Affinity Labeling of *Escherichia coli* B Deoxyribonucleic Acid Dependent Ribonucleic Acid Polymerase†

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ABSTRACT: Many laboratories have extensively studied the subunit structure of the purified bacterial enzyme DNA-dependent RNA polymerase. We have synthesized a pseudo-substrate 6-methyl[^{35}S]thioinosinedicarboxaldehyde (MMPR-OP) and have shown that this compound binds to ϵ-amino groups of lysine residues. Through binding studies, we have determined that radiolabeled MMPR-OP can act as an affinity label and will bind to *Escherichia coli* RNA polymerase in a 1:1 molar ratio at a drug concentration of 5×10^{-4} M. Separation of the subunits of the [^{35}S]MMPR-OP-bound enzyme by urea denaturation and polyacrylamide gel electrophoresis indicates that the [^{35}S]MMPR-OP is bound to the β subunit of the enzyme.

The core enzyme of DNA-dependent RNA polymerase from *Escherichia coli* has been shown to be composed of three major proteins having a stoichimetric relationship of ($\alpha_2\beta\beta'$) (Burgess, 1969a). Our laboratory has been involved in determining which of these subunits contains the catalytic center of the enzyme and in the mapping of the amino acids directly involved in the catalysis. Rifampicin, an inhibitor of initiation, has been reported to bind to the β subunit of the enzyme (Rabussay and Zillig, 1969; Zillig *et al.*, 1970). Further evidence associating the β subunit with the catalytic center of RNA polymerase was provided by Heil and Zillig (1970) when they were able to resconstitute an active enzyme from separated subunits.

It has been postulated that the catalytic center of the enzyme contains three subsites; namely, a DNA template subsite, an initiation subsite (2′,3′-OH terminis subsite), and an elongation subsite (nucleoside triphosphate subsite) (Spoor *et al.*, 1970; Krakow and Fronk, 1969). In addition, the amino acids, histidine, cysteine and lysine have been shown to be essential for catalytic activity (Spoor *et al.*, 1970; Ishihama and Hurwitz, 1969).

In our previous report, we showed that the periodate oxidation product of β-D-ribosyl-6-methylthiopurine (MMPR-OP)[1] inhibited *E. coli* RNA polymerase presumably by binding to the initiation subsite by forming a Schiff's base with the ϵ-NH_2 group of an essential lysine residue (Spoor *et al.*, 1970). In this communication, we report that we have successfully affinity labeled the enzyme by covalently binding MMPR-OP to an essential lysine residue in the active center.

Materials and Methods

Enzyme Purification and Assay. DNA-dependent RNA polymerase (holoenzyme) was extracted from *E. coli* B (General Biochemical) by a modification (Spoor, 1970) of previous methods (Burgess, 1969b; Alberts and Herrick, 1971). The activity of the enzyme was assayed in a total volume of 0.5 ml with a reaction mixture containing 400 nmol each of ATP, GTP, CTP, and [5′-^{3}H]UTP; 15 μg of purified enzyme; 50 μmol of Tris-HCl (pH 7.9), which was 8 μM in $MgCl_2$ and 0.2 M in KCl. Activity was measured as the amount of [^{3}H]-UMP incorporated into an acid-insoluble product during a 15-min incubation period at 37°.

Synthesis of [^{35}S]MMPR-OP. Using ^{35}S rhombic sulfur, 6-mercaptopurine ribonucleoside (Nutritional Biochemicals) was labeled by sulfur exchange (Bennett *et al.*, 1965). The 6-[^{35}S]Mercaptopurine ribonucleoside was then methylated with methyl iodide to produce 6-[^{35}S]methylmercaptopurine ribonucleoside ([^{35}S]MMPR). The periodate oxidation product of [^{35}S]MMPR was prepared by the method of Kimball *et al.* (1968). The radiolabeled drug was then checked for purity by paper chromatography using Whatman No. 3MM paper and a solvent system composed of ethanol–saturated sodium tetaborate–ammonium acetate (5.0 M, pH 9.5)–0.5 M Versene (220:80:20:5). The specific activity of the purified drug was determined to be 3.72×10^{12} cpm/mole.

Binding [^{35}S]MMPR-OP to Enzyme. In a typical experiment, 679 μg of enzyme was incubated for 45 min at 37° with 5×10^{-4} M [^{35}S]MMPR-OP in 1.0 ml of 0.1 M $KHCO_3$ buffer (pH 7.9), which was 8 mM in $MgCl_2$ and 0.2 M in KCl. After incubation, the reaction mixture was cooled to 4° and 15 mg of $NaBH_4$ in 1.0 ml of $KHCO_3$ buffer was added. Reduction of the Schiff's base to a stable covalent bond was allowed to occur for 12 hr at 4°. Non-protein-bound [^{35}S]MMPR-OP and $NaBH_4$ were dialyzed away against excess 0.1 M $KHCO_3$ buffer. The [^{35}S]MMPR-OP-bound enzyme was then stored in a 50% glycerol storage buffer at −20°.

Disc Electrophoresis. Electrophoresis on polyacrylamide gel was performed according to method described by Burgess (1969a) and Burgess *et al.*(1969). Enzyme was denatured with either 8 M urea or 3% sodium dodecyl sulfate. Approximately 250 μg of denatured protein was layered on 7.5% polyacrylamide gels, 1.3 cm in diameter prepared with 8 M urea and buffered with a Tris-glycine buffer at a pH of 9.3. Gels were run for 15 min at 7 mA/tube then the amperage was increased to 15 mA/tube until completion, usually 4 hr. The gels were then stained with Amido Black 10B (1.0%) in 7.0% acetic acid for 90 min; then destained with 7.0% acetic acid until gels had cleared and bands were visible.

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[1] Abbreviations used are: MMPR-OP, β-D-ribosyl-6-methylthiopurine oxidation product, thioinosinedecarboxaldehyde; [^{35}S]MMPR, 6-[^{35}S]methylmercaptopurine ribonucleoside.

TABLE I: Determining the Concentration at Which [^{35}S]-MMPR-OP Binds in a 1:1 Molar Ratio with RNA Polymerase.[a]

RNA Polymerase (μmol/Reaction)	[^{35}S]MMPR-OP		Molar Binding Ratio Drug: Enzyme
	Concn (M)	μmol Bound	
1.357×10^{-3}	5×10^{-6}	1.53×10^{-5}	0.01:1
1.357×10^{-3}	5×10^{-5}	0.88×10^{-4}	0.07:1
1.357×10^{-3}	5×10^{-4}	1.05×10^{-3}	0.77:1

[a] RNA polymerase (1.375 mmol/reaction mixture) was incubated with varying concentrations of [^{35}S]MMPR-OP in a 0.1 M $KHCO_3$ buffering system (pH 7.9), containing 8 μmol of $MgCl_2$ and 0.2 M with respect to KCl. No substrates or template was present. The incubation period was 15 min at 37°. The reaction mixture was then cooled to 3° and 2 mg of $NaBH_4$ was added in 0.2 ml of water. The reaction mixtures were left in an ice bath under reducing conditions for 3 hr. The reaction mixtures were then treated with ice-cold trichloroacetic acid (3 ml of a 5% solution). This treated mixture was then filtered through a double layer of Whatman GF/C glass fiber filter paper and unbound radiolabeled drug was washed through with ten volumes of 5% trichloroacetic acid. After washing, the glass filters were removed, dried at 55° for 2 hr, and placed directly into a toluene base, liquid scintillation system for counting.

Results

Table I summarizes the results of incubating an increasing range of concentrations of [^{35}S]MMPR-OP with purified RNA polymerase (0.68 mg/reaction). After an incubation period of 45 min, the ^{35}S-labeled drug-bound enzyme was trapped on a double layer of Whatman GF/C glass fiber filters and unbound drug was removed by washing with cold trichloroacetic acid. It can be seen that at a concentration of 5×10^{-4} M, the radiolabeled drug was binding in an approximately 1:1 molar ratio with RNA polymerase.

Utilizing the observation that the substrate analog, [^{35}S]-MMPR-OP, was binding to RNA polymerase at a drug concentration of 5×10^{-4} M, large quantities of RNA polymerase were labeled according to the procedure described previously. At various intervals during the binding study and reduction period, aliquots of the reaction were removed and assayed for activity. Inhibition of [^{3}H]UMP incorporation into an acid-insoluble product by the presence of [^{35}S]MMPR-OP is shown in Figure 1. These results indicate that the control reaction mixture was inhibited by less than 10% after the incubation and reduction period. In contrast, the [^{35}S]MMPR-OP-treated enzyme steadily lost activity and was inhibited by over 70% at the end of the reduction period.

A portion of this ^{35}S-labeled drug-bound enzyme was digested enzymatically with Pronase at 37° from 12 to 72 hr. A control experiment was conducted analogous to the binding study in which free L-lysine was substituted for enzyme and was incubated with an equimolar concentration of [^{35}S]-MMPR-OP reduced under the same conditions and exposed to the same hydrolytic activity as described above. This lysine-bound drug mixture was then cochromatographed with the hydrolyzed enzyme by a thin-layer chromatographic technique. Strip counts of the chromatogram are shown in Figure 2. The lysine-bound drug was also identified in two paper chromatographic (Whatmann No. 3MM) systems: butanol–acetic acid–water, 4:1:5 (lysine, R_F 0.12; MMPR-OP, R_F 0.95; lysine-MMPR-OP, R_F 0.36), and pyridine–

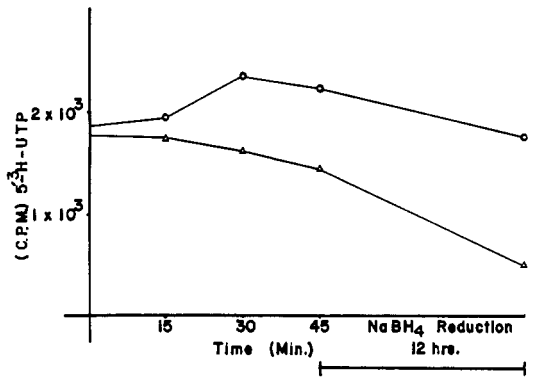

FIGURE 1: A monitoring of the binding of (^{35}S)MMPR-OP to RNA polymerase. The overall binding procedure for affinity-labeling RNA polymerase with [^{35}S]MMPR-OP is described in the Materials and Methods portion of the text. A control mixture containing enzyme, water substituted for drug, and $NaBH_4$ was carried through this procedure. During the binding incubation period, aliquots (25 μl, approximately equal to 0.5 μg of enzyme) were taken from both the control and drug-treated reaction mixtures. These were kept at 3° until $NaBH_4$ postreduction aliquots could be taken. When all samples had been collected, other components of the normal assay mixture were added and the reaction mixtures were incubated for 10 min at 37°. Activity was measured by cross channel counting of the incorporation of [5′-^{3}H]UMP into an acid-insoluble product. (○) Control, no [^{35}S]MMPR-OP and (△) [^{35}S]MMPR-OP at 5×10^{-4} M.

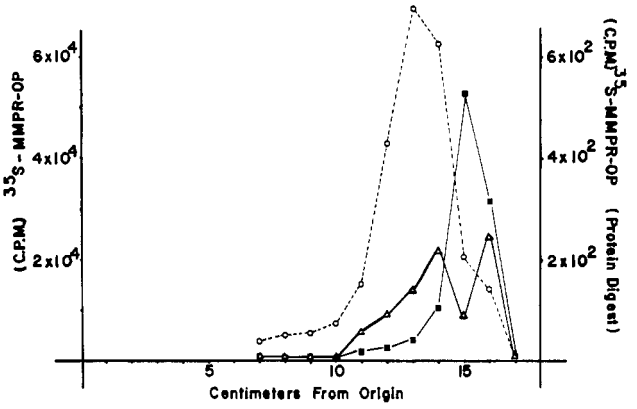

FIGURE 2: Strip counting of a proteolytic digest of [^{35}S]MMPR-OP-bound RNA polymerase and controls separated by thin-layer chromatography. The [^{35}S]MMPR-OP–polymerase complex was digested with Pronase for 72 hr at 37° and this enzymatic digest was separated by thin-layer chromatography. Two controls were run with the enzyme digest on the same chromatogram. The first control was 25 μg of free [^{35}S]MMPR-OP and the second control consisted of [^{35}S]MMPR-OP-lysine prepared under the same conditions that were used in binding the drug to RNA polymerase. Silica G plates (Eastman) were spotted and a 95% ethanol–15% ammonia (4:1) solvent system was used. Two distinct peaks were observed in the strip count of the "free" lysine-bound [^{35}S]MMPR-OP. The first, R_F 0.82, is lysine bound to drug, and the second corresponds to free drug at an R_F value of 0.92. (■) Free [^{35}S]MMPR-OP and (△) lysine-bound [^{35}S]MMPR-OP (both scaled to left); (○) RNA polymerase bound [^{35}S]MMPR-OP Pronase digest (scaled to right).

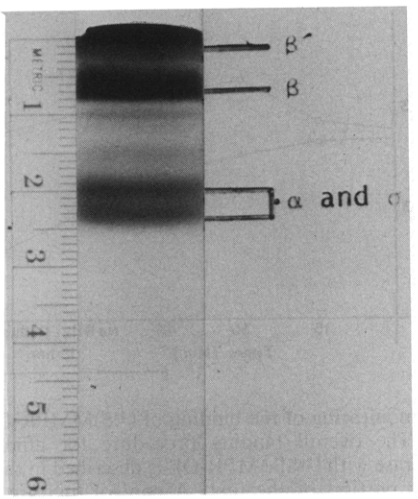

Figure 3: Electrophoretic separation of the subunits composing the [^{35}S]MMPR-OP-bound RNA polymerase complex. A polyacrylamide gel electrophoretic separation of approximately 250 μg of RNA polymerase–[^{35}S]MMPR-OP complex was performed on 8 M urea gels at pH 9.3. Gels were run for 15 min at 7 mA/tube, then the amperage was increased to 15 mA/tube until completion, usually 4 hr. Gels were run at room temperature. The stain used was Amido Black 10B (1.0%) solution in 7% acetic acid. Destaining was done with a 7% acetic acid solution until bands were visible.

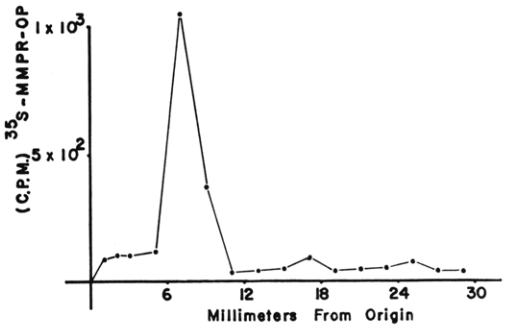

FIGURE 4: A strip count of the polyacrylamide gel in Figure 3 containing separated subunits of the [^{35}S]MMPR-OP–RNA polymerase complex. Gels, like those described in Figure 3, were sliced and either the 1.0- or 2.0-mm slices were digested with 0.4 ml of H_2O_2 in a water bath at 55° for 6 hr. The clear digests were counted in the standard Triton X-100–toluene scintillation fluor (Materials and Methods). A strip count of the gel indicated that 84% of the radioactivity was located in two consecutive slices which overlapped the β-subunit band on the gel (Figure 3).

isoamyl alcohol–water–diethylamine, 10:10:7:3 (lysine, R_F 0.07; MMPR-OP, R_F 0.88; lysine-MMPR-OP, R_F 0.72). These results support the studies of Loy (1970) and imply that enzyme inhibition by this drug results from binding of MMPR-OP to an ϵ-amino group of a lysine residue in or near the catalytic center of RNA polymerase (Spoor *et al.*, 1970). In addition, the extensive studies of Loy (1970) have shown that, under our conditions, MMPR-OP binds to only one other amino acid, ornithine, a non-protein amino acid that could be thought of as an analog of lysine.

A quantity of the [^{35}S]MMPR-OP-bound enzyme was denatured in 8 M urea and separated by polyacrylamide disc electrophoresis as described in the Methods section. Large acrylamide gels were used and could separate quantities of protein up to 500 μg. The subunits of the separated holoenzyme are shown in Figure 3. Sectioning the gels and strip counting indicated that 84% of the radio-labeled ^{35}S could be found in two consecutive slices which overlapped the β-subunit band on the gel, 5–8 mm from the origin Figure 4.

Discussion

The MMPR-OP has previously been shown by Spoor *et al.* (1970) to be a potent inhibitor of *E. coli* DNA-dependent RNA polymerase demonstrating noncompetitive inhibition kinetics. This drug has also previously been shown to be an inhibitor of DNA and protein synthesis in Ehrlich ascites tumor tissue (Kimball *et al.*, 1968). At a drug concentration of 5×10^{-4} M, MMPR-OP has an inhibition constant (K_i) very close to the K_m of the uninhibited reaction (Spoor *et al.*, 1970). This implies that at this concentration, the drug is binding to the enzyme with an affinity equal to that of the normal substrate. Spoor *et al.* (1970) also postulate that the binding of this substrate analog was *via* a Schiff's base with an ϵ-amino group of a lysine residue in the initiation subsite. The results presented in this report confirm that if [^{35}S]MMPR-OP is incubated with RNA polymerase, it does bind to a lysine residue on the enzyme. Inhibition of enzyme activity is demonstrated upon binding of the analog and evidence indicates that the drug is binding in a 1:1 molar ratio with the enzyme. All of this evidence supports the fact that there is one critical lysine residue near the triphosphate binding subsite in the active center of the enzyme which will bind MMPR-OP.

After mild reduction, the carbon–nitrogen bond of the Schiff's base becomes a stable covalent bond. The discovery of [^{35}S]MMPR-OP bound to the β subunit of RNA polymerase is in agreement with work done previously with radioactive rifampicin by Zillig *et al.* (1970). The kinetic studies by Spoor *et al.* (1970) indicate that MMPR-OP binds at the initiation subsite adjacent to the elongation site and inhibits noncompetitively. The results of the study presented here further support the study by Spoor *et al.* (1970) and suggests that a critical lysine bound by MMPR-OP is in the catalytic center of DNA-dependent RNA polymerase which is located on the β subunit of the enzyme.

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Effect of Sonication on the Structure of Lecithin Bilayers†

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ABSTRACT: The properties of dipalmitoyllecithin vesicles of varying sizes have been compared employing proton and ^{31}P nuclear magnetic resonance spectroscopy and dilatometry. These studies indicate that small vesicles of about 250 Å in diameter are inherently disordered and that the well-known spectral differences generally observed for vesicles of different curvatures such as lecithin multilayers and sonicated bilayers arise from variations in the molecular packing of phospholipid molecules in the bilayer phase.

The increased interest in membrane structure and biological transport has stimulated the development of easily prepared model membrane systems, which can be readily manipulated to elucidate some of the physical and chemical properties of biological membranes. In particular there has been much interest in phospholipid bilayers. A simple suspension of bilayer-forming lipids in water produces a multilamellar structure containing a number of concentric vesicles of various sizes (Bangham and Horne, 1964). Although many structural questions concerning the bilayer phase of these model membranes can be deduced from studies of these multilamellar structures (Papahadjopoulos and Miller, 1967), they are unsuitable for transport studies (Bangham, 1972). Moreover, each multilamellar unit contains an unknown number of phospholipid bilayers separated by an unknown amount of water, whose state of order is unclear. It is, however, possible to obtain homogeneous phosphatidylcholine vesicles by prolonged ultrasonic irradiation. These vesicles are closed spheres about 250 Å in diameter, each composed of a single continuous lipid bilayer membrane enclosing a volume of aqueous solution (Huang, 1969). Such vesicles have been widely used in recent years as model systems for structural studies of the bimolecular lipid lamellar phase and, in contrast to the unsonicated preparation of Bangham (1968), have been used in numerous physiochemical and transport studies (Kornberg and McConnell, 1971a,b; Lee *et al.*, 1972; Papahadjopoulos and Watkins, 1967; Bangham, 1972).

During the last several years a number of attempts have been made to characterize phospholipid bilayers by nuclear magnetic resonance spectroscopy (Penkett *et al.*, 1968; Finer *et al.*, 1972). As a result of these studies it is now known that the nuclear magnetic resonance (nmr) spectrum of phospholipid bilayers is very different depending upon whether the bilayers are in a multilamellar state or whether they are in vesicles of 250 Å in diameter. Unsonicated lecithin suspensions (multilayers) exhibit only broad resonances (Chan *et al.*, 1971) in contrast to the proton magnetic resonance (pmr) spectra of sonicated vesicles which contain sharp resonances. The sharper resonances are presumably a manifestation of a faster and more complete averaging of the nuclear dipole–dipole interactions of the proton spins, but the way in which the sonication of lecithin leads to this motional averaging is not understood despite numerous attempts.

This paper describes an attempt to elucidate those factors responsible for the spectral differences between lecithin multilayers and vesicles of 250 Å in diameter. In this work dipalmitoyllecithin vesicles were prepared with a wide range of diameters and their nmr spectral as well as molal volume characteristics were studied under a variety of experimental conditions. These studies indicate that the small vesicles of about 250 Å in diameter are inherently disordered and that the observed differences associated with vesicles of different curvatures arise from differences in local mobility through variations in molecular packing.

Experimental Section

Preparation of Dipalmitoyllecithin Samples. L-α-Dipalmitoylphosphatidylcholine from Nutritional Biochemicals was checked for purity by thin-layer chromatography (tlc) and found to contain less than 1% impurity. It was also shown to have no effect on the conductivity of deionized and distilled water. This lecithin was used without further purification in the preparation of the following types of bilayers.

UNSONICATED BILAYERS. Dipalmitoyllecithin samples in the range of 100–300 mg/ml of lecithin in H_2O were suspended by repeated passage at 60° through a 6-in. long 20-gauge needle.

VESICLES CONTAINING HIGH SALT. A suspension of about 50 mg/ml of lecithin in D_2O (0.1 M NaCl–2 mM PO_4 at pD 7.8) was sonicated for five minutes at power level 6 with a Bronson sonifier Model S-75. Initially the temperature was 20° but during sonication the temperature of the sample rose above 50°. The sample was then centrifuged at 20,000*g* in a Sorvall RC-2 centrifuge for 30 min and the lower layer was drawn off since the larger particles centrifuged to the surface. The

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