Fluorescent Affinity Labeling of Initiation Site on Ribonucleic Acid Polymerase of *Escherichia coli*†

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ABSTRACT: A fluorescent analog of 6-methylthioinosinedicarboxaldehyde (MMPR-OP) has been synthesized in which the methyl group is replaced by *N*-(acetylaminoethyl)-1-naphthylamine-5-sulfonate. This fluorescent nucleotide analog (AMPR-OP) is a much more potent inhibitor of DNA dependent RNA polymerase of *Escherichia coli* than MMPR-OP. The concentration of AMPR-OP required to inhibit 50% of RNA polymerase activity is 7×10^{-6} M as compared to 5×10^{-4} M for MMPR-OP. The noncompetitive inhibition of AMPR-OP with respect to nucleoside triphosphate suggests that AMPR-OP binds to a site on the enzyme involved in the initiation of RNA chains. The inhibition of DNA dependent [^{32}P]PP$_i$ exchange reaction by low concentrations of AMPR-OP further support the contention that this compound primarily inhibits the initiation of RNA chains. When RNA polymerase was incubated with excess AMPR-OP followed by $NaBH_4$ reduction, the dye was stoichiometrically bound to the enzyme. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the denatured, labeled enzyme indicates that AMPR-OP is covalently attached to the β subunit of the enzyme. Although the labeled enzyme is essentially inactive, fluorescence studies show that it still retains the ability to bind DNA template and nucleoside triphosphates. The binding of nucleoside triphosphates is presumably to the second nucleotide site (the polymerization site) on the enzyme and its specificity is dependent on the template. Furthermore, upon binding of the template and nucleoside triphosphates, the enzyme undergoes conformational changes. Energy transfer measurements indicate that the initiation site and rifampicin binding site are at least 37 Å apart. Thus the inhibitory effects of rifampicin on initiation of RNA chains is indirectly mediated through enzyme molecule.

Fluorescent probes have been used to provide insight into the structure, interactions, and dynamics of macromolecules. By systematically labeling multiple active sites of DNA dependent RNA polymerase of *Escherichia coli* with various fluorescent probes, we have examined the molecular mechanism of gene transcription (Wu and Wu, 1973a–c).

Recently, Spoor *et al.* (1970) have shown that the periodate oxidation product of 6-methylmercaptopurine ribonucleoside (MMPR-OP)[1] inhibited *Escherichia coli* RNA polymerase by covalently binding to the initiation site (the first NTP site, Wu and Goldthwait, 1969; or the product terminus site, Krakow and Fronk, 1969) on the enzyme. The binding site has been shown to be an ϵ-amino group of a lysine residue in the β subunit of RNA polymerase.

We report here the affinity labeling of *Escherichia coli* RNA polymerase with a fluorescent analog of MMPR-OP in which the methyl group is replaced by *N*-(acetylaminoethyl)-1-naphthylamine-5-sulfonate (AMPR-OP, Figure 1). Like MMPR-OP, AMPR-OP also binds to the initiation site on the β subunit. Although the AMPR-OP-labeled enzyme is catalytically inactive, it still interacts with DNA template and nucleoside triphosphates. A model of the active sites of RNA polymerase consistent with the fluorescence spectroscopic results is discussed. Furthermore, energy transfer measurements have been carried out to elucidate spatial and functional relationships between the initiation site on the enzyme and the binding site of rifampicin, which is a specific inhibitor of RNA chain initiation.

Materials and Methods

Materials. Unlabeled ribonucleoside triphosphates were purchased from P-L Biochemicals. ^{3}H-Labeled ribonucleoside triphosphates and ^{32}P-labeled sodium pyrophosphate were obtained from New England Nuclear Corp. Poly[d-(A-T)] and calf thymus DNA were products of Miles Laboratories, Inc., and Worthington Biochemical Corp., respectively. 6-Mercaptopurine ribonucleoside (6-MPR) was obtained from Cyclochemicals and sodium periodate from Fisher Scientific Co. Tricine, sodium borohydride, and dithiothreitol were purchased from Sigma. Unlabeled and [^{14}C]-rifampicin were gifts of Drs. R. White and L. Sylvestri of Gruppo-Lepetit Laboratories. *N*-(Iodoacetylaminoethyl)-1-naphthylamine-5-sulfonate (1,5 I-AENS) was synthesized by the method of Hudson and Weber (1973). Silica gel plates for thin-layer chromatography were obtained from Eastman Organic Chemicals. Sephadex G-75 was the product of Pharmacia Fine Chemicals. Inc.

RNA Polymerase. RNA polymerase was purified from *E. coli* as described by Wu and Wu (1973c). The enzyme was 98% pure, and contained all subunits (α, β, β', and σ) as shown by sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis.

RNA Polymerase Activity Assay. Enzyme activities of the labeled and unlabeled RNA polymerase were assayed by the

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[1] Abbreviations used are: NTP, nucleoside triphosphate; PP$_i$, inorganic pyrophosphate; 6-MPR, 6-mercaptopurine ribonucleoside; MMPR, 6-methylmercaptopurine ribonucleoside; MMPR-OP, oxidation product of MMPR, *i.e.*, methylthioinosinedicarboxaldehyde; 1,5 I-AENS, *N*-(iodoacetylaminoethyl)-1-naphthylamine-5-sulfonate; AENS, *N*-(acetylaminoethyl)-1-naphthylamine-5-sulfonate group; AMPR, *N*-(acetylaminoethyl)-1-naphthylamine-5-sulfonate derivative of 6-MPR, 5-[[2-[[[(9-β-D-ribofuranosyl-9*H*-purin-6-yl)thio]acetyl]amino]-ethyl]amino]-1-naphthalenesulfonate; AMPR-OP, oxidation product of AMPR.

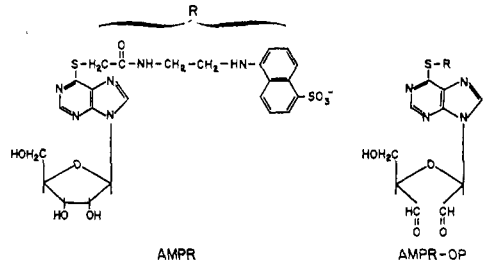

FIGURE 1: Structures of AMPR and AMPR-OP.

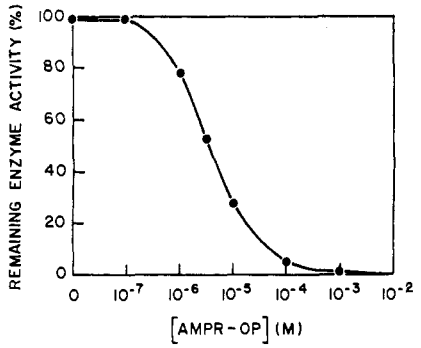

FIGURE 2: The dependence of RNA synthesis on the concentration of AMPR-OP. The per cent of enzyme activity remaining after exposure to AMPR-OP is plotted against the concentration of AMPR-OP added. The reaction mixture and conditions of the activity assay were as described in the Materials and Methods section.

incorporation of ^{3}H-labeled ribonucleoside monophosphate into acid-insoluble material using the procedure described previously (Wu and Wu, 1973c). The enzyme (5 μg) was preincubated at 37° for 15 min with 80 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, and various concentrations of nucleoside analog inhibitor or water. After preincubation the reaction mixtures were cooled to 4° and to these were added 0.12 mM calf thymus DNA, 0.4 mM each of ATP, CTP, UTP, and GTP (one labeled with ^{3}H, 8×10^3 cpm/nmol), 1.6 mM Na_2HPO_4, and 4 mM β-mercaptoethanol. When poly[d(A-T)] was used as a template, GTP and CTP were omitted and 0.2 M KCl was added to the reaction mixture. The complete system (0.25 ml) was further incubated at 37° for 20 min and reactions were terminated by cooling the mixtures to 4°, adding 0.1 ml of 0.1 M sodium pyrophosphate and 5 ml of 5% trichloroacetic acid. The acid-insoluble precipitates were collected on a glass fiber filter (Whatman GF/C, 2.4 cm) and washed with cold 1% trichloroacetic acid and 95% ethanol. The filter was dried and the radioactivity was measured with a liquid scintillation spectrophotometer.

Synthesis of AMPR and AMPR-OP. Equimolar quantities of 1,5 I-AENS (22 mg) and 6-MPR (14 mg) in 0.1 N NaOH (1 ml) were reacted in the dark at room temperature for 3 hr and then at 4° overnight. Quantitative yield of the addition product, AMPR, was obtained after evaporation to dryness and recrystallization from acetone. AMPR (28 mg) was then oxidized with an equimolar amount of sodium periodate (10.7 mg) in H_2O (3 ml) in the dark at room temperature for 5 hr. After addition of 95% ethanol, the white precipitate was filtered. The alcohol solution was evaporated to dryness and brown crystals of AMPR-OP were obtained in 50% yield after recrystallization from ethanol–water. Thin-layer chromatography of the reactants and products on silica gel in 5% Na_2HPO_4 solvent system gave a single spot having R_F values of 0.50, 0.59, 0.65, and 0.80 for 1,5 I-AENS, AMPR-OP, AMPR, and 6-MPR, respectively. The molar absorption coefficients of AMPR-OP in aqueous solution (pH 7) are 4.7×10^3 M^{-1} cm^{-1} at 355 nm (shoulder), 3.5×10^4 M^{-1} cm^{-1} at 285 nm, and 2.7×10^4 M^{-1} cm^{-1} at 265 nm (shoulder). The fluorescent emission maximum of AMPR-OP is at 485 nm.

DNA Dependent [^{32}P]PP$_i$ Exchange Reaction. The DNA dependent [^{32}P]PP$_i$ exchange reaction catalyzed by RNA polymerase was measured as described by Krakow and Fronck (1969).

Labeling of RNA Polymerase with AMPR-OP. In labeling experiments, 1.2 mg of enzyme was first dialyzed overnight against 0.1 M $NaHCO_3$ or Tricine buffer (pH 7.9) containing 8 mM $MgCl_2$, 0.2 M KCl, and 0.1 mM dithiothreitol to remove Tris normally present in the enzyme storage buffer. The enzyme was then incubated for 45 min at 37° with a 10- to 300-fold molar excess of AMPR-OP. After incubation, the reaction mixture was cooled to 4° and 2–20 mg of $NaBH_4$ in 1 ml of $NaHCO_3$ buffer was added. Reduction of the Schiff base to a stable covalent bond was allowed to occur for 16 hr at 4°. The entire reaction mixture was then passed through a Sephadex G-75 column (1 × 14 cm) to remove unreacted AMPR-OP and $NaBH_4$. The labeled enzyme was eluted with 50 mM Tris-HCl buffer (pH 8) containing 0.5 M KCl, 0.1 mM EDTA, and 0.1 mM dithiothreitol. This step was followed by extensive dialysis against the same buffer.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis Analysis. Electrophoresis on polyacrylamide gel was performed according to the method described by Weber and Osborn (1969). The AMPR-OP-bound enzyme was denatured with 3% sodium dodecyl sulfate or 7 M urea. Approximately 50 μg of denatured protein was layered on 7.5% polyacrylamide gels. Gels were run for 4–5 hr at 8 mA/tube. The gels were stained with Coomassie Brilliant Blue (0.2% methanol–acetic acid–H_2O, 5:1:5) overnight; then destained with the same solvent mixture as above until the gels yielded clear, visible bands.

Spectroscopic measurements were carried out with a Cary 118C recording spectrophotometer in a 1-cm light-path quartz cell. All spectroscopic measurements were carried out at 22 ± 0.1°.

Fluorescence excitation and emission spectra were recorded in a Hitachi-Perkin-Elmer fluorescence spectrophotometer, Model MPF-3, equipped with a corrected spectra accessory. The solutions used for fluorescence studies had absorbances of less than 0.05 at the excitation wavelength to obviate inner filter effect.

Quantum yield ϕ of a sample was calculated from absorbance (A) and the area enclosed by the corrected emission spectrum using the relationship (Parker and Rees, 1960)

$$\phi_S = \phi_R \frac{(1-10^{-A_R})}{(\text{area})_R} \frac{(\text{area})_S}{(1-10^{-A_S})} \frac{n_R^2}{n_S^2} \quad (1)$$

where n is the refractive index of the solvent, and S and R refer to sample and reference, respectively. 5-Anilinonaphthalene-1-sulfonate in ethanol was used as a reference of quantum yield 0.37 (Stryer, 1965). The areas of the corrected emission spectra were obtained by planimetry.

Results

Inhibition of RNA Polymerase Activities by AMPR-OP. Figure 2 shows the effect of various concentrations of AMPR-OP on the DNA dependent RNA synthesis catalyzed by *Escherichia coli* polymerase. Virtually no inhibition occurred up to 10^{-7} M, while complete inhibition was achieved at about

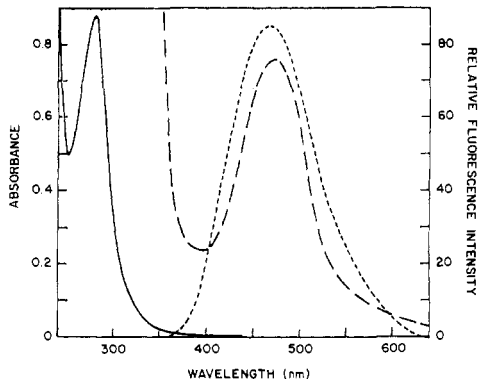

FIGURE 3: Absorption and corrected fluorescence emission spectra of AMPR-OP labeled polymerase and the overlap with the absorption spectrum of rifampicin: (—) absorption spectrum of 1.5×10^{-6} M labeled RNA polymerase in 0.5 M KCl–0.05 M Tris-HCl (pH 8)–0.1 mM EDTA–0.1 mM dithiothreitol; (- - - - - -) corrected fluorescence emission spectrum of 5×10^{-7} M labeled RNA polymerase in the same buffer, the excitation wavelength was at 330 nm; (– – –) absorption spectrum of 7.2×10^{-6} M rifampicin in the same buffer.

10^{-4} M. The concentration of AMPR-OP required for 50% inhibition was about 7×10^{-6} M, which is 100 times smaller than that of MMPR-OP for the same extent of inhibition.

The effect of nucleotide concentration on AMPR-OP inhibition was studied using poly[d(A-T)] as template. At a saturating concentration of UTP (0.4 mM), variation of the concentration of the alternate nucleoside triphosphate, ATP, yielded a linear double reciprocal plot. The results showed that the V_{max} decreased in the presence of AMPR-OP (the values of V_{max} are 1.0, 0.83, and 0.43 nmol/min at AMPR-OP concentrations of 0, 4×10^{-6}, and 1×10^{-5} M), while the apparent K_m remained unchanged (7×10^{-5} M), suggesting that AMPR-OP is a noncompetitive inhibitor with respect to ATP binding to RNA polymerase. The K_i value obtained for AMPR-OP was 7.4×10^{-6} M.

The type of inhibition produced by AMPR-OP is the same as that by MMPR-OP (Spoor *et al.*, 1970). This suggests that like MMPR-OP, AMPR-OP might bind to the initiation site on the enzyme. (If it binds to the polymerization site, a competitive type of inhibition would be expected.) To further support this contention, the effect of AMPR-OP on the poly[d(A-T)]-dependent [^{32}P]PP$_i$ exchange reaction was examined. As shown in Table I, at 0.1 mM AMPR-OP the incorporation of [^{32}P]PP$_i$ was almost completely inhibited. Thus, AMPR-OP primarily inhibits the initiation of RNA chains in the RNA polymerase reaction.

Affinity Labeling of RNA Polymerase with AMPR-OP. When the holoenzyme of RNA polymerase was incubated with excess AMPR-OP and then reduced by $NaBH_4$, AMPR-OP was bound to the enzyme in about 1:1 molar ratio. Prolonged incubation with a large excess of the dye did not significantly alter this stoichiometry. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the labeled enzyme (Weber and Osborn, 1969) showed a fluorescent band corresponding to the β subunit; bands corresponding to other subunits were nonfluorescent. This indicated that AMPR-OP was covalently bound to the β subunit of RNA polymerase.

The labeled RNA polymerase was essentially inactive (<1%) in DNA dependent polymerization and PP$_i$ exchange reactions. The absorption and fluorescence emission spectra of the labeled enzyme are shown in Figure 3. The absorption maximum for the modified protein was at 280 nm with a tail at 300–350 nm due to the bound dye. The fluorescence excitation and emission maxima were at 335 (not shown) and 470 nm, respectively.

TABLE I: Effect of AMPR-OP on Poly[d(A-T)] Dependent [^{32}P]PP$_i$-Exchange Reaction.[a]

AMPR-OP Added (M)	[^{32}P]PP$_i$ Incorp (nmol)	Inhibition (%)
0	37.4	0
1×10^{-5}	9.9	74
1×10^{-4}	3.0	92

[a] The incorporation of [^{32}P]PP$_i$ into nucleoside triphosphates was measured by adsorption to activated charcoal (Krakow and Fronk, 1969). The complete system (0.25 ml) contained 80 mM Tris-HCl (pH 7.8), 40 mM β-mercaptoethanol, 4 mM $MgCl_2$, 0.4 mM UTP, 1 mM sodium [^{32}P]-pyrophosphate (1.4×10^4 cpm/nmol), 0.1 A_{260} unit of poly[d-(A-T)], and 5 μg of RNA polymerase. The incubation was for 10 min at 37° and reactions were stopped by addition of 0.2 ml of 0.1 M EDTA (pH 6.0), and 0.1 ml of 0.1 M sodium pyrophosphate (pH 6.0), followed by addition of 0.5 ml of a 10% suspension of acid-washed, activated charcoal in 0.01 M sodium pyrophosphate (pH 6.0). After mixing, 3 ml of 0.01 M sodium pyrophosphate was added and the mixture was filtered through glass-fiber filters. The filters were washed with 40 ml of 0.01 M sodium pyrophosphate, dried, and counted.

Interaction of the Labeled Enzyme with DNA and Nucleotides. When calf thymus DNA (100 μg) or poly[d(A-T)] (same amount) was added to a solution containing 10^{-8} M labeled polymerase, 10 mM $MgCl_2$, 0.2 M KCl, 0.1 mM dithiothreitol, and 0.05 M Tris-HCl (pH 7.8) there was a 20–30% increase in the fluorescence intensity of the labeled enzyme and a small blue shift (3 nm) of the emission maximum. In the absence of DNA, addition of 0.4 mM of a single nucleoside triphosphate (ATP, GTP, UTP, or CTP) to 10^{-8} M labeled enzyme in the same buffer brought about a 5-nm blue shift of the emission maximum and a small (about 5–7%) enhancement of the fluorescence intensity. These observations occurred with any one of the four nucleoside triphosphates, and the effect of more than two nucleoside triphosphates was less than additive. In the presence of calf thymus DNA, however, the observed fluorescence enhancement was much larger, about 20% increase by each nucleoside triphosphate. If poly[d(A-T)] was present instead of calf thymus DNA, the situation was quite different. Addition of ATP (0.4 mM) did not significantly alter the fluorescent properties of the probe, whereas addition of UTP (0.4 mM) markedly enhanced the fluorescence intensity (30%). Addition of AMP, UMP, ADP, or UDP has no effect on the fluorescent properties of the labeled enzyme in the presence of either calf thymus DNA or poly[d(A-T)].

Energy Transfer from the Initiation Site to the Rifampicin Binding Site on RNA Polymerase. Rifampicin, a specific inhibitor of RNA chain initiation, has been shown to bind to a single site on RNA polymerase (Zillig *et al.*, 1970). The modification of RNA polymerase by AMPR-OP did not significantly alter its ability to bind rifampicin. By use of a gel filtration technique (Yarbrough and Wu, 1974), we found that the labeled enzyme bound 0.5 mol of [^{3}H]rifampicin per mole of enzyme while the unlabeled enzyme bound 0.6 mol of [^{3}H]rifampicin per mole of enzyme under the same experimental conditions. Thus energy transfer measurements were carried out to estimate the distance between the rifampicin

binding site and the initiation site on RNA polymerase because rifampicin, which possesses an absorption maximum at 470 nm, is an ideal energy acceptor of the emission of AMPR-OP (Figure 3).

In Förster's theory of dipole–dipole energy transfer (Förster, 1947), the transfer efficiency (E) is related to the distance (r) between the donor and acceptor by

$$E = r^{-6}/(r^{-6} + R_0^{-6}) \quad (2)$$

R_0, the distance (in Å) at which the transfer efficiency is 50%, is given by

$$R_0 = (JK^2Q_0n^{-4})^{1/6}(9.79 \times 10^3) \quad (3)$$

where K^2 is the orientation factor for dipole–dipole transfer, Q_0 is the quantum yield of the donor in the absence of transfer, n is the refractive index of the medium, and J is the spectral overlap integral calculated from the emission spectrum of the donor and the absorption spectrum of the acceptor. Q_0, the quantum yield of the enzyme-bound AMPR-OP, was determined to be 0.05 with 8-anilino-1-naphthalenesulfonate in ethanol as a reference of quantum yield 0.37 (Stryer, 1965). The value of J was 3.3×10^{-14} cm^3 M^{-1} as estimated from the corrected fluorescence emission spectrum of the enzyme-bound AMPR-OP and the absorption spectrum of the enzyme-bound rifampicin (Figure 3) using the equation

$$J = \frac{\int F(\bar{\nu})\epsilon(\bar{\nu})\bar{\nu}^4 \, d\bar{\nu}}{\int F(\bar{\nu})d\bar{\nu}} \quad (4)$$

where $F(\bar{\nu})$ is the fluorescence intensity of the donor at wave number $\bar{\nu}$, and $\epsilon(\bar{\nu})$ is the extinction coefficient of the energy acceptor at that wave number. Though n cannot be measured directly, there is little uncertainty as to its value, which we assume to be 1.4. The problem arises in assuming a value to the orientation factor K^2. If the relative orientation of donor–acceptor pairs is completely randomized during the excited state lifetime, K^2 equals $^2/_3$. Although the orientation of the energy acceptor is not known, nanosecond emission anisotropy measurements show that the energy donor used in this study has local rotational mobility (C.-W. Wu and F. Y.-H. Wu, to be published). The rapid decrease in emission anisotropy (from 0.32 to 0.08) within 7 nsec indicates that the energy donor rotates over an angle of the order of 60°. (The excited state lifetime of the enzyme-bound AMPR-OP was 15 nsec.) The value of R_0 was calculated to be 25 Å for the AMPR-OP and rifampicin pair on RNA polymerase using the experimentally observed values of J and Q_0 and assuming that n = 1.4 and $K^2 = {}^2/_3$. If an energy donor has complete rotational freedom and an energy acceptor is fixed, K^2 can range from $^1/_3$ to $^4/_3$. Since R_0 is proportional to $^1/_6$ power of K^2, the factor of 2 variations in K^2 will result in 12% error in the value of R_0.

The transfer efficiency, E, was determined from quantum yields of the donor in the presence and absence of energy acceptor (Q and Q_0, respectively). When rifampicin (6 μM)

$$E = 1 - (Q/Q_0) \quad (5)$$

was added to a solution of the labeled enzyme, a 10% decrease in quantum yield was observed. Thus an apparent distance between the initiation site and the rifampicin binding site on RNA polymerase was calculated to be 37 Å according to eq 2.

Discussion

Spoor *et al.* (1970) have shown that MMPR-OP is a potent inhibitor of *Escherichia coli* DNA dependent RNA polymerase. By substituting the methyl group of MMPR-OP with a fluorescent chromophore, *N*-(acetylaminoethyl)-1-naphthylamine-5-sulfonate (AENS), we found that the new pseudosubstrate, AMPR-OP, was a much more potent inhibitor of RNA polymerase. The K_i of AMPR-OP (7.4×10^{-6} M) was two orders of magnitude smaller than that of MMPR-OP (5.1×10^{-4} M). Although AMPR-OP is a larger molecule, the result indicates that the fluorescent chromophore somehow stabilizes the inhibitor–enzyme complex.

Since noncompetitive inhibition was observed for MMPR-OP with respect to polymerization of nucleoside triphosphates (Spoor *et al.*, 1970), it was proposed that the inhibitor bound at the initiation site, a nucleotide binding site on the enzyme other than the polymerization (elongation) site. Similar noncompetitive kinetics was observed for AMPR-OP, suggesting that AMPR-OP may also bind to the initiation site.

The binding site of MMPR-OP has been shown to consist of an ε-amino group of a lysine residue in the β subunit of RNA polymerase (Nixon *et al.*, 1972). If AMPR-OP and MMPR-OP were bound to the same site, AMPR-OP should also form a Schiff base with the ε-amino group of the lysine. In fact, after sodium borohydride reduction, a stable covalent linkage between AMPR-OP and the β subunit of RNA polymerase was obtained as shown by the sodium dodecyl sulfate polyacrylamide gel electrophoresis of the labeled enzyme.

The labeled polymerase was essentially inactive in catalyzing RNA synthesis and the DNA dependent PP_i exchange reaction. However, the altered enzyme still could interact with DNA template and nucleoside triphosphates as demonstrated by the enhancement of fluorescence intensity and the blue shifts of the emission maximum. Since the fluorescent probe used here is sensitive to the environment (Hudson and Weber, 1973), these observations indicate that the surrounding of the AMPR-OP binding site has become less polar upon binding to DNA or nucleoside triphosphate, *i.e.*, a template- or substrate-induced conformational change of the enzyme has taken place. (No direct interaction between AMPR-OP and DNA or NTP could be detected by fluorescence measurements.) In addition, the emission maximum of free AMPR-OP is at 485 nm and that of the bound AMPR-OP is at 470 nm. This implies that the environment of the initiation is slightly more hydrophobic than that of the aqueous media.

Wu and Goldthwait (1969) have demonstrated two nucleoside triphosphate binding sites on *Escherichia coli* RNA polymerase (in the absence of DNA): a weak binding site (K_s = 1.5×10^{-4} M) with preferential affinity for purine nucleotides, and a strong binding site ($K_s = 1.5 \times 10^{-5}$ M) for all four nucleoside triphosphates. Kinetic analysis (Anthony *et al.*, 1969) has suggested that the weak binding site is the initiation site and the strong binding site, the polymerization site. In this paper, we have shown that the affinity-label very probably binds to the initiation site of the enzyme (although it is not really proved). If AMPR-OP were bound to the initiation site by the affinity labeling as suggested above, then nucleoside triphosphate must interact with the other binding site, the polymerization site on the labeled enzyme. This is consistent with the observation that in the absence of DNA, all four nucleoside triphosphates produce similar fluorescence changes of the labeled enzyme.

The most interesting findings are the interactions between nucleoside triphosphates and the labeled enzyme in the presence of DNA. The further increase in fluorescence intensity of the labeled enzyme by binding nucleoside triphosphates in the presence of calf thymus DNA, as compared to

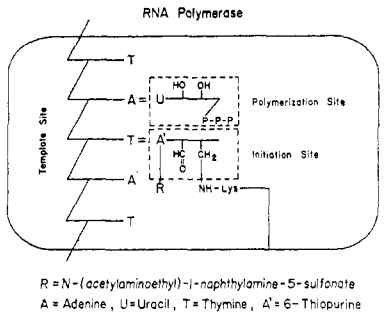

FIGURE 4: Model for active sites on RNA polymerase of *Escherichia coli*.

that in the absence of DNA, may reflect some structural alteration of the polymerization site (on the enzyme) by the template. This is best demonstrated when d(A-T) copolymer was used as the template. As shown in the model presented in Figure 4, AMPR-OP is covalently attached to a lysine residue at or near the initiation site. When d(A-T) copolymer occupies the template site on the enzyme, the purine moiety of AMPR-OP may form hydrogen bonds with a thymine base of the template. The specificity of the polymerization site is then governed by the adjacent adenine base of the template due to base complementation (A-U hydrogen bonding), or alternatively, the adenine moiety of the template may induce a conformational change of the enzyme so that the polymerization site binds UTP preferentially. Our observation that in the presence of poly[d(A-T)] UTP but not ATP markedly enhanced the fluorescence of the labeled enzyme can be readily explained by this model.

Rifampicin is a known inhibitor of *Escherichia coli* RNA polymerase (Hartmann *et al.*, 1967). It binds to a single site on the enzyme and genetic evidence suggests that the rifampicin binding site is on the β subunit of the enzyme (Rabussay and Zillig, 1969; Zillig *et al.*, 1970; Heil and Zillig, 1970). Since rifampicin specifically inhibits initiation of RNA chains, and the initiation site may also be located on the β subunit, it was of interest to determine the structural and functional relationship between the initiation site and the rifampicin binding sites. To this end, energy-transfer measurements were carried out to estimate the distance between these two sites. The results indicate that these two sites are at least 37 Å apart. Therefore, although both the initiation site and the rifampicin binding site are on the β subunit, they are not adjacent to each other. This suggests that the effect of rifampicin on RNA chain initiation is indirectly mediated through the enzyme molecule.

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