Katze, J. R., & Farkas, W. R. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3271–3275.
Katze, J. R., Basile, B., & McCloskey, J. A. (1982) *Science (Washington, D.C.) 216*, 55–56.
Katze, J. R., Beck, W. T., Cheng, C. S., & McCloskey, J. A. (1983) *Recent Results Cancer Res. 83*, 146–159.
Kinoshita, T., Schram, K. H., & McCloskey, J. A. (1981) *J. Labeled Compd. Radiopharm. 9*, 525–534.
Nishimura, S. (1983) *Prog. Nucleic Acid Res. Mol. Biol. 28*, 49–80.
Noguchi, S., Nishimura, V., Hirota, Y., & Nishimura, S. (1982) *J. Biol. Chem. 257*, 6544–6550.
Okada, N., & Nishimura, S. (1977) *Nucleic Acids Res. 4*, 2931–2937.
Okada, N., & Nishimura, S. (1979) *J. Biol. Chem. 254*, 3061–3066.
Okada, N., Shindo-Okada, N., & Nishimura, S. (1977) *Nucleic Acids Res. 4*, 415–423.
Okada, N., Noguchi, S., Kasai, H., Shindo-Okada, N., Ohgi, T., Goto, T., & Nishimura, S. (1979) *J. Biol. Chem. 254*, 3067–3079.
Reyniers, J. P., Pleasants, J. R., Wostmann, B. S., Katze, J. R., & Farkas, W. R. (1981) *J. Biol. Chem. 256*, 11591–11594.
Sethi, S. K., Crain, P. F., & McCloskey, J. A. (1983a) *J. Chromatogr. 254*, 109–116.
Sethi, S. K., Katze, J. R., Brizgys, L., Cheng, C. S., Crain, P. F., Kinoshita, T., Smith, D. L., Yu, C. T., & McCloskey, J. A. (1983b) *Int. J. Mass Spectrom. Ion Phys. 48*, 121–124.
Shindo-Okada, N., Okada, N., Ohgi, T., Goto, T., & Nishimura, S. (1980) *Biochemistry 19*, 395–400.
Singhal, R. P., & Vakharia, V. N. (1983) *Nucleic Acids Res. 11*, 4257–4277.
Walden, T. L., Jr., Howes, N., & Farkas, W. R. (1982) *J. Biol. Chem. 257*, 13218–13222.

Affinity Labeling of *Escherichia coli* DNA Polymerase I with Thymidine 5′-Triphosphate and 8-Azidoadenosine 5′-Triphosphate: Conditions for Optimum Labeling, Specificity, and Identification of the Labeling Site†

Kakkudiyil I. Abraham and Mukund J. Modak*

ABSTRACT: We find that *Escherichia coli* DNA polymerase I, unlike other template-dependent DNA polymerases, has a unique ability to covalently link the bound deoxynucleoside triphosphate as well as 8-azido-ATP upon exposure to ultraviolet light energy. The conditions for the optimum cross-linking of deoxynucleoside triphosphate as well as 8-azido-ATP, a photoaffinity analogue of ATP, to *Escherichia coli* DNA polymerase I have been established. The cross-linking produced via the photoaffinity probe is at least 1 order of magnitude higher than those produced with unsubstituted deoxynucleoside triphosphates. However, in spite of these quantitative differences in the efficiency of cross-linking, the requirements for and characteristics of the cross-linking reactions with both probes are quite similar and corroborate results obtained from the equilibrium dialysis studies of Kornberg and colleagues [Englund, P. T., Huberman, J. A., Jovin, T. M., & Kornberg, A. (1969) *J. Biol. Chem. 244*, 3038–3044]. Thus, the cross-linking of thymidine 5′-triphosphate (dTTP) and 8-azido-ATP to *Escherichia coli* DNA polymerase I is (1) strictly dependent on the presence of a divalent cation, (2) competitively inhibited in the presence of deoxynucleoside and nucleoside triphosphates, (3) only slightly inhibited in the presence of deoxynucleoside monophosphate or pyrophosphate, and (4) sensitive to pyridoxal 5′-phosphate, a triphosphate binding site directed inhibitor of DNA polymerases. Tryptic peptide analysis of *Escherichia coli* DNA polymerase I, labeled with [^{32}P]dTTP and 8-azido[^{32}P]ATP, revealed that the major site of cross-linking of both dTTP and 8-azido-ATP resides in a neutral peptide.

The enzymatic synthesis of DNA is a complex process that involves multiple components (Kornberg, 1980). Although the general features of this catalytic process have been clarified, the mechanism of template-directed base selection is not yet understood. *Escherichia coli* DNA polymerase I (pol I)[1] is the prototype DNA polymerase, and extensive characterization and physical analysis of this enzyme have been carried out (Kornberg, 1980; Lehman & Uyemura, 1976). Recently, through genetic manipulation, the gene coding for pol I has been cloned and sequenced (Joyce et al., 1982), and producer lysogen has been constructed (Kelley et al., 1977; Kelley & Stump, 1979). This development resulted, in turn, in the elucidation of the entire primary amino acid sequence of pol I (Joyce et al., 1982; Brown et al., 1982). These advances, together with the fact that homogeneous preparations of pol I may now be obtained in relatively large quantities, made this enzyme attractive for structure–function studies of substrate binding sites of template-dependent DNA polymerases. Earlier, we had initiated such studies for the development of site-specific reagents using reverse transcriptase from avian

† From the Sloan-Kettering Institute for Cancer Research, Rye, New York 10580. *Received June 21, 1983.* This work was supported in part by U.S. Public Health Service Grant CA-21404 and Research Career Development Award 1 K04-CA-545 to M.J.M.

[1] Abbreviations: pol I, *Escherichia coli* DNA polymerase I; AMV, avian myeloblastosis virus; RLV, Rauscher leukemia virus; dNTPs, deoxynucleoside triphosphates; UV, ultraviolet light; PyP, pyridoxal 5′-phosphate; SDS, sodium dodecyl sulfate; TPCK, tosylphenylalanine chloromethyl ketone; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/84/0423-1176$01.50/0

myeloblastosis virus (AMV) and Rauscher leukemia virus (RLV) as sources of model DNA polymerases, and pol I was used in many of these studies, which was found to react with site-specific inhibitors in a manner analogous to reverse transcriptase (Modak, 1976; Modak & Marcus, 1977a; Modak & Srivastava, 1979; Srivastava & Modak, 1980; Srivastava & Modak, 1982). In our continuing effort to identify the substrate binding site in these enzymes, we have utilized a protocol whereby deoxynucleoside triphosphates (dNTPs) are covalently cross-linked to the terminal deoxynucleotidyltransferase binding site upon activation by ultraviolet light (UV) (Modak & Gillerman-Cox, 1982). Our results indicate that pol I has the unique ability to cross-link to dNTPs as well as to 8-azido-ATP in the absence of template-primer. The characterization and identification of the site of cross-linking of the two triphosphate probes in pol I are the topics of this paper.

Experimental Procedures

Reagents. α-^{32}P-Labeled dNTPs, 8-azido[γ-^{32}P]ATP, and Na^{125}I were purchased from New England Nuclear Corp. while [γ-^{32}P]dTTP was obtained from I.C.N. Corp. Nonradioactive nucleoside mono- and triphosphates as well as synthetic template-primers were products of P-L Biochemicals, Inc. Pyridoxal 5′-phosphate (PyP) was purchased from Sigma Chemical Co., while cellulose thin-layer chromatography plates were obtained from E. Merck. Iodobeads (chloramine T derivatized polystyrene beads) were the product of Pierce Chemical Co. All other reagents were of analytical grade. The high-intensity UV illumination lamp (1300 μW/cm^2 at 254 nm at a distance of 15 cm) was the product of Ultraviolet Products, Inc., and was obtained through Fisher Scientific Products Co.

Enzymes. Homogeneous pol I used in the initial experiments was the kind gift of Dr. L. A. Loeb of the University of Washington, Seattle, WA. In later experiments, pol I was purified from *Escherichia coli* strain CM 4935 lysogenized with λpolA phage NM857 (grown from a single colony) according to the procedure of Kelley & Stump (1979). Enzyme preparations from the latter procedure revealed a single protein band on SDS–polyacrylamide gel electrophoresis, corresponding to a molecular weight of 109 000. Results from both enzyme preparation were identical. Reverse transcriptase from AMV and RLV was purified by poly(rC)–agarose chromatography as previously described (Marcus & Modak, 1976; Modak & Marcus, 1977b). Terminal deoxynucleotidyltransferase purified from calf thymus according to the procedure of Chang & Bollum (1971) was the generous gift of Dr. Robert Ratliff of Los Alamos. DNA polymerase β was a byproduct of terminal deoxynucleotidyltransferase purification and was further purified by using phosphocellulose, Sephadex, and poly(rC)–agarose chromatography (Modak, 1979). TPCK–trypsin was obtained from Worthington Biochemical Corp.

DNA Polymerase Assay. The standard assay condition for DNA polymerase I was carried out as described earlier (Modak et al., 1974) using poly(dA)·poly(dT)$_{12-18}$ as a template-primer.

UV-Mediated Cross-Linking of dNTPs and Ribo-NTPs and SDS–Polyacrylamide Gel Electrophoresis. The standard irradiation mixture in a final volume of 50 μL contained 20 mM Hepes buffer (pH 7.8), 10% glycerol, 0.1 mM 2-mercaptoethanol, 1–2 mM $MnCl_2$, 2–5 μCi of ^{32}P-labeled NTPs (corresponding to final concentrations of approximately 0.1 μM for dNTPs and 4 μM for 8-azido-ATP), and 0.2–1 pmol of purified enzyme, except in the case of terminal deoxynucleotidyltransferase where 10 pmol of enzyme was used. UV irradiation was carried out in plastic Eppendorf tubes at 4 °C for a period of 15 min at a distance of 5 cm (1080 ergs mm^{-2} s^{-1}) for cross-linking of dNTPs, whereas a 10-cm distance (560 ergs mm^{-2} s^{-1}) was maintained for the cross-linking of 8-azido-ATP to enzyme. Upon termination of UV exposure, 10 μL of 20 mM ATP and 10 μL of protein-solubilizing solution (containing 1% SDS, 20 mM EDTA, 25 mM Tris-HCl, pH 7.8, and 20 mg/mL bromophenol blue) were added to the irradiation mixture, which was briefly heated in a boiling water bath. The entire irradiation mixture was then loaded onto a SDS–polyacrylamide gel of 8% polyacrylamide with an overlay of 4% polyacrylamide. Electrophoresis was carried out by using a Tris–glycine buffer system according to the method of Laemmli (1970) until the dye front reached the gel bottom. The gel was washed several times with distilled water and/or 50% methanol containing 50 mM sodium phosphate (pH 5.0) and was exposed to Kodak X-ray film.

Iodination of pol I. Iodination of homogeneous preparations of pol I with ^{125}I was carried out at pH 7.0 using Iodobeads according to the procedure supplied by the Pierce Chemical Co. Separation of ^{125}I-labeled proteins from the reactants was achieved by SDS–polyacrylamide gel electrophoresis.

Tryptic Digestion and Separation of Tryptic Peptides. The radiolabeled protein bands were excised from the polyacrylamide gel, soaked in 250 mM Tris–glycine buffer (pH 8.0) containing 0.5% SDS, and subjected to electroelution at pH 8.0 using 10 mM Tris–glycine buffer containing 0.02% SDS for a period of 2 h. The electroeluted protein samples were subjected to dialysis against distilled water for 3–4 h to remove buffer ions, lyophilized, further extracted with 80% methanol containing 20 mM sodium phosphate (pH 5.0) to remove SDS, and dried. Recovery of labeled protein at this stage is approximately 80%. The labeled protein was then digested at 37 °C with a 10 μg/mL solution of TPCK-treated trypsin in 50 mM ammonium bicarbonate (pH 8.5). After 2 h of digestion, another aliquot of trypsin was added, and digestion was continued for 2 h. The digest was lyophilized repeatedly to remove ammonium bicarbonate. The peptide samples were dissolved in 10 μL of electrophoresis buffer, pH 8.0 (Michl, 1958) (pyridine–acetic acid–water, 1:1:48 v/v), spotted on a 20 cm × 20 cm cellulose plate, and subjected to electrophoresis at 50 V/cm for 1 h. The plates were then air-dried and subjected to chromatography in a second dimension (Light & Smith, 1962) in a butanol–pyridine–acetic acid–water (75:50:15:60 v/v) solvent system until the solvent front reached 10 cm from the origin. The plates were then exposed to X-ray film for the desired time.

Results

UV-Mediated Cross-Linking of Substrate dNTPs to DNA Polymerases. Our success at cross-linking dNTPs to terminal deoxynucleotidyltransferase (Modak & Gillerman-Cox, 1982) encouraged us to apply that technology to other DNA polymerases in order to achieve specific labeling of the putative substrate binding site in these enzymes. We therefore examined the ability of the template-dependent DNA polymerases, AMV and RLV reverse transcriptase, pol I, and calf thymus DNA polymerase β to cross-link to [^{32}P]dTTP and dGTP by using conditions that we had previously standardized for terminal transferase. The results of this experiment are presented in the form of an autoradiogram in Figure 1. It is interesting to note that, besides terminal transferase, pol I was the only other DNA polymerase that exhibited cross-linking to substrate dNTPS. The failure of the other template-dependent enzymes to cross-link dNTPs was not due to

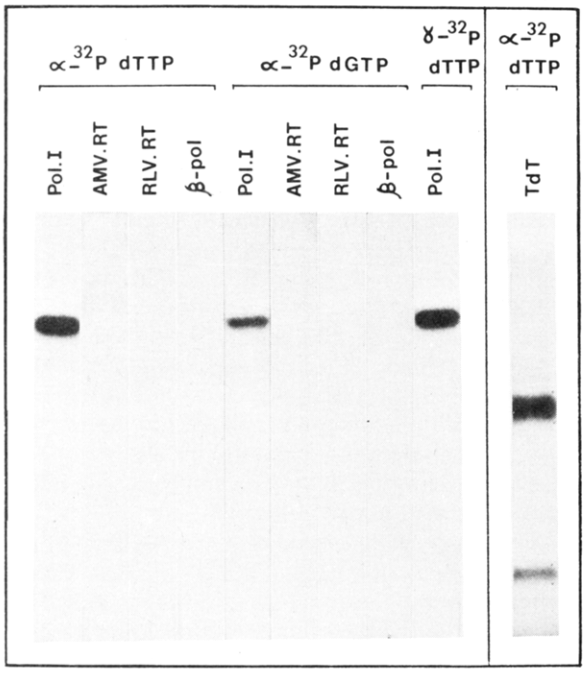

FIGURE 1: Cross-linking of [α-^{32}P]- and [γ-^{32}P]dTTP and [α-^{32}P]-dGTP to various DNA polymerases. The standard irradiation mixture contained 5 μCi of the desired ^{32}P-labeled dNTP and 0.2–1 μg of the desired DNA polymerase. Irradiation of this mixture was carried out as described under Experimental Procedures, and the extent of cross-linking of dNTP to enzyme was determined by SDS–polyacrylamide gel electrophoresis followed by autoradiography of the gel. Terminal deoxynucleotidyltransferase was included as a positive control (with [α-^{32}P]dTTP only) and was analyzed on a 12% SDS–polyacrylamide gel. No cross-linking was noted with AMV and RLV reverse transcriptase as well as DNA polymerase β with [γ-^{32}P]dTTP (data not shown).

contaminating phosphatase activities, since mixing pol I with any one of the other enzymes in a cross-linking mixture promptly produced pol I–dNTP complexes (data not shown).

Since pol I is the only template-dependent DNA polymerase that can bind and cross-link substrate dNTPs in the absence of template-primer, the dNTP binding site in pol I seemed amenable to physical analysis for the first time through its ability to form a covalent linkage with a radiolabeled dNTP probe. In previous equilibrium dialysis studies, ATP, which neither serves as a substrate nor competes with dNTPs during polymerization reactions, was shown to compete with dNTPs for binding to pol I (Englund et al., 1969). Since the photoaffinity analogue of ATP, 8-azido-ATP, is now commercially available, and because the efficiency of photoaffinity labeling has been found to be much higher than that of affinity labeling with unsubstituted dNTPs for terminal deoxynucleotidyltransferase (Abraham et al., 1983), we also included this reagent as a probe to label the triphosphate binding site in pol I.

Optimization of Conditions for Cross-Linking of dTTP and 8-Azido-ATP to pol I. A typical time course of irradiation (or photoactivation) time vs. the extent of cross-linking of [α-^{32}P]dTTP and 8-azido[γ-^{32}P]ATP to pol I is shown in autoradiographic form in Figure 2. The actual amount or radioactivity incorporated into pol I as a result of cross-linking at each time point, measured by excising the appropriate bands from the gel, is also graphically illustrated in Figure 2. It should be pointed out here that the distances used for UV-mediated cross-linking of dTTP and UV-mediated photoactivation of 8-azido-ATP are different.

Photoactivation of 8-azido-ATP is achieved with relatively mild UV energy (distance of 10 cm from sample surface), with maximum labeling occurring within 15 min. The activation energy required for dTTP cross-linking, on the other hand, is 2-fold higher (a distance of 5 cm from the sample surface), and maximum cross-linking is achieved in approximately 20 min (Figure 2). The quantitative superiority of the photoaffinity labeling probe is quite apparent from these results. Some of the salient features of cross-linking reactions with both dTTP and 8-azido-ATP are listed below and are shown in Tables I and II: (1) Cross-linking of both dTTP and 8-azido-ATP is strictly dependent on UV energy and the presence of a divalent cation (Table I); (2) the quantity of 8-azido-ATP

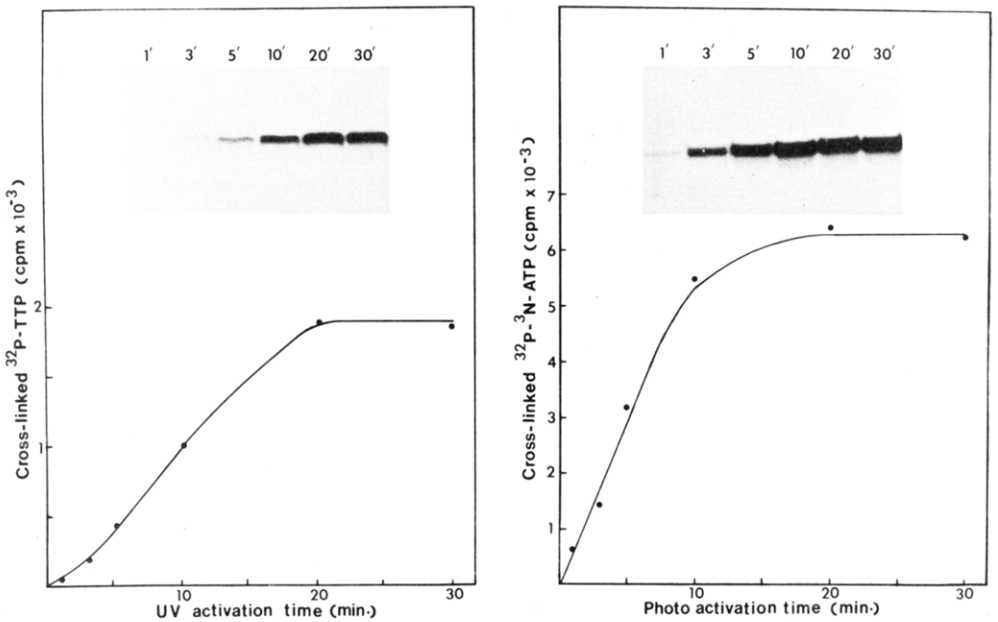

FIGURE 2: Time course of cross-linking of [α-^{32}P]dTTP and 8-azido[γ-^{32}P]ATP to pol I. A standard reaction mixture that contained 0.2 μg of pol I and 5 μCi of [α-^{32}P]dTTP (0.125 μM, specific activity 800 Ci/mmol) or 8-azido[γ-^{32}P]ATP (4.5 μM, specific activity 23 Ci/mmol) was exposed to UV light at 5 and 10 cm, respectively, for the desired amount of time. Various samples were then subjected to SDS–polyacrylamide gel electrophoresis on 8% polyacrylamide gels, and autoradiography was then performed on the gels. The numbers at the top of the autoradiogram represent minutes of UV exposure. The graph was drawn from the absolute radioactivity determined for each cross-linking time by excising the labeled protein bands from the gel and determining the incorporated radioactivity directly with a scintillation counter.

Table I: Requirements for the Cross-Linking of [α-^{32}P]dTTP and 8-Azido[γ-^{32}P]ATP to pol I[a]

addition and/or irradiation condition	nucleotide cross-linking (fmol)	
	dTTP	azido-ATP
no metal	0.0	0.0
5 mM Mg^{2+}	0.97	32.56
10 mM Mg^{2+}	0.5	23.16
1 mM Mn^{2+}	0.7	30.5
2 mM Mn^{2+}	1.19	109.4
1 mM Co^{2+}	0.65	15.9
2 mM Mn^{2+}, no UV light	0.0	0.0
2 mM Mn^{2+} + 5 mM EDTA	0.0	0.0
background[b]	0.05	0.3

[a] The cross-linking of [α-^{32}P]dTTP or 8-azido[γ-^{32}P]ATP to pol I was carried out in a standard irradiation mixture containing 5 μCi of dTTP or azido-ATP, 1 pmol of pol I, and the desired divalent cation at the indicated concentration. The quantitation of cross-linking of radiolabeled probe to pol I was calculated from the amount of radioactivity present in pol I under the different conditions. The labeled pol I was localized after cross-linking on SDS–polyacrylamide gels by autoradiography. [b] Background values were determined by counting the radioactivity in a gel piece corresponding to the area of enzyme migration, but which was never exposed to UV light prior to electrophoresis. Background has been subtracted from all values reported in this table.

Table II: Extent of Cross-Linking of Various dNTPs and 8-Azido-ATP to pol I and the Effect of Varying Enzyme Concentration[a]

labeled substrate	extent of cross-linking (fmol/pmol of enzyme)
[α-^{32}P]dTTP	1.15
[γ-^{32}P]dTTP	1.05
[^{3}H]dTTP[b]	0.85
[α-^{32}P]dATP	0.39
[α-^{32}P]dCTP	0.3
[α-^{32}P]dGTP	0.24
8-azido[γ-^{32}P]ATP	106.0
prephotolyzed 8-azido[γ-^{32}P]ATP	1.17
[α-^{32}P]dTTP[c]	0.3
[α-^{32}P]dTTP + 0.81 pmol of pol I	1.0[d]
[α-^{32}P]dTTP + 1.62 pmol of pol I	1.7[d]
[α-^{32}P]dTTP + 2.44 pmol of pol I	2.5[d]
[α-^{32}P]dTTP + 3.25 pmol of pol I	3.2[d]
[α-^{32}P]dTTP + 3.25 pmol of heat-denatured pol I	<0.05[d]

[a] The standard irradiation mixture contained 5 μCi of the desired dNTP (specific activity approximately 800 Ci/mmol for [^{32}P]dNTPs, 30 Ci/mmol for 8-azido[^{32}P]ATP, and 30 Ci/mmol for [^{3}H]dTTP) and 1 pmol of pol I and was irradiated under appropriate conditions (see Experimental Procedures), and the extent of cross-linking achieved between substrate and pol I was determined as described in the legend to Figure 4. [b] Because of the low specific activity of [^{3}H]dTTP, the amount of enzyme used in this sample was 20-fold higher than in other samples. [c] Represents cross-linking of [α-^{32}P]dTTP under 8-azido-ATP cross-linking conditions (distance of sample to UV light of 10 cm). [d] This section of the table shows that cross-linking is proportional to enzyme concentration and that heat-denatured (70 °C for 5 min) enzyme does not cross-link dNTP.

found cross-linked to enzyme is 1–2 orders of magnitude higher than that of dTTP; (3) although Mg^{2+}, Mn^{2+}, and Co^{2+} served as divalent cations required in the cross-linking reactions, optimal cross-linking with both dTTP and 8-azido-ATP occurred in the presence of 2 mM Mn^{2+}; (4) photolyzed 8-azido-ATP (exposure of 8-azido-ATP alone to UV light for 10 min at 10 cm) is not effectively cross-linked to pol I, suggesting that the azido group was required for efficient cross-linking (Table II); (5) cross-linking of both dTTP and 8-azido-ATP was proportional to enzyme concentration (Table II); and (6) heat inactivation of pol I (3 min at 70 °C) prior to cross-linking resulted in the loss of cross-linking ability (Table II). When enzyme inactivation as a consequence of cross-linking was measured, the loss of catalytic activity amounted to only 10–20% over that observed in the absence of triphosphate (data not shown). The inactivation of control enzyme as a result of UV exposure under the 8-azido-ATP and dTTP labeling conditions, however, showed significant differences. The inactivation of control enzyme under 8-azido-ATP labeling conditions (15 min at 10 cm) was less than 5%, whereas under dTTP cross-linking conditions (15 min at 5 cm) enzyme inactivation amounted to nearly 40% (data not shown).

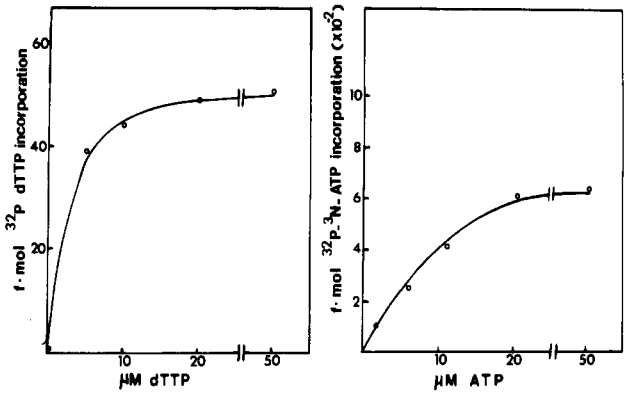

FIGURE 3: Saturation kinetics for the cross-linking of [α-^{32}P]dTTP and 8-azido[γ-^{32}P]ATP to pol I. The standard irradiation mixture containing 1 pmol of enzyme and 5 μCi of [α-^{32}P]dTTP (specific activity 800 Ci/mmol) or 8-azido[γ-^{32}P]ATP (specific activity 30 Ci/mmol) was adjusted to provide the indicated final triphosphate concentrations by the addition of nonradioactive triphosphates. Irradiation of individual mixtures, SDS–polyacrylamide gel analysis, autoradiography, and excision of labeled protein bands were carried out essentially as described in the legend to Figure 2. The incorporation of dTTP and 8-azido-ATP into enzyme protein was directly quantitated as a function of substrate concentration.

Stoichiometry of Cross-Linking of dTTP and 8-Azido-ATP to pol I. Despite the similarity of conditions required for the cross-linking and dNTP binding, the stoichiometry of cross-linking of both dTTP and 8-azido-ATP probes was rather low. Approximately 10% of the input enzyme molecules were estimated to be linked to 8-azido-ATP as compared to less than 1% cross-linked to dTTP. The low efficiency of cross-linking of bound dNTP to enzyme was certainly in part due to the suboptimal concentrations of labeled triphosphates used, which are nonsaturating. Increasing the concentration of the radioactive probe (or the addition of unlabeled probe) resulted in an increase of the absolute amount of triphosphate cross-linked to enzyme up to a concentration of 10 μM for dTTP and 20 μM for 8-azido-ATP (Figure 3). However, even at saturating concentrations of triphosphate, the stoichiometry of cross-linked triphosphate to enzyme did not exceed 0.3 mol of 8-azido-ATP and 0.03 mol of dTTP per mol of enzyme. At saturating concentrations of 8-azido-ATP, 30% inhibition of enzyme activity was observed, following cross-linking. We, however, continued to use suboptimal concentrations of radioactive probes for economic reasons.

Cross-Linking of Other dNTPs. Since pol I has previously been shown, by using equilibrium dialysis studies (Englund et al., 1969), to bind all four dNTPs at a single site, we de-

Table III: Effect of the Addition of Other dNTPs and ATP on the Cross-Linking of dTTP or 8-Azido-ATP to pol I[a]

addition (20 μM)	% of control cross-linking	
	dTTP	8-azido-ATP
none	100	100
dTTP	9.9	54.3
dATP	4.8	39
ATP	6.7	55.1
dGTP	3	26.7
dCTP	17.6	61

[a] The effect of the addition of individual dNTP or ATP at a final concentration of 20 μM on the cross-linking of ^{32}P-labeled dTTP or 8-azido-ATP was assessed by including the indicated triphosphate in the standard irradiation mixture. Assuming all four dNTPs and ATP, at 20 μM concentration, compete for binding to a single site, with similar affinity, the expected decrease in the radiolabeling with dTTP and 8-azido-ATP would amount to 90% and 50%, respectively. The above values are determined after considering the decrease in specific activity as well as the increase in cross-linking of NTPs at higher substrate concentration (see Figure 3). One hundred percent cross-linking represents 2400 cpm for dTTP and 6000 cpm for 8-azido-ATP cross-linked to pol I under standard conditions.

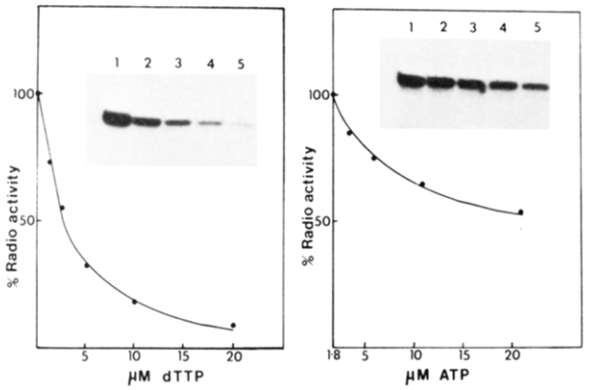

FIGURE 4: Effect of the addition of nonradioactive dTTP or ATP on the cross-linking of [α-^{32}P]dTTP or 8-azido[γ-^{32}P]ATP to pol I. The experimental design was the same as that described in the legend to Figure 3. One hundred percent radioactivity is the amount present in the enzyme sample (1) that was cross-linked to ^{32}P-labeled probe in the absence of unlabeled NTP. Samples 2–5 represent cross-linking observed in the presence of increasing concentrations of dTTP or ATP and 8-azido-ATP. Reduction in the cross-linking of 8-azido[γ-^{32}P]ATP to pol I was identical regardless of whether ATP or 8-azido-ATP was used, and only data for ATP addition are shown. The maximum expected decrease in radiolabeling by the addition of 20 μM dTTP or ATP is estimated to be 90% and 50%, respectively. These values are calculated after considering the decrease in specific activity as well as the increase in NTP cross-linking at higher substrate concentration (see Figure 3). One hundred percent radioactivity in the case of dTTP is equal to 2200 cpm, while that for 8-azido-ATP is 6000 cpm.

termined the extent of cross-linking of various dNTPs to pol I (Table II). The results illustrate that all four dNTPs can indeed be cross-linked to pol I, although the cross-linking efficiencies of the four dNTPs differ significantly.

Effect of Homologous and Heterologous dNTPs on the Cross-Linking of dTTP and 8-Azido-ATP to pol I. The addition of any unlabeled dNTP to the cross-linking reaction promptly reduces the cross-linking of the labeled dNTP (Table III). The competitive nature of such a reduction in cross-linking by homologous labeled dNTPs is shown in Figure 4. As the amount of exogenously added unlabeled dNTP is increased, a progressive decrease in labeling efficiency by either [^{32}P]dTTP or 8-azido[^{32}P]ATP is observed.

Characteristics of Cross-Linking of dTTP and 8-Azido-ATP to pol I. The cross-linking of both triphosphates was

Table IV: Effect of Various dNMPs, Pyrophosphate (PP_i), Pyridoxal 5′-Phosphate (PyP), and KCl on Cross-Linking of dTTP and 8-Azido-ATP to pol I[a]

addition	% of control cross-linking	
	dTTP	8-azido-ATP
none (control)	100	100
dTMP (200 μM)	89.7	73.8
dGMP (200 μM)	58.4	60.4
AMP (200 μM)	59	59.7
PP_i (50 μM)	100	100
PP_i (500 μM)	69	68
PyP (100 μM)	11	2.4
KCl (0.5 M)	5	4.5

[a] The standard irradiation mixture containing 5 μCi of ^{32}P-labeled dTTP of 8-azido-ATP and 1 pmol of pol I was irradiated in the presence of compounds at the final concentrations described above, and the effects of these compounds on the cross-linking of dTTP and 8-azido-ATP were determined as described in the figure legends.

proportional to enzyme concentration (Table II) and exhibited reaction requirements that are known to exist for dNTP binding. For example, the cross-linking of triphosphate to pol I is strictly dependent on the presence of divalent cations and is sensitive to inhibition by pyridoxal 5′-phosphate (Table IV), which is known to be a substrate binding site directed inhibitor of DNA polymerases (Modak, 1976). Since dNTP cross-linking in pol I is likely to represent the substrate binding reactions during catalysis, the effects of several deoxynucleoside monophosphates, pyrophosphate, and pyridoxal phosphate on this process were examined in order to confirm the apparent site specificity. Table IV shows clearly that the substrate binding site specific inhibitor pyridoxal phosphate strongly inhibits the cross-linking of both dTTP and 8-azido-ATP to pol I, whereas dNMPs and pyrophosphate have only moderate inhibitory effects even at concentrations 100–400-fold higher than the dNTP probe. Furthermore, it was interesting to note that addition of 0.5 M salt completely blocked the cross-linking of both 8-azido-ATP and dTTP, implying that their binding to pol I is ionic in nature (Table IV).

Site of Cross-Linking of [α-^{32}P]dTTP and 8-Azido[γ-^{32}P]ATP to pol I. In order to determine the site(s) within pol I involved in the cross-linking of both dTTP and 8-azido-ATP, we utilized tryptic digestion of the labeled protein, followed by separation of tryptic peptides and identification of labeled peptides by autoradiography. To establish a standard tryptic peptide map of pol I, we used ^{125}I-labeled enzyme. The results of this analysis are presented in Figure 5. An uncharged (neutral) peptide species (as judged by its lack of migration during electrophoresis) appeared to contain the major binding site for the cross-linking of both dTTP and 8-azido-ATP. In the case of dTTP, however, three additional minor peptide species were also noted. While there exist a few neutral peptide species in the tryptic digest of ^{125}I-labeled pol I (Figure 5), the majority of ^{125}I-labeled peptides were seen to migrate toward the cathode in this buffer system. In tryptic digests prepared from 8-azido-ATP-labeled pol I, similar to those we have prepared from 8-azido-ATP-labeled terminal transferase (Abraham et al., 1983), the appearance of a distinct negatively charged species migrating far toward the anode has been noted (data not shown).

Discussion

The technology of UV-mediated cross-linking of substrate dNTPs as well as photoaffinity analogues to their acceptor site in terminal deoxynucleotidyltransferase provided the means of producing stable, covalent linkages between substrate and

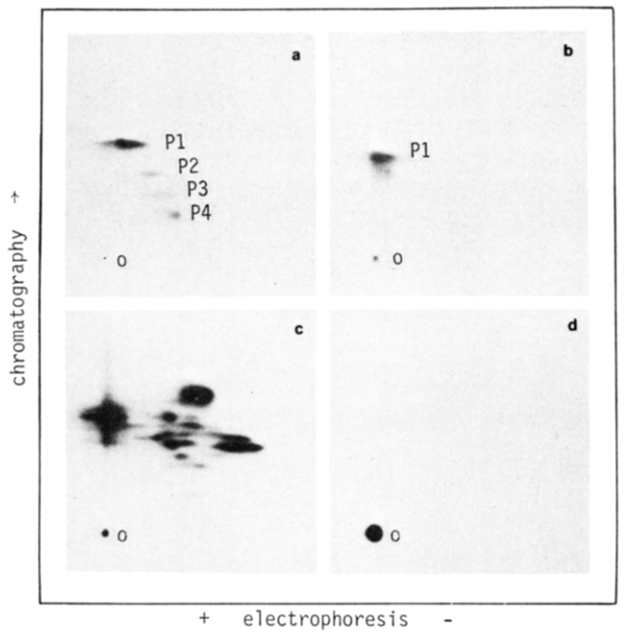

FIGURE 5: Tryptic peptide patterns of [α-^{32}P]dTTP-labeled, 8-azido[γ-^{32}P]ATP-labeled, and ^{125}I-labeled pol I. [α-^{32}P]dTTP and 8-azido[γ-^{32}P]ATP as well as ^{125}I were used to label pol I samples as described under Experimental Procedures. Labeled samples were subjected to SDS–polyacrylamide gel electrophoresis, and the radiolabeled protein band was excised and processed for tryptic digestion (see Experimental Procedures). An undigested but identically treated control for [α-^{32}P]dTTP-labeled enzyme was also included (panel d). Panels a, b, and c represent tryptic peptide maps of [α-^{32}P]dTTP, 8-azido[γ-^{32}P]ATP, and ^{125}I-labeled pol I, respectively. P1 represents the major peptide obtained from [^{32}P]dTTP and azido[^{32}P]ATP-labeled pol I. In the case of [α-^{32}P]dTTP-cross-linked pol I, three minor peptide species designated P2, P3, and P4 are also noted (panel a). The radioactivity incorporated into peptides P1, P2, P3, and P4 was 1270, 36, 325, and 390 cpm, respectively. Polarity and direction of electrophoresis and chromatography are indicated by appropriate symbols. The point of application of samples was marked as the origin (O).

DNA polymerase (Modak & Gillerman-Cox, 1982; Abraham et al., 1983). Our examination of four template-dependent DNA polymerases, namely pol I, AMV and RLV reverse transcriptase, and DNA polymerase β from calf thymus, for their ability to cross-link to unsubstituted dNTPs revealed that except for pol I, template-dependent enzymes were unable to cross-link to substrate dNTPs (Figure 1). The steric hindrance and/or unfavorable chemical selectivity at the binding region in these polymerases may result in the failure of these enzymes to establish cross-linking with the bound substrate. Alternatively, such an inability may also arise from the failure of these enzymes to bind to a dNTP residue in the absence of an appropriate template-primer. Our attempts to demonstrate cross-linking of these enzymes with substrate dNTPs in the presence of template-primer are inconclusive since nonhydrolyzable analogues of ^{32}P-labeled dNTP substrate are not available at present. pol I therefore appears to be unique in its ability to bind and cross-link all four dNTPs in the absence of template-primer. The ability of pol I to bind all four dNTPs as well as ATP to a single site in the absence of template-primer was demonstrated in the well-known equilibrium dialysis studies of Englund et al. (1969). The ability to produce covalent complexes of a desired dNTP with pol I now allows the identification as well as analysis of a site involved in triphosphate binding. Since, in the absence of template-primer, all four dNTPs and ATP were shown to compete for binding to pol I (Englund et al., 1969), we also attempted labeling of pol I with a photoaffinity analogue of ATP, 8-azido-ATP, and carried out a comparative analysis and characterization of cross-linking reactions that occurred between pol I and the photoaffinity analogue of ATP and dTTP. One of the most striking differences between dNTP cross-linking and photoaffinity labeling, both of which require UV energy for activation, is that the efficiency of cross-linking with the azido group mediated linkages is significantly higher (Figure 2) than that obtained with unsubstituted dTTP. Furthermore, the intensity of UV energy required for the activation of the azido group is much lower than that required for activation of heterocyclic purine/pyrimidine ring structures of unsubstituted nucleotides. It was, therefore, not surprising that a significant amount of enzyme inactivation, as a result of UV exposure, occurs under the conditions used for dNTP cross-linkings, whereas it is minimal under 8-azido-ATP cross-linking conditions. In both cases, however, optimal cross-linking occurred in 15–20 min (Figure 2). That the participation of the azido group indeed increases the efficiency of cross-linking is further shown by the fact the prephotolyzed (exposure of 8-azido-ATP to UV light for 10 min prior to its use) 8-azido-ATP or [α-^{32}P]dTTP, under identical cross-linking conditions, amounted to less than 1% of control cross-linkings (Table II). Estimates of the stoichiometry of cross-linking of the moles of dTTP and the moles of 8-azido-ATP per mole of pol I, calculated from the amount of each triphosphate cross-linked to enzyme when the individual triphosphate was present at saturating concentration (Figure 3), were 0.03 and 0.3 mol of ligand/mol of enzyme, respectively. The reason for the low stoichiometry of cross-linking compared to the established stoichiometry of binding of 1 mol of dNTP to 1 mol of enzyme is not clear at the present time.

Since cross-linking assays depend heavily on autoradiographic analysis as a measure of cross-linking, the amount of radioactivity rather than the absolute concentration of radioactive triphosphates was an important factor in our studies. Even at nonsaturating concentrations, cross-linking reactions exhibited all the characteristics of triphosphate binding as reported in equilibrium dialysis studies (Englund et al., 1969). Thus, cross-linking reactions with both dTTP and 8-azido-ATP to pol I were strictly dependent on the presence of a divalent cation (Table I) and were proportional to enzyme concentration (Table II). The fact that equivalent amounts of [α-^{32}P]dTTP, [γ-^{32}P]dTTP, and ring-^{3}H-labeled dTTP are cross-linked to pol I suggests that the entire dTTP molecule is incorporated into the enzyme structure (Table II). Inclusion of other dNTPs as well as ATP reduced the labeling of radioactive triphosphate (Figure 4, Table III), whereas the addition of deoxynucleoside monophosphate or pyrophosphate had minimal effect (Table IV). The ability of other dNTPs to reduce cross-linking of [^{32}P]dTTP showed a qualitatively similar pattern of competition to previous binding studies (Englund et al., 1969) (Table III). Furthermore, cross-linking reactions exhibited strong sensitivity to the presence of pyridoxal 5′-phosphate, a substrate binding site directed inhibitor of many DNA polymerases (Table IV) (Modak, 1976). We therefore conclude that the cross-linking reactions between dTTP or 8-azido-ATP and pol I truly represent triphosphate binding reactions.

To further characterize the cross-linking site for dTTP and 8-azido-ATP in pol I, we carried out tryptic digest mapping of pol I protein labeled with approriate triphosphate. Some of the difficulties experienced in the analysis of terminal transferase labeled with 8-azido-ATP (Abraham et al., 1983) also existed in the analysis of pol I labeled with this probe. One example of such a difficulty was the appearance of a

non-peptide-associated breakdown product of 8-azido-ATP that is generated during the tryptic digestion procedure. This product is easily identified by its unique migration toward the anode (data not shown). When tryptic digest patterns obtained from 8-azido-ATP and dTTP-labeled pol I were compared with that obtained from ^{125}I-labeled pol I, it was clear that cross-linking of 8-azido-ATP was restricted to a single peptide species which also was the site of dTTP cross-linking. However, with dTTP, three additional minor peptides were also apparent. With the exception of a few neutral peptides, most of the ^{125}I-labeled peptides migrated toward the cathode during the first-dimensional electrophoretic run (which is subsequently resolved during the chromatographic run in the second dimension). The peptide that showed cross-linking to both 8-azido-ATP and dTTP, on the other hand, did not exhibit any migration during electrophoresis. It is possible that the cross-linking of a negatively charged triphosphate moiety may negate the normally positive charge on this peptide, although we have tentatively classified it as a neutral peptide. This peptide is similar to the neutral peptide species observed in the case of terminal deoxynucleotidyltransferase cross-linked to 8-azido-ATP, except that definitive chromatographic migration differences exist between the azido-ATP-labeled peptides from pol I and terminal transferase (Abraham et al., 1983). We are unable to explain the appearance of the three additional peptides that show cross-linking to dTTP, but we suspect that the strong UV irradiation used may have caused some interenzyme–protein linkages which may have altered the migration pattern of tryptic peptides. In any event, these studies have identified one major peptide that exhibits cross-linking to both dTTP and 8-azido-ATP, and it probably contains the site for triphosphate binding. However, it is not known if the site that we have identified through the cross-linking studies described above is the true substrate binding site (i.e., the one used in accepting a substrate dNTP in the presence of a template-primer). Clarification of this point must await the development of technology which will allow the exclusive labeling of the substrate binding site in the presence of template-primer.

Acknowledgments

We thank Dr. S. Marcus for general counsel and gratefully acknowledge the technical assistance of E. Gillerman-Cox.

Registry No. dTTP, 365-08-2; 8-azido-ATP, 53696-59-6; pol I, 9012-90-2.

References

Abraham, K. I., Haley, B., & Modak, M. J. (1983) *Biochemistry 22*, 4197–4203.

Brown, W. E., Stump, K. G., & Kelley, W. S. (1982) *J. Biol. Chem. 257*, 1965–1972.

Chang, L. M. S., & Bollum, F. J. (1971) *J. Biol. Chem. 246*, 909–916.

Englund, P. T., Huberman, J. A., Jovin, T. M., & Kornberg, A. (1969) *J. Biol. Chem. 244*, 3038–3044.

Joyce, C. M., Kelley, W. S., & Grindley, N. D. F. (1982) *J. Biol. Chem. 257*, 1958–1964.

Kelley, W. S., & Stump, K. H. (1979) *J. Biol. Chem. 254*, 3206–3210.

Kelley, W. S., Chalmers, K., & Murray, N. E. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5632–5636.

Kornberg, A. (1980) in *DNA Replication*, W. H. Freeman, San Francisco, CA.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lehman, I. R., & Uyemura, D. G. (1976) *Science (Washington, D.C.) 193*, 963–969.

Light, A. L., & Smith, E. L. (1962) *J. Biol. Chem. 237*, 2537–2542.

Marcus, S. L., & Modak, M. J. (1976) *Nucleic Acids Res. 3*, 1473–1486.

Michl, H. (1958) *J. Chromatogr. 1*, 93–106.

Modak, M. J. (1976) *Biochemistry 15*, 3620–3626.

Modak, M. J. (1979) *Biochemistry 18*, 2679–2684.

Modak, M. J., & Marcus, S. L. (1977a) *J. Virol. 22*, 243–246.

Modak, M. J., & Marcus, S. L. (1977b) *J. Biol. Chem. 252*, 11–19.

Modak, M. J., & Srivastava, A. (1979) *J. Biol. Chem. 254*, 4756–4759.

Modak, M. J., & Gillerman-Cox, E. (1982) *J. Biol. Chem. 257*, 15105–15109.

Modak, M. J., Marcus, S. L., & Cavalieri, L. F. (1974) *J. Biol. Chem. 249*, 7373–7376.

Srivastava, A., & Modak, M. J. (1980) *J. Biol. Chem. 255*, 917–921.

Srivastava, S. K., & Modak, M. J. (1982) *Biochemistry 21*, 4633–4639.