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Biochemistry of Terminal Deoxynucleotidyltransferase: Characterization and Properties of Photoaffinity Labeling with 8-Azidoadenosine 5′-Triphosphate†

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ABSTRACT: We have found that 8-azidoadenosine 5′-triphosphate (8-azido-ATP) and its photolyzed product are competitive inhibitors of terminal deoxynucleotidyltransferase with respect to substrate deoxynucleoside triphosphates. A detailed characterization of the inhibitory effect of 8-azido-ATP revealed that its mechanism of inhibition is identical with that reported for ATP [Modak, M. J. (1978) *Biochemistry 17*, 3116–3120]. Photoactivation of the azido-ATP–enzyme complex results in the covalent binding of azido-ATP to terminal deoxynucleotidyltransferase. No significant incorporation of prephotolyzed azido-ATP or unsubstituted ATP into enzyme protein is noted when complexes of these nucleotides with enzyme were exposed to identical photoactivation conditions. The majority of incorporated analogue was associated with the 26 000-dalton subunit of terminal deoxynucleotidyltransferase. Incorporation of azido-ATP was further found to be absolutely dependent on the presence of a divalent cation. All four deoxyribonucleoside triphosphates as well as ATP and guanosine 5′-triphosphate were able to compete with azido-ATP during the incorporation experiment as judged by the competitive reduction in the cross-linking of the photoaffinity analogue to terminal deoxynucleotidyltransferase (TDT). In addition, substrate binding site directed inhibitors, pyrophosphate and pyridoxal 5′-phosphate, effectively blocked the incorporation of azido-ATP into enzyme protein, while several other inhibitors of TDT catalysis, namely, ethylenediaminetetraacetic acid, α,α'-dipyridyl, 1,10-phenanthroline, *p*-(chloromercuri)benzoate, Rose Bengal, and the presence of 0.5 M KCl, influenced the cross-linking reaction to varying degrees. A tryptic peptide analysis of the azido-ATP-labeled 26K subunit of TDT revealed that the majority of the incorporated photoaffinity analogue was present in two peptides.

All known DNA polymerases including terminal deoxynucleotidyltransferase (TDT)[1] bind substrate deoxynucleoside triphosphate (dNTP) in their metal chelate forms and subsequently incorporate the bound substrate into a DNA chain with concomitant release of pyrophosphate (Bollum, 1974). In addition, most of these enzymes also catalyze reversal of the synthetic reaction, namely, PP_i exchange and pyrophosphorolysis reactions (Kornberg, 1980; Srivastava & Modak, 1980a,b). TDT, however, is unique among DNA polymerases in that it does not obey template direction, and, consequently, all four dNTPs with the exception of Mn-dATP readily 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 another unique feature of this enzyme (Kato et al., 1967; Bhalla et al., 1977; Modak, 1978, 1979). Thus, the substrate binding site in this enzyme appeared to be amenable to binding both dNTPs and rNTPs. With the development and availability of photoaffinity analogues of rNTPs in general and ATP and GTP in particular (Czarnecki et al., 1979; Geahlen & Haley, 1979; Khatoon et al., 1983) in recent years, it appeared plausible that specific photoaffinity labeling of the triphosphate binding site in TDT could be accomplished provided that the photoaffinity analogue has inhibitory properties identical with those of its parent compound. This report describes the results of the characterization of TDT inhibition by the photoaffinity analogue of ATP, 8-azido-ATP, and shows that it is identical with that inhibition caused by ATP as judged by kinetic as well as physical binding studies. Furthermore, we find that covalent binding of 8-azido-ATP to TDT may be achieved via photoactivation. Thus, the requirements and restrictions for covalent binding

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[1] Abbreviations: TDT, terminal deoxynucleotidyltransferase; dNTP, deoxynucleoside triphosphate; rNTP, ribonucleoside triphosphate; PP_i, sodium pyrophosphate; TLC, thin-layer chromatography; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-pressure liquid chromatography; SDS, sodium dodecyl sulfate; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; UV, ultraviolet; Tris, tris(hydroxymethyl)aminomethane; *p*CMB, *p*-(chloromercuri)benzoate.

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as well as the site of binding of azido-ATP in TDT could be determined.

Materials and Methods

Materials. Both nonradioactive and azido[γ-^{32}P]ATP used in the standardization of labeling protocols were prepared in Dr. Haley's laboratory at the University of Wyoming essentially as described by Czarnecki et al. (1979). However, this compound has now become commercially available and was also purchased from New England Nuclear, Inc. Photolyzed azido-ATP was prepared by exposing the Hepes-buffered solution of azido-ATP to UV light for a period of 5 min. Complete photolysis was achieved by this treatment as judged by the shift in the absorption spectrum (Potter & Haley, 1982). We have occasionally observed that commercially obtained, HPLC-purified batches of azido[^{32}P]ATP contain a reactive impurity of higher molecular weight species (presumably a tetramer of ATP) which can also bind to the enzyme protein. This results in the appearance of a minor doublet of the 26K band upon SDS gel electrophoresis and autoradiography (data not shown). [α-^{32}P]dTTP was purchased from New England Nuclear, while nonradioactive dNTPs, ATP, and synthetic primers were the products of P-L Biochemicals, Inc. Rose Bengal, *p*-(chloromercuri)benzoate, 1,10-phenanthroline, 2,9-dimethyl-1,10-phenanthroline, and α,α'-dipyridyl were obtained from Sigma. TPCK–trypsin and thin-layer chromatographic plates were purchased from E. Merck while all other reagents were of analytical grade. A mineral lamp (model R52), manufactured by Ultraviolet Products Inc., was obtained through Fisher Scientific Products Co. This is a high-intensity UV illumination lamp (1300 μW/cm^2 at a distance of 15 cm at 254 nm).

Enzymes. TDT from calf thymus glands was purified essentially as described by Chang & Bollum (1971) with minor modifications. The purified enzyme displayed two subunits corresponding to molecular weights of 26000 and 10000 on a SDS–polyacrylamide gel. For comparison, we also included a batch of TDT kindly provided by Dr. Ratliff of Los Alamos Scientific Laboratories, Los Alamos, NM, which displayed an identical subunit composition and gave identical results.

Enzyme Assays. The standard assay conditions used for TDT determinations have been described (Modak, 1978, 1979).

Photoactivation of the Azido-ATP–TDT Complex by UV Treatment and SDS–Polyacrylamide Gel Electrophoresis. The irradiation mixture in a final volume of 50 μL contained 50 mM Tris-HCl or Hepes–KOH buffers (pH 7.8), 1 mM $MnCl_2$, 5 μCi of azido[γ-^{32}P]ATP (specific activity 15–30 Ci/mmol) in place of substrate dNTPs, and 20% glycerol. Each reaction contained 3–30 pmol of purified TDT, and photoactivation of the reaction mixture was carried out by exposing the reaction mixture to a UV source at a distance of 10 cm for a period of 5 min, unless stated otherwise. The time of exposure and the distance from the UV source should be carefully standardized since different UV sources may have different outputs of photoenergy. Relatively longer exposure times or shorter distances from the UV source are required for the cross-linking of unsubstituted dNTPs or prephotolyzed analogues to TDT (Modak & Gillerman-Cox, 1982). The reaction temperature was maintained at or below 4 °C during photooxidation. It is essential that reducing agents such as dithiothreitol or mercaptoethanol be absent from the irradiation mixtures containing photoaffinity analogues, since azido groups are rapidly reduced by these compounds. Pretreatment of enzymes with inhibitors, whenever needed, was carried out prior to UV exposure, and inhibitors were present in the irradiation mixture during photoactivation. Upon completion of the desired UV exposure, 10 μL each of 10 mM ATP and protein solubilizing solution (containing 1% SDS, 2 mM EDTA, 25 mM Tris-HCl, pH 7.8, and 20 mg/mL bromophenol blue) was added to each sample. The samples were briefly boiled in a water bath and loaded onto a slab gel consisting of 12% polyacrylamide with an overlay of 4% polyacrylamide. Electrophoresis was carried out in a Tris–glycine buffer system essentially as described by Laemmli (1970). The gel was then washed several times with 50% methanol containing 50 mM sodium phosphate (pH 5) and exposed to X-ray film for autoradiography.

Iodination of TDT. Iodination of homogeneous TDT was carried out with ^{125}I by using the lactoperoxidase procedure essentially as described previously (Modak & Marcus, 1977). Separation of 26K and 10K subunits from ^{125}I-labeled TDT was achieved by SDS–polyacrylamide gel electrophoresis.

Tryptic Digestion and Separation of Tryptic Peptides by High-Voltage Electrophoresis and Chromatography. The radiolabeled 26K subunit of TDT was excised from the polyacrylamide gel and washed successively with sodium phosphate buffer (pH 5) containing 50% methanol and then with 80% methanol in order to remove SDS from the gel. The gel pieces were then washed with 20 mM ammonium bicarbonate solution for 2 h, dried under vacuum, and resuspended in freshly prepared 50 mM ammonium bicarbonate buffer (pH 8.5) and 10 μg/mL TPCK–trypsin. The digestion was continued for 2 h by gentle shaking at room temperature, and another aliquot of trypsin (20 μg) was added. After the solution stood for 2 h, supernatant fluid from the gel pieces was collected by centrifugation and lyophilized until all the ammonium bicarbonate was removed. Recovery of approximately 60–70% of the labeled protein is achieved in the final digest by using this procedure (Planck et al., 1980). The lyophilized digest was dissolved in 10 μL of electrophoresis buffer consisting of pyridine–acetic acid–water (1:1:48), pH 4.8, spotted on a 20 cm × 20 cm cellulose plate, and electrophoresed in the same buffer system (Michl, 1958) at 50 V/cm for 1 h. A chromatographic resolution of peptides in the second dimension was carried out in a solvent system consisting of 1-butanol–pyridine–acetic acid–water (75:50:15:60) as described by Light & Smith (1962). Subsequent autoradiography of the celluose plate revealed the location of the labeled peptides. We have noted that exposure of photoaffinity-labeled samples to solutions with a pH of lower than 2 causes a significant loss of label from azido-ATP-bound enzyme, presumably due to a depurination type of reaction. It is probably for this reason that we could not visualize cyanogen bromide mediated fragments of azido-ATP-labeled 26K subunit. Similarly, when conventional electrophoretic separation of tryptic digests that utilized buffer systems at pH 1.9 was attempted, significant loss of label was consistently encountered. A strong radioactivity-containing spot that migrated toward the anode, presumably as a result of depurination of the cross-linked labeled azido-ATP residue, was consistently observed. Further support for the observation that a strong radioactivity-containing spot migrating toward the anode is a breakdown product of nucleotide is obtained by the fact that when free azido-ATP was subjected to acidic pH treatment an identical spot was noted upon electrophoresis (data not shown).

Results

Properties and Characterization of 8-Azido-ATP-Mediated Inhibition of TDT. A typical dose–response pattern of azido-ATP concentration and the activity of TDT using oligomeric $(dA)_{12-18}$ and activated DNA as primers are shown in

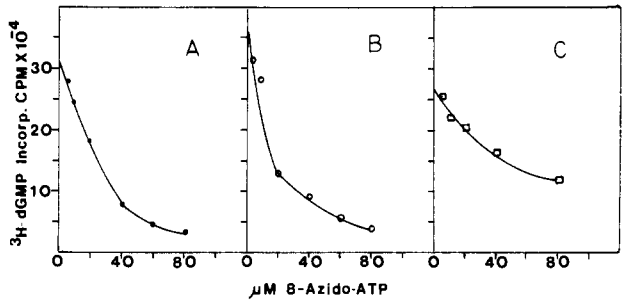

FIGURE 1: Dose–response pattern of 8-azido-ATP on the catalysis by TDT by using (A) $(dA)_{12-18}$ as a primer, (B) activated DNA as a primer, or (C) oligo(dA) primer with Mg^{2+} (5 mM) as the effective divalent cation in place of Mn^{2+}. The standard reaction mixtures contained the desired primer and the indicated amount of 8-azido-ATP, and the amount of incorporation of [^{3}H]dGTP in 30 min was used as an assay for enzyme activity.

Table I: Effect of Increasing Primer, Enzyme, and Substrate Concentrations on the Inhibition of TDT by 8-Azido-ATP[a]

component	concn	activity (pmol/30 s) control	activity (pmol/30 s) with 8-azido-ATP	% inhibition
$(dA)_{12-18}$	0.14 μg	71	18	75
$(dA)_{12-18}$	0.35 μg	149	28	81
$(dA)_{12-18}$	0.7 μg	157	37	76
$(dA)_{12-18}$	1.4 μg	146	36	74
enzyme	2 ng	181	30	83
enzyme	5 ng	322	61	81
enzyme	10 ng	630	108	83
enzyme	20 ng	1270	286	77
dGTP	50 μM	330	115	65
dGTP	100 μM	360	210	42
dGTP	200 μM	350	280	20
dGTP	400 μM	360	270	25
dGTP + 100 μM dATP		4	2.7	32

[a] The inhibitory effect of 8-azido-ATP (20 μM) on the catalysis of poly(dG) synthesis was determined as a function of increasing concentrations of the desired component listed above. The assays were carried out by using dGTP as a labeled precursor which was present at 20 μM concentration in all the reaction mixes. Addition of excess dGTP decreases the specific activity of [^{3}H]dGTP for both control and test sets.

Figure 1. At equimolar concentrations of the substrate dGTP and the inhibitor azido-ATP, approximately 60–70% inhibition persists irrespective of the synthetic or natural DNA primer used. The inhibitory effect of azido-ATP is more pronounced in the presence of Mn^{2+} (Figure 1A,B) than in the presence of Mg^{2+} (Figure 1C). Photolyzed azido-ATP (azido-ATP that has been exposed to UV light) was also as effective an inhibitor of TDT catalysis as intact azido-ATP (data not shown). The apparent K_i both for 8-azido-ATP and for its photolyzed product was calculated to be in the range of 15–20 μM. In order to gain some insight into the probable site of azido-ATP action, we assessed the inhibitory effect of azido-ATP as a function of increasing enzyme, primer, and substrate concentrations. A 10-fold change in primer as well as enzyme concentration did not alter the degree of inhibition, but increases in the substrate concentrations significantly protected the enzyme from azido-ATP inhibition (Table I). A kinetic study of the inhibition mediated by azido-ATP confirmed that the inhibition was competitive with respect to substrate dGTP but was noncompetitive with respect to primer concentrations (data not shown). Furthermore, the addition of azido-ATP

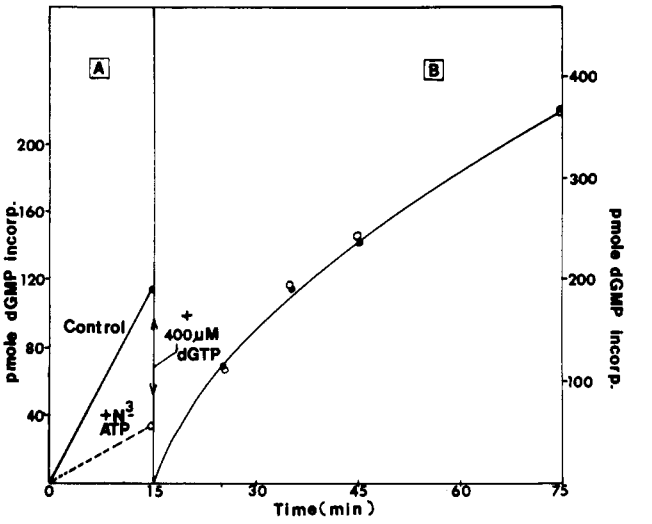

FIGURE 2: Reversal of the inhibitory effect of azido-ATP by addition of excess dGTP. In this experiment, two sets of standard reaction mixtures (except for [^{3}H]dGTP) were incubated in the presence and absence (control) of 20 μM azido-ATP for 15 min. 400 μM dGTP together with 5 μCi of [^{3}H]dGTP was then added to each set, and 0.1-mL aliquots were removed at the desired times to determine the acid-insoluble radioactive product formation. The left panel shows the effect of 20 μM azido-ATP on TDT catalysis during a 15-min preincubation (determined by addition of 5 μCi of [^{3}H]dGTP to an aliquot from each set). The right panel represents the rates of polymerization of azido-ATP-treated and control sets upon addition of 400 μM dGTP.

prior to or after the initiation of catalysis produced identical degrees of inhibition (data not shown) similar to that found for ATP (Modak, 1978). The inhibition of TDT mediated by rNTPs had been thought to be an effect of modification of the primer terminus due to the incorporation of the ribo residue by TDT (Bollum, 1974). However, we clearly demonstrated that ATP-mediated inhibition of TDT is due to blockage of the substrate binding process by ATP and did not result from modification of the primer terminus via ATP incorporation (Modak, 1978). A similar mechanism appears to account for the azido-ATP-mediated TDT inhibition as judged by the ready reversibility of inhibition by the addition of excess dGTP as detailed in Figure 2.

Covalent Binding of Azido[γ-^{32}P]ATP to TDT. The above-described kinetic studies strongly suggested that a specific binding site for ATP or its photoaffinity analogue may indeed exist in TDT. Since photoaffinity compounds are readily activated by exposure to ultraviolet light energy, prospects of establishing covalent linkage between azido-ATP and its acceptor site in TDT appeared quite feasible. We therefore investigated the conditions required for the optimal covalent binding of azido-ATP to TDT. Since TDT from calf thymus is a two-subunit enzyme, it was also interesting to determine if one or both subunits were able to link azido-ATP. In cross-linking studies, the use of high specific activity azido[γ-^{32}P]ATP is required to visualize the relatively low number of azido-ATP molecules that are incorporated into TDT protein. Therefore, the actual concentration of azido[^{32}P]ATP used in these studies was well below its K_i value. A typical time course of the covalent binding of azido-ATP to TDT as a function of UV irradiation (photoactivation) time is shown in Figure 3. The inset in the figure is an autoradiogram of the SDS–polyacrylamide gel containing aliquots of photoaffinity-labeled TDT at various times. It is clear that the majority of azido-ATP incorporation into TDT occurred in the 26K subunit and that a plateau of covalent modification is reached in about 8 min. Approximately 2% of the 26K subunit

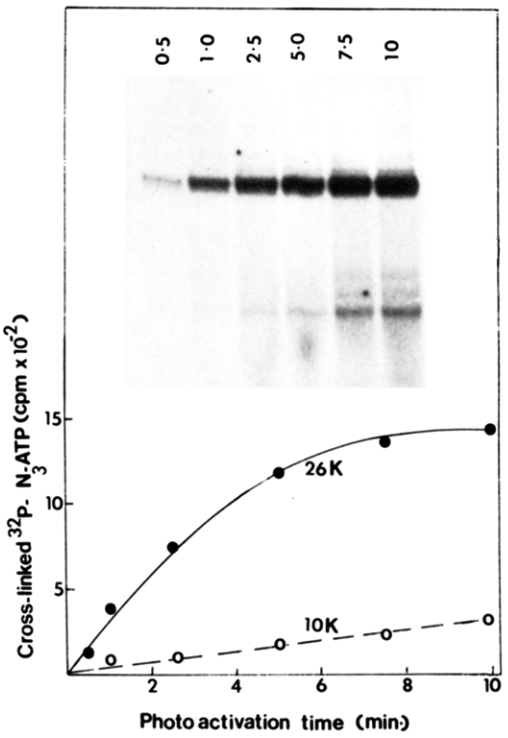

FIGURE 3: Time course of cross-linking of azido[γ-^{32}P]ATP to TDT. The standard photoactivation mixture, in a final volume of 100 μL, contained 1 μg of TDT and 5 μCi of azido[^{32}P]ATP (final concentration 5 μM) together with buffer, divalent cation, and glycerol as described under Materials and Methods. The mixture was exposed to ultraviolet light, and 15-μL aliquots were removed at desired time intervals and stored on ice. At the end, all the aliquots were mixed with 10 μL each of 10 mM ATP and protein solubilizing solution, briefly boiled, and subjected to electrophoresis on a 12% SDS–polyacrylamide gel. The gel was then washed successively with 50 mM sodium phosphate (pH 5) buffer, with the same buffer containing 50% methanol, and finally with 80% methanol in phosphate buffer. The washed gel was subsequently wrapped in a plastic sheet, exposed to X-ray film, and autoradiographed. The positions of the 26K and 10K subunits of TDT on the gel were localized with the help of the autoradiogram, the bands were excised, and the radioactivity contained in the individual band was determined directly in a scintillation counter by Cerenkov counting. The results are graphically presented. It is important to point out that the radioactivity present in each lane is shown as counts per minute. In the absence of scintillation fluid, methanol soaked polyacrylamide gel counts with an efficiency of 60%.

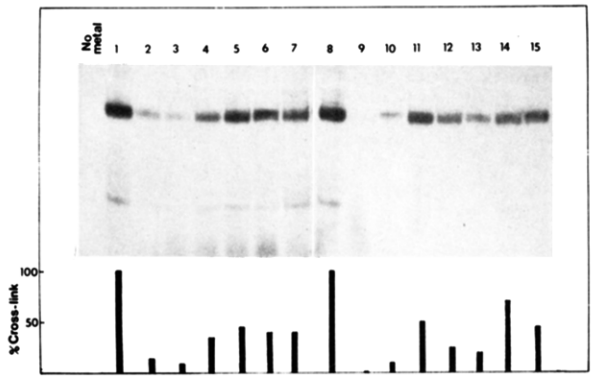

FIGURE 4: Effect of various inhibitors and deoxynucleoside triphosphates on the extent of cross-linking of azido[γ-^{32}P]ATP to TDT. The effect of pretreatment of TDT with various known inhibitors of the polymerization reaction as well as the presence of ATP and other dNTPs on the ability of TDT to cross-link to azido-ATP was determined by using 0.1 μg of TDT that was treated for 15 min on ice with the desired inhibitor at the concentrations noted below in a standard reaction mixture in a final volume of 45 μL. The final concentration of Mn^{2+} in this experiment was increased to 2 mM in order to not deplete the reaction of essential divalent cations via chelation through some of the inhibitors. Five microcuries of azido[^{32}P]ATP (effective final concentration 5 μM) was then added to the above mixture, and cross-linking was effected by exposure to UV light for 5 min. Each sample was then electrophoresed on 12% SDS–polyacrylamide gels and autoradiographed as described under Materials and Methods. Lanes 1–15 represent analyses performed on two slab gels, each containing an independent control (lanes 1 and 8). Other lanes represent the effect of pretreatment or addition of (2) 200 μM pyrophosphate, (3) 200 μM pyridoxal phosphate, (4) 20 μM ATP, (5) 20 μM dATP, (6) 20 μM dTTP, (7) 20 μM Rose Bengal, (8) control, (9) 1 mM EDTA, (10) 1 mM 1,10-phenanthroline, (11) 1 mM 2,9-dimethyl-1,10-phenanthroline, (12) 1 mM α,α'-dipyridyl, (13) 1 mM *p*-(chloromercuri)benzoic acid, (14) 0.5 μg of $(dA)_{12-18}$, or (15) 0.5 M KCl. The reaction mixture lacking divalent cation is labeled appropriately. The bar graph is constructed by counting the radioactivity in individual 26K bands, with the control representing 100% cross-linking (approximately 1900 cpm). Incorporation of azido-ATP in the 10K subunit (control sample) amounted to only 15% of that found in its 26K counterpart, and upon various inhibitor treatments, some decrease in the labeling of this subunit is also noted. However, no reliable quantitation of this pattern could be obtained due to low quantities of radioactivity incorporated.

is modified under these conditions. We have noted that there is some variation within different batches of commercially obtained radiolabeled azido-ATP and incorporation as high as 4%, under identical conditions, has been achieved with some batches. A certain degree of cross-linking of azido-ATP to the 10K subunit of TDT is also noted. However, labeling of this subunit amounted to about 10–20% of that found in the 26K subunit. The fact that prephotolyzed azido[γ-^{32}P]ATP is poorly incorporated into TDT protein, under identical labeling conditions (data not shown), further indicates that the covalent incorporation of azido-ATP into TDT occurs via activation of the azido group. Similarly, cross-linking of unsubstituted α-^{32}P- and γ-^{32}P-labeled ATP as well as [α-^{32}P]-dTTP appeared to occur to a minor extent (approximately 10% of that found for azido-ATP) under the photoactivation conditions used for azido-ATP labeling (data not shown). Longer exposures or reducing the distance between the UV source and the reaction mixtures containing unsubstituted nucleotide–TDT complexes, however, permits successful cross-linkings of nucleotides to TDT, and a detailed analysis of this phenomenon has already appeared (Modak & Gillerman-Cox, 1982). It is important to point out that photoactivation energy via a UV source is absolutely required for the formation of covalent complexes between the photoaffinity analogue and TDT.

Factors Influencing the Incorporation of Azido-ATP into TDT. While the presence of a divalent cation is obligatory for the expression of the catalytic activity of TDT (Bollum, 1974), the presence of a reducing agent such as dithiothreitol or mercaptoethanol (Bollum, 1974) and potassium ions (Chirpich, 1977) have been reported to enhance the catalytic activity. We therefore examined the influence of these components on the incorporation of azido[γ-^{32}P]ATP into TDT protein and found that covalent incorporation of azido-ATP into TDT required the presence of a divalent cation (Figure 4) and that Mn^{2+} is twice as effective as Mg^{2+} or Co^{2+} in supporting the cross-linking reactions (data not shown). Addition of reducing agents such as dithiothreitol or mercaptoethanol was found to render the photoaffinity analogue quite ineffective, presumably because the azido group of the photoaffinity probe is rapidly reduced by these compounds (data not shown). In fact, pretreatment of TDT with SH-directed reagents does not significantly block the incorporation of azido-ATP (see below), implying nonrequirement of SH groups in TDT protein for this reaction. Addition of KCl, ranging in concentration from 10 to 100 mM, had very little influence on the extent of cross-linking of azido-ATP to TDT (data not shown).

Table II: Nature of the Inactivation of TDT Activity by Azido-ATP under Photoreactive Conditions[a]

preincubation conditions	pmol of dGMP incorporated/15 min
enzyme + 20 μM dGTP	650
enzyme + 400 μM azido-ATP + 20 μM dGTP	1
enzyme + 1 mM dGTP	830
enzyme + 400 μM azido-ATP + 1 mM dGTP	620
enzyme + 400 μM azido-ATP + UV + 20 μM dGTP	1
enzyme + 400 μM azido-ATP + UV followed by 1 mM dGTP	540

[a] A reaction mixture containing 20 ng of TDT and 400 μM azido-ATP was exposed to ultraviolet light on ice under conditions where complete photolysis of azido-ATP occurs. Inactivation of enzyme activity was measured by using the standard assay with activated DNA as a primer. Reversibility of inactivation was measured by the addition of 1 mM dGTP to azido-ATP-treated samples.

Stoichiometry of Covalent Binding of Azido-ATP to TDT. When limiting concentrations of azido-ATP were used in the photoaffinity labeling of TDT, approximately 2% of the total 26K subunits present were modified (Figure 3). Since the concentration of azido-ATP used in this experiment was 3–4-fold below the K_i value for azido-ATP (as determined in kinetic experiments), we expected a significant increase in the extent of photoaffinity labeling upon increasing the concentration of azido[γ-^{32}P]ATP in reaction mixtures. Based on this expectation, we determined the extent of TDT labeling by using 20 and 50 μM concentrations of azido[γ-^{32}P]ATP. The results indicated that a net increase of approximately 1.7- and 2.5-fold occurred as a result of the above increases in the concentrations of the radiolabeled probe (data not shown). Simultaneous enzyme inactivation studies, using as high a concentration as 400 μM nonradioactive azido-ATP, revealed that the permanent inactivation of TDT resulting from the covalent incorporation of azido-ATP into TDT upon UV exposure amounted to only about 10–20% (Table II) of the control. It therefore appears that incorporation of the photoaffinity analogue of ATP into TDT obtained by the procedures used may be rather low. This observation also explains that the fractional modification of the total enzyme, observed in the time course experiment, was not due to a limiting concentration of radiolabeled probe.

Specificity of Incorporation of Azido-ATP into TDT. Kinetic studies with ATP (Modak, 1978) and azido-ATP had clearly indicated that the process of substrate binding in TDT was affected by these inhibitors in a competitive manner. It was therefore reasonable to assume that the process of incorporation of a photoaffinity analogue of ATP may also exhibit requirements and limitations that have been known to exist for the process of substrate binding, such as the requirement for the presence of a divalent cation to form a nucleotide chelate (Bollum, 1974). This requirement is also observed in the cross-linking of azido-ATP to TDT since no cross-linking of azido-ATP to TDT occurs in the absence of a metal ion (Figure 4). We therefore conclude that ATP (substituted or unsubstituted) exerts its inhibitory effect via its metal chelate form. Reduction in the incorporation of azido-ATP into TDT, upon inclusion of any one of the four dNTPs or rNTPs as well as other substrate binding site directed inhibitors, also confirmed the expected competitive nature of binding of azido-ATP to TDT (Figure 4; data for some nucleotides not shown). It is also interesting that the incorporation of azido-ATP into TDT, similar to the binding of substrate to enzyme, is independent of the binding of primer to enzyme.

Effect of Inhibitors of TDT Catalysis on the Cross-Linking of Azido-ATP to TDT. The two known substrate site directed inhibitors, namely, pyrophosphate and pyridoxal 5′-phosphate (Bollum, 1974; Deibel & Coleman, 1980; Modak, 1976; Srivastava & Modak, 1979, 1980b), severely reduce cross-linking of azido-ATP to TDT (Figure 4). However, the effects of several other inhibitors of TDT catalysis on the binding of azido-ATP to TDT (as judged by its incorporation into TDT) are not known. For example, *p*-(chloromercuri)benzoic acid is a sulfhydryl group specific reagent that can block the reactive sulfhydryl groups present on TDT. Similarly, 1,10-phenanthroline, a zinc-directed chelator, and other metal chelators such as EDTA and α,α′-dipyridyl have been shown to inhibit the catalytic activity of TDT (Chang & Bollum, 1970; Deibel & Coleman, 1980). Rose Bengal is another potent inhibitor of DNA polymerases, including TDT (Srivastava & Modak, 1982, 1983), whose site of action is not known. The effect of the various inhibitors listed above on the incorporation of azido-ATP into TDT is shown in Figure 4. Results indicate that the inhibitors that react with a specific structural component of the enzyme do exert a moderate to severe inhibitory effect, implying the importance of the involved component in the binding of azido-ATP. Thus, the metal chelators EDTA and 1,10-phenanthroline (but not its nonchelating analogue) severely inhibit incorporation of azido-ATP into TDT, while the sulfhydryl group specific reagent, *p*CMB, the presence of high salt concentration, and treatment of TDT with Rose Bengal have only a moderate inhibitory effect. The general patterns of the inhibitor effects observed above are similar to those found for direct cross-linking of unsubstituted dNTP to TDT (Modak & Gillerman-Cox, 1982).

Analysis of the Azido-ATP Binding Site in TDT. In spite of the fact that a relatively small percentage of input TDT is covalently modified by azido-ATP, the amount of ^{32}P label introduced into the 26K subunit appeared sufficient to determine the specificity of binding through peptide analysis. We therefore processed the ^{32}P-labeled 26K subunit for tryptic peptide analysis (see Materials and Methods). The tryptic peptides were separated on a thin-layer cellulose plate by electrophoresis in the first dimension and chromatography in the second (Planck et al., 1980). For comparison, an undigested ^{32}P-labeled 26K subunit (which underwent a mock tryptic digestion protocol) and also digests prepared from an ^{125}I-labeled 26K subunit preparation were processed simultaneously. The results of these analyses are presented in the form of an autoradiogram of a thin-layer plate (Figure 5). While the ^{125}I-labeled 26K subunit exhibited 8–10 distinct peptides (Figure 5, panel A), the azido[^{32}P]ATP-labeled sample displayed several radioactive spots marked a–f (Figure 5, panel B). Examination of the undigested (control) ^{32}P-labeled 26K subunit revealed that spots d–f do not represent true peptides and are probably the breakdown products generated during the tryptic digestion protocol (namely, extensive exposure to ammonium bicarbonate and repeated lyophilizations). The quantitation of radioactivity present in peptide spots a–c revealed that approximately 90% of the radioactivity is associated with peptides a and b. It is also curious that the ^{125}I-labeled digest contains two peptides that exhibit migration patterns similar to ^{32}P-labeled peptides a and b. The relationship between these two sets of peptides, if any, is not clear at present.

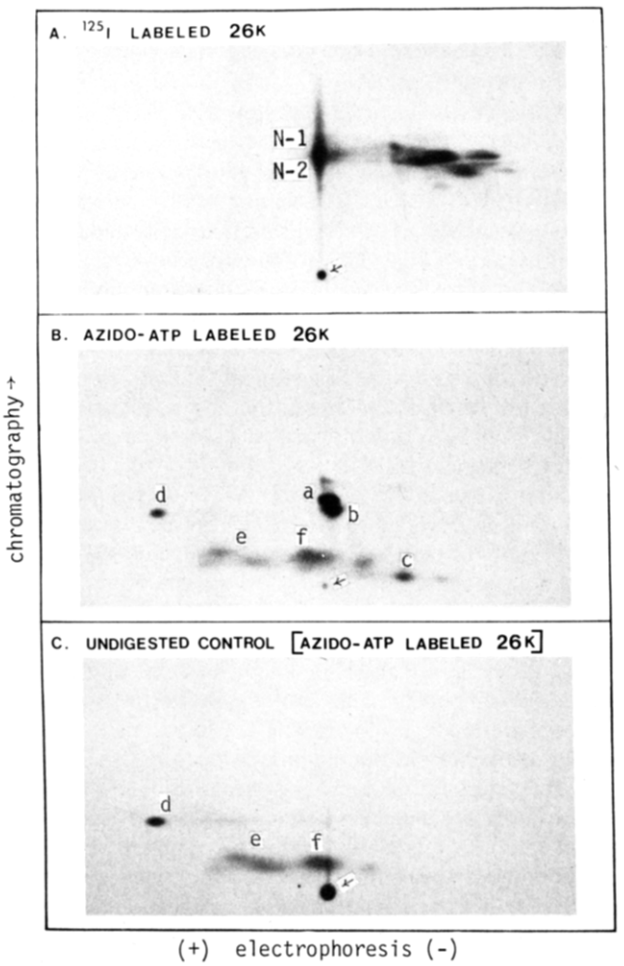

FIGURE 5: Tryptic peptide analyses of ^{125}I-labeled and azido[^{32}P]-ATP-labeled 26K subunit of TDT. ^{125}I-Labeled and photoaffinity-labeled TDT was subjected to SDS–polyacrylamide gel electrophoresis, and the gel piece containing 26K subunit was excised and processed for tryptic digestion as described under Materials and Methods. An undigested but identically treated control for azido-ATP-labeled enzyme was also included (panel C). The point of sample application is shown by the arrow in the autoradiogram. N-1 and N-2 markings in panel A indicate neutral peptides (as judged by their nonmigration during electrophoresis) present in ^{125}I-labeled digests, while peptides marked a and b are their counterparts in the digest prepared from azido[^{32}P]ATP-labeled 26K subunit. Spots d–f appear to be breakdown products (compare panels B and C) and are presumably not associated with peptides. Polarity and the direction of electrophoresis are indicated by appropriate signs.

Lability of Azido[γ-^{32}P]ATP-Labeled Protein. Traces of phosphatases in the reagents and exposure to acidic media during processing may result in some loss of the [^{32}P]phosphate moiety. Traces of phosphatases are likely contaminants in many proteolytic enzymes, while exposure to acidic media may occur during electrophoresis and chromatography of peptides. The loss of label through depurination-type reactions under acidic conditions may be minimized by replacing strongly acidic buffer systems (the conventionally used pH is 1.9) with somewhat milder systems, such as pH 4.8 (Michl, 1958), which do not affect the peptide separation. The sugar phosphate moiety of the nucleotide and other breakdown products, released as a result of a depurination type of reaction, appear to migrate toward the anode during electrophoresis and could be identified by simultaneous processing of an undigested control as seen in Figure 5 (panel C).

Discussion

In this report, we have shown that a photoaffinity analogue of ATP is a potent inhibitor of TDT catalysis and that it could be covalently linked to the 26K subunit of TDT. Using synthetic as well as natural DNA primers and with time course and kinetic studies of the inhibitory effect of azido-ATP, we noted a remarkable similarity between the mechanisms of inhibition of TDT by the photoaffinity analogue and its parent compound ATP (Modak, 1978). The fact that photolyzed azido-ATP is as effective an inhibitor as its nonphotolyzed counterpart implies that a distortion of the five-membered ring of purine nucleotide does not affect the process of ATP recognition and binding by TDT. We have ruled out the possibility that azido-ATP-mediated inhibition was due to its incorporation into the primer terminus, for only increases in the substrate but not the primer concentration in the inhibited reaction provided a prompt reversal of inhibition (Table I). Furthermore, azido-ATP-mediated inhibition of the ongoing synthetic reaction could be readily reversed by addition of excess substrate dGTP (Figure 2). Kinetic studies have clearly shown a competitive mode of inhibition with respect to substrate dNTP. We therefore conclude that azido-ATP-mediated inhibition primarily affects the substrate binding by TDT.

It is quite interesting to note that the triphosphate moiety of nucleotide structure appears to be the major recognition signal for binding by TDT. Modifications or distortions of heterocyclic ring structures, as in azido-ATP, photolyzed azido-ATP, or etheno-dATP (Liu & Deibel, 1983), or modifications in the sugar moiety, such as dialdehyde and dialcohol derivatives of ATP (Srivastava et al., 1983) or *ara*-ATP (Dicioccio & Srivastava, 1977), do not affect binding of such nucleotides as judged by their strong inhibitory effect on TDT catalysis.

Since photoaffinity analogues can be covalently linked at or near the site of their binding, specific labeling of that site may be achieved by using an appropriate radiolabeled compound. A time course study of photactivation time vs. covalent binding of γ-^{32}P-labeled azido-ATP to TDT revealed that the major site of incorporation of the photoaffinity analogue was contained in the 26K subunit of TDT and that a plateau in the incorporation was reached in 7–8 min of UV exposure. Under these conditions, approximately 2% of the input enzyme (or 26K subunit) was modified. However, even when the concentration of azido[^{32}P]ATP was increased 10-fold, from 5 to 50 μM, the incorporation of azido-ATP into 26K subunit amounted to only about 5%. The relatively low covalent modification of TDT by the photoaffinity analogue, therefore, may be attributed to an unfavorable local environment around the binding site of azido-ATP on the 26K subunit and not to suboptimal concentrations of radiolabeled probe. Further support for this contention is obtained from the enzyme inactivation studies discussed below. Azido-ATP and its photolyzed analogue strongly but reversibly inhibit TDT catalysis. For example, a totally inhibited TDT reaction due to the presence of 400 μM azido-ATP may be almost completely reversed by the addition of 1 mM substrate dGTP (Table II). However, when an azido-ATP–TDT complex was first exposed to photoactivation conditions, covalent incorporation of azido-ATP into TDT may be expected to block the subsequent reversal of inhibition by the addition of 1 mM dGTP. Results of such an experiment, however, indicated that permanent inactivation of TDT upon photoactivation of azido-ATP–TDT complexes amounted to only about 10–15% (Table II). These results strongly suggest that the covalent modification of TDT with the photoaffinity probe, under the conditions used, is rather limited.

In spite of the restricted modification of TDT that could be achieved, the process of modification appeared to exhibit

all the characteristics that one would expect from binding of a competitive inhibitor. The ability of all four dNTPs as well as ATP to reduce the incorporation of azido-ATP together with the sensitivity of this process to known substrate binding site directed inhibitors strongly indicates that incorporation of azido-ATP occurs at a specific site and that this site may directly (as a substrate binding site) or indirectly (as an effector site) be involved in the catalysis. We favor the former possibility for the following reasons. (1) Under specific assay conditions, and in spite of greatly reduced rates of catalysis, ATP and other rNTPs have been shown to serve as substrates in TDT-catalyzed ribonucleotide addition reactions [Roychoudhury et al., 1976). (2) In our investigation of cross-linking of unsubstituted dNTPs to TDT, ATP has been found to block the substrate cross-linking to TDT in a competitive manner (Modak & Gillerman-Cox, 1982). (3) Incorporation of both azido-ATP and substrate dNTPs into TDT protein requires the presence of a divalent cation and is sensitive to substrate binding site directed inhibitors. (4) Cross-linking of substrate as well as the photoaffinity analogue to TDT has exhibited similar sensitivities to a variety of inhibitors that react with a specific structural component of TDT. (5) The presence of a primer in the irradiation mixture does not interfere in the cross-linkings of dNTPs or the photoaffinity analogue. To identify the peptide(s) that are involved in the covalent binding of azido-ATP, we carried out a two-dimensional fingerprinting analysis of the tryptic digest. Identification of true peptide spots from the affinity-labeled 26K subunit was somewhat difficult since some unidentified breakdown products were invariably generated during the tryptic digestion and separation protocols. An undigested but identically treated azido-ATP-labeled 26K subunit clearly aided in the unambiguous identification of two peptides that were the major site of binding of azido-ATP (Figure 5, panels B and C). The site specificity of azido-ATP binding is also apparent from the fact that of the several structural peptides derived from ^{125}I-labeled 26K subunit, only two peptides contained the azido-ATP binding site. These two peptides do not migrate during electrophoresis and therefore may be classified as neutral peptides. However, the possibility that a triphosphate moiety present on these peptides may have retarded their migration in an electric field has not been ruled out. The appearance of two neutral peptides in the ^{125}I-labeled tryptic digest of the 26K subunit, with migration properties similar to those of two peptides containing azido-ATP, is quite intriguing. The eventual amino acid composition and sequence determination of the two ^{32}P-labeled and the two ^{125}I-labeled neutral peptides is needed to ascertain whether these two sets of peptides are related to each other.

In conclusion, this study has demonstrated that photoaffinity labeling of the ATP binding site in TDT can be successfully accomplished with a high degree of specificity. Many properties of photoaffinity labeling and direct cross-linking of unsubstituted dNTPs to TDT (Modak & Gillerman-Cox, 1982) are quite comparable. However, the use of a photoaffinity reagent has a distinct advantage in that no damage to the enzyme protein due to UV exposure is encountered in the photoaffinity labeling protocol. This technology should permit elucidation of the amino acid sequence and the geometry of the ATP binding site and clarify its relationship to the substrate binding site in TDT.

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Registry No. TdT, 9027-67-2; 8-azido-ATP, 53696-59-6; pCMB, 59-85-8; PP_i, 7722-88-5; dGTP, 2564-35-4; dATP, 1927-31-7; dTTP, 365-08-2; ATP, 56-65-5; EDTA, 60-00-4; KCl, 7447-40-7; pyridoxal phosphate, 54-47-7; Rose Bengal, 11121-48-5; 1,10-phenanthroline, 66-71-7; 2,9-dimethyl-1,10-phenanthroline, 484-11-7; α,α'-dipyridyl, 366-18-7.

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