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# Biochemistry of Terminal Deoxynucleotidyltransferase (TdT): Characterization and Mechanism of Inhibition of TdT by $P^1,P^5$-Bis(5′-adenosyl) Pentaphosphate†

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ABSTRACT: The catalysis of DNA synthesis by calf thymus terminal deoxynucleotidyltransferase (TdT) is strongly inhibited in the presence of $Ap_5A$, while replicative DNA polymerases from mammalian, bacterial, and oncornaviral sources are totally insensitive to $Ap_5A$ addition. The $Ap_5A$-mediated inhibition of TdT seems to occur via its interaction at both the substrate binding and primer binding domains as judged by (a) classical competitive inhibition plots with respect to both substrate deoxynucleoside triphosphate (dNTP) and DNA primer and (b) inhibition of ultraviolet light mediated cross-linking of substrate dNTP and oligomeric DNA primer to their respective binding sites. Further kinetic analyses of $Ap_5A$ inhibition revealed that the dissociation constant of the $Ap_5A$–enzyme complex, with either substrate binding or primer binding domain participating in the complex formation, is approximately 6 times higher ($K_i$ = 1.5 $\mu$M) compared to the dissociation constant ($K_i$ = 0.25 $\mu$M) of the $Ap_5A$–TdT complex when both domains are available for binding. In order to study the binding stoichiometry of $Ap_5A$ to TdT, an oxidized derivative of $Ap_5A$, which exhibited identical inhibitory properties as its parent compound, was employed. The oxidation product of $Ap_5A$, presumably a tetraaldehyde derivative, binds irreversibly to TdT when the inhibitor–enzyme complex is subjected to borohydride reduction. The presence of aldehyde groups in the oxidized $Ap_5A$ appeared essential for inhibitory activity since its reduction to alcohol via borohydride reduction or its linkage to free amino acids prior to use as an inhibitor rendered it completely ineffective. With use of a tritiated oxidation product of $Ap_5A$, a binding stoichiometry of 1 mol of $Ap_5A$ to 1 mol of TdT was observed. Thus, a single $Ap_5A$ molecule seems to span across both the substrate and primer binding site domains in TdT.

Enzymatic synthesis of DNA is a complex reaction that involves multiple components. Of the various DNA polymerases that catalyze this reaction, terminal deoxynucleotidyltransferase (TdT)[1] is a relatively simple polymerase since it does not require template direction, and consequently, all four deoxyribonucleoside triphosphates (dNTPs), with the exception of Mn-dATP, compete for binding to TdT (Bollum, 1974; Modak, 1979). The ability of ribonucleoside triphosphates (rNTPs) to compete with dNTPs for binding to TdT with subsequent enzyme inhibition is a rather unique feature of TdT (Kato et al., 1967; Bhalla et al., 1977; Modak, 1978, 1979). Thus the substrate binding site in this enzyme appeared to be able to bind both dNTPs and rNTPs. Using ultraviolet (UV) light mediated cross-linking of substrate dNTPs and photoaffinity labeling with azido-ATP to TdT, we demonstrated that the 26-kDa subunit of TdT was responsible for binding of both dNTPs and ATP and that the cross-linking of these triphosphates exhibited all the characteristics and requirements that were known to form enzyme–substrate complex (Modak & Gillerman-Cox, 1982; Abraham et al., 1983). While affinity labeling in this manner provides a

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[1] Abbreviations: $Ap_5A$, $P^1,P^5$-bis(5′-adenosyl) pentaphosphate; TdT, terminal deoxynucleotidyltransferase; dNTP, deoxynucleoside triphosphate; $K_i$, dissociation constant; rNTP, ribonucleoside triphosphate; UV, ultraviolet; o-$Ap_5A$, $Ap_5A$ oxidized at the 2′- and 3′-positions of ribose moieties; $Gp_3A$, guanosine(5′)triphospho(5′)adenosine; $Gp_3G$, $P^1,P^3$-bis(5′-guanosyl) triphosphate; $mGp_3G$, 7-methylguanosine(5′)triphospho(5′)guanosine; $Ap_4A$, $P^1,P^4$-bis(5′-adenosyl) tetraphosphate; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; BSA, bovine serum albumin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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convenient means of specifically labeling the substrate binding site in TdT as well as *Escherichia coli* DNA polymerase I (Abraham & Modak, 1984), the extent of labeling is generally low. Further susceptibility of the labeled nucleotide residue in the cross-linked enzyme to procedures used in protein chemistry has made peptide and sequence studies somewhat difficult. As an alternate to this approach, we were searching for chemical modifications, which are usually quantitative, and had tested ATP and bis(5′-adenosyl) tetra- and pentaphosphates ($Ap_4A$ and $Ap_5A$) together with their oxidation (usually aldehyde derivative) products. Earlier, we had noted the sensitivity of TdT and other DNA polymerases to an aldehyde- and phosphate-containing compound, namely, pyridoxal phosphate, and had inferred that a lysine residue was present at the substrate binding site of these enzymes (Modak, 1976; Srivastava & Modak, 1980a,b). Of the various ATP analogues, $Ap_5A$ and its oxidation product (o-$Ap_5A$) appeared to be the most potent inhibitors of TdT catalysis, and a detailed analysis of the inhibitory action of these compound was therefore undertaken. Surprisingly, $Ap_5A$ is found to react at both substrate and primer binding domains in TdT while it has absolutely no reactivity toward other DNA polymerases. Results of this investigation are the subject matter of this paper.

## Materials and Methods

*Materials.* dNTPs, rNTPs, $Gp_3A$, $Gp_3G$, $mGp_3G$, and synthetic primers were obtained from P-L Biochemicals; $Ap_5A$, $Ap_4A$, and ADP morpholidate were the products of Sigma Chemical Co. Purity of commercial preparations of $Ap_4A$ and $Ap_5A$ was adjudged to be ~95% by thin-layer chromatographic analysis. The inhibitory activity was associated with the major UV-absorbing material (i.e., $Ap_4A$ and $Ap_5A$) as judged by the dose–response pattern of eluted material. [$^{32}$P]dTTP was purchased from Amersham Corp. while [$^3$H]ATP and [$^3$H]dGTP were obtained from ICN.

*Enzymes.* Reverse transcriptases from avian myeloblastosis virus and from Rauscher murine leukemia virus (supplied through the courtesy of Cancer Cause and Prevention Division of the National Cancer Institute) were purified by poly(rC)–agarose chromatography as described before (Marcus et al., 1974; Modak & Marcus, 1977). The large fragment of *E. coli* DNA polymerase I was purified from a high-producer *E. coli* (Joyce & Grindley, 1983), and DNA polymerase $\alpha$, from calf thymus, was the kind gift of Dr. S. Wilson of National Cancer Institute. DNA polymerase $\beta$ from the same source was a byproduct of TdT purification (Modak, 1979). The 45-kDa TdT was purified from a chromatin extract of calf thymus to homogeneity by a simplified purification procedure (to be published elsewhere).

*Enzyme Assay.* The standard conditions used for the various enzyme assays have been described as follows: TdT (Modak & Gillerman-Cox, 1982), *E. coli* DNA polymerase I (Abraham & Modak, 1984), reverse transcriptase (Srivastava & Modak, 1980a), DNA polymerase (Modak & Marcus, 1977), and DNA polymerase $\beta$ (Srivastava & Modak, 1983). Activated DNA was used as template primer for all the enzymes, except for reverse transcriptase where poly(rC)·$(dG)_{12-18}$ was the template primer (Modak & Marcus, 1977).

*Preparation of Primer with [$^{32}$P]dTMP at the 3′ End.* A reaction mixture (50 $\mu$L) containing 50 mM Tris-HCl, pH 7.8, 1 mM $MnCl_2$, 10% glycerol, 250 pmol of oligo(pdT)$_6$, 250 pmol of [$\alpha$-$^{32}$P]dTTP (200 $\mu$Ci/nmol), 10 $\mu$g of BSA, 1 mM DTT, and 150 units of TdT (~1 $\mu$g) was incubated at 37 °C for 30 min followed by 2 h of incubation at room temperature. The reaction was terminated by placing the tube in an 80 °C water bath for 2–3 min. The reaction mixture was chilled in ice, adjusted to 0.3 M KCl, and passed through a small column (300 $\mu$L) of oligo(rA)–agarose preequilibrated with 0.3 M KCl solution at 4 °C. After the column was washed with 5 mL of 0.3 M KCl, $^{32}$P-labeled oligo(pdT) was eluted with water. Fractions of 200 $\mu$L were collected and monitored for radioactivity by Cerenkov counting. Active fractions were pooled, lyophilized, and redissolved in 50 $\mu$L of 50 mM Tris-HCl buffer, pH 7.8, and stored at −20 °C.

*Synthesis of [$^3$H]$Ap_5A$.* [$^3$H]$Ap_5A$ was synthesized by a modification of the procedure reported for the preparation of nonradioactive dinucleoside 5′-polyphosphates (Reiss & Moffatt, 1965). [8-$^3$H]$Na_2$ATP (100 nmol, 6 $\mu$Ci/nmol) was passed through a 0.5-mL column of Dowex-50 resin ($H^+$ form). The eluate and waterwash were concentrated to 50 $\mu$L and diluted with 300 $\mu$L of dry pyridine containing 200 nmol of tributylamine. The clear solution was evaporated to dryness under vacuum. The residue was redissolved in dry pyridine and evaporated to dryness. Separately, the 100 nmol of ADP morpholidate, which was completely freed of $H_2O$ by repeated evaporations with dry pyridine, was added to the tributylamine derivative of [$^3$H]ATP in 100 $\mu$L of dry pyridine. The mixture was evaporated to dryness by three additions of 200-$\mu$L portions of dry pyridine and stored in dry pyridine (50 $\mu$L) for 3 days at room temperature to form the condensation product $Ap_5A$. The solvent was then evaporated, and residual pyridine was removed by repeated evaporations with water. The product, $Ap_5A$, thus formed was dissolved in 5 mM ammonium bicarbonate, pH 7.8 (200 $\mu$L), and applied to a 1 × 40 cm column of Sephadex G-10 preequilibrated with 5 mM ammonium bicarbonate and was eluted with the same solution. The portions of the first three-fourths of the radioactive peak containing $Ap_5A$ were pooled, evaporated to dryness, and freed from residual bicarbonate by repeated evaporations with methanol. The product was then dissolved in 70% methanol and checked for purity by thin-layer chromatography with 2 M LiCl as the solvent system. The final radiochemical yield was 27% with respect to [$^3$H]ATP as the starting material. The purity of isolated [$^3$H]$Ap_5A$ was greater than 96%.

*Synthesis of Oxidized $Ap_5A$ (o-$Ap_5A$).* The oxidized $Ap_5A$ was prepared by mixing $Ap_5A$ and sodium periodate in a 1:4 molar ratio as described by Esterbrook-Smith et al. (1976) for the synthesis of dialdehyde ATP. The reaction mixture was applied to a Sephadex G-10 column (2 × 30 cm) equilibrated with $H_2O$. The column was eluted with water, and 0.5-mL fractions were collected. Each fraction was checked for traces of periodate contamination as described before (Srivastava et al., 1983), and those fractions containing o-$Ap_5A$ but free from periodate were pooled, lyophilized, dissolved in 70% methanol, and stored at −20 °C. The oxidized derivative of [$^3$H]$Ap_5A$ was also prepared in a similar manner.

*UV-Mediated Cross-Linking of $^{32}$P-Labeled dNTP Substrate and Primer to TdT Protein.* UV-mediated cross-linking of $^{32}$P-labeled substrate or primer to TdT protein was performed on ice in a reaction mixture containing 20 mM Hepes–KOH, pH 7.8, 0.1 mM 2-mercaptoethanol, 5% glycerol, 1 mM MnCl, 2 $\mu$g of TdT, and 5 $\mu$Ci of [$\alpha$-$^{32}$P]dTTP in a total volume of 50 $\mu$L. Reaction mixtures were incubated at 0 °C for 15 min and then irradiated, as a drop, on Parafilm with high-intensity UV illumination (Modak & Gillerman-Cox, 1982). Cross-linking of [$^{32}$P]oligo(dT) to TdT was carried out in a similar manner (Abraham et al., 1983). The extent of cross-linking was determined by SDS–polyacrylamide gel electrophoresis and autoradiography as described before (Abraham & Modak, 1984).

Table I: Effect of $Ap_5A$ on Activity of Various DNA Polymerases[a]

| DNA polymerases | pmol of [$^3$H]dGMP incorporation/10 min: control | 10 $\mu$M $Ap_5A$ | % inhibition |
|---|---|---|---|
| *E. coli* DNA polymerase I | 153 | 150 | 2 |
| DNA polymerase $\alpha$ (calf thymus) | 159 | 160 | 0 |
| DNA polymerase $\beta$ | 56 | 47 | 16 |
| AMV reverse transcriptase | 64 | 58 | 8 |
| RLV reverse transcriptase | 69 | 59 | 15 |
| terminal deoxynucleotidyltransferase (calf thymus) | | | |
| 32 kDa | 360 | 25 | 93 |
| 45 kDa | 370 | 22 | 94 |

[a] DNA polymerase assays were carried out with activated DNA as a template primer and other standard reaction components while reverse transcriptase assays were performed with poly(rC)·$(dG)_{12-18}$ as a template primer (Modak & Marcus, 1977).

## Results

*General Properties of $Ap_5A$-Mediated Inhibition of TdT.* A comparative study of inhibitory effects of ATP, $Ap_3A$, $Ap_4A$, $Ap_5A$, and $Ap_6A$ on the activity of TdT revealed that $Ap_5A$ was nearly a 10 times more effective inhibitor than the rest of the compounds (data not shown). Furthermore, two different molecular weight species of TdT (32 and 45 kDa) showed very similar sensitivity to above compounds. Of the various adenine dinucleotides mentioned above, $Ap_4A$ is reported to be present in most cells (Rapaport & Zamecnik, 1976). Since $Ap_5A$ is structurally related to the 5' cap structure of eukaryotic messenger RNAs, we also examined effects of other cap analogues such as $Gp_3A$, $Gp_3G$, and $mGp_3G$. All these compounds, when present in relatively high concentrations (50–100 $\mu$M), inhibited TdT (data not shown).

*Effect of $Ap_5A$ on DNA Synthesis Catalyzed by Replicative DNA Polymerases.* Earlier we had reported that ATP was a selective inhibitor of TdT catalysis and had no effect on template-dependent DNA polymerases (Bhalla et al., 1977; Modak, 1979). A similar examination of eukaryotic DNA polymerase $\alpha$ and $\beta$, *E. coli* DNA polymerase I, and reverse transcriptases from avian myeloblastosis virus and Rauscher leukemia virus revealed that $Ap_5A$ had no significant effect on the DNA synthesis catalyzed by replicative polymerases. Both species of TdT (32 and 45 kDa), however, were selectively inactivated by its presence (Table I).

Furthermore, preincubation of DNA polymerases with as high as a 100 $\mu$M concentration of $Ap_5A$ had no inhibitory effect on their activity (data not shown). In contrast, preincubation of TdT with as little as 10 $\mu$M $Ap_5A$ resulted in irreversible inactivation (see below).

*Characterization of $Ap_5A$-Mediated Inhibition of TdT.* Since both prototype 32-kDa and high molecular weight 45-kDa forms showed similar response to $Ap_5A$, we have used a homogeneous preparation of 45-kDa TdT as a test enzyme for all further studies. Preincubation of TdT with $Ap_5A$, regardless of the temperature of incubation (4 or 30 °C), resulted in complete inactivation of enzyme activity (Table II). Addition of excess substrate (400 $\mu$M dGTP) or primer (10 $\mu$g of activated DNA) did not reverse the inactivation. However, when high concentrations of both substrate and primer were added, a partial reversal of enzyme inhibition was apparent (Table II).

To further evaluate the effect of substrate and primer additions on the reversibility of enzyme activity, the protective effect of each component on the enzyme that was presaturated with the other component was examined. Results of this experiment are presented in Table III. It should be pointed out here that substrate or primer presaturation does not protect the enzyme from inactivation when subjected to subsequent exposure to $Ap_5A$. However, addition of the other component (i.e., substrate to primer-saturated enzyme and primer addition to substrate-saturated enzyme) completely reverses the $Ap_5A$ inhibition (Table III). These results strongly suggest that $Ap_5A$ may affect functions of both substrate and primer binding domains. The double-reciprocal plots shown in Figures 1 and 2 represent inhibition kinetics of TdT by $Ap_5A$ with varying concentration of either substrate or primer. A classical competitive type of inhibition is revealed with respect to both the substrate and the primer concentration. The dissociation constants ($K_i$) of enzyme–substrate–$Ap_5A$ complex and of enzyme–primer–$Ap_5A$ complex were calculated from the secondary plots of slopes vs. $Ap_5A$ concentrations as well as from Dixon plots (Dixon, 1953). Values of $K_i$ for both substrate-saturated and primer-saturated enzyme–$Ap_5A$ com-

Table II: Reversibility of Inactivation of TdT by Preincubation with $Ap_5A$[a]

| addition in preincubation mix | pmol of [$^3$H]dGMP incorporation/30 min: control set | $Ap_5A$-treated set | % inhibition |
|---|---|---|---|
| none | 459 | 14 | 97 |
| +400 $\mu$M dGTP | 974 | 77 | 92 |
| +10 $\mu$g of activated DNA | 486 | 53 | 89 |
| +400 $\mu$M dGTP and 10 $\mu$g of activated DNA | 1368 | 533 | 61 |
| +400 $\mu$M dGTP and 5 $\mu$g of oligo(dA) | 1080 | 367 | 66 |

[a] Six micrograms of TdT was preincubated in a final volume of 80 $\mu$L containing standard reaction components, except primer and substrate, in the presence of 10 $\mu$M $Ap_5A$. Preincubations were carried out at 4 °C for 15 min, and the desired quantity of substrate or primer either individually or together was added to the indicated tubes. Upon further incubation for 15 min on ice, 10-$\mu$L aliquots from individual tubes were removed and assayed with the standard reaction mix. The set containing no $Ap_5A$ served as a control for the corresponding $Ap_5A$-containing set.

Table III: Effect of $Ap_5A$ on Substrate- or Primer-Saturated TdT[a]

| preincubation condition | assay conditions (addition to assay mix): primer ($\mu$g) | dGTP ($\mu$M) | % inhibition |
|---|---|---|---|
| TdT + $Ap_5A$ | 1.5 | 20 | 97 |
| | 2.5 | 100 | 67 |
| | 5.0 | 400 | 56 |
| primer saturated, TdT + $Ap_5A$ | 1.5 | 20 | 82 |
| | 1.5 | 100 | 0 |
| | 1.5 | 400 | 0 |
| substrate saturated, TdT + $Ap_5A$ | 1.5 | 20 | 86 |
| | 2.5 | 20 | 20 |
| | 5.0 | 20 | 0 |

[a] Saturation of TdT with substrate or primer was carried out by incubating 4.0 $\mu$g of enzyme with 400 $\mu$M dGTP or 8 $\mu$g of oligo$(dA)_{18}$, respectively, in a reaction mixture (80 $\mu$L) containing 50 mM Tris-HCl, pH 7.8, 1 mM $Mn^{2+}$, 1 mM DTT, and 20 $\mu$g of albumin. A set containing all the components except substrate or primer was also incubated under similar conditions. After 45 min of incubation on ice, 20 $\mu$L of solution containing 2 nmol of $Ap_5A$ was added to each tube and incubated further for 30 min on ice. At the end of the incubation, 10-$\mu$L aliquots were removed from each tube and assayed for enzyme activity with varying final concentrations of substrate and/or primer as indicated in the table. Each set had its own control with similar additions, but without $Ap_5A$, and was assayed simultaneously. The results are expressed as percent inhibition with respect to their controls.

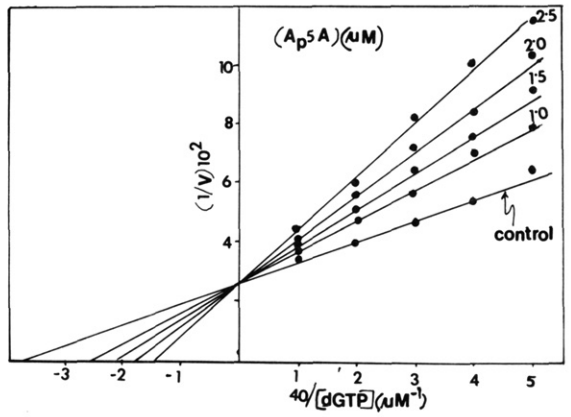


FIGURE 1: Competitive inhibition of the TdT-catalyzed reaction by $Ap_5A$ with varying concentrations of dGTP as the substrate. The concentration of activated DNA as the primer was held constant at 5 $\mu$g/100 mL of reaction volume. The concentration of $Ap_5A$ is as indicated. All the points shown in the figure are the averages of three sets of experiments.

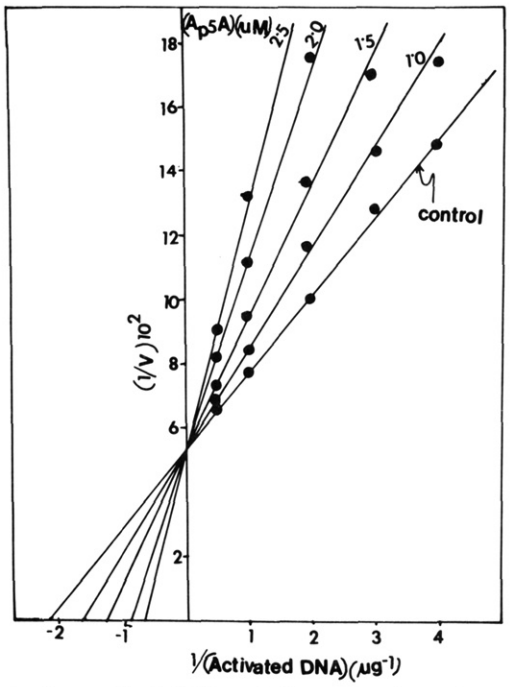


FIGURE 2: Competitive inhibition of TdT-catalyzed reaction by $Ap_5A$ with varying concentrations of activated DNA as the primer. The concentration of dGTP was held constant at 50 $\mu$M. The concentration of $Ap_5A$ is as shown. All the points shown in the figure are the averages of three sets of experiments.

plexes were approximately 1.5 $\mu$M. The sociation constant of the $Ap_5A$-TdT complex in the absenc f primer and substrate was obtained by binding stoichiom ry (see below) and was found to be one-sixth of the above value.

*Effect of $Ap_5A$ on Photoaffinity Labeling of TdT with Substrate and Primer.* The dependence of TdT on the presence of both primer and substrate for protection from $Ap_5A$ inhibition and the kinetic studies showing classical competitive inhibition strongly supported the notion that $Ap_5A$ was reacting at both substrate and primer binding domains. In order to directly assess such an effect of $Ap_5A$, we examined the sensitivity of UV-mediated cross-linking of substrate dNTP and oligomeric DNA primer to TdT in the presence of $Ap_5A$.

Earlier, we had shown that UV-mediated cross-linking of substrate dNTP represents the process of substrate binding in TdT (Modak & Gillerman-Cox, 1982; Abraham et al., 1983) while oligomeric DNA cross-linking to TdT provided

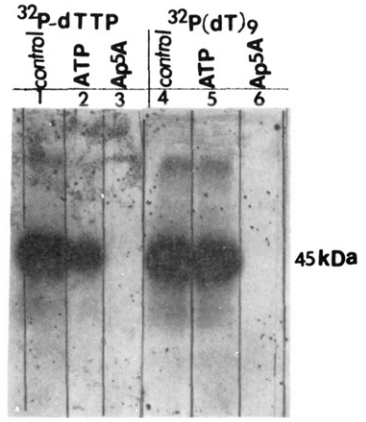


FIGURE 3: Effect of $Ap_5A$ on UV-mediated cross-linking of TdT with the substrate [$^{32}$P]dTTP and with the primer $(dT)_9$. The standard reaction mixture contained 5 $\mu$Ci of [$^{32}$P]dTTP or 0.5 $\mu$Ci of [$^{32}$P]$(dT)_9$ and 2–3 $\mu$g of TdT. ATP or $Ap_5A$ was present in indicated samples at 10 $\mu$M concentration. Irradiation of these mixtures was carried out as described in the text, and the extent of cross-linking of substrate or primer to the enzyme was determined by SDS–polyacrylamide gel electrophoresis followed by autoradiography of the gel.

the qualitative test for the primer binding function. Results shown in Figure 3 clearly show that the cross-linking of both [$^{32}$P]dTTP and 3′ end labeled oligo$(dT)_{6-9}$–[$^{32}$P]dT is totally abolished in the presence of $Ap_5A$. In contrast, ATP, which we have shown to be a competitive inhibitor of dNTP substrate binding (Modak, 1978) and whch can also be cross-linked to TdT (Abraham et al., 1983), does not block primer cross-linking to TdT (Figure 3, lane 5).

*Binding Stoichiometry of $Ap_5A$ to TdT Using Oxidized $Ap_5A$ as a Binding Probe.* In order to determine the stoichiometry of binding of $Ap_5A$ to TdT, it was essential that a stable adduct between enzyme and inhibitor, which can withstand the subsequent analytical protocol, be formed. A chemically reactive oxidation product of $Ap_5A$ (o-$Ap_5A$) seemed capable of forming a covalent complex with TdT. Therefore, the binding estimates using o-$Ap_5A$ could be used in place of those of $Ap_5A$ if the oxidation product of $Ap_5A$ is found to be equally inhibitory to TdT catalysis as the parent compound. Earlier we had noted that the oxidation product of ATP (dialdehyde ATP) was as potent an inhibitor as ATP, and therefore, the oxidation product of $Ap_5A$ was examined for its inhibitory characteristics and compared to those of $Ap_5A$. The results indicated that o-$Ap_5A$-mediated inhibition of TdT is very similar to that observed with its parent compound with respect to kinetic parameters and inhibition characteristics (data not shown).

*Some Properties of o-$Ap_5A$-Mediated Inhibition of TdT.* Oxidation of $Ap_5A$ with periodate treatment most likely results in the oxidation of 2′- and 3′-hydroxyl groups of both ribose moieties of $Ap_5A$ to their aldehyde form. In the case of ATP, a quantitative conversion of ATP to dialdehyde ATP with periodate has been demonstrated (Srivastava et al., 1983), and therefore, oxidation of $Ap_5A$ may be expected to yield a tetraldehyde derivative. When requirements for the inhibitory properties of o-Ap5A were examined, we found that further derivatization of the aldehyde group via reduction or modification results in complete loss of its inhibitory activity. For example, reduction of o-Ap5A with borohydride as well as derivatization of aldehyde to other amino acids (followed by borohydride treatment) results in the loss of its inhibitory activity (Table IV).

*Binding Studies.* Use of o-$Ap_5A$ as an irreversible binding reagent to a specific site depends on the fact that the desired site (or domain) must contain a free amino group such as the

Table IV: Effect of Periodate-Oxidized $Ap_5A$ (Tetraldehyde $Ap_5A$) and Its Derivatives on the Activity of TdT[a]

| compd (10 μM) | % inhibition | compd (10 μM) | % inhibition |
|---|---|---|---|
| $Ap_5A$ | 96 | argininated $Ap_5A$ | 0 |
| o-$Ap_5A$ | 93 | lysinated $Ap_5A$ | 14 |
| o-$Ap_5A$ reduced with borohydride | 0 | glycinated $Ap_5A$ | 14 |

[a] Preparation of o-$Ap_5A$ has already been described under Materials and Methods. The alcohol or amino acid derivatives of o-$Ap_5A$ were generated in situ by incubating 2 nmol of o-$Ap_5A$ with 10 nmol of the respective amino acids in 50 mM Hepes buffer, pH 7.8, in a final volume of 10 μL. After incubation for 45 min on ice, 1 μmol of a freshly prepared solution of borohydride in 10 mM NaOH was added to covalently link the amino acids to the 2′–3′ positions of o-$Ap_5A$. The reaction was allowed to continue for another 20 min on ice. The alcohol derivative of $Ap_5A$ was generated by reducing an aliquot of o-$Ap_5A$ with borohydride. The standard TdT assay mix was then added directly to each of the tubes, and TdT activity was determined. Each set has its own control containing all the components except o-$Ap_5A$ or its derivatives and was treated in a similar manner. The results are expressed as percent of inhibition with respect to their controls.

Table V: Stoichiometry of o-$Ap_5A$ Binding to TdT[a]

| enzyme incubated with | % enzyme bound to o-$Ap_5A$ (or inactivated enzyme) | % enzyme free or unbound to o-$Ap_5A$ | mol of o-$Ap_5A$ bound/mol of enzyme |
|---|---|---|---|
| no additions | 0 | 100 | 0 |
| [$^3$H]o-$Ap_5A$ | | | |
| 4 μM | 79 | 21 | 0.8 |
| 2 μM | 68 | 32 | 0.7 |
| 1 μM | 59 | 41 | 0.58 |
| 0.33 μM | 51 | 49 | 0.51 |
| 0.25 μM | 38 | 62 | 0.42 |
| 0.2 μM | 37 | 63 | 0.37 |

[a] TdT (4.5 μg, 0.1 nmol) in a total volume of 100 μL containing 50 mM Hepes–KOH buffer, pH 7.8, 0.5 mM $Mn^{2+}$, and the indicated concentration of [$^3$H]o-$Ap_5A$ (80 000 cpm/nmol) was incubated for 4 h on ice. A freshly prepared solution of borohydride in 10 mM NaOH was added to a final concentration of 500 μM, and individual tubes were further incubated for 1 h on ice. Ten microliter aliquots from individual tubes were removed for enzyme activity, and the remainder was precipitated directly with 10% TCA. The enzyme activity data were used to determine the percentage of free (active) and o-$Ap_5A$-bound enzyme (inactive species). Trichloroacetic acid insoluble radioactivity present in the enzyme–o-$Ap_5A$ complex was used to determine the mole fraction of $Ap_5A$ bound to enzyme.

amino group of lysine, which will react with aldehyde groups via Schiff base formation. Our earlier studies with pyridoxal 5′-phosphate (Modak, 1976; Srivastava & Modak, 1980) had indicated the presence of lysine at the substrate binding domain of TdT. The Schiff base thus formed between lysine and an aldehyde group could be reduced to covalent linkage via borohydride reduction. Therefore, determination of covalently bound [$^3$H]o-Ap5A provides a quantitation of inhibitor binding to TdT. With this protocol, binding of $Ap_5A$ to TdT at different inhibitor concentrations was determined. The fraction of enzyme inactivated at a given concentration of inhibitor was determined by activity measurement, while incorporation of [$^3$H]o-Ap5A into protein (as judged by TCA precipitation) provided quantitation of the $Ap_5A$ binding to enzyme (Table V).

Kinetic treatment of these data in the form of a double-reciprocal plot [1/(moles of Ap5A bound per mole of enzyme) vs. 1/($Ap_5A$) concentration)] permits the deduction of a dissociation constant for the enzyme–inhibitor complex (in the absence of substrate and primer). The intercept on the abscissa (1/[$Ap_5A$] axis) (Dixon & Webb, 1979) indicates this value to be 0.25 μM (Figure 4). Similarly, the intercept on the vertical axis [1/(moles of $Ap_5A$ bound per mole of enzyme)]

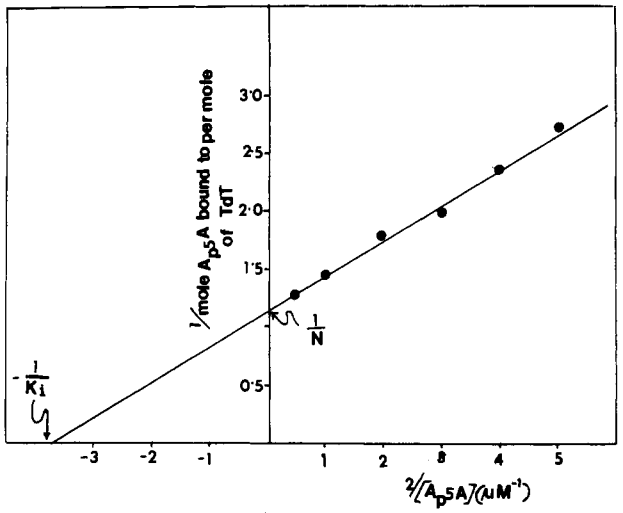


FIGURE 4: Binding of o-$Ap_5A$ by TdT. The data obtained in Table V are plotted according to the equation $1/r = K_i/N + 1/I + 1/N$ (Dixon & Webb, 1979), in which $r$ = the number of moles of o-$Ap_5A$ bound per equivalent of enzyme (45 000 g), $K_i$ = the dissociation constant of the TdT–$Ap_5A$ complex, $N$ = the number of binding sites, and $I$ = the concentration of o-$Ap_5A$.

provides the estimate of the number of binding site(s) for the inhibitor. The stoichiometry of $Ap_5A$–TdT binding was calculated to be 1:1. When an enzyme presaturated with either substrate or primer was used in these experiments, there was no significant change in binding stoichiometry; however, the $K_i$ value of $Ap_5A$ under these conditions increased nearly 6-fold (data not shown), which is consistent with the kinetic constants obtained from Figures 2 and 3. These results strongly suggest that a single molecule of $Ap_5A$ can block both the substrate and primer binding domains in TdT.

## Discussion

To our knowledge this is the first paper that describes an inhibitor that reacts at both substrate and primer binding sites in TdT. Several interesting observations have been made with this unique "dinucleotide"-type inhibitor. Indeed, this synthetic compound, $Ap_5A$, has been used as an ATP analogue in a study of adenylate kinase where it has been shown to react at ATP and AMP binding sites (Lienhard & Secemski, 1973). Since ATP is a substrate binding site directed inhibitor of TdT (Modak, 1978), $Ap_5A$ was expected to act in a manner similar to that of ATP. In fact, $Ap_4A$, another analogue of ATP (Ono et al., 1980), as well as other ATP derivatives such as etheno-ATP (Deibel et al., 1985), azido-ATP (Abraham et al., 1983), and *ara*-ATP (Dicioccio & Srivastava, 1977), has been reported to inhibit TdT by competing with substrate triphosphates.

Thus, the basic requirement for the inhibitory activity of adenine nucleotide appears to be the presence of a minimum of three phosphate groups, while the modifications at the sugar or purine ring do not seem to interfere with the recognition of these compounds by TdT. Of the many adenine nucleotides, ATP and $Ap_4A$ are commonly found cellular components and are therefore likely to play some biological role in the regulation of TdT. We had earlier proposed such a regulatory role for ATP in TdT catalysis (Modak, 1978). Since $Ap_4A$ typically represents a cap structure found in eucaryotic messenger RNAs, we also examined $Ap_3G$, $Gp_3G$ and its methyl analogue, and $Gp_3A$ for their inhibitory activity on TdT catalysis. All these compounds inhibited TdT catalysis, and the degree of inhibition was comparable to that of ATP (data not shown). In contrast, $Ap_5A$-mediated inhibition was nearly an order of

magnitude higher. Its activity toward both primer and substrate binding domains in TdT may be responsible for the high degree of inhibition. It is quite interesting that the presence of only one additional phosphate moiety in $Ap_5A$, compared to $Ap_4A$, appears to be responsible for its recognition at the primer binding domain in TdT.

There is some evidence that substrate and primer binding sites are separate entities in TdT since substrate binding site directed reagents such as pyridoxal phosphate (Srivastava & Modak, 1980), ATP (Figure 4, lane 2), and $Ap_4A$ (Ono et al., 1980) do not affect primer binding. Yet the inactivation of either one of the two domains will result in the inactivation of the catalysis of DNA synthesis. The binding of substrate or primer to enzyme as judged by UV-mediated cross-linking provides the way to determine the precise site of inhibitor action. The sensitivity of both domains of TdT to $Ap_5A$ is inferred not only from the competitive kinetics of inhibitor with respect to both primer and substrate but also from the observation that cross-linking of these two components to TdT is blocked by $Ap_5A$ (Figure 3).

When both domains are available for reaction with inhibitor, the binding of inhibitor to enzyme seems to be very strong as judged by the very low dissociation constant ($K_i$ = 0.25 $\mu$M) whereas when one of the two domains is blocked by presaturating the enzyme with substrate or primer the dissociation constant is increased by 6-fold ($K_i$ = 1.5 $\mu$M). Nonetheless, these constants are still an order of magnitude lower than the $K_m$ for the substrate ($\sim$20 $\mu$M).

Use of the oxidation product of $Ap_5A$ to investigate binding stoichiometry has provided further insight into inhibitor action as well as active site orientation in TdT. The oxidation product of $Ap_5A$ is most likely a tetraaldehyde derivative, since ATP under similar conditions is known to be quantitatively converted to dialdehyde ATP (Srivastava et al., 1983). Our results show that to be an effective inhibitor an aldehyde group must be present in o-$Ap_5A$ since reduction of o-$Ap_5A$ prior to enzyme addition or the derivatization of o-$Ap_5A$, which blocks aldehyde groups, results in loss of its inhibitory activity. Sensitivity of TdT to an aldehyde derivative of $Ap_5A$ strongly suggests that the basic amino acids (such as arginine or lysine) must be present at one or both active site domains. The fact that presaturation with either substrate or primer does not alter binding stoichiometry strongly suggests that basic amino acid residues (capable of forming a Schiff base) are present at both domains.

The results presented here are consistent with the notion that the two domains are in molecular proximity such that two adenosine moieties, which are separated by a string of five phosphoryl groups, can form a Schiff base with basic amino acids present at either one or both domains. Such an assumption will explain (a) the 1:1 stoichiometry of $Ap_5A$–enzyme binding resulting in functional inactivation of both domains and (b) the absence of reduction in the binding stoichiometry when enzyme is presaturated with either primer or substrate (Table V). In conclusion, $Ap_5A$ is a multisite reagent for TdT that could be used to radiolabel both the substrate and the primer binding site in TdT. Prospects of defining the active site domains as well as their geometry thus seem to be attainable.

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