Affinity Labeling of the 3′-OH Terminal Binding Site of the Ribonucleic Acid Chain on Deoxyribonucleic Acid Dependent Ribonucleic Acid Polymerase from *Escherichia coli*†

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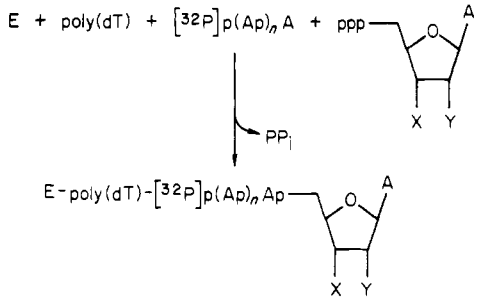
ABSTRACT: Nucleoside triphosphates modified at the 3′-OH are chain terminators for RNA polymerase. They form inactive ternary complexes with the enzyme, poly(dT), and oligoadenylate, the stabilities of which depend upon the length of the oligonucleotide. Employing [5′-^{32}P]p(Ap)$_{10}$A, together with the reactive analogues 3′-(bromoacetamido)-3′-deoxyadenosine triphosphate or 3′-(isothiocyanato)-2′,3′-dideoxyadenosine triphosphate, as well as 3′-amino-3′-deoxyadenosine triphosphate, followed by cross-linking with glyoxal, we labeled RNA polymerase primarily at the β' subunit. The latter therefore appears to contain at least in part the 3′-OH terminus of the nascent RNA chain when the enzyme is in the form of the ternary complex.

Our understanding of the roles of the various subunits in procaryotic DNA-dependent RNA polymerase is still far from complete [for reviews see Zillig et al. (1976) and Krakow et al. (1976)]. The technique of affinity labeling has been employed to determine the subunits(s) containing the catalytic center of this enzyme. Labeling of the β subunit was observed with periodate oxidized nucleosides (Nixon et al., 1972; Wu & Wu, 1974) and with the modified nucleoside triphosphates α-5-formyluridine triphosphate (Armstrong et al., 1976) and 4-thiouridine triphosphate (Frischauf & Scheit, 1973). In the case of the latter compound, labeling of the β' subunit was also observed, but this was suppressed in the presence of poly[d-(A-T)].[1] In contrast to the nucleosides, the periodate oxidized nucleoside triphosphates were found to primarily label the α subunit (Malcolm & Moffat, 1978).

These investigations have involved interaction of the respective affinity label with the enzyme alone or in some cases with the binary complex, that is, the enzyme–DNA complex. They do not provide information as to the situation in the actively transcribing ternary complex: enzyme–DNA–product RNA. Recently, an affinity labeling method involving this complex has been reported (Sverdlov et al., 1978), in which photolabile γ-phosphate-modified triphosphates were employed as initiators for RNA polymerase. With various triphosphate combinations, short oligonucleotide sequences could be synthesized at different DNA promoters, and after photolysis their location on the enzyme could be determined. Under the conditions employed, α, σ, and β/β' were labeled, the extent depending upon the promoter employed and the length of the oligonucleotide sequence. Although this procedure does not affinity label the active site directly, since the photoreactive group is attached to the 5′ end of the growing RNA chain, it should provide useful information as to the topography of the subunits in the vicinity of the catalytic center. We have also devised an affinity labeling procedure utilizing the catalytic competence of RNA polymerase but which selectively labels the 3′-OH terminus of the product RNA chain. It is based on the observations that 3′-modified nucleoside triphosphates, such as 3′-deoxyadenosine triphosphate (3′-dATP), are chain terminators for RNA polymerase (Shigeura & Boxer,1964) and that a stable chain terminated ternary complex can be isolated over a gel column (Sentenac et al., 1968).

Scheme I



(a) Y = OH; X = $NHCOCH_2Br$
(b) Y = H; X = NCS
(c) Y = OH; X = NH_2; cross-link with glyoxal

Our approach is outlined in Scheme I. Incorporation of a suitably reactive 3′-modified ATP analogue into a radioactively labeled oligoadenylate primer (Van Kreijl et al., 1977) in the presence of poly(dT) and RNA polymerase leads to an inactive ternary complex, in which the reactive functional group can react at the 3′-OH terminus site of the product RNA. This is similar to the suicidal inactivation of DNA polymerase I observed with 2′,3′-epoxide 5′-triphosphate (Aboud et al., 1978), the latter elongating the DNA chain and then reacting with the enzyme. Three different functional groups were employed for the affinity labeling of RNA polymerase: (a) the bromoacetylamido group; (b) the isothiocyanate group; (c) the amino group followed by cross-linking to the enzyme with glyoxal or acrolein. We have concentrated our studies on RNA polymerase core enzyme, since this is the form of the enzyme during elongation.

Experimental Procedure

Materials

Polynucleotide kinase and $(Ap)_4A$ were purchased from Boehringer Mannheim; $(Ap)_{10}A$ was a generous gift of Dr.

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[1] Abbreviations used: poly(dT), poly(deoxythymidylic acid); poly[d-(A-T)], copolymer of alternating deoxyadenylate and thymidylate; $(Ap)_4A$, [adenylyl(3′–5′)]$_4$adenosine; $(Ap)_{10}A$, [adenylyl(3′–5′)]$_{10}$-adenosine; DTE, dithioerythritol.

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G. Weimann (Boehringer). Poly(dT) was a product of Miles Laboratories. 3′-Amino-3′-deoxyadenosine triphosphate was a kind gift of Dr. M. Morr, and 3′-amino-2′,3′-dideoxyadenosine triphosphate (Imazawa & Eckstein, 1978) was generously donated by Dr. M. Imazawa. 3′-(Bromoacetamido)-3′-deoxyadenosine triphosphate was synthesized from the respective 3′-amino derivative by using the *N*-hydroxysuccinimide ester of bromoacetic acid (Wilchek & Givol, 1977). 3′-(Isothiocyanato)-2′,3′-dideoxyadenosine triphosphate was obtained from reaction of the corresponding 3′-amino compound with *N,N′*-(thiocarbonyl)diimidazole (Staab & Walther, 1962). Full details of the syntheses of these two nucleotide analogues will be published elsewhere. [γ-^{32}P]-3′-Amino-3′-deoxyadenosine triphosphate was synthesized by the method of Glynn & Chappell (1964).

Methods

Enzyme Purification and Assay. RNA polymerase holoenzyme was a generous gift of Dr. H. Sternbach (Sternbach et al., 1975). The core enzyme was isolated according to Burgess (1969) over a phosphocellulose column. Enzyme activity was assayed in a reaction mixture (0.1 mL) containing 40 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 50 mM KCl, 0.1 mM EDTA, 0.1 mM DTE, 1.25 A_{260} units of poly(dT), and 1 mM [^{14}C]ATP (2000 cpm/nmol). Incubation was at 37 °C, and a 50-μL aliquot was removed after 5 min and streaked onto a Whatman 3 MM paper strip (2 × 13.5 cm) which had been pretreated with 50 μL of 0.3 M EDTA. The strip was then developed in 1 M ammonium acetate–ethanol (1:1 v/v) by descending chromatography. After drying, the origin (2 × 2 cm) was cut out and the radioactivity measured in 6 mL of a toluene-based scintillation fluid.

Synthesis of [5′-^{32}P]Oligoadenylates. The procedure of Richardson (1965) was used to phosphorylate $(Ap)_4A$ and $(Ap)_{10}A$ at the 5′ position. The oligoadenylate (50–100 nmol) was incubated in a solution (2 mL) containing 100 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 300 nmol of [γ-^{32}P]ATP (200–600 cpm/pmol), and 50 units of polynucleotide kinase for 60 min at 37 °C. Reaction was stopped by heating in a boiling water bath for 2 min. In the case of $(Ap)_4A$, the product was purified by concentrating the solution to approximately 0.4 mL and applying to Schleicher & Schüll 2043b paper. After developing in 1-propanol–ammonia–water (55:10:35 v/v/v) by descending chromatography, we eluted the product from the paper. The solution from the $(Ap)_{10}A$ reaction was applied directly to a Bio-Gel P6 column (30 × 3 cm) equilibrated in 50 mM triethylammonium bicarbonate (TEAB) solution. After elution with the same buffer, the first peak from the column was collected, concentrated, and rechromatographed over a Sephadex G-50 column (110 × 1 cm) equilibrated with 50 mM TEAB.

Determination of Radioactive Label Covalently Bound to Core Enzyme. The enzyme was incubated in a solution containing 40 mM Bicine, pH 8.0, 10 mM $MgCl_2$, 50 mM KCl, 0.1 mM EDTA, 5 A_{260} units/mL poly(dT), labeled oligoadenylate, and 3′-modified nucleotide as described in the legends to the figures. Aliquots of the labeled enzyme were then diluted in 1 mL of 20 mM Tris-HCl, pH 8.0, and 50 mM KCl and filtered over a nitrocellulose filter (Schleicher & Schüll; 0.45 μm, 2-mm diameter). The filters were washed with 10 mL of the same buffer and were then stirred for 30 min in 6 M urea, 20 mM Tris-HCl, pH 8.0, and 250 mM KCl (two changes of buffer) and finally for 10 min in the previous buffer system. After drying, we measured the amount of radioactivity bound to the filters in 6 mL of a toluene-based scintillation fluid.

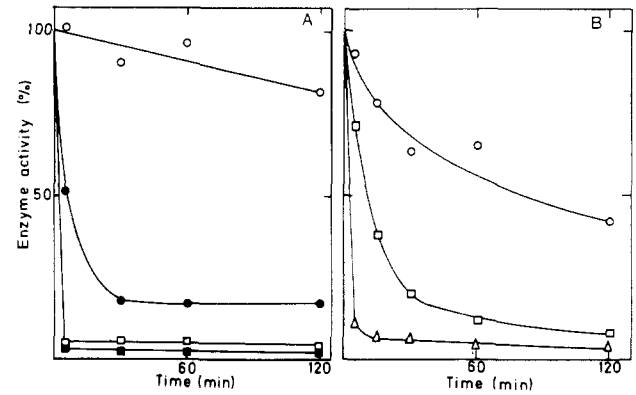

FIGURE 1: (A) Formation of an inactive ternary complex with 3′-amino-3′-deoxyadenosine triphosphate and oligoadenylates of different length. The enzyme (27 μg) was incubated in a solution (0.1 mL) containing 40 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 50 mM KCl, 0.1 mM DTE, 0.1 mM EDTA, 0.5 A_{260} unit of poly(dT), 0.05 mM 3′-amino-3′-deoxyadenosine triphosphate, and 1 nmol of $(Ap)_4A$ (●, ○) or 0.6 nmol of $(Ap)_{10}A$ (■, □). Aliquots were removed after various times, and enzyme activity was determined as described under Methods, either in the absence (closed symbols) or in the presence (open symbols) of 1 nmol of the respective oligoadenylate. (B) Formation of an inactive ternary complex with 2′-deoxy analogues and $(Ap)_{10}A$. The enzyme was incubated as described above but omitting the 3′-amino-3′-deoxyadenosine triphosphate and including 0.6 nmol of $(Ap)_{10}A$ and either (a) 0.2 mM 3′-amino-2′,3′-dideoxyadenosine triphosphate (△), (b) 0.2 mM 3′-(isothiocyanato)-2′,3′-dideoxyadenosine triphosphate (□), or (c) 0.2 mM 2′-dATP (○). The enzyme was assayed after various times, as described above. 100% activity corresponded to 4–5 nmol of triphosphate polymerized per μg of enzyme.

Cellulose Acetate Electrophoresis of Labeled Enzyme. Labeled core enzyme was separated into its subunits by electrophoresis (Rabussay & Zillig, 1969) on cellulose acetate strips (5.7 × 14 cm; Chemetron). The electrophoresis was performed in 6 M urea, 0.15 M ammonium bicarbonate, pH 9.0, 0.01 M magnesium acetate, 0.02 M 2-mercaptoethanol, 1 mM EDTA, and 5% glycerol (v/v) at 500 V for 3–4 h. After staining and destaining, we cut the strips into 2- or 3-mm sections and determined the radioactivity in 10 mL of Brays solution.

Results

Formation of an Inactive Ternary Complex. Oligoadenylates are able to function as primers for the transcription of poly(dT) by RNA polymerase (Van Kreijl et al., 1977). We therefore chose to investigate the effect on enzyme activity of preincubation of 3′-amino-3′-deoxyadenosine triphosphate and either $(Ap)_4A$ or $(Ap)_{10}A$ together with core enzyme and poly(dT). After addition of 3′-amino-3′-deoxyadenosine triphosphate to an enzyme–poly(dT)–primer complex, aliquots of the reaction mixture were removed after various times and the enzyme activity was determined.

In the case of $(Ap)_4A$, a gradual loss in activity was observed, but this could be mitigated by the presence of $(Ap)_4A$ in the assay solution (Figure 1A). This suggests an unstable ternary complex, the 3′-aminoadenylate being incorporated into the 3′ end of the $(Ap)_4A$ to yield an inactive primer with which the excess unmodified $(Ap)_4A$ competes in the assay.

With $(Ap)_{10}A$, enzyme activity was rapidly reduced to less than 5% of the initial value and remained as such over a period of at least 2 h (Figure 1A). Excess $(Ap)_{10}A$ in the enzyme assay did not affect these results, indicating the stability of the inactivated ternary complex. This was further substantiated by the following experiment. The inactivated ternary complex was formed as above and then diluted with the assay

Table I: Turnover of Pyrophosphate with [γ-^{32}P]-3′-Amino-3′-deoxyadenosine Triphosphate, Oligo(A), Poly(dT), and Core Enzyme[a]

oligo(A)	nmol of PP_i
	2.3
$(Ap)_{10}A$	2.1
$(Ap)_4A$	11.0

[a] The core enzyme (27 μg) was incubated at 37 °C in a solution (0.2 mL) containing 40 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 50 mM KCl, 0.1 mM DTE, 0.1 mM EDTA, 2.5 A_{260} units of poly(dT), 50 nmol of [γ-^{32}P]-3′-NH_2-ATP and 10 nmol of oligo(A). Aliquots were removed after 120 min, and the amount of non-charcoal-adsorbable radioactivity was determined (Krakow & Fronk, 1969).

mixture containing excess $(Ap)_{10}A$ but omitting the [^{14}C]ATP. Incubation was continued at 37 °C for 60 min, and then the enzyme was assayed by addition of the triphosphate. No recovery in activity was observed. Suitable controls in which 3′-amino-3′-deoxyadenosine triphosphate was omitted indicated that the enzyme normally retained full activity under these conditions.

Release of [^{32}P]Pyrophosphate. Further evidence for the differing stabilities of the $(Ap)_4A$ and $(Ap)_{10}A$ ternary complexes was obtained by use of [γ-^{32}P]-3′-amino-3′-deoxyadenosine triphosphate in formation of the ternary complex, i.e., in the latter's stability. Core enzyme, poly(dT), and either $(Ap)_4A$ or $(Ap)_{10}A$ were incubated together with the labeled nucleotide, and the inorganic [^{32}P]pyrophosphate released was determined as non-charcoal-adsorbable material. The results are presented in Table I.

In the absence of primer, a small release of phosphate occurred over the 2-h period. The figure obtained with $(Ap)_{10}A$ hardly differed from that of the background, whereas almost 11 nmol of radioactive pyrophosphate was produced in the presence of $(Ap)_4A$, amounting to approximately 87% (after correction for the background) of the oligonucleotide employed and corresponding to approximately 129 turnovers of the substrate. The assay would not have been sensitive enough to detect a low turnover with the longer primer. It may therefore be inferred that the ternary complex formed with $(Ap)_4A$ is far less stable than that observed by using $(Ap)_{10}A$. Rifampicin completely suppressed the formation of pyrophosphate with $(Ap)_4A$ (data not shown), in accord with the observations of McClure & Cech (1978) that only di- and trinucleotides can be synthesized in the presence of this antibiotic.

Labeling of RNA Polymerase with 3′-(Bromoacetamido)-3′-deoxyadenosine Triphosphate. For the purposes of the affinity labeling experiments, $(Ap)_{10}A$ and $(Ap)_4A$ were phosphorylated at the 5′ position with [^{32}P]phosphate by using [γ-^{32}P]ATP and polynucleotide kinase (Richardson, 1965). In a series of control experiments, $p(Ap)_{10}A$ was shown to form an inactive ternary complex with 3′-(bromoacetamido)-3′-deoxyadenosine triphosphate when incubated with core enzyme and poly(dT), the data (not shown) being virtually identical with those observed for $(Ap)_{10}A$ and 3′-amino-3′-deoxyadenosine triphosphate. The amount of label covalently bound to the enzyme on incubation with the labeled oligonucleotide, poly(dT), and the bromoacetyl analogue was determined on a nitrocellulose filter (Krakow & Goolsby, 1971; Armstrong et al., 1976) as described under Experimental Procedure. It was necessary to remove noncovalently bound material by stirring the filter in a 6 M urea solution in order to denature the enzyme and dissociate the ternary complex. The protein remained bound to the filter under these conditions as was shown by filtering known quantities of the enzyme and determining the amount of protein bound before and after the urea treatment by the method of Schaffner & Weissmann (1973). Less than 5% of the enzyme was lost from the filter under these conditions.

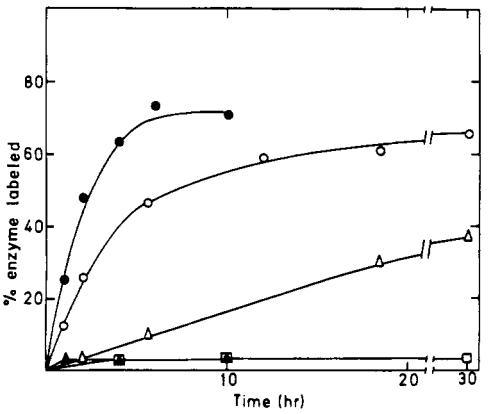

FIGURE 2: Incorporation of label into core polymerase with 3′-(bromoacetamido)-3′-deoxyadenosine triphosphate or 3′-(isothiocyanato)-2′,3′-dideoxyadenosine triphosphate. Enzyme (132 μg) was incubated in a solution (0.4 mL) as described under Methods containing, in addition, (a) 0.2 mM 3′-(bromoacetamido)-3′-deoxyadenosine triphosphate and 1.6 nmol of [^{32}P]$p(Ap)_{10}A$ (○), (b) 0.2 mM 3′-(bromoacetamido)-3′-deoxyadenosine triphosphate and 1.8 nmol of [^{32}P]$p(Ap)_4A$ (△), (c) 0.2 mM 3′-(bromoacetamido)-3′-deoxyadenosine triphosphate, 0.1 mM 3′-amino-3′-deoxyadenosine triphosphate, and 1.6 nmol of [^{32}P]$p(Ap)_{10}A$ (□), (d) 0.2 mM 3′-(isothiocyanato)-2′,3′-dideoxyadenosine triphosphate and 1.1 nmol of [^{32}P]$p(Ap)_{10}A$ (●), and (e) 0.2 mM 3′-(isothiocyanato)-2′,3′-dideoxyadenosine triphosphate, 0.1 mM 3′-amino-3′-deoxyadenosine triphosphate, and 1.1 nmol of [^{32}P]$p(Ap)_{10}A$ (▲).

The results of the labeling experiments are given in Figure 2. Employing [^{32}P]$p(Ap)_{10}A$, label was covalently incorporated into the enzyme until approximately 60% of the protein had been modified. Complete labeling of the enzyme was not achieved, possibly due to the protein not initially containing 100% active enzyme or due to some inactivation of the enzyme by the nucleotide during the long incubation period. When 3′-(bromoacetamido)-3′-deoxyadenosine triphosphate at a concentration of 1.8 mM was incubated with enzyme alone, a 50% inactivation was observed in 4.5 h. As would be expected, 3′-amino-3′-deoxyadenosine triphosphate suppressed the amount of label incorporated. With the shorter primer, [^{32}P]$p(Ap)_4A$, the rate of incorporation of label was much lower compared to that of [^{32}P]$p(Ap)_{10}A$. Presumably, this reflects the decreased stability of the ternary complex with the former. Furthermore, it is also an indication that unspecific labeling caused by reaction of the bromoacetylated oligonucleotides at sites on the enzyme other than the active site is low. Greater unspecific labeling would be expected with [^{32}P]$p(Ap)_4A$ since higher concentrations of modified oligonucleotide will be produced in this case.

The location of the label on the various subunits of RNA polymerase was ascertained by cellulose acetate electrophoresis (Rabussay & Zillig, 1969) of the labeled enzyme, as described under Experimental Procedure. The majority of the label was found to migrate with the β' subunit (Figure 3), although the radioactive peak was displaced slightly in front of the major protein band. This phenomenon was also observed by Sverdlov et al. (1978) and may be explained by the extra negative charges that the labeled protein carries on account of the attached oligonucleotides. Small amounts of label were also associated with the α and β subunits, but this amounted to less than 20% of the total.

Labeling of RNA Polymerase with 3′-(Isothiocyanato)-2′,3′-dideoxyadenosine Triphosphate. We had originally in-

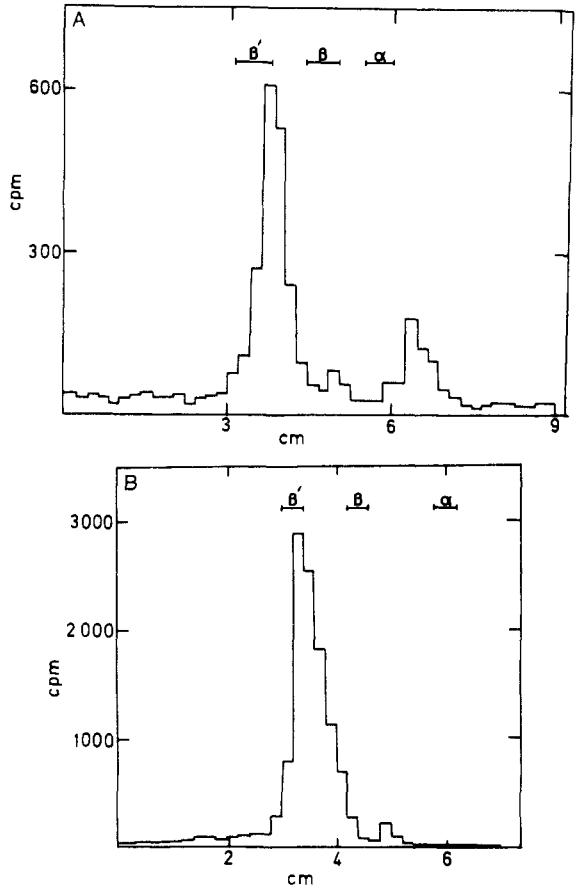

FIGURE 3: (A) Identification of the subunit(s) labeled by using 3′-(bromoacetamido)-3′-deoxyadenosine triphosphate. Enzyme (340 μg) was incubated for 5 h at 37 °C in a solution (0.5 mL) as described under Methods, which also contained 0.2 mM 3′-(bromoacetamido)-3′-deoxyadenosine triphosphate and 2 nmol of [^{32}P]p(Ap)$_{10}$A. The reaction was stopped by precipitation of the enzyme with an equal volume of saturated ammonium sulfate, pH 8.0. After centrifugation, the supernatant was discarded, and the pellet was dissolved in 0.05 mL of 20 mM Tris-HCl, pH 8.0, and 30 mM KCl. The enzyme solution was dialyzed for 2 h against the same buffer and was then subject to cellulose acetate electrophoresis as described under Methods. (B) Identification of the subunit(s) labeled by using 3′-(isothiocyanato)-2′,3′-dideoxyadenosine triphosphate. The procedure was the same as that described above except that the bromoacetyl analogue was replaced by 0.2 mM 3′-(isothiocyanato)-2′,3′-dideoxyadenosine triphosphate and incubation was for 4 h at 37 °C.

tended to use the corresponding ribose derivative, 3′-(isothiocyanato)-3′-deoxyadenosine triphosphate, as a potential affinity label. However, in this compound the isothiocyanate group forms an unreactive thiocarbamate with the neighboring 2′-hydroxyl (details will be published elsewhere). Since 2′-deoxynucleoside triphosphates are known to be substrates for RNA polymerase (Hurwitz et al., 1972), albeit poor ones, we decided to investigate the deoxy derivative, 3′-(isothiocyanato)-2′,3′-dideoxyadenosine triphosphate. The ability of this triphosphate to form an inactive ternary complex with core enzyme, poly(dT), and (Ap)$_{10}$A was initially tested (Figure 1B) along with 3′-amino-2′,3′-dideoxyadenosine triphosphate and 2′-deoxyadenosine triphosphate (2′-dATP). The amino derivative rapidly formed an inactive complex in contrast to 2′-dATP which only caused a slow reduction in enzyme activity. Either the ternary complex with 2′-dATP is much less stable than that with the amino derivative or the former is a much poorer substrate than the latter. This was not investigated further. The isothiocyanate also formed an inactive ternary complex, although at a slower rate than the amino compound. By use of [^{32}P]p(Ap)$_{10}$A and 3′-(isothiocyanato)-2′,3′-dideoxyadenosine triphosphate, the rate of incorporation of label into enzyme was determined (Figure 2) as described for the 3′-bromoacetyl analogue. The rate of labeling was slower than the rate of inactivation (Figure 1), indicating that the latter was due to the initial formation of a ternary complex, followed by a slower chemical reaction at the active site. Approximately 70–80% of the protein could be labeled, and protection was afforded by 3′-amino-3′-deoxyadenosine triphosphate. The subunit labeled was determined as described previously and was again found to be β′ (Figure 3B).

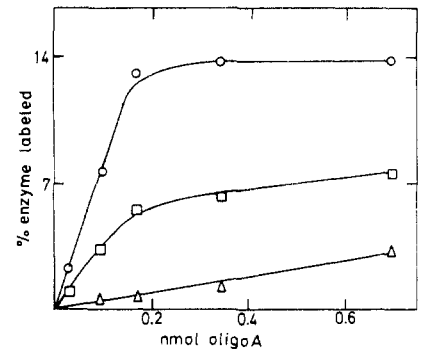

FIGURE 4: Incorporation of label into core polymerase using 3′-amino-3′-deoxyadenosine triphosphate and glyoxal. The standard labeling conditions were employed, as described under Methods. The incubation solution (0.05 mL) contained, in addition, core enzyme (34 μg), 0.05 mM 3′-amino-3′-deoxyadenosine triphosphate, and (a) [^{32}P]p(Ap)$_{10}$A (○), (b) [^{32}P]p(Ap)$_{10}$A and 0.05 mM 3′-deoxyadenosine triphosphate (□), and (c) [^{32}P]p(Ap)$_4$A (△). After 15 min at 37 °C, 5 μL of a 2 M glyoxal solution was added and incubation was continued for a further 5 min. The amount of label bound to the enzyme was then determined as described under Methods.

Labeling of RNA Polymerase with 3′-Amino-3′-deoxyadenosine Triphosphate and Glyoxal. The inactive ternary complex with a 3′-amino-modified nucleotide at the 3′-OH terminal position of the RNA chain provided a further opportunity for the affinity labeling of RNA polymerase. It was envisaged that a suitable cross-linking reagent such as acrolein or glyoxal would covalently couple the modified oligonucleotide to the active site of the enzyme. The data obtained with glyoxal are shown in Figure 4. A high concentration (0.2 M) of glyoxal was required in order to observe significant labeling of the enzyme. At a ratio of 2:1 of [^{32}P]p(Ap)$_{10}$A/enzyme, no further increase in the percentage of enzyme labeled occurred, indicating the enzyme to be saturated with oligonucleotide. Similar data (not shown) were obtained with acrolein, although in this case approximately 22% of the enzyme was labeled under saturating conditions. The amount of enzyme labeled in both cases was, however, rather low compared to that of the bromoacetyl and isothiocyanate analogues. This presumably depends upon the rate at which the oligonucleotide is linked to the enzyme and the rate at which dissociation of the ternary complex occurs due to modification and inactivation of the enzyme at the high concentration of the reactive species employed. When [^{32}P]p(Ap)$_4$A was used instead of [^{32}P]p(Ap)$_{10}$A, the extent of labeling was much reduced (Figure 4), providing evidence for the specificity of the labeling in the case of the latter, as was described for the bromoacetyl analogue. 3′-dATP also suppressed the incorporation of label with [^{32}P]p(Ap)$_{10}$A and 3′-amino-3′-deoxyadenosine triphosphate, presumably competing with the latter for incorporation into the primer.

The cross-linking with glyoxal must occur through formation of Schiff's bases. It was, however, not necessary to reduce with sodium borohydride, as is usual, the data obtained both with

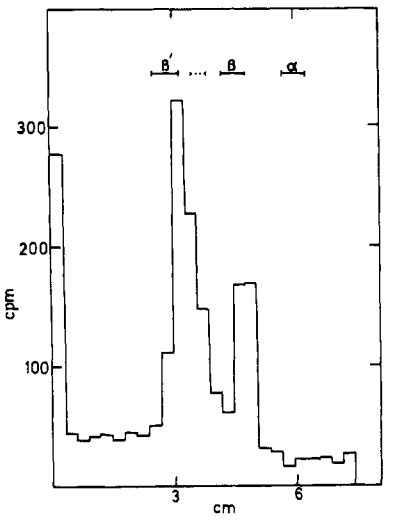

FIGURE 5: Location of subunit(s) labeled by using 3′-amino-3′-deoxyadenosine triphosphate and glyoxal. Enzyme (270 μg) was incubated under the standard labeling conditions as described under Methods. The incubation solution (0.05 mL) contained, in addition, 0.05 mM 3′-amino-3′-deoxyadenosine triphosphate and 70 nmol of [^{32}P]p(Ap)$_{10}$A. After 15 min at 37 °C, 0.05 mL of a 2 M glyoxal solution was added and incubation was continued at 37 °C for a further 5 min. The enzyme was then precipitated, and the enzyme was subjected to cellogel electrophoresis as described in the legend to Figure 3.

and without treatment of this reagent being identical. Possibly, the conjugation afforded by this system stabilizes the Schiff's bases, or further reaction with other groups on the enzyme occurs.

Bifunctional reagents are normally employed to cross-link multisubunit enzymes, and it was therefore necessary to investigate the effect of acrolein and glyoxal on RNA polymerase. $NaDodSO_4$ gel electrophoresis of the enzyme after treatment with 0.2 M acrolein demonstrated that this reagent was extremely effective at cross-linking the enzyme; hardly any protein was observed to migrate into a 5% gel. It was therefore not possible to use this reagent to locate the labeled subunit. Glyoxal, on the other hand, produced very little cross-linking of the enzyme.

Cellulose acetate electrophoresis of the enzyme labeled by the glyoxal procedure once again indicated that the β′ subunit was the main target for the affinity labeling, although a small proportion of the label was also associated with the β subunit (Figure 5). A faint protein band was observed between the β′ and β bands, possibly representing cross-linked subunits; this band was also seen when enzyme alone was treated with glyoxal.

Discussion

RNA polymerase exists as a ternary complex during chain elongation consisting of enzyme, double-stranded helical DNA, and the nascent RNA. Such complexes are extremely stable to salt dissociation and insensitive to the effects of inhibitors of the free enzyme or binary complexes [for a review see Krakow et al. (1976)]. In the case of single-stranded RNA, however, the complex is sensitive to the effects of heparin (Walter et al., 1967), and extensive termination and reinitiation of RNA chains occur (Maitra et al., 1967). Nevertheless, under the conditions that we employed, the inactive ternary complex, formed after single addition of a 3′-modified adenylate residue to an oligoadenylate primer in the presence of poly(dT), was extremely stable to dissociation, provided that the oligoadenylate was of sufficient length.

In the present affinity labeling procedure, introduction of the radioactive label into the primer rather than the modified ATP analogue containing the reactive species offers obvious advantages with regard to the specificity of labeling. Most importantly, only active RNA polymerase molecules able to incorporate this analogue into the primer will be labeled. The differing stabilities of the ternary complexes formed from $(Ap)_4A$ and $(Ap)_{10}A$ also provide further evidence for the specificity. If this labeling were to be unspecific, it would be expected to occur to a greater extent with the shorter primer since the higher turnover of substrate in this case would produce a higher concentration of modified oligonucleotide in the enzyme solution. In actual fact, the labeling observed by using the shorter oligonucleotide was always much less than that with the longer.

The β′ subunit was the primary target for modification in all three cases of 3′-modified nucleotide that we employed, suggesting that the 3′-OH binding site is located on this subunit. From previous studies it has been postulated that the initiation site, and therefore the 3′-OH binding site for the nascent RNA chain, is on the β subunit (Nixon et al., 1972; Wu & Wu, 1974). This supposition derives from kinetic studies with the nucleosides 6-(methylmercapto)purine ribonucleoside, its periodate-oxidized analogue, and 6-(methylmercapto)purine arabinonucleoside (Spoor et al., 1970). The ribose derivative and its oxidized analogue were observed to be noncompetitive inhibitors of RNA polymerase, whereas the arabinonucleoside was a competitive inhibitor. The conclusion was drawn that the latter must therefore bind to the elongation site of RNA polymerase and the ribonucleoside must bind to the initiation site. However, in the case of the oxidized analogue, the feature which is supposed to be specific for the binding to this site, viz. the cis diol moiety, is no longer present, and the fact that this derivative also produces noncompetitive inhibition does not necessarily imply that it also binds to the same site. As we have already noted (Armstrong et al., 1976), kinetic studies with aldehydes may be complicated by their ability to form Schiff's bases, the equilibrium of formation of which would influence the type of inhibition observed. The evidence that the β subunit may also contain in part the initiation site is therefore as yet insufficient; protection studies with purine triphosphates which bind specifically to this site (Wu & Goldthwait, 1969) may help to resolve this problem. Affinity labeling experiments with modified nucleoside 5′-triphosphates (Frischauf & Scheit, 1973; Armstrong et al., 1976) have demonstrated that there is a triphosphate binding site, possibly the elongation site, located on the β subunit. This subunit is also involved in the binding of rifampicin and streptolydigin [see references in Zillig et al. (1976)], inhibitors of RNA polymerase, and this was confirmed in the case of the former by affinity labeling (Stender et al., 1975). The β′ subunit is the only subunit that can bind the DNA template in the absence of the other subunits [see references in Zillig et al. (1976)], and this was the subunit labeled on photolysis of the enzyme with a modified template, poly(deoxy-4-thymidylic acid) (Frischauf & Scheit, 1973). On photolysis of unspecific complexes of T7 DNA and RNA polymerase (Hillel & Wu, 1978), the β, β′, and σ subunits were found to be attached to the template, but when specific complexes were employed in which the enzyme was bound to the RNA polymerase promoter sites only β and σ were labeled.

The results to date, therefore, implicate both the β and β′ subunits in some function of the catalytic mechanism of RNA polymerase. Since cross-linking experiments (King et al., 1974; Coggins et al., 1977; Hillel & Wu, 1977) have shown that

there is a large contact area betweeen the two subunits, we suggest that the catalytic center is located at the interface between the two. We would caution, however, that the various data have been derived from studies with different forms of the enzyme, i.e., free enzyme, enzyme–template binary complex, and ternary complex. The form of the active center and the subunits involved may differ, depending upon which stage of the transcription cycle the enzyme has reached. In the case of the ternary complex, our results show that the 3′-OH terminus of the nascent RNA chain is located at least in part on the β' subunit.

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