Interaction of Aspartate Transcarbamylase with 5-Bromocytidine 5′-Tri-, Di-, and Monophosphates†

Christian Tondre‡ and Gordon G. Hammes*

ABSTRACT: The interaction of the 5-bromo derivatives of CTP, CDP, and CMP with aspartate transcarbamylase from *Escherichia coli* was studied by binding, inhibition, and temperature-jump kinetic experiments in the presence of saturating concentrations of carbamyl phosphate and succinate, an aspartate analog. Almost the entire range of fractional saturation of regulatory sites could be studied with these three inhibitors. All three inhibitors display apparent negative cooperativity in their binding isotherms. The binding isotherms can be quantitatively described by a model consisting of two sets of three identical sites per enzyme or by a mathematically equivalent model of six identical sites per enzyme molecule with occupation of the first three sites weakening ligand binding to the last three sites (*i.e.*, negative cooperativity). The maximum inhibition and binding affinity of the ligands vary in the order 5-bromo-CTP > 5-bromo-CDP > 5-bromo-CMP. The results obtained from binding and inhibition experiments show that the fractional extent of inhibition is directly proportional to the fraction of regulatory sites occupied. A single relaxation process is observed for the enzyme–inhibitor interaction with all three inhibitors. The simplest mechanism consistent with the concentration dependence of the relaxation times postulates that the six regulatory sites are truly identical, with negative cooperativity occurring in each regulatory dimer, and that the same conformational change follows the binding of each ligand.

The enzyme aspartate transcarbamylase from *Escherichia coli* is an allosteric enzyme inhibited by CTP and activated by ATP (Gerhart and Pardee, 1962). It contains distinct regulatory and catalytic subunits (Gerhart and Schachman, 1965). The native enzyme consists of six catalytic 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; Gray *et al.*, 1973). Equilibrium binding studies have shown that CTP and ATP bind to the regulatory sites heterogeneously (Winlund and Chamberlin, 1970; Buckman, 1970; Matsumoto and Hammes, 1973; Gray *et al.*, 1973). The data are consistent with a model postulating the existence of two classes of regulatory binding sites, three having a high affinity for the nucleotide effectors and three having a relatively low affinity. A second, equivalent, model is the postulation of six identical sites with the binding of the first effector molecule inducing negative cooperativity in the binding of a second molecule to a regulatory dimer. The amount of inhibition and activation appears to be directly proportional to the number of regulatory sites occupied (Matsumoto and Hammes, 1973).

Kinetic investigations of the interaction of aspartate transcarbamylase with 5-bromocytidine 5′-triphosphate (Eckfeldt *et al.*, 1970), CTP (Harrison and Hammes, 1973), the ATP analog 6-mercapto-9-β-D-ribofuranosylpurine 5′-triphosphate (Wu and Hammes, 1973), carbamyl phosphate (Hammes and Wu, 1971a), and succinate and malate (Hammes and Wu, 1971b) have been carried out. The results obtained suggest at least three conformational changes are involved in the control process. The binding of effector molecules can be explained by a mechanism assuming two classes of binding sites. However, in the cases of 5-bromo-CTP and CTP, kinetic experiments could not be carried out at low degrees of saturation because of the tightness of binding.

In this work, the interaction of aspartate transcarbamylase with a series of inhibitors, 5-bromo-CTP, 5-bromo-CDP, and 5-bromo-CMP, was studied by binding, inhibition, and temperature-jump kinetic experiments. With these ligands the entire range of fractional saturation of regulatory sites is experimentally accessible and the inhibitory capabilities of the ligand varies. The use of bromo derivatives permits the direct kinetic detection of spectral changes during the binding process. The results obtained strengthen the hypothesis that inhibition is directly proportional to the fraction of regulatory sites occupied. The kinetic studies suggest that the six regulatory sites are identical, with negative cooperativity in the regulatory dimer, and that the conformational change following binding is identical for all six sites.

Experimental Section

Enzyme. Aspartate transcarbamylase was prepared according to the procedure of Gerhart and Holoubek (1967). The mutant bacteria were obtained from the New England Enzyme Center. Aliquots of the purified enzyme were usually dialyzed against 100 volumes of 0.04 M imidazole-HCl (pH 7.0), 1 mM dithiothreitol, and 0.2 mM ethylenediaminetetraacetic acid, and the buffer was changed once during the course of dialysis. The solution was then centrifuged at 27,000 rpm for 45 min at 4° in order to obtain a clear solution. Concentrations were calculated from the absorbance at 280 nm using an extinction coefficient of 0.59 cm^2/mg (Gerhart and Holoubek, 1967).

Chemicals. The CTP, CDP, CMP, carbamyl phosphate, succinic acid, imidazole, aspartate, and diacetyl monoxime were obtained from the Sigma Chemical Co. Diphenylamine-*p*-sulfonate was obtained from Eastman Chemicals and BRIJ from Atlas Chemical Industries. Other chemicals used were the best available commercial grades. Dilithium carbamyl phosphate was further purified by precipitation from 50% ethanol (Gerhart and Pardee, 1962); carbamyl phosphate solutions were prepared just before use and kept on ice. Imidazole was recrys-

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‡ North Atlantic Treaty Organization Postdoctoral Fellow 1972–1973; on leave from the C.N.R.S., Centre de Recherches sur les Macromolecules, Strasbourg, France.

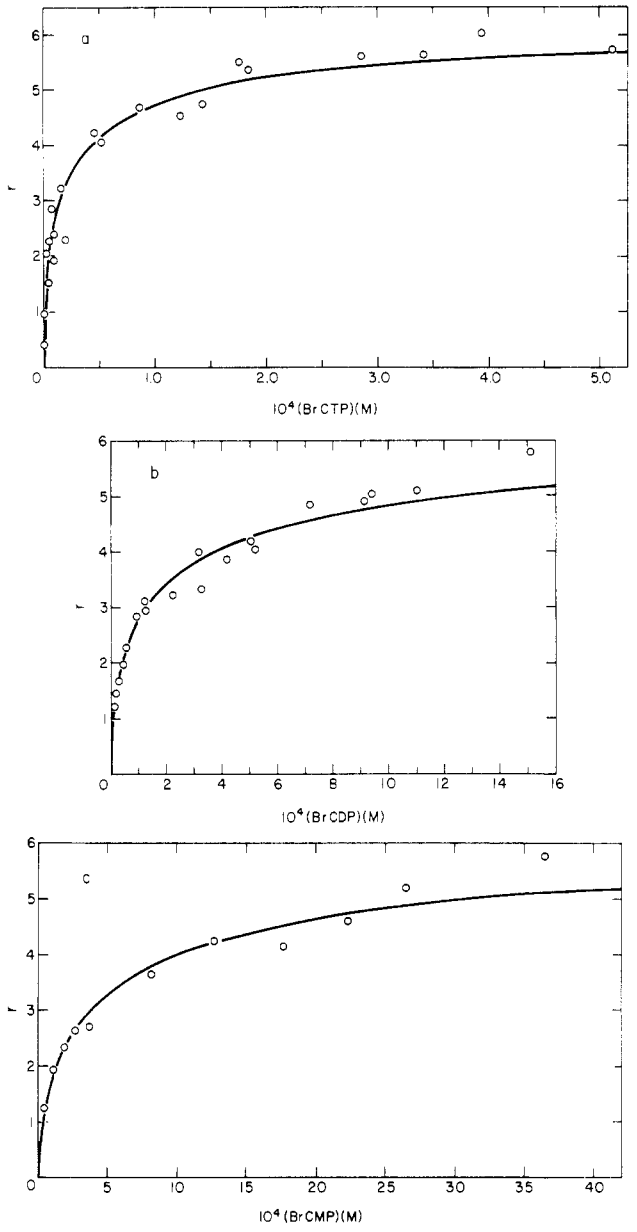

FIGURE 1: A plot of the moles of ligand bound per mole of enzyme, r, *vs.* the free-ligand concentration at 4°, 0.03 M imidazole-HCl (pH 7.0), 0.15 M KCl, 2 mM carbamyl phosphate, and 10 mM succinate. The solid lines were calculated with eq 1 and the equilibrium binding constants in Table I. The ligands are (a) 5-bromo-CTP, (b) 5-bromo-CDP, and (c) 5-bromo-CMP.

tallized twice from benzene. Aqueous solutions of succinic acid were neutralized to pH 7.0 with potassium hydroxide.

The 5-bromocytidine nucleotides were synthesized by bromination of CTP, CDP, and CMP, respectively, in formamide as previously described for 5-bromo-CTP (Bessman *et al.*, 1958; Eckfeldt *et al.*, 1970). For the CMP derivative the product was precipitated with two volumes of 95% ethanol, since almost no precipitate occurs with the normally used 0.1 M barium acetate. The final reaction products were purified by column chromatography at 4° on DEAE-cellulose using a linear gradient of 0.01–0.7 M ammonium acetate for elution. The fractions were monitored and collected with a LKB 7000 UltroRac. After removing most of the solvent by evaporation below 30°, the products were lyophilized. The lyophilized powder was used to prepare aqueous stock solutions of 14–40 mM adjusted to pH 7. The purity of the compounds was checked by thin-layer chromatography in the solvent system *n*-butyl alcohol–acetone–acetic acid–5% NH_4OH–water (v/v, 450:150:100:100:200) and by ultraviolet absorption spectrum. All compounds showed the expected ultraviolet absorption peaks at 289 nm at neutral pH and at 299 nm in 0.1 N HCl. Concentrations were calculated from the known molar extinction coefficient of 5-bromo-dCTP in 0.1 N HCl at 299 nm, 9200 M^{-1} cm^{-1} (Bessman *et al.*, 1958). The extinction coefficients of nucleotides do not depend significantly on the number of attached phosphates.

TABLE I: Aspartate Transcarbamylase-Nucleotide Binding Constants.[a]

Nucleotide	5-Bromo-CTP	5-Bromo-CDP	5-Bromo-CMP
K_1 (M^{-1})	3.23×10^5	3.58×10^4	1.0×10^4
K_2 (M^{-1})	1.58×10^4	1.82×10^3	7.23×10^2

[a] The constants are defined according to eq 1 and were determined at 4°, 0.15 M KCl, 0.03 M imidazole chloride (pH 7.0), 2 mM carbamyl phosphate, and 10 mM succinate.

Binding measurements were carried out at 4° with the equilibrium dialysis method, using Plexiglas microcells having a volume of 100 μl on each side of the membrane. The membrane used was cellulose dialysis tubing which had been boiled in distilled water and in 0.5 M $NaHCO_3$ and 5×10^{-4} M ethylenediaminetetraacetic acid. A 90-μl aliquot of enzyme solution (60–87 μM in 0.03 M imidazole-HCl, pH 7.0) was introduced on one side of the membrane with a Hamilton syringe. The other side contained 90 μl of 0.03 M imidazole-HCl (pH 7.0), 4 mM carbamyl phosphate, 20 mM succinate, 0.3 M KCl, and variable amounts of 5-bromocytidine nucleotides. The free ligand concentrations were measured by ultraviolet absorption on the side of the membrane not containing the enzyme after 15- to 16-hr equilibration. For each set of experiments, the background ultraviolet absorption was determined by carrying out experiments in the absence of nucleotides. Experiments with buffer solution replacing the enzyme solution indicated 15 hr was sufficient for complete equilibration of the dialysis cells and that adsorption of nucleotide on the dialysis cell surfaces was not significant at the concentrations employed for binding measurements. The amount of bound ligand was calculated from the difference between the initial concentration and the concentration of free ligand.

Inhibition Measurements. The production of carbamyl aspartate was measured using the colorimetric method of Gerhart and Pardee (1962). The assay mixture contained 5 mM aspartate, 3.7 M carbamyl phosphate, 0.03 M imidazole-HCl (pH 7.0), 0.15 M KCl, and varying concentrations of 5-bromocytidine nucleotides in a total volume of 0.5 ml. The enzyme concentration was about 10^{-9} M. The reaction was stopped after 30-min incubation at 4°. The final absorption, measured at 560 nm 20 min after adding $K_2S_2O_8$ to the quenched reaction mixture, was corrected for the background absorption obtained from samples run without enzyme.

Kinetic Measurements. The equipment used for temperature-jump relaxation measurements has been described elsewhere (Faeder, 1970). A 10-kV discharge through the 0.2-ml cell was used, giving a temperature rise of 7.5°. The final temperature was 13° in all cases. Concentration changes were monitored at 310 nm, which corresponds approximately to a maximum in the ultraviolet difference spectrum for the interaction of enzyme with BrCTP (Eckfeldt *et al.*, 1970). Although pH indicators have been used to study the kinetics of CTP binding to aspartate transcarbamylase (Harrison and

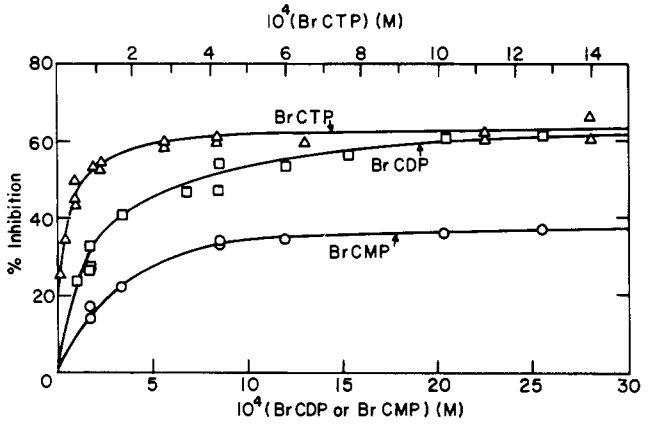

FIGURE 2: A plot of the per cent of inhibition *vs.* the concentration of inhibitor at 4°, 0.03 M imidazole–HCl (pH 7.0), 0.15 M KCl, 5 mM aspartate, and 3.7 mM carbamyl phosphate. Upper abscissa: 5-bromo-CTP (△); lower abscissa: 5-bromo-CDP (□) and 5-bromo-CMP (○).

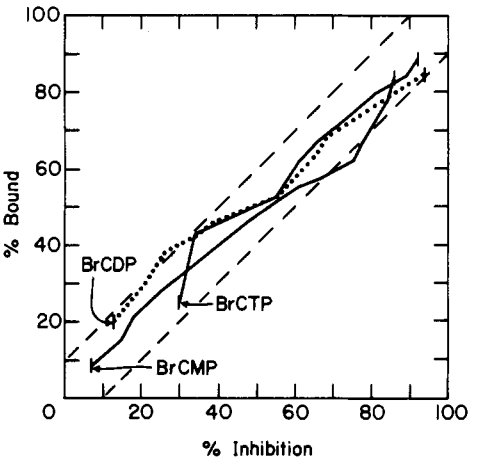

FIGURE 3: A plot of the per cent of saturation of regulatory sites versus the per cent of maximum inhibition for the three 5-bromo nucleotides. The lines were obtained as explained in the text. The dashed lines delineate the approximate width of a 45° line due to the experimental uncertainties.

Hammes, 1973), they cannot be used with CDP and CMP as ligands because of coupling between intermolecular proton transfer and enzyme–ligand relaxation effects. The enzyme concentrations used were in the range 11–27 μM, based on an enzyme molecular weight of 310,000 (Gerhart and Schachman, 1965). The cytidine nucleotide concentrations were 0.019–0.98 mM for 5-bromo-CMP, 0.025–1.43 mM for 5-bromo-CDP, and 0.035–0.53 mM for 5-bromo-CTP. All solutions contained 2 mM carbamyl phosphate, 10 mM succinate, 0.15 M KCl, and 0.02 M imidazole-HCl (pH 7) in a final volume of 2 ml. The relaxation times and amplitudes were calculated using a PDP-11 digital computer on-line to the temperature-jump apparatus (Hilborn *et al.*, 1973; Harrison and Hammes, 1973). The relaxation times reported are averages of five or six experiments.

Results and Treatment of Data

Binding Measurements. The results of the binding measurements are presented in Figure 1, where the moles of ligand bound per mole of enzyme, r, is plotted *vs.* the free-ligand concentration for the binding of 5-bromo-CTP, 5-bromo-CDP, and 5-bromo-CMP to the enzyme at 4° (0.03 M imidazole-HCl (pH 7.0)–0.15 M KCl) in the presence of saturating concentrations of carbamyl phosphate and the aspartate analog, succinate. The small amount of carbamyl phosphate decomposition during the course of the dialysis was assumed to not influence the results. In all cases, r approaches six at high ligand concentrations, indicating six regulatory binding sites are present per enzyme molecule. The binding isotherms display apparent negative cooperativity. The data can be well fit by assuming two sets of three independent binding sites, exactly as found for the binding of CTP and ATP to the enzyme in the presence of phosphates (Winlund and Chamberlin, 1970; Matsumoto and Hammes, 1973; Gray *et al.*, 1973). For this model

$$r = \frac{3K_1(\mathrm{I})}{1 + K_1(\mathrm{I})} + \frac{3K_2(\mathrm{I})}{1 + K_2(\mathrm{I})} \qquad (1)$$

where K_1 and K_2 are the intrinsic binding constants for the two sets of sites and (I) is the free-ligand concentration. The values of the constants obtained by a nonlinear least-squares fit of the data are reported in Table I, and the lines in Figure 1 have been calculated with these constants and eq 1. The precision in K_1 is relatively lower than in K_2 due to the difficulty in measuring low concentrations of free ligand with ultraviolet absorption spectroscopy. Although the standard deviation in the constants according to the least-squares analysis is about ±25%, this is an overestimate of the accuracy, and the constants are probably not more reliable than a factor of two.

Inhibition Measurements. The variation of the per cent of inhibition of the reaction catalyzed by aspartate transcarbamylase with the concentrations of the 5-bromocytidine nucleotides at 4° is shown in Figure 2. The buffer was identical with that used in the binding experiments. The maximum inhibition is very hard to assess precisely, but appears to be in the order 5-bromo-CTP > 5-bromo-CDP > 5-bromo-CMP with maximum per cent inhibition values of approximately 64, 62, and 44, respectively.

A plot of the per cent of the maximum inhibition *vs.* the per cent of saturation of regulatory sites for the three nucleotides is shown in Figure 3. The lines were obtained from smooth curves drawn through the data in Figures 1 and 2; the curves in Figure 3 span only the actual concentration ranges of the measurements. A straight line with a slope of unity would be expected if the per cent of inhibition is directly proportional to the per cent of regulatory sites occupied. A line with a slope of unity is outlined by the two dashed lines in Figure 3; the breadth of the line takes into account the estimated errors for both sets of measurements. At high concentrations of inhibitor (>1 mM), part of the nucleotide inhibition may be due to competitive inhibition of carbamyl phosphate binding.

Kinetic Measurements. A single relaxation process was associated with the interaction of the brominated nucleotides with the enzyme in the presence of saturating concentrations of carbamyl phosphate and succinate at 13°. A relaxation process was not observed when either the inhibitor or enzyme was not present. Some typical relaxation effects are shown in Figure 4.

The dependence of the reciprocal relaxation time on the concentration of free ligand is shown in Figure 5. The total enzyme concentration was 13 μM with 5-bromo-CTP as ligand, 11–22 μM with 5-bromo-CDP as ligand, and 27 μM with 5-bromo-CMP as ligand. The equilibrium binding constants determined at 4° (Table I) were multiplied by 0.5 and were used to calculate the free-ligand concentration. This assumes the temperature coefficient for the binding constants is similar to that found for the enzyme–CTP binding constants (Matsumoto and Hammes, 1973). [Unfortunately temperature-jump experiments cannot be conveniently done at final temperatures much lower than 13°, while dialysis cells cannot be conveniently thermostated at other than room temperatures (4 or 23°).] Previous temperature-jump studies with 5-bromo-CTP were

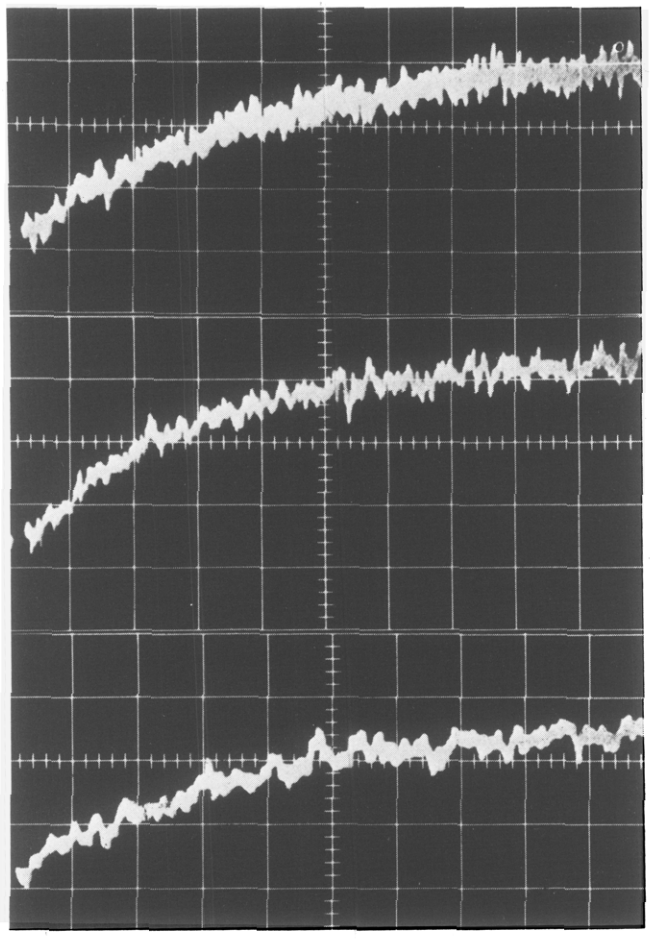

FIGURE 4: Oscilloscope traces of temperature-jump relaxation effects associated with the interaction of native aspartate transcarbamylase with 5-bromo nucleotides at 13°, 0.15 M KCl, 0.02 M imidazole-HCl (pH 7.0), 2 mM carbamyl phosphate, and 10 mM succinate. The wavelength for observations was 310 nm. Vertical scale, 2 mV/large division. (bottom) 0.19 mM 5-bromo-CMP, 27.1 μM enzyme; abscissa, 50 μsec/large division; (middle) 0.12 mM 5-bromo-CDP, 21.9 μM enzyme; abscissa, 100 μsec/large division; (top) 0.17 mM 5-bromo-CTP, 13.1 μM enzyme; abscissa, 200 μsec/large division.

carried out at 25° (Eckfeldt *et al.*, 1970); however, the relaxation amplitudes are significantly greater at 13° than at 25°, especially with the mono- and diphosphates. Although the results reported here cannot be directly compared with those from earlier work, they are qualitatively similar.

The simplest mechanism found in previous studies (Harrison and Hammes, 1973; Wu and Hammes, 1973) to be consistent with the kinetics of the binding of ligands to the regulatory site is shown in eq 2. In this mechanism E_T represents the "tight"

$$\begin{matrix} E_T + I \underset{L_2}{\overset{L_1}{\rightleftharpoons}} \\ E_L + I \end{matrix} X_1 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} X_2 \qquad (2)$$

binding enzyme sites, E_L is the "loose" binding sites, I is the inhibitor, X_1 and X_2 are different conformations of the enzyme–inhibitor complex, which are assumed to be the same for tight and loose sites, L_1 and L_2 are binding constants for the steps indicated, and k_2 and k_{-2} are rate constants. If the binding steps are assumed to equilibrate fast relative to the conformational change of the complex, the smallest reciprocal relaxation time is

$$1/\tau = k_{-2} + \frac{k_2}{1 + \dfrac{L_1 + L_2}{(E_T) + (E_L) + (I)}} \qquad (3)$$

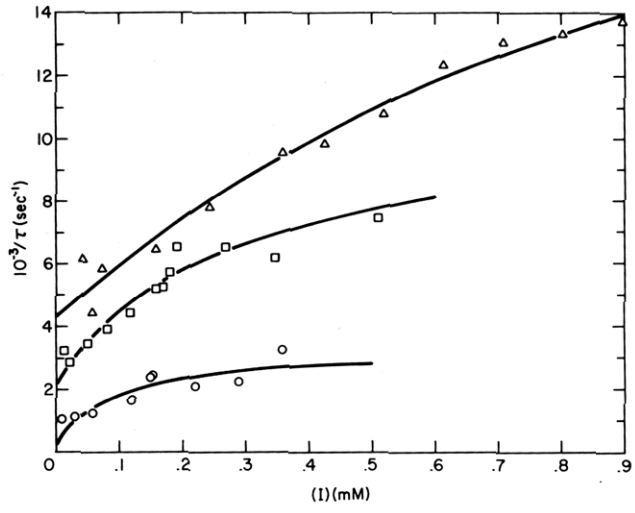

FIGURE 5: A plot of the reciprocal relaxation times *vs.* the free inhibitor concentration at 13°, 0.15 M KCl, 0.02 M imidazole-HCl (pH 7.0), 2 mM carbamyl phosphate, and 10 mM succinate: 5-bromo-CMP (△), 5-bromo-CDP (□), 5-bromo-CTP (○). The solid lines were calculated with eq 6 and the parameters in Table II.

The kinetic data for the binding of 5-bromo-CDP and 5-bromo-CMP to the enzyme cannot be fit to this equation within the experimental precision, while those for 5-bromo-CTP binding are reasonably consistent with eq 3. (In fact, extrapolation of the data to zero ligand concentration according to eq 3 leads to negative values of k_{-2} for the mono- and diphosphates.) With 5-bromo-CDP and 5-bromo-CMP as ligands, both classes of sites go from unoccupied to occupied over the concentration range employed, whereas in the case of 5-bromo-CTP the tight sites are always nearly fully occupied. Thus the concentration dependence of the relaxation times for 5-bromo-CDP and 5-bromo-CMP is a more sensitive test of mechanism than the corresponding data with 5-bromo-CTP as ligand.

A relatively simple alternative mechanism consistent with the data is to assume that the regulatory sites are all equivalent, that is, preexisting tight and loose sites do not exist. Instead negative cooperativity occurs, with the cooperative unit being a dimer. In this case, the binding isotherm is given by

$$r/3 = \frac{K_1'(I) + 2K_1'K_2'(I)^2}{1 + K_1'(I) + K_1'K_2'(I)^2} \qquad (4)$$

where K_1' and K_2' are the binding constants for the first and second ligand, respectively, binding to the dimer. This is equivalent to eq 1 if $K_1' = K_1 + K_2$ and $K_2' = K_1K_2/(K_1 + K_2)$, so that the equilibrium binding data are equally consistent with both mechanisms. The relaxation process can then be attributed to a conformational change or isomerization of the enzyme–inhibitor complexes. The detailed mechanism is presented in

$$\begin{matrix} E + 2I \overset{L_1'}{\rightleftharpoons} X_1 + I \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} X_2 + I \\ \quad L_2' \downarrow\uparrow L_2' \\ X_1I \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} X_2I \end{matrix} \qquad (5)$$

In this equation E is the dimeric cooperative unit with two unoccupied inhibitor sites, X_1 and X_2 are two different conformations of the dimer with one site occupied, and X_1I and X_2I are two different conformations of the totally liganded species. The actual conformational transition is assumed to be independent of whether one or two sites are occupied so that the rate constants for the reaction $X_1 \rightleftharpoons X_2$ are identical with those for the reaction $X_1I \rightleftharpoons X_2I$. If the binding steps are assumed to equili-

brate rapidly relative to the conformational transitions and the inhibitor is assumed to be buffered ($\delta(I) \approx 0$, where $\delta(I)$ is the change in inhibitor concentration caused by the temperature perturbation), the smallest reciprocal relaxation time is

$$1/\tau = k_{-2} + k_2\frac{L_1'(I) + L_1'L_2'(I)^2}{1 + L_1'(I) + L_1'L_2'(I)^2} \quad (6)$$

This equation, which is derived in the Appendix, only has two parameters, k_2 and k_{-2}, since $L_1' = K_1'/(1 + k_2/k_{-2})$ and $L_2' = K_2'/(1 + k_2/k_{-2})$. The data shown in Figure 5 were fit to eq 6 using a nonlinear least-squares procedure and the lines in the figure were calculated with eq 6 and the best fit parameters given in Table II. The fit is very good for the data with 5-bromo-CDP and 5-bromo-CMP as ligands, but is not as good with 5-bromo-CTP as ligand, principally at low concentrations. The associated rate constants are precise to about ±25% in the first two cases and ±50% in the last case. The primary reason for this difference in precision is that the relaxation time could only be measured over a very restricted range of ligand concentrations in the case of 5-bromo-CTP.

A more general expression for the relaxation time, not assuming buffering of inhibitor, is presented in the Appendix. Detailed examination of this more general equation indicates that with the exception of the few lowest inhibitor concentrations, eq 6 is a very good approximation over the range of enzyme and inhibitor concentrations studied. A fit of the data using the general expression for the relaxation time is not warranted by the precision of the available data.

Discussion

The binding of the mono-, di-, and triphosphates to aspartate transcarbamylase all conform well to the model of two classes of binding sites, with three sites of each class. However, the equilibrium measurements cannot distinguish between the preexistence of two classes of sites before nucleotide binding and the creation of a second class of sites by the binding of nucleotides to the first three sites (true negative cooperativity). The presence of carbamyl phosphate and succinate at high concentrations effectively prevents the binding of nucleotides at the active site. The values of the two binding constants for the enzyme–5-bromo-CTP interaction are within a factor of two to three of those found for the enzyme–CTP interaction under essentially identical conditions ($K_1 = 7.1 \times 10^5$ M^{-1} and $K_2 = 4.4 \times 10^3$ M^{-1} for CTP; Matsumoto and Hammes, 1973), which is probably within the experimental uncertainties. The constants characterizing CTP binding provide a good fit to the 5-bromo-CTP binding at low inhibitor concentrations, but significant deviations occur at high concentrations (>30 μM). The binding clearly becomes weaker as phosphates are removed from 5-bromo-CTP—removal of the first phosphate reduces K_1 and K_2 by about a factor of ten, while removal of the second phosphate further reduces these constants by about a factor of three. The degree of negative cooperativity appears to decrease slightly as phosphates are removed: $K_1/K_2 = 22$, 20, and 14 for the tri-, di-, and monophosphates, respectively, but this is within the experimental uncertainties.

All three nucleotides are effective allosteric inhibitors. While the maximum inhibition is difficult to assess, it is clearly similar for 5-bromo-CTP and 5-bromo-CDP and significantly lower for 5-bromo-CMP. The loss of the second phosphate from 5-bromo-CTP obviously has a much greater effect on the inhibition than the loss of the first. No obvious correlation exists between the binding strength of the allosteric ligands to the enzyme and the maximum inhibition. The maximum inhibition for 5-bromo-CTP, about 64%, agrees quite well with that for CTP, about 70% (Matsumoto and Hammes, 1973), determined under identical conditions; the correlation between the per cent of maximum inhibition and the per cent of regulatory sites occupied is quite good (Figure 3) suggesting each ligand bound is equally effective in causing inhibition. The deviation of the experimental data from a straight line at 45° to the abscissa appears to be random. The experimental curves are particularly sensitive to the choice of the per cent maximum inhibition, and in addition reflect the experimental uncertainties in the individual inhibition and binding measurements. By combining the data for all three inhibitors almost the complete range from 0 to 100% of binding and inhibition is accessible.

TABLE II: Rate Parameters for the Mechanism of Eq 5.[a]

Nucleotide	5-Bromo-CTP	5-Bromo-CDP	5-Bromo-CMP
k_2 (sec^{-1})	2900	8310	21,200
k_{-2} (sec^{-1})	270	2130	4320
k_2/k_{-2}	9.8	3.9	5.0
L_1' (M^{-1})	1.57×10^4	3.8×10^3	9.0×10^2
L_2' (M^{-1})	6.6×10^2	1.76×10^2	5.6×10^1

[a] Determined at 13°, 0.15 M KCl, 0.02 M imidazole chloride (pH 7.0), 2 mM carbamyl phosphate, and 10 mM succinate.

As in previous temperature-jump studies of the binding of inhibitors to the regulatory subunit of aspartate transcarbamylase (Eckfeldt *et al.*, 1970; Harrison and Hammes, 1973), only a single relaxation process can be observed, and the concentration dependence of the relaxation times indicates that a conformational change of the enzyme–inhibitor complex is involved in the relaxation process. The tight binding of CTP (and 5-bromo-CTP) to the enzyme has previously prevented studying the kinetics of binding appreciably below 50% saturation. However, in the cases of 5-bromo-CDP and 5-bromo-CMP, kinetic studies can be readily carried out at very low degrees of saturation. The fact that only a single relaxation process is observed and the observed concentration dependence of the relaxation time requires that the conformational change which occurs is essentially the same for all sites; therefore the negative cooperativity is reflected only in the initial binding step before the conformational transition. Furthermore, the bimolecular reaction apparently is fast compared to the conformational transition; a lower bound of about 10^8 M^{-1} sec^{-1} can be estimated from the values of the observed relaxation times and the concentration ranges studied. Thus the initial inhibitor–enzyme reaction is essentially diffusion controlled.

The simple mechanism previously proposed (eq 2), which assumes two different types of preexisting regulatory sites are present on the enzyme, is inadequate to explain the results in Figure 5. However, the assumption of six identical sites per enzyme molecule with negative cooperativity in each regulatory dimer and an identical conformational change following binding of each ligand (eq 5) quantitatively describes the data. This is the first experimental evidence suggesting the six regulatory sites are truly identical. (Of course, the possibility of a more complex mechanism cannot be excluded.) It was hoped to establish some quantitative correlation between the extent of the conformational change, as measured by the ratio $(X_2)/(X_1) = k_2/k_{-2}$, and the maximum inhibition, but the experimental uncertainties in the kinetic parameters are too large for this to be possible. This ratio is the same order of magnitude for all three nucleotides, although it appears to be significantly larger for 5-bromo-CTP relative to the other two ligands. The maxi-

mum per cent inhibition does not differ greatly for the three inhibitors so that a correlation would be fortuitous. For all three ligands the ratio L_1'/L_2', which is a measure of the negative cooperativity, lies between 16 and 24. These constants decrease by a factor of 3–4 in going from the tri- to diphosphate and from the di- to monophosphate. The variation of these constants as the number of phosphates changes is more regular than the variation of the overall binding constants.

The rate constants associated with the conformational change, k_2 and k_{-2}, increase significantly as each phosphate is removed. In the case of 5-bromo-CTP, the rate constants are similar in magnitude to the turnover number of the enzyme, while for 5-bromo-CMP and 5-bromo-CDP the rate constants are significantly greater than the turnover number.

In summary, the binding and inhibition studies indicate that 5-bromo-CTP, 5-bromo-CDP, and 5-bromo-CMP display negative cooperativity in binding to aspartate transcarbamylase and that the extent of inhibition is directly proportional to the fraction of regulatory sites occupied. The kinetic studies indicate that the same conformational transition follows the initial binding at each site. If the inhibition is attributed to this conformational change, the inhibition would be proportional to the fraction of sites occupied, as observed. A simple mechanism assuming identical sites and negative cooperativity in the regulatory dimers is consistent with all of the data.

Acknowledgment

We are greatly indebted to Miss Jean Hsu for her technical assistance.

Appendix

For the reaction mechanism of eq 5, the rate equation in the neighborhood of equilibrium for the slowest relaxation process, assuming the ligand binding steps are rapid, is (*cf.* Hammes and Schimmel, 1970)

$$\frac{-d\delta[(X_2) + (X_2I)]}{dt} = \left[k_{-2} + \frac{k_2}{1 + \frac{\delta(E)}{\delta(X_1)} + \frac{\delta(X_1I)}{\delta(X_1)}} + \frac{k_2}{1 + \frac{\delta(E)}{\delta(X_1)}\frac{\delta(X_1)}{\delta(X_1I)} + \frac{\delta(X_1)}{\delta(X_1I)}}\right] \times \delta[(X_2) + (X_2I)] = (1/\tau)\delta[(X_2) + (X_2I)] \quad (A1)$$

where δ designates the deviation from equilibrium of the concentrations and the above equations define the reciprocal relaxation time, $1/\tau$.

In order to complete the evaluation of the relaxation time, the quantities $\delta(E)/\delta(X_1)$ and $\delta(X_1I)/\delta(X_1)$ must be evaluated from the mass conservation equations (A2) and (A3) and the derivatives of the equilibrium relationships $L_1' = (X_1)/[(E)(I)]$ and $L_2' = (X_1I)/[(X_1)(I)] = (X_2I)/[(X_2)(I)]$, eq A4–A6.

$$\delta(E) + \delta(X_1) + \delta(X_2) + \delta(X_1I) + \delta(X_2I) = 0 \quad (A2)$$

$$\delta(I) + \delta(X_1) + \delta(X_2) + 2\delta(X_1I) + 2\delta(X_2I) = 0 \quad (A3)$$

$$L_1'(I)\delta(E) + L_1'(E)\delta(I) = \delta(X_1) \quad (A4)$$

$$L_2'(X_1)\delta(I) + L_2'(I)\delta(X_1) = \delta(X_1I) \quad (A5)$$

$$L_2'(X_2)\delta(I) + L_2'(I)\delta(X_2) = \delta(X_2I) \quad (A6)$$

In eq A4–A6 the concentrations are the equilibrium concentrations at the final temperature after the temperature jump. If $\delta(I) = 0$ (the buffering approximation)

$$\delta(E)/\delta(X_1) = 1/L_1'(I) \quad (A7)$$

$$\delta(X_1I)/\delta(X_1) = L_2'(I) \quad (A8)$$

Substitution of eq A7 and A8 into eq A1 yields the expression for the relaxation time in eq 6. If eq A2–A6 are solved simultaneously, then

$$\delta(E)/\delta(X_1) = \frac{1 + L_2'[(I) + (X_1) + (X_2)]}{L_1'(I)\{1 + L_2'[(I) + (X_1) + (X_2) + 2(E)]\} + L_1'(E)} \quad (A9)$$

$$\delta(X_1I)/\delta(X_1) = [L_2'(I)\{1 + L_2'[(X_1) + (X_2) + (I) + 2(E)]\} + L_2'(E) + L_2'(X_1)/L_1'(I) + 2L_2'^2(X_1)/L_1']/[1 + L_2'[(I) + (X_1) + (X_2) + 2(E)] + (E)/(I)] \quad (A10)$$

Equations A9 and A10 can be substituted into eq A1 to obtain a general expression for $1/\tau$. When the total inhibitor concentration is much greater than the total enzyme concentration, eq A9 and A10 reduce to eq A7 and A8, as expected.

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