Biochemistry

 Volume 23, Number 13 June 19, 1984

*Articles*

# Photoaffinity Labeling of DNA-Dependent RNA Polymerase from *Escherichia coli* with 8-Azidoadenosine 5′-Triphosphate†

A-Young M. Woody, Connie R. Vader, Robert W. Woody,* and Boyd E. Haley

ABSTRACT: A photoaffinity analogue of adenosine 5′-triphosphate (ATP), 8-azidoadenosine 5′-triphosphate (8-$N_3$ATP), has been used to elucidate the role of the various subunits involved in forming the active site of *Escherichia coli* DNA-dependent RNA polymerase. 8-$N_3$ATP was found to be a competitive inhibitor of the enzyme with respect to the incorporation of ATP with $K_i$ = 42 $\mu$M, while uridine 5′-triphosphate (UTP) incorporation was not affected. UV irradiation of the reaction mixture containing RNA polymerase and [$\gamma$-$^{32}$P]-8-$N_3$ATP induced covalent incorporation of radioactive label into the enzyme. Analysis by gel filtration and nitrocellulose filter binding indicated specific binding. Subunit analysis by sodium dodecyl sulfate and sodium tetradecyl sulfate gel electrophoresis and autoradiography of the labeled enzyme showed that the major incorporation of radioactive label was in $\beta'$ and $\sigma$, with minor incorporation in $\beta$ and $\alpha$. The same pattern was observed in both the presence and absence of poly[d(A-T)] and poly[d(A-T)] plus ApU. Incorporation of radioactive label in all bands was significantly reduced by 100–150 $\mu$M ATP, while 100–200 $\mu$M UTP did not show a noticeable effect. Our results indicate major involvement of the $\beta'$ and $\sigma$ subunits in the active site of RNA polymerase. The observation of a small extent of labeling of the $\beta$ and $\alpha$ subunits, which was prevented by saturating levels of ATP, suggests that these subunits are in close proximity to the catalytic site.

RNA polymerase is a multisubunit enzyme, but as yet we do not fully understand the functions of each subunit, especially with regard to their relationship with the catalytic center of the enzyme. Various chemical affinity analogues of nucleosides and nucleotides have been utilized with enzyme alone (Nixon et al., 1972; Wu & Wu, 1974; Armstrong et al., 1976; Malcolm & Moffat, 1978; Miller et al., 1980) or with a ternary complex (Armstrong & Eckstein, 1979) to resolve this problem. An analogue of the initiation inhibitor rifamycin (Stender et al., 1975) has also been used. A photoaffinity analogue, 4-thiouridine 5′-triphosphate ($S^4$-UTP), which, when photoactivated, reacts with a nucleophile in the vicinity, was used with the enzyme and the enzyme–DNA complex by Frischauf & Scheit (1973). Photoaffinity analogues of nucleotides, which react via a photochemically produced nitrene, have also been utilized with a ternary complex (Sverdlov et al., 1978, 1980; DeRiemer & Meares, 1981).

In this study we have utilized a photoaffinity analogue of adenosine 5′-triphosphate, 8-azidoadenosine 5′-triphosphate (8-$N_3$ATP), to study the topology of the active site of RNA polymerase. The advantage of using this photoaffinity label is that the nitrene photochemically generated in the active site is capable of reacting fairly nonselectively with nearby groups, not requiring nucleophilic or electrophilic groups (Knowles, 1972). 8-$N_3$ATP has been successfully used in probing ATP-binding proteins (Potter & Haley, 1982), e.g., the ($Na^+$,$K^+$)-ATPase of the red cell membrane (Haley & Hoffman, 1974) and bacterial $F_1$ ATPase (Scheurich et al., 1980).

## Materials and Methods

Poly[d(A-T)], calf thymus DNA, and unlabeled ribonucleoside triphosphates were obtained from Miles Laboratories and Sigma Chemical Co. $^3$H-Labeled ATP and $\gamma$-$^{32}$P-labeled ATP were purchased from ICN and New England Nuclear, respectively. $\gamma$-$^{32}$P-Labeled 8-$N_3$ATP and unlabeled 8-$N_3$ATP were prepared as described by Czarnecki et al. (1979). All other reagents were of the highest purity available from commercial sources. *Escherichia coli* B (3/4 log phase, enriched medium) was obtained as a frozen cell paste from

† From the Department of Biochemistry, Colorado State University, Fort Collins, Colorado 80523 (A-Y.M.W., C.R.V., and R.W.W.), and the Department of Biochemistry, University of Wyoming, Laramie, Wyoming 82071 (B.E.H.). *Received September 26, 1983.* This research has been supported in part by U.S. Public Health Service Grants GM-23697 at Colorado State University and GM-21998 at the University of Wyoming.

0006-2960/84/0423-2843$01.50/0 

Grain Processing Co., Muscatine, IA.

RNA polymerase was prepared according to the procedure of Burgess & Jendrisak (1975) with slight modification as described by Reisbig et al. (1979). Holoenzyme was used throughout the experiments. Enzyme activity and purity were determined by the method of Reisbig et al. (1979). Enzyme activity on calf thymus DNA was 600 nmol of AMP incorporation per milligram of enzyme per 10 min. $\sigma$ saturation was (100 ± 10)%, and purity was greater than 95% according to sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis.

Enzyme concentration was determined by absorbance at 280 nm after correction for light scattering (Leach & Scheraga, 1960). An extinction coefficient at 280 nm of 305 $mM^{-1}$ $cm^{-1}$ was used (Reisbig et al., 1979). Absorption spectra were obtained on a Cary 118C scanning spectrophotometer. The buffer used throughout the experiments was 10 mM tris(hydroxymethyl)aminomethane (Tris-HCl), pH 7.9, containing 10 mM $MgCl_2$, 100 mM KCl (TMK buffer), and 500 $\mu$M $\beta$-mercaptoethanol ($\beta$ME).

*Inhibition Kinetics.* For the inhibition studies, solution A contained enzyme (0.1 $\mu$M), poly[d(A-T)] (100 $\mu$M), and ApU (100 $\mu$M) and solution B contained the inhibitor 8-$N_3$ATP, UTP, and [$^3$H]ATP (or [$^3$H]UTP) in desired proportions. Solutions A and B were preincubated separately for 5 min at 37 °C, and the reaction was started by adding 1 volume of solution A to 1 volume of solution B. For a control, identical experiments were performed without inhibitor. After the desired time interval, an aliquot of 25 $\mu$L was pipetted and placed on a Whatman DE-81 circle as described by Reisbig et al. (1979). The effect of 8-$N_3$ATP on the incorporation of [$\gamma$-$^{32}$P]ATP was measured in the same way as above except that ApU was omitted and the [$\gamma$-$^{32}$P]ATP concentration was varied from 100 to 400 $\mu$M in the presence and absence of 20 or 40 $\mu$M 8-$N_3$ATP.

*Photolysis.* An appropriate amount of 8-$N_3$ATP in methanol was added to each well of a serological plate and air-dried. A total of 100 $\mu$L of 0.1 $\mu$M (or 0.5 $\mu$M) holoenzyme in TMK buffer plus 500 $\mu$M $\beta$ME containing in some experiments 100 $\mu$M poly[d(A-T)] or 100 $\mu$M poly[d(A-T)] and 100 $\mu$M ApU was placed in the wells, mixed, and incubated a few minutes. Adenosine was added to the enzyme solutions to maintain identical absorbances at 254 nm in most experiments. A control experiment was performed in which ATP replaced 8-$N_3$ATP during photolysis, and this led to no detectable inactivation. Reaction mixtures were irradiated for the desired time interval with a UVSL-25 mineral light (254 nm) at a distance of 7.5 cm or under a UV S-11 mineral light (254 nm) (Ultra-Violet Products, Inc., 630 $\mu W/cm^2$ at the filter surface) at a distance of 20 cm for 8 min with 90° rotation of the plates every 2 min to ensure uniform irradiation. All solutions were kept under cover before and after photolysis, and all subsequent steps were performed as quickly as possible. No degradation or cross-linking of enzyme was observed under these conditions, according to SDS–polyacrylamide gel electrophoresis. To study the effect of ATP and UTP on photoincorporation, ATP (or UTP) was mixed with the enzyme solution and incubated for 1 min, followed by the addition of [$\gamma$-$^{32}$P]-8-$N_3$ATP. After being mixed for 30 s, the solution was photolyzed.

*Gel Electrophoresis and Autoradiography.* SDS–polyacrylamide gel electrophoresis was done immediately after the photolysis according to the procedure of Owens & Haley (1976) (electrophoresis condition I). This method gave good resolution for the $\sigma$ and $\alpha$ subunits from each other and from the $\beta$ and $\beta'$ subunits, but $\beta$ and $\beta'$ were not resolved from each other, presumably because of their similar molecular weights. A method was used to resolve $\beta$ and $\beta'$ in which both molecular weight and intrinsic charge would determine the mobility. This condition (electrophoresis condition II) involved dissociating the subunits in the presence of 2.5% SDS and running the electrophoresis on a gradient gel of 6–12% polyacrylamide (or in some cases a simple 8% gel) containing 0.1% SDS in the gel and 0–0.01% SDS in the running buffer. Since the order of the migration of $\beta$ and $\beta'$ was not established under our experimental conditions, we have also subjected our photolabeled enzyme to sodium tetradecyl sulfate (STS) gel electrophoresis according to the method of Fisher & Blumenthal (1980) (electrophoresis condition III). The gels were dried on a Hoffer Scientific Instruments slab gel dryer. The autoradiogram was developed according to the procedure of Owens & Haley (1976). Radioactive incorporation was quantitated by slicing gel bands and counting them in the toluene-based scintillation fluid, as well as tracing the autoradiogram with a Joyce-Loebl densitometer or a Beckman DU-8B spectrophotometer with gel scanner.

*Nitrocellulose Filter Binding and P2 Column Chromatography.* Nitrocellulose filter-binding assays followed the method of Hinkle & Chamberlin (1972). A total of 50 $\mu$L of 0.2 $\mu$M enzyme was added to 50 $\mu$L of a solution containing the desired amount of 8-$N_3$ATP. The mixture was photolyzed at room temperature at a distance of 7.5 cm for 3 min. The mixture was immediately diluted with 1 mL of TMK buffer, filtered through a nitrocellulose filter with gentle suction, and washed with 50 mL of TMK buffer. The filter was dried and counted in a toluene-based scintillation fluid. For a control, the experiment was performed as above except that photolysis was omitted. The control values were subtracted from the sample values for the calculation of the extent of incorporation. For P2 column chromatography, immediately after photolysis the mixture was placed on ice and filtered through a P2 column equilibrated with TMK buffer. The protein peak was collected, and the concentration was determined by absorbance at 280 nm. Radioactivity was counted, after mixing 1 mL of protein solution with 4 mL of Bray's solution, in a Tri-Carb scintillation counter. A known concentration of radioactive 8-$N_3$ATP was treated in the same way, as a control.

## Results

*Kinetics of Inactivation.* We investigated the effects of 8-$N_3$ATP on RNA polymerase activity before using the nucleotide analogue as a photoaffinity label. In all our experiments, poly[d(A-T)] was used as a template. RNA synthesis was inhibited by 8-$N_3$ATP in the presence and absence of an initiating dinucleotide, ApU. We used ApU to bypass the initiation step (Downey & So, 1970). The initial rate of RNA synthesis was determined at saturating levels of UTP (400 $\mu$M) while varying the concentration of ATP. The apparent Michaelis parameters $K_m^{app}$ and $V^{app}$ were obtained from a double-reciprocal plot. A replot of $K_m^{app}$ vs. the inhibitor concentration gives $K_i$ and $K_m$ values of 42 $\mu$M and 36 $\mu$M, respectively. These results are shown in Figure 1 No inhibition by 8-$N_3$ATP was observed when UTP was the variable substrate at a saturating level of ATP (data not shown). A Hill plot (Cornish-Bowden, 1979) of the kinetic data yields a value of $n = 1.0 \pm 0.1$ for the Hill constant. This is consistent with a 1:1 complex and also shows that 8-$N_3$ATP does not bind cooperatively.

The effect of 8-$N_3$ATP on the initiation step was studied by measuring the initial velocity of [$\gamma$-$^{32}$P]ATP incorporation into poly(A-U) in the presence and absence of 8-$N_3$ATP. Two concentrations of 8-$N_3$ATP were used (20 and 40 $\mu$M) while

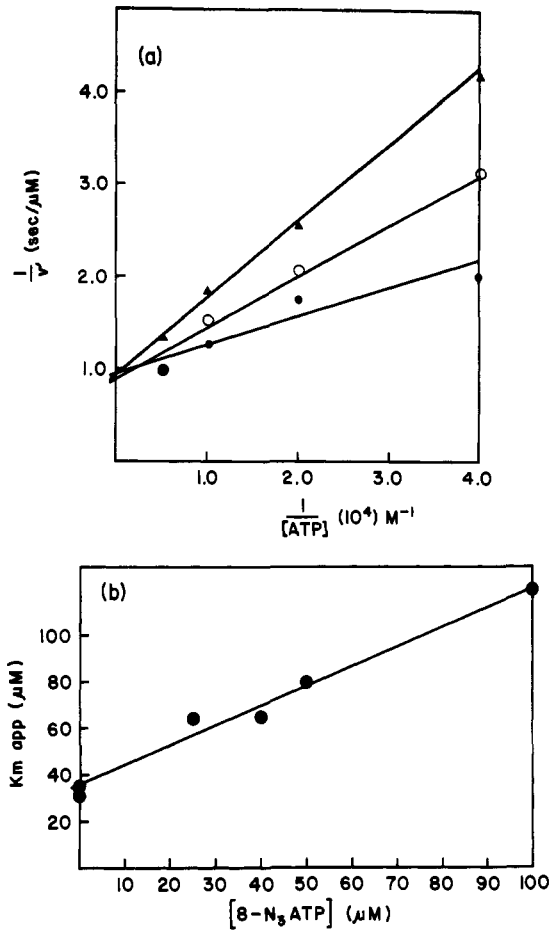


FIGURE 1: (a) Inhibition of RNA synthesis by 8-$N_3$ATP at 37 °C. Double-reciprocal plot of initial velocity vs. ATP concentration. The reaction mixture contained 0.1 μM holoenzyme, 100 μM poly[d(A-T)], 100 μM ApU, 400 μM UTP, varying concentrations of ATP, [$^3$H]ATP, 500 μM β-mercaptoethanol, and 8-$N_3$ATP. The buffer used throughout the experiments was TMK buffer (10 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 0.1 M KCl). 8-$N_3$ATP concentration: 0 μM (●); 25 μM (○); 50 μM (▲). (Combination of three separate experiments.) (b) Replot of $K_m^{app}$ vs. 8-$N_3$ATP concentration.

the [γ-$^{32}$P]ATP concentration was varied from 100 to 400 μM. Within experimental error, there was no inhibition of the incorporation of [γ-$^{32}$P]ATP. Analysis of [$^{32}$P]$PP_i$ formation from [γ-$^{32}$P]-8-$N_3$ATP in a reaction mixture containing poly[d(A-T)] and 400 μM UTP by means of the charcoal extraction procedure (Heinz & Hoffman, 1969) revealed that 8-$N_3$ATP is not a substrate of RNA polymerase under our experimental conditions. 8-$N_3$ATP has a strong preference for the syn conformation (Evans & Kaplan, 1976). This probably accounts for the inability of 8-$N_3$ATP to serve as a substrate. The ability of 8-$N_3$ATP to inhibit elongation but not initiation may reflect more exacting steric requirements on the nucleotide conformation for binding at the initiation site.

*Irreversible Inactivation of Enzyme as a Measure of Covalent Modification of RNA Polymerase.* RNA polymerase was covalently modified by 8-$N_3$ATP upon photolysis with 254-nm UV light, as described under Materials and Methods. The extent of modification was estimated by the irreversible inactivation of the enzyme as a function of exposure time (Figure 2). The enzyme activity was determined on a poly[d(A-T)] template at saturating concentrations of ATP and UTP. We do not see complete inactivation because of the low efficiency of photoincorporation of 8-$N_3$ATP into the enzyme. No degradation or cross-linking of the enzyme was detectable by SDS gel electrophoresis under the irradiation conditions.

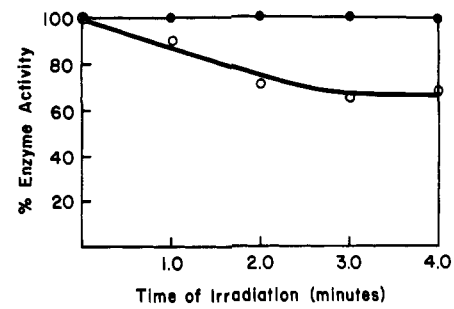


FIGURE 2: Light-induced inactivation of RNA polymerase in the presence of 8-$N_3$ATP. The 100-μL reaction mixture contained 0.2 μM holoenzyme, 50 μM 8-$N_3$ATP, and 500 μM β-mercaptoethanol in TMK buffer. The reaction mixture was irradiated at a distance of 7.5 cm with 254-nm UV light for the desired time. The activity of holoenzyme was assayed immediately by adding 25 μL of the photolyzed solution to 25 μL of the poly[d(A-T)]–nucleotide mixture and analyzed according to the assay procedure described under Materials and Methods. (○) Holoenzyme plus 8-$N_3$ATP; (●) holoenzyme plus an appropriate amount of ATP to give the same absorbance of 254 nm as in the case of 8-$N_3$ATP.

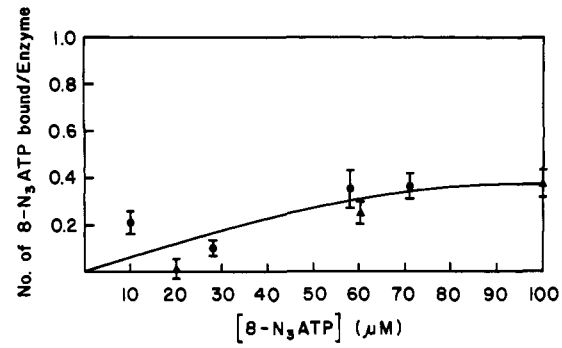


FIGURE 3: Number of [γ-$^{32}$P]-8-$N_3$ATP molecules bound per enzyme molecule vs. 8-$N_3$ATP concentration: gel filtration (●) and nitrocellulose filter-binding studies (▲). Irradiation was for 3 min and the conditions are as described under Materials and Methods.

During the 2-min irradiation less than 10% of the 8-$N_3$ATP was hydrolyzed as determined by the loss of the terminal phosphate. In the absence of UV light, incubation of 8-$N_3$ATP with enzyme produced no inhibition under the above assay conditions.

*Gel Filtration and Nitrocellulose Filter-Binding Studies.* Gel filtration and nitrocellulose filter-binding studies were carried out to quantitate the extent of covalent attachment of 8-$N_3$ATP to the enzyme after photolysis of the mixture containing enzyme and 8-$N_3$ATP. Figure 3 shows the results. According to these results, even at 100 μM 8-$N_3$ATP, only 0.4 molecule of 8-$N_3$ATP is incorporated per enzyme molecule. This agrees very well with the extent of covalent modification determined by enzyme activity shown in Figure 2.

*Photochemical Labeling of RNA Polymerase and Subunit Analysis.* UV irradiation of the reaction mixture containing RNA polymerase and [γ-$^{32}$P]-8-$N_3$ATP in the presence or absence of poly[d(A-T)] and poly[d(A-T)] plus ApU induced covalent incorporation of radioactive 8-$N_3$ATP into RNA polymerase. The total radioactive label incorporated into the enzyme leveled off as the 8-$N_3$ATP concentration was increased, indicating a saturation phenomenon (Figure 4). In the absence of the UV light no photoincorporation was observed. Analysis of the labeled enzyme by SDS gel electrophoresis and autoradiography revealed that the radioactive label was distributed among all of the subunits but to a differing extent ($\beta + \beta' = 52\%$, $\sigma = 35\%$, $\alpha = 13\%$). Poly[d(A-T)] and poly[d(A-T)] plus ApU do not seem to have a noticeable effect on this distribution or the extent of incor-

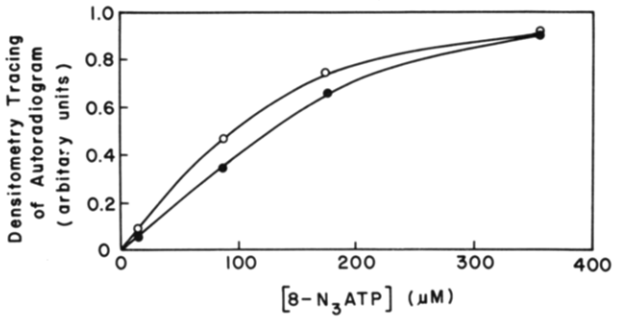


FIGURE 4: Densitometric tracing of total radioactivity incorporated into RNA polymerase vs. [$\gamma$-$^{32}$P]-8-$N_3$ATP concentration: (●) enzyme (0.1 $\mu$M) alone; (○) enzyme (0.1 $\mu$M) plus poly[d(A-T)] (100 $\mu$M).

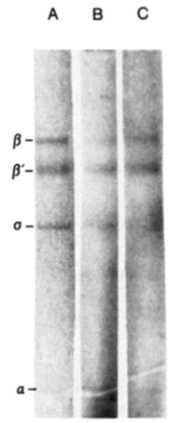


FIGURE 5: Autoradiogram showing the photoincorporation of [$\gamma$-$^{32}$P]-8-$N_3$ATP into subunits fractionated by STS gel electrophoresis: (A) Coomassie Blue stained gel showing the order of migration of each subunit of RNA polymerase (0.5 $\mu$M); (B) RNA polymerase (0.5 $\mu$M) plus 20 $\mu$M [$\gamma$-$^{32}$P]-8-$N_3$ATP; (C) RNA polymerase (0.5 $\mu$M)–poly[d(A-T)] (100 $\mu$M) complex plus 20 $\mu$M [$\gamma$-$^{32}$P]-8-$N_3$ATP. Photolysis was for 3 min at 25 °C.

poration. Initially, the separation of $\beta$ and $\beta'$ on SDS gel electrophoresis was not adequate to permit quantitation of the two bands individually. When we were able to separate the $\beta$ and $\beta'$ subunits, and the order of migration of $\beta$ and $\beta'$ was ascertained by STS gel electrophoresis (Figure 5), the specificity of radioactive incorporation into subunits was analyzed by densitometric tracing of the autoradiogram. The following order of incorporation of radioactivity was observed: $\beta' > \sigma \gg \beta > \alpha$. A band due to the $\alpha$ subunit is not detectable following STS gel electrophoresis. In general, the detectable bands are more diffuse on STS gels than the corresponding ones on SDS gels. Apparently the $\alpha$ band, which is relatively weak in any case, is so diffuse on STS gels that it is not detectable above background. The results are shown in Figure 5 and Table I. With some enzyme preparations, though the method of preparation is identical, we have observed a band below $\sigma$, which was very faintly stained by Coomassie Blue and yet was labeled quite strongly even at 5 $\mu$M [$\gamma$-$^{32}$P]-8-$N_3$ATP. In the presence of 30 $\mu$M ATP, this band disappeared, indicating that it was a protein impurity that bound ATP very tightly and copurified with RNA polymerase. This protein could be the ATPase ($M_r$ 68 000) that Paetkau & Coy (1972) observed in their preparation of RNA polymerase from *E. coli*.

*Effect of ATP and/or UTP on Photoincorporation of* [$\gamma$-$^{32}$*P*]-*8-*$N_3$*ATP into RNA Polymerase.* The effect of ATP and/or UTP was estimated by measurements of the protection of RNA polymerase from (1) inactivation and (2) radioactive labeling after photolysis. ATP (400 $\mu$M) reduced the inactivation of RNA polymerase resulting from covalent modification. In terms of percent protection as defined in Table II, 30–50% protection was observed in all three systems studied. Although the percent error is large, the protection was observed consistently. The protection by ATP was also supported by the analysis of the autoradiogram of the radioactively labeled enzyme (Figure 6 and Table III). The experiments on protection by ATP based on enzyme activity are less convincing because the extent of photoincorporation is 40% at most. Any reduction in photoincorporation by ATP has to be relative to this 40% value. As a consequence, when enzyme activity is determined, because of the large portion of the unmodified enzyme present, the numerical values were less than impressive. On the other hand, in autoradiography, ATP protection against photoincorporation is much more convincing because we are observing only the labeled enzyme.

Table I: Distribution of Radioactivity into Subunits[a]

| electrophoresis condition[b] | [$\gamma$-$^{32}$P]-8-$N_3$ATP concn ($\mu$M) | template[c] | % distribution of radioactivity in subunits[d] $\beta'$ | $\beta$ | $\sigma$ | $\alpha$ |
|---|---|---|---|---|---|---|
| II | 15 | no | 48 | 10 | 33 | 9 |
| | 30 | no | 42 | 16 | 30 | 12 |
| | 20 | no[e] | 32 | 15 | 29 | 25 |
| | 25 | no[e] | 40 | 6 | 36 | 18 |
| III | 20 | no | 56 | 16 | 28 | |
| | 20 | yes | 57 | 12 | 33 | |

[a] Photolysis was performed at 25 °C except for footnote *e*. [b] As described under Materials and Methods. [c] Poly[d(A-T)] (100 $\mu$M) was used. [d] Relative mass of subunits (%): $\beta'$, 36; $\beta$, 33; $\sigma$, 15; $\alpha$, 16. Subunit molecular weights used in kilodaltons: $\beta'$, 165 (Burgess, 1969); $\beta$, 155 (Ovchinnikov et al., 1981); $\sigma$, 70 (Burton et al., 1981); $\alpha$, 36.5 (Ovchinnikov et al., 1977). [e] Temperature of photolysis was 0 °C.

Table II: Photoinactivation of RNA Polymerase by 8-$N_3$ATP and Protection by ATP

| 8-$N_3$ATP | other additions | activity (%)[a] enzyme | E–poly-[d(A-T)] | E–poly[d-(A-T)] + ApU |
|---|---|---|---|---|
| 0 | 394 $\mu$M Ado | 100 | 100 | 100 |
| 40 $\mu$M | 370 $\mu$M Ado | 76 ± 5 | 74 ± 6 | 76 ± 13 |
| 40 $\mu$M | 400 $\mu$M ATP | 82 ± 8 | 87 ± 8 | 83 ± 14 |
| | | protection (%)[b] | | |
| | | 29 ± 18 | 52 ± 13 | 48 ± 27 |

[a] Average of three experiments. Photolysis was at 25 °C for 2 min and enzyme activity measured at 37 °C for 2 min. ATP and 8-$N_3$ATP were mixed first. [b] Expressed as percent of control (line 1): % protection = [(line 3 − line 2)/(line 1 − line 2)] × 100.

Table III: Effect of ATP and UTP on Photoincorporation of [$\gamma$-$^{32}$P]-8-$N_3$ATP into Subunits[a]

| electrophoresis condition | concn ($\mu$M) [$\gamma$-$^{32}$P]-8-$N_3$ATP | ATP | UTP | % of value in absence of ATP or UTP $\beta'$ | $\beta$ | $\sigma$ | $\alpha$ |
|---|---|---|---|---|---|---|---|
| II | 20 | 0 | 0 | 100 | 100 | 100 | 100 |
| | 20 | 35 | 0 | 75 | 40 | 66 | 57 |
| | 20 | 150 | 0 | 20 | 11 | 15 | 11 |
| III | 20 | 0 | 0 | 100 | 100 | 100 | |
| | 20 | 100 | 0 | 30 | 42 | 35 | |
| III | 20 | 0 | 0 | 100 | 100 | 100 | |
| | 20 | 0 | 100 | 100 | 120 | 110 | |
| | 20 | 0 | 200 | 90 | 98 | 94 | |

[a] All the experiments were performed at 25 °C.

Table IV: Subunits Labeled by Base-Substituted Affinity Analogues

| affinity analogue | substrate or inhibitor[a] | subunits labeled | reference |
|---|---|---|---|
| 5-formyl-1-(α-D-ribofuranosyl)uracil 5′-triphosphate | NC ($K_i$ = 0.54 mM)[b]<br>C ($K_i$ = 3.33 mM)[c] | β | Armstrong et al. (1976) |
| 4-thiouridine 5′-triphosphate | S | β′ > β[d]<br>β > β′[e] | Frischauf & Eckstein (1973) |
| 9-(β-D-arabinofuranosyl)-6-thiopurine | C (elongation subsite) | β′ | Miller et al. (1980) |
| 8-azidoadenosine 5′-triphosphate | C (elongation subsite) | β′ > σ >> β > α | present work |

[a] C = competitive inhibitor; NC = noncompetitive inhibitor; S = substrate. [b] At low concentrations. [c] At high concentrations. [d] Core enzyme used. [e] Core enzyme and poly[d(A-T)] used.

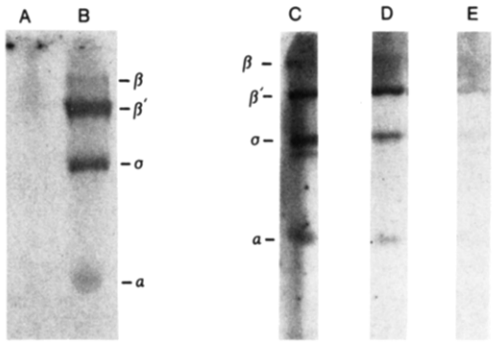


FIGURE 6: Photoincorporation of [γ-$^{32}$P]-8-$N_3$ATP into various subunits and protection against photoincorporation by ATP. Subunits were separated on low SDS–polyacrylamide gel (gel electrophoresis condition II). (A) Enzyme alone (0.1 μM), no light, 8-$N_3$ATP (20 μM); (B) enzyme alone (0.1 μM), photolysis at 25 °C for 3 min, 8-$N_3$ATP (25 μM); (C) enzyme alone, photolysis at 0 °C, 8-$N_3$ATP (20 μM); (D) enzyme plus 35 μM ATP, photolysis at 0 °C, 8-$N_3$ATP (20 μM); (E) enzyme plus 150 μM ATP, photolysis at 0 °C, 8-$N_3$ATP (20 μM). The TMK buffer containing βME (500 μM) was used throughout, and the irradiation time for photolysis was 3 min.

8-$N_3$ATP is not an inhibitor of UTP incorporation under our experimental conditions, and this is supported by the lack of a protective effect on the photoincorporation of [γ-$^{32}$P]-8-$N_3$ATP by UTP (Table III). If anything, photoincorporation may be slightly enhanced.

## Discussion

The advantage of 8-$N_3$ATP as a photoaffinity probe is the ability of the photochemically generated nitrene to react fairly nonselectively with the groups that are in the proximity of the active site, unlike chemical affinity labels that require particular nucleophiles or electrophiles.

Some of the basic requirements for a photoaffinity probe are as follows. (1) It must yield reversible kinetic parameters such as $K_m$ or competitive $K_i$ values that show that it is interacting with the active site. (2) It must absolutely require light for covalent incorporation. (3) It must exhibit saturation of photoincorporation commensurate with its binding constant. (4) Its photoincorporation must be prevented by concentrations of its natural counterpart, in agreement with the affinity of the natural compound for the active site.

We have demonstrated in Figure 1 that 8-$N_3$ATP is a competitive inhibitor of DNA-dependent RNA polymerase with a $K_i$ of approximately 42 μM. Covalent incorporation of 8-$N_3$ATP is light dependent (Figure 2) and shows saturation effects that are in agreement with its $K_i$ value (see Figure 3). The data in Tables II and III and Figure 6 demonstrate that ATP prevents [γ-$^{32}$P]-8-$N_3$ATP photoincorporation at concentrations within the micromolar range. Analysis of the ATP protection curve (figure not shown) shows that $K_m$ for protection is 50–60 μM.

Under our experimental conditions all of the subunits were labeled to some extent, with β′ and σ being labeled most heavily. This labeling pattern suggests that all the subunits are in close proximity of the active site or contain other nucleotide binding sites. The number of active-site amino acid residues that may react with a given photoaffinity probe is primarily dependent on two factors. First is the occupancy time within the active site relative to the reactivity of the generated nitrene, and the second is the geometry of the photoprobe–protein complex (i.e., is the reactive nitrene near a reactive or nonreactive residue or possibly held in the aqueous phase?). If a photoprobe is held rigidly within the active site, it will have potentially fewer residues to react with within the half-life of the nitrene than if it is loosely held. Loosely bound probes may be in several different positions when the nitrene is generated and finds a residue to react with.

8-$N_3$ATP binds to DNA-dependent RNA polymerase relatively loosely, as demonstrated by a $K_i$ value of 42 μM. It would be expected, therefore, that photoincorporation of [γ-$^{32}$P]-8-$N_3$ATP could occur in several amino acid residues within the binding region. These residues could exist on different subunits if more than one subunit makes up the binding site. The photoincorporation and protection data presented in Tables I and III support the hypothesis that β, β′, σ, and α are all near the binding region, with β′ and σ being labeled the most heavily and therefore representing a potentially more defined active site.

The fact that 8-$N_3$ATP is a competitive inhibitor of ATP incorporation in the elongation phase implies that β′ and σ may play a significant role in the elongation step. We have tabulated the existing data on the affinity labeling of RNA polymerase with affinity analogues whose functional groups are on the base in Table IV. It can be seen that the three previous studies utilizing reactive groups on the base have implicated both β and β′ subunits. In the various affinity labeling studies of RNA polymerase thus far reported, all of the subunits have been implicated by labeling with one or more reagents. Current models for RNA polymerase based on small-angle neutron scattering (Stöckel et al., 1980a,b) and X-ray scattering (Meisenberger et al., 1981) have all the subunits in close contact, with the possible exception of the α subunit. Thus, we may anticipate close interaction among the subunits, and the active site may involve all of the subunits to a greater or lesser degree.

Our results on the photoaffinity labeling pattern are consistent with this model and indicate that β′ and σ are extensively involved in the makeup of the active site. We are in the process of locating the exact binding site of the photolabel in each subunit.

Our kinetic results on a poly[d(A-T)] template with ApU as an initiator show that 8-$N_3$ATP inhibits ATP incorporation only. The effect of ATP and UTP on the photoincorporation of [γ-$^{32}$P]-8-$N_3$ATP supports the kinetic data (Table III). This suggests that 8-$N_3$ATP can only bind to the elongation site when a T is present in the template site, not when an A is present. An alternative interpretation is that two nucleotide binding sites participate in elongation, one specific for purines, the other for pyrimidines.

**Registry No.** 8-$N_3$ATP, 53696-59-6; ATP, 56-65-5; RNA polymerase, 9014-24-8.

References


Armstrong, V. W., & Eckstein, F. (1979) *Biochemistry 18*, 5117–5122.

Armstrong, V. W., Sternbach, H., & Eckstein, F. (1976) *Biochemistry 15*, 2086–2091.

Burgess, R. R. (1969) *J. Biol. Chem. 244*, 6168–6176.

Burgess, R. R., & Jendrisak, J. J. (1975) *Biochemistry 14*, 4634–4638.

Burton, Z. F., Burgess, R. R., Lin, J., Moore, D., Holder, S., & Gross, C. (1981) *Nucleic Acids Res. 9*, 2889–2903.

Cornish-Bowden, A. (1979) *Fundamentals of Enzyme Kinetics*, pp 152–154, Butterworths, London.

Czarnecki, J., Geahlen, R., & Haley, B. E. (1979) *Methods Enzymol. 56*, 642–653.

DeReimer, L. H., & Meares, C. F. (1981) *Biochemistry 20*, 1612–1617.

Downey, K. M., & So, A. G. (1970) *Biochemistry 9*, 2520–2525.

Evans, F. E., & Kaplan, N. O. (1976) *J. Biol. Chem. 251*, 6791–6797.

Fisher, R., & Blumenthal, T. (1980) *J. Biol. Chem. 255*, 11046–11062.

Frischauf, A. M., & Scheit, K. H. (1973) *Biochem. Biophys. Res. Commun. 53*, 1227–1233.

Haley, B. E., & Hoffman, J. F. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 3367–3371.

Heinz, E., & Hoffman, J. F. (1969) *J. Cell. Comp. Physiol. 65*, 31–43.

Hillel, Z., & Wu, C.-W. (1978) *Biochemistry 17*, 2954–2961.

Hinkle, D. C., & Chamberlin, M. J. (1972) *J. Mol. Biol. 70*, 157–185.

Knowles, J. R. (1972) *Acc. Chem. Res. 5*, 155–160.

Leach, S. J., & Scheraga, H. A. (1960) *J. Am. Chem. Soc. 82*, 4790–4792.

Malcolm, A. D. B., & Moffat, J. R. (1978) *Biochem. J. 175*, 189–192.

Meisenberger, O., Huemann, H., & Pilz, I. (1981) *FEBS Lett. 123*, 22–24.

Miller, J. A., Serio, G. G., Bear, J. L., Howard, R. A., & Kimball, A. P. (1980) *Biochim. Biophys. Acta 612*, 286–294.

Nixon, J., Spoor, T., Evans, J., & Kimball, A. (1972) *Biochemistry 11*, 4570–4572.

Ovchinnikov, Yu. A., Lipkin, V. M., Modyanov, N. N., Chertov, O. Yu., & Smirnov, Yu. V. (1977) *FEBS Lett. 76*, 108–110.

Ovchinnikov, Yu., Monastryskara, G., Gubanov, V., Guriev, S., Chertov, O., Modyanov, N., Grikevich, V., Marchenko, T., Polovnikova, F., Lipkin, V., & Sverdlov, E. (1981) *Eur. J. Biochem. 116*, 621–629.

Owens, J. R., & Haley, B. E. (1976) *J. Supramol. Struct. 5*, 81–102.

Paetkau, V., & Coy, G. (1972) *Can. J. Biochem. 50*, 142–150.

Potter, R. L., & Haley, B. E. (1982) *Methods Enzymol. 91*, 613–633.

Reisbig, R. R., Woody, A-Y. M., & Woody, R. W. (1979) *J. Biol. Chem. 254*, 11208–11217.

Scheurich, P., Schäfer, H. J., & Dose, K. (1978) *Eur. J. Biochem. 88*, 253–257.

Stender, W., Stüts, A. A., & Scheit, K. H. (1976) *Eur. J. Biochem. 56*, 129–136.

Stöckel, P., May, R., Strell, I., Cejka, Z., Hoppe, W., Huemann, H., Zillig, W., & Crespi, H. (1980a) *Eur. J. Biochem. 112*, 411–417.

Stöckel, P., May, R., Strell, I., Cejka, Z., Hoppe, W., Huemann, H., Zillig, W., & Crespi, H. (1980b) *Eur. J. Biochem. 112*, 419–423.

Sverdlov, E. D., Tsarev, S. A., Modyanov, N. N., Lipkin, V. M., Grachev, M. S., Zaychikov, E. F., & Plentnyov, A. G. (1978) *Bioorg. Khim. 4*, 1278–1280.

Sverdlov, E. D., Tsarev, S. A., & Kuznetsova, N. F. (1980) *FEBS Lett. 112*, 296–298.

Wu, F. Y.-H., & Wu, C.-W. (1974) *Biochemistry 13*, 2562–2565.