Allosteric Transitions Associated with the Binding of Substrate and Effector Ligands to T_2 Phage Induced Deoxycytidylate Deaminase†

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ABSTRACT: The binding characteristics of T_2 phage induced deoxycytidylate deaminase were examined through the use of ultrafiltration and equilibrium dialysis. The positive effectors, 5-(hydroxymethyl)deoxycytidine 5′-triphosphate and deoxycytidine 5′-triphosphate, were bound in a highly cooperative manner, which is consistent with the allosteric effects promoted by these compounds. Their respective $S_{0.5}$ values were 8 and 2 μM. A similar degree of cooperativity was associated with the binding of such competitive inhibitors of deoxycytidylate deaminase as dGMP, 4-*N*-hydroxydeoxycytidine 5′-monophosphate, and tetrahydrodeoxyuridylate. The negative effector, dTTP, also inhibited the binding of dCTP in a pH-dependent manner, which is consistent with its previously demonstrated inhibition of catalysis [Maley, G. F., Guarino, D. U., & Maley, F. (1972) *J. Biol. Chem. 247*, 931–939]. The binding of dTTP could be demonstrated only at low phosphate concentrations and did not appear to be cooperative. The number of binding sites for the allosteric ligands, substrate, and substrate inhibitors was shown to be six, which coincides with the number of enzyme subunits. It was established by CD difference spectroscopy that dCTP, at concentrations normally employed to demonstrate enzyme activation, effects a dramatic conformation transition in the deaminase, as indicated by a sharp decrease in ellipticity at about 280 nm. The nature of this response suggests that the microenvironment of some of the enzyme's tyrosyl residues had been perturbed by the presence of this allosteric nucleotide.

The allosteric properties of deoxycytidylate deaminase (EC 3.4.3.12), first suggested in studies with crude chick embryo enzyme (Maley & Maley, 1962a,b), have since been more clearly delineated with homogeneous enzyme from chick embryo (Maley & Maley, 1970), donkey spleen (Scarano et al., 1967a), and T_2 phage infected *Escherichia coli* (Maley et al., 1972a). With the exception of the deoxycytidylate deaminase from SP-8-infected *Bacillus subtilis* (Nishihara et al., 1967), the enzyme from numerous other sources has also proven to be finely regulated in vitro by the interplay of its allosteric effectors, dCTP[1] and dTTP (Maley & Maley, 1972). Because of the deaminase's potential role in supplying dUMP, a substrate for thymidylate synthetase, the extent to which it contributes to the overall formation of dUMP has become important for assessing how chemotherapeutic agents act, as well as for improving their effectiveness.

Thus as shown recently by Jackson (1980), thymidine rescue of certain cells treated with methotrexate results from its conversion to dTTP, a potent feedback inhibitor of deoxycytidylate deaminase. A consequence of this inhibition is the prevention of a detrimental depletion of the intracellular deoxycytidine nucleotide pool, an effect reminiscent of the sparing effect of thymidine on deoxycytidine utilization described earlier by us (Maley & Maley, 1962a,b, 1963).

While the kinetics of catalysis has been useful in clarifying the regulation of the deaminase by its substrate and allosteric ligands (Maley & Maley, 1972), the extent to which these compounds interact with the enzyme directly to effect this control has not been clearly defined. Previous attempts to obtain such binding parameters with the donkey spleen enzyme (Scarano et al., 1967b) are suspect because of the marginal amounts of enzyme available and the lack of sensitivity of the technique employed. Since binding data have been difficult to obtain with enzyme from an animal source because of its limited quantity, our attention was directed to the more available homogeneous deaminase from T_2 phage infected *E. coli*. Although the chick and phage enzymes differ somewhat in their enzymic properties (Maley & Maley, 1972), they have similar molecular weights (about 120 000), are composed of six subunits (unpublished data for chick embryo), and are allosterically regulated, which suggests that the binding properties of these enzymes may be comparable. The present studies clearly define these parameters for the phage-induced deaminase and demonstrate that the enzyme undergoes a conformation transition on being exposed to the positive allosteric effector, dCTP.

Experimental Procedures

Materials

Homogeneous deoxycytidylate deaminase, with a specific activity of 610 μmol of dUMP formed min^{-1} (mg of protein)$^{-1}$ (determined by amino acid analysis) at 37 °C was isolated from T_2-infected *E. coli* B as described previously (Maley et al., 1972a). Enzyme solutions were prepared from the protein precipitated on dialysis against 10 mM potassium phosphate, pH 8.0, and 20 mM 2-mercaptoethanol by dissolving it in 0.2 M potassium phosphate, pH 7.1, and 0.1 M 2-mercaptoethanol. Where indicated, other buffers of different pH were used to dissolve the enzyme. Protein concentrations were measured by amino acid analysis (Spackman et al., 1958) with a Beckman 119CL amino acid analyzer and by the method of Lowry et al. (1951) with bovine serum albumin as a standard. The latter method was used routinely for protein measurements. However, when it was necessary to obtain a true measure of protein concentration, amino acid analysis was used, since the Lowry method gave results which were 30% higher. Throughout these studies a molecular weight of 124 000 for T_2 deoxycytidylate deaminase (Maley et al., 1972b) was used to calculate the molar ratio of bound ligand

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[1] Abbreviations: HM-dCMP and HM-dCTP, 5-(hydroxymethyl)-deoxycytidine 5′-monophosphate and 5′-triphosphate, respectively; 4-*N*-HO-dCMP, 4-*N*-hydroxydeoxycytidine 5′-monophosphate; H_4dUMP, tetrahydrodeoxyuridylate; APB, wide-range acetate–phosphate–borate buffer.

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to enzyme. The enzyme was stored in an ice bath as a solution of 4–5 mg of protein/mL at 0 °C. No loss in activity was detected during the course of the study.

Binding was measured with ^{14}C-labeled nucleotides at specific activities of 2–20 dpm/pmol. All labeled nucleotides were obtained from Schwarz/Mann, except for 5-[^{14}C]HM-dCMP, [^{14}C]HM-dCTP, H_4[2-^{14}C]dUMP, and 4-*N*-HO-[2-^{14}C]dCMP, which were prepared chemically from [2-^{14}C]-dCMP as described previously (Maley & Maley, 1971, 1964).

Methods

Ultrafiltration. The method of Paulus (1969) utilizing the apparatus produced by Metalloglass, Inc., was used for ultrafiltration. Samples (100 μL) containing radioactive ligand, buffer A, and deoxycytidylate deaminase were filtered through UM-10 Diaflo membranes at 40 psi. Buffer A contained 0.1 M acetate–phosphate–borate wide-range buffer (APB) (Gerwin et al., 1966), 25 mM 2-mercaptoethanol, and 5 mM $MgCl_2$. After filtration was complete in all channels, ethylene glycol (5 mL) was injected through the rinse channels to wash the bottom side of the membranes. The membranes were transferred to counting vials containing 0.5 mL of water and, after addition of 10 mL of Aquasol, counted in a Packard Tri-Carb scintillation counter. Blank values were determined for each sample in the absence of protein in order to correct for the small volume of solution retained by the membrane. This correction was usually less than 10% of the count bound by the protein.

Equilibrium Dialysis. The Kontron-Diapack apparatus described by Weder et al. (1971) was used for the equilibrium dialysis experiments, and the experimental approach was similar to that described earlier by us (Galivan et al., 1976). Regenerated cellulose membranes were boiled for 15 min in glass-distilled water, rinsed thoroughly in water, and soaked in 0.01 M potassium phosphate, pH 7.5, with several changes. Membranes were stored in the same buffer at 0–4 °C, rinsed with fresh buffer containing 20 mM 2-mercaptoethanol, and blotted prior to use. All experiments were conducted at 25 °C. Equilibration of [2-^{14}C]dCTP vs. buffer was attained within 3 h, but 4 h was routinely allowed for each experiment. Buffer mixture B was used unless noted; it contained 20% ethylene glycol, 0.1 M potassium phosphate, pH 8.0, 25 mM 2-mercaptoethanol, and 5 mM $MgCl_2$. Protein and ligand solutions (50 μL) were added to each cell, one on each side of the dialysis membrane. After equilibrium was reached, the ligand concentration was determined on duplicate aliquots from each cell compartment. Samples of 10 or 20 μL were counted in 10 mL of Aquasol in a Packard Tri-Carb scintillation counter.

The difference in radioactivity between the ligand and protein cells was considered to represent enzyme-bound ligand, while activity in the ligand compartment was used to calculate the concentration of unbound ligand. Recovery of added radioactivity after equilibrium was reached was 98–100%.

Protein concentrations in equilibrium dialysis experiments were 1.5–6.0 μM (0.2–0.8 mg/mL); nucleotide concentrations were 2.0–500 μM. No corrections for Donnan effects were made.

Enzyme Assay. Deoxycytidylate deaminase was assayed spectrophotometrically as described previously (Maley, 1967). In most experiments a sample of enzyme was removed from the protein part of the dialysis chamber after equilibration and assayed for enzymatic activity. Because of the presence of such stabilizing agents as ethylene glycol and 2-mercaptoethanol, no decrease in activity was observed unless an added inhibitor was present.

Data Analysis. A graphic analysis of the binding characteristics of the ligands to protein was employed, based on the Scatchard equation (Scatchard, 1949):

$$r/L = (n - r)/K \quad (1)$$

where r is the number of moles of ligand bound per mole of enzyme at a given L, L is the concentration of free ligand, n is the number of binding sites per mole of enzyme at infinite L, and K is the dissociation constant of the enzyme–ligand complex. Where plots of r/L vs. r were nonlinear, which is indicative of cooperative interactions among the protein subunits, they were linearized through the use of an appropriate exponent for L (Kuehn et al., 1971). The exponent was determined from a Hill plot of the binding data:

$$r/(n - r) = L^m/K' \quad (2)$$

which is basically a variant of eq 1. Similar results can be obtained from the kinetic form of the Hill equation:

$$v/(V_m - v) = S^m/K \quad (3)$$

In both equations m is the slope of the line obtained from a log plot of the data and provides a measure of the cooperativity introduced by the allosteric ligands. When the binding becomes infinitely cooperative, $m = n$.

Aside from the graphic analysis of the binding data, n was also calculated by using an algorithm for linear regression that provided least-squares and robust estimates (Nicklin & Paulsen, 1981). For these analyses a PDP 11/45 Digital Equipment computer was employed.

CD Measurements. CD spectra were recorded at 25 °C with a Cary 61 spectropolarimeter that had been calibrated with a standard solution of 10-camphorsulfonic acid. The samples (3.0 mL) were placed in quartz cuvettes with a 1-cm light path and were scanned at 3 nm/min at a pen period of 10 s and a slit multiplier of 2. Ellipticity values obtained at a dynode voltage above 0.4 were usually excluded. The results are expressed in molar ellipticity, [Θ], deg·cm^2·dmol^{-1}, calculated from the equation

$$[\Theta] = 100 \cdot \Theta'/[(\text{cm})(\text{M})]$$

where Θ′ is the observed ellipticity for each sample using a full scale of 0.05°, cm is the light path, and M is the molarity of deoxycytidylate deaminase in the cuvette based on a molecular weight of 1.24×10^5 (Maley et al., 1972b). The quantity of enzyme used in each experiment was determined by amino acid analysis and varied from 4×10^{-6} to 7×10^{-6} M.

Results

Binding of dCTP. The binding of dCTP to deoxycytidylate deaminase was initially studied by the ultrafiltration technique (Paulus, 1969) because of this technique's rapidity. When the data were analyzed in the form of a double-reciprocal plot and extrapolated to infinite dCTP concentration, a maximum binding of 6 mol of dCTP/mol of enzyme was found. Essentially similar results were found when HM-dCTP was used as the ligand, but the binding of this nucleotide was even more cooperative than that of dCTP. As in the case of dCTP, 6 mol of HM-dCTP was bound/mol of enzyme. However, it was found that more precise measurements on the binding interactions of substrate and allosteric ligands with deoxycytidylate deaminase could be obtained by the method of equilibrium dialysis.

A typical binding curve for dCTP ranging in concentration from 0.5 to 100 μM at 25 °C is presented in Figure 1, which

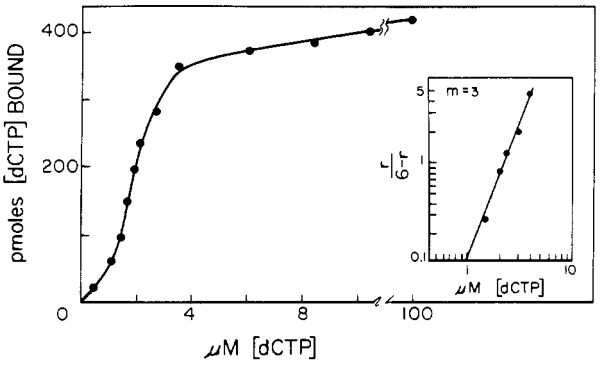

FIGURE 1: Binding of dCTP by T_2 deoxycytidylate deaminase as determined by equilibrium dialysis. The ligand cell contained initially varying concentrations of [2-^{14}C]dCTP (20 dpm/pmol), 20 μL of buffer B at pH 8.0, and water to a final volume of 50 μL. The protein cell contained 88 μg (71 pmol) of enzyme, 20 μL of buffer B at pH 8.0, and water to a final volume of 50 μL. The inset depicts the same data in the form of a Hill plot, using $n = 6$ as suggested by the results in Figure 3.

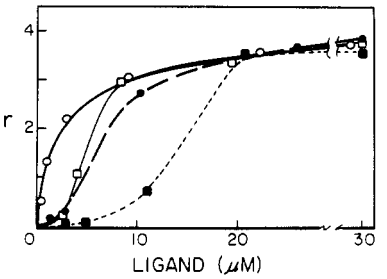

FIGURE 2: Effect of pH on binding of dCTP and HM-dCTP by T_2 deoxycytidylate deaminase. The ligand cells contained initially varying concentrations of [2-^{14}C]dCTP (20 dpm/pmol) or [2-^{14}C]HM-dCTP (3.1 dpm/pmol). The protein cells contained 188 μg (152 pmol) of enzyme. Both cells contained 20 μL of buffer A in a final volume of 50 μL. Ligand binding was measured as follows: dCTP, pH 6 (○) and pH 10 (●); HM-dCTP, pH 6 (□) and pH 10 (■).

Table I: Effect of Mg^{2+} on dCTP Binding to T_2 Deoxycytidylate Deaminase at Various pHs[a]

pH	dCTP bound (pmol)	
	$-Mg^{2+}$	$+Mg^{2+}$
6	57.6	175.2
7	48.1	184.3
8	43.1	203.6
9	4.8	116.0

[a] Each sample (100 μL) contained 4.1 μM [2-^{14}C]dCTP (5.24 dpm/pmol), 10 mM 2-mercaptoethanol, 25 mM APB at the indicated pH, 1 mM $MgCl_2$ where indicated, and 13 μg of enzyme. The data were obtained by the ultrafiltration technique (Paulus, 1969).

as shown earlier in kinetic studies (Maley, 1967; Maley et al., 1972a) and confirmed in the binding studies (Table I) is optimal between pH 6 and pH 8 and requires Mg^{2+}. The sigmoidal nature of this binding is even more clearly evident when Figure 1 is replotted as fractional sites ($\bar{Y}$) occupied vs. the log of dCTP concentration. Although equilibrium dialysis was found to be much more sensitive than ultrafiltration in demonstrating the cooperative nature of dCTP binding, the data in both instances yielded half-saturation ($S_{0.5}$) values for dCTP binding of 2–3 μM. The extent of binding is clearly pH dependent (Figure 2) and is more so for HM-dCTP than for dCTP. These results are consistent with those obtained when the pH dependence of the kinetics of activation of deoxycytidylate deaminase was determined in the presence of dCTP and HM-dCTP (Maley et al., 1972a).

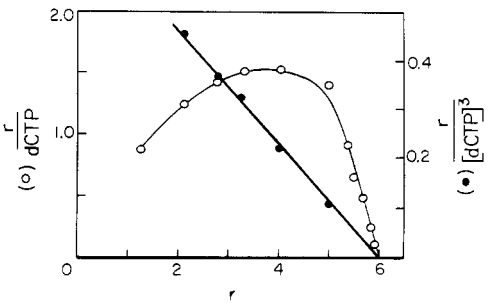

FIGURE 3: Scatchard plot of binding of dCTP by T_2 deoxycytidylate deaminase. The results in Figure 1 were plotted (○) by the usual Scatchard analysis (eq 1) and (●) by using the linearizing exponent $m = 3$ in eq 2. The binding constant, n, was determined by least-squares (5.50 ± 0.44) and robust analyses (5.56 ± 0.42) using the values for (○) on the linear portion of this curve.

Table II: Effect of Buffers on Ligand Binding to T_2 Deoxycytidylate Deaminase[a]

buffer	mM	pmol bound		
		dCTP	HM-dCTP	dTTP
APB	50	515	702	0
phosphate	20	511	693	344
	50	546	685	245
Tris-HCl	10			254
	40	511	670	205
	50			163

[a] The protein cell contained 152 pmol of enzyme. The ligand cell contained [2-^{14}C]dCTP [1.8 nmol (22 dpm/pmol)], [2-^{14}C]HM-dCTP [5.7 nmol (3.1 dpm/pmol)], or [2-^{14}C]dTTP [14 nmol (5.6 dpm/pmol)]. Except for buffer concentration the conditions were as described under Experimental Procedures.

Scatchard analysis (eq 1) of the binding data for the positive effector, dCTP (Figure 3), yielded nonlinear plots, indicative of cooperative binding. Similar results were obtained with HM-dCTP (data not shown). The linearization procedure of Kuehn et al. (1971) gave a value of $m = 3$ (Figure 1) for each nucleotide. By employment of this value as an exponent for the free ligand concentration in the modified Scatchard equation (eq 2), an extrapolated value was obtained of 6 mol of dCTP (Figure 3) and 6 mol of HM-dCTP bound/mol (data not shown) of T_2 deoxycytidylate deaminase, or 1 mol of nucleotide/subunit of enzyme. The sigmoidal nature of these curves clearly indicates that a high degree of positive cooperativity is associated with the binding of dCTP and HM-dCTP.

Binding of dTTP. Under the same conditions that were successful in demonstrating the binding of dCTP and HM-dCTP to the deaminase, the binding of dTTP to the enzyme could not be detected by using either equilibrium dialysis or ultrafiltration. Further study indicated that this effect was related to ionic strength, which apparently impaired the binding of dTTP more so than that of dCTP or HM-dCTP. Thus as shown in Table II, the wide-range buffer APB, normally used to measure dCTP binding, completely inhibited the binding of dTTP to the deaminase but not that of dCTP or HM-dCTP. However, low concentrations of phosphate and Tris buffer supported the binding of dTTP to the deaminase (Figure 4, Table II), but in contrast to dCTP and HM-dCTP the binding of dTTP did not appear to be cooperative. Because the binding of dTTP is about 100-fold lower than that of dCTP it was found necessary to use about 5 times more enzyme than in the case of dCTP to obtain measurable differences between the ligand and protein parts of the dialysis cell.

Linearizing the Scatchard plot with a Hill interaction coefficient of $m = 2$ (Figure 4) gave an n value of 6 similar

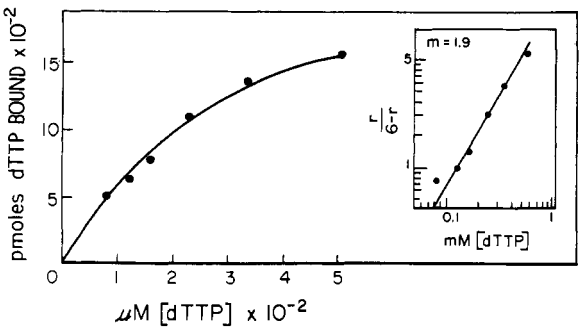

FIGURE 4: Equilibrium dialysis study of binding of dTTP by T_2 deoxycytidylate deaminase. The ligand cell contained initially varying concentrations of [2-^{14}C]dTTP (5.4 dpm/pmol); the protein cell contained 376 μg (304 pmol) of enzyme. Each cell contained 20 μL of buffer B in a final volume of 50 μL. The inset depicts the same data in the form of a Hill plot, using $n = 6$ as suggested from the linearized plot of Figure 5.

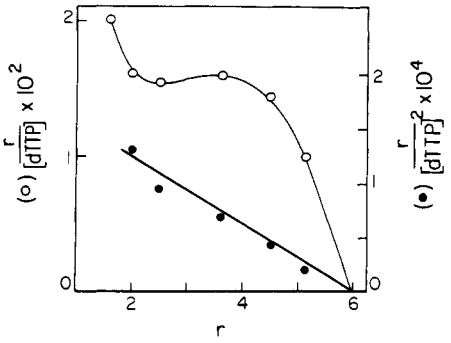

FIGURE 5: Scatchard plot of binding of dTTP by T_2 deoxycytidylate deaminase. The results were plotted as described for dCTP in Figure 3 but are based on the data in Figure 4. The least-squares and robust analyses for n, obtained from (○), were 6.30 ± 0.76 and 6.04 ± 0.31, respectively.

Table III: Effect of dTTP on dCTP Binding[a]

addition	μM	dCTP bound (pmol) pH 6.0	pH 10.0
0		201	47
dTTP	17	159 (21)	22 (53)
	34	127 (37)	20 (58)
	170	20 (90)	10 (79)

[a] The protein cell contained 152 pmol of enzyme; the ligand cell initially contained 7.2 μM [2-^{14}C]dCTP (20 dpm/pmol). In addition each cell contained APB buffer at pH 6.0 or 10.0 as indicated, 1 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and 7% ethylene glycol. Also present in the ligand cell was dTTP at the initial concentrations shown above. The figures in parentheses represent percent inhibition.

to that for the positive effectors (Figure 5). The binding of dTTP could be completely prevented by an equal concentration of dCTP, but 25-fold higher concentrations of dTTP were necessary to impair dCTP binding, an effect that is pH dependent (Table III) as noted previously in kinetic studies (Maley et al., 1972b). Neither the substrate, HM-dCMP, nor the inhibitor, 4-*N*-HO-dCMP, affected the binding, even at concentrations 10–15-fold higher than that of dTTP.

Binding of Substrate. The relatively high concentration of active enzyme required for these studies limited the ability to measure the binding of dCMP, which was mostly deaminated (70%) during the time required for equilibration. Thus the binding curve most probably reflects the binding of dUMP (Figure 6), but even so a value of five to six binding sites was obtained. Since dUMP is a competitive inhibitor of dCMP these results signify that the latter also binds to six sites.

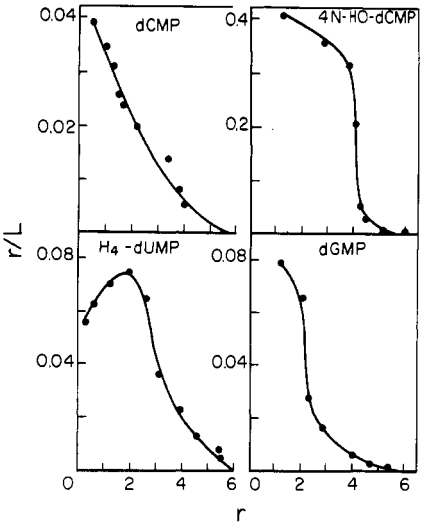

FIGURE 6: Binding of dCMP, 4-*N*-HO-dCMP, H_4dUMP, and dGMP by T_2 deoxycytidylate deaminase. In each case the ligand cell contained initially varying concentrations of the nucleotide: [2-^{14}C]dCMP, 10.0 dpm/pmol; 4-*N*-HO-[2-^{14}C]dCMP, 8.4 dpm/pmol; [2-^{14}C]H_4dUMP, 7.8 dpm/pmol; [8-^{14}C]dGMP, 4.2 dpm/pmol. The protein compartment contained 164 μg (132 pmol) of enzyme. Each cell also contained 40 mM potassium phosphate, pH 8.0, 1 mM $MgCl_2$, and 20 mM 2-mercaptoethanol in a final volume of 50 μL.

Similar problems were encountered with HM-dCMP, which is normally not a substrate (Maley & Maley, 1966), but at the enzyme concentrations used in the dialysis experiments deamination probably occurred. In an attempt to circumvent this problem, the binding curves of 4-*N*-HO-dCMP and dGMP (Maley & Maley, 1964), both competitive inhibitors of dCMP, were determined in the hope that they might clarify the number and type of substrate binding sites. As indicated in Figure 6 these compounds appear to bind two to four sites most readily and then gradually fill the others. The binding of H_4dUMP, another competitive inhibitor (Maley & Maley, 1971), although apparently cooperative, is somewhat more complex in that it fills its first site more effectively than the second. It is of interest to note that all of these compounds, as in the case of the allosteric effectors, bind to six sites on the enzyme, strongly implying that the substrate does too.

CD Spectral Changes Associated with dCTP Binding. To determine whether conformational changes are associated with the binding of dCTP by deoxycytidylate deaminase, we employed a technique similar to that used for measuring allosteric transitions in aspartate transcarbamylase (Griffin et al., 1972), that of CD difference spectroscopy. Since no apparent differences were found in the low-UV region, where changes in α helix and β structure are measured, the near-UV region from 250 to 310 nm was examined. As shown in Figure 7, a striking spectral change at 277 nm occurred in the presence of dCTP at concentrations normally employed to measure the allosteric activation of T_2 deoxycytidylate deaminase (Maley et al., 1972a). Consistent with this observation was the finding that versene eliminated the cotton effect at this wavelength, indicating that Mg^{2+} is not only required for the activation of the enzyme by dCTP but also associated with the spectral changes observed. These effects could have been anticipated from the fact that Mg^{2+} is required for the binding of dCTP (Table I).

Although similar spectral shifts could not be demonstrated with dTTP alone, those effected by dCTP could be reversed by dTTP at pH 9.0, where the binding of dCTP is so diminished (Maley et al., 1972a) that the spectral effects of dTTP are measurable. At pH 7.0, however, the concentration of

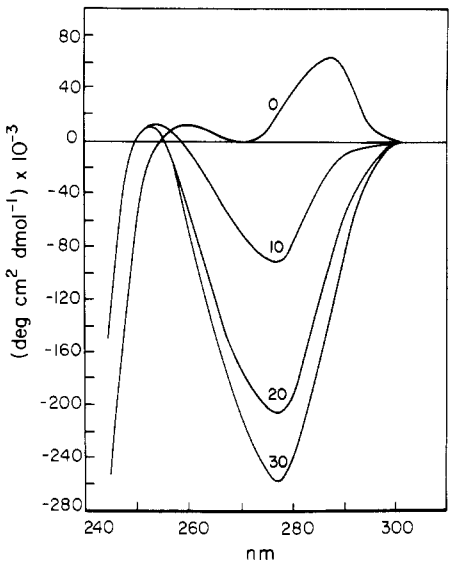

FIGURE 7: CD changes associated with binding of dCTP to T_2 deoxycytidylate deaminase. The basic solution on which the CD analyses were performed contained 0.2 M potassium phosphate, pH 7.1, 10 mM 2-mercaptoethanol, 1 mM $MgCl_2$, 4.34 μM deoxycytidylate deaminase, and, as indicated, 0, 10, 20, or 30 μL of 4.08 mM dCTP. The total volume of the solution was 3.0 mL. The CD patterns represent the difference spectra solutions containing identical components except for the omission of enzyme from the second cell.

dTTP required to reverse the CD spectral shifts of dCTP is too high to permit sufficient light through the CD cell to be measured accurately.

Although the difference spectrum in Figure 7 is one in which the spectral contributions of dCTP have been discounted, the absorbance due to the enzyme protein was not subtracted from the spectra at 10, 20, and 30 μM dCTP. If this were done, the negative CD peak would be intensified and shifted more toward 280 nm, a spectral region associated mainly with tyrosine. Although other chromophoric groups, such as disulfides, are absent from this protein (Maley et al., 1972a), tryptophan is present, and its contribution to the spectrum in this region cannot be neglected. However, the observed CD change effected by dCTP is most likely due to an alteration in the environment of several of the enzyme's tyrosyl residues (Horwitz et al., 1970). Similar spectral perturbations were observed with UV difference spectra in the presence and absence of dCTP (data not shown) but were much less dramatic than those obtained by the CD measurements. Whether tyrosyl residues are involved directly in the binding of dCTP or merely reflect conformational perturbations in the protein's structure remains to be clarified, but recent studies demonstrating a marked sensitivity of the deaminase to tetranitromethane, a tyrosine modifying reagent (Sokolovsky et al., 1966), support a functional role for tyrosine in the catalytic process.

Discussion

Deoxycytidylate deaminase, at least in the case of the animal enzyme (Maley & Maley, 1972), has proven to be one of the most sensitive allosteric enzymes studied to date, in that it is subject to extreme changes in activity as a result of small changes in effector concentration. Thus the enzyme is finely regulated by the end products of its metabolic pathway, dCTP and dTTP, where dCTP is both an activator and stabilizing agent and dTTP acts as a negative effector. Although direct physical evidence linking conformational changes to the allosteric transitions effected by the interaction of the regulatory nucleotides and substrate with the enzyme subunits has been difficult to obtain, due to insufficient quantities of enzyme, indirect evidence for this relationship was suggested in earlier studies. Thus dCTP markedly stabilizes the enzyme against heat denaturation, protects against thiol reagents and denaturation by sodium dodecyl sulfate, and prevents proteolytic inactivation (Maley & Maley, 1968). In addition dCTP maintains the enzyme in its hexameric form, whereas dTTP promotes a dissociation of the enzyme to its inactive monomeric state. In the case of the T_2 enzyme, however, the most probable positive natural effector is HM-dCTP, since dCMP is replaced completely in T even phage DNA by HM-dCMP. This phenomenon occurs as a consequence of the induction by the phage genome of such enzymes as dCTPase (Koerner et al., 1960), dCMP hydroxymethylase (Pizer & Cohen, 1962), and deoxycytidylate deaminase (Keck et al., 1960), which does not readily deaminate HM-dCMP (Maley & Maley, 1966). The latter property may be important for the survival of the T even phage by limiting the depletion of this essential DNA component.

T_2 deoxycytidylate deaminase is much more responsive to regulation in the presence of its natural effector, HM-dCTP, than in the presence of dCTP. The ratio of dTTP to HM-dCTP required to effect a 50% inhibition of the deaminase was constant from pH 6 to pH 8 and was in all cases considerably lower than that of dTTP to dCTP, which varied 4-fold over this pH range (Maley et al., 1972a). This observation is consistent with HM-dCTP's potentially more significant role as a physiologic modifier than dCTP, considering the environment in which it was designed to operate. Nonetheless dCTP has proven useful in clarifying the binding properties of the enzyme because of its availability relative to HM-dCTP and because much of the information obtained with dCTP has been confirmed with HM-dCTP.

Despite the fact that the chick embryo and phage deaminases differ somewhat in their catalytic properties, their physical and allosteric attributes are strikingly similar. Both are hexamers of comparable molecular weight and kinetic plots obtained with dCTP as the effector have yielded Hill coefficients characteristic of cooperative interactions among the enzyme subunits as a result of dCTP binding, particularly at pH 10 (n = 5.6) (Maley et al., 1972a). This type of cooperativity is in good agreement with the quantitative binding results for dCTP and HM-dCTP.

While previous experiments had suggested that there are four ligand-binding sites on deoxycytidylate deaminase from donkey spleen (Scarano et al., 1967a,b), the present studies clearly implicate six binding sites for substrate, competitive inhibitors, and allosteric ligands. Binding estimates obtained by least-squares and robust analyses (Nicklin & Paulsen, 1981) were as follows: dCTP, 5.50 ± 0.44 and 5.56 ± 0.42, respectively; dTTP, 6.30 ± 0.76 and 6.04 ± 0.31, respectively. Although the earlier study (Scarano et al., 1967b) may be correct in its assessment of the number of binding sites, which suggests that the spleen enzyme is composed of four subunits, a recent isolation of the human spleen deaminase (Ellims et al., 1981) claims the presence of 2 subunits/110000 daltons. However, a gross discrepancy, which remains to be explained in this study, is the fact that the specific activity of the homogeneous spleen deaminase is only $^1/_{60}$ that of the pure enzyme from other sources, both animal (Scarano et al., 1967a; Maley & Maley, 1972) and bacterial (Maley et al., 1972a). In the case of the T_2 deaminase, the subunits appear to be identical, which is based on dimethylsuberimidate analysis (Maley et al., 1972b) and more recent sequencing data (unpublished experiments).

Whether this enzyme fits the sequential model of allosteric regulation (Kuehn et al., 1971; Koshland et al., 1966) or the more restrictive concerted model (Monod et al., 1965) will be difficult to evaluate because the complex interactions between substrate and modifier with the enzyme subunits are pH dependent. Thus there appears to be an absolute requirement for dCTP in the enzyme reaction above pH 7.0, which becomes markedly cooperative in both binding and catalysis with pH. This effect is particularly true for HM-dCTP (Figure 2), with both compounds appearing to conform to a model in which the binding of the allosteric ligand to an enzyme subunit sequentially enhances its binding to the next subunit (Koshland et al., 1966). Similarly, the substrate, on the basis of the binding of its analogues, appears also to be bound in a cooperative manner (Figure 6). In any event a complex network of heterotropic interactions appears to exist between substrate and effector, which may add another dimension to the regulatory aspects of this enzyme.

As a result of the availability of adequate quantities of pure deaminase it was possible to determine whether a conformational change is associated with the binding of dCTP (Figure 7). Since these changes occurred at concentrations of dCTP associated with the activation of the enzyme, it is highly probable that a significant change in the enzyme's structure occurs on the binding of dCTP. It is possible that the observed CD perturbation is due in part to an alteration in the conformation of dCTP, but the nature of the observed effect suggests that a change in the hydrophobic environment of some of the enzyme's tyrosyl residues is a more likely explanation (Horwitz et al., 1970). In comparison with the CD transitional effects associated with the binding of allosteric ligands and substrates to aspartate transcarbamylase (Griffin et al., 1972), the ellipticity change effected by dCTP at 280 nm is at least 30 times greater and appears to be the most dramatic seen to date in response to an allosteric modifier. The ability of dTTP to reverse this effect is consistent with its being an allosteric response. Because of dTTP's relatively low affinity for the enzyme in comparison to that of dCTP, it was not possible to demonstrate a comparable optical transition effected by this nucleotide.

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