INHIBITION OF E. COLI DNA DEPENDENT RNA POLYMERASE BY NOVEL NUCLEOTIDES, DSI and DSII*

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SUMMARY The unusual nucleotides DSI and DSII inhibit the activity of E. coli RNA polymerase in vitro. At saturating concentrations, DSI decreased the RNA polymerase activity by 55%. Preincubation of DSI with the DNA-RNA polymerase mixture in the absence of the nucleoside triphosphates increased the level of inhibition. The inhibitory activity was unstable during storage above -76°C. Some intramolecular change in DSI which also causes a change in the UV spectrum may explain the loss of inhibitory activity. The inhibition was shown not to be the result of a simple oxidant effect. The cooperative binding of DSI either to the DNA-RNA polymerase complex or to just the DNA was directly measured by nitrocellulose filters.

INTRODUCTION The isolation and partial characterization of two novel nucleotides from E. coli, designated DSI and DSII, was recently reported (1). The nucleotides were found to be present in low amounts during all phases of growth, but upon a shift from aerobic to anaerobic growth and during growth into stationary phase, the level of DSI could rise as much as ten-fold. DSI was found to consist of coenzyme A and glutathione joined by a disulfide bridge. DSII was found to be a coenzyme A dimer modified by the addition of two equivalents of glutamic acid (2). This report describes the effects of DSI and DSII upon the in vitro activity of E. coli RNA polymerase. Evidence is presented that the nucleotides inhibit the polymerase activity and that binding of DSI to the RNA polymerase - DNA complex may be involved in the inhibition.

MATERIALS AND METHODS All biochemicals were obtained from Sigma or P-L Biochemicals. Nitrocellulose filters were purchased from Gellman. DSI and DSII were prepared as described previously (1) and stored at either -20°C or -76°C in distilled water. Concentrations were estimated on the basis of absorbance at 260nm assuming an extinction coefficient of 15,300. E. coli RNA polymerase holoenzyme was obtained from Sigma. Before use, the enzyme stock was diluted ten fold in buffer containing 50mM Tris, pH 7.9, 10mM $MgCl_2$, 100mM KCl, 0.1mM EDTA, 50% glycerol, 1 mg/ml BSA and either 0.1mM or no dithiothreitol depending

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on the experiment. Each assay contained 50 units/ml of the polymerase. Assays were done according to Burgess (3) except that [^{3}H] UTP (New England Nuclear) was used with any modifications to the procedure being described in the Legends.

RESULTS: Inhibition kinetics. The typical response of RNA polymerase activity to increasing concentrations of DSI can be seen in Figure 1. In the low concentration range (up to 30μM), a slight stimulation was observed. The same pattern was observed when the assay was conducted with dithiothreitol (DTT) excluded from the enzyme dilution buffer, thus eliminating the possibility that the stimulation was due to its presence affecting the structure of DSI. The stimulatory effect could be eliminated by raising the concentration of UTP in the assay from 2μM to 100μM. Inhibition by DSI began with DSI concentrations above 40μM and was maximal at 60-70μM when incorporation had fallen by 50-60%. Raising DSI levels as high as 150μM produced no further inhibition. DSII was found to inhibit RNA polymerase as well, but never more than 35%. DSII was not studied further because of its low yield during purification.

The pattern of inhibition described above was not changed either when T4 bacteriophage DNA was used instead of calf thymus DNA or when the assay was conducted at low salt (no KCl) or high salt (0.2M KCl). The level of incorporation however, was much higher with calf thymus DNA and low salt than with T4 DNA and high salt (Figure 2).

Inactivation of DSI on storage. Storage of DSI affected the extent to which it would inhibit maximally. When DSI was stored at -20°C with frequent short

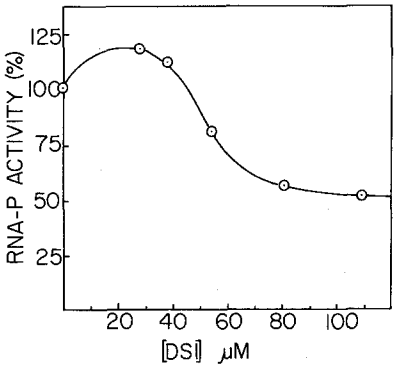

Figure 1. Effect of increasing concentrations of DSI on E. coli RNA polymerase activity. Assay mixtures contained 50 mM Tris, pH 7.6, 10 mM $MgCl_2$, 200 μg/ml calf thymus DNA, 50 units/ml RNA polymerase (RNA-P), 200 μM of ATP, GTP and CTP and 2 μM of [^{3}H] UTP at 1 μCi/μ mole. DSI was premixed with the four nucleotides before addition to the reaction mixture. All other conditions were as described by Burgess (3). Relative activity refers to the amount of [^{3}H] UMP incorporated, compared to the control with no DSI.

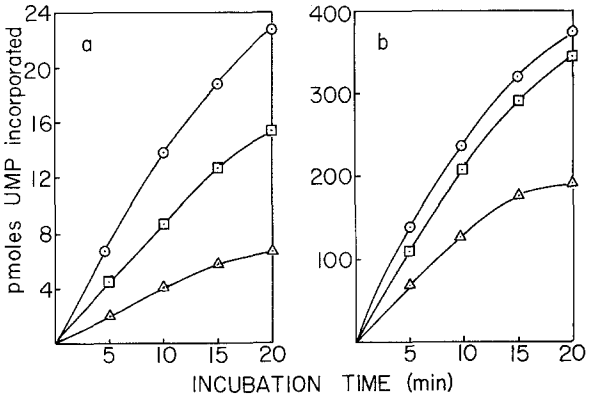

Figure 2. Effect of preincubation of DSI in the reaction mixtures. Assay mixtures contained 50 mM Tris, pH 7.6, 10 mM $MgCl_2$, 0.2 M KCl, 50 units/ml RNA polymerase, 20 µg/ml native T4 DNA and 200 µM ATP, GTP and CTP. [^{3}H] UTP, 1 µCi/µ mole, was present at 4 µM in (a) and 100 µM in (b). Assay conditions were as described by Burgess (3) with 10 µl aliquots being sampled at each time point. a) Incorporation of UMP in the absence of DSI (o), with DSI added at 0 minutes (Δ) and with DSI added to premixed DNA and RNA polymerase (with salts but no nucleotides) 5 minutes before the start of the reaction (□). b) The DNA-RNA polymerase-salts mixture was preincubated in the absence of DSI with 400 µM ATP for 10 minutes prior to starting the reaction with the remaining nucleoside triphosphates (o). DSI (75 µM) was added 5 minutes after ATP (400 µM) but 5 minutes before the start of the reaction (□). DSI (75 µM) was added to the DNA-RNA-polymerase-salts mixture 10 minutes before and ATP (400 µM) was added 5 minutes before the start of the reaction (Δ).

periods at 5°C, its inhibitory power dropped from 55% to less than 10% in five weeks. Over the same period there were no changes in the physical composition or chromatographic properties of DSI (2). Only the UV absorbance spectrum change, noted previously (1), had occurred with the A250/260 ratio having dropped from 1.05 to 0.85. The loss of inhibitory activity and the change of spectrum of DSI could both be retarded by storage at -76°C. A sample of DSI initially capable of 65% inhibition of RNA polymerase was still able to inhibit the enzyme activity by 45% after 4 weeks at -76°C.

Effect of preincubation. The order in which assay components were mixed was found to be important for the degree of inhibition achieved. When DSI was allowed to interact with the DNA-RNA polymerase complex before the addition of nucleotides, instead of simply being added with the nucleotides, inhibition was markedly increased (Figure 2-a). The effect of preincubation with DSI was the same at both low (2µM) and high (100µM) UTP concentrations. Preincubation with 400µM ATP largely abolished DSI inhibition even when DSI was also preincubated after the ATP preincubation was begun (Figure2-b). A variety of prein-

Table 1. Effect of the order of addition of reaction components on UMP incorporation by RNA polymerase (RNA-P).

Time of Addition				Relative Activity
-15	-10	-5	0	(%)
DNA	RNA-P	DSI	-	30
RNA-P	DSI	DNA	-	62
DNA	DSI	RNA-P	-	68
DNA	RNA-P	-	DSI	86
DNA	RNA-P	H_2O	-	100

Components were added at 5 minute intervals and incubated at 37°C. The times indicate minutes before addition of the four nucleoside triphosphates. The relative activity refers to the amount of [^{3}H] UMP incorporated compared to the control using water in place of 75 μM DSI. Salts were added with DNA and assay conditions were as described in the legend to Figure 1.

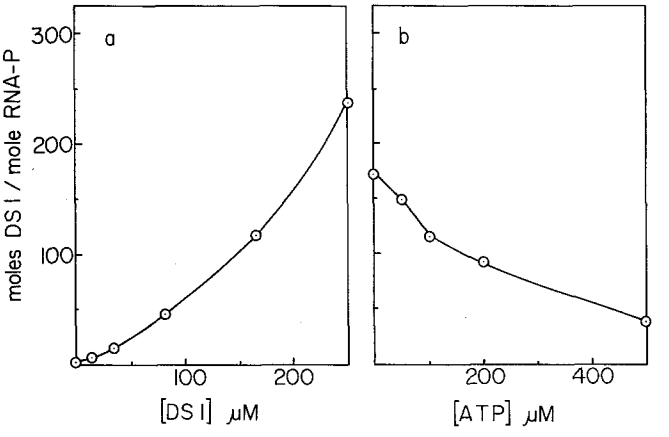

Figure 3. Retention of [^{32}P] DSI on nitrocellulose filters. The reaction mixture contained 50 mM Tris pH 7.6, 10 mM $MgCl_2$, 200 μg/ml calf thymus DNA and 5 units/ml of RNA polymerase (RNA-P). (a) RNA polymerase, DNA and salts were incubated for 5 minutes at 30°C, then [^{32}P] DSI (1000 cpm/nmole) at various concentrations was added followed by another 10 minutes incubation at 30°C. The total mixture was passed through a nitrocellulose filter and washed with 15 ml of cold buffer containing 50 mM Tris pH 7.6, 10 mM $MgCl_2$, and 1 mg/ml BSA. The radioactivity was then counted in Brays scintillant in a Beckman LS 230 scintillation counter. No DSI bound to the filter in the absence of RNA polymerase. (b) RNA polymerase, DNA, salts and various concentrations of ATP were incubated for 5 minutes at 30°C, then [^{32}P] DSI was added to 200 μM followed by another 10 minutes incubation at 30°C. The mixture was then treated as in (a).

Table 2. Effect of various compounds on RNA polymerase activity.

Experiment	Addition	Concentration μM	Relative Activity (%)
1	- (control)	-	100
2	DSI	75	68
3	GSH	1000	155
4	GSH + DSI	1000 + 75	214
5	DTT	1000	202
6	DTT + DSI	1000 + 75	250
7	CoA	100	173
8	GSSG	100	100

The assay conditions were as described in the legend to Figure 1, except that the indicated compounds were present. The relative activity refers to the amount of [^{3}H] UMP incorporated compared to the control in experiment 1. The order of addition at 5 minute intervals was DNA, test compound, RNA polymerase and nucleotides. Salts were added with DNA and assay conditions were as described in the legend to Figure 1.

cubation conditions were therefore studied (Table 1). Inhibition by DSI was greater as long as it was added prior to initiation and elongation, but the greatest effect was acheived when the nucleotide was allowed to interact with the DNA-RNA polymerase complex. DSI which inhibited only 10% when added with the nucleotides could inhibit 50% if preincubation was allowed. Freshly prepared DSI which inhibited 50% without preincubation inhibited more than 90% of the polymerase activity upon preincubation.

Effect of CoA and reducing agents. Since DSI was found to be composed of coenzyme A and glutathione, although in a modified form, the effects of these molecules and of dithiothreitol on transcription was studied (Table 2). Both glutathione (GSH) and DTT stimulated RNA polymerase activity greatly (55% and 102% respectively) saturating at 0.1 to 5 mM. The addition of normally inhibiting levels of DSI together with either of the reducing agents resulted in additional stimulation. Coenzyme A at a concentration of 100μM also stimulated polymerase

activity by 73% in the absence of reducing agent. Glutathione dimer (GSSG) at 100μM had no effect upon the polymerase activity.

Binding studies. The direct association of DSI with the DNA-RNA polymerase complex was studied using nitrocellulose filters. Figure 3-a shows that approximately 60 molecules of DSI bind per molecule of RNA polymerase at 100μM DSI. This binding increased to over 150 molecules per polymerase molecule at 200μM DSI. The presence of RNA polymerase was required for binding of DSI to the filter and the addition of DSI to the DNA solution before RNA polymerase resulted in no DSI being retained on the filter. The DNA-RNA polymerase-DSI complex was not sufficiently stable to be isolated by gel filtration on Sephadex G100. Hence, whether the binding was to the DNA-RNA polymerase complex or just to the DNA was not clear The shape of the binding curve suggested that the binding was cooperative similar to the binding exhibited by such proteins as the T4induced gene 32 protein and the fd induced gene 5 protein (13,14).

The addition of ATP to the DNA-RNA polymerase complex before the addition of DSI reduced DSI binding in proportion to the concentration of ATP (Figure 3-b). ATP can therefore abolish both DSI inhibition of the polymerase and DSI binding to the DNA-RNA polymerase complex or DNA. In addition, Figure 4 shows that DSI is a non-competitive inhibitor for the binding of ATP to the polymerase since the V_{max} is decreased but the K_M is unchanged by DSI. Since DSI and ATP do not compete for the same site on the polymerase, the active complex formed with ATP, RNA polymerase and DNA may be less sensitive to DSI.

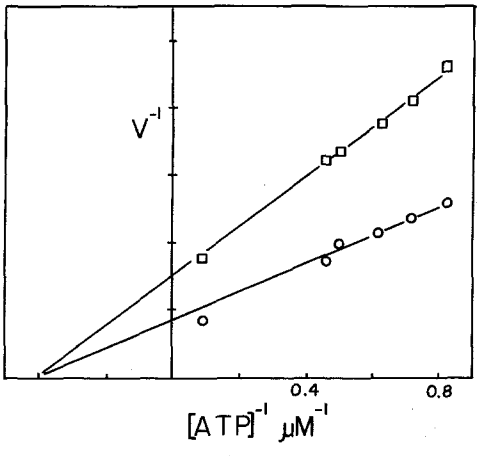

Figure 4. Effect of ATP concentration on the inhibition of E. coli RNA polymerase by 75 μM DS-I, presented in Lineweaver-Burk form. Assay conditions were as in Figure 1 except 20 μg/ml coliphage T4 DNA and high salt (200 mM KCl) were used. DNA-RNA polymerase complex was incubated 5 minutes with DS-I (□) or with water (o) before starting the reaction. The plot represents the average of triplicate assays.

DISCUSSION DSI has previously been shown to be formed when there is a shift from aerobic to anaerobic growth or at the onset of stationary phase growth. These experiments show that DSI also has an inhibitory effect on in vitro RNA polymerase activity which is enhanced by preincubation of the nucleotide with the DNA-RNA polymerase complex. The direct binding of DSI to either DNA or the DNA-RNA polymerase complex also suggests that a DSI complex is formed which blocks the polymerase. Maximum inhibition occurs at 40-70μM DSI corresponding to 20-40 moles of DSI per mole of enzyme-DNA complex. Below this concentration, insufficient DSI may be bound to block the polymerase and above this concentration, the extra binding may act to reinforce the inhibition reflecting the cooperative nature of the binding.

The mechanism of inactivation of DSI would seem to involve a change in the structure of the molecule to form an unmodified CoASSG mixed disulfide. No change in the physical composition of DSI could be found which would account for the spectrum change and inactivation. A seemingly unmodified CoASSG has previously been identified in yeast (10) and liver (11,12) but no metabolic role aside from a storage form of the two components was proposed. The inhibitory effect of DSI could not be explained as a simple oxidant effect of the disulfide bond since inactive DSI still contained the disulfide link and GSSG had no effect on the polymerase activity. Several reports have appeared implicating nucleotides in various regulatory roles (4-9). DSI seems to be another such molecule which combines two molecules very important in other metabolic roles and each with a stimulatory effect on RNA polymerase into a form which upon modification can inhibit the polymerase activity.

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