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PROBES OF EUKARYOTIC DNA-DEPENDENT RNA POLYMERASE II—II

COVALENT BINDING OF TWO PURINE NUCLEOSIDE DIALDEHYDES TO THE INITIATION SUBSITE*

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Abstract—The catalytic center of wheat germ DNA-dependent RNA polymerase II (nucleosidetriphosphate:RNA nucleotidyltransferase, EC 2.7.7.6) as a model eukaryotic enzyme system was probed with two purine nucleoside dialdehydes, 6-methylthioinosinedicarboxaldehyde (MMPR-OP) and a derivative 6-[(acetylaminoethyl)-1-naphthylamine-5-sulfonyl]thioinosinedicarboxaldehyde (AMPR-OP). Both drugs gave noncompetitive inhibition with respect to [^{3}H]UMP incorporations into RNA, and inhibitor bindings were reversed with initiation substrates. The K_i values for MMPR-OP and AMPR-OP were determined to be 0.64 mM and 1.0 μM respectively. The drugs were covalently bound to the catalytic center by $NaBH_4$ reduction. Both were found bound to the largest enzyme subunit, IIa. It is tentatively concluded that MMPR-OP and AMPR-OP inhibit RNA polymerase II by binding to an essential lysine in the initiation subsite of the catalytic center located on the IIa subunit.

The design and synthesis of more specific and potent inhibitors of the eukaryotic DNA-dependent RNA polymerases II (or B) will require detailed information of the microenvironments in and about the catalytic center. In this vein, Brodner and Wieland [1] affinity-labeled the elongation subsite of an eukaryotic RNA polymerase with an amatoxin derivative. The amatoxins are known to block RNA chain elongation in eukaryotes [2]. Therefore, the catalytic center of eukaryotic RNA polymerase II, like *Escherichia coli* RNA polymerase, can probably be divided into several subsites [3–6]: an initiation subsite which binds the initiating purine nucleoside 5′-triphosphates and later the 3′-OH terminus of a growing RNA chain; an elongation subsite which binds the propagating nucleoside 5′-triphosphates; and, finally a DNA template subsite binding the DNA strand running in the 3′ → 5′ direction. Previously, 6-methylthioinosinedicarboxaldehyde (MMPR-OP)** and a derivative 6-[(acetylaminoethyl) - 1 - naphthylamine - 5 - sulfonyl]thioinosinedicarboxaldehyde (AMPR-OP) were used to bind to and affinity-label the initiation subsite in the catalytic center of *E. coli* RNA polymerase [4, 5]. MMPR-OP, known to have anticancer activity [7], also inhibits RNA synthesis in tumor cells [7]. In this report, MMPR-OP and AMPR-OP (Fig. 1) were used to probe the initiation subsite of the catalytic center of wheat germ RNA polymerase II as a model eukaryotic enzyme system.

EXPERIMENTAL

Isolation and purification of wheat germ RNA polymerase II. Wheat germ DNA-dependent RNA polymerase II was isolated and purified to homogeneity by the method of Jendrisak and Burgess [8].

RNA polymerase assay. Enzyme activity was assayed by the procedure of Jendrisak and Burgess [8]. The reaction mixture (0.25 ml) contained 2.5 μmoles Tris buffer (pH 7.9); 0.25 μmoles $MnCl_2$; 5 μmoles $MgCl_2$; 12.5 μmoles $(NH_4)_2SO_4$; 100 nmoles each of ATP, GTP and CTP; 0.1 nmole [5,6-^{3}H]UTP (sp. act. 1 μCi/0.1 nmole); 50 μg of heat-denatured calf thymus DNA; 125 μg of bovine serum albumin; and various concentrations of enzyme. The assay mixtures were incubated for 15 min at 25°, and the reaction was stopped in an ice-water bath by the addition of 2 ml of ice-cold 5% TCA containing 25 mM sodium pyrophosphate. After 5 min in the ice bath, the acid-insoluble materials were collected on Whatman GF/C (2.4 cm) glass fiber filters and were washed five times, each time with 5 ml of ice-cold 2% TCA containing 10 mM sodium pyrophosphate. Finally the filters were each washed with 2 ml of 95% ethanol, dried, and counted in a Beckman LS 9000 scintillation counter after the addition of 10 ml of toluene-based fluor.

Synthesis of MMPR-OP, [6-^{35}S]MMPR-OP, and AMPR-OP. The chemical syntheses of MMPR-OP and its radiolabeled derivative have been described

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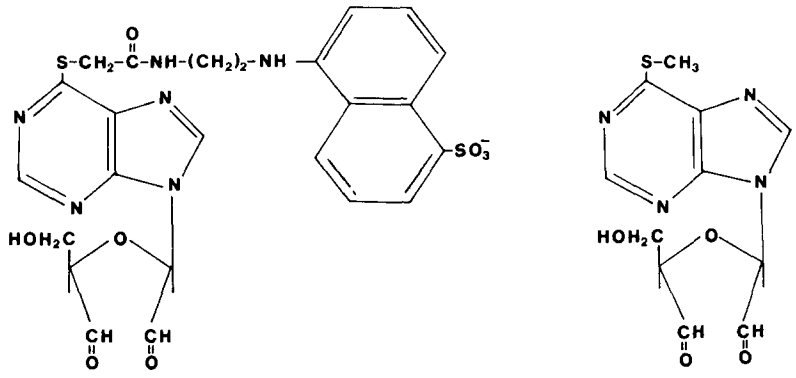
** Abbreviations: MMPR-OP, 6,methylthioinosinedicarboxaldehyde; AMPR-OP, 6-[(acetylaminoethyl)-1-naphthylamine - 5 - sulfonyl]thioinosinedicarboxaldehyde; TCA, trichloroacetic acid; SDS, sodium dodecylsulfate; and ara-6-MP, 9-β-D-arabinofuranosyl-6-mercaptopurine.



AMPR-OP MMPR-OP

Fig. 1. Structures of AMPR-OP and MMPR-OP.

in detail elsewhere [9]. The initial specific activity of the radiopure [6-^{35}S]MMPR-OP was determined to be 2.24×10^4 cpm/nmole. AMPR-OP was prepared by the method of Wu and Wu [5].

Inhibition studies. For inhibition studies, 12 μg (22 pmoles) of enzyme was preincubated for 10 min at 25° with various concentrations of MMPR-OP or AMPR-OP before the addition of ATP, GTP, CTP, [5,6-^{3}H]UTP, and DNA. The assay of enzyme activity was then carried out as described above. Protection from enzyme inhibition by MMPR-OP or AMPR-OP using 0.4 mM ATP or GTP was done by preincubating ATP or GTP with the enzyme for 10 min before the addition of 0.8 mM MMPR-OP or 7.0 μM AMPR-OP. Preincubations were continued for 10 min before assay.

Irreversible binding of [6-^{35}S]MMPR-OP. [^{35}S]MMPR-OP was bound irreversibly to the enzyme in the following manner. RNA polymerase II, 0.55 nmole, was incubated for 45 min at 25° with 2.50 mM [^{35}S]MMPR-OP in 1.0 ml of 0.1 M $KHCO_3$ buffer (pH 7.9) containing 8 mM $MgCl_2$ and 0.2 M KCl. The reaction mixture was then cooled to 4° and 15 mg of $NaBH_4$ in 1.0 ml of 0.1 M $KHCO_3$ buffer was added. The reduction of the Schiff's base to the stable amine bond was allowed to occur for 21 hr at 4°. Non-bound [^{35}S]MMPR-OP and unreacted $NaBH_4$ were dialyzed away at 4° against excess 0.1 M $KHCO_3$ buffer (pH 7.9) containing 50% glycerol until the radioactivity of the dialyzing buffer dropped to background level. The enzyme–[^{35}S]MMPR-OP complex was stored at −20° awaiting further analysis.

Irreversible binding of AMPR-OP and radiolabeling. Labeling of wheat germ RNA polymerase II with AMPR-OP in order to determine which subunit(s) was bound was carried out by the procedure of Wu and Wu [5]. The enzyme (0.47 nmole) was incubated for 45 min at 25° with 1 mM AMPR-OP in 0.1 M $KHCO_3$ buffer (pH 7.9) containing 8 mM $MgCl_2$ and 0.2 M KCl. After incubation, the resulting mixture was cooled at 4° and then 1 mM NaB^3H_4 (sp. act. 100 mCi/mmole) was added. The sample was incubated for 16 hr at 4° to reduce the Schiff's base to the stable amine. The reaction mixture was then applied to a Sephadex G-75 column (1.2 × 15 cm) to remove unreacted reagents. The labeled enzyme complex was then eluted with 0.1 M $KHCO_3$ buffer (pH 7.9) containing 0.1 mM EDTA, 0.1 mM dithiothreitol and 0.5 M KCl. The pooled fractions were dialyzed against excess 0.1 M $KHCO_3$ buffer (pH 7.9) containing 50% glycerol and then stored at −20°.

Gel electrophoresis. Wheat germ RNA polymerase II and RNA polymerase II-labeled complexes were run on SDS–polyacrylamide gels by the method of Laemmli [10] with slight modification by replacing methylenebisacrylamide with *N,N'*-diallyltartardiamide. The gels were preelectrophoresed for 15 min at a current of 1.5 mA/gel tube before adding the samples. The samples were prepared by denaturing the enzyme with the sample preparation buffer (1:1, v/v) containing 0.125 M Tris–HCl (pH 6.8); 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.01% bromophenol blue for 5 min at 95°. The gels were run with the anode in the lower reservoir at a current of 3 mA/gel tube until the tracking dye eluted. The gels were stained for at least 8 hr in 0.05% Coomassie brilliant blue R-250 in ethanol–acetic acid–water (5:1:5, v/v). They were destained with shaking in 7.5% acetic acid solution containing 3 g of mixed bed resin (AG 501-X8, Bio-Rad Laboratories, Richmond, CA) per gel in screw-capped culture tubes for about 12 hr at room temperature. The resulting band patterns were scanned at 550 nm in a Beckman 25 spectrophotometer equipped with a gel scanning apparatus. The radiolabeled gels were then cut into 2 mm slices and digested with 0.4 ml of 50% H_2O_2 in tightly capped scintillation vials at 55° for 5 hr. The vials were cooled to room temperature, 15 ml of Triton X-100 based scintillation fluor (Ready-Solv Solution VI, Beckman) was added, and the samples were counted in a Beckman scintillation counter (LS9000).

Protein determinations. Protein concentrations were determined by the procedure of Lowry *et al.* [11] using bovine serum albumin as a standard or by the method of Richardson [12].

Materials. Wheat germ, calf thymus DNA, bovine serum albumin, nucleosides and nucleotides were purchased from the Sigma Chemical Co., St. Louis, MO. [^{3}H]UTP was obtained from ICN Pharmaceuticals Inc., Irvine, CA; [^{35}S]rhombic sulfur in

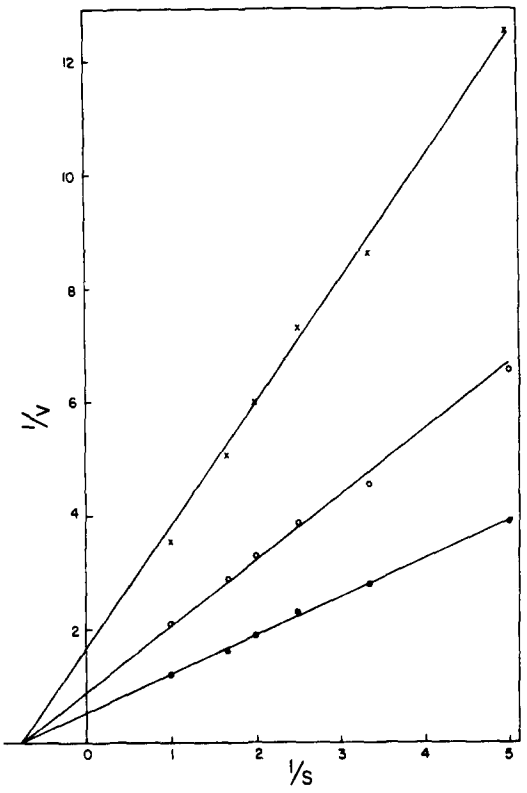


Fig. 2. Lineweaver–Burk plot of the inhibition of RNA synthesis by MMPR-OP. The reaction mixtures were those described in the Experimental section, except that UTP concentrations were varied. The reaction was terminated, and the RNA was precipitated, filtered and counted as outlined in the Experimental section. Key: (●——●) control; (○——○) 0.5 mM MMPR-OP; and (X——X) 1 mM MMPR-OP. The reaction rate was measured as dpm incorporated into an acid-insoluble material in 15 min. *S* represents [UTP] × 10^{-3} M.

powdered form was bought from the Amersham Corp., Arlington Heights, IL. The following electrophoretic reagents were purchased from Bio-Rad: acrylamide, methylenebisacrylamide, ammonium persulfate, sodium dodecylsulfate, Tris base, glycine, bromophenol blue, and Coomassie brilliant blue R-250. Ultrapure urea and ammonium sulfate were obtained from Schwarz/Mann, Orangeburg, NY. The following molecular weight markers were purchased from Pharmacia Fine Chemicals Inc., Piscataway, NJ: thyroglobulin, ferritin, catalase (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6.), lactate dehydrogenase (L-lactate:NAD^+ oxidoreductase, EC 1.1.1.27), and albumin.

RESULTS

Inhibition studies. MMPR-OP and AMPR-OP inhibited wheat germ RNA polymerase II. Figure 2 gives the Lineweaver–Burk plot which shows that MMPR-OP gave noncompetitive inhibition with respect to [^{3}H]UMP incorporation into RNA. The K_i for MMPR-OP was determined to be 0.64 mM (the K_m for UTP was 1.4 mM). AMPR-OP was also found to give noncompetitive inhibition with a K_i value of 1.0 μM (Fig. 3). Since [^{3}H]UMP incorporation is a measure of chain elongation, the noncompetitive inhibitions given by MMPR-OP or AMPR-OP implied that the inhibitors were not binding in the elongation subsite but elsewhere.

Reversal of inhibitions by initiation substrates. If MMPR-OP or AMPR-OP were binding in the initiation subsite, then preincubation with the initiation substrates, ATP and GTP, should protect this site from inhibitor bindings. Data presented in Table 1 show that if the enzyme was first preincubated with either 0.4 mM ATP or 0.4 mM GTP before the addition of 0.8 mM MMPR-OP or 7 μM AMPR-OP, almost no inhibition (13–19%)

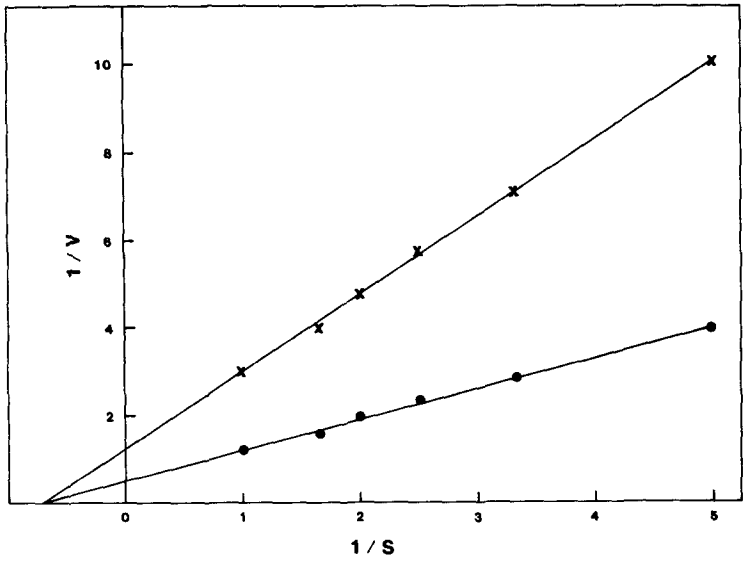


Fig. 3. Lineweaver–Burk plot of the inhibition of RNA synthesis by AMPR-OP. The samples were assayed as described in the legend to Fig. 2 and in the Experimental section. The reaction rate was measured as dpm incorporated into an acid-insoluble product in 15 min. *S* represents [UTP] × 10^{-3} M. Key: (●) controls; and (X) 10 μM AMPR-OP added.

Table 1. Protection of RNA polymerase II from MMPR-OP and AMPR-OP inhibition by preincubation with ATP and GTP*

Drug	[^{3}H]UMP incorporated (cpm)	Inhibition (%)
Control	17,500	
MMPR-OP (0.8 mM)	5,800	67
ATP (0.4 mM) + MMPR-OP (0.8 mM)	15,320	13
GTP (0.4 mM) + MMPR-OP (0.8 mM)	14,210	19
Control	17,420	
AMPR-OP (7.0 μM)	8,750	50
ATP (0.4 mM) + AMPR-OP (7.0 μM)	14,790	15
GTP (0.4 mM) + AMPR-OP (7.0 μM)	14,390	17

* RNA polymerase (22 pmoles/reaction mixture) was preincubated with either ATP or GTP for 10 min at 25° before the addition of MMPR-OP or AMPR-OP. Preincubations were continued for an additional 10 min before assay.

occurred. These results, coupled with the noncompetitive (with respect to UTP) inhibition, indicated that MMPR-OP and AMPR-OP were binding in the initiation subsite.

Irreversible binding of [6-^{35}S]MMPR-OP to RNA polymerase II. MMPR-OP has been shown to produce a Schiff's base with the epsilon amino group of essential lysines in the catalytic centers of several enzymes [4, 13, 14]. The slope of the line in a Hill plot [15] of MMPR-OP inhibition, therefore, would yield the number of essential lysine residues bound [3, 4, 13, 14]. The result of a typical experiment for this determination with MMPR-OP and RNA polymerase II is depicted in Fig. 4. The slope of the line was 1.8 and constant over the range of 0.38 to 5.0 mM which indicated that two essential lysines in the initiation subsite were forming Schiff's bases with MMPR-OP. At very high concentrations, MMPR-OP is known to modify nonessential lysine groups [14]. However, since essential amino acid residues in enzyme active sites presumably are more reactive than other identical amino acids [16], affinity-labels will modify the catalytic amino acid side chains with a one-to-one stoichiometry. The stoichiometry of MMPR-OP binding to RNA polymerase II was ascertained using an application [9] of the Freundlich adsorption isotherm [17]. These results are given in Table 2. From these data, it was determined that [6-^{35}S]MMPR-OP at 3.6 mM or less would bind to 0.55 nmole RNA polymerase II in a one-to-one molar ratio. The [6-^{35}S]MMPR-OP was then covalently bound to the enzyme by mild reduction with $NaBH_4$, and the enzyme–[6-^{35}S]MMPR-OP complex subjected to polyacrylamide gel electrophoresis. These results are shown in Fig. 5. Initial electrophoresis in 12.5% gels (data not shown) showed that 80% of the radioactivity was associated with the two largest subunits, IIa and IIb, which were not resolved. However, electrophoresis in 4% gels (Fig. 5) showed that only the largest subunit, IIa (mol. wt 220,000 daltons), was labeled by [6-^{35}S]MMPR-OP.

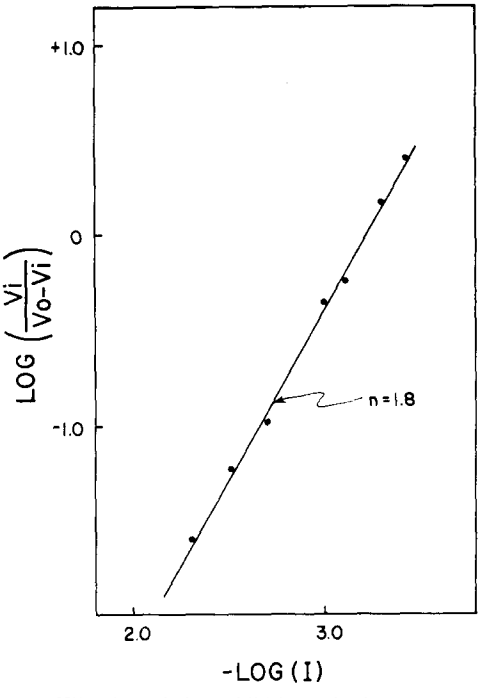


Fig. 4. Hill plot of the inhibition of wheat germ RNA polymerase II by MMPR-OP. The RNA polymerase II (22 pmoles) was incubated with various concentrations of MMPR-OP ranging from 0.38 to 5.0 mM. As described under the Experimental section, all substrates and template (in a final volume of 0.25 ml) were then added, and the samples were incubated for 15 min at 25°. The reaction was stopped, and the RNA was precipitated, filtered and measured as described in the Experimental section. (I) represents various concentrations of MMPR-OP; Vo and Vi represent the uninhibited and inhibited reaction rates respectively.

Irreversible binding of [^{3}H]AMPR-OP. AMPR-OP has also been shown to produce a Schiff's base with an essential lysine in the catalytic center of the *E. coli* RNA polymerase [5]. Stable covalent amine bond formation and radiolabeling can be accomplished in one step by NaB^3H_4 reduction. This procedure was carried out with the wheat germ RNA polymerase II using 1.0 mM AMPR-OP, 0.47 nmole enzyme, and 1.0 mM NaB^3H_4. Polyacrylamide gel electrophoretic studies were performed on the purified enzyme–[^{3}H]AMPR-OP complex. Again, initial electrophoresis in 12.5% gels ruled out radiolabeling of any subunits except subunits IIa and IIb which were not adequately resolved. Finally, electropho-

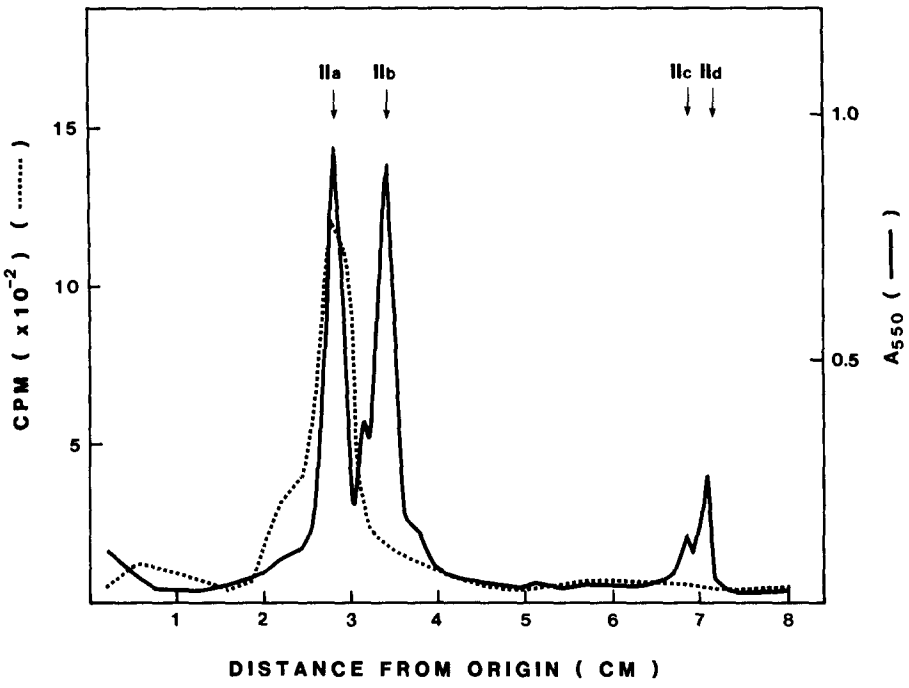


Fig. 5. Subunit localization of [^{35}S]MMPR-OP bound to RNA polymerase II. The methods and procedures were the same as those given in the Experimental section. Electrophoresis of the denatured enzyme–[^{35}S]MMPR-OP complex was carried out in 4% polyacrylamide gels. Key: subunit IIa, 220,000 daltons; subunit IIb 140,000 daltons; subunit IIc 42,000 daltons; and subunit IId 40,000 daltons. On this 4% gel, subunits below IId ran off the gel. The dotted line denotes radioactivity, and the solid line represents the absorbance at 550 nm.

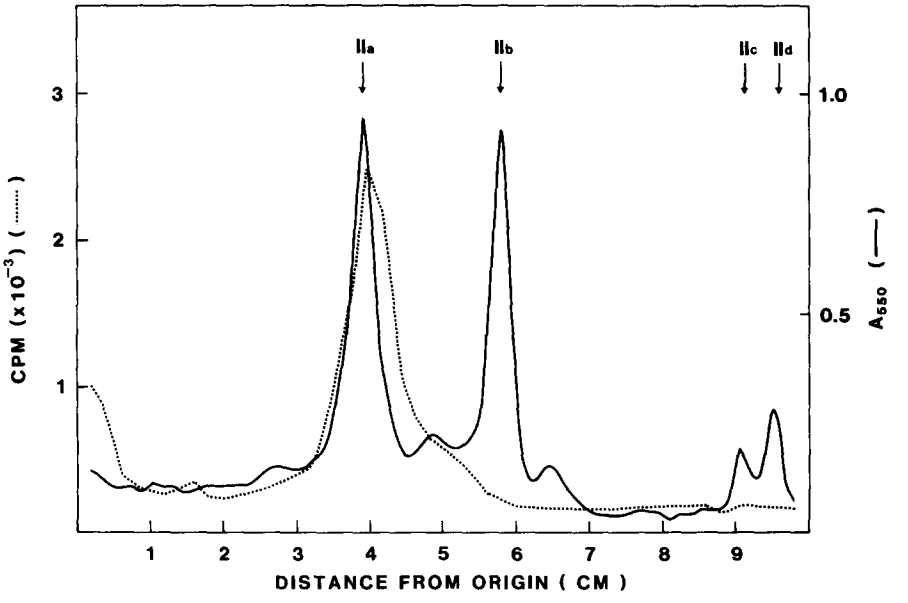


Fig. 6. SDS–polyacrylamide gel (5%) electrophoresis of RNA polymerase II–AMPR-OP complex after reduction with NaB^3H_4. (Initial electrophoresis in 12.5% gels showed only one major radioactive peak localized to the IIa and IIb subunits with the other subunits unlabeled.) The solid line represents the absorbance profile of a 5% gel at 550 nm after staining with Coomassie brilliant blue R-250; the broken line shows the radioactivity.

Table 2. Determination of the concentration at which [^{35}S]MMPR-OP binds in a 1:1 molar ratio with RNA polymerase*

RNA polymerase (nmole/reaction)	[^{35}S]MMPR-OP		Molar binding ratio (drug/enzyme)
	Concn (M)	nmoles/bound	
0.55	1×10^{-4}	0.023	0.042
0.55	5×10^{-4}	0.127	0.231
0.55	1×10^{-3}	0.182	0.331
0.55	2×10^{-3}	0.238	0.433
0.55	5×10^{-3}	0.720	1.309

* RNA polymerase (0.55 nmole/reaction mixture) was incubated for 15 min at 25° in a final volume of 0.25 ml with various concentrations of [^{35}S]MMPR-OP (sp. act. 2.24×10^4 cpm/nmole) in 0.1 M $KHCO_3$ buffer (pH 7.9) containing 8 μM $MgCl_2$ and 0.2 M KCl. No substrates and/or template were present. The reaction mixtures were cooled to 4°, and the reactions were terminated by the addition of 2 ml of ice-cold 10% TCA. The treated mixtures were then filtered through Millipore membranes (RAWP 2.5 cm, Millipore Corp.), and the unbound radiolabeled compound was washed through with 20 ml of ice-cold 2% TCA and 2 ml of ethanol. The filters were allowed to air dry for 2 hr. Finally, they were placed directly into scintillation vials containing 10 ml of a toluene-based scintillation fluid, and the bound [^{35}S]MMPR-OP was counted in a liquid scintillation spectrometer.

resis in 5% gels (Fig. 6) showed that subunit IIa was labeled by [^{3}H]AMPR-OP which was consistent with the labeling of IIa by [^{35}S]MMPR-OP (Fig. 5).

DISCUSSION

Wheat germ DNA-dependent RNA polymerase II provides an excellent model of the eukaryotic enzyme for inhibition studies since it apparently exists in only one "isoenzyme" form unlike other eukaryotic RNA polymerases II [18], and can be isolated in homogeneous form in high yield from a convenient source. The enzyme has a molecular weight of 5.5×10^5 daltons and is composed of eleven subunits [19]. Our laboratory has been engaged in probing the catalytic center of the enzyme with the long-range goal of developing specific and potent inhibitors. This end requires detailed knowledge of the amino acid residues in and about the catalytic center as well as the microenvironments (i.e. hydrophobicity) about the binding sites of inhibitors. In other research reported in this journal [20], the anticancer drug 9-β-D-arabinofuranosyl-6-mercaptopurine (ara-6-MP) was found to bind to the elongation subsite and to interact closely with an essential cysteine residue. Since the radiolabeled ara-6-MP was found bound to the IIb subunit, the elongation subsite was assigned to that subunit. MMPR-OP, another anticancer agent, as well as a derivative, AMPR-OP, have been used to study the initiation subsite of the *E. coli* RNA polymerase [3–5]. MMPR-OP and AMPR-OP apparently bind, as well, to the initiation subsite of wheat germ RNA polymerase II. The [6-^{35}S]MMPR-OP and [^{3}H]AMPR-OP were covalently bound to the largest subunit of the enzyme, IIa. Thus, the catalytic center of this eukaryotic RNA polymerase II is possibly divided between two subunits of unequal size—a situation noted for the catalytic center of the *E. coli* enzyme [21, 22]. It was of interest to compare the K_i values of MMPR-OP ($K_i = 0.64$ mM) and AMPR-OP ($K_i = 1.0$ μM). Wheat germ RNA polymerase II, therefore, binds AMPR-OP about 500 times more strongly than MMPR-OP. MMPR-OP has a methyl group attached to the sulfur at the purine sixth position while AMPR-OP has an acetylaminoethyl-1-naphthylamine-5-sulfonate group replacing the methyl group (Fig. 1). Since this large side chain on AMPR-OP could be considered to be hydrophobic, it raises the intriguing possibility that the purine base moiety of an initiating purine nucleoside 5′-triphosphate molecule may bind in a hydrophobic pocket in the initiation subsite. A series of hydrophobic derivatives of thioinosinedicarboxaldehyde will be prepared to test this possibility.

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