canalco standard gel, pH 9.5) indicated that the final product was at least 90% pure. In addition to the major band, there was a minor band corresponding to the catalytic subunit, a very faint band migrating at the position of the regulatory subunit, and an unidentified band moving slightly ahead of the major band. The purified enzyme could be crystallized, and after crystallization the catalytic subunit band was absent and the unknown contaminant was very much reduced. The experiments reported here were performed with uncrystallized enzyme. Concentrations were calculated from the absorbance at 280 nm and the published extinction coefficient, 0.59 cm^2/mg (Gerhart and Holoubek, 1967). The ratio of absorbance at 280 nm to the absorbance at 260 nm was always 1.75 or greater. For experiments in imidazole buffer, the purified enzyme after centrifugation at 80,000*g* for 15 min was dialyzed against 50 volumes of 0.04 M imidazole-HCl, pH 7.0, 0.001 M dithiothreitol, and 0.0002 M EDTA. This buffer was changed once during the course of dialysis.

The regulatory subunit of ATCase was prepared by the method of Gerhart and Holoubek (1967) with the exception that 0.001 M dithiothreitol was substituted for 2-mercaptoethanol in the final dialysis step. A_{280}/A_{260} ratios were 1.24–1.27 for these preparations. Concentrations of regulatory subunits were determined by the Lowry procedure (Lowry *et al.*, 1951) with native ATCase as a standard.

Chemicals. Cytidine triphosphate was purchased from P-L Biochemicals. 5-Bromocytidine triphosphate was synthesized by bromination of CTP in formamide as described for BrdCTP (Bessman *et al.*, 1958). The product was isolated by precipitation with two volumes of 0.1 M barium acetate, treatment with Dowex 50 (NH_4^+) to remove barium ion, and column chromatography at 4° on DEAE-cellulose. A linear gradient of 0.01–0.7 M ammonium acetate was used for elution, and peak fractions were lyophilized. Paper chromatography on DEAE-cellulose paper in 0.6 M ammonium formate, pH 3.1 (Morrison, 1968), showed that no breakdown to di- or monophosphate occurred during preparation of BrCTP or after storage of solutions for several months at −20°. The ultraviolet absorption spectrum of BrCTP at neutral pH showed the expected peak at 289 nm. Concentrations of BrCTP were calculated from the published molar extinction coefficient (9200 M^{-1} cm^{-1} at 299 nm) for BrdCTP in 0.1 N HCl (Bessman *et al.*, 1958). Succinic acid was obtained from Sigma Chemical Co. Aqueous solutions of succinic acid were neutralized with KOH to pH 7.0. Dilithium carbamyl phosphate, also from Sigma Chemical Co., was further purified by the method described by Gerhart and Pardee (1962). All carbamyl phosphate solutions were freshly prepared, kept in ice, and used within 2 hr. Imidazole, purchased from Eastman Chemical Corp., was recrystallized once from benzene.

Ultraviolet Difference Spectroscopy. Difference spectra were measured in rectangular quartz tandem cells (Pyrocell Manufacturing Co.) having either 1.0-cm or 0.44-cm path length in each chamber. All spectra were determined in the same buffers that were used for temperature-jump experi-

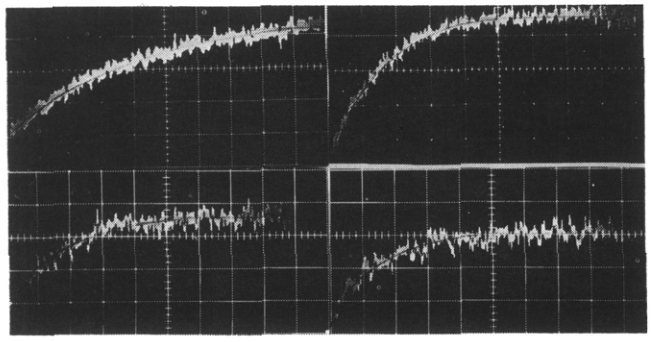

FIGURE 2: Temperature-jump relaxation effects of BrCTP with ATCase and regulatory subunit in 0.15 M KCl and 0.02 M imidazole-HCl, pH 7.0. The vertical scale is absorbancy (arbitrary scale) and the horizontal scale is time: upper, native ATCase + BrCTP. The concentrations of enzyme and BrCTP used were 6.05×10^{-5} M and 2.68×10^{-4} M, respectively. The time scale is 200 μsec/large division: left, no substrate; right, with 2 mM carbamyl phosphate and 10 mM succinate; lower, regulatory subunit + BrCTP. The concentration of the regulatory subunit was 1.0×10^{-4} M and the concentration of BrCTP was 11.8×10^{-4} M. The time scale is 100 μsec/large division: left, no substrate; right, with 2 mM carbamyl phosphate and 10 mM succinate.

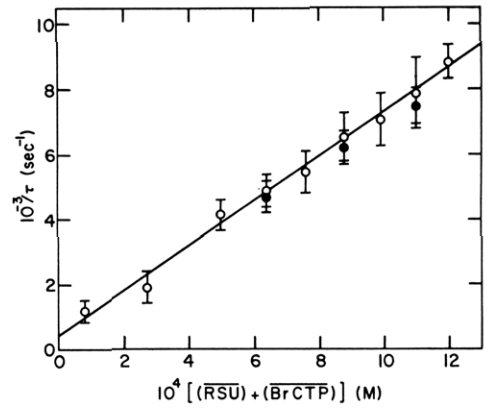

FIGURE 3: The reciprocal relaxation time for the interaction of BrCTP with the regulatory subunit of ATCase as a function of the sum of equilibrium concentrations of the regulatory subunit and BrCTP. The initial concentration of the regulatory subunit was 1.0×10^{-4} M based on a molecular weight of 17,000. The initial concentration of BrCTP varies from 0.8 to 12×10^{-4} M. The open circles represent relaxation times measured in the absence of the substrate, while the filled circles are relaxation times measured in the presence of 2 mM carbamyl phosphate and 10 mM succinate. The error bars represent the standard deviation for the traces analyzed. The straight line was determined by a least-squares analysis of the data.

ments; 0.15 M KCl and 0.02 M potassium phosphate, pH 7.0, or 0.02 M imidazolium chloride, pH 7.0. A Zeiss PMQ II spectrophotometer was used for initial difference spectra and for spectrophotometric titrations. Other difference spectra were measured on a Cary 14 recording spectrophotometer using an 0–0.1 absorbance slide-wire. Titration experiments and calculations to determine the dissociation constant were performed essentially as described by Anderson *et al.* (1968), using an 0.1-mm slit width at a wavelength of 308 nm. For each titration several enzyme solutions were used to avoid making more than 3–4 sequential additions of ligand to any given solution and thereby minimizing accumulated volumetric errors. Six to twelve points were taken for each titration. Enzyme concentrations of 3–7 mg/ml were employed. BrCTP concentrations ranged from 1×10^{-5} to 5×10^{-4} M. All experiments were conducted at 25 ± 0.1°.

Kinetic Measurements. The temperature-jump apparatus used is described in detail elsewhere (Faeder, 1970). The reaction volume of this instrument is slightly less than 0.2 ml and signal to noise ratios of 1000–1500 were routinely obtained. Concentration changes were monitored at 308 nm. A 10-kV discharge through the cell was used to obtain a temperature rise of 7.5° with a heating time constant of 8 μsec. The final temperature was 25° in all cases. Solutions were prepared from freshly boiled, distilled, deionized water and contained 0.15 M KCl. Twelve to fifteen jumps were made on the same solution with no detectable change in time constant or amplitude of relaxation effects. Enzyme concentrations varied from 2 to 10 mg per ml and BrCTP concentrations from 2×10^{-5} to 2×10^{-3} M. For each solution at least 6 oscilloscope traces were photographed. The relaxation time was evaluated from logarithmic plots of the amplitude *vs.* time. An IBM 1800 computer with a curve tracer was used to process the data.

Stopped-flow measurements were made with a Gibson–Durrum apparatus equipped with a Kel-F syringe block and mixing chambers.

Results and Treatment of Data

Thermodynamic Measurements. The binding of the allosteric inhibitor, CTP, or the inhibitor analog, BrCTP, to native ATCase produces difference spectra such as those shown in Figure 1. The maxima of the difference absorbance are at 290 and 240 nm for CTP, and at 308 and 252 nm for BrCTP. In order to reduce the interference caused by the absorption of protein, BrCTP was substituted for CTP in all of the following experiments. The absorbance at 308 nm was monitored to measure concentration changes. Addition of 2 mM carbamyl phosphate (a substrate) and 10 mM succinate (an aspartate analog) did not alter the BrCTP–ATCase difference spectrum, except for a slight change in the difference extinction coefficient. The difference spectrum characterizing the binding between regulatory subunit and BrCTP is essentially identical with that of the BrCTP–ATCase interaction.

The dissociation constants were determined by spectrophotometric titrations. The difference absorption between BrCTP–ATCase complex (or BrCTP–regulatory subunit complex) and free BrCTP plus free ATCase (or free regulatory subunit) was measured as a function of the concentration of BrCTP. In all cases the dissociation constants were calculated using the equation (Hammes and Schimmel, 1966)

$$K = \frac{(\overline{E})(\overline{BrCTP})}{(\overline{E\text{–}BrCTP})} = \frac{[(E)_0 - \Delta a/\Delta\epsilon][(BrCTP)_0 - \Delta a/\Delta\epsilon]}{\Delta a/\Delta\epsilon} \quad (1)$$

where $(\overline{E})$, $(\overline{BrCTP})$, and $(\overline{E\text{–}BrCTP})$ are equilibrium concentrations of the enzyme, BrCTP, and enzyme–BrCTP complex, respectively, $(E)_0$ and $(BrCTP)_0$ are total concentrations of the enzyme and BrCTP, Δa is the measured difference absorbance at 308 nm, and $\Delta\epsilon$ is the molar difference extinction coefficient at the same wavelength. After assuming a value of $\Delta\epsilon$, K was

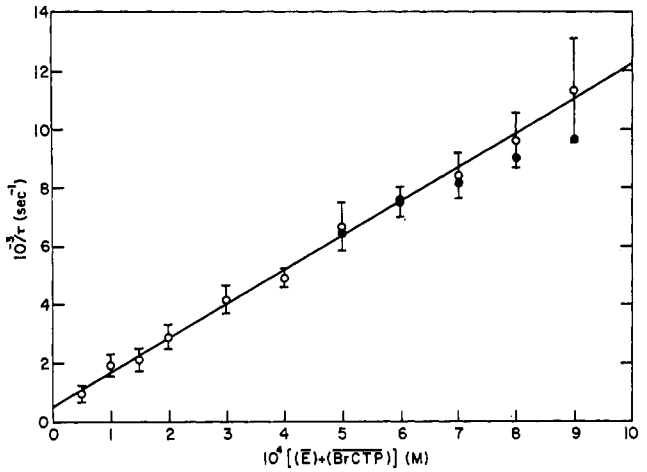

FIGURE 4: The reciprocal relaxation time, $1/\tau$, for ATCase–BrCTP interactions in imidazole buffer in the absence of substrates as a function of the sum of equilibrium concentrations of the enzyme and BrCTP. The initial enzyme concentration varied from 0.6 to 3×10^{-5} M and the BrCTP concentration from 0.3 to 10×10^{-4} M. The buffer was 0.15 M KCl and 0.02 M imidazole-HCl, pH 7.0. Each data point is the average of at least 6 determinations. The error bars represent the standard deviation for the traces analyzed. The straight line was obtained by least-squares analysis of the data. The sum of equilibrium concentrations of the enzyme and BrCTP was calculated from the initial concentrations of the reactants and the spectrophotometrically measured dissociation constant, $K = 0.52 \times 10^{-4}$ M. The filled circles are data points obtained with a different preparation of enzyme, and were not used in determining the straight line.

TABLE II: Summary of Kinetic Data (25°).

System (Substrates[a])	$10^{-6} k_1$ (M^{-1} sec^{-1})	k_{-1} (sec^{-1})	$10^5 K$ (M)
Bimolecular Mechanism			
RSU + BrCTP(±)[b]	6.88	449	6.5
ATCase + BrCTP (−)[b]	11.7	513	4.4
ATCase + BrCTP (−)[c]	7.44	914	12.3
	$10^4 k_{-1}/k_1$ (M)	$10^{-3} k_2$ (sec^{-1})	$10^{-3} k_{-2}$ (sec^{-1})	$10^5 K$ (M)
Isomerization Mechanism				
ATCase + BrCTP (+)[b]	13.2	11.7	1.45	14.0
ATCase + BrCTP (+)[c]	4.8	6.07	1.20	7.9

[a] (+) 2 mM carbamyl phosphate and 10 mM succinate; (−) no substrates. [b] 0.15 M KCl and 0.02 M imidazole-HCl, pH 7.0. [c] 0.15 M KCl and 0.02 M potassium phosphate, pH 7.0.

calculated for all BrCTP concentrations using the measured values of Δa. The best value of $\Delta\epsilon$ was taken to be that which minimized the per cent standard deviation of K. Typical average per cent standard deviations were in the range of 8–10%. A summary of the values of the dissociation constants and $\Delta\epsilon_{308}$ is given in Table I.

Kinetic Measurements. A single relaxation process was seen with the temperature jump for solutions containing BrCTP and native ATCase, both in the presence and absence of carbamyl phosphate and succinate, and for solutions containing BrCTP and the regulatory subunit. No relaxation effects were seen unless both the modifier and enzyme (or regulatory subunit) were present. Some typical relaxation effects are shown in Figure 2 under a variety of experimental conditions. The accessible time range on the temperature jump is from about 10 μsec to 100 msec. Mention should be made of the fact that an initial very rapid absorbancy change could be seen following the temperature jump, which indicates some relaxation processes are occurring that are more rapid than the resolution time of the apparatus. Stopped-flow and hand-mixing experiments were performed over a wide range of concentrations of BrCTP, carbamyl phosphate, and succinate in order to explore the time range from 100 msec to several minutes. In all cases no absorbancy changes could be detected.

The concentration dependence of the reciprocal relaxation time as a function of the sum of the free BrCTP and free regulatory subunit concentrations ($\overline{RSU}$) is shown in Figure 3. A molecular weight of 17,000 per binding site was used to calculate the subunit concentration (Weber, 1968; Wiley and Lipscomb, 1968; Hammes *et al.*, 1970). The straight line obtained suggests a simple bimolecular binding mechanism

$$\text{BrCTP} + \text{RSU} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{RSU–BrCTP} \qquad (2)$$

The reciprocal relaxation time for this mechanism is given by

$$1/\tau = k_1[(\overline{\text{BrCTP}}) + (\overline{\text{RSU}})] + k_{-1} \qquad (3)$$

where the bars designate equilibrium concentrations. The rate constants obtained from the data in Figure 4 are given in Table II. The equilibrium binding constant, k_{-1}/k_1, determined kinetically is 6.5×10^{-5} M, in excellent agreement with that determined spectrophotometrically, 6.7×10^{-5} M. The presence of succinate and carbamyl phosphate has no effect on the relaxation time within experimental error (see Figure 3).

In the absence of substrates, the reciprocal relaxation time for the BrCTP–ATCase interaction in imidazole buffer was also a linear function of the sum of the equilibrium concentrations of enzyme, E, and BrCTP (Figure 4). The amplitude of the relaxation effect was too small to permit determination of the relaxation time at BrCTP concentrations greater than approximately 10^{-3} M. The data obtained over the accessible concentration range again suggest a simple bimolecular mechanism similar to eq 3. The rate constants determined from the data in Figure 5 are included in Table II. The kinetically determined equilibrium constant in imidazole buffer is 4.4×10^{-5} M, which is consistent with the value obtained from difference spectra, 5.2×10^{-5} M.

When 2 mM carbamyl phosphate and 10 mM succinate are present, the plot of $1/\tau$ *vs.* $[(\overline{E}) + (\overline{BrCTP})]$ does not give a straight line. As shown in Figure 5, the reciprocal relaxation time tends to reach a limiting value at high concentrations

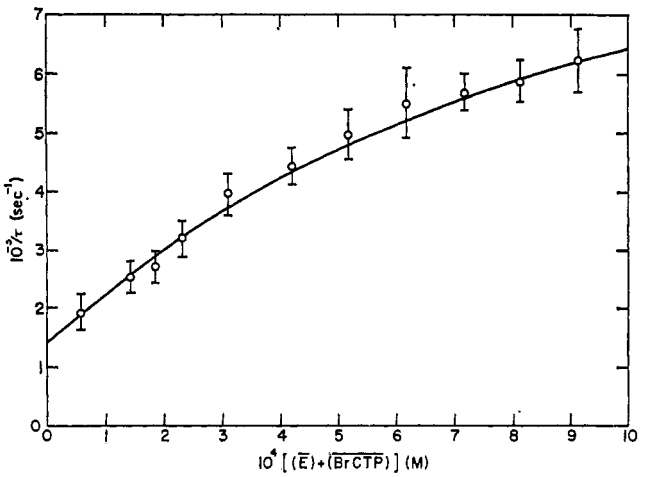

FIGURE 5: The reciprocal relaxation time, $1/\tau$, for ATCase–BrCTP interactions in imidazole buffer in the presence of 2 mM carbamyl phosphate and 10 mM succinate as a function of the sum of equilibrium concentrations of the enzyme and BrCTP. The error bars represent the standard deviation for the traces analyzed. Conditions of the experiments were the same as described in Figure 4, except for the addition of succinate and carbamyl phosphate. The spectrophotometrically measured dissociation constant used for the calculation of equilibrium concentrations was 10.2×10^{-5} M. The curve represents the theoretical values of the reciprocal relaxation times calculated with the parameters given in Table II according to eq 5.

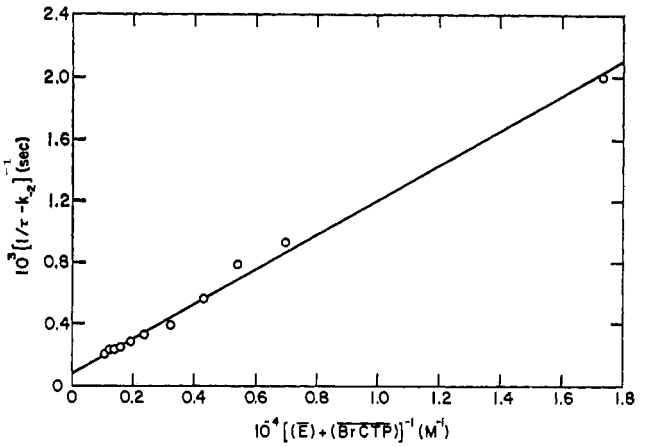

FIGURE 6: A plot of $[1/\tau - k_{-2}]^{-1}$ *vs.* $[(\bar{E}) + (\overline{BrCTP})]^{-1}$ with $k_{-2} = 1450$ sec^{-1}. The data points are the same as those plotted in Figure 5. The straight line was determined by a least-squares analysis of the data.

of ATCase and BrCTP. Two relatively simple mechanisms are quantitatively consistent with the data.

One mechanism postulates a rapid bimolecular reaction followed by a relatively slow isomerization of the complex formed, with each binding site assumed to be equivalent and independent

$$E + BrCTP \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (E\text{–}BrCTP)^1 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (E\text{–}BrCTP)^2 \qquad (4)$$

The slowest relaxation time can be written as

$$\frac{1}{\tau} = k_{-2} + \frac{k_2}{1 + \dfrac{k_{-1}}{k_1[(\bar{E}) + (\overline{BrCTP})]}} \qquad (5)$$

This equation can be rearranged to give

$$[1/\tau - k_{-2}]^{-1} = 1/k_2 + (k_{-1}/k_1k_2)\,[(\bar{E}) + (\overline{BrCTP})]^{-1} \qquad (6)$$

Various values of k_{-2} can be assumed in a trial and error process until a linear fit of the data is obtained, and k_2 and k_{-1}/k_1 can be determined from the value of the slope and intercept. The straight line obtained from the data plotted accordingly to eq 6 with $k_{-2} = 1450$ sec^{-1} is shown in Figure 6. The kinetic parameters obtained are included in Table II and the curve in Figure 5 is calculated with these parameters according to eq 5. The overall binding constant is equal to $k_{-1}/[k_1(1 + k_2/k_{-2})]$, or 1.4×10^{-4} M, in reasonable agreement with the equilibrium constant determined spectrophotometrically (1.02×10^{-4} M).

A second mechanism consistent with the data is that proposed by Monod *et al.* (1965) for allosteric regulation. For an enzyme with 6 binding sites this mechanism can be formulated as

$$\begin{array}{ccc}
6M + R_0 & \underset{k_{-0}}{\overset{k_0}{\rightleftharpoons}} & T_0 + 6M \\
\downarrow\uparrow & & \downarrow\uparrow \\
5M + R_1 & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & T_1 + 5M \\
\downarrow\uparrow & & \downarrow\uparrow \\
4M + R_2 & \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} & T_2 + 4M \\
\downarrow\uparrow & & \downarrow\uparrow \\
3M + R_3 & \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} & T_3 + 3M \\
\downarrow\uparrow & & \downarrow\uparrow \\
2M + R_4 & \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} & T_4 + 2M \\
\downarrow\uparrow & & \downarrow\uparrow \\
M + R_5 & \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} & T_5 + M \\
\downarrow\uparrow & & \downarrow\uparrow \\
R_6 & \underset{k_{-6}}{\overset{k_6}{\rightleftharpoons}} & T_6
\end{array} \qquad (7)$$

Here M is the modifier (BrCTP) and R and T designate different conformational states of the enzyme; the subscripts designate the number of bound modifier molecules per molecule of enzyme. If the assumption is made that the vertical steps equilibrate rapidly relative to the horizontal steps, *i.e.*, the binding of the modifier to the enzyme is very fast, the slowest relaxation time is determined by the rates of interconversion of the R and T states of the enzyme. The general expression for the slowest relaxation time is quite complex even in this case. A very simple limiting case consistent with the data is as follows. (1) The modifier is buffered, *i.e.*, $\delta(M)$, the deviation of the modifier concentration from its equilib-

TABLE III: Kinetic Parameters for Concerted Mechanism (eq 7).

	With Substrates[a,c]	With Substrates[b,c]	Without Substrate[b]
c	2.27	1.72	2.85
K_T (M)	0.52×10^{-4}	0.5×10^{-4}	0.5×10^{-4}
K_R (M)	1.18×10^{-4}	0.86×10^{-4}	1.42×10^{-4}
L_0 [$=(T_0)/(R_0)$]	0.030	0.102	0.028
k_{-0} (sec^{-1})[d]	1650	1540	778
k_0 (sec^{-1})	49	158	22
k_1 (sec^{-1})	111	272	63
k_2 (sec^{-1})	253	467	179
k_3 (sec^{-1})	575	804	509
k_4 (sec^{-1})	1310	1380	1,450
k_5 (sec^{-1})	2970	2380	4,140
k_6 (sec^{-1})	6750	4100	11,800
$K_{overall}$[e] (M)	0.93×10^{-4}	0.705×10^{-4}	

[a] 0.15 M KCl and 0.02 M imidazole-HCl, pH 7.0. [b] 0.15 M KCl and 0.02 M potassium phosphate, pH 7.0. [c] 2 mM carbamyl phosphate and 10 mM succinate. [d] $k_{-0} = k_{-1} = k_{-2} = \ldots = k_{-6}$. [e] Apparent overall binding constant, *i.e.*, the concentration of free BrCTP when $\bar{Y} = 0.5$.

rium value, is equal to zero. This is a good approximation if the modifier concentration is much higher than that of the enzyme, a situation which is valid for most of the data obtained. (2) The rate of conversion of all transitions from the T into the R states are characterized by identical rate constants, *i.e.*, $k_{-0} = k_{-1} = k_{-2} = \ldots = k_{-6}$. If the ratio of the microscopic binding constants for the R and T states, $K_R/K_T = c$, then detailed balance requires that $k_0 = k_1/c = k_2/c^2 = \ldots = k_6/c^6$. The slowest relaxation time characterizing the interconversion of R and T states can then be written as (see Appendix)

$$1/\tau = k_{-0} + k_0\left[\frac{1 + (M)/K_T}{1 + (M)/K_R}\right]^6 \qquad (8)$$

The value of K_T was taken to be the apparent overall binding constant in the absence of carbamyl phosphate and succinate (5.2×10^{-5} M); virtually no R form of the enzyme is present in this situation. A plot of $1/\tau$ *vs.* ($\overline{BrCTP}$) (see Figure 7) is then a function of only three constants c, k_0, and k_{-0}. An additional constraint on the system is the overall binding constant in the presence of carbamyl phosphate and succinate. The curve in Figure 7 has been calculated from eq 8 and the parameters given in Table III. The fit of the data is quite good. Both eq 5 and 8 predict that when (M) is constant, the relaxation time should be essentially independent of enzyme concentration. This has been verified experimentally at several different concentrations of enzyme and substrate where the modifier concentration is much higher than that of the enzyme so that M is approximately constant.

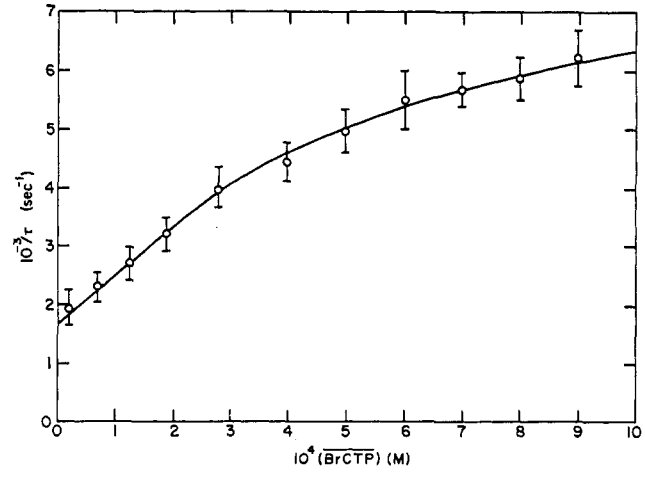

FIGURE 7: A plot of the reciprocal relaxation time, $1/\tau$, as a function of the equilibrium concentration of BrCTP in the presence of 2 mM carbamyl phosphate and 10 mM succinate. The curve was calculated according to eq 8 with the parameters given in Table III. Conditions of the experiments were the same as described in Figure 5.

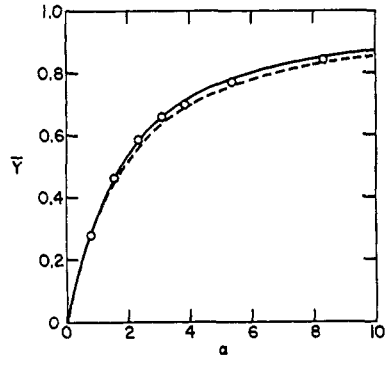

FIGURE 8: The calculated and observed binding isotherms for ATCase–BrCTP interactions in the presence of 2 mM carbamyl phosphate and 10 mM succinate. The dashed curve is the theoretical hyperbolic binding isotherm calculated from the concentrations of ATCase and BrCTP used in the titration experiments described above assuming 6 independent binding sites with the same intrinsic binding constant. The binding constant used in the calculation was 1.02×10^{-4} M. The solid curve represents the theoretical binding isotherm calculated from eq 9 according to the mechanism of eq 7 with the parameters given in Table III.

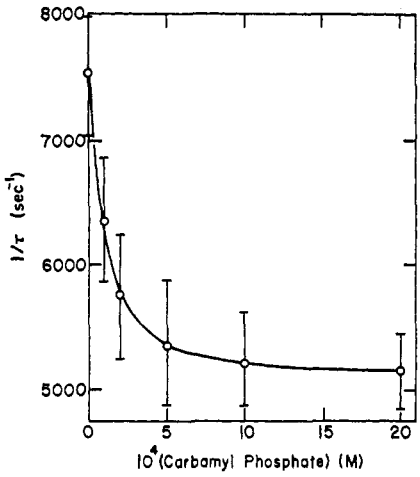

FIGURE 9: Effect of different concentrations of carbamyl phosphate on the reciprocal relaxation time for the ATCase–BrCTP interaction in the presence of 10 mM succinate. The error bars represent the standard deviation for the traces analyzed. The sum of the equilibrium concentrations of ATCase and BrCTP was held constant at 8×10^{-4} M. The buffer was 0.15 M KCl–0.02 M imidazole-HCl, pH 7.0.

A theoretical binding isotherm for this mechanism can be calculated from the saturation function, $\bar{Y}$ (Monod *et al.*, 1965)

$$\bar{Y} = \frac{L_0\alpha(1 + \alpha)^5 + (\alpha/c)(1 + \alpha/c)^5}{L_0(1 + \alpha)^6 + (1 + \alpha/c)^6} \qquad (9)$$

where $\alpha = (M)/K_T$ and $L_0 = (T_0)/(R_0)$. Figure 8 shows the experimentally measured binding isotherm (circles), the theoretical binding isotherm calculated according to eq 9 and the parameters in Table III (solid line), and the theoretical binding isotherm for six independent sites with the same intrinsic binding constant (dashed line). Both calculated curves fit the experimental data adequately. The apparent overall binding constant (the value of M when $\bar{Y} = 0.5$) estimated from eq 9 is 0.93×10^{-4} M, in good agreement with the value obtained from spectrophotometric titration. Thus the mechanism of eq 7 is consistent with available kinetic and thermodynamic data.

In order to determine whether carbamyl phosphate or succinate was causing the observed change in the relaxation time, kinetic measurements were made at a constant value of $[(\bar{E}) + (\overline{BrCTP})]$ of 8×10^{-4} M, with carbamyl phosphate or succinate or both. The results are summarized in Table IV. From these results it can be seen that both succinate and carbamyl phosphate alter the relaxation time to a different extent, and that the effects of the two ligands are not additive. If the concentration of succinate is held constant at 10 mM, titration with carbamyl phosphate results in a continuous decrease in the reciprocal relaxation time as shown in Figure 9. The concentration of carbamyl phosphate required to achieve half of the maximal effect was approximately 1.4×10^{-4} M, which is comparable to the apparent K_m of carbamyl phosphate, 2.0×10^{-4} M (Bethell *et al.*, 1968).

Many of the earlier investigations with ATCase have been carried out in phosphate buffer, although this is not an ideal medium since phosphate is a product of the reaction and

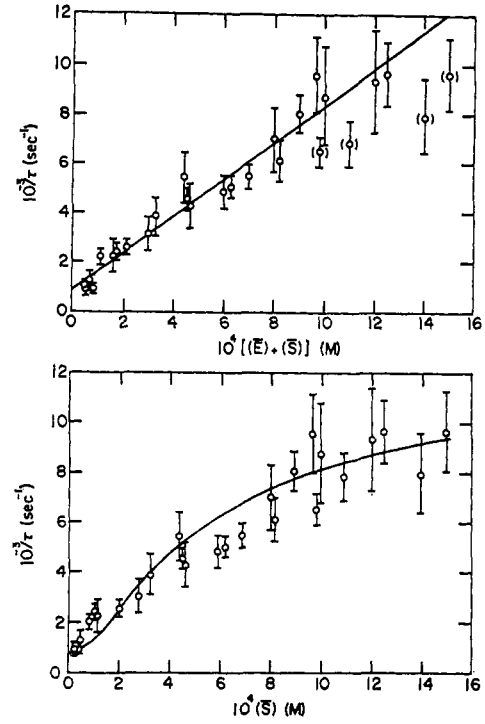

FIGURE 10: Concentration dependence of $1/\tau$ for native ATCase and BrCTP in phosphate buffer in the absence of substrates. Conditions are the same as described in Figure 4 except the buffer used was 0.15 M KCl and 0.02 M potassium phosphate, pH 7.0: top, a plot of $1/\tau$ *vs.* $[\bar{E})\ (\overline{BrCTP})]$. The straight line was obtained by a least-squares analysis of the data, neglecting the points in parentheses: bottom, a plot of $1/\tau$ *vs.* $(\overline{BrCTP})$. The curve was calculated according to eq 8 with the parameters given in Table III. The error bars represent the standard deviation for the traces analyzed.

binds to the same site on the enzyme as carbamyl phosphate (Kleppe, 1966; Porter *et al.*, 1969). Temperature-jump experiments have been carried out in 0.15 M KCl and 0.02 M phosphate buffer, pH 7.0. The results are summarized in Figure 10 (in the absence of carbamyl phosphate and succinate) and Figure 11 (in the presence of carbamyl phosphate and succinate). The latter figure is virtually identical with that obtained in the absence of phosphate buffer (Figures 5 and 7) and can be analyzed in terms of the two mechanisms (eq 4 and

TABLE IV: Effect of Succinate and Carbamyl Phosphate on the Relaxation Time of the ATCase–BrCTP Interaction.[a]

Conditions	$1/\tau$ (sec^{-1})
No substrate	9650 (±960)
10 mM Succinate	7510 (±570)
2 mM Carbamyl phosphate	6370 (±600)
10 mM Succinate + 2 mM carbamyl phosphate	5170 (±460)

[a] Experiments were performed at a constant sum of the equilibrium concentrations of ATCase and BrCTP of 8×10^{-4} M. The buffer was 0.15 M KCl–0.02 M imidazole-HCl, pH 7.0.

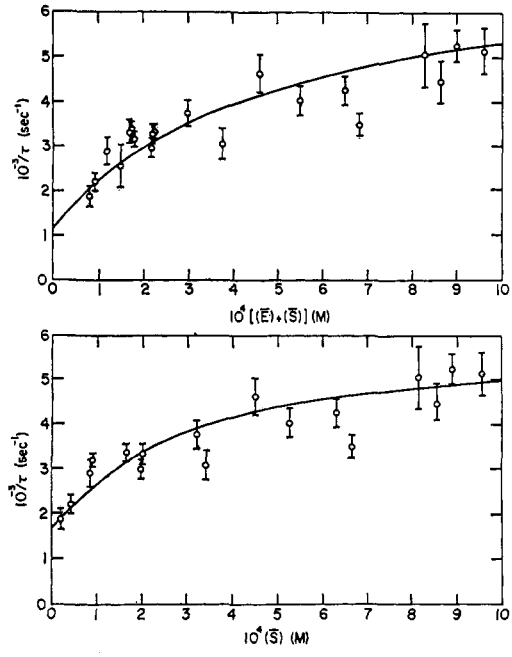

FIGURE 11: Concentration dependence of $1/\tau$ for native ATCase and BrCTP in phosphate buffer in the presence of 2 mM carbamyl phosphate and 10 mM succinate. Conditions are the same as described in Figure 6 except the buffer used was 0.15 M KCl–0.02 M potassium phosphate, pH 7.0; top, a plot of $1/\tau$ *vs.* [(Ē) + ($\overline{\text{BrCTP}}$)]. The curve was calculated according to eq 5 with the parameters given in Table II: bottom, a plot of $1/\tau$ *vs.* ($\overline{\text{BrCTP}}$). The curve was calculated according to eq 8 with the parameters given in Table III. The error bars represent the standard deviation for the traces analyzed.

7) previously discussed. The kinetic and thermodynamic parameters characterizing the data are summarized in Table II (sequential mechanism according to eq 4) and Table III (concerted mechanism according to eq 7) and the curves in Figure 11 have been calculated with these parameters for both mechanisms. The apparent overall binding constants predicted from these parameters are 7.9×10^{-4} M and 7.0×10^{-4} M according to the mechanism of eq 4 and 7, respectively, in good agreement with that measured spectrophotometrically (6.25×10^{-4} M).

The data in the upper part of Figure 10 are difficult to interpret: a fairly straight line is observed at low values of [(Ē) + ($\overline{\text{BrCTP}}$)], but the reciprocal relaxation time appears to level off when the sum of these two concentrations are greater than 10^{-3} M. A qualitative difference observed in the presence of phosphate buffer relative to imidazole buffer is that the amplitude of the relaxation process is significantly larger at high concentrations of BrCTP ($>10^{-3}$ M). Two relatively simple interpretations of these data are possible. One mechanism postulates that a bimolecular reaction is being observed and that the deviation from linearity at high concentrations of BrCTP is related to the binding of BrCTP at the catalytic site (Porter *et al.*, 1969). However, if the straight-line portion of a plot of $1/\tau$ *vs.* [(Ē) + ($\overline{\text{BrCTP}}$)] is used to calculate the rate constants, the overall binding constant determined kinetically is over twice as large as that measured spectrophotometrically (see Table II for a summary of the kinetic constants). This difference appears to be just outside

TABLE V: Relaxation Time as a Function of the Degree of Saturation at Constant [(Ē) + ($\overline{\text{BrCTP}}$)].[a]

10^4 [(Ē) + ($\overline{\text{BrCTP}}$)] (M)	$\bar{Y}$ = ($\overline{\text{EBrCTP}}$)/(E_0)	$1/\tau$ (sec^{-1})
1.73	0.671	2470 (±110)
1.73	0.289	2390 (±320)
1.64[b]	0.456	3330 (±270)
1.74[b]	0.341	3420 (±140)
1.78[b]	0.589	3190 (±140)

[a] 0.15 M KCl–0.02 M potassium phosphate, pH 7.0. [b] In the presence of 2 mM carbamyl phosphate and 10 mM succinate.

the estimated experimental uncertainties. A second mechanism postulates that phosphate alters the enzyme conformation such that a conformational change is rate limiting in the binding process. In this case a simple mechanism such as eq 4 is incompatible with the overall binding constant determined spectrophotometrically, but a concerted mechanism (eq 7) fits the data fairly well. The curve in the lower part of Figure 10 has been drawn according to eq 8 with the parameters given in Table III. For this mechanism a comparison of the calculated overall binding constant with that observed experimentally is not meaningful. This is because an independent estimate of K_T cannot be made for this system; a value of 5×10^{-5} M was chosen because of the data in imidazole buffer, but this constant, and therefore the calculated overall binding constant, can be varied considerably, while still fitting the experimental data adequately. This mechanism is also consistent with the equilibrium binding isotherm determined from difference spectroscopy.

Still another possible interpretation of the results obtained is that the mechanism of eq 4 pertains, but that the exact relaxation times for the coupled processes must be used. The pertinent relaxation time is then a complex function of all four rate constants. The equilibrium and kinetic data can be fit reasonably well by suitable variation of these four constants, and the rate constants are similar in magnitude to those given in Table II. However, the second relaxation process anticipated for this situation was not observed, although the calculated relaxation time is within the time resolution of the apparatus used. This is only weak evidence against the proposed mechanism since the amplitude of the relaxation process cannot be anticipated.

Experiments have been done which demonstrate that in the system under consideration, τ is primarily a function of [(Ē) + ($\overline{\text{BrCTP}}$)], rather than of $\bar{Y}$. Some of these results are presented in Table V. A simple bimolecular or sequential mechanism predicts that $1/\tau$ is a function of [(Ē) + ($\overline{\text{BrCTP}}$)]. The concerted conformational change mechanism (eq 7) is an extremely complex function of (Ē) and ($\overline{\text{BrCTP}}$) if $\delta(M) \neq 0$, and it has not proved practical to consider this general case. The data in Figures 7, 10, and 11 were all obtained under the condition ($\overline{\text{BrCTP}}$) > (Ē) so that the assumption $\delta(M) = 0$ is reasonable. Although the situation is not unambiguous, the concerted mechanism of eq 7 seems most compatible with

available data for the binding of BrCTP to ATCase in the presence of phosphate.

Discussion

The equilibrium dissociation constants for BrCTP binding to ATCase reported here are slightly larger than those previously determined with equilibrium dialysis by Changeux *et al.* (1968). However, in this work six BrCTP binding sites were assumed per enzyme molecule of molecular weight 300,000, whereas Changeux *et al.* reported four BrCTP binding sites per enzyme molecule. Recent evidence has indicated that six regulatory subunits exist per enzyme molecule (Weber, 1968; Wiley and Lipscomb, 1968), and that each of these subunits binds a BrCP molecule (Hammes *et al.*, 1970). In view of this discrepancy, some difference in reported binding constants is expected.

The reaction between BrCTP and regulatory subunit can be associated with a simple bimolecular mechanism, and the rate constants reported in Table II are similar to those normally encountered for enzyme–substrate reactions (Hammes, 1968). A similar statement applies to the reaction between BrCTP and ATCase in imidazole buffer.

Probably the most surprising result is that only a single relaxation process can be detected in such a complex system. If other processes are of importance, they must have associated relaxation times of less than 10 μsec. Both the simple sequential mechanism (eq 4) and the concerted mechanism (eq 7) are fairly consistent with the data, although as indicated earlier the data in phosphate buffer can be best reconciled with a concerted mechanism. However, both of these mechanisms pose a similar problem; namely, why is the bimolecular reaction between BrCTP and enzyme not observed in the presence of carbamyl phosphate and succinate, and perhaps in the presence of phosphate. In the case of the sequential mechanism, this can be readily rationalized by simply assuming that the nature of the initial interaction of BrCTP with enzyme is different in the presence of carbamyl phosphate and succinate or phosphate, and that the associated relaxation time is too short to be measured. For the concerted mechanism since BrCTP binds more favorably to the T form than to the R form, the assumption can be made that in the absence of carbamyl phosphate and succinate or phosphate, the T form of the enzyme predominates so that the observed relaxation process in imidazole buffer characterizes the bimolecular reaction of BrCTP and the enzyme in the T form. On the other hand, in the presence of substrates the R form predominates so that the amplitude of the relaxation process for the BrCTP–T form reaction is too small to observe. In this case the relaxation time for the reaction of BrCTP with the R form would be too short to measure.

This analysis is only partially borne out by the data. If the parameters in Table III are taken literally then the ratio $(T_i)/(R_i) > 1$ when $i \geq 5$ (in imidazole buffer), and it is apparent the amount of T form is not negligible at high degrees of saturation of BrCTP. Therefore, at high degrees of saturation one might expect to observe the bimolecular reaction of enzyme and substrate, although the expected amplitude would be very small. Unfortunately the concerted mechanism used for analysis of the data is quite a simple one so that the calculated ratios between the R and T forms cannot be taken too literally. The assumption that all of the k_i are equal is particularly questionable. In fact, the parameters in Table III should not be interpreted quantitatively. *The analysis that has been carried out unequivocally shows that a mechanism of the general type of eq 7 is consistent with the data, i.e., a mechanism in which two different conformations of the enzyme exist and the ligand binds preferentially to one of the forms.* However, fourteen rate constants and two dissociation constants obviously cannot be ascertained from these data. Thus, for example, the values of L_0 given should only be regarded as approximations. The parameters in Table III should be regarded as one particular set, among many, that is consistent with the data. If the mechanism of Monod *et al.* (1965) was quantitatively supported by this mechanism, c would be identical for all three cases in Table III. The fact that the value of c is similar in all three cases should be considered as being quite satisfactory.

Another alternative is to postulate that the relaxation process observed in imidazole buffer actually is associated with the conformational change of the concerted mechanism, and that the consistency of the data with a bimolecular mechanism is fortuitous. The concerted mechanism can be fit to the data within the experimental uncertainty, but systematic differences between the calculated and observed behavior occur. Therefore, this alternative explanation is not an entirely satisfactory one.

A general sequential mechanism such as the scheme of Koshland *et al.* (1966) predicts a spectrum of relaxation times which is clearly not consistent with the experimental observations (unless the processes are extremely rapid).

Thus a concerted mechanism seems to be favored by the available data. Special note should be taken of the fact that the apparently "hyperbolic" binding isotherm observed experimentally is consistent with a concerted mechanism.

Two proteins possessing sigmoidal binding isotherms have been studied with relaxation methods. The detailed mechanism for the binding of O_2 to hemoglobin is still not clear, but a spectrum of relaxation times is observed (Schuster and Ilgenfritz, 1968; Brunori and Schuster, 1969). The binding of NAD to yeast D-glyceraldehyde 3-phosphate dehydrogenase is characterized by three relaxation times (Kirschner *et al.*, 1966), which can be quantitatively analyzed in terms of the mechanism of Monod *et al.* (1965). The observed rates of the R–T interconversions are considerably slower than the rate of the enzymatic reaction, and whether or not a biological control function is related to the observed phenomena is still an open question.

In the case of ATCase, a biological control function is clearly established. The results reported here are most consistent with a concerted mechanism similar to that of Monod *et al.* (1965), although a simple sequential mechanism cannot be excluded. The rates of the conformational changes are quite rapid and similar to those observed in simple enzyme systems (Hammes, 1968). Moreover, the relaxation process associated with the conformational change was observed only when the structure of native enzyme was intact. Other kinetic experiments are now in progress which should further elucidate the allosteric mechanism.

Acknowledgment

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Appendix

If the horizontal steps in the mechanism of eq 7 equilibrate much more slowly than the vertical steps, the slowest relaxation time for the system characterizes the interconversion of the R and T states. The relaxation time can be derived from the rate law

$$-\frac{d\sum_{i=0}^{n}(R_i)}{dt} = \frac{d\sum_{i=0}^{n}(T_i)}{dt} = \sum_{i=0}^{n} k_i(R_i) - \sum_{i=0}^{n} k_{-i}(T_i)$$

In the neighborhood of equilibrium this can be written as (*cf.* Eigen, 1967)

$$-\frac{d\sum_{i=0}^{n}\delta(R_i)}{dt} = \left[\frac{\sum_{i=0}^{n} k_i\delta(R_i)/\delta(R_0)}{\sum_{i=0}^{n}\delta(R_i)/\delta(R_0)} + \frac{\sum_{i=0}^{n} k_{-i}\delta(T_i)/\delta(T_0)}{\sum_{i=0}^{n}\delta(T_i)/\delta(T_0)}\right]\times$$

$$\sum_{i=0}^{n}\delta(R_i) = \sum_{i=0}^{n}\delta(R_i)/\tau$$

where δ represents the deviation from equilibrium and the mass conservation relationship

$$\sum_{i=0}^{n}\delta(R_i) = -\sum_{i=0}^{n}\delta(T_i)$$

has been utilized. The above equation defines the relaxation time. If the assumption is made that $\delta(M) = 0$, the equilibrium dissociation constants can be used to evaluate the ratios $\delta(R_i)/\delta(R_0)$

$$\frac{K_R}{(M)} = \frac{(n-i)\delta(R_i)}{(i+1)\delta(R_{i+1})} \quad i = 0, 1 \ldots n-1$$

$$\frac{K_T}{(M)} = \frac{(n-i)\delta(T_i)}{(i+1)\delta(T_{i+1})} \quad i = 0, 1 \ldots n-1$$

The reciprocal relaxation time can then be written as

$$\frac{1}{\tau} = \frac{\sum_{i=0}^{n} k_i \frac{n!}{(n-i)!i!}[(M)/K_R]^i}{[1+(M)/K_R]^n} + \frac{\sum_{i=0}^{n} k_{-i}\frac{n!}{(n-i)!i!}[(M)/K_T]}{[1+(M)/K_T]^n}$$

If the further assumptions are made that $n = 6$ and $k_{-0} = k_{-1} = \ldots k_{-n}$ and $K_R/K_T = c$, eq 8 is obtained.

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