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Photoaffinity Labeling of Terminal Deoxynucleotidyl Transferase. 1. Active Site Directed Interactions with 8-Azido-2′-deoxyadenosine 5′-Triphosphate†

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ABSTRACT: A photoaffinity analogue of dATP, 8-azido-2′-deoxyadenosine 5′-triphosphate (8-azido-dATP), was used to probe the nucleotide binding site of the non-template-directed DNA polymerase terminal deoxynucleotidyl transferase (EC 2.7.7.31). The Mg^{2+} form of 8-azido-dATP was shown to be an efficient enzyme substrate with a K_m of 53 μM. Loss of enzyme activity occurred during UV photolysis only in the presence of 8-azido-dATP. At saturation (120 μM 8-azido-dATP), 54% of the protein molecules were modified as determined by inhibition of enzyme activity. Kinetic analysis of enzyme inhibition induced by photoincorporation of 8-azido-dATP indicated an apparent K_d of $\sim$38 μM. Addition of 2 mM dATP to 120 μM 8-azido-dATP resulted in greater than 90% protection from photoinduced loss of enzyme activity. In contrast, no protection was observed with the addition of 2 mM dAMP. Enzyme inactivation was directly correlated with incorporation of radiolabeled 8-azido-dATP into the protein and UV-induced destruction of the azido group. Photoincorporation of 8-azido-dATP into terminal transferase was reduced by all purine and pyrimidine deoxynucleoside triphosphates of which dGTP was the most effective. The α and β polypeptides of calf terminal transferase were specifically photolabeled by [γ-^{32}P]-8-azido-dATP, and both polypeptides were equally protected by all four deoxynucleoside triphosphates. This suggests that the nucleotide binding domain involves components from both polypeptides.

Terminal deoxynucleotidyl transferase (deoxynucleoside triphosphate:oligodeoxynucleotide deoxynucleotidyl transferase, EC 2.7.7.31) (terminal transferase)[1] is a DNA-polymerizing enzyme independent of DNA templates. Terminal transferase catalyzes a linear condensation polymerization reaction in vitro. Like all known DNA polymerases, terminal transferase requires an acceptor 3′-OH group (Kato et al., 1967) and metal chelate forms of (deoxy)nucleoside triphosphates. Unlike other polymerases, terminal transferase has a specialized tissue localization in prelymphocytes of mammalian bone marrow and thymus and in their differentiation-arrested counterparts (Coleman et al., 1974; Chang, 1971; McCaffrey et al., 1973). The expression of terminal transferase is correlated with the insertion of extra random nucleotides between the V, D, and J regions that accompany the rearrangement of immunoglobulin heavy chain genes or T cell receptor β genes during lymphoid ontogeny (Alt et al., 1982; Desiderio et al., 1984). Terminal transferase is synthesized as a catalytically active single polypeptide (60 kDa); however, lower M_r forms can be detected in crude tissue extracts and during enzyme purification (Chang et al., 1982; Deibel et al., 1983). These other two catalytically active enzyme forms result from specific

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[1] Abbreviations: terminal transferase, terminal deoxynucleotidyl transferase; 8-azido-dATP, 8-azido-2′-deoxyadenosine 5′-triphosphate; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; SDS, sodium dodecyl sulfate; BBOT, 2,5-bis(5-*tert*-butylbenzoxazo-2-yl)-thiophene.

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cleavage to produce a 44-kDa or a two-polypeptide enzyme consisting of 11-kDa (α) and 33-kDa (β) polypeptides, for which the amino acid sequence is known (Peterson et al., 1984, 1985; Beach et al., 1985). We have used the two-polypeptide enzyme species to initiate studies to define the nucleotide substrate binding region of terminal transferase.

Photoactive nucleotide analogues containing aryl azides have been used to characterize nucleotide binding domains in many different proteins (Haley, 1983; Colman, 1983). The azido group, when exposed to a short duration of low-energy UV light, releases N_2 and forms the electron-deficient nitrene, which will react to form a covalent bond with moieties rich in electrons. Because of the high reactivity of the aryl nitrene, these compounds tend to form covalent bonds in an indiscriminate manner, and therefore, the probability of obtaining cross-linking is high since labeling is not dependent upon the presence of a particular amino acid side chain in the active site.

Several types of amino acids have been identified as active site residues in a variety of nucleotide binding proteins by use of 8-azidoadenosine-containing nucleotides. Recent studies have identified lysine and tryptophan (Hegyi et al., 1986); tyrosine, proline, leucine, and isoleucine (Garin et al., 1986); and threonine (Palczewski & Kochman, 1987) as residues that can form isolable cross-links with aryl azide nucleotide photoprobes. Because of the wide variety of amino acids identified with aryl azide photoprobes, we felt this group of analogues would be well suited to study the active site of terminal transferase.

The purpose of the present study was to characterize the binding of 8-azido-dATP to calf terminal transferase $\alpha\beta$ polypeptides and to optimize the conditions that favor specific photoincorporation at the enzyme catalytic site. In the accompanying paper (Evans et al., 1989), two active site peptides were isolated and identified.

Materials and Methods

Materials

[^{3}H]dATP was from New England Nuclear (Boston, MA). Oligo[d(pA_{50})] was synthesized in our laboratory (Coleman & Hutton, 1981). Nonradioactive 8-azido-dATP and [α-^{32}P]-8-azido-dATP were synthesized according to Czarnecki et al. (1979) and were generous gifts from Dr. Boyd Haley (Lucille P. Markey Cancer Center, University of Kentucky). The purity of [α-^{32}P]-8-azido-dATP was assessed spectrophotometrically as well as by chromatographic techniques (Czarnecki et al., 1979) and was >97%. [γ-^{32}P]-8-Azido-dATP and -ATP were synthesized according to a standard procedure (Glynn & Chappell, 1964). Other biochemicals were of reagent grade or higher from commercial sources.

Enzyme. Terminal transferase was purified in our laboratory as described by Deibel and Coleman (1980). The purified enzyme exhibited a specific activity of greater than 100 000 units/mg. The enzyme consisted of two polypeptides corresponding to molecular weights of 33 000 (β) and 11 000 (α) on SDS–polyacrylamide gels. To determine enzyme concentration, the protein was hydrolyzed in 6 N HCl with the addition of a crystal of phenol for 24 h at 110 °C followed by amino acid analysis according to Beach et al. (1985).

Methods

Enzyme Assays. The assay conditions for calf thymus terminal transferase have been described in detail (Coleman, 1977). Briefly, the components of the assay were 1 mM [^{3}H]dATP (15–50 cpm/pmol), 0.01 mM d(pA_{50}), 0.2 M potassium cacodylate, pH 7.5, 1 mM 2-mercaptoethanol, and 8 mM $MgCl_2$. When [α-^{32}P]-8-azido-dATP was employed as a substrate, similar reaction components were used. Reactions were incubated at 35 °C; aliquots were removed and analyzed as described previously (Deibel & Coleman, 1980).

Photoaffinity Labeling. The irradiation mixture was in a final volume of 50 μL in white porcelain spot plates (Coors) or in polystyrene 96-well microtest assay plates (Becton Dickinson Labware) at 4 °C. Each photolabeling reaction contained 50 mM Bis-Tris-HCl, pH 7.1, 25 mM NaCl, and 8 mM $MgCl_2$, along with various concentrations of [γ-^{32}P]-8-azido-dATP and 40–50 pmol of homogeneous terminal transferase. Each reaction was photolyzed for 1 min with a 302-nm UV lamp (Spectronics Corp., Model EB-28, 1500 $\mu W/cm^2$ at 15 cm) from a distance of 0.5 cm. All minus photolysis controls were exposed to the same conditions, and treatment was as with the photolyzed samples.

SDS–Polyacrylamide Gel Electrophoresis. Reactions were quenched with 25 μL of protein-solubilizing mix containing 200 mM dithiothreitol, 75 mM 3-mercaptopropionic acid, 40% glycerol, 2.5% SDS, 50 μg/mL pyronin-Y, and 125 mM Tris-HCl buffer, pH 8.0. Electrophoresis in SDS gels was carried out in 12% polyacrylamide gels (Laemmli, 1970). Following electrophoresis, gels were rinsed with destaining solution (5% methanol, 7.5% acetic acid) followed by distilled water to ensure removal of excess radioactivity. After staining with Coomassie Blue and extensive destaining, the gels were dried and autoradiographed. The dried gels were sliced, and the gel slices corresponding to radioactive protein bands were counted in a liquid scintillation cocktail containing 0.4% BBOT in toluene.

Quantification of Incorporated Photoprobe. A filter precipitation method was also used to quantitate the amount of 8-azido-dATP incorporated by covalent modification of the protein. The reactions (described above) containing various concentrations of photoprobe were photolyzed as previously described. After UV photolysis, dATP was added to a final concentration of 2 mM. Aliquots were then removed and spotted on glass fiber filters (Whatman, GF/C). The filters were dipped in 5% trichloroacetic acid at 4 °C for 10 s, placed on a vacuum block, and washed with 25 mL of cold 5% trichloroacetic acid. Following batch washing with cold ethanol and diethyl ether, the filters were air-dried and counted by liquid scintillation counting. To quantitate background radioactivity on the filters not due to photoincorporation of [γ-^{32}P]-8-azido-dATP, control reactions were performed. Each control was treated as before except the reactions were photolyzed without terminal transferase present. After UV photolysis the terminal transferase was added as well as dATP to a final concentration of 2 mM. Aliquots were then spotted and analyzed as before.

Results

The photoanalogue 8-azido-dATP was tested as a competitive inhibitor and enzyme substrate. Polymerization of [^{3}H]dATP in the presence of varying concentrations of 8-azido-dATP revealed a pattern consistent with competitive inhibition with a K_i of 50 μM (data not shown). [α-^{32}P]-8-Azido-dATP was tested as an enzyme substrate under optimal assay conditions (high enzyme concentration and inorganic pyrophosphatase to remove PP_i, an inhibitory reaction product). Greater than 40% of the substrate analogue was converted to polymer product, while under similar conditions the polymerization of dATP was about 90% and that of dGTP was 57%. The specific activity of the enzyme with azido-dATP as substrate was 33 000 units/mg as compared with a specific activity of 100 000 units/mg with dATP as the substrate. A

Table I: Characterization of Photoincorporation of 8-Azido-dATP into Terminal Transferase

conditions[a]	inhibition of enzyme activity (%)	enzyme ^{32}P labeled (%)[b]	enzyme active sites ^{32}P labeled (%)[c]
(1) standard (1 μM enzyme)	55	65	21
(2) no 8-azido-dATP, $^{+}h\nu$ for 3 min	<5		
(3) 160 μM prephotolyzed 8-azido-dATP, no $h\nu$	<5		
(4) 160 μM prephotolyzed 8-azido-dATP	<5	nd[d]	
(5) 5 μM enzyme	45	62	21
(6) 7.5 μM enzyme	45	63	19
(7) 10 μM enzyme	40		
(8) 15 μM enzyme	39		
(9) second addition of 8-azido-dATP	62		
(10) third addition of 8-azido-dATP	68		

[a] Conditions for the standard reaction were 50 mM Bis-Tris, 25 mM NaCl, pH 7.1 at 4 °C, 8 mM $MgCl_2$, 120 μM 8-azido-ATP or 120 μM [γ-^{32}P]-8-azido-dATP, and 1 μM terminal transferase in a final volume of 50 μL. All reactions were photolyzed for 1 min unless otherwise indicated. [b] Terminal transferase molecules labeled is moles of incorporated [γ-^{32}P]-8-azido-dATP expressed as a mole percent of terminal transferase molecules present (determined by filter precipitation as under Methods). [c] Active sites labeled with ^{32}P is the difference in moles of [γ-^{32}P]-8-azido-dATP incorporated between the control reaction and the same reaction with 2 mM dATP present, expressed as a mole percent of terminal transferase (determined by filter precipitation as under Methods). [d] nd = no detectabe ^{32}P incorporation.

Lineweaver–Burk kinetic analysis of 8-azido-dATP polymerization yielded a K_m of 53 μM (x intercept = −0.019 μM^{-1}, y intercept = 2.2×10^{-5} h/nmol, slope = 1.2). In contrast, the K_m of dATP is 140 μM (Deibel et al., 1985). These results indicated that 8-azido-dATP interacts with the enzyme active site.

Comparison of several different buffers (Tris-HCl, Hepes, phosphate, cacodylate, and Bis-Tris) at concentrations ranging from 10 to 100 mM showed that 50 mM Bis-Tris gave optimum levels of photoincorporation of [γ-^{32}P]-8-azido-dATP. Conditions for modification of terminal transferase photolyzed in the presence of 8-azido-dATP (120 μM) were first evaluated by measuring enzyme activity remaining after a 1-min exposure to 302-nm light. Terminal transferase exhibited a broad pH optimum between pH 6.8 and pH 7.7 in Bis-Tris (data not shown) for enzyme inactivation by UV photolysis in the presence of 8-azido-dATP. We selected pH 7.1 as the standard labeling condition (Table I) at which 120 μM photoprobe gave 55% inhibition of terminal transferase activity. In order to assess the effectiveness of 8-azido-dATP as an active site probe, several control reactions were carried out. Photolysis for an extended time (3 min) under the standard conditions caused no loss of enzyme activity (Table I). Prephotolyzed 8-azido-dATP was not a potent inhibitor of terminal transferase activity (Table I), suggesting that the loss of activity observed with the standard condition was not due to an inhibitory photolysis reaction product. As an additional control, prephotolyzed [γ-^{32}P]-8-azido-dATP or unlabeled probe was added to the reaction containing terminal transferase (Table I). This latter experiment indicated that the postphotolysis product of [γ-^{32}P]-8-azido-dATP was not cross-linked or inhibitory to terminal transferase even if rephotolyzed in the presence of the enzyme.

Our initial experiments indicated that interaction of the photoanalogue with the enzyme was extremely sensitive to buffers and salt concentration. In order to develop a condition for labeling sufficient quantities of the enzyme for peptide isolation, the effect of terminal transferase concentration on both enzyme inactivation and incorporation of radioactive label was investigated. As shown in Table I, between 1 and 7.5 μM protein, photoinactivation in the presence of 8-azido-dATP resulted in the loss of about 50% enzyme activity. Increasing the concentration of protein to 15 μM decreased inhibition of enzyme activity by about 16%. An alternate strategy for increasing the percentage of protein molecules modified by incorporation of probe was to photolabel the protein multiple times. A modest increase in enzyme inhibition was observed when two or three sequential photolabeling reactions were carried out after addition of fresh 8-azido-dATP to the reaction (Table I). The rather moderate increases in enzyme inhibition on the second and third photolabeling event were due to the competitive interference of the photolyzed probe from the previous photolysis event (data not shown).

The correlation between inhibition of enzyme activity and covalent modification of the protein was determined by precipitating terminal transferase after photolabeling with [γ-^{32}P]-8-azido-dATP and comparing the precipitated radioactivity with that of an unphotolyzed sample or with a reaction photolyzed before the terminal transferase addition. Using this technique, we found that the standard labeling condition (120 μM probe) gave an incorporation of 32.5 pmol of [γ-^{32}P]-8-azido-dATP into 50 pmol of enzyme. Thus, 65% of the terminal transferase molecules were apparently modified (Table I). In order to determine the extent of modification at the active site, the same experiment was performed in the presence of 2 mM dATP or 2 mM dAMP. With dATP in the reaction, labeling by [γ-^{32}P]-8-azido-dATP decreased by 32%. This indicated that 32% of the 32.5 pmol of protein-bound label was at the nucleoside triphosphate binding site. In contrast, with 2 mM dAMP in the photolabeling reaction no decrease in protein modification was observed. Identical results were obtained at 5 and 7.5 μM enzyme concentrations. These data suggested that at saturating probe concentrations a minimum of 21% of the terminal transferase molecules in solution was photolabeled at the active site (Table I). This result was unanticipated since 55% enzyme inactivation was observed following photolabeling. As indicated earlier, Table I shows that the additional loss in enzyme activity over the amount of active site labeling was not due to the effect of UV light alone, to the effect of an inhibitor produced by UV light, or to the effect of the photolyzed probe in the presence of UV light.

In order to correlate the rate of loss of enzyme activity with activation of the photoanalogue by UV light, the time course of [γ-^{32}P]-8-azido-dATP incorporation into terminal transferase was monitored and compared to the time course of enzyme inactivation as well as with the change in UV absorbance of the photoanalogue (Figure 1). All three events occurred on the same time scale with the time for half-maximal photoincorporation ($t_{1/2}$) of the analogue being ~5 s. The decrease in 8-azido-dATP UV absorbance caused by photolysis is due to loss of the azido group (Potter & Haley, 1982). Therefore, the correlation of these three events shows that photoincorporation of [γ-^{32}P]-8-azido-dATP and the UV-induced loss of terminal transferase activity are due solely to the activation of 8-azido-dATP by UV light.

To determine if the loss of terminal transferase activity due to photolabeling with 8-azido-dATP was saturable, reactions were prepared with increasing concentrations of 8-azido-dATP and assayed with and without photolysis. Data in Figure 2

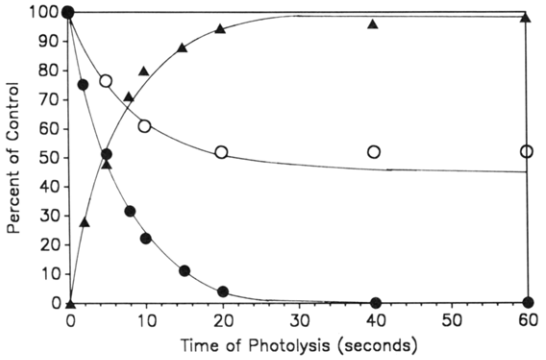


FIGURE 1: Effect of UV photolysis on photoincorporation of 8-azido-dATP into terminal transferase. All reactions included standard conditions (Table I) and 160 μM 8-azido-dATP. The terminal transferase activity remaining after UV photolysis (○) was determined from aliquots removed from the photolabeling reaction after the indicated photolysis time. The remaining terminal transferase activity was determined as described under Methods and is expressed as a percent of the unphotolyzed control. When [γ-^{32}P]-8-azido-dATP was employed, incorporation of ^{32}P into terminal transferase (▲) was quantitated as described under Methods and is expressed as a percent of maximum photoinsertion at 60 s of photolysis. Loss of 8-azido-dATP UV absorbance (●) was monitored by determining the optical density of a reaction (without terminal transferase) at the λ_{max} of 8-azido-dATP (280 nm) after the indicated photolysis time. Loss of 8-azido-dATP absorbance is expressed as a percent of the maximum loss of UV absorbance (1.17 OD units) at 60 s.

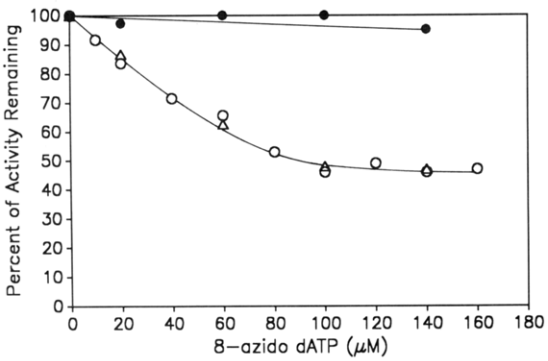


FIGURE 2: Loss of terminal transferase enzyme activity caused by photolabeling with 8-azido-dATP. Standard reaction conditions were used (Table I) with varying concentrations of 8-azido-dATP. After a 1-min UV photolysis, aliquots were removed and assayed for terminal transferase activity as described under Methods. The remaining activity for all reactions is expressed as a percent of the unphotolyzed control. The control reaction (○) included only 8-azido-dATP whereas the protection reactions included either 2 mM dATP (●) or 2 mM dAMP (△).

illustrate that loss of enzyme activity was maximal (54%) at ~120 μM 8-azido-dATP. A K_d of ~38 μM for 8-azido-dATP under the standard photolabeling conditions was estimated from the curve. This value is similar to the K_m (53 μM) that was determined for 8-azido-dATP. When 2 mM dATP was included in the reaction mixtures, greater than 90% protection was observed at saturating concentrations of 8-azido-dATP. However, 2 mM dAMP was an ineffective competitor of photolabeling (Figure 2).

In order to determine the relative binding affinities of 8-azido-dATP and a natural substrate (dATP) to the enzyme, a competition experiment was performed with constant radiolabeled probe (20 μM) and increasing dATP concentrations. The photolyzed reaction products were subjected to electrophoresis in SDS–polyacrylamide gels. Following autoradiography to locate radiolabeled α and β polypeptides, gel slices were excised and counted by liquid scintillation counting. As seen in Figure 3 the protection of 8-azido-dATP photolabeling was nearly identical on the α and β polypeptides, which

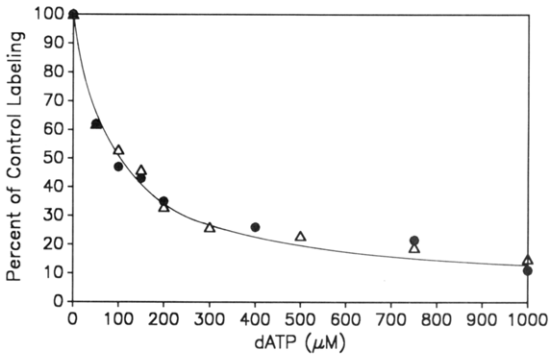
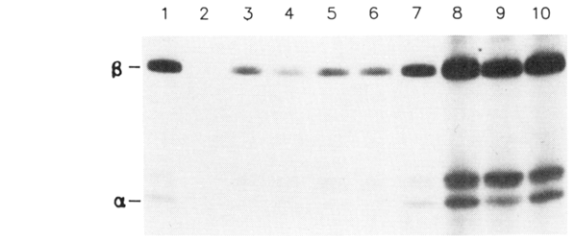


FIGURE 3: Protection of [γ-^{32}P]-8-azido-dATP photoincorporation into terminal transferase by dATP. Standard reaction conditions were used (Table I) with 20 μM [γ-^{32}P]-8-azido-dATP (specific activity 9.6 mCi/μmol) and varying concentrations of dATP. The amount of [γ-^{32}P]-8-azido-dATP photoincorporation into the α (△) and β (●) polypeptides of terminal transferase was determined by liquid scintillation counting of radioactive bands excised from an SDS–polyacrylamide gel and is expressed as a percent of the control reaction lacking dATP. The line represents a curve that fits the data for the β polypeptide.



UV hν	+	–	+	+	+	+	+	+	+	+
COMPETITOR	NONE	NONE	dATP	dGTP	dCTP	dTTP	dAMP	NONE	dATP	dAMP
% of CONTROL – β	100	–	29	15	31	28	94	100	63	90
% of CONTROL – α	100	–	29	8	32	33	100	100	67	100

FIGURE 4: Protection of [γ-^{32}P]-8-azido-dATP photoincorporation into terminal transferase by various competing nucleotides. Figure 4 is an autoradiogram of an SDS–polyacrylamide gel showing the level of [γ-^{32}P]-8-azido-dATP (specific activity 11.6 mCi/μmol) incorporated with 20 (lanes 1–7) or 120 μM [γ-^{32}P]-8-azido-dATP (lanes 8–10). The positions corresponding to the α and β polypeptides of terminal transferase are shown as well as the percent of control photolabeling on each polypeptide. In protection reactions all competing nucleotides were present at a final concentration of 500 μM (lanes 1–7) or 2 mM (lanes 8–10).

strongly suggests the involvement of both polypeptides in formation of the active site. The data indicate that a 4-fold excess of dATP over 8-azido-dATP was required for a 50% reduction in photolabeling of terminal transferase, suggesting a 4-fold higher binding affinity for 8-azido-dATP over dATP. The K_d of 8-azido-dATP binding to terminal transferase was found to be 38 μM (Figure 2). A 4-fold increase would yield a K_d for dATP of about 150 μM. This value agrees very well with the K_m of dATP (141 ± 9 μM) that has been determined from substrate saturation experiments (Deibel et al., 1985).

The photolabeling of α and β polypeptides of terminal transferase with [γ-^{32}P]-8-azido-dATP is shown in the autoradiogram of an SDS–polyacrylamide gel (Figure 4). Photoincorporation into the protein was totally dependent upon UV photolysis (lanes 1 and 2). Protection of photoincorporation at subsaturating photoprobe concentrations (20 μM azido-dATP) occurred with all deoxynucleoside triphosphates but not with dAMP (lanes 3–7). Furthermore, when the protection from photolabeling of each polypeptide was compared, it was clear that both the pattern and extent of labeling inhibition were similar for each polypeptide. These data suggest that components of each polypeptide contribute to the formation of the active site. When 120 μM [γ-^{32}P]-8-azido-dATP was used in the photolabeling reaction, protection by

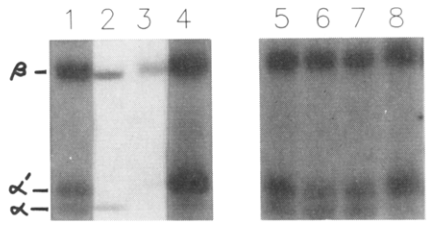


FIGURE 5: Effect of photolabeling on migration of terminal transferase α polypeptide during SDS–polyacrylamide gel electrophoresis. Lanes 1–4 show the effect of 50 (lanes 1 and 2) and 200 μM (lanes 3 and 4) [γ-^{32}P]-8-azido-dATP on the autoradiogram (lanes 1 and 4) and Coomassie blue staining profile (lanes 2 and 3) of an SDS gel. Lanes 5 and 6 show the results when 100 (lane 5) or 30 μM (lane 6) [γ-^{32}P]-8-azido-ATP was used to label terminal transferase. Lane 7 shows the autoradiogram profile when a control reaction using 40 μM [γ-^{32}P]-8-azido-dATP was used while lane 8 shows the change in migration of the α band when the control reaction (lane 7) was subjected to a second photolysis following the addition of 200 μM 8-azido-dATP. Specific activity of [γ-^{32}P]-8-azido-dATP and [γ-^{32}P]-8-azido ATP was 2.5 and 3.6 mCi/μmol, respectively.

dATP (2 mM) was less effective (lanes 8 and 9, Figure 4). However, the protection at 120 μM probe (35%, lanes 8 and 9) agrees well with the extent of protection as measured by the filter precipitation assay (Table I) with the same ratio of 8-azido-dATP to dATP in the reaction.

SDS gel electrophoresis of the photolabeled protein revealed the presence of two radioactive bands migrating in a region consistent with the molecular weight of the α polypeptide (Figure 4). The identity of both protein bands was confirmed by an experiment in which the protein was photolabeled with 50 or 200 μM [γ-^{32}P]-8-azido-dATP (Figure 5, lanes 1–4). Following electrophoresis in an SDS–polyacrylamide gel, protein bands were stained with Coomassie blue and subjected to autoradiography. In the presence of 50 μM photoprobe, the α polypeptide appeared as a single Coomassie blue staining band (lane 2). However, doublet bands were observed on the autoradiogram (lane 1). One of the autoradiogram bands (α) comigrated with the Coomassie blue staining α polypeptide while the other autoradiogram band (α') migrated higher in the gel. When the concentration of the photoprobe was increased to 200 μM, the α' autoradiogram band became more prominent (lane 4) while a new staining band appeared on the gel which comigrated with α' (lane 3). Concomitant with the appearance of the new staining band at α' was the disappearance of the stained band correspondong to the α polypeptide (lane 3). The protection experiments shown in Figure 4 illustrate that the fast-migrating α band was protected from photolabeling by dATP while the band corresponding to α' was not (lanes 8 and 9). This slow-migrating α' band was apparently generated by photolabeling that was nonspecific.

To show that the α' band was not due to an artifact produced by labeling with 8-azido-dATP, two concentrations of the nonsubstrate analogue (8-azido-ATP) were employed (Figure 5). Lanes 5 and 6 show the autoradiogram from an SDS gel in which 100 (lane 5) or 30 μM (lane 6) [γ-^{32}P]-8-azido-ATP was used to photolabel terminal transferase. It is evident from the data that 8-azido-ATP produces the same nonspecifically labeled band (α') produced by [γ-^{32}P]-8-azido-dATP labeling (lane 7).

To show that the α' band is generated by labeling at a site distinct from the nucleoside triphosphate binding site, a double-labeling experiment was performed. Lane 8 of Figure 5 shows that the α band produced by photolabeling with 50 μM [γ-^{32}P]-8-azido-dATP migrates to the α' position when a second photolabeling event is performed with nonradioactive 8-azido-dATP at high concentration (200 μM). Apparently, the specifically labeled α polypeptide (band α of lane 7) can be modified at a second site which results in a change in migration of the labeled polypeptide. The change in migration of the labeled α polypeptide seen between lanes 7 and 8 was also observed following a second photolabeling event with 200 μM 8-azido-dAMP (data not shown). This result indicates that the second photolabeled site on the α polypeptide is not specific for nucleoside triphosphates.

Discussion

The objective of this study was to establish conditions for active site labeling of terminal transferase with use of the photoactive substrate analogue 8-azido-dATP. We have evaluated several different photolabeling conditions to optimize photoinsertion efficiency and specificity in order to isolate and identify active site peptides from limited amounts of enzyme. Previous attempts to photolabel terminal transferase had been performed with high concentrations (400 μM) of the nonsubstrate analogue 8-azido-ATP. In these experiments only 10–20% of the enzyme was inactivated, and 5% of the protein molecules were covalently labeled (Abraham et al., 1983). In this study, optimization of buffer conditions, coupled with use of the substrate analogue, 8-azido dATP, resulted in the inhibition of 55% of the enzyme activity and active site covalent modification of at least 21% of the protein molecules at saturation (120 μM). These differences in photolabeling efficiency between 8-azido-dATP and 8-azido-ATP probably reflect the relative capacity of the buffer ions to scavenge the nitrene on the photoactivated reagent (Ruoho et al., 1973), since these analogues gave comparable photoincorporation results in our hands (Figure 5, lanes 6 and 7).

By several criteria, 8-azido-dATP was shown to photolabel the active site of terminal transferase and to interact specifically with the protein in a reversible manner until activated by UV light. This photoprobe was a competitive inhibitor of the polymerization reaction and an efficient substrate. The apparent binding affinity of 8-azido-dATP to terminal transferase was 4-fold higher than that of dATP. This difference in apparent binding affinity was also reflected in a 3-fold lower K_m value for 8-azido-dATP (53 μM) as compared to dATP (141 ● 9 μM; Deibel et al., 1985). However, the V_{max} values for 8-azido-dATP were 3-fold lower than for dATP (33000 units/mg and 100000 units/mg, respectively). Similar binding differences have also been reported (Deibel et al., 1985) for another substrate analogue, 1,N^6-ethenodeoxyadenosine triphosphate (ϵdATP) with respect to its interactions with terminal transferase. The large modification of the adenine ring in ϵdATP also produced a nucleotide with a much lower K_m (52 μM), but a 2.5-fold lower catalytic rate.

The enhanced binding affinity of 8-azido-dATP for terminal transferase permitted saturation of the catalytic site with low concentrations of the photoprobe. However, the relative differences in affinity for 8-azido-dATP as compared to the natural substrate required extremely high concentrations of dATP in competition experiments. The high nucleotide concentration in the photolabeling reaction can decrease the efficiency of photoprobe activation due to screening of UV light. This potential problem was circumvented in this study by the use of a medium-wavelength UV lamp (maximum output at 302 nm). In the 290–310-nm range, the protecting nucleotide (dATP λ_{max} = 260 nm) did not absorb light appreciably while 8-azido-dATP (λ_{max} = 280 nm) was photolyzed effectively.

In the course of this study, we found that under optimal photolabeling conditions there was a difference between the amount of terminal transferase activity lost and the percent of enzyme molecules labeled with radioactivity at the active

site (as calculated from protection experiments). The control experiments ruled out trivial explanations such as enzyme inactivation by UV light alone, production of an enzyme inhibitor during UV photolysis, or enzyme inactivation by a combination of UV light and photolyzed 8-azido-dATP. Other control experiments revealed that although the nonspecific labeling was not reduced by 2 mM dATP, the loss of enzyme activity with photolabeling could be almost completely prevented by 2 mM dATP but not by 2 mM dAMP. The difference between enzyme inhibition and active site labeling could be due to the abstraction of a hydrogen atom from the protein by the nitrene on activated 8-azido-dATP, which could cause enzyme inactivation without covalent insertion. Such hydrogen abstraction is known to occur with nitrenes (March, 1977). Another potential explanation could be the formation of an extremely labile photogenerated bond between the activated probe and an amino acid side chain. It is also theoretically possible that only 50% of the protein molecules are catalytically active. However, we have no other evidence suggesting that this may be so. Whatever the explanation for this effect, it is clear that a minimum of 21% of the terminal transferase molecules in solution was labeled at the active site by 8-azido-dATP and greater than 90% of the active site labeling was protected by 2 mM dATP.

Previous active site labeling studies on terminal transferase using the nonsubstrate analogue 8-azido-ATP indicated active site labeling on the β polypeptide and nonsaturable labeling on the α polypeptide (Abraham et al., 1983). Our studies revealed some nonspecific photolabeling of the α polypeptide with the substrate analogue 8-azido-dATP, as well as with 8-azido-ATP. Fortunately, the electrophoretic conditions used herein resulted in complete separation of the specifically labeled α polypeptide from the nonspecifically labeled protein. The altered migration in SDS of the nonspecifically photolabeled α polypeptide (labeling not protected by dATP) was probably due to altered binding of SDS. Although the difference in labeling efficiency between the β and α polypeptides using 8-azido-dATP cross-linking was appreciable ($\sim$6-fold higher for β), experiments using substrate nucleotides to protect against 8-azido-dATP photoincorporation showed the involvement of the α polypeptide in forming the active site. However, since the cross-linking efficiency is dependent upon several variables, (i) distance from nitrene to amino acid side chain, (ii) electron density or types of amino acids within cross-linking distance, (iii) exposure of nitrene to solvent and buffer components, and (iv) stability of bonds formed, the importance of the participation of each polypeptide or peptide within the active site cannot be based solely on relative photolabeling efficiencies.

Using the conditions established in this study for active site labeling and protection from 8-azido-dATP labeling by dATP, we have isolated two active site peptides from terminal transferase and identified their amino acid sequences in the accompanying paper (Evans et al., 1989).

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