An Equilibrium Binding Study of the Interaction of Aspartate Transcarbamylase with Cytidine 5′-Triphosphate and Adenosine 5′-Triphosphate†

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ABSTRACT: Equilibrium binding studies of the interaction of aspartate transcarbamylase from *Escherichia coli* with its nucleotide effectors, cytidine 5′-triphosphate and adenosine 5′-triphosphate, have been carried out at 4 and 23° in the presence and absence of the substrate, carbamyl phosphate, and the substrate analog, succinate, using gel filtration and ultrafiltration methods. Extensive binding data were obtained for cytidine 5′-triphosphate over a wide range of enzyme saturation levels at both temperatures, while more limited data were obtained for adenosine 5′-triphosphate because of the weakness of the ligand binding. For both nucleotides, at 4 and 23°, in the presence of carbamyl phosphate (2 mM), with or without succinate (10 mM), the binding isotherms can be described in terms of two sets of three independent binding sites which have affinities differing by one to two orders of magnitude. The binding is considerably weaker at the higher temperature. Carbamyl phosphate and succinate have only a small effect on the binding of nucleotides to these two sets of sites, but in their absence binding also occurs at the catalytic sites. The difference spectra associated with the binding of the effector analogs 5-bromocytidine 5′-triphosphate and 6-mercaptoadenosine 5′-triphosphate to enzyme show a reasonably linear correlation with the extent of binding. The total per cent saturation of both classes of sites by the inhibitor and activator nucleotides parallels the per cent inhibition and activation, respectively, of the enzymatic reaction. These results suggest a sequential change of subunit conformations is involved in the binding of effectors.

An understanding of the binding characteristics of enzymes is essential for the elucidation of enzyme mechanisms. This is especially true for enzymes involved in metabolic regulation which bind several ligands since protein-mediated interactions between ligands may be of importance. Aspartate transcarbamylase from *Escherichia coli* (EC 2.1.3.2) is a well-known control enzyme in pyrimidine biosynthesis which utilizes carbamyl phosphate and aspartate as substrates (Yates and Pardee, 1956; Gerhart and Pardee, 1962). Its activity is inhibited by CTP, the end product of the pyrimidine pathway, and it is activated by the purine nucleotide ATP. Not only are the substrates and nucleotide effectors structurally different, but they act on distinct subunits of the enzyme. One type of subunit having catalytic activity can be separated from another type which has no activity but binds nucleotides (Gerhart and Schachman, 1965). However, a number of discrepancies exist among the studies which have been made of effector binding to the enzyme (Changeux *et al.*, 1968; Eckfeldt *et al.*, 1970; Hammes *et al.*, 1970; Winlund and Chamberlin, 1970; Buckman, 1970). In order to provide quantitative data for mechanistic analysis, the binding of CTP and ATP to aspartate transcarbamylase is reexamined in this work. A wide range of effector concentrations at 4 and 23° is covered and the effects of carbamyl phosphate and the nonreactive aspartate analog succinate on nucleotide binding also are reported.

Experimental Section

Materials. Aspartate transcarbamylase was prepared according to the procedure of Gerhart and Holoubek (1967). The overproducing mutant of *Escherichia coli* was grown by the New England Enzyme Center. Enzyme concentrations were determined by absorbance measurements at 280 nm assuming an extinction coefficient of 0.59 cm^2/mg (Gerhart and Holoubek, 1967) and a molecular weight of 310,000 (Gerhart and Schachman, 1965). The CTP, ATP, carbamyl phosphate, succinate, imidazole, aspartate, antipyridine, and diacetylmonoxime were obtained from the Sigma Chemical Co. Carbamyl phosphate was further purified by precipitation from 50% ethanol (Gerhart and Pardee, 1962). Imidazole was twice recrystallized from benzene. The radioactive nucleotides [^{14}C]CTP (0.2 Ci/mmol), [^{3}H]CTP (20 Ci/mmol), and [^{3}H]-ATP (20 Ci/mmol) were Schwarz/Mann products and were purified by paper chromatography in the solvent system isobutyric acid–1 N NH_4OH (100:60, v/v) and elution of the appropriate parts of the chromatogram with water. All solutions were made from distilled water and contained 0.1 M imidazole acetate, 1 mM dithiothreitol, and 0.5 mM EDTA. At 23° the buffer used had a pH of 7.0 and at 4° the same buffer had a pH of 7.3. Radioactive samples were counted in a naphthalene–dioxane scintillation fluid (Bray, 1960). The dioxane used was spectroscopic grade and the scintillation fluors were obtained from Packard.

Binding Measurements. Two filtration methods were used to study the binding of CTP and ATP to the enzyme. These methods were selected because their short execution time prevented appreciable hydrolysis of carbamyl phosphate. In the gel filtration method (Hummel and Dryer, 1962), the effluent from a thermostated column of Sephadex G-25 (0.6 cm^2 × 60 cm or 3 cm^2 × 50 cm) was monitored with a micro-flow cell (Hellma 178) in a Cary 14 spectrophotometer equipped with a 0–0.1 absorbance slide-wire. The nucleotide concentration in the column effluent was determined by its absorbance at a wavelength of 272 nm for CTP or 259 nm for ATP. Approximately 1 × 10^{-8} mol of enzyme was applied in a

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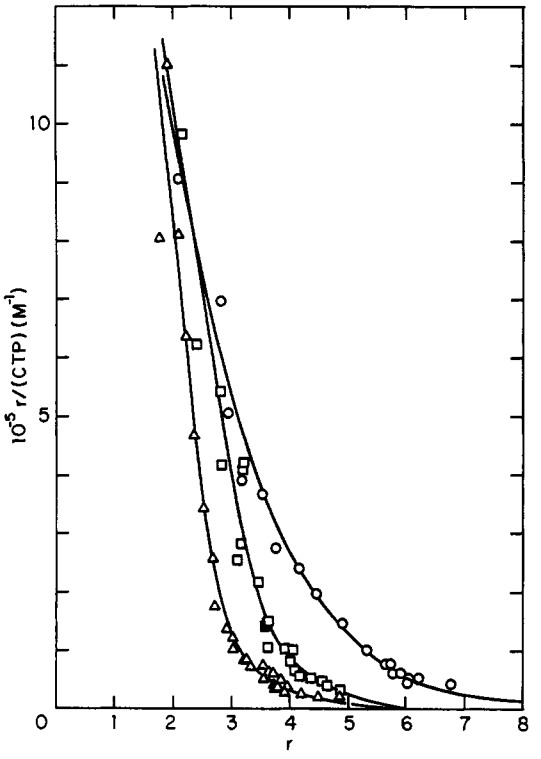

FIGURE 1: CTP binding isotherms at 4° and pH 7.3 (0.1 M imidazole acetate–1 mM dithiothreitol–0.5 mM EDTA) plotted as $r/(CTP)$ *vs.* r, where r is the number of moles of ligand bound per mole of enzyme of mol wt 310,000 and (CTP) is the concentration of free ligand: (○) buffer alone; (□) buffer plus 2 mM carbamyl phosphate; (Δ) buffer plus 2 mM carbamyl phosphate and 10 mM succinate. The curves are calculated according to eq 1 and the parameters in Table I with the n_i as variables except in buffer alone.

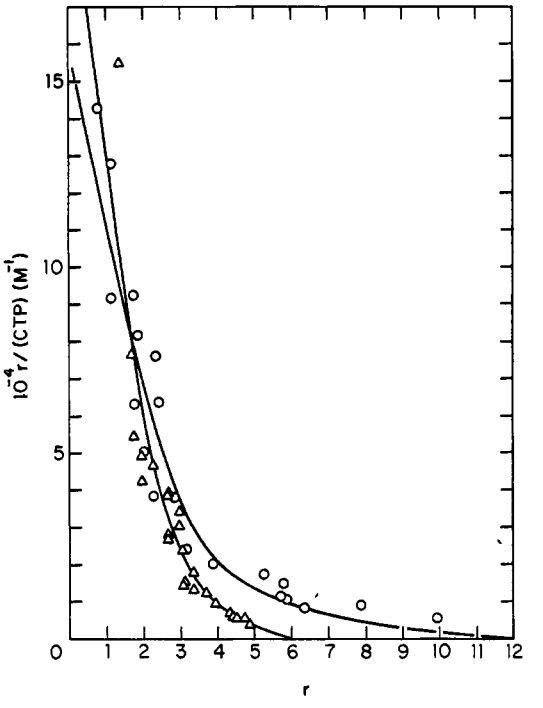

FIGURE 2: CTP binding isotherms at 23° and pH 7.0 (0.1 M imidazole acetate–1 mM dithiothreitol–0.5 mM EDTA) plotted as $r/(CTP)$ *vs.* r, where r is the number of moles of ligand bound per mole of enzyme of mol wt 310,000 and (CTP) is the concentration of free ligand: (○) buffer alone; (Δ) buffer plus 2 mM carbamyl phosphate and 10 mM succinate. The curves are calculated according to eq 1 and the parameters in Table I with $n_1 = n_2 = 3$, $n_3 = 6$ in the former case and n_1 and n_2 as variables in the latter case.

volume of 100 μl to a column which had been equilibrated with ligand and buffer. The number of moles of ligand bound to the enzyme was determined by measuring the area of the absorbance trough following the elution of the protein. The applied enzyme volume contained no ligand; therefore the product of this volume multiplied by the ligand concentration of the elution buffer was subtracted from the trough area. The range of CTP concentrations employed was from 2×10^{-6} to 2×10^{-4} M and that of ATP from 1×10^{-5} to 3×10^{-4} M. The error in r, the number of moles of ligand bound per mole of enzyme, is less than ±10% in the middle of the range of ligand concentrations employed and is slightly larger at the extremes of high and low concentrations. In the former case this is due to the small fraction of ligand bound, while in the latter case it is due to the difficulty in measuring small concentration changes.

The second technique used to measure binding was an ultrafiltration method (Paulus, 1969). An apparatus manufactured by Metaloglass and Diaflow UM-10 membrane filters were used. The number of moles of ligand bound was determined by measuring the difference in radioactivity between filters obtained after filtration (under pressure) of solutions with and without protein. The radioactivity of the blank filter is due to the fact that a small amount of liquid is retained by the filter. Binding of nucleotide to the filter was not observed. In most cases two or more filtrations were simultaneously run on each solution and the results averaged before calculating r. The amount of protein per sample compartment ranged from 8×10^{-11} to 5.5×10^{-10} mol in a volume of 0.25–0.45 ml. The range of CTP concentrations covered was 5×10^{-5} to 1×10^{-3} M and that for ATP was 2×10^{-5} to 2×10^{-3} M. The error in r is estimated as ±10 to 20%, with larger errors at the extremes of ligand concentration.

For both methods individual runs could be completed approximately two hours after preparation of a carbamyl phosphate solution. The ultrafiltration method was used only at 23° and the column method was used at both 4 and 23°. At the higher temperature the two methods gave comparable results.

Enzyme Assay. The production of carbamyl aspartate was measured using the colorimetric assay of Prescott and Jones (1969). The assay mixture contained varying concentrations of substrates and CTP or ATP in 0.5 ml of 0.1 M imidazole acetate buffer (pH 7.0) without dithiothreitol or EDTA. After incubation at 23° for 0.5 hr, the reaction was stopped by addition of 1 ml of the antipyrene–oxime color mix and the yellow color was developed by Method II of Prescott and Jones (1969).

Results and Treatment of Data

The binding data obtained are summarized in Figures 1–4 in plots of $r/(L)$ *vs.* r, where r is the number of moles of ligand bound per mole of enzyme and (L) is the concentration of free ligand (Scatchard, 1949). Extensive data were obtained for the binding of CTP to the enzyme at 4° in buffer, in buffer plus 2 mM carbamyl phosphate and in buffer plus 2 mM carbamyl phosphate and 10 mM succinate, an aspartate analog (Figure 1). At 23° data were obtained in buffer and in buffer plus 2

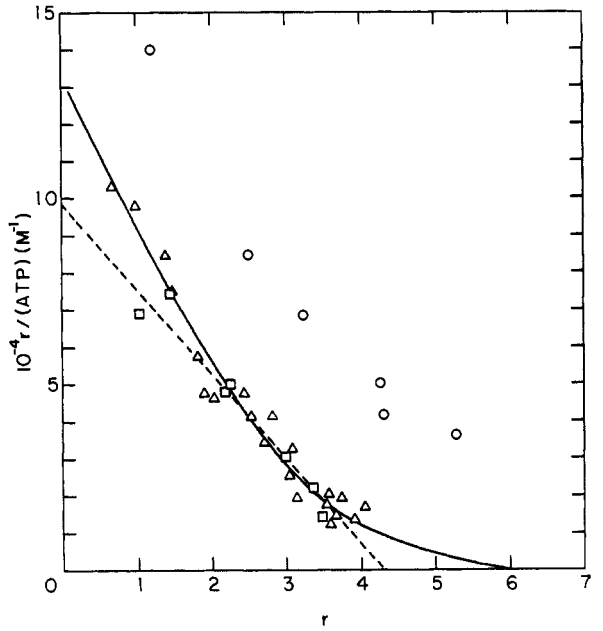

FIGURE 3: ATP binding isotherms at 4° and pH 7.3 (0.1 M imidazole acetate–1 mM dithiothreitol–0.5 mM EDTA) plotted as $r/(ATP)$ *vs.* r, where r is the number of moles of ligand bound per mole of enzyme of mol wt 310,000 and (ATP) is the concentration of free ligand: (○) buffer alone; (□) buffer plus 2 mM carbamyl phosphate; (Δ) buffer plus 2 mM carbamyl phosphate and 10 mM succinate. The solid curve is calculated according to eq 1 and the parameters in Table I. The dashed line is a least-squares fit of the data in the presence of carbamyl phosphate and succinate assuming a single class of sites.

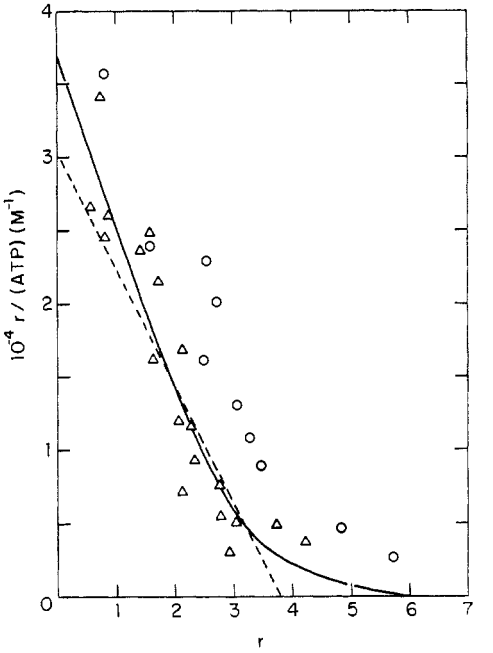

FIGURE 4: ATP binding isotherms at 23° and pH 7.0 (0.1 M imidazole acetate–1 mM dithiothreitol–0.5 mM EDTA) plotted as $r/(ATP)$ *vs.* r, where r is the number of moles of ligand bound per mole of enzyme of mol wt 310,000 and (ATP) is the free ligand concentration: (○) buffer alone; (Δ) buffer plus 2 mM carbamyl phosphate and 10 mM succinate. The solid curve is calculated according to eq 1 and the parameters in Table I. The dashed line is a least-squares fit of the data in the presence of carbamyl phosphate and succinate assuming a single class of sites.

mM carbamyl phosphate and 10 mM succinate (Figure 2). In the case of ATP binding, less extensive data were obtained, primarily due to the fact that ATP binds much more weakly to the enzyme than CTP so that a wide range of r could not be studied. Most of the data for ATP were obtained in buffer plus 2 mM carbamyl phosphate and 10 mM succinate, although some results were obtained in buffer alone and in buffer plus 2 mM carbamyl phosphate (Figures 3 and 4).

The data obtained for CTP binding to aspartate transcarbamylase are not consistent with binding to a single set of sites. If this were the case, the plots in Figures 1 and 2 would be straight lines intersecting the abscissa at the number of ligand binding sites per enzyme molecule. For ATP the plot at 4° is clearly curved, although the maximum value of r could not be approached experimentally because of the weakness of the binding. At 23° the curvature in the Scatchard plot is barely detectable for ATP binding.

The simplest model found to fit all of the experimental data was based on two sets of independent binding sites in the presence of 2 mM carbamyl phosphate and three sets of independent sites in buffer alone. For this model r can be written as

$$r = \sum_{i=1}^{3} \frac{n_i K_i(\mathrm{L})}{1 + K_i(\mathrm{L})} \qquad (1)$$

Here n_i is the number of sites having binding constant K_i, which is defined as $K_i = (\mathrm{E}_i\mathrm{L})/[(\mathrm{E})(\mathrm{L})]$, where (E) is the concentration of unliganded enzyme and $(\mathrm{E}_i\mathrm{L})$ is the concentration of enzyme–ligand complexes involving a particular site of type i. The CTP binding curves in the presence of carbamyl phosphate were analyzed using the first two terms of eq 1 by a nonlinear least-squares procedure with n_1, n_2, K_1, and K_2 as unknown parameters. The values of the parameters obtained are given in Table I and the curves in Figures 1 and 2 have been calculated based on these parameters and eq 1. Within experimental uncertainties $n_1 = n_2 = 3$, indicating a total of six regulatory sites. The binding constants obtained by assuming $n_1 = n_2 = 3$ also are included in Table I. In the absence of carbamyl phosphate the CTP binding isotherms were analyzed assuming $n_1 = n_2 = 3$ and $n_3 = 6$. This was based on the supposition that in addition to binding at the six regulatory sites, binding of CTP also occurs at the six catalytic sites (*cf.* Porter *et al.*, 1969, and Hammes *et al.*, 1970). The binding constants obtained from a nonlinear least-squares analysis of the data with these assumptions is included in Table I, and the appropriate curves are shown in Figures 1 and 2.

The ATP binding isotherms in the presence of carbamyl phosphate were fit to the first two terms of eq 1 assuming $n_1 = n_2 = 3$. Owing to the lack of data at high degrees of saturation the n_i could not be taken as adjustable parameters. The values of the binding constants obtained are given in Table I and the calculated binding curves are shown in Figures 3 and 4. Although the Scatchard plots show a slight, but definite, curvature at both 4 and 23°, the data can be fit reasonably well assuming a single class of sites. The theoretical curve for such an analysis is included in Figures 3 and 4. Marked deviations from linearity occur at low ligand concentrations. The values of n and K obtained are 4.3 and 2.3×10^4 M^{-1} respectively at 4° and 3.8 and 8×10^3 M^{-1} respectively at 23°. No attempt was made to fit the ATP binding data in the absence of carbamyl phosphate.

Although the model based on two independent classes of regulatory sites and one class of catalytic sites fits the data

TABLE I: Parameters for CTP and ATP Binding to Aspartate Transcarbamylase.[a]

Site Class No.	n_i[b]	K_i (M^{-1})	Ligands
CTP, 4°, pH 7.3			
1	3	$7 (\pm 1) \times 10^5$	—
2	3	$4.3 (\pm 0.6) \times 10^4$	
3	6	$1.0 (\pm 0.2) \times 10^3$	
1	3.4 (±0.2)	$7 (\pm 2) \times 10^5$	2 mM carbamyl phosphate
2	2.9 (±0.8)	$7 (\pm 4) \times 10^3$	
1	3	$1.3 (\pm 0.2) \times 10^6$	
2	3	$1.1 (\pm 0.1) \times 10^4$	
1	2.7 (±0.2)	$1.1 (\pm 0.3) \times 10^6$	2 mM carbamyl phosphate, 10 mM succinate
2	3.2 (±0.5)	$6 (\pm 3) \times 10^3$	
1	3	$7.1 (\pm 0.7) \times 10^5$	
2	3	$4.4 (\pm 0.3) \times 10^3$	
CTP, 23°, pH 7.0			
1	3	$5 (\pm 1) \times 10^4$	—
2	3	$1.4 (\pm 0.9) \times 10^3$	
3	6	$6 (\pm 1) \times 10^2$	
1	2.5 (±0.4)	$8 (\pm 4) \times 10^4$	2 mM carbamyl phosphate, 10 mM succinate
2	3.5 (±0.4)	$2 (\pm 1) \times 10^3$	
1	3	$4.8 (\pm 0.5) \times 10^4$	
2	3	$1.3 (\pm 0.1) \times 10^3$	
ATP, 4°, pH 7.3			
1	3	$4.2 (\pm 0.4) \times 10^4$	2 mM carbamyl phosphate, 10 mM succinate
2	3	$2.1 (\pm 0.2) \times 10^3$	
ATP, 23°, pH 7.0			
1	3	$1.2 (\pm 0.2) \times 10^4$	2 mM carbamyl phosphate, 10 mM succinate
2	3	$3.3 (\pm 0.9) \times 10^2$	

[a] 0.1 M imidazole acetate–1 mM dithiothreitol–0.5 mM EDTA. Standard deviations of parameters are given in parentheses. [b] Assumed values if standard deviations are not given.

very well, a more complex model of ligand binding in the presence of substrate also was examined. In this model, six regulatory sites were assumed, each with its own characteristic binding constant, so that

$$r = \frac{K_1(L) + 2K_1K_2(L)^2 + \cdots + 6K_1K_2K_3K_4K_5K_6K(L)^6}{1 + K_1(L) + K_1K_2(L)^2 + \cdots + K_1K_2K_3K_4K_5K_6(L)^6} \quad (2)$$

where $K_1 = (EL)/[(E)(L)]$, $K_2 = (EL_2)/[(E)(L)]$, ... The procedure of Cornish-Bowden and Koshland (1970) was used to obtain the six constants. (We are grateful to Professor D. Koshland for providing us with the necessary computer program.) This model did not fit the data any better than the simpler model described above. In general the individual intrinsic constants again could be divided into two classes, but it was difficult to determine K_1 and K_6 due to the absence of data at very low and high values of r.

Discussion

The results for the binding of regulatory nucleotide triphosphates to aspartate transcarbamylase are consistent with the previous studies. Two types of CTP sites have been reported in phosphate buffer at 4° (Winlund and Chamberlin, 1970) and at 23° (Buckman, 1970). For the class of tight binding sites, good quantitative agreement exists with regard to both the number of sites and their binding affinity. The differences in the parameters for the second weaker class of sites can be ascribed to the difficulty in measurement at high ligand concentrations and to the partial competition between phosphate and CTP binding at the catalytic site. As discussed by Winlund and Chamberlin (1970), the presence of six sites divided into two classes of three sites each could be explained by the dimeric structure of the three regulatory subunits in aspartate transcarbamylase (Weber, 1968; Rosenbusch and Weber, 1971). Whether the observed nonequivalence of sites is due to direct or indirect interactions between sites still is unknown. In earlier studies of CTP binding, which had assumed a single class of sites, the single constant reported always is between the two constants reported here (Gerhart and Schachman, 1965; Changeux *et al.*, 1968; Eckfeldt *et al.*, 1970). Some type of average constant, valid over the limited concentration range investigated, apparently was determined. The variation in the number of sites from four to eight in the earlier reports probably was due to extrapolation from an incomplete segment of a binding curve which is very complex in its entirety (*cf.* the incomplete binding isotherms for ATP in Figures 3 and 4). Previously the binding of ATP had only been studied by indirect methods. Changeux *et al.* (1968) used ATP competition with CTP binding to calculate an ATP binding constant which is very close to that obtained above when a single class of sites is assumed. Buckman (1970) reports a somewhat lower value as the reciprocal concentration at which the electron paramagnetic resonance (epr) signal of the spin-labeled enzyme has half its maximum height. In the case of ATP it has not been possible to independently determine the number of

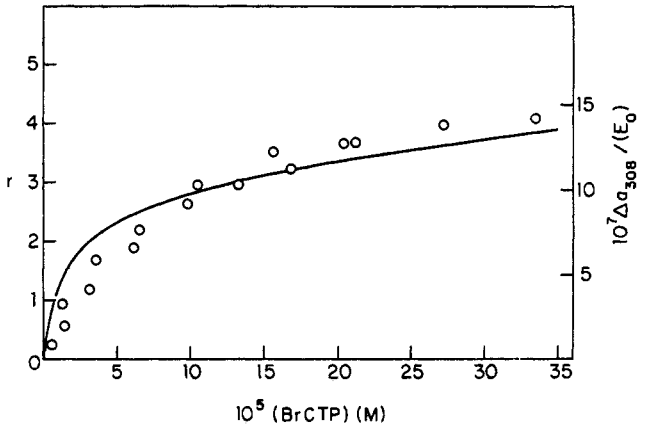

FIGURE 5: Correlation between 5-bromoCTP difference spectral titration at 25° and CTP binding at 23°. The curve is the calculated value of r (left-hand ordinate), the number of moles of ligand bound per mole of enzyme of mol wt 310,000, as a function of the free 5-bromoCTP concentration (BrCTP). The points refer to the right-hand ordinate which is the difference absorbance at 308 nm, Δa_{308}, divided by the total enzyme concentration, (E_0). The values $n_1 = 2.5$, $K_1 = 8 \times 10^4$ M^{-1}, $n_2 = 3.5$ $K_2 = 2 \times 10^3$ M^{-1} from Table I were used to calculate the curve and the concentrations of free 5-bromoCTP. The difference spectral data are from Eckfeldt *et al.* (1970) in 0.15 M KCl and 0.02 M imidazole-HCl (pH 7.0), with 2 mM carbamyl phosphate and 10 mM succinate at 25°.

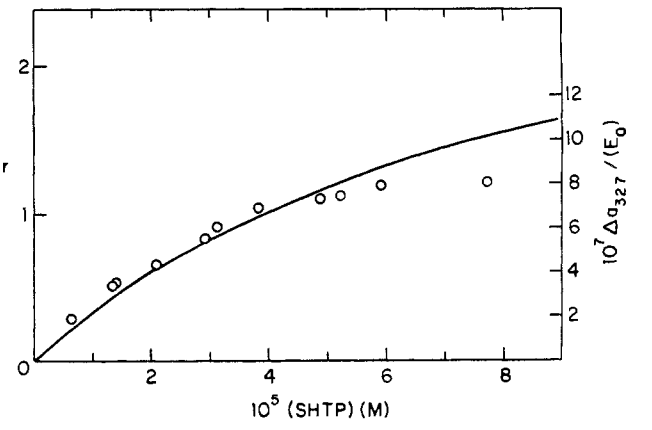

FIGURE 6: Correlation between the 6-mercaptoATP difference spectral titration at 25° and ATP binding at 23°. The curve is the calculated value of r (left-hand ordinate), the number of moles of ligand bound per mole of enzyme of mol wt 310,000, as a function of the free 6-mercaptoATP concentration (SHTP). The points refer to the right hand ordinate which is the difference absorbance at 327 nm, Δa_{327}, divided by the total enzyme concentration (E_0). The values $n_1 = 3$, $K_1 = 1.2 \times 10^4$ M^{-1}, $n_2 = 3$, $K_2 = 3.3 \times 10^2$ M^{-1} from Table I were used to calculate the curve and the concentrations of free SHTP. The difference spectral data are from Wu and Hammes (1973) in 0.15 M potassium acetate and 0.04 M imidazole acetic acid (pH 7.0), with 2 mM carbamyl phosphate and 10 mM succinate at 25°.

regulatory sites, but the data can be fit extremely well by assuming the same stoichiometry as found for CTP.

Binding isotherms for the binding of 5-bromoCTP and 6-mercaptoATP to the enzyme have been determined using difference spectroscopy (Eckfeldt *et al.*, 1970; Wu and Hammes, 1972). These measurements can be correlated with the direct binding studies by assuming that the measured change in absorbancy is directly proportional to the extent of binding and that 5-bromoCTP and 6-mercaptoATP have the same binding isotherms as CTP and ATP, respectively. This latter assumption is strongly suggested by comparative studies of these compounds (Changeux *et al.*, 1968; Gerhart and Schachman, 1968; Wu and Hammes, 1973). In this case the difference absorbance, Δa, divided by the total enzyme concentration, (E_0), should be directly proportional to r. Therefore, if the proportionality constant is fixed at one point on the binding isotherm, a plot of $\Delta a/(E_0)$ *vs.* the free ligand concentration should coincide exactly with a plot of r versus free ligand concentration as determined by direct binding. A comparison of the difference spectroscopy and direct binding measurements on this basis is shown in Figures 5 and 6 for 5-bromoCTP and 6-mercaptoATP, respectively. The solid lines represent the direct binding measurements and the points are derived from the difference spectral measurements. In the case of 5-bromoCTP, the two methods are in reasonable agreement, although some deviations occur. It should be noted that binding isotherms obtained from difference spectroscopy do not have a high precision, especially at very low and very high ligand concentrations. Good agreement is observed in Figure 6 in the case of 6-mercaptoATP, but only a very small range of r is accessible to difference spectral measurements.

Binding at two classes of sites is observed at both 4 and 23° for both CTP and ATP. The difference in affinity for the two classes of regulatory sites is appreciable; the binding constants differ by factors of 20–100. The temperature dependence of the binding constants also is large, although a quantitative thermodynamic interpretation cannot be given until pH profiles of the binding constants are available at two temperatures. In the case of CTP, the binding constants for both tight and loose sites decrease an order of magnitude on raising the temperature from 4 to 23°, while the decrease is less in the case of ATP. This suggests the enthalpy changes associated with CTP binding may be larger (more negative) than those associated with ATP binding. Also the temperature coefficients, and therefore the enthalpy changes, for the binding constants of the tight and loose sites are quite similar suggesting that the differences in binding constants between the two types of sites may be largely entropic in nature.

The major effect of the substrate carbamyl phosphate and the substrate analog succinate on nucleotide triphosphate binding is to eliminate binding at the catalytic site. Apparently very little change in affinity occurs at the regulatory sites. In the case of CTP binding at 4°, within the experimental uncertainties the binding constants for the sites of class one (Table I) are unchanged by the presence of carbamyl phosphate and succinate. The binding constants for the class two sites decrease by about a factor of four in the presence of carbamyl phosphate and decrease an additional factor of two to three when both carbamyl phosphate and succinate are present. However, at 23° carbamyl phosphate and succinate have no observable effect on CTP binding to either class of sites. These findings are similar to those of Changeux *et al.* (1968) who found that the binding of 5-bromoCTP in the presence of high carbamyl phosphate concentrations was not greatly altered by succinate. The effect of carbamyl phosphate and succinate is not so clear for ATP binding, but identical values of r are obtained in the presence of 2 mM carbamyl phosphate with or without 10 mM succinate. Whatever the detailed mechanism may be, if it is true that carbamyl phosphate and succinate have little effect on CTP (or ATP) binding, then this necessarily implies that CTP has only a small effect on carbamyl phosphate and succinate binding. This conclusion has not yet been tested experimentally. (It has been shown that 5-bromoCTP only partially reduces the effect of succinate on

TABLE II: Correlation between Inhibition and CTP Binding.

(CTP) (μM)	% of Max. Inhibn[a]	% Satn of Tight Sites[b]	% Satn of Loose Sites[b]	% Satn of Total Sites[b]
2000	86 (94)	99	80	88
500	77 (82)	98	50	70
50	44 (49)	80	9	39
25	36 (33)	67	5	31

[a] These values were measured as described in the Experimental Section at 5 mM aspartate–3.6 mM carbamyl phosphate–0.1 M imidazole acetate (pH 7.0) and 23° using an extrapolated maximum inhibition of 70%. The values in parentheses are taken from Gerhart and Pardee (1962) assuming a maximum inhibition of 90% at 5 mM aspartate–3.6 mM carbamyl phosphate–0.04 M potassium phosphate at pH 7.0 and 28°. [b] Based on the parameters in Table I for CTP binding in the presence of 2 mM carbamyl phosphate and 10 mM succinate at 23° with $n_1 = 2.5$ and $n_2 = 3.5$.

TABLE III: Correlation between Activation and ATP Binding.

(ATP) (mM)	% of Max. Inhibn[a]	% Satn of Tight Sites[b]	% Satn of Loose Sites[b]	% Satn of Total Sites[b]
2	65 (73)	96	40	68
1	60 (60)	92	25	59
0.5	51 (40)	86	14	50
0.25	41 (22)	75	8	41

[a] These values were measured as described in the Experimental Section at 2 mM aspartate–10 mM carbamyl phosphate–0.1 M imidazole acetate (pH 7.0) and 23° using an extrapolated maximum activation of 170%. The values in parentheses are estimated from data given by Gerhart (1970) assuming a maximum activation of 130% at 2 mM aspartate–3.6 mM carbamyl phosphate–0.04 M potassium phosphate (pH 7.0). [b] Based on the parameters in Table I for ATP binding in the presence of 2 mM carbamyl phosphate and 10 mM succinate at 23°.

the reaction of the enzyme with *p*-hydroxymercuribenzoate (Gerhart and Schachman, 1968).) This poses a dilemma since CTP and ATP are known to strongly inhibit and activate the enzyme, presumably by altering the affinity of the enzyme for aspartate (Gerhart and Pardee, 1962). Several explanations for these observations are possible. First, the experimental error in the binding measurements may be masking significant changes. Second, the enzymatic reaction velocity may not be directly related to the equilibrium binding of aspartate, that is, a kinetic effect may be involved. This question might be resolved by detailed steady state studies, although it is unlikely a definitive answer could be obtained for such a compelx mechanism. Finally succinate may not be an appropriate analog of aspartate so that it does not mimic the effect of asparate on effector binding. For example, a known difference between succinate and aspartate is that the former binds much more tightly to the active site than the latter (Porter *et al.*, 1969; Collins and Stark, 1969). Insufficient information is available to resolve this problem at the present time.

The nucleotide binding isotherms can be compared with the inhibition and activation of enzymic activity by nucleotides, assuming the binding isotherms are not appreciably altered by the substitution of aspartate for succinate and do not depend significantly on the aspartate concentration. The percentage inhibition or activation at a given nucleotide concentration depends on the aspartate concentration so that the data at a particular aspartate concentration were normalized by dividing the percentage activity change at a given nucleotide concentration by the estimated maximal percentage change. Some uncertainty exists in assessing the maximal percentage changes because of inhibition at high nucleotide concentrations due to their binding at the catalytic site. As shown in Table II, the percentage of maximum inhibition parallels the overall percentage of saturation by CTP, while no obvious correlation is observed with saturation of a single class of sites. The activation by ATP also apparently involves both sets of regulatory sites (Table III). Thus the extent of binding of effectors can be correlated with metabolic control and indicates all six regulatory sites are involved in regulation.

Since a regulatory protein is most sensitive to effector concentrations approximately equal to the dissociation constant for the protein–effector complex, the presence of two binding sites with affinities differing by one or two orders of magnitude greatly extends the sensitivity of regulation. This complements the effect of positive cooperativity which narrows the range of ligand concentration between completely saturated and completely unsaturated states. The mechanism of enzyme–effector interactions is discussed further in accompanying papers concerned with kinetic studies of CTP and 6-mercaptoATP binding to aspartate transcarbamylase (Harrison and Hammes, 1973; Wu and Hammes, 1973). However, the correlation observed between nucleotide binding and inhibition or activation and the negative cooperativity found in the effector binding isotherms suggest that a sequential change in subunit conformation, rather than a concerted one, accompanies the binding of nucleotides (Levitzki and Koshland, 1969).

Addendum

After this report was submitted for publication, a study of CTP binding to aspartate transcarbamylase was reported by Cook (1972). At both 4 and 23° the binding was shown to be negatively cooperative for r greater than one and positively cooperative when r had fractional values. The behavior at high CTP concentrations ($r > 1$) is in qualitative agreement with our results, but the extent of binding is slightly lower at 23° and much lower at 4° in comparison with our results and previous studies. We were unable to obtain reproducible values of r at concentrations of free CTP of less than 1 μM, where positive cooperativity is reported by Cook. At these concentrations, the gel filtration and ultrafiltration methods involve measuring a small difference between two large quantities. Although this problem is alleviated in the equilibrium dialysis method used by Cook, whether or not the observed positive cooperativity is a real phenomenon is still uncertain because of another complication. High enzyme concentrations were used which exceeded the free ligand concentration by a factor of 100 or more in the range where positive cooperativity occurred. Assuming three tight sites and a

binding constant of 10^5–10^6 M^{-1}, as suggested by our data, 5–1%, respectively, of the total ligand is unbound at a concentration of 1 μM free CTP, with even smaller percentages at lower free-ligand concentrations. Under these conditions, freely equilibrating radioactive impurities would result in an apparent decrease in binding strength as the ligand concentration is lowered, thus giving the appearance of positive cooperativity. This problem requires further investigation, but in any event, the analysis presented here quantitatively describes all of the binding data we have been able to obtain.

Added in Proof

We are indebted to Drs. Gray and Chamberlin for sending us the results of an investigation of the interaction of CTP and ATP with aspartate transcarbamylase prior to publication (C. W. Gray, M. J. Chamberlin, D. M. Gray, submitted for publication). Although the experimental conditions are somewhat different, the equilibrium binding results are in quite good agreement with those reported here.

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