Mechanism of Inhibition of RNA Polymerase II and Poly(adenylic acid) Polymerase by the *O*-*n*-Octyloxime of 3-Formylrifamycin SV†

Kathleen M. Rose,* Patricia A. Ruch, and Samson T. Jacob

ABSTRACT: Factors affecting the inhibition of RNA polymerase II from rat liver by the *O*-*n*-octyloxime of 3-formylrifamycin SV (AF/013) were investigated. Using either native or denatured calf-thymus DNA as template, almost complete inhibition of RNA polymerase II was observed when AF/013 was added directly to the enzyme. Considerable resistance to AF/013 was observed when RNA polymerase II was preincubated with denatured DNA at either 0 or 37°. However, under similar conditions, no resistance was observed when enzyme was preincubated with native DNA. Only when AF/013 was added to the ongoing reaction using native DNA did a resistance to AF/013 occur. The inhibition of RNA polymerase II by AF/013 was competitive with respect to all four nucleoside triphosphate substrates. The inhibition by AF/013 remaining after enzyme–DNA complex formation also appeared competitive with nucleoside triphosphate levels. The effect of exogenous protein (bovine serum albumin, BSA) on the inhibition of RNA polymerase II was also investigated. BSA reduced the extent of inhibition by AF/013, but did not alter the competitive nature of inhibition. Concurrently, the inhibition of highly purified nuclear poly(A) polymerase from rat liver, a template independent enzyme which incorporates AMP in a chain elongation reaction, was examined. As in the case of RNA polymerase, poly(A) polymerase was inhibited by AF/013 in a manner competitive with the nucleoside triphosphate substrate. The competitive nature of inhibition of RNA polymerase by AF/013 with respect to all four nucleoside triphosphate substrates, before and after enzyme–DNA complex formation, as well as the competitive nature of inhibition of poly(A) polymerase with respect to ATP tend to indicate that the major effect of AF/013 on RNA polymerase II is at the level of the substrate binding as opposed to a specific inhibition of initiation.

The antibiotic rifamycin and its semisynthetic derivatives have been shown to inhibit many nucleotidyl transferases from prokaryotic and eukaryotic cells (Hartmann et al., 1967; Chambon et al., 1970; Hinkle et al., 1972; Riva et al., 1972; Gerard et al., 1973; diMauro and Mezzina, 1974; Jacob et al., 1974; Jacob and Rose, 1974). In particular, these drugs have served as powerful tools in elucidating the molecular mechanism of RNA synthesis catalyzed by DNA-dependent RNA polymerase. While rifamycin itself is a specific inhibitor of the bacterial RNA polymerase (Hartmann et al., 1967) and of some mitochondrial RNA polymerases (Kuntzel and Schafer, 1971; Gadaleta et al., 1970; Reid and Parsons, 1971), several of the potent derivatives of this compound can inhibit mammalian RNA polymerases as well (Meilhac et al., 1972; Onishi and Muramatsu, 1972; Juhasz et al., 1972).

The major effect of rifamycin and/or its derivatives on the reaction catalyzed by DNA-dependent RNA polymerase is to inhibit the reaction prior to initiation of RNA synthesis (Bautz and Bautz, 1970; Hinkle et al., 1972; Meilhac and Chambon, 1973). The complexity of the RNA polymerase reaction makes studies on post-initiation events relatively difficult. Several factors, such as reinitiation of the enzyme on the template, altered initiation sites on different templates, effects of divalent ions and salt concentrations (Meilhac and Chambon, 1973; Lill and Hartmann, 1973; Tsai and Saunders, 1973; Kerrich-Santo and Hartmann, 1974), and the possible nonspecific binding of the rifamycin derivatives to other proteins (Riva et al., 1972; diMauro and Mezzina, 1974) contribute to the difficulties in interpreting data on inhibition by these drugs.

Recent studies in our laboratory (Jacob et al., 1974; Jacob and Rose, 1974) have demonstrated that derivatives of rifamycin can also inhibit the elongation of polyribonucleotides in the template-independent synthesis of poly(A) catalyzed by mitochondrial poly(A) polymerase. The inhibition of poly(A) polymerase can be reversed by elevated levels of substrate. The present study compares the kinetics of the inhibition of the template-dependent reaction catalyzed by rat liver RNA polymerase II and the primer dependent reaction catalyzed by nuclear poly(A) polymerase by one of the most potent rifamycin derivatives, the 1-*n*-octyloxime of 3-formylrifamycin SV (AF/013). The results suggest that the inhibition of both nuclear polymerases by AF/013 occurs in a manner which is competitive with substrate.

Experimental Section

Materials. Highly polymerized calf-thymus DNA was obtained from Sigma Chemical Co.; poly(A) and recrystallized bovine serum albumin were from Miles Laboratories and the nucleoside triphosphates were from Calbiochem. [2,8-^{3}H]ATP (35 Ci/mmol), [5,6-^{3}H]UTP (35 Ci/mmol), [8-^{3}H]GTP (6 Ci/mmol), and [5-^{3}H]CTP (22 Ci/mmol) were purchased from New England Nuclear Corporation.

Preparation of Enzymes. Enzymes were extracted from rat liver nuclei by a modification of the procedure developed by Jacob et al. (1970). Rat liver nuclei from 200 g of tissue were suspended in an alkaline buffer containing 50 m*M* Tris-HCl (pH 8.9), 50 m*M* KCl, 2 m*M* dithiothreitol,

† From the Department of Pharmacology, Chemical Carcinogenesis Unit, The Milton S. Hershey Medical Center, College of Medicine, The Pennsylvania State University, Hershey, Pennsylvania 17033. *Received March 11, 1975.* Supported in part by U.S. Public Health Service Grant No. CA-15733 from the National Cancer Institute.

0.1 m*M* EDTA, 1 m*M* $MgCl_2$, 0.5 m*M* $PhMeSO_2F$,[1] 0.12 mg/ml of BSA,[1] and 40% glycerol (v/v). The ratio of buffer to nuclei was 1 ml/g original wet weight of liver. The suspension was sonicated in 60-ml batches for 4 × 15 sec at full output (20 kHz) in a Branson sonifier (Model W 140). The sonicated suspension was diluted with an equal volume of the above buffer from which glycerol was omitted, incubated for 5 *min* at 37°, and precipitated with $(NH_4)_2SO_4$ (0.42 g/ml). After 1 hr of stirring at 4°, the suspension was centrifuged at 105,000*g* for 40 min. The pellet was suspended in a small volume of buffer containing 50 m*M* Tris-HCl (pH 7.9), 25% glycerol (v/v), 5 m*M* $MgCl_2$, 0.1 m*M* EDTA, 0.5 m*M* dithiothreitol, and 30 m*M* $(NH_4)_2SO_4$ (TGMED buffer) and dialyzed overnight against the same buffer. The dialysate was centrifuged at 80,000*g* for 40 min and the supernatant was subjected to DEAE-Sephadex chromatography (2.4 × 12 cm column) as described previously (Rose and Jacob, 1974). Poly(A) polymerase was eluted in the void volume and RNA polymerase II eluted from a linear gradient at 0.2 *M* $(NH_4)_2SO_4$–TGMED. Only the very peak fractions containing RNA polymerase II activity were pooled and utilized for these studies. The specific activity of RNA polymerase II varied between 6 and 10 nmol of AMP incorporated/mg of protein depending on the preparation. RNA polymerase II was 100% sensitive to α-amanitin, completely dependent on DNA template, and had no detectable RNase or polynucleotide phosphorylase activity. RNA polymerase II was stored in the absence of bovine serum albumin, was stable for 2 weeks at −70°, and was thawed immediately before use. Poly(A) polymerase was further purified by phosphocellulose and hydroxylapatite chromatography (manuscript in preparation). Poly(A) polymerase had a specific activity of 500 nmol/mg of protein, was approximately 80% pure as estimated by acrylamide gel electrophoresis, and contained no serum albumin.

RNA Polymerase Assay. The standard RNA polymerase assay contained 1.6 m*M* $MnCl_2$, 3.3 m*M* NaF, 8 m*M* KCl, 60 m*M* $(NH_4)_2SO_4$, 1.5 m*M* $MgCl_2$, 60 m*M* Tris-HCl (pH 8.0), 0.2 m*M* dithiothreitol, 0.64 m*M* CTP, GTP, and UTP, 27–550 μ*M* [^{3}H]ATP (as indicated), with a specific activity not less than 4000 cpm/nmol, 50 μg of calf-thymus DNA (native or denatured, as indicated), and 0.1 ml of enzyme containing 8–10 μg of protein in a total reaction volume of 0.35 ml. The picomoles of AMP incorporated into Cl_3CCOOH insoluble material in 10 min at 37° were determined as described previously (Rose and Jacob, 1974). Dimethylformamide or AF/013 dissolved in dimethylformamide was added in a volume of 10 μl.

Poly(A) Polymerase Assay. The standard assay mixture contained 70 m*M* Tris-HCl (pH 8.0), 0.5 m*M* $MnCl_2$, 0.12–1.1 m*M* [^{3}H]ATP (as indicated) with a specific activity not less than 1000 cpm/nmol, 180 μg of poly(A), and 10 μg of enzyme protein in a final volume of 0.3 ml. The nanomoles of AMP incorporated in 60 min at 37° were determined as described previously (Rose and Jacob, 1974). Five microliters of dimethylformamide or AF/013 dissolved in dimethylformamide was added.

Results

Effect of Template on the Inhibition of RNA Polymerase II by AF/013. Before investigating the mechanism of inhibition of RNA polymerase II by the rifamycin derivative AF/013, the effect of the nature of the template (native vs. denatured DNA) on the inhibition was first determined at saturating levels of DNA. Table I summarizes these results. Almost complete inhibition of RNA polymerase was observed at 21 μ*M* AF/013 when the drug was added directly to the enzyme using native or denatured DNA as template. Since RNA polymerase must first complex with DNA before initiation of RNA synthesis, the effect of preincubation of the enzyme and template upon inhibition by AF/013 was investigated. When AF/013 was added to the enzyme after preincubation at 0°, only the reaction utilizing denatured DNA was resistant to inhibition by AF/013, presumably by forming an enzyme–DNA complex insensitive to the drug (the activity increased from 17 to 74% of the control). No significant resistance to AF/013 was observed when RNA polymerase was preincubated with native DNA either at 0 or 37° (11% vs. 7%). However, when the nucleoside triphosphates and cofactors were present in the preincubation medium with native DNA and enzyme, considerable resistance (81% of the control) to AF/013 inhibition occurred. However, under these conditions utilizing denatured DNA there was only a small increase over the resistance developed in the absence of nucleotides and cofactors (74 and 86% in the absence and presence of nucleotides and cofactors, respectively).

Table I: Effect of Template and BSA on the Inhibition of RNA Polymerase II by AF/013.[a]

Conditions of Preincubation	Activity (% of Control)	
	Native DNA	Denatured DNA
AF/013 + enzyme, then NTP + DNA	11	17
Enzyme + DNA, 15 min at 0°, then NTP + AF/013	7	74
Enzyme + DNA, 20 min at 37°, then NTP + AF/013	8	Not tested
Enzyme + DNA + NTP, 1 min at 37°, then AF/013	81	86
AF/013 + enzyme + BSA, then NTP + DNA	51	67
Enzyme + BSA + DNA, 15 min at 0°, then NTP + AF/013	83	94

[a] AF/013 was used at a concentration of 21 μ*M*, BSA at 400 μg/ml, and DNA at 145 μg/ml. NTP was a mixture of nucleoside triphosphates and cofactors as described in the Experimental Section containing 100 μ*M* [^{3}H]ATP (20,000 cpm/nmol). Control activities were 50 and 250 pmol of AMP incorporated using native and denatured DNA, respectively.

The effect of increasing concentrations of AF/013 upon RNA synthesis catalyzed by RNA polymerase II using denatured DNA (in excess) as template is shown in Figure 1. When the drug was added directly to the enzyme (in the presence of 350 μg/ml of BSA) the incorporation of AMP into product was completely inhibited at a drug concentration at 85 μ*M*. However, when the enzyme was preincubated with denatured DNA, considerable resistance to inhibition by AF/013 was observed and 10% of the original activity remained even at 340 μ*M* AF/013. The concentration of AF/013 required to achieve 50% inhibition without preincubation was 34 μ*M*, whereas 136 μ*M* of the drug was needed to achieve the same degree of inhibition under conditions of preincubation. It should be pointed out that the

[1] Abbreviations used are: BSA, bovine serum albumin; $PhMeSO_2F$, phenylmethylsulfonyl fluoride.

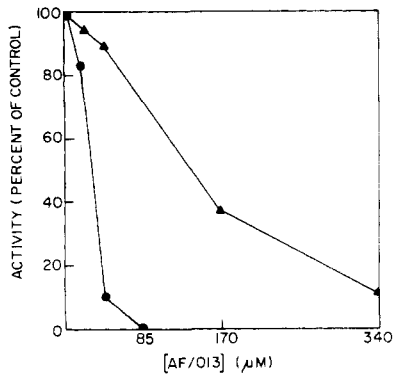

FIGURE 1: Inhibition of RNA polymerase II by AF/013. AF/103 was added in increasing concentrations to RNA polymerase II either directly to the enzyme or after preincubation of the enzyme with denatured DNA. (●) Ten microliters of dimethylformamide or AF/013 (as indicated) was added to assay tubes containing the enzymes, cofactors, and nucleoside triphosphates. The reaction was started by addition of denatured DNA. One-hundred percent activity corresponded to 0.35 nmol of AMP incorporated in 15 min at 37°. (▲) After preincubation of the enzyme with denatured DNA for 15 min at 0°, the reaction was started by addition of cofactors and nucleoside triphosphates. Either 10 μl of dimethylformamide or AF/013 as indicated was added simultaneously with the nucleotides. Dimethylformamide itself had no apparent inhibitory effect. One-hundred percent activity corresponded to 0.4 nmol of AMP incorporated. The standard reaction mixture contained 100 μM [^{3}H]ATP (20,000 cpm/nmol).

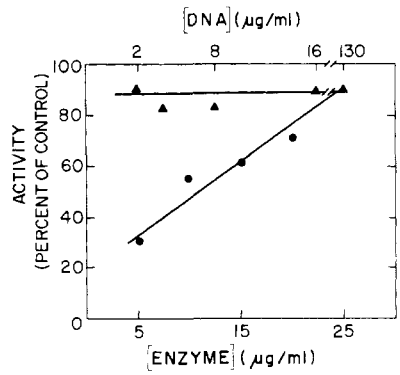

FIGURE 2: Effect of template and enzyme concentrations on the inhibition by AF/013. RNA polymerase II activity was determined in the presence and absence of AF/013 (12 μM) by varying the enzyme concentration at a fixed concentration (130 μg/ml) of native DNA (●) or by varying the DNA concentration at fixed levels (25 μg/ml) of enzyme (▲). The drug was added directly to the enzyme and the reaction was started by the addition of DNA. The standard reaction mixture contained 80 μM [^{3}H]ATP (20,000 cpm/nmol). One-hundred percent activity for both the lowest DNA and enzyme concentrations was 15 pmol and increased linearly to 60 pmol. Product formation increased linearly for all concentrations of enzyme and at least up to 10 μg/ml of DNA.

absolute amount of drug necessary to inhibit the enzyme varied slightly from preparation to preparation. However, the relative degree of inhibition by AF/013 before and after preincubation with denatured DNA remained constant. In a typical experiment 50% inhibition of 8 μg of RNA polymerase II (350 pmol of AMP incorporated) in the presence of 350 μg/ml of BSA occurred at a drug concentration of 25–35 μg/ml (30–40 μM) when the drug was added prior to enzyme–DNA complex formation.

In order to determine whether the inhibitory action of AF/013 was altered by enzyme or DNA levels an experiment was carried out such that RNA synthesis was measured in the presence and absence of the rifamycin derivative when (1) the enzyme concentration was altered keeping the template in excess or (2) the DNA levels were varied with RNA polymerase in excess. Figure 2 shows the results obtained when native DNA was used as template and AF/013 was added prior to initiation of RNA synthesis. Increasing levels of enzyme reduced the effect of the drug whereas decreasing the DNA to as low as 2 μg/ml had no effect on the percent inhibition by AF/013. Similar results were obtained when the drug was added to the ongoing reaction, using a higher concentration of the drug.

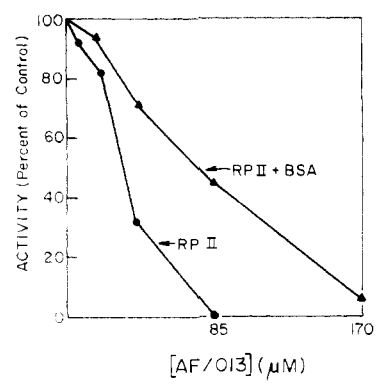

FIGURE 3: Effect of BSA on the inhibition of RNA polymerase II by AF/013. RNA polymerase II (RPII) isolated and stored in the absence of BSA was assayed with increasing amounts of AF/013 in the presence (400 μg/ml; ▲) and absence (●) of BSA. The nanomoles of AMP incorporated in 15 min were determined as described in the text at a [^{3}H]ATP concentration of 130 μM (10,000 cpm/nmol). The reaction was started by addition of denatured DNA. One-hundred percent activity corresponded to 0.42 and 0.32 nmol of AMP incorporated in the presence and absence of BSA, respectively.

Effect of BSA on the Inhibition of RNA Polymerase II by AF/013. Since AF/013 appears to bind to extraneous proteins such as BSA (Riva et al., 1972) and BSA is routinely used for stabilization of mammalian RNA polymerase (see Jacob, 1973), the effect of BSA on the inhibition of RNA polymerase II was also tested. As shown in Table I, when AF/013 was added directly to the enzyme the inhibition in the presence of BSA was reduced for both native and denatured DNA and the corresponding activities increased from 11 to 51% and from 17 to 67%, respectively. Figure 3 shows the profile of inhibition of RNA polymerase II by AF/013 (using denatured DNA as template) in the presence and absence of BSA. At all concentrations tested, the potency of the drug was reduced in the presence of BSA.

The effect of BSA on the preincubation of enzyme and template at 0° was then investigated. As shown in Table I, when enzyme (plus BSA) was preincubated at 0° with denatured DNA a complex almost completely resistant to inhibition by AF/013 was formed (67% activity vs. 94% activity when preincubated in the presence of BSA). RNA polymerase preincubated with native DNA at 0° in the presence of BSA also further increased its resistance to AF/013 inhibition (51% activity vs. 83%). The formation of a resistant complex at 0° in the presence of BSA using native DNA was also investigated at a higher AF/013 concentration (42 μM). When 42 μM of AF/013 was added directly to the enzyme in the presence of BSA only 1% of the control activity was observed (not shown). When the enzyme, BSA, and native DNA were preincubated at 0°, the activity after addition of 42 μM AF/013 increased to 34% of the control. Thus, in addition to the type of template used, BSA itself appeared to affect the formation of a complex resistant to inhibition by AF/013. This increase in resistance to AF/

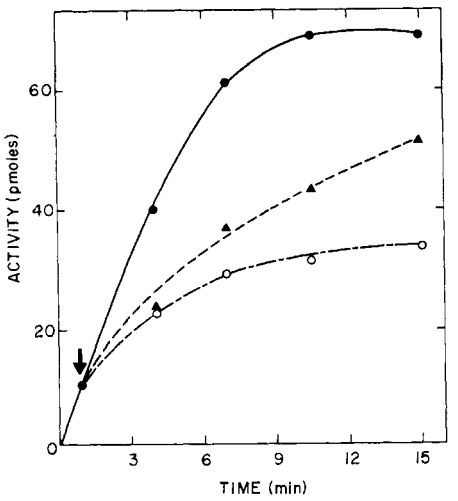

FIGURE 4: The effect of AF/013 on the time course of RNA synthesis by RNA polymerase II. Dimethylformamide or AF/013 was added 1 min after the start of the reaction using 130 μg/ml of native DNA as template. Reactions were terminated at times indicated: dimethylformamide (●-●); 50 μM AF/013 (▲- - -▲); 100 μM AF/013 (○- - -○). The standard reaction mixture contained 80 μM [^{3}H]ATP (20,000 cpm/nmol).

013 by preincubation at 0° with native DNA was in contrast to the lack of resistance obtained after preincubation in the absence of BSA.

The effect of AF/013 on the kinetics of RNA synthesis resistant to the drug was tested by adding AF/013 to the ongoing reaction (in the absence of BSA). The results, shown in Figure 4, indicate that RNA synthesis does not completely cease after addition of the drug but occurs at a slower rate, and that an increased AF/013 concentration further reduces the rate of product formation.

Effect of Increasing ATP Concentration on the Inhibition of RNA Polymerase II by AF/013. To elucidate the mechanism of inhibition of RNA polymerase II by AF/013, the possibility of competitive inhibition with respect to substrate was explored. In order to test this possibility, the velocity of the reaction catalyzed by RNA polymerase was measured at varying (limiting) ATP concentrations (keeping GTP, CTP, and UTP at saturating levels) for several different drug concentrations. The template (denatured DNA) was also used in excess. Figure 5 shows the double reciprocal plot of the incorporation of AMP into product as a function of ATP concentration at three AF/013 concentrations when the drug was added directly to the enzyme (in the presence of 350 μg/ml of BSA). The inhibition by the drug was markedly affected by the ATP concentration and appeared to be competitive with ATP. Thus, the V_m^{app} of the reaction remained unaltered in the presence of the drug. Similar results were obtained using native DNA as template (not shown). As observed with certain other polymerases (Chamberlin and Ring, 1972; Gurgo et al., 1974), the potency of the rifamycin derivative was not proportional in a linear manner to its concentration and the inhibition appeared to vary as an exponential function of the drug concentration.

To ascertain that BSA alters only the extent of inhibition of RNA polymerase by AF/013, but not the mechanism, the effect of ATP concentration on the inhibition by AF/013 was measured in the absence of BSA (see Figure 6, lower left). As in the presence of BSA, AF/013 was a competitive inhibitor with respect to ATP.

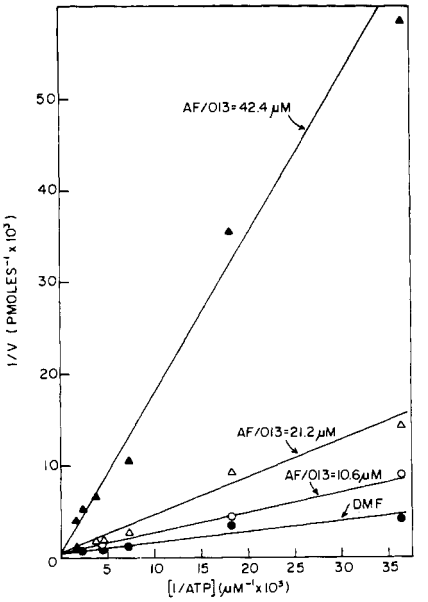

FIGURE 5: Effect of AF/013 on RNA polymerase II: the double reciprocal plot of the velocity of AMP incorporation vs. ATP concentration. Dimethylformamide (●) or AF/013 at 10.6 μM (○), 21.2 μM (△), and 42.4 μM (▲) was added to the enzyme simultaneously with the cofactors and nucleoside triphosphates at varying ATP concentrations. The [^{3}H]ATP concentration ranged from 27.5 μM (65,000 cpm/nmol) to 550 μM (4000 cpm/nmol). The reaction was started by addition of denatured DNA.

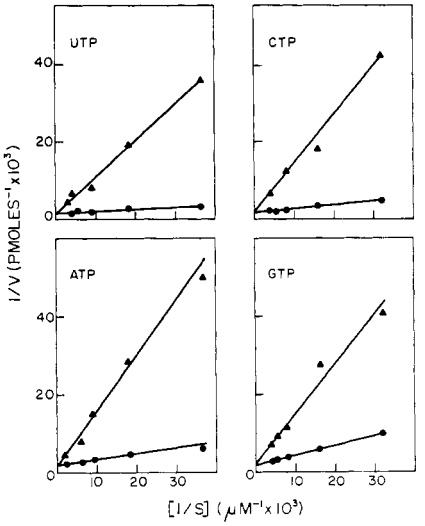

FIGURE 6: The double reciprocal plot of the reaction velocity in the absence of BSA as a function of nucleoside triphosphate concentration in the presence and absence of AF/013. Each of the nucleosides triphosphates labeled with ^{3}H was added to the RNA polymerase reaction in increasing concentrations as described in the legend to Figure 5. Ten microliters of dimethylformamide (●) or AF/013 (21 μM; ▲) was added directly to RNA polymerase prepared in the absence of BSA. The reaction was started by the addition of denatured DNA.

In order to determine if the competitive nature of inhibition by AF/013 was specific for the substrate ATP or occurred with all four nucleoside triphosphates, the effect of the concentration of GTP, CTP, and UTP upon inhibition of RNA polymerase II by AF/013 was also determined. The velocity of the reaction (in the presence and absence of AF/013) was measured by using one of the nucleotide triphosphates (labeled with ^{3}H) in limiting quantities while keeping the other three nucleotides and denatured DNA at saturating levels. The double reciprocal plots of the enzy-

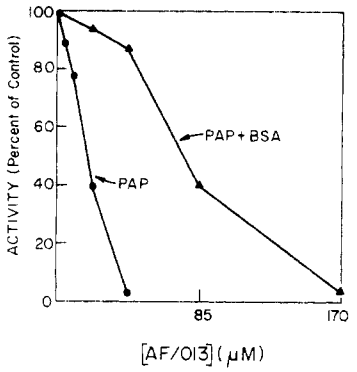

FIGURE 7: Effect of BSA on the inhibition of poly(A) polymerase by AF/013. Increasing amounts of AF/013 were added to the poly(A) polymerase (PAP) reaction in the presence (400 μg/ml; ▲) or absence (●) of BSA. The nanomoles of AMP incorporated in 1 hr were determined as described in the Experimental Section, using 250 μM ATP (2000 cpm/nmol). The reaction was started by addition of poly(A); 100% activity corresponded to 5.0 and 4.5 nmol of AMP incorporated in the presence and absence of BSA, respectively.

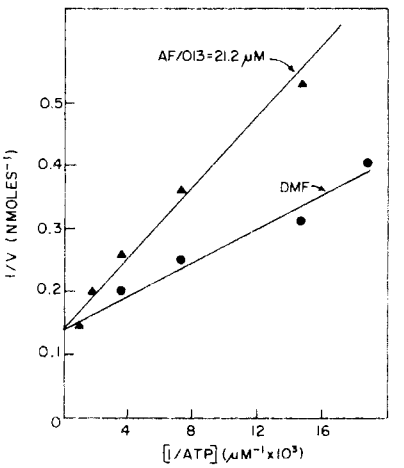

FIGURE 8: The double reciprocal plot of the reaction velocity vs. ATP concentration catalyzed by poly(A) polymerase in the presence and absence of AF/013. Five microliters of dimethylformamide (●) or AF/013 (▲; 21.2 μM) was added to the poly(A) polymerase reaction mixture simultaneously with varying ATP concentrations. The [^{3}H]ATP concentration ranged from 70 μM (20,000 cpm/nmol) to 1100 μM (1400 cpm/nmol). The reaction was started by addition of poly(A) as the primer.

matic activity as a function of each of the nucleotide triphosphate concentrations for the controls (dimethylformamide) and with 21 μM AF/013 are shown in Figure 6. In all cases, inhibition by the drug appeared competitive with the nucleoside triphosphate measured.

Inhibition of Nuclear Poly(A) Polymerase by AF/013. The demonstration of the inhibition by AF/013 of the elongation reaction catalyzed by mitochondrial poly(A) polymerase has been previously reported (Jacob et al., 1974; Jacob and Rose, 1974). To determine whether the mechanism of inhibition of the template independent ATP polymerization catalyzed by poly(A) polymerase occurred in the same manner as the inhibition of RNA polymerase II, the very active rat liver nuclear poly(A) polymerase was extensively purified for use in these studies. Figure 7 shows the profile of inhibition of poly(A) polymerase in the presence and absence of BSA. As for RNA polymerase, addition of BSA to the poly(A) polymerase reaction also reduced the potency of inhibition by AF/013.

In order to determine whether AF/013 also inhibits this poly(A) polymerase in a manner competitive with substrate, the velocity of the reaction at several ATP concentrations in the presence and absence of drug was measured. The Lineweaver-Burk plot of the inhibition by AF/013 (21 μM) is shown in Figure 8. As with RNA polymerase, the poly(A) polymerase was inhibited by AF/013 in a manner competitive with ATP. Addition of the drug after the reaction was started altered neither the extent of inhibition nor the effect of substrate concentration. The mechanism of inhibition of the poly(A) polymerase elongation reaction thus appeared to be the same as the mechanism of inhibition of RNA polymerase II.

Discussion

The present studies show that the inhibition of RNA polymerase II as well as of poly(A) polymerase by AF/013 occurs at the level of the substrate binding. That the inhibition of the RNA polymerase reaction is not due to a specific effect on initiation is supported by the following observations. (a) All four nucleoside triphosphates successfully compete with AF/013 for binding sites on the "free" enzyme. This presumably would not occur if initiation (by ATP or GTP) sites were the major targets of the inhibition. (b) The elongation reaction catalyzed by poly(A) polymerase is inhibited in a manner analogous to the template-dependent reaction catalyzed by RNA polymerase.

The competitive nature of the inhibition of polymerase II with respect to nucleoside triphosphate levels has not been reported by other workers. We have found that the inhibition by AF/013 is related to the substrate levels whether the drug is added prior to the start of the reaction or to the ongoing reaction (not shown). This observation is not in disagreement with other studies (Meilhac et al., 1972; Tsai and Saunders, 1973) which have shown that rifamycin derivatives bind to the polymerase molecule and thereby prevent the binding of the enzyme to DNA. The presence of saturating levels of substrates may compete with AF/013 for sites on the enzyme which, when occupied with drug (but not with substrate), may prevent the complexing of enzyme to DNA or may alter the configuration of the enzyme molecule such that its affinity for AF/013 is reduced. It should be pointed out that the level of AF/013 necessary to detect the competition with substrate must be monitored very carefully so that the drug concentration is not too high. At high concentrations of AF/013, the proportionally higher levels of ATP necessary to reverse the inhibition significantly reduce the sensitivity of the assay. The molar concentration ratio of ATP to AF/013 should be in the range of 2-20.

The inhibition of the RNA polymerase reaction by AF/013 probably occurs primarily by binding to "free" enzyme (not complexed with DNA). The reduction in the level of inhibition of RNA polymerase by AF/013 after enzyme-DNA complex formation (see Figure 1) suggests that only a small proportion of the enzyme is available for inhibition by the drug at this stage of the reaction. This inhibition may reflect binding of AF/013 to enzyme molecules which have been released from the template after chain termination, thereby rendering them accessible to the drug. Binding of the drug to "free" enzyme molecules is also indicated by the continuation of RNA synthesis after addition of AF/013 (see Figure 4), since enzyme engaged in active transcription is not susceptible to drug action. However, if only reinitia-

tion of RNA synthesis was prevented by the binding of AF/013 to enzyme which had been released from the template one would not expect to find accentuated inhibition of the RNA polymerase reaction at higher drug levels as is shown in Figures 1 and 4. It is possible that at very high AF/013 levels, the drug may effectively displace the enzyme from the template. Indeed, Tsai and Saunders (1973) have shown that rifamycin derivative AF/013 at a concentration of 120 μM can displace human RNA polymerase II from homologous DNA. Displacement of polymerase II from the DNA template at high concentrations of AF/013 could also explain the inhibition of chain elongation by AF/013 reported by Juhasz et al. (1972) for rat liver polymerase II since the reduction in chain length was found only at drug concentrations of 250 μM. The inability of Meilhac and coworkers (Meilhac et al., 1972; Meilhac and Chambon, 1973) to detect a displacement of calf-thymus polymerase II from SV40 DNA with AF/013 may be a reflection of (1) the relatively low levels of drug used in the DNA binding studies, (2) an extremely high affinity of their enzyme preparation for DNA, or (3) the result of the large amounts of BSA used in their assay which appears to stabilize the enzyme–DNA complex (see Table I). It should be noted that the inhibition at high levels of AF/013 when the drug is added to the ongoing reaction with double-stranded DNA as template also appears competitive with regard to ATP (not shown).

Previous studies (Riva et al., 1972) have shown that AF/013 can bind to proteins (including BSA) other than nucleotidyl transferases. The present studies show that the *extent, but not the mechanism,* of inhibition by AF/013 is altered by BSA. This effect is most marked in very highly purified enzymes. Accordingly, the inhibition of less purified enzyme preparations by rifamycin derivatives is not substantially altered by further addition of exogenous proteins such as BSA (Jacob et al., 1974). However, less pure enzymes also require increased levels of AF/013 to attain the same extent of inhibition. Thus, partially purified mitochondrial poly(A) polymerase requires 100 μg/ml to attain 50% inhibition of 2 units of the enzyme (nanomoles/milligram per hour) whereas an activity of 500 units of highly purified nuclear poly(A) polymerase can be inhibited to the same extent by AF/013 concentrations as low as 30 μg/ml. Thus, studies on partially purified enzymes retain their validity, even though increased levels of the drug may be necessary to observe the inhibiton. The role of BSA in the formation of a complex resistant to AF/013 (at 0°) using RNA polymerase II and native DNA remains unclear (see Table I). It is possible that BSA stabilizes the enzyme–DNA complex which may be readily dissociable in its absence.

In addition to the effect of exogenous proteins, several other factors affect the absolute number of AF/013 molecules necessary to inhibit an enzyme. Since AF/013 binds to the enzyme molecule, the amount of enzyme present in the reaction will affect the level of drug necessary for inhibition. Also, the levels of substrate can alter the inhibition profile. Comparisons of the amount of drug necessary to inhibit nucleotidyl transferases must therefore be approached with caution. In spite of these difficulties, it is interesting to note that the AF/013 inhibition profiles for the two nuclear polymerases, RNA polymerase II and poly(A) polymerase, are strikingly similar.

The physiological implications of the current study are imminent. Reduced nucleoside triphosphate levels in the cell can lead to an increased sensitivity of the nucleotidyl transferases to AF/013 or its derivatives. This is of particular importance with regard to ATP levels, since the poly(A) polymerase is not protected from inhibition by the drugs by an enzyme–DNA complex as could be the case for RNA polymerase. (Preincubation of poly(A) polymerase with primer also does not form a resistant complex.) Since poly(A) is involved in the proper processing of messenger RNA (Darnell et al., 1973; Brawerman, 1974) the sensitivity of poly(A) polymerase to inhibition by rifamycin derivatives could partially explain the toxicity of these drugs used clinically as antiviral agents.

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Studies on Interaction between Poly(L-lysine^{58}, L-phenylalanine^{42}) and Deoxyribonucleic Acids†

Regina M. Santella and Hsueh Jei Li*

ABSTRACT: A random copolymer of 58% L-lysine and 42% L-phenylalanine, poly(Lys^{58}Phe^{42}), was used as a model protein for studying the role of phenylalanine residues in protein–DNA interaction. Complexes between this copolypeptide and DNA, made by direct mixing, were studied by absorbance, circular dichroism (CD), fluorescence, and thermal denaturation. Complex formation results in an increase in absorbance, and an enhancement, red-shift, and broadening of phenylalanine fluorescence. The fluorescence enhancement is opposite to the quenching observed when a tyrosine copolypeptide is bound to DNA (R. M. Santella and H. J. Li (1974), *Biopolymers 13,* 1909). The positive CD band of DNA near 275 nm is reduced and red-shifted by the binding of the phenylalanine copolypeptide to a greater extent than by the tyrosine copolypeptide. Thermal denaturation of the complexes in 2.5×10^{-4} *M* EDTA (pH 8.0) shows three characteristic melting bands. For complexes with calf thymus DNA, free base pairs melt at $T_{m,I}$ (47–49°) and copolypeptide-bound base pairs show two melting bands ($T_{m,II}$ at 73–75°, and $T_{m,III}$ at 88 –90°). Similar thermal denaturation results have been observed for complexes with *Micrococcus luteus* DNA. The fluorescence intensity of the complexes is greatly increased when the temperature is raised to the $T_{m,II}$ region. In addition to fluorescence measurements, the effects of increasing temperature on absorption and CD spectra of the complexes were also studied. Stacking interaction between the phenylalanine chromophore and DNA bases, either partial or full intercalation, is implicated by the experimental results. Several mechanisms are proposed to describe the reaction between the copolypeptide and DNA, and thermal denaturation of the complex.

Many biological functions directly involve interactions between DNA and proteins, such as histones and non-histone proteins (Johnson et al., 1974, Hnilica, 1972), repressors, unwinding proteins, repair enzymes, and DNA and RNA polymerases (Kornberg, 1974). At least two classes of interactions can be distinguished, nonspecific and highly specific. Perhaps the best example of nonspecific interactions are those between DNA and histones, protamine, polylysine, or polyarginine, in which the main interaction is between anionic phosphates of DNA and the cationic amino acid residues of proteins. For highly specific interactions, the most extensively studied system is between the lactose repressor and operator, in which interaction occurs between a specific protein and a specific DNA sequence (Jacob and Monod, 1961; Gilbert and Müller-Hill, 1967; Beckwith and Zipser, 1970).

During the initial stage of studies on protein–DNA interactions, both proteins and DNA were considered as two interacting macromolecules. Beyond this stage, one must look into those factors which are directly involved in the binding between these two macromolecules, such as amino acid residues and bases and the physical and chemical environments in both proteins and DNA. These questions have been dealt with from several laboratories, using polytyrosine (Friedman and Ts'o, 1971), oligopeptides (Helene et al., 1971a,b; Gabbay et al., 1972, 1973; Novak and Dohnal, 1973; Dimicoli and Helene, 1974a,b; Brun et al., 1975, Durand et al., 1975), copolypeptides with given sequences (Sponar et al., 1974; G. D. Fasman, private communication), and random sequences (Santella and Li, 1974; Pinkston and Li, 1974).

Our recent studies on the interaction between DNA and poly(Lys^{40}Ala^{60}) emphasize the effects of α-helical proteins in binding to DNA (Pinkston and Li, 1974). Studies on the interaction between DNA and poly(Lys^{50}Tyr^{50}), on the other hand, emphasize the role played by the aromatic amino acid residues (Santella and Li, 1974). As an extension of the latter report, we have studied the interaction between DNA and poly(Lys^{58}Phe^{42}). For the tyrosine chromophore, in addition to the aromatic ring, there is a hydroxyl group. The former favors stacking with bases while the latter favors hydrogen bonding with hydrogen-bond acceptors on the surface of DNA. It seems that outside interaction is favored over intercalation (Santella and Li, 1974).

† From the Department of Chemistry, Brooklyn College of the City University of New York, Brooklyn, New York 11210. *Received February 7, 1975.* Supported by Grant GM21481 from the National Institutes of Health.

* Address correspondence to Division of Cell and Molecular Biology, State University of New York at Buffalo, Buffalo, N.Y. 14214.