# Identification of a Site Necessary for Allosteric Regulation in T4-Phage Deoxycytidylate Deaminase†

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ABSTRACT: An allosteric inhibitor of dCMP deaminase, dTTP, forms a photolabile covalent bond with T4-phage dCMP deaminase in the presence of UV light at 254 nm. The importance of the methyl group in this process is supported by the findings that dUTP, also an allosteric inhibitor, does not photofix to the enzyme and that tritium is released from [*methyl*-$^3$H]dTTP during the course of the photofixation. That the bond formed is photolabile is demonstrated by the fact that tritium is released by about 10-fold over the amount of nucleotide that is photofixed. The amino acid that covalently binds dTTP in T4-dCMP deaminase was identified as $Phe_{112}$. On conversion of $Phe_{112}$ to an alanine by site-directed mutagenesis, there was a dramatic change in the enzyme's response to its allosteric effectors when measured early in the reaction, in that the mutant enzyme was as active as the wild-type even in the absence of dCTP and was only weakly inhibited by dTTP. However, after 10–15% of the substrate had been deaminated, the reaction rate fell off rather markedly, indicating either that an inhibitor was being accumulated on the enzyme or that the enzyme was being irreversibly inactivated with time. That the latter was not the case was shown by the addition of dCTP to the reaction, which restored the rate to that expected when it was present initially. Furthermore, we showed that, consistent with the observed loss of allosteric regulation by dCTP and dTTP, the affinity of the mutant enzyme for dTTP and dCTP as determined by binding studies was greatly reduced relative to the wild-type enzyme. Interestingly, $Phe_{112}$ resides within a peptide motif previously shown to be conserved in several dTTP-binding proteins [McIntosh, E. M., & Haynes, R. H. (1986) *Mol. Cell. Biol. 6*, 1711–1721].

Deoxycytidylate deaminase (EC 3.5.4.12) is an allosteric enzyme containing six identical subunits that converts dCMP to dUMP (Maley & Maley, 1972, 1990). Since this enzyme is located at a critical branchpoint in pyrimidine deoxynucleotide metabolism, it is not surprising that the end products of this pathway, dCTP and dTTP, regulate the deaminase, with the former behaving as an activator and the latter as an inhibitor (Maley & Maley, 1962). By this means, a balance of dCTP and dTTP is maintained (Maley & Maley, 1972; Jackson, 1980; de Saint Vincent et al., 1980) which, if disrupted, as in the case of dCMP deaminase deficient cells, can result in deleterious increases in the mutation rate (Weinberg et al., 1981; Sargent & Mathews, 1987). To understand how the deaminase interacts with its allosteric effectors, we have undertaken a study to define the binding sites of dCTP and dTTP, which was facilitated by our previous finding that dTTP can be photofixed to the dCMP deaminase from T2-phage in the presence of ultraviolet light at 254 nm (Maley & Maley, 1982). The formation of this linkage potentially allows a delineation of the amino acid residue bound directly to dTTP, which could shed light on the composition of the allosteric region of dCMP deaminase. Similar to this enzyme, we observed that photofixation of dTTP also occurs with the T4-dCMP deaminase, an enzyme that differs from the T2-deaminase in only nine amino acids, including a five amino acid deletion at the carboxyl end (Maley et al., 1990). The T4-deaminase was chosen for this study since it was recently cloned and amplified in a high-expression system (Moore et al., 1993b), making it more available than the T2-deaminase for large-scale studies. Using the T4-dCMP deaminase, we have been able to characterize both the nature and the location of the dTTP photolabeling site, which will be shown in this study to be involved in the allosteric regulation of the deaminase.

## MATERIALS AND METHODS

*Materials.* [*methyl*-$^3$H]dTTP (50 Ci/mmol) in 50% ethanol/water was purchased from Moravek Biochemicals Inc. (Brea, CA). [5-$^3$H]dUTP (10 Ci/mmol) in 50% ethanol/water was obtained from Amersham (Arlington Heights, IL). [$\alpha$-$^{32}$P]dCTP (>3000 Ci/mmol), [$\alpha$-$^{35}$S]dATP (1000 Ci/mmol), and [$\alpha$-$^{32}$P]dTTP (>3000 Ci/mmol) in Tricine buffer were purchased from NEN Research Products, Dupont Corp. (Boston, MA). Unlabeled deoxynucleoside 5′-triphosphates and SDS–PAGE[1] molecular weight markers were purchased from Pharmacia/LKB Biotechnology (Piscataway, NJ). TPCK-treated trypsin was purchased from Worthington Biochemical Corporation (Freehold, NJ). Endoproteinase lys-C was from Promega Corp. (Madison, WI). 4-Vinylpyridine (98%) was obtained from Aldrich (Milwaukee, WI), while ultrapure urea was from Bethesda Research Laboratories (Gaithersburg, MD). T4 DNA polymerase (3000 units/mL)

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[1] Abbreviations: *E. coli*, *Escherichia coli*; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HMdCTP, 5-(hydroxymethyl)-dCTP; BSA, bovine serum albumin; HPLC, high-pressure liquid chromatography; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; $AC_{50}$, concentration of activator at which 50% maximal activation is achieved.

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and T4 DNA ligase (400 000 units/mL) were purchased from New England Biolabs (Beverly, MA). Ultrafree-MC filter units containing polysulfone membranes with a 30 000 nominal molecular weight limit were obtained from Millipore Corp. (Bedford, MA).

*Purification of T4-dCMP Deaminase.* To overexpress the T4-dCMP deaminase gene product using the pET system (Studier et al., 1990), a 581 base pair fragment encoding the T4-dCMP deaminase was subcloned into the plasmid vector pET3c and transformed into the *Eschericia coli* strain BL21-(DE3)/pLysS (Moore et al., 1993b). Soluble, active enzyme was obtained under conditions where inclusion body formation could be avoided (Moore et al., 1993b). Using this procedure, approximately 20 mg of homogeneous deaminase was purified per liter of bacteria. Human dCMP deaminase was cloned and overexpressed similarly, as described by Weiner et al. (1993). Protein concentrations were determined using Bio-Rad protein reagents (Bio-Rad, Richmond, CA). Deoxycytidylate deaminase activity was assayed spectrophotometrically as described earlier (Maley, 1967), with 1 unit being defined as the amount of enzyme required to convert 1 μmol of dCMP to dUMP at 30 °C. SDS–PAGE was performed by the method of Laemmli (1970). The separating gel contained 15% acrylamide and the stacking gel contained 4.5% acrylamide.

*Photofixation.* The photofixation reaction was similar to that described previously (Maley & Maley, 1982a) and contained the following components in 100 μL: 10 mM potassium phosphate (pH 7.5), 10 mM $MgCl_2$, 0.5 mM [$\alpha$-$^{32}$P]-dTTP ((1–5) × $10^7$ cpm) or 0.5 mM [*methyl*-$^3$H]dTTP (5 × $10^7$ cpm), and 60 μg of T4-dCMP deaminase (0.5 nmol of holoenzyme or 3 nmol of enzyme subunits). Other deoxynucleotides were added to, or substituted for, dTTP as indicated in the text. The reaction mixture was placed on Parafilm over a small lead block that was surrounded with ice. Irradiation was performed with an overhead 254-nm light source, which was approximately 9 cm above the sample. The intensity of the surface of the lead block, measured with a YSI-Model 65 radiometer, was about 3 mW/cm$^2$. Samples of 10–30 μL were removed at various times after the start of ultraviolet illumination and added to 100 μg of BSA in 0.5 mL of cold $H_2O$, followed by 1.0 mL of ice-cold 10% TCA. The suspension was filtered through a 24-mm Whatman GF/A glass microfiber filter. The filter was washed three times with 3.0 mL of ice-cold 5% TCA and placed in a glass scintillation vial with 6 mL of Aquasol scintillation liquid (Dupont, Boston, MA) for measurement in a Beckman Model 3801 scintillation counter.

*Tritium Release from [methyl-$^3$H]dTTP.* Samples (10 μL) were taken from a standard photofixation reaction containing 12.5–15.0 μCi of [*methyl*-$^3$H]dTTP and transferred to 1.5-mL Eppendorf tubes. Protein was precipitated by adding 0.5 mL of 1 mg/mL BSA to each tube, followed by 1.0 mL of ice-cold 10% TCA. After incubation on ice for 5 min, the precipitate was centrifuged at 14000*g* for 10 min in the cold (4 °C), using an Eppendorf microfuge. A 50-μL aliquot of the supernatant was combined with 200 μL of 100 mg/mL of acid-washed Norit A in 2% TCA to adsorb free nucleotides. The mixture was allowed to stand on ice for 5 min and then centrifuged as above for 6 min, after which time 50 μL of the supernatant fraction was assayed for radioactivity in a scintillation counter.

*Proteolysis Conditions.* A large-scale reaction (about 0.8 mL) containing 480 μg of T4-dCMP deaminase (24 nmol of enzyme subunits) and 120 μCi of [$\alpha$-$^{32}$P]dTTP or 500 μCi of [*methyl*-$^3$H]dTTP (see Photofixation section above) was placed on the Parafilm-covered lead block in 100-μL volumes and irradiated for 1.5 min. It was noted that longer photofixation times reduced the efficiency of the subsequent proteolysis, an effect possibly due to enzyme aggregation or to subunit cross-linking. Immediately after the reaction, a 15-μL aliquot was taken to determine the amount of acid-precipitable material, as described above. The remaining sample was lyophilized to dryness and resuspended in 400 μL of 6 M guanidine hydrochloride (ultrapure), 0.25 M Tris Cl (pH 8.5), 1 mM EDTA, and 4 μL of 10% 2-mercaptoethanol. This solution was incubated at 37 °C for 1 h under $N_2$, followed by the addition of 8 μL of 4-vinylpyridine to convert the cysteinyl residues to *S*-(β-4-pyridylethyl)cysteines (Freidman et al., 1970). After incubation for 3 h at ambient temperature under $N_2$, the reaction was stopped by the addition of 100 μL of 1 M dithiothreitol followed by dialysis of the sample against three 1-L changes of $H_2O$. The protein that precipitated during the dialysis was collected in a microfuge by centrifugation at 14000*g* for 10 min. It was noted that >90% of the incorporated label was present in the precipitate, which was solubilized after centrifugation in 125 μL of a solution of 8 M urea and 0.4 M $NH_4HCO_3$. The protein solution was then diluted to 500 μL with $H_2O$ to reduce the urea concentration to 2 M. These conditions were a modification of those described by Stone et al. (1989). Trypsin (1 mg/mL) in a solution of 1 mM HCl and 11.5 mM $CaCl_2$ was added at an enzyme/substrate ratio of 1/50 and incubated at 37 °C, for 18 h. The samples were then lyophilized and resuspended in 500 μL of 0.1% TFA for HPLC analysis.

For the lys-C digestion, 480 μg of T4-dCMP deaminase was photofixed with 500 μCi of [*methyl*-$^3$H]dTTP and treated as described by Wilkinson (1986). Solid urea was added to the samples to give a final concentration of 6.4 M, and they were incubated for 60 min at 60 °C. The solutions were then diluted 4-fold with 25 mM Tris Cl (pH 7.7) and 1 mM EDTA to give a final urea concentration of 1.6 M. Endoproteinase lys-C (suspended according to Promega's directions) was added to the sample at an enzyme/substrate ratio of 1/50 and incubated at 37 °C for 18 h, followed by lyophilization.

*Isolation of [methyl-$^3$H]dTTP- or [$\alpha$-$^{32}$P]dTTP-Labeled Peptides by HPLC.* The lyophilized peptides were dissolved in 0.5 mL of 0.1% TFA and separated by reverse-phase HPLC using a Vydac/Protein (C4-330 Å) column (4.6 × 250 mm) (Rainin Instruments). The solvent system consisted of 0.1% TFA in water (solvent A) and 0.1% TFA in acetonitrile (solvent B). After injection of the sample onto the reverse-phase column that had been equilibrated with solvent A, the column was washed with solvent A until the radioactivity of the eluate fell to near-background levels. A nonlinear gradient of 0–100% solvent B was used to effect the desired separation of the peptides. The flow rate was 0.5 mL/min, and 1-mL fractions were collected. The eluate was monitored by absorbance at 230 nm, and a 100-μL aliquot from each fraction was counted to identify those fractions containing radioactivity.

*Peptide Sequence Analysis and Peptide Synthesis.* Peptide sequencing was carried out by automated Edman degradation with a Model 477A Applied Biosystems pulsed liquid sequencer equipped with an on-line (phenylthio)hydantoin–amino acid analyzer and fraction collector (Applied Biosystems Model 120A). The PTH–amino acid fractions were counted for radioactivity. Peptides were synthesized on an Applied Biosystems 431A automated peptide synthesizer. Fluorenylmethyl chloroformate chemistry was used, and cleavage was performed with TFA in the presence of the appropriate

Table 1: Degree of Photofixation of dTTP to Various dCMP Deaminases[a]

| enzyme | dTTP fixed per dCMP deaminase subunit (nmol/nmol) |
|---|---|
| T4-dCMP deaminase | 0.17 ± 0.03 |
| T4-dCMP deaminase ($Phe_{112}Ala$) | 0.03 ± 0.01 |
| human dCMP deaminase | 0.05 ± 0.02 |

[a] See Materials and Methods for details.

scavengers. The amino acid sequences of the peptides were confirmed by amino acid analysis and peptide sequencing.

*Site-Directed Mutagenesis.* Plasmid pET3c (Studier et al., 1990) containing the CD5 insert encoding T4-dCMP deaminase was mutagenized by a modification (Moore et al., 1993a) of the unique-site-elimination method (Deng & Nickoloff, 1992). Mutagenesis was carried out with the following oligonucleotides: 5′-GTGCCACCTGTCGAC-TAAGAAACC-3′, which eliminates the unique *Aat*II site in pET3c-CD5, and 5′-CCATTTCGTGCAGCAGCCAAAAT-TGCATTTAG-3′, which changes bases 872 and 873 (TT to GC). The latter change converts the codon representing phenylalanine 112 (TTT) to an alanine codon (GCT). Correct identification of the phenylalanine to alanine mutation was carried out by dideoxy-DNA sequencing (Sanger et al., 1977). Since the plasmid mutagenesis was carried out in the expression vector, pET3c-CD5, the resulting mutant plasmid was immediately ready for use in protein overexpression in *E. coli* BL21(DE3)/pLysS. The $Phe_{112}Ala$ mutant of T4-dCMP deaminase was purified as described above for the wild-type enzyme.

*Nucleotide Binding Assays.* Equilibrium dialysis was conducted by a filter binding assay described by Örmo and Sjöberg (1990). Experiments were carried out at ambient temperature in a mixture containing 10–50 mM Tris or phosphate buffer (pH 7.1), 10 mM 2-mercaptoethanol, 5 mM $MgCl_2$, and 10% (v/v) ethylene glycol. [$\alpha$-$^{32}$P]dCTP and [*methyl*-$^3$H]dTTP were adjusted to a concentration of 5–500 $\mu$M, with specific activities of 50–150 dpm/pmol and 5–60 dpm/pmol, respectively. About 190 $\mu$g of protein was used for each data point in a volume of 150 $\mu$L, giving a final concentration of 10 $\mu$M. After the components were mixed (enzyme added last), the samples were incubated for 20 min at ambient temperature. Aliquots of 30 $\mu$L were taken to determine the total radioactivity. The remaining 120 $\mu$L was transferred to the upper sample reservoir of ultrafree-MC filter units. The samples were centrifuged immediately for 30–40 s at 16000*g* in an Eppendorf centrifuge (Model 5415) at ambient temperature. Aliquots of 10–25 $\mu$L were withdrawn from the filtrate for scintillation counting and for calculation of free nucleotide concentration.

## RESULTS

*Optimization of T4-dCMP Deaminase Photolabeling Conditions.* By measuring acid-precipitable radioactivity after photolabeling with [$\alpha$-$^{32}$P]dTTP, optimal conditions for $MgCl_2$, pH, and time were determined. It was found that maximal fixation of dTTP to T4-dCMP deaminase occurred between 60 and 90 s of exposure to UV light at $\lambda$ = 254 nm and that the maximal amount of dTTP incorporated occurred at concentrations $\geq$200 $\mu$M, where 0.17 ± 0.03 nmol of dTTP/nmol of T4-dCMP deaminase subunit was fixed (Table 1). These findings are similar to those reported using T2-dCMP deaminase except for the magnitude of dTTP incorporation (Maley & Maley, 1982a), which was somewhat lower than that reported for the T2 enzyme. This may be due, in part, to the differences in the primary structures of the two enzymes. It should be noted, as shown below, that the photolabeling of the deaminase by dTTP is also photosensitive and appears to represent a steady-state level of fixation, which would explain why a 1/1 level of incorporation is not achieved, even at saturating levels of dTTP.

*Binding of dTTP to T4-dCMP Deaminase Is Nucleotide- and Methyl-Specific.* Although fixation of dTTP to T4-dCMP deaminase is relatively low (about 20% that which should be fixed maximally per subunit), the labeling is highly specific. Thus, when the photofixation reactions containing [$\alpha$-$^{32}$P]dTTP were carried out in the absence of T4-dCMP deaminase or in the presence of 28 $\mu$g of BSA, the amount of label fixed to the filter was 0.01 ± 0.01 and 0.02 ± 0.01, respectively (data not shown). The significance of the 5-methyl group of dTTP in the photofixation is emphasized by the fact that dUTP acts as an allosteric inhibitor similar to dTTP, but it cannot be photofixed to the enzyme. In addition, adding purine deoxyribonucleoside 5′-triphosphates had little effect on the degree of dTTP fixation. However, on the addition of the positive allosteric effector, dCTP, complete inhibition of dTTP fixation to the deaminase was obtained, or if dCTP was added to dTTP containing deaminase and irradiated, the dTTP was released. These findings are similar to those reported earlier for the T2-deaminase (Maley & Maley, 1982a). In addition, the incorporation at higher wavelengths (300 and 350 nm) was much less efficient. As reported for the T2-dCMP deaminase, the incorporated [$\alpha$-$^{32}$P]dTTP appears to be covalently bound since much of the radioactivity remained with a 21-kDa subunit after boiling in 1% SDS and electrophoresis for SDS–PAGE (Maley & Maley, 1982a).

*Significance of the 5-Methyl Group of dTTP in the Binding of dTTP to T4-dCMP Deaminase.* Since dUTP also inhibits T4-dCMP deaminase but does not photofix, it is logical to assume that the methyl of dTTP is responsible for this difference, as indicated above. To verify this assumption, [*methyl*-$^3$H]dTTP was used in place of [$\alpha$-$^{32}$P]dTTP, and at various times after initiation of the photolabeling reaction, the reactions were stopped by TCA and the extent of [*methyl*-$^3$H]dTTP fixation was measured until a steady-state level of [*methyl*-$^3$H]dTTP fixed to the deaminase was obtained. As indicated above, when [$\alpha$-$^{32}$P]dTTP was used in the photolabeling reactions, a maximum of 0.17 ± 0.03 nmol of dTTP/nmol of T4-dCMP deaminase subunit was photofixed, while with [*methyl*-$^3$H]dTTP a maximum of 0.14 ± 0.02 nmol was obtained (data not shown) or about two-thirds that of [$\alpha$-$^{32}$P]-dTTP. These findings suggest that the photofixation of dTTP is associated with the loss of a single hydrogen atom from the 5-methyl, therefore reducing the amount of radioactivity incorporated by one-third relative to $^{32}$P. Similar results were reported by Kierdaszuk and Eriksson (1988) with ribonucleotide reductase.

*Photofixation of dTTP to T4-dCMP Deaminase Represents a Dynamic Equilibrium.* In contrast to measurements on the fixation of dTTP to the deaminase, which plateau after about 3 min, when the release of tritium from [*methyl*-$^3$H]-dTTP was measured by the addition of charcoal to the reactions to absorb the labeled unfixed nucleotide, an almost linear increase in $^3$H release occurred over a period of 40 min in the presence of 254-nm light (Figure 1). In the absence of enzyme or in the presence of a nonspecific protein, this release was minimal. The continual release of tritium during the photolabeling reaction suggests that the methyl group of [*methyl*-$^3$H]dTTP is in constant flux with the deaminase, which is supported by the fact that 25 of the 50 nmol of dTTP in the

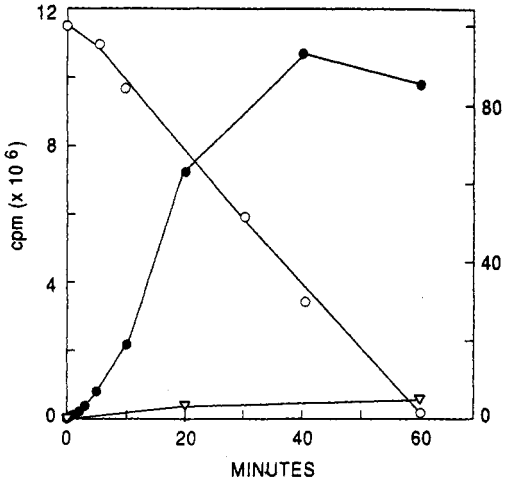


FIGURE 1: Time course of tritium release from [*methyl*-$^3$H]dTTP when exposed to $UV_{254}$ in the presence and absence of T4-dCMP deaminase. Solutions (see Materials and Methods) containing 60 $\mu$g of T4-dCMP deaminase and 0.5 mM [*methyl*-$^3$H]dTTP ($5 \times 10^7$ cpm) in 100 $\mu$L were used to determine the rate of tritium release during the course of photolysis. Aliquots of the photolabeling reactions (10 $\mu$L) were analyzed for tritium release at various times between 0 and 60 min after the start of exposure of the samples to $UV_{254}$ by adding activated charcoal to the samples in 1.5-mL Eppendorf tubes and centrifuging. The tritium released to the medium was determined by measuring aliquots in a scintillation counter. After background radioactivity at 0 min was subtracted, values were adjusted to represent the amount of tritium released per 100-$\mu$L reaction. The photolabeling reactions consisted of T4-dCMP deaminase (●), no T4-dCMP deaminase (▽), and relative activity of T4-dCMP deaminase during the course of the reaction (○). The ordinate on the right side of the graph represents relative enzyme activity (0–100%).

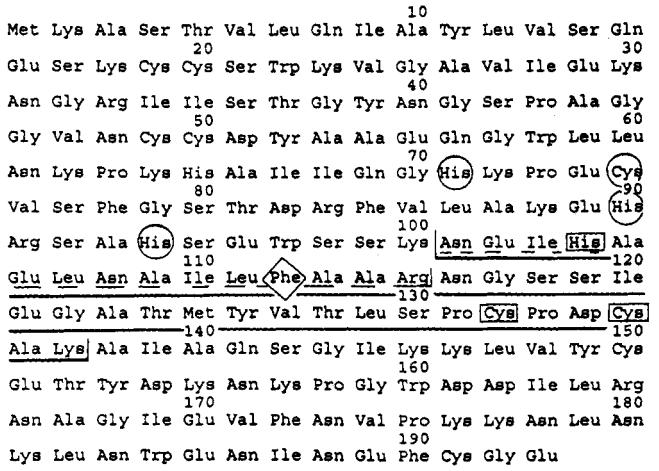


FIGURE 2: Amino acid sequence of T4-dCMP deaminase [taken from Maley et al. (1990)]: ○, amino acids involved in a putative zinc finger (Moore et al., 1993c); □, amino acids binding zinc in the catalytic site; ◇, amino acid involved in binding of dTTP; - - -, isolated tryptic peptide from Figure 2; (—), isolated endo-lysC peptide from Figure 4.

reaction had been turned over during this period by only 3 nmol of T4-dCMP deaminase subunits (assuming that 1 mol of tritium was released per mole of [*methyl*-$^3$H]dTTP). Since acid-precipitable counts (either [*methyl*-$^3$H]dTTP or [$\alpha$-$^{32}$P]-dTTP) reached a maximum in less than 3 min while tritium continued to be released over a 40-min period, the binding reaction appears to represent a dynamic equilibrium, with the UV light promoting the binding and release of dTTP at a steady-state value of 0.17 mol of [$\alpha$-$^{32}$P]dTTP bound/mol of deaminase subunit. The observed plateau in tritium release at 40 min most likely occurs as a result of enzyme inactivation by the UV light, since only about one-half of the [*methyl*-$^3$H]dTTP had reacted with the enzyme during this period. Probably more tritium would have been released had the enzyme not been inactivated or if active enzyme had been added at this point. Since the amount of dTTP fixed to the deaminase appears to represent a steady-state level, the linkage between the two must be photolabile, and thus is supported by the fact that continuing the exposure after 3 min in the presence of unlabeled dCTP or dTTP results in the complete loss of radioactivity from the enzyme (data not shown). Aside from being photolabile, the bond between dTTP and dCMP deaminase appears to be labile in general, as will be described below in attempts to isolate the amino acid to which the dTTP was fixed.

*Analysis of Tryptic Peptides from [$\alpha$-$^{32}$P]dTTP-Photolabeled T4-dCMP Deaminase.* To locate the site on the deaminase to which dTTP was photofixed, the labeled enzyme was subjected to trypsin digestion, and an attempt was made to isolate a specific peptide containing dTTP. However, the lability of the bond between dTTP and the deaminase made it difficult to identify a proteolytic fragment containing dTTP since, during the several days it took to isolate the desired labeled peptide, most of the attached label was lost. This occurred regardless of whether [*methyl*-$^3$H]dTTP or [$\alpha$-$^{32}$P]-dTTP was utilized in the photofixation reaction. For this reason, these experiments were scaled up to utilize much greater amounts of radioactivity in the initial photolabeling reaction, a factor that turned out to be critical. Starting with 4 nmol (480 $\mu$g) of photolabeled deaminase, only about 10% of the original bound radioactivity could be recovered associated with a *tryptic* peptide. Part of the poor recovery was due in part to incomplete digestion by trypsin, but the main loss in recovery appeared due to the release of dTTP or a degradation product of this compound from the protein during HPLC. The lability of photofixed compounds to peptides is not uncommon and has been encountered by other investigators (Catalano et al., 1990; King et al., 1991). Another difficulty associated with photolabeled peptides is that they often chromatograph with lower resolution than unlabeled peptides (Rush & Konigsberg, 1990; King et al., 1991).

However, we were able to isolate sufficient amounts of a pure, labeled peptide from the tryptic digest (data not shown) to obtain a tentative sequence of the isolated, labeled peptide, which was found to represent amino acids 101–115 of T4-dCMP deaminase (Figure 2). This sequence was found to identify with a putative dTTP-binding motif previously suggested by McIntosh and Haynes (1986).

*Comparison of the HPLC Profile of $^3$H-Labeled Deaminase Peptide with That of the Corresponding Unlabeled Peptide.* The HPLC migration of the labeled peptide was compared to the migration of a synthetic peptide of the same sequence. This approach was taken because it seemed reasonable to propose that the labeled and synthetic peptides might migrate differently on the HPLC column due to the presence of the bound dTTP on the labeled peptide. In these experiments, [*methyl*-$^3$H]dTTP-labeled deaminase was digested with trypsin, and the labeled peptide was purified by HPLC as described above. This peptide was then mixed with a synthetic peptide of the sequence N-E-I-H-A-E-L-N-A-I-L-F-A-A-R and chromatographed by HPLC using the same conditions that were used to isolate the tryptic peptide originally. It was found that the synthetic peptide eluted from the column 15 min prior to the labeled peptide, verifying that photofixed dTTP did indeed alter the elution of the above peptide (data not shown).

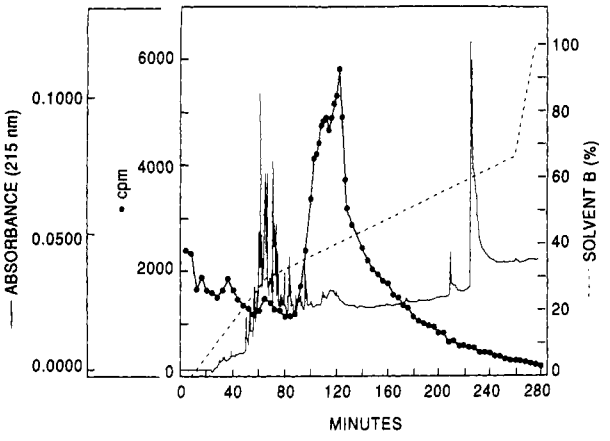


FIGURE 3: HPLC analysis of endo-lysC digestion of [*methyl*-$^3$H]-dTTP-labeled dCMP deaminase. After the reaction, the protein was denatured in 6.4 M urea, and the solution was diluted to 1.6 M urea prior to digestion with lys-C for 18 h. The peptides were separated by HPLC as described in Materials and Methods.

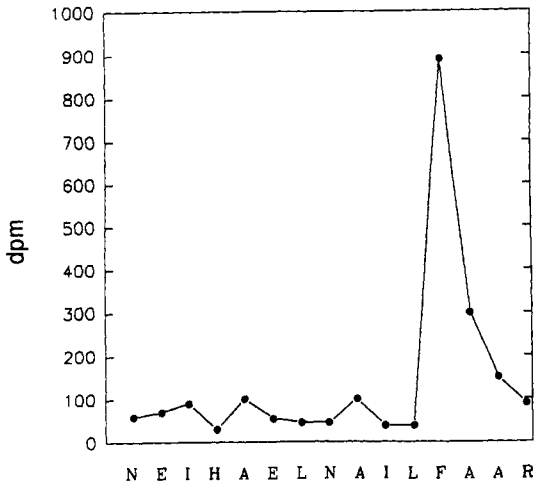


FIGURE 4: Location of radioactivity in the $^3$H-labeled lys-C peptide isolated in Figure 3. After this pooled peptide was sequenced on an Applied Biosystem 477A protein sequencer, individual fractions were counted in a scintillation counter, and as noted, most of the tritium is associated with a fraction corresponding to $Phe_{112}$ of the T4-dCMP deaminase (Figure 2).

*Analysis of lys-C Peptides from [methyl-$^3$H]dTTP-Photolabeled T4-dCMP Deaminase.* To confirm the identity of the dTTP-binding peptide obtained in limited amounts from the tryptic digest, we conducted experiments using lys-C digests of [*methyl*-$^3$H]dTTP-labeled deaminase. This digestion appeared to be somewhat more efficient than that with trypsin, as reflected by the recovery of nearly 20% of the incorporated radioactivity in a single peptide (Figure 3). Sequence analysis of the $^3$H-labeled lys-C peptide fractions revealed that most of the radioactivity was associated with amino acid 12 of the isolated lys-C peptide (Figure 4), which corresponds to $Phe_{112}$ of the deaminase (Figure 2). It is interesting to note that this peptide, although 22 amino acids larger than the labeled tryptic peptide in Figure 2, elutes somewhat faster (125 vs 142 min) using similar elution conditions.

*Effect of the Mutation of Phenylalanine-112 to Alanine on Properties of dCMP Deaminase.* If $Phe_{112}$ is involved in the binding of dTTP, it might be predicted that the replacement of $Phe_{112}$ by an alanine could alter the photofixation of dTTP. This possibility was suggested by comparing the binding of dTTP to the human dCMP deaminase (Weiner et al., 1993), since this enzyme contains a two amino acid deletion in the putative dTTP-binding motif, with one of the missing amino acids being the phenylalanine corresponding to phenylalanine-112 of T4-dCMP deaminase. It is shown in Table 1 that both the human dCMP deaminase and $Phe_{112}$Ala (prepared by a modified USE mutagenesis procedure; Moore et al., 1993a) photofix dTTP to a much lesser degree than the T4 wild-type deaminase, indicating that the phenylalanine in the dTTP-binding site is essential for efficient photofixation, a result supported by the nucleotide-binding studies below.

Table 2: Evidence for Reduced Allosteric Regulation of $Phe_{112}$Ala Relative to Wild-Type dCMP Deaminase[a]

| | effect of nucleotide addition on initial velocity ($\Delta A_{290}$/min) | | |
|---|---|---|---|
| enzyme | none | dCTP | dTTP |
| wild type | 0.005 | 0.050 | 0.007 |
| $Phe_{112}$Ala | 0.035 | 0.035 | 0.040 |

[a] Wild type and $Phe_{112}$Ala dCMP deaminases (60 munits each) were assayed spectrophotometrically over a full-scale range of 0.0–0.2 absorbancy units at $\lambda$ = 290 nm. The reaction solutions contained 0.5 mM dCMP, 1.0 mM $MgCl_2$, 10 mM Tris Cl (pH 8.0), 20 mM mercaptoethanol, and 10 $\mu$M dCTP when present; dTTP, when added, was at a final concentration of 0.24 mM.

*Response of $Phe_{112}$Ala to Its Negative Allosteric Effector, dTTP.* The nature of the feedback inhibition of the T-even-phage deaminase by dTTP is well-documented (Maley et al., 1972), and although the specific activity of $Phe_{112}$Ala was 10–20 times lower than that of the wild-type deaminase at 10 $\mu$M levels of the activator dCTP, comparable amounts of enzyme units[2] in each case were expected to show similar allosteric properties. However, as shown in Table 2, $Phe_{112}$-Ala, when diluted to approximately the same activity as the wild-type deaminase, was unaffected by dTTP concentrations ranging from 60 to 120 $\mu$M. Only at higher concentrations (240 $\mu$M) was a slight inhibition observed. By contrast, the wild-type enzyme is potently inhibited by this level of dTTP.

*Response of $Phe_{112}$Ala to Its Positive Allosteric Effector, dCTP.* We next sought to determine whether $Phe_{112}$Ala was also altered in its response to dCTP. In these experiments, the enzymatic activity of wild type and the $Phe_{112}$Ala mutant dCMP deaminase were compared directly in the presence and absence of different concentrations of dCTP. As shown in Table 2, the wild-type enzyme possesses little activity in the absence of dCTP, but is fully activated by as little as 2 $\mu$M dCTP. The mutant, in contrast, is as active in the presence of 10 $\mu$M dCTP as in its absence (Table 2). However, at much higher concentrations of dCTP (100 $\mu$M) some activation could be seen. This is reflected in the fact that, at pH 8.0, the $AC_{50}$ for dCTP with the wild-type deaminase is about 12 $\mu$M, whereas for $Phe_{112}$Ala it is about 560 $\mu$M. At pH 5.7, however, where dCTP binds more tightly to the enzyme, the $AC_{50}$ for the wild-type enzyme is 0.6 $\mu$M and is 10 times higher for $Phe_{112}$Ala. Thus, although the specific activity of the mutant enzyme is 10–20 times less than that of the wild-type enzyme at 10 $\mu$M dCTP, this difference can be reduced by one-half by raising the concentration of dCTP to >200 $\mu$M when assaying the $Phe_{112}$Ala deaminase. The allosteric responsiveness of the $Phe_{112}$Ala deaminase would therefore appear to be greatly reduced relative to the wild-type T4-deaminase.

A clear-cut distinction in the catalytic properties of the two enzymes is seen in Figure 5, where measurement of the

[2] $Phe_{112}$Ala requires much higher levels of dCTP for activation. When measured at 200 $\mu$M dCTP, the specific activity of $F_{112}$A is about one-half that of the wild type. The units of activity of the mutant are determined at this concentration of dCTP.

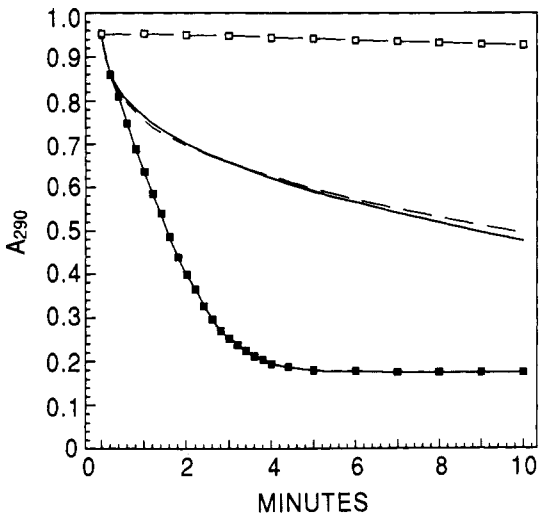


FIGURE 5: Kinetics of deamination of dCMP by wild type and mutant dCMP deaminases. The reaction conditions are similar to those described in Materials and Methods, except for the following changes: in the case of the wild-type enzyme, 0.12 unit (0.38 $\mu$g) was used in the presence (■) and absence (□) of 10 $\mu$M dCTP, while for the mutant enzyme ($Phe_{112}Ala$), 0.52 unit (3.7 $\mu$g) was added to the assay solutions in the presence (- - -) and absence (—) of 10 $\mu$M dCTP. The absorbancy scale used was from 0 to 1.0, which is 5 times that in Table 2. In the case of $Phe_{112}Ala$, a unit is defined as that for the wild type, except that at least 200 $\mu$M dCTP is required to obtain an optimal rate. Under these conditions, the specific activity of the $Phe_{112}Ala$ deaminase is about one-half that of the wild-type enzyme.

activities of the mutant and wild-type deaminases over a 5-fold greater range in absorbancy (1.0 vs 0.2) reveals some interesting properties of $Phe_{112}Ala$. Thus, while the wild-type deaminase shows no activity in the absence of dCTP, the mutant starts out initially with the same kinetics as the wild-type enzyme in the presence or absence of 10 $\mu$M dCTP, but after a minute or so the activity falls off rapidly (Figure 5). On the addition of about 200 $\mu$M dCTP at 3–5 min, the activity of the mutant assumes a rate comparable to that of the wild type in 10 $\mu$M dCTP (data not shown). The rapid fall-off in activity would appear to be due to the accumulation of an inhibitor, but it does not appear to be due to dUMP as the addition of this end product had no effect on the initial rate of the mutant enzyme's activity.

*Binding of dCTP to the Wild Type and $Phe_{112}Ala$ Mutant of T4-dCMP Deaminase.* Because $Phe_{112}Ala$ responded so poorly to activation and inhibition, we undertook studies to determine whether binding of the effectors was compromised in the mutant (Figure 6). dCTP binding to wild-type T4-dCMP deaminase at a pH of 7.5–7.8 showed cooperativity in both phosphate and Tris buffers, which resulted in a curvature of the Scatchard plots. In both cases the Hill plots were linear, with Hill coefficients of 1.22 in phosphate and 1.38 in Tris. The most significant difference in binding between phosphate and Tris at the same pH was the number of binding sites per hexamer estimated after linearization of the Scatchard plots, where it was shown that there are six binding sites/hexamer in phosphate, similar to that described earlier for the T2-dCMP deaminase (Maley & Maley, 1982b), but only three in Tris buffer (data not shown). This suggests that, in Tris buffer at pH 8.0, only one-half of the subunits are available for dCTP binding. At pH 5.7, dCTP was found to bind to six sites per hexamer, but it seems that in this case there are two different types of binding sites: three of very high affinity and three of much lower affinity ($K_D$ = 42 $\mu$M). Also, the binding of dCTP is not cooperative, reflecting the tighter binding of dCTP at this pH relative to pH 8.0. The $Phe_{112}Ala$ mutant clearly binds dCTP with a much lower affinity than wild type (Figure 6A vs 6B) at the higher pH.

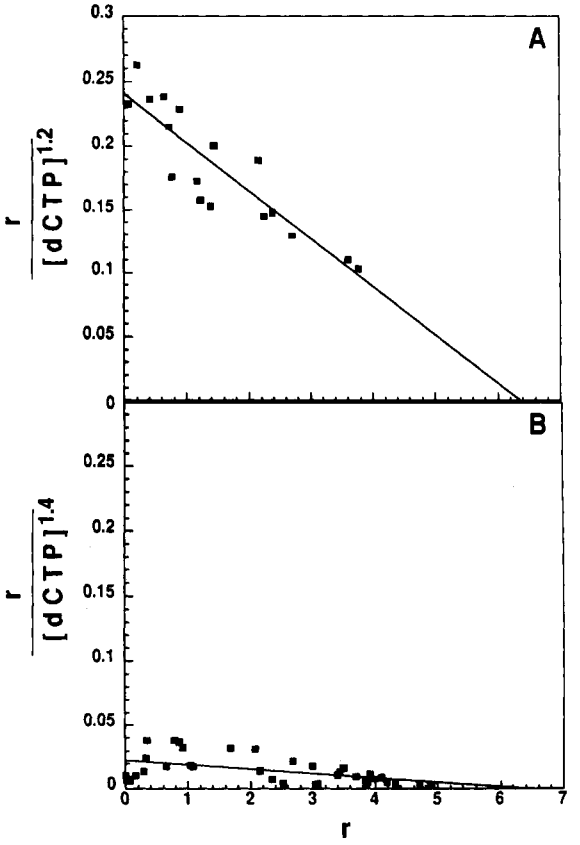


FIGURE 6: Comparison of the extent of binding of the positive allosteric effector dCTP to (A) wild type and (B) $Phe_{112}Ala$ T4-dCMP deaminases. See Materials and Methods for conditions.

*Binding of dTTP to the Wild Type and $Phe_{112}Ala$ Mutant of T4-dCMP Deaminase.* At pH 7.5–7.8, dTTP appears to bind to the wild-type enzyme with a much higher affinity than the $Phe_{112}Ala$ deaminase (Figure 7A,B). Thus, there are two different types of binding sites per hexamer molecule in phosphate buffer: three of high affinity ($K_D$ = 22 $\mu$M) and three of low affinity ($K_D$ = 100 $\mu$M). In Tris buffer, as was the case for dCTP, three dTTP molecules were found to bind to the hexamer ($K_D$ = 55 $\mu$M). The $Phe_{112}Ala$ mutant enzyme was a very poor binder of dTTP, with a $K_D$ of greater than 200 $\mu$M (Figure 7B). Decreasing the pH value of phosphate buffer to 5.7 affects dTTP binding for wild-type enzyme only slightly, in that there are still two types of binding sites per hexamer, three with a $K_D$ of 7.5 $\mu$M and three with a $K_D$ of 140 $\mu$M.

## DISCUSSION

Several studies have shown that dTTP can be photofixed to proteins when exposed to UV light, particularly when this nucleotide is a specific ligand for that protein (Eriksson et al., 1982; Maley & Maley, 1982a; Modak & Gillerman-Cox, 1982; Pandey & Modak, 1988; Cheng et al., 1993). If dTTP is bound tightly enough, the amino acid associated with the binding can be identified. Thus, cysteine-292 was found in the case of ribonucleotide reductase (Kierdaszuk & Eriksson, 1988), cysteine-227 and cysteine-234 were found in terminal deoxyribonucleotidyl transferase (Pandey & Modak, 1988), histidine-881, was in *E. coli* DNA polymerase, and lysine-73 was in reverse transcriptase (Cheng et al., 1993). The nature of the reaction is not entirely clear, but probably involves a

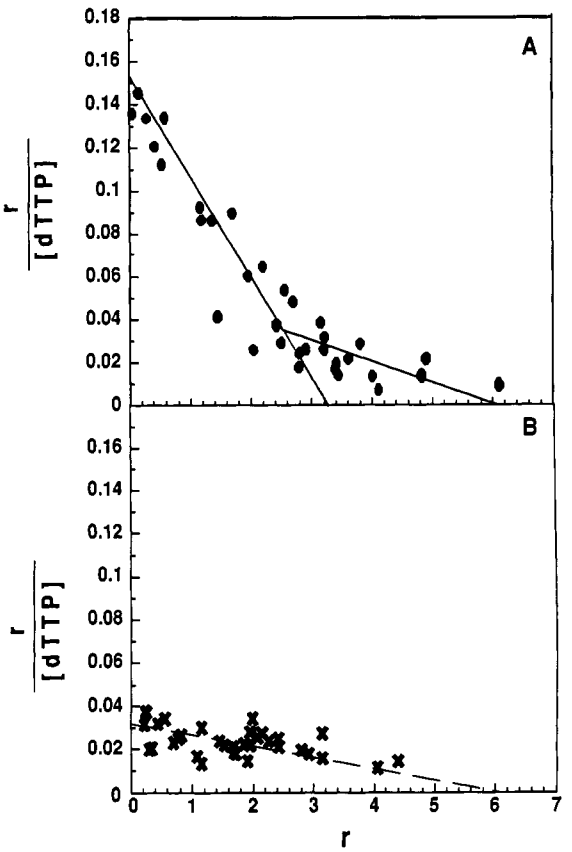


FIGURE 7: Comparison of the extent of binding of the negative allosteric effector dTTP to (A) wild type and (B) $Phe_{112}Ala$ T4-dCMP deaminases. The filter-binding assay of Örmo and Sjöberg (1990) was used to measure the affinity of these enzymes for dTTP. For a description of the reaction conditions, see Materials and Methods. The results are illustrated using a Scatchard plot.

free radical mechanism, one that does not have to include a protein or nucleic acid, since it has been found that, of several of the natural nucleosides, thymidine alone could be photofixed to lysine or other alkylamines (Saito et al., 1983a,b).

In this article, we have clearly shown that, similar to the above findings, another amino acid, $Phe_{112}$ of T4-dCMP deaminase, is capable of being photofixed to a thymidine derivative, dTTP. Previously, it was shown with the T2-dCMP deaminase (Maley & Maley, 1982a) that [$\alpha$-$^{32}$P]dTTP photofixed to the amino end of one of the two peptides obtained on CNBr cleavage of T2-dCMP deaminase, which is where a comparable phenylalanine residue would be found. Although the mechanism of this interaction is not known, it apparently involves the methyl group of thymidine since there is a displacement of tritium from [*methyl*-$^{3}$H]dTTP. This suggests, but does not prove, that the methyl group is bound covalently to the ring of phenylalanine in a photolabile linkage. Further studies are currently underway to verify this assumption.

The data in Figure 1 reveal that more than 1 mol of $^{3}$H can be released per mole of dTTP, indicating either that dTTP is in a state of dynamic equilibrium with the enzyme or that more than 1 mol of $^{3}$H can be released while dTTP is bound to the enzyme. Even when saturated with dTTP only about 0.2 mol of this nucleotide is fixed per mole of enzyme subunit, suggesting that the dTTP fixed to the protein turns over by being released by the UV light. An alternate possibility is that only one subunit is involved in the binding of an equivalent of dTTP, but equilibrium dialysis studies on the corresponding T2-deaminase (Maley & Maley, 1982b), as well as the filtration studies described in Figure7, reveal six binding sites for dTTP, or one per subunit. Similar conclusions were drawn when the complex of the gene 5 protein of bacteriophage fd and fd DNA was photofixed and a maximal value of only 22% was obtained (Paradiso et al., 1979). This reaction was shown to be reversible. Also, the maximal value obtained on photofixing ATP to adenosine-5-triphosphatase was 0.15 mol/mol of enzyme (Yue & Schimmel, 1977), while only 0.1 mol of ATP was photolabeled to *Acanthamoeba* myosin IA (Maruta & Korn, 1981). In the case of the *E. coli* B1 subunit of ribonucleotide reductase, 0.12 mol of dTTP was fixed to each subunit (Kierdaszuk & Eriksson, 1988). All of the above enzymes were found to bind 1 mol of ligand/mol of protein in equilibrium dialysis experiments, indicating that the low level of photofixation of dTTP to T4-dCMP deaminase is not an isolated case.

Additional evidence for the involvement of $Phe_{112}$ in the binding was provided by mutating this amino acid to an alanine residue, which resulted in the deaminase basically losing its capacity to bind both dCTP and dTTP (Figures 6 and 7), except at very high levels of these regulators. Furthermore, the importance of the methyl group in the photofixation is supported by the fact that, while such allosteric regulators as dCTP and dUTP can prevent dTTP binding, they themselves cannot be photofixed. Finally, we have found recently that 5-methyl-dCTP, which also activates the deaminase, can be photofixed to the enzyme (data not shown), again emphasizing the importance of the methyl group in the fixation. Whether $Phe_{112}$ or another amino acid is involved in the photofixation is under investigation.

It is of interest to note that, while dTTP photofixes to T4-dCMP deaminase, it does not also photofix to the human deaminase (Table 1), although dTTP still acts as an allosteric inhibitor (Maley et al., 1993). The inhibition occurs despite the fact that this protein does not possess a phenylalanine comparable to $Phe_{112}$, suggesting that other amino acids must compensate for the absence of phenylalanine in promoting the binding of dTTP.

Conversion of $Phe_{112}$ to an alanine residue appears to result in a loss in allosteric activation by dCTP or inhibition by dTTP, an effect clearly seen in Table 2 and Figure 5. Although much of this attenuation in regulation appears due to a marked diminution in binding of dCTP and dTTP to this mutant (Figures 6 and 7), little or no regulation by dCTP or dTTP could be observed, at least at an early stage in the reaction (Table 2). However, when viewed in its totality (Figure 5), the reaction falls off gradually as if an inhibitor is accumulating. This apparent inhibition could be reversed by adding relatively large amounts of dCTP to the reaction (>200 $\mu$M). Our supposition that the mutant enzyme is not regulated, at least initially, is based on the fact that, although this enzyme is active in the absence of dCTP, little or no activity is obtained with a comparable amount of wild-type dCMP deaminase under these conditions (Figure 5). The proposed inhibitor could be identical or similar to the transition-state intermediate suggested by Frick et al. (1989), which in the presence of dCTP is rapidly converted to dUMP. Thus, as indicated,

$$E + S \rightleftharpoons ES \overset{k_3}{\rightleftharpoons} ES' \overset{k_5}{\rightarrow} E + P$$

S′ represents the transition-state intermediate that gradually accumulates to inhibit the enzyme. On addition of dCTP, the deaminase is converted to its active form, wherein $k_5$ becomes much greater than $k_3$, preventing ES′ from accumulating. In the case of the wild-type enzyme, $k_3$ can be assumed to be

greater than $k_3$ in the mutant, resulting in an immediate inhibition as seen in Figure 5. The inhibition is prevented from occurring by the presence of dCTP at the beginning of the reaction.

Although other proposals can be presented to explain the results in Figure 5, it is of interest to note that a transition-state analogue based on the intermediate proposed by Frick et al. (1989) has been shown to be a potent inhibitor of dCMP deaminase (Maley et al., 1993). It is unlikely that the inhibition is due to dUMP, as the addition of dUMP to the reaction cuvettes at higher concentrations than could be expected to accumulate at the active site yielded little or no inhibition. In a similar vein, the human deaminase shows a much more pronounced hysteretic effect in the presence of dCTP than the T4 enzyme, which is consistent with the possibility that the former enzyme does not release the inhibitor as rapidly as the latter.

It would be of interest to repeat these studies with HMdCTP, the natural positive regulator of T4-phage dCMP deaminase. However, the synthesis of sufficient amounts of this compound for such a study is not a trivial matter, so we elected to use dCTP as the activator. While providing the desired information for this article, dCTP does not reveal the potential regulation of the deaminase *in situ*, which probably occurs as a consequence of the interaction of the deaminase with HMdCTP and dTTP. Thus, as shown earlier by us (Maley et al., 1972), HMdCTP is much less effective than dCTP as an activator of T2-dCMP deaminase, particularly above pH 7.0. As an example, the ratio of dTTP to dCTP required to effect a 50% inhibition of the enzyme at this pH was found to be 10/1, whereas the ratio of dTTP to HMdCTP required to achieve this degree of inhibition under the same conditions was 1.6/1. At pH 8.0, this ratio was reduced to 5/1 for dTTP to dCTP, but remained at about 1.6 for dTTP to HMdCTP. From these studies, it would appear that the T-even-phage deaminase is much more sensitive to regulation in the presence of HMdCTP than dCTP.

McIntosh and Haynes (1986) identified a conspicuous site of sequence similarity on comparing the T2-phage and *Saccharomyces cerevisiae* dCMP deaminases. This region is identical in the T2 and T4 enzymes. They also showed that, when this conserved region was compared to the amino acid sequence of another dTTP-binding protein, the M1 subunit of mouse ribonucleotide reductase (Caras et al., 1985), a region of similar identity was found. Interestingly, when the sequence was compared to the ribonucleotide reductase of the Epstein–Barr virus, which is insensitive to allosteric regulation by dTTP (Caras et al., 1985), the putative dTTP-binding motif was absent. Furthermore, it was found that this region is remarkably similar to a block of conserved sequences that is common to a variety of eukaryotic retrovirus reverse transcriptases and viral DNA polymerases (McIntosh & Haynes, 1986). These include DNA polymerases from human T-cell leukemia virus, Moloney murine leukemia virus, Rous sarcoma virus (RSV), cauliflower mosaic virus, hepatitis B virus (Patarca & Haseltine, 1983), and the yeast Tyb 912 gene product (Clare & Farabaugh, 1985). All of these enzymes share the property of dTTP binding.

Figure 2 presents information on sites other than the dTTP photofixation site at $Phe_{112}$ that contribute to the enzyme reaction. From what has been established earlier (Moore et al., 1993c), it is clear that zinc is involved in the catalytic mechanism involving $His_{104}$, and $Cys_{132}$ and $Cys_{135}$, since mutations involving any of these amino acids result in the loss of zinc, as well as enzyme activity (J. T. Moore, G. F. Maley, and F. Maley, unpublished results). This region is similar to those described for *E. coli* cytidine deaminase (Yang et al., 1992) and human adenosine deaminase (Wilson et al., 1991). In addition, $His_{71}$, $Cys_{75}$, $His_{90}$, and $His_{94}$ contribute to an unusual zinc-binding motif, which when modified results in the loss of zinc, but not enzyme activity. The role of this site, which resembles a zinc finger, is not entirely clear yet, but X-ray crystallography and DNA-binding studies currently underway should shed light on this subject.

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