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

Cheng-Wen Wu and Gordon G. Hammes*

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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Previous kinetic studies of the binding of the inhibitors 5-bromocytidine 5′-triphosphate[1] and CTP, the substrate carbamyl phosphate and the aspartate analogs succinate and L-malate to the enzyme have suggested that at least three conformational transitions are involved in the control process (Eckfeldt *et al.*, 1970; Hammes and Wu, 1971a–c; Harrison and Hammes, 1973). The rates of all of the conformational changes are dependent on the concentrations of inhibitors, carbamyl phosphate and aspartate analogs. The conformational changes associated with carbamyl phosphate and aspartate–analog binding appear to be concerted in nature (*cf.* Monod *et al.*, 1965), while that associated with inhibitor binding can be described by a sequential mechanism (*cf.* Koshland *et al.*, 1966).

This paper presents the results of a study of the interaction of the activator 6-mercapto-9-β-D-ribofuranosylpurine 5′-triphosphate (sRTP) with aspartate transcarbamylase utilizing difference spectroscopy and the temperature-jump method. The results obtained indicate that a conformational change is rate limiting in the binding of sRTP both in the presence and absence of carbamyl phosphate and succinate, although the rate of the conformational change is altered by the presence of carbamyl phosphate and succinate. This conformational transition apparently is the same as that involved in the binding of BrCTP to the native enzyme. A simple two-state allosteric control mechanism for the binding of effectors is consistent with all of the data. However, a multiconformational model involving both concerted and stepwise conformational transitions is required to account for the complete regulatory mechanism.

Experimental Section

Chemicals. The sRTP was synthesized from the barium salt of the monophosphate (Sigma Chemical Corp.) by method 2 of Murphy *et al.* (1970). The concentration of sRTP was determinated spectrophotometrically at 322 nm using an extinction coefficient of 23,000 cm^{-1} M^{-1} at pH 4.6. The ATP and CTP were purchased from P-L Biochemicals. The BrCTP was synthesized as previously described (Eckfeldt *et al.*, 1970). Dilithium carbamyl phosphate (Sigma Chemical Corp.) was further purified by precipitation from aqueous solution by addition of cold ethanol (Gerhart and Pardee, 1962). All carbamyl phosphate solutions were kept in ice and used within 2 hr of preparation. Sodium succinate also was obtained from Sigma Chemical Corp. Imidazole, purchased from Eastman Chemical Corp., was recrystallized once from benzene before use.

Aspartate Transcarbamylase and Its Subunits. Aspartate transcarbamylase from *Escherichia coli* and its dissociated subunits were prepared according to the method of Gerhart and Holoubek (1967). The mutant bacteria were obtained from the New England Enzyme Center. The purity of the enzyme has been described previously (Eckfeldt *et al.*, 1970). Protein concentrations were determined spectrophotometrically at 280 nm, using a specific absorbance of 0.59 cm^2 mg^{-1} for native aspartate transcarbamylase (Gerhart and Holoubek, 1967) and of 0.7 cm^2 mg^{-1} for the catalytic subunit (Collins and Stark, 1969). Protein concentration of the regulatory subunit was determined by the Lowry procedure (Lowry *et al.*, 1951) using native aspartate transcarbamylase as the protein standard.

Enzyme Assay. Aspartate transcarbamylase activity was assayed by two different methods. A colorimetric method similar to that described by Gerhart and Pardee (1962) was used with an assay mixture consisting of 4 mM carbamyl phosphate, 25 mM L-aspartate, and 0.1 M imidazole acetate (pH 7.0). The enzyme was incubated in 0.5 ml of the assay mixture for 30 min at 28° and the formation of carbamyl L-aspartate was determined by the method of Prescott and Jones (1969).

A more convenient assay utilizes the pH-Stat technique (Radiometer Type TTT 1b and Type TTT A31 microtitration assembly). The assay solutions contained 0.1 M potassium acetate, 4 mM carbamyl phosphate, and various concentrations of L-aspartate in a final volume of 2 ml. The final pH of the solutions was adjusted with NaOH or acetic acid to pH 8.0. A 25-μl aliquot of the enzyme was introduced into the reaction mixture to initiate the reaction. Initial velocities were obtained from the resulting plots of base added *vs.* time. In general, linear plots could be obtained over a convenient period of time (15 min or more).

Spectral Measurements. Ultraviolet absorption spectra and difference spectra were obtained by use of a Cary 14 recording spectrophotometer. For difference absorbance measurements, rectangular quartz tandem cells with a path length of 0.44 cm/cell compartment (Pyrocell Manufacturing Co.) were employed. A Zeiss PMQ II spectrophotometer was used for spectrophotometric titrations with a fixed slit width at the desired wavelength. The spectrophotometric cells were thermostatted at 25°. Titration experiments were performed as described previously (Eckfeldt *et al.*, 1970).

Kinetic Measurements. The temperature-jump apparatus used is described elsewhere (Faeder, 1970). The reaction volume is about 0.2 ml and 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, deionized, distilled water and contained 0.15 M potassium acetate. Concentration changes were monitored at 335 nm for sRTP interactions with aspartate transcarbamylase and at 308 nm for BrCTP interactions with the enzyme. For each solution at least six oscilloscope traces were photographed. The photographed oscilloscope traces were analyzed by means of a curve tracer interfaced with a PDP-9 digital computer. The relaxation time was calculated from a least-squares analysis of the logarithm of the signal amplitude *vs.* time and has an uncertainty of about ±10%.

Results and Treatment of Data

Effect of sRTP on ATCase Activity. The effect of the allosteric activator ATP and its structural analog sRTP on the activity of aspartate transcarbamylase is shown in Figure 1. Although small quantitative differences may exist, both substances activate the enzyme to a similar extent and in a similar manner. In both cases the sigmoidicity of the aspartate saturation curve is reduced. This similarity in the effect of ATP and sRTP on the enzymic activity indicates the latter is a good activator analog. This analog is especially convenient to utilize in a direct study of the interaction between enzyme and activator since the absorption maximum of sRTP is at 322 nm, which is quite far removed from the absorption maximum of the protein.

An Attempted Affinity Label. sRTP has been shown to be an

[1] Abbreviations used are: BrCTP, 5-bromocytidine 5′-triphosphate; sRTP, 6-mercapto-9-β-D-ribofuranosylpurine 5′-triphosphate; pHMB, *p*-hydroxymercuribenzoate.

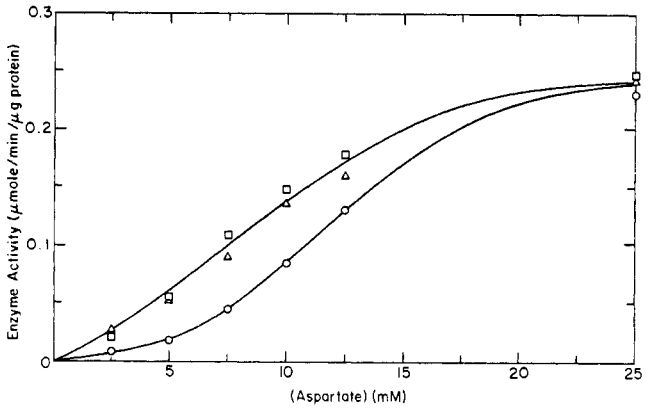

FIGURE 1: Effect of sRTP and ATP on the initial velocity saturation curve for aspartate. The pH-Stat assay was used as described in the text. The reaction mixture contained 0.1 M potassium acetate (pH 8), 4 mM carbamyl phosphate, 1.25–10 μg of native aspartate transcarbamylase, and L-aspartate as indicated at 25°: (○) control; (△) 2 mM sRTP added; (□) 2 mM ATP added.

affinity label for active sites with appropriately located sulfhydryl groups (Murphy and Morales, 1970). To test the possibility that sRTP may undergo disulfide formation with an amino acid side chain in aspartate transcarbamylase, a 200-fold excess of sRTP (8×10^{-3} M) was incubated with the enzyme (4×10^{-5} M) at 4° for 72 hr in the presence and absence of 2 mM carbamyl phosphate and 10 mM succinate. The incubations were carried out both at pH 7 and 8. After the incubation, the enzyme was separated from the incubation mixture by passing the solution through a Sephadex G-50 column. Spectrophotometric measurements indicated that no tightly bound sRTP was associated with the enzyme. Furthermore, the treated enzyme was subject to CTP inhibition and ATP activation to an extent comparable to that of the untreated enzyme.

Binding Measurements. The binding of sRTP to the isolated catalytic subunit produces a difference spectrum with a maximum at 336 nm and a minimum at 310 nm as shown in Figure 2. This difference spectrum may be attributed to the binding of sRTP to the carbamyl phosphate site since, as

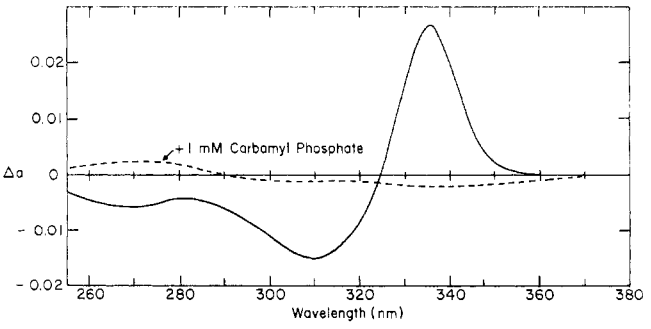

FIGURE 2: Difference spectra for the interaction of sRTP with the isolated catalytic subunit at 25°. Here Δa is the absorbance of the sample cell which contains the catalytic subunit and sRTP in compartment a and the buffer in compartment b, minus the absorbance of the reference cell which contains enzyme alone in compartment a and sRTP in compartment b. The buffer was 0.15 M potassium acetate–0.04 M imidazole acetate (pH 7.0). The concentration of the catalytic subunit was 1.81×10^{-4} M and that of the sRTP was 2.5×10^{-4} M: ——, no substrate; – – –, plus 1 mM carbamyl phosphate.

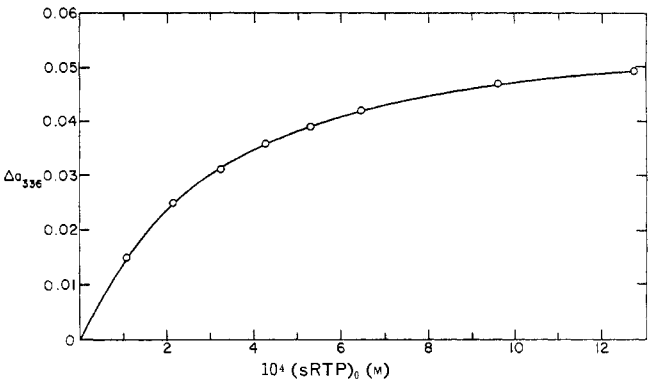

FIGURE 3: A plot of the difference absorbance at 336 nm, Δa_{336}, *vs.* the total concentration of sRTP, $(sRTP)_0$, in the presence of 0.55×10^{-4} M catalytic subunit. A microsyringe was used to add microliter increments of sRTP to the enzyme in a cuvet.

shown in Figure 2, it disappeared when 2 mM carbamyl phosphate was added.

A difference spectral titration of the isolated catalytic subunit was carried out with sRTP (Figure 3): the difference absorbance between the sRTP–catalytic subunit complex and free sRTP plus free catalytic subunit was measured as a function of the concentration of sRTP. The dissociation constant of the sRTP–catalytic subunit complex was determined from the titration curve assuming six equivalent sites and using the equation (Eckfeldt *et al.*, 1970)

$$K = \frac{(\bar{E})(\overline{sRTP})}{(\overline{EsRTP})} = \frac{[(E)_0 - \Delta a/(\Delta\epsilon l)][(sRTP)_0 - \Delta a/(\Delta\epsilon l)]}{\Delta a/(\Delta\epsilon l)} \quad (1)$$

where $(\bar{E})$, $(\overline{sRTP})$, and $(\overline{EsRTP})$ are equilibrium concentrations of the catalytic subunit, sRTP, and the enzyme–sRTP complex, respectively; $(E)_0$ and $(sRTP)_0$ are total concentrations of the enzyme (*i.e.*, the catalytic subunit) and sRTP; Δa is the measured difference absorbance at 335 nm; $\Delta\epsilon$ is the molar difference extinction coefficient at the same wave length; and l is the path length. After assuming a value of $\Delta\epsilon$, K was calculated for all sRTP 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. The final values of $\Delta\epsilon$ and K obtained are 2300 M^{-1} cm^{-1} and 2.5×10^{-4} M. The minimum per cent standard deviation was 14%.

The number of sRTP binding sites, n, also can be evaluated from the titration data. If the concentration of the sRTP-bound enzyme is written as $p = (\Delta a/\Delta a_{max})(E)_0$, where Δa_{max} is the difference absorbance when the enzyme is completely saturated with sRTP, the dissociation constant can be written as

$$K = \frac{[n(E)_0 - np][(sRTP)_0 - np]}{np} \quad (2)$$

which can be rearranged to give

$$(sRTP)_0/p = K/[(E)_0 - p] + n \quad (3)$$

Thus a plot of $(sRTP)_0/p$ *vs.* $1/[(E)_0 - p]$ has a slope equal to the dissociation constant and an intercept equal to the number of binding sites per enzyme unit. The size of the enzyme unit

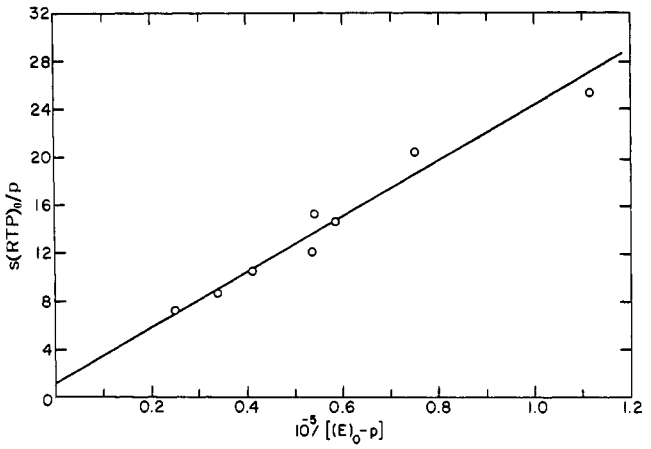

FIGURE 4: Graphical determination of the dissociation constant and number of binding sites for sRTP binding to catalytic subunit. Here $(sRTP)_0$ and $(E)_0$ designate the total concentration of sRTP and catalytic subunit and $p = (\Delta a/\Delta a_{max})(E)_0$. The catalytic subunit concentration was based on an equivalent molecular weight of 33,000. The data were taken from Figure 3 and $\Delta a_{max}/(E)_0 = \Delta\epsilon$.

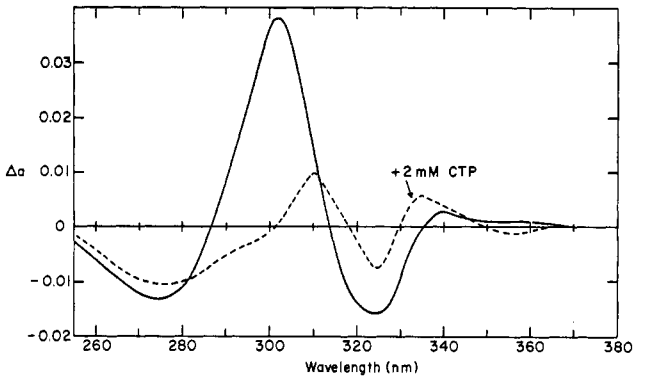

FIGURE 5: Difference spectra for the interaction of sRTP with the isolated regulatory subunit at 25°. Difference spectra were measured as described in the legend of Figure 2 except that regulatory subunit was substituted for catalytic subunit. The concentration of the regulatory subunit was 0.62×10^{-4} M and that of sRTP was 2.64×10^{-4} M: ——, no CTP; – – – –, plus 2 mM CTP.

is determined by the molecular weight of the enzyme assumed to calculate $(E)_0$. In the present case, an equivalent molecular weight of 33,000 was assumed, and the resultant plot is shown in Figure 4. A least-squares analysis of the data gives $n = 1.08$, $K = 2.5 \times 10^{-4}$ M. A similar plot assuming a molecular weight of 100,000 gave $n = 3$ and $K = 3 \times 10^{-4}$ M. Since one carbamyl phosphate site exists per 33,000 molecular weight of catalytic subunit (Hammes *et al.*, 1970), these results are consistent with the hypothesis that sRTP binds to the carbamyl phosphate site or alternatively suggests an equivalent molecular weight (per active site) of 33,000.

In order to carry out the above analysis Δa_{max} was set equal to $\Delta\epsilon(E)_0 l$. This biases the value of n obtained somewhat since $\Delta\epsilon$ was obtained by already assuming a value of n. Therefore, several different equivalent molecular weights were assumed and the difference spectra data were analyzed according to eq 1 to give values of $\Delta\epsilon$. These $\Delta\epsilon$ values were then used to analyze the data according to eq 2. In all cases, within the experimental uncertainty one site per 33,000 molecular weight of protein was obtained. Thus the determination of the number of binding sites appears to be free from any bias in the method of data analysis.

The difference spectrum characterizing the interaction of sRTP with the enzyme regulatory subunit is presented in Figure 5. In contrast to that associated with the sRTP–catalytic subunit interaction, it shows a negative trough at 324 nm and a positive peak at 302 nm. Titration with sRTP increases the amplitude of the positive peak but causes little change in the negative peak. Very likely the negative trough is due to the binding of sRTP to residual pHMB which could not be dialyzed out during the preparation. In an independent experiment, it was found that pHMB binds sRTP and produces a difference spectrum with a negative trough at 324 nm, but not a positive peak at 302 nm. As shown in Figure 5, the amplitude of both the 302-nm peak and 324-nm trough decreases when CTP is added. The observed changes are difficult to interpret since the CTP–regulatory subunit interaction also gives rise to a difference spectrum. The absorbance changes, measured at 302 nm, increased linearly in the range of sRTP concentrations used (3.75×10^{-5}–2.6×10^{-4} M). The binding must be quite weak; however, a reliable evaluation of binding parameters could not be obtained from these data. Experiments could not be carried out at higher sRTP concentrations because of the large absorbancies of the solutions.

As shown in Figure 6, in the absence of substrates the difference spectrum of the sRTP–native enzyme interaction is very similar to that for the sRTP–catalytic subunit interaction. In the presence of 2 mM carbamyl phosphate, the maximal difference absorbance shifts from 332 to 327 nm. No further shift of the maximal difference absorbance occurred when 10 mM succinate was added; however the absolute magnitude of the difference spectrum greatly decreased. As would be expected, the difference absorbance at 327 nm in the presence of carbamyl phosphate was considerably reduced in the presence of ATP and CTP.

The difference spectral titrations of native enzyme with sRTP in the absence and presence of carbamyl phosphate and carbamyl phosphate–succinate also are shown in Figure 7. When saturating amounts of carbamyl phosphate are present, sRTP binds only to the regulatory sites of the native enzyme. As shown in an accompanying paper (Matsumoto and Hammes, 1973), the binding of ATP to the regulatory sites of the

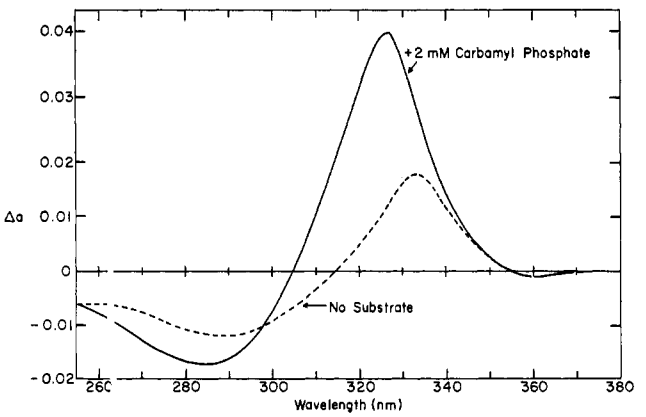

FIGURE 6: Difference spectra for the interaction of sRTP with native aspartate transcarbamylase. Difference spectra were measured as desribed in figure legend 2 except that native enzyme was substituted for catalytic subunit. ——, no substrate; $(E)_0 = 5.5 \times 10^{-5}$ M; $(sRTP)_0 = 9.55 \times 10^{-5}$ M; – – – –, 2 mM carbamyl phosphate, $(E)_0 = 5.8 \times 10^{-5}$M $(sRTP)_0 = 9.55 \times 10^{-5}$ M.

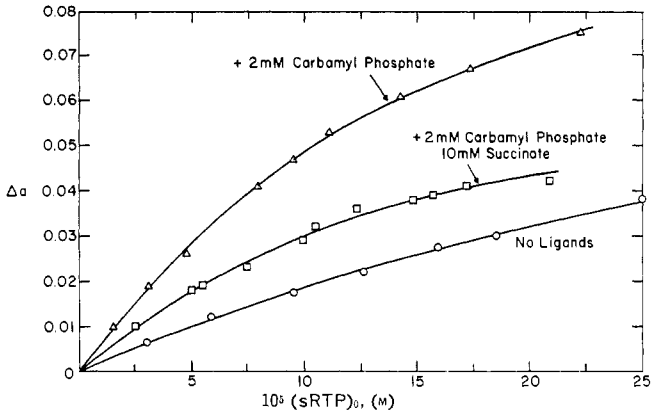

FIGURE 7: A plot of the difference absorbance, Δa, *vs.* the total concentration of sRTP, $(sRTP)_0$, for the native enzyme. (○) No substrate, $(E)_0 = 7.2 \times 10^{-5}$ M, wavelength = 336 nm; (□) 2 mM carbamyl phosphate–10 mM succinate, $(E)_0 = 8.7 \times 10^{-5}$ M, wavelength = 327 nm; (Δ) 2 mM carbamyl phosphate, $(E)_0 = 5.8 \times 10^{-5}$ M, wavelength = 327 nm.

native enzyme cannot be described well with a single binding constant. If, as suggested by the results shown in Figure 1, ATP and sRTP have essentially the same binding characteristics, then in the presence of saturating amounts of carbamyl phosphate or of carbamyl phosphate and succinate, the difference spectrum binding isotherm can be described by assuming two classes of three sites each with association constants of 1.2×10^4 and 3.3×10^2 M^{-1}, respectively. The linear correlation between difference spectral changes and binding is shown in an accompanying paper (Matsumoto and Hammes, 1973). A complete analysis of the difference spectral titration in the absence of carbamyl phosphate and succinate is not possible at this time.

Kinetic Measurements. No relaxation process could be detected with the temperature-jump method for sRTP binding

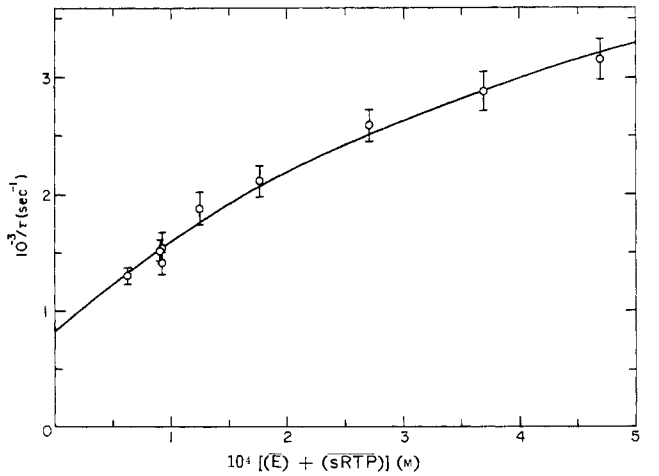

FIGURE 8: The reciprocal relaxation time for the interaction of sRTP with native enzyme as a function of the sum of the equilibrium concentrations of the enzyme and sRTP in the absence of carbamyl phosphate and succinate. The initial enzyme concentrations were 0.38–0.92 M, and the initial concentrations of sRTP varied from 0.53 to 5.3×10^{-4} M. The buffer was 0.15 M potassium acetate–0.04 M imidazole acetate (pH 7.0). The error bars represent the standard deviation for the traces analyzed. The equilibrium concentrations were calculated as described in the text. The solid line was calculated with eq 5 and the constants in Table I.

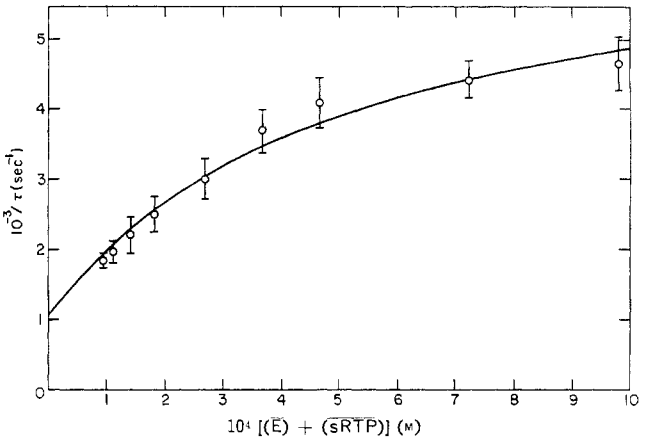

FIGURE 9: The reciprocal relaxation time for the interaction of sRTP with native enzyme as a function of the sum of the equilibrium concentrations of the enzyme and sRTP in the presence of 2 mM carbamyl phosphate. The initial enzyme concentration varied from 0.58 to 0.88×10^{-4} M, and the initial concentrations of sRTP varied from 0.53 to 10.6×10^{-4} M. Other conditions were the same as in Figure 8. The equilibrium concentrations were calculated by assuming a dissociation constant of 0.8×10^{-4} M. The error bars represent the standard deviation for traces analyzed. The solid line was calculated with eq 5 and the constants in Table I.

to the isolated regulatory subunit under the experimental conditions tested. For sRTP binding to the isolated catalytic subunit, however, a very fast relaxation process ($\tau \sim 20$–30 μsec) of low amplitude was observed which disappeared when a saturating amount of carbamyl phosphate was added to the solution. Because of its overlap with the absorption changes associated with the heating of the solution, this fast relaxation process cannot be reliably analyzed.

The addition of sRTP to a solution of native enzyme in 0.15 M potassium acetate–0.04 M imidazole acetate (pH 7.0) causes the appearance of two relaxation processes: a very fast process with a relaxation time (τ_1) in a time range similar to that observed for the interaction of sRTP with the isolated catalytic subunit and a slower one with a relaxation time (τ_2) in the time range 0.3–1 msec. No relaxation process was seen for solutions containing the enzyme or sRTP alone in the buffer.

The relaxation process associated with τ_1 is very likely due to the binding of sRTP to the catalytic (carbamyl phosphate) site on the enzyme, not only because it occurs in a time range very similar to that observed for sRTP binding to the isolated catalytic subunit, but also due to the fact that addition of carbamyl phosphate (2 mM) completely abolished the relaxation process. However, the slower relaxation process still appeared in the presence of saturating carbamyl phosphate. Therefore, it is reasonable to assume that τ_2 is related to the binding of sRTP to the regulatory site. The following kinetic analysis of τ_2 is based on this assumption. In addition it is assumed that binding to the regulatory site is independent of binding to the catalytic site and, since $1/\tau_1 \gg 1/\tau_2$, is a much slower process.

In Figure 8, $1/\tau_2$ is shown as a function of the sum of the equilibrium concentrations of sRTP and enzyme. The equilibrium concentrations were calculated assuming six catalytic sites with a dissociation binding constant of 2.5×10^{-4} M, three regulatory sites with a dissociation constant of 0.8×10^{-4} M and three regulatory sites with a dissociation constant of 3×10^{-3} M (Matsumoto and Hammes, 1973).

TABLE I: Kinetic Parameters for the Two-Step Mechanism of Equation 4.

	No Substrate	Carbamyl Phosphate (2 mM)	Carbamyl Phosphate (2 mM) and Succinate (10 mM)
k_2 (sec^{-1})	6260	6860	17,600
k_{-2} (sec^{-1})	828	1060	1,590
k_2/k_{-2}	7.6	6.5	11
$10^4\ k_{-1}/k_1$ (M)	6.05	5.20	9.67

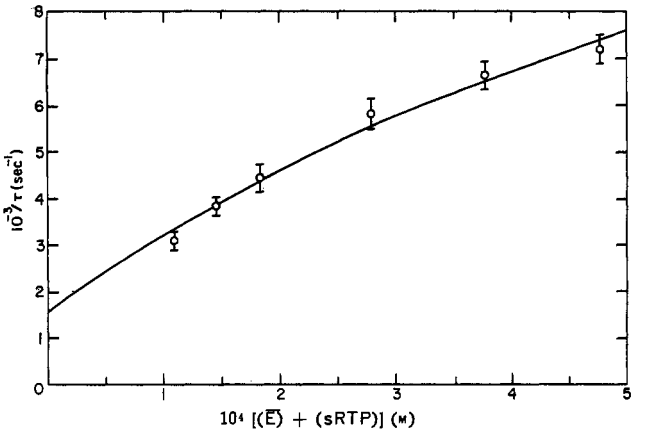

FIGURE 10: The reciprocal relaxation time for the interaction of sRTP with native enzyme as a function of the sum of the equilibrium concentrations of the enzyme and sRTP in the presence of 2 mM carbamyl phosphate and 10 mM succinate. The initial enzyme concentration varied from 0.72 to 0.91 $\times$ 10^{-4} M and that of sRTP from 1.06 to 5.27 $\times$ 10^{-4} M. Other conditions were the same as in Figure 8. The equilibrium concentrations were calculated by assuming a dissociation constant of 0.8 $\times$ 10^{-4} M. The error bars represent the standard deviation of the traces analyzed. The solid line was calculated with eq 5 and the constants in Table I.

However, binding to the three weak sites could be neglected over the accessible concentration range. As shown in the figure, $1/\tau_2$ increases with increasing [(Ē) + (sRTP̄)] and reaches a limiting value at high concentrations. A simple mechanism consistent with this concentration dependence is a bimolecular step followed by a relatively slow isomerization or conformational changes of the sRTP–enzyme complex (eq 4). In this equation (EsRTP) and (EsRTP)′ represent two

$$E + sRTP \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (EsRTP) \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (EsRTP)' \qquad (4)$$

different forms of the enzyme–sRTP complex. If the first step equilibrates very rapidly compared to the second step, the slow relaxation time is

$$1/\tau_2 = k_{-2} + \frac{k_2}{1 + k_{-1}/\{k_1[(\bar{E}) + (\overline{sRTP})]\}} \qquad (5)$$

The data in Figure 9 were fit to eq 5 by a computer program using an iterative trial and error procedure. The overall binding constant for the three regulatory sites, $K = k_{-1}/[k_1 \cdot (1 + k_2/k_{-2})] = 0.8 \times 10^{-4}$ M, was assumed to be known in the analysis. The best-fit parameters (using a least-squares criteria) obtained are summarized in Table I and the curve in Figure 8 was calculated with eq 5 and these parameters. The calculated curve is in reasonable agreement with the experimental data.

As mentioned above, in the presence of 2 mM carbamyl phosphate, only one relaxation process (0.2–1 msec) could be detected in solutions containing sRTP and native enzyme. The relaxation time was similar in magnitude to τ_2 observed in the absence of substrates, but was systematically shorter. The concentration dependence of the reciprocal relaxation time as a function of [(Ē) + (sRTP̄)] is given in Figure 9. Again only binding to three of the regulatory sites is appreciable under these conditions and a dissociation constant of 0.8 $\times$ 10^{-4} M was assumed in calculating the equilibrium concentrations. In Figure 9, $1/\tau$ increases as [(Ē) + (sRTP̄)] increases and reaches a limiting value at high concentrations, and the data were analyzed in terms of the two-step mechanism (eq 4 and 5). The best fit curves are shown as solid lines in the figure and the parameters obtained are included in Table I.

If 10 mM succinate is present in addition to 2 mM carbamyl phosphate a single relaxation process with further enhancement of the relaxation rate was observed, but the relaxation amplitude became much smaller, apparently due to a decrease in $\Delta\epsilon$ under these conditions (*cf.* Figure 7). Figure 10 shows the concentration dependence of the reciprocal relaxation time. Again the data can be analyzed by the two-step mechanism and the best fit parameters obtained are included in Table I.

Regardless of the detailed mechanism, these results suggest that a conformational change of the enzyme occurs when sRTP is bound to enzyme. Other studies (Eckfeldt *et al.*, 1970; Harrison and Hammes, 1973) indicate that when carbamyl phosphate and succinate are present the inhibitors BrCTP and CTP also induce a conformational change of the native enzyme which can be quantitatively analyzed in terms of a two-step mechanism. If a simple two-state mechanism is correct, BrCTP and sRTP should induce the conformational transition between the same two states, and only one relaxation process should be observed in the presence of both sRTP and BrCTP. On the other hand, if the conformational transition induced by BrCTP is different from that induced by sRTP, two relaxation processes should be seen. Unfortunately the relaxation times are quite similar, but in order to reduce the overlap as much as possible, a relatively low concentration of sRTP and a relatively high concentration of BrCTP were used. Within the experimental uncertainty, a single relaxation process was observed in the presence of both activator and inhibitor. This is illustrated in Figure 11 where

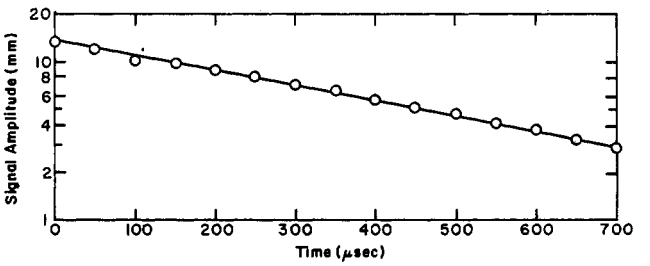

FIGURE 11: Plot of the logarithm of the signal amplitude (absorbancy change), at 335 nm *vs.* time for the relaxation process associated with BrCTP and sRTP binding to the native enzyme. Experimental conditions as described in Table II.

TABLE II: Relaxation Times for Enzyme–sRTP–BrCTP Solutions at 25°.[a]

10^4(sRTP)$_0$ (M)	10^4(BrCTP)$_0$ (M)	$1/\tau$ (sec^{-1})
2.50	0	2800[b]
0	8.0	5800[c]
2.11[e]	8.0	4560[d]

[a] Reaction mixture: 0.72 × 10^{-4} M enzyme–0.15 M potassium acetate–0.04 M imidazole acetate–2 mM carbamyl phosphate (pH 7.0). [b] Monitored at 335 nm. [c] Monitored at 308 nm. [d] Monitored at 335 nm. [e] The total concentration of sRTP was adjusted to maintain the concentration of unbound sRTP at roughly the same concentration in the experiment where no BrCTP was present.

TABLE III: Effect of sRTP on the Succinate–Enzyme Interaction.

10^4·(Succinate)$_0$ (M)	10^4(sRTP)$_0$ (M)	$1/\tau_1$ (sec^{-1})	$1/\tau_2$ (sec^{-1})
5	0		77[a]
5	1.055		65[b]
5	2.110	2370	48[b]
5	5.275	3620	45[b]
10	0		47[a]
10	5.275	4320	45[b]

[a] Reaction mixture: 0.94 × 10^{-4} M enzyme–2 mM carbamyl phosphate–0.1 M potassium acetate–2 × 10^{-5} M Phenol Red (pH 7.5). Concentrations of succinate as indicated; monitored at 560 nm. [b] Reaction mixture: 0.61 × 10^{-4} M enzyme–0.15 M potassium acetate–0.04 M imidazole acetate–2 mM carbamyl phosphate (pH 7.5); succinate and sRTP concentrations as indicated; monitored at 335 nm.

a plot of the logarithm of the signal amplitude *vs.* time is shown for a typical oscilloscope trace. A comparison of the relaxation times is presented in Table II. In practical terms, this means that if two relaxation processes occur in the presence of BrCTP and sRTP, the relaxation times probably differ by less than a factor of two.

The key role of an allosteric effector is to alter the substrate affinity to the enzyme. Therefore, it is important to examine the effect of sRTP on the substrate–enzyme interactions. In the experimental results presented thus far, no relaxation process due to the interaction of substrates and enzyme has been observed since carbamyl phosphate and succinate concentrations were used which saturated the enzyme. When the concentration of succinate was lowered to the level of the enzyme concentration, in the presence of sRTP, a relatively slow relaxation process, not seen previously, could be observed by monitoring the reaction progress at 335 nm. This process could be attributed to the succinate–enzyme interaction previously observed (Hammes and Wu, 1971a), with the enzyme–sRTP difference spectrum serving as an indicator. As shown in Table III, when the succinate concentration is held constant, the reciprocal relaxation time decreases as the sRTP concentration increases. The faster relaxation process expected for the sRTP–native enzyme interaction also was observed. A study of the enzyme–carbamyl phosphate interaction (Hammes and Wu, 1971b) in the presence of sRTP could not be carried out because of the very small relaxation amplitudes found.

Discussion

The difference spectra produced by the binding of sRTP to aspartate transcarbamylase and the catalytic subunit imply an appreciable interaction between the purine moiety of the nucleotide and the protein. They could be related to a change in the ionization constant of the sulfhydryl group, which is 10^{-8} M for the unbound nucleotide, since the absorption changes appreciably when ionization occurs (Murphy and Morales, 1970). The large difference between the difference spectrum associated with the sRTP–regulatory subunit interaction and that associated with binding to the regulatory sites of the native enzyme (when carbamyl phosphate is saturating the catalytic site) suggests a significant change of structure of the regulatory subunit is caused by the catalytic subunit. This also is suggested by the marked difference in the affinity of sRTP for the regulatory site on the native enzyme and for the isolated regulatory subunit (also see Changeux *et al.*, 1968). However, this should be contrasted to the situation when CTP is the ligand: the difference spectra and the binding constants are quite similar for the regulatory subunit and the native enzyme in the presence or absence of substrates (Changeux *et al.*, 1968; Eckfeldt *et al.*, 1970). Perhaps the purine and pyrimidine sites on the regulatory subunit do not overlap appreciably, whereas the sugar and triphosphate binding sites may be identical for CTP and ATP. The nucleotides are competitive for the same regulatory site since the sRTP–enzyme difference spectra are markedly reduced in the presence of CTP and carbamyl phosphate, which blocks the catalytic site (Figures 5 and 6). This is consistent with the finding of Changeux *et al.* (1968) that CTP binding to native aspartate transcarbamylase is markedly reduced by the addition of ATP.

The sRTP is also a potential "affinity label" since the sulfhydryl group can undergo mixed-disulfide formation with a sulfhydryl group of a protein amino acid side chain. It has been used to label the active site of guanosine-5′-phosphate reductase (Brox and Hampton, 1968) and the adenosine triphosphatase sites of myosin (Murphy and Morales, 1970). The lack of success in this work indicates a properly oriented sulfhydryl group near the catalytic or regulatory site is not present.

The fact that sRTP binds to the carbamyl phosphate site on the catalytic subunit is not surprising since other nucleotide triphosphates are competitive inhibitors of the catalytic subunit enzymatic activity (Porter *et al.*, 1969). Although spectral titration is not an accurate method for determining binding stoichiometry, the one catalytic site per equivalent molecular weight of 33,000 is consistent with that obtained by a study of carbamyl phosphate binding to the isolated catalytic subunit (Hammes *et al.*, 1970). The dissociation constant for the sRTP–catalytic subunit complex is similar to the competitive inhibition constants for other triphosphates (Porter *et al.*, 1969) and to the concentration of ATP required to achieve 50% inhibition of the catalytic subunit (Bigler and Atkinson, 1969).

Unfortunately useful equilibrium and kinetic data could not be obtained with the isolated regulatory subunit. Even in the case of the isolated catalytic subunit, where the binding constant is more favorable, a detailed study could not be made because of the rapidity of the binding process and the smallness of the relaxation amplitude. However, the shortness of the observed relaxation time implies the bimolecular reaction is probably diffusion controlled. A similar kinetic process has been observed in the binding of CTP to the isolated catalytic subunit and to the native enzyme (Harrison and Hammes, 1973).

The binding of ATP, and by analogy that of sRTP, to the native enzyme is very complex as at least three types of sites are present in the absence of carbamyl phosphate and at least two types of sites in the presence of carbamyl phosphate (Matsumoto and Hammes, 1973). In view of this, the simplicity of the relaxation spectrum is not expected. The concentration dependence of the single reciprocal relaxation time associated with sRTP binding to the regulatory sites of the native enzyme appears to be similar both in the presence and absence of either carbamyl phosphate or carbamyl phosphate and succinate: it increases with increasing sRTP concentration and approaches a limiting value at higher concentrations. This implies an intramolecular process is rate limiting in the binding process. Very similar behavior has been observed for the binding of BrCTP and CTP to the native enzyme in the presence of carbamyl phosphate and succinate (Eckfeldt *et al.*, 1970; Harrison and Hammes, 1973).

Since the bimolecular step in the binding process is not observed, either the associated relaxation amplitude is too small or the associated relaxation time is too short. The assumption that this step equilibrates much faster than the conformational change step requires that the second-order rate constant be greater than about 10^8 M^{-1} sec^{-1}, which is not at all unreasonable (*cf.* Hammes and Schimmel, 1970).

Some aspects of the nature of the conformational change can be delineated with the available data. The negative cooperativity observed in the binding isotherms and the approximately linear correlation between binding and the extent of changes in the difference spectra (Matsumoto and Hammes, 1973) suggest that a sequential change of subunit conformations is occurring. The kinetic data are adequately described by the two step mechanism of eq 4 and do not require the postulation of homotropic interactions. The fact that only a single relaxation process is observed is most readily explained by the proposition that the conformational change is largely confined to a single subunit and is the same for all six regulatory subunits. The suggestion that the conformational change is similar for all sites in spite of the binding heterogeneity is consistent with the finding that the inhibition and activation of the enzyme correlate with the total number of sites occupied (Matsumoto and Hammes, 1973). In spite of the circumstantial evidence favoring the stepwise change of subunit conformations, the involvement of a concerted conformational change in the control mechanism strictly cannot be excluded. This would require, of course, that the mechanisms for the negative cooperativity and control be different.

The kinetic parameters for the two-step mechanism are summarized in Table I. The dissociation constant k_{-1}/k_1 is roughly the same in the presence or absence of carbamyl phosphate and succinate. Moreover, this value ($\sim 10^{-3}$ M) is very similar to the dissociation constant for the association of ATP and the isolated regulatory subunit (1.5×10^{-3} M; Changeux *et al.*, 1968). This could mean that the conformational change which stabilizes binding in the native enzyme cannot occur in the isolated regulatory subunit. The rate constant k_2 markedly increases in the presence of carbamyl phosphate and succinate with a corresponding increase in k_2/k_{-2}. Experimentally this is manifested by an increase in the reciprocal relaxation time in the presence of carbamyl phosphate and succinate at high sRTP concentrations.

A simple two-state model postulates that control is exerted by shifting the equilibrium between two conformations of the enzyme (or subunit). If this is correct, only one relaxation process associated with the conformational transition would be observed in the presence of both sRTP and BrCTP. In fact, this is the case. As shown in Table II, the relaxation time in the presence of both BrCTP and sRTP is faster than that associated with sRTP binding but slower than that associated with BrCTP binding. This finding, however, is consistent with both sequential and concerted models.

A simple schematic mechanism which accommodates the results of binding studies of both sRTP and CTP is shown in eq 6. This mechanism indicates that the binding of ATP and

$$\begin{aligned} E + ATP &\rightleftharpoons X_1 \rightleftharpoons X_2 \\ E + CTP &\rightleftharpoons X_2 \rightleftharpoons X_1 \end{aligned} \tag{6}$$

CTP causes the formation of the two enzyme–effector conformations, X_1 and X_2. However, the binding of CTP favors the formation of X_1, while the binding of ATP favors the formation of X_2. Furthermore, the binding of carbamyl phosphate and succinate favors the formation of X_2; this is observed for both CTP and ATP binding. (Experimentally this is manifested by the observation that at high effector concentrations, the addition of carbamyl phosphate and succinate increases $1/\tau$ in the case of sRTP and decreases it in the case of CTP.) Thus this rather simple two state mechanism appears to be consistent with all of the known experimental results for ATP and CTP binding to aspartate transcarbamylase.

The situation is quite different for the relatively slow conformational change induced by succinate binding (at saturating concentrations of carbamyl phosphate) and that due to sRTP binding. As indicated in Table III, two coupled but distinct relaxation processes are observed which indicates two distinct, but coupled, conformational transitions are induced by sRTP and succinate. However, in contrast to the effect of BrCTP, increasing the sRTP concentration reduces the reciprocal relaxation time associated with the succinate-induced conformational change. The antagonistic effects of CTP and ATP on the rate of this conformational transition parallels their effects on the aspartate binding isotherm (Gerhart and Pardee, 1962).

The overall picture of the control processes for aspartate transcarbamylase which emerges from these studies can be summarized as follows. Both ATP and CTP appear to carry out their control functions by altering essentially the same conformational equilibria in the enzyme. On the other hand, the conformational change involved in control that is induced by succinate binding is quite distinct from that induced by CTP and ATP. In addition carbamyl phosphate binding appears to be associated with still a different conformational change (Hammes and Wu, 1971b). A definite interaction between these various conformational changes occurs: the associated rates depend on the concentrations of all ligands. The nature of the conformational changes can be specified to some extent: those associated with succinate and carbamyl phosphate binding seem most consistent with concerted con-

formational changes, whereas that associated with ATP and CTP binding probably involves a stepwise change in the conformation of the subunits. A detailed molecular picture of the control processes remains to be specified as a better understanding of the structure of the enzyme emerges.

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Aggregation of Rabbit Muscle Phosphofructokinase†

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ABSTRACT: Rabbit skeletal muscle phosphofructokinase has been found to consist of polypeptide chains of essentially identical molecular weight. This molecular weight was found to be 75,000 (±10,000) by acrylamide gel electrophoresis in sodium dodecyl sulfate and 85,000 (±10,000) by gel chromatography in 6 and 7 M guanidine-HCl–0.1 M β-mercaptoethanol. The aggregation state of the enzyme was studied by frontal gel chromatography in 0.1 M phosphate buffer over the pH range 6.0–8.0 and the protein concentration range 0.001–1.0 mg/ml. The results obtained indicate that a stable tetramer with a Stokes' radius of 67 Å and a molecular weight of approximately 320,000 exists over an appreciable concentration range at pH 8.0. This tetrameric aggregate can be broken down into smaller species by lowering either the protein concentration or pH. The data at concentrations less than 0.2 mg/ml can be described by a mechanism involving a pH-dependent equilibrium between a dimer and tetramer, and information about the shape of the aggregates has been inferred. The enzymatic activity appears to be dependent on the aggregation state of the enzyme, with aggregates smaller than the tetramer not possessing appreciable enzymatic activity.

Rabbit skeletal muscle phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) exhibits kinetic properties in the physiological pH range which are indicative of an allosteric enzyme (Hofer and Pette, 1968b). Sigmoidal initial velocity–substrate concentration isotherms are observed, and the enzymatic reaction is susceptible to activation and inhibition by a number of nonsubstrate effectors (Passonneau and Lowry, 1962). Because of these properties and its strategic location in the metabolic process, phosphofructokinase is considered to be of primary importance in the regulation of glycolysis.

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