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AFFINITY LABELING OF A CYSTEINE AT OR NEAR THE CATALYTIC CENTER OF *ESCHERICHIA COLI* B DNA-DEPENDENT RNA POLYMERASE

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Summary

9-β-D-Arabinofuranosyl-6-thiopurine was used to affinity label DNA-dependent RNA polymerase isolated from *Escherichia coli* B. This substrate analogue displayed competitive type inhibition which could be reversed by addition of a thiol reagent, such as dithiothreitol, while exposure to hydrogen peroxide, a mild oxidizing agent, caused an increase in both the inhibitory and enzyme binding capability of arabinofuranosyl thiopurine. Chromatographic analysis of the products obtained by pronase digestion of the 9-β-D-arabinofuranosyl-6-[^{35}S]thiopurine-enzyme complex suggests that disulfide bond formation occurs between the inhibitor and a cysteine residue located in or near the active center of the enzyme. In addition, polyacrylamide gel electrophoresis indicated that the arabinofuranosyl thiopurine moeity was bound to the β′ subunit of the enzyme.

Introduction

The complex multiple subunit composition of DNA-dependent RNA polymerase together with the complicated nature of the polymerization reactions it catalyzes makes it a formidable task to isolate and identify the specific amino acids that participate in the catalytic process. Two techniques which have been used to help resolve this problem are chemical modification and affinity label-

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ing [1—4]. Chemical modification has been employed previously to identify amino acid residues having a specific subfunction in the overall polymerization reaction. Selective modification of the amino acids lysine, cysteine, histidine, and arginine, inhibit, either partially or completely, enzymatic activity [5—9].

In a previous communication we reported on a radioactive substrate analogue, [^{35}S]methylthioinosine dicarboxaldehyde, capable of reacting irreversible with a lysine situated in the initiation subsite of the active center [6]. A fluorescent derivative of this reagent was prepared and was also found to bind in the initiation subsite [10]. In this paper, we report on the radioactive substrate analogue 9-β-D-arabinofuranosyl-6-[^{35}S]thiopurine which was used to affinity label the active site of RNA polymerase.

Materials and Methods

Reagents. Escherichia coli cells were purchased from Grain Processing Corp., [^{3}H]UTP from ICN Pharmaceuticals Inc. rhombic [^{35}S]sulfur, Aquasol II and Protosol from New England Nuclear; arabinofuranosyl thiopurine and pronase from Calbiochem, ultrapure urea from Schwartz/Mann, [^{14}C]cysteine from Amersham-Searle. All other reagents were purchased from Sigma Chemical Company unless otherwise stated.

Enzyme purification and standard assay. DNA-dependent RNA polymerase was extracted from *E. coli* cells and purified using the procedures described by Burgess and Jendrisak [11]. Enzymatic activity was measured as the amount of [^{3}H]UMP incorporated an acid-insoluble product during a 10 min incubation period at 37°C. The concentration of agents in a standard 0.50 ml assay were 8 μmol $MgCl_2$, 100 μg calf thymus DNA, 400 μmol ATP, 400 μmol GTP, 400 μmol CTP, 400 μmol [^{3}H]UTP (spec. act. of $5.5 \cdot 10^6$ dpm/μmol) 100 μmol KCl, 50 μmol Tris-HCl (pH 7.9). For inhibition studies, the enzyme was preincubated for 10 min at 37°C with the appropriate quantity of arabinofuranosyl thiopurine and then assayed. For substrate and dithiothreitol protection experiments, the enzyme (15 μg) was preincubated for 10 min at 37°C with the appropriate protecting agent (400 μmol ATP or CTP) prior to preincubation with arabinofuranosyl thiopurine.

Synthesis of 9-β-D-arabinofuranosyl-6-[^{35}S]thiopurine. Rhombic [^{35}S]sulfur was used to label arabinofuranosyl thiopurine by sulfur exchange [14,15]. The radiolabeled drug was checked for radiopurity by thin-layer chromatography using a solvent composed of ethanol/saturated sodium tetraborate/ammonium acetate (5.0 M, pH 9.5)/0.5 M Versene (220 : 80 : 20 : 5). The initial specific activity of pure arabinofuranosyl-6-[^{35}S]thiopurine was determined to be $2.5 \cdot 10^{13}$ dpm/mol.

Arabinofuranosyl-6-[^{35}S]thiopurine binding assay. 49 μg RNA polymerase were incubated with arabinofuranosyl-6-[^{35}S]thiopurine for 10 min at 37°C in a total volume of 0.5 ml. The resulting enzyme-inhibitor complex was pelleted with bovine serum albumin by addition of 3 ml 5% trichloroacetic acid in water. The supernatant was decanted, fresh 5% trichloroacetic acid added, and the sample repelleted. This procedure was repeated for a total of four washes. The pellet was then air-dried, dissolved in 0.3 ml Protosol and counted for radioactivity. Experiments investigating the prevention of arabinofuranosyl-6-

[^{35}S]thiopurine binding by substrates were conducted by preincubating 5 μg enzyme for 10 min at 37°C with the appropriate substrate (400 nmol ATP, 400 nmol CTP or 100 μg calf thymus DNA) prior to the treatment outlined above.

Protein determination. Protein concentration was measured by the procedure of Lowry et al. [12], using crystalline bovine serum albumin as a standard. In addition, the protein concentration of RNA polymerase was determined assuming a specific absorbance at 280 nm of 0.65 mg/ml [13].

Acid urea polyacrylamide gel electrophoresis. Gels were made employing an adaptation of the procedure of Pastewake et al. [16], using a final acrylamide concentration of 5.5% and a final urea concentration of 8 M. Gels were layered over with 6 M urea solution and allowed to polymerize. The gels were electrophoresed for 15 min before the samples were added. The samples were prepared by denaturing the enzyme with 8 M urea solution in a formate buffer (pH 3.2) (1 : 4, v/v) for 20 min at 30°C. For every 50 μl of denatured enzyme 10 μl tracking dye, basic fuchsin (0.01% in 8 M urea) was included. The gels were run with the anode inserted into the upper reservoir at a current of 2.5 mA/tube until the dye entered the lower reservoir. The gels were stained in 0.05/Coomassie blue in 7% acetic acid for 3—5 h and destained in 7% acetic acid overnight at room temperature. The resulting band patterns were scanned at 500 nm in a Beckman 25 spectrophotometer equipped with a gel scanning apparatus. For radioactive counting, the gels were cut into 2-mm slices dissolved in 0.2 ml 60% $HClO_4$, and digested with 0.4 ml 30% H_2O_2 in tightly capped glass scintillation vials at 60°C for 3—5 h [17]. The vials were cooled to room temperature, 5 ml Aquasol II added, and the samples counted for radioactivity in a model 3375 Packard scintillation spectrophotometer.

Results and Discussion

The nucleoside analogue, arabinofuranosyl thiopurine, was found to be an inhibitor of DNA-dependent RNA polymerase activity. Least-squares analysis of the kinetic data indicated that the inhibition was competitive (UTP as the substrate) and that the K_i and K_m values of the system were $8.5 \cdot 10^{-4}$ and $2.9 \cdot 10^{-4}$ M, respectively. A Hill plot of the inhibition data yielded a line of slope 1, thus indicating that arabinofuranosyl thiopurine was binding in or near the active center of the enzyme in a molar ratio of 1 : 1 [18]. The 1 : 1 binding ratio was corroborated by using radiolabeled arabinofuranosyl-6-[^{35}S]thiopurine to directly monitor the number of inhibitor molecules bound per enzyme as a function of the concentration of arabinofuranosyl-6-[^{35}S]thiopurine. The results of this experiment showed that concentrations of less than $3 \cdot 10^{-4}$ M arabinofurabosyl-6-[^{35}S]thiopurine resulted in a 1 : 1 binding stoichiometry betweeen inhibitor and enzyme. However, above this critical concentration the slope of the binding curve changed to 2.6 indicating that binding of the initial inhibitor molecule probably induced a comformation change in the enzyme which exposed 1 or 2 additional binding sites.

Substrate protection against inhibition of RNA polymerase activity by arabinofuranosyl thiopurine was demonstrated by preincubation of the enzyme with either ATP or CTP (data not presented). The results of this experiment showed that although arabinofuranosyl thiopurine inhibited the unprotected

enzyme by 39%, preincubation of the enzyme with CTP or ATP decreased the inhibition by a factor of two. In order to determine whether substrate protection was a direct consequence of decreasing the amount of enzyme-inhibitor complex formed, the binding of inhibitor was monitored using arabinofuranosyl-6-[^{35}S]thiopurine (data not shown). The results of this study show that preincubation of the enzyme with ATP or CTP diminished inhibitor binding by 32 and 46%, respectively. In addition, preincubation of the enzyme with calf thymus DNA produced a similar but less pronounced effect decreasing inhibitor binding by only 14% (control enzyme 1988 dpm). A suitable model to describe the binding of arabinofuranosyl thiopurine to RNA polymerase that is consistant with competitive type inhibition and substrate protection by ATP and CTP involves placing the binding site of the nucleoside analogue in or very near the elongation subsite of the enzyme. The fact that ATP and CTP strongly inhibited the binding of arabinofuranosyl-6-[^{35}S]thiopurine, while template DNA had a much smaller effect, is added support to such a conclusion. The effect of thiol reagents, such as dithiotheritol, on arabinofuranosyl thiopurine inhibition of RNA polymerase is shown in Table I. When arabinofuranosyl thiopurine was preincubated with the enzyme for 10 min and 10^{-3} M dithiothreitol was added with the substrate, arabinofuranosyl thiopurine inhibition was reversed by 50%. In addition, when dithiothreitol at varying concentrations was incubated with the enzyme prior to the preincubation with arabinofuranosyl thiopurine, no inhibition by arabinofuranosyl thiopurine was observed. These data suggested that the 6-thiol group of the purine nucleoside analogue was involved in enzyme inhibition and that, perhaps, the inhibitor interacted with the sulfhydryl group of a cysteine residue located in the vicinity of the inhibitor binding site. Presumptive disulfide bond formation between arabinofuranosyl thiopurine and a cysteine in the region of the catalytic center of RNA polymerase was determined by preincubating the enzyme with $3 \cdot 10^{-4}$ arabinofuranosyl thiopurine and 10^{-4} M H_2O_2, and then assaying enzyme activity as a function of time (Fig. 1). The inhibition by arabinofuranosyl thiopurine plus H_2O_2 was progressive with time, indicating that H_2O_2 was promot-

TABLE I

EFFECT OF DITHIOTHREITOL UPON INHIBITION OF RNA POLYMERASE BY ARABINOFURANOSYL THIOPURINE

10 μg RNA polymerase were incubated for 10 min at 37°C with varying concentrations of arabinofuranosyl thiopurine in 0.1 M Tris-HCl buffer (pH 7.9), containing (in a final volume of 0.5 ml) 8 μmol $MgCl_2$ and 100 μmol KCL. The samples were assayed by the standard system.

Concentration of arabinofuranosyl thiopurine (M)	Percent inhibition				
	no dithiothreitol	10^{-3} M thiothreitol added with substrates	Dithiothreitol preincubated with enzymes		
			10 mM	1 mM	100 μM
$1 \cdot 10^{-3}$	70.2	33.8	0	0	0
$5 \cdot 10^{-4}$	42.6	25.2	2.4	0	0
$1 \cdot 10^{-4}$	18.2	7.1	5.8	0	0

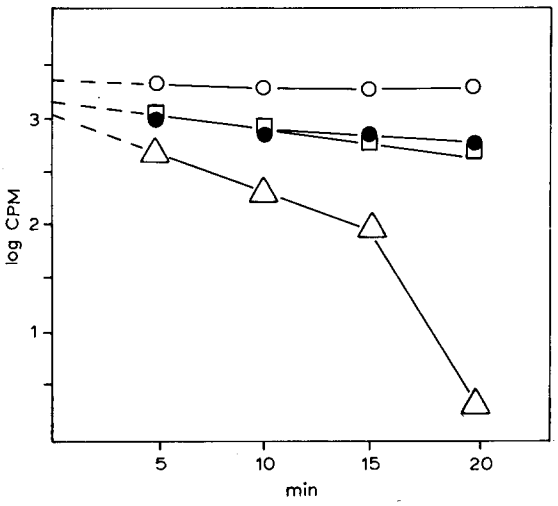

Fig. 1. Effect of H_2O_2 on the inhibition of RNA polymerase by arabinofuranosyl thiopurine. 5 μg enzyme alone (○), 5 μg enzyme plus 10^{-4} M H_2O_2 (●), 5 μg enzyme plus $3 \cdot 10^{-4}$ M arabinofuranosyl thiopurine (■), and 5 μg enzyme plus $3 \cdot 10^{-4}$ M arabinofuranosyl thiopurine with 10^{-4} M H_2O_2 (△), were incubated at 37°C. At specified times, 0.3-ml aliquotes were removed and assayed by the standard system. The reaction causing the inhibition of RNA polymerase in each of the three systems appears to be pseudo-first-order for the first 15 min of exposure. Least-squares analysis of that portion of the data yielded the following rate constants; $k_● = 6.7 \cdot 10^{-2}$ min^{-1}; $k_□ = 4.6 \cdot 10^{-2}$ min^{-1}; $k_△ = 0.17$ min^{-1}.

ing the formation of a disulfide bridge between the thiol group of the analogue and a catalytically essential cysteine.

In order to determine conclusively whether the arabinofuranosyl thiopurine was interacting with a cysteine residue, enzymatic digestion of the H_2O_2 promoted arabinofuranosyl-6-[^{35}S]thiopurine-enzyme complex was carried out with pronase under slightly acidic conditions (pH 5.0). The digestion product was chromotographed on Whatman No. 1 paper (Fig. 2A) and subjected to thin-layer high-voltage electrophoresis (Fig. 2B). In both of these experiments the major radioactive peak from the pronase digestion overlapped with the peak corresponding to the arabinofuranosyl thiopurine-cysteine complex. Since the H_2O_2-catalyzed reaction between arabinofuranosyl thiopurine and cysteine produced several products, [^{14}C]cysteine was used in a third system (Fig. 2C). Extensive overlap of the main radioactive peak from the pronase digest and the arabinofuranosyl thiopurine [^{14}C]cysteine complex was again observed. As an added precaution, peroxidase was used in this preparation to decompose excess H_2O_2 prior to pronase digestion.

Several attempts to use alkaline gel electrophoresis on the arabinofuranosyl-6-[^{35}S]thiopurine-RNA polymerase complex failed to isolate the particular subunit to which the analogue was bound. For example, electrophoresis of the labeled complex on SDS gels with or without β-mercaptoethanol at several different alkaline pH values consistently resulted in the label not being associated with any of the separated protein bands. Apparently, the label was lost because of the inherent instability of disulfide bonds in an alkaline environment [28]; a liability which is probably exaggerated by the conditions of gel electrophoresis.

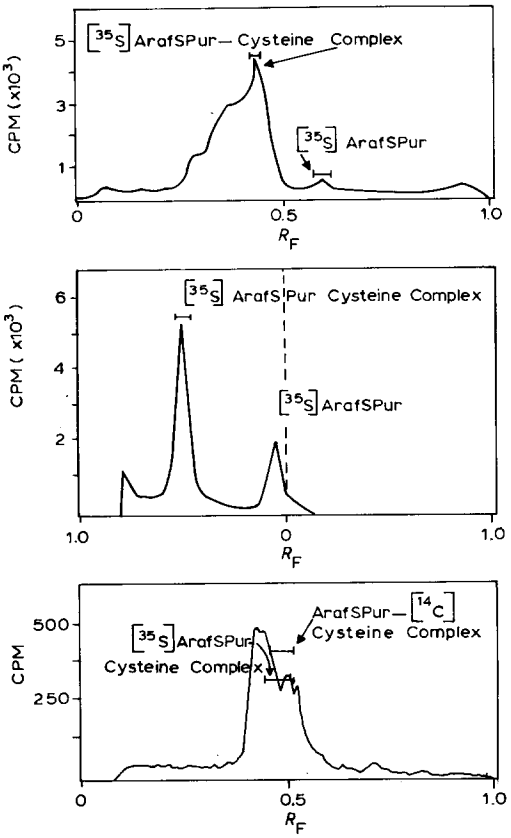

Fig. 2. Chromatograms of pronase-digested arabinofuranosyl-6-[^{35}S]thiopurine treated RNA polymerase. 500 μg enzyme in 0.01 M Tris-HCl buffer (pH 7.9) containing 0.01 mM EDTA, 0.01 mM dithiothreitol and 5% (v/v) glycerol was incubated for 15 min at 37°C with $3 \cdot 10^{-5}$ M arabinofuranosyl-6-[^{35}S]thiopurine. 10^{-4} M H_2O_2 was then added and the mixture incubated for another 15 min. At this point, 500 μg peroxidase was added to the 'B' mixture followed by another 15 min incubation. Following this, 3.2 μg of pronase (pH 5) was mixed with each of the solutions and the resulting mixture incubated for 6 days at 37°C to insure complete digestion of the polymerase protein. From this point, each of the three mixtures received different treatment. 'A' mixture: The sample was applied to Whatman No. 1 paper (50 cm) and run as ascending chromatography in an ethanol/*tert*-butanol/water/formic acid (60 : 20 : 15 : 5, v/v) solvent. The paper was then cut into 1-cm strips and analyzed for radioactivity in a toluene base scintillation fluid. R_F values of various known substances in this solvent are: arabinofuranosyl-6-[^{35}S]thiopurine, 0.59; disulfide of arabinofuranosyl-6-[^{35}S]thiopurine, 0.58; cysteine, 0.60; cysteine-arabinofuranosyl-6-[^{35}S]thiopurine complex, 0.42. 'B' mixture: The sample was applied to cellulose 300 plates and subjected to thin-layer high-voltage electrophoresis at 1000 V, 3.5 mA, 1 W, for 40 min in a pyridine/acetic acid/water (0.5 : 50 : 49.5, v/v) solvent system. Migration toward the cathode is indicated by positive R_F values and the anode by negative R_F values. The R_F values of known controls in this solvent are: arabinofuranosyl-6-[^{35}S]thiopurine, 0.05; disulfide of arabinofuranosyl-6-[^{35}S]thiopurine, 0.60; cysteine, 0.82; cysteine-arabinofuranosyl-60[^{35}S]thiopurine complex, 0.60. 'C' mixture: The sample was applied to a cellulose 300 plate and subjected to ascending chromotography in an ethanol/*tert*-butanol/water (60 : 20 : 20, v/v) solvent system. The R_F values of known controls in this solvent are: arabinofuranosyl-6-[^{35}S]thiopurine, 0.50; disulfide of arabinofuranosyl-6-[^{35}S]thiopurine, 0.71; cysteine, 0.54; cysteine-arabinofuranosyl-6-[^{35}S]thiopurine complex, 0.46.

Similar difficulties were previously encountered by Cranston and Rudden [29] with 6-chloro-8-aza-9-cyclopentylpurine, a substrate analogue also targeted at a cysteine residue. In order to circumvent this problem, we developed an acidic

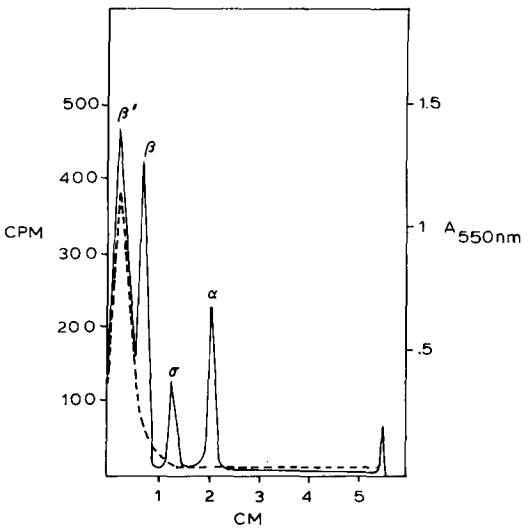

Fig. 3. Subunit localization of arabinofuranosyl-6-[^{35}S]thiopurine on RNA polymerase. RNA polymerase (99 μg) was incubation with $3 \cdot 10^{-4}$ M arabinofuranosyl-6-[^{35}S]thiopurine for 15 min. 10^{-4} H_2O_2 was added and the sample incubated for another 15 min at 37°C. This mixture was then dialyzed for 4 h at 4°C in 0.01 M Tris-HCl buffer (pH 7.0). The sample was denatured and electrophoresed as indicated in Materials and Methods. The solid line indicates the subunit positions as determined by gel scanning at 550 nm, and the broken line indicates radioactive counting of the sliced gel. Subunit positions were verified by the electrophoretic patterns of the isolated subunits.

urea polyacrylamide gel which adequately separated the various subunits of RNA polymerase. The electrophoretic separation on this gel of denatured affinity-labeled enzyme complex (Fig. 3) revealed that the radioactivity was confined to the β'-subunit fraction.

We [6,8], Krakow and Fronk [20], and Wu and Wu [10], have proposed a multi-subsite model for the catalytic center of RNA polymerase. Briefly, the active center is postulated to be composed of an initiation subsite or 3'-OH terminus subsite for a growing RNA molecule, an elongation subsite or triphosphate subsite for incoming ribonucleoside-5'-triphosphate substrate molecules, and a DNA template subsite. Nixon et al. [6] and Wu and Wu [10] have shown unequivocally that the initiation subsite is located on the β-subunit of the enzyme. Finding the radiolabel from arabinofuranosyl-6-[^{35}S]thiopurine on the β'-subunit and determining the analogue to be bound in the vicinity of the elongation subsite presents some interesting possibilities concerning the physical location of the subsites of the catalytic center of RNA polymerase. Several interpretations present themselves. The elongation subsite could be divided between the β- and β'-subunits; that is to say, the β- and β'-subunits could act in concert with both containing elements of the elongation subsite. This type of subunit interaction was suggested many years ago [21] and is known to be the case for the NAD^+ binding site of dogfish lactate dehydrogenase [22].

In recent studies conducted by Coggins et al. [23] and Hillel and Wu [24] on *E. coli* RNA polymerase it was suggested that the β- and β'-subunits do interface. Because DNA has been reported to bind selectively to the β'-subunit [25,

26], this would, probably place the template subsite on that subunit. Together, these allow for a multi-subsite catalytic center shared between the β- and β′-subunits, which would be consistent with our findings. If the template subsite is also shared between the β- and β′-subunits, as was suggest above for the elongation subsite, then the template DNA could be 'sandwiched' between the two subunits.

Another interpretation is also possible. The elongation subsite is seen to be composed to three recognition sites: a triphosphate binding site, a binding site for ribose, and a base recognition (or binding) site. The cysteine, affinity-labelled by arabinofuranosyl thiopurine and located on the β′-subunit, would not be a part per se of the elongation subsite but would be located within a few angströms [27] of position 6 of the purine ring. The purine ring would be bound in the elongation subsite. The formation of a disulfide bond between arabinofuranosyl thiopurine and a cysteine residue necessitates the close proximity of the two sulfhydryl groups. Thus, this cysteine could also be close to or part of the template subsite.

In summary, the substrate analogue arabinofuranosyl thiopurine has been used to affinity label one cysteine which lies in or near the elongation subsite of *E. coli* DNA-dependent RNA polymerase. In addition, this cysteine has been localized on the β′-subunit of the enzyme.

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