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INHIBITION OF RNA POLYMERASE BY CAPTAN AT BOTH DNA AND SUBSTRATE BINDING SITES

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Abstract—RNA synthesis carried out *in vitro* by *Escherichia coli* RNA polymerase was inhibited irreversibly by captan when T_7 DNA was used as template. An earlier report and this one show that captan blocks the DNA binding site on the enzyme. Herein, it is also revealed that captan acts at the nucleoside triphosphate (NTP) binding site, and kinetic relationships of the action of captan at the two sites are detailed. The inhibition by captan via the DNA binding site of the enzyme was confirmed by kinetic studies and it was further shown that [^{14}C]captan bound to the β' subunit of RNA polymerase. This subunit contains the DNA binding site. Competitive-like inhibition by captan versus UTP led to the conclusion that captan also blocked the NTP binding site. In support of this conclusion, [^{14}C]captan was observed to bind to the β subunit which contains the NTP binding site. Whereas, preincubation of RNA polymerase with both DNA and NTPs prevented captan inhibition, preincubation with either DNA or NTPs alone was insufficient to protect the enzyme from the action of captan. Furthermore, the interaction of [^{14}C]captan with the β and β' subunits was not prevented by a similar preincubation. Captan also bound, to a lesser extent, to the α and σ subunits. Therefore, captan binding appears to involve interaction with RNA polymerase at sites in addition to those for DNA and NTP; however, this action does not inhibit the polymerase activity.

A single enzyme, RNA polymerase (EC 2.7.7.6), is responsible for the transcription of genetic sequences in *Escherichia coli*. The overall enzymatic reaction catalyzed by *E. coli* RNA polymerase consists of several major steps including promotor binding, initiation, elongation and termination. The synthetic reaction employs ribonucleoside triphosphates as substrates and requires the presence of DNA to serve as a template in the reaction. *E. coli* RNA polymerase is a large molecule of 454,000 daltons [1]. It is composed of five subunits, $\alpha_2\beta\beta'\sigma$, each with specific functions for RNA synthesis. The β subunit has the nucleotide binding site, the β' subunit contains the DNA template binding site, and the σ subunit is involved in promotor recognition and transcription initiation [2]. The function of the α subunit has not been defined clearly. The elucidation of the functions of these subunits has been facilitated by many experimental approaches including the application of various inhibitors to the analysis of the enzyme-catalyzed reactions.

Captan, *N*-trichloromethylthio-4-cycloheximide, is an inhibitor of a number of DNA and RNA polymerases. Enzyme activities shown to be inhibited by captan include DNA polymerase activity in isolated bovine liver nuclei [3], RNA polymerase I and II activities in bovine liver nuclei [4], DNA polymerase and ribonuclease H activities of AMV reverse transcriptase [5], and both the DNA polymerizing and exonuclease activities of *E. coli* DNA polymerase I [6–9]. Captan was found to alter RNA synthesis catalyzed by *E. coli* RNA polymerase in toluenized *E. coli* cells [10], and *E. coli* RNA polymerase activity was inhibited by captan in *in vitro* assays [11]. The latter study showed that captan inhibited the binding of T_7 DNA to *E. coli* RNA polymerase. Therefore, the DNA template binding site on the enzyme is at least one of the target sites of captan's action.

The purpose of the present study is to further examine the mechanism by which captan inhibits *E. coli* RNA polymerase, with a focus on the interaction of captan at sites other than the DNA binding site of the enzyme. This and future studies will be directed to better understand how captan inhibits *E. coli* RNA polymerase and to use that information to elucidate characteristics of the enzyme-catalyzed reaction.

MATERIALS AND METHODS

Materials. The Sigma Chemical Co. was the supplier of ATP, CTP, GTP, UTP, *E. coli* RNA polymerase, yeast RNA and Kodak XAR-5 film. [^{3}H]UTP (30 Ci/mmol) and [^{3}H]ATP (30 Ci/mmol) were synthesized by ICN. Bacteriophage T_7 and its host *E. coli* strain (NCIB 11595) were purchased from the American Type Culture Collection. Bacteriophage T_7 DNA was isolated and purified from phage T_7-infected host *E. coli* cells according to the method designed for the purification of bacteriophage lambda DNA, with minor modifications [12]. Captan was a gift of the Chevron Corp., Richmond, CA. The Stauffer Chemical Co. gratuitously supplied [^{14}C]captan (56 Ci/mmol).

RNA polymerase assays. E. coli RNA polymerase activity was assayed by a modification of the method of Chamberlin *et al.* [13]. The incubation mixture contained 20 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 5 μg of T_7 DNA, 0.10 U (0.5 μg) of *E. coli* RNA polymerase, 0.4 mM each of ATP, CTP and GTP, and 0.04 mM [^{3}H]UTP (1.0 μCi) in a total volume

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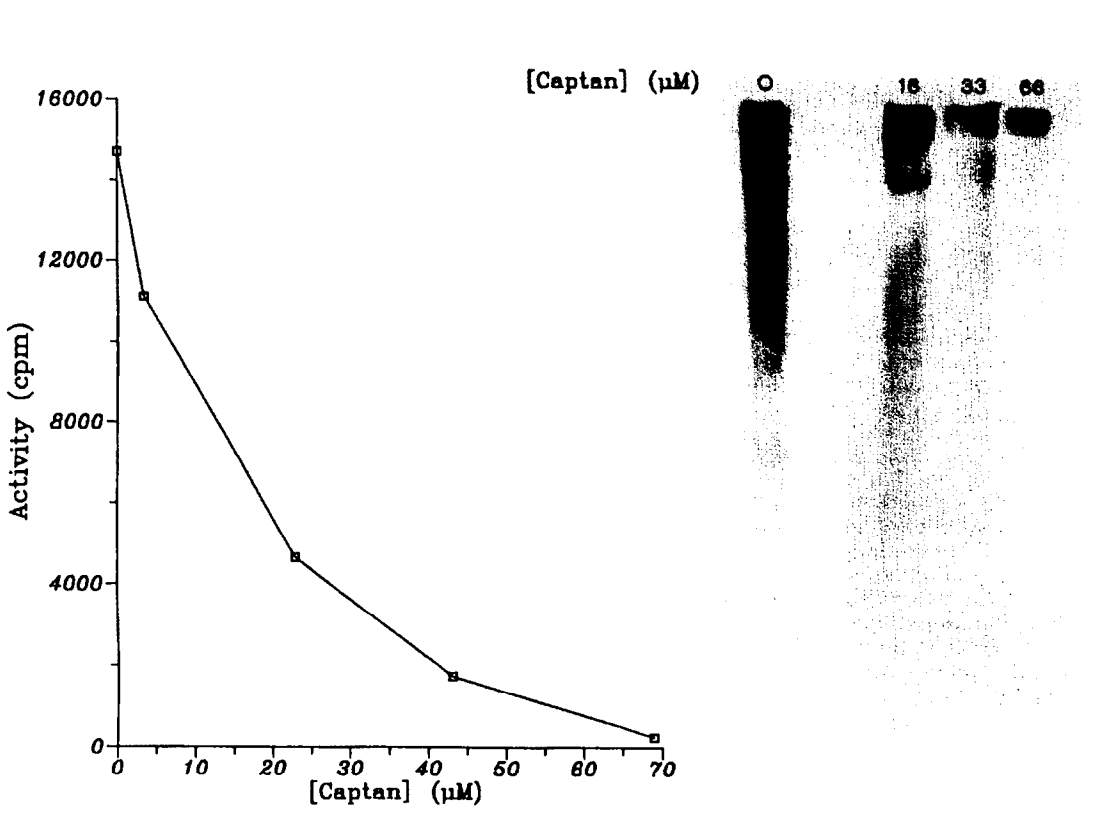

Fig. 1. Inhibition of *E. coli* RNA polymerase activity by captan. RNA polymerase activity was determined as described in Materials and Methods. Captan was added to the reaction solution containing both the polymerase and all four NTPs, and incubated for 2 min on ice before the final addition of T_7 DNA. Reactions proceeded for 30 min at 37°. Final concentrations of ATP, CTP and GTP were 0.4 mM and that of [^{3}H]UTP was 0.04 mM. (A) RNA polymerase activity was determined by measuring the amount of radioactivity incorporated into the acid-insoluble material. (B) RNA products were analyzed by gel electrophoresis and fluorography.

of 100 μL. The various conditions for preincubation are described in the legends to the corresponding figures. In certain experiments [^{3}H]ATP was substituted for [^{3}H]UTP. In some reactions the concentration of [^{3}H]UTP ranged from 0.00033 to 0.4 mM (final specific radioactivity of [^{3}H]UTP ranged from 30 to 0.024 Ci/mmol). The effect of captan on RNA synthesis was determined by adding captan to the incubation solution under different preincubation conditions. In all cases, the time period between the addition of captan and the addition of the other component(s) was 2 min. RNA synthesis was initiated by moving the incubation solution which contained all necessary components from either ice or 25° to 37°. After incubation, reactions were stopped by adding 200 μL of carrier solution containing 50 mM sodium pyrophosphate, 50 mM EDTA and 0.5 mg/mL yeast RNA. Three milliliters of ice-cold solution of 10% trichloroacetic acid (TCA) was then added to precipitate the RNA product. After standing on ice for at least 1 hr, labeled RNA molecules were collected by filtration on Whatman GF/A filters. Each filter was washed with 18 mL of ice-cold 10% TCA solution and 5 mL of ice-cold 95% ethanol and counted in a scintillation counter. Electrophoresis using a 3.0% acrylamide–0.5% agarose composite gel was carried out as described by Peacock and Dingman [14]. *In vitro* RNA synthesis was stopped by the addition of TE saturated phenol. After two phenol extractions the RNA products were precipitated with 2.5 vol. of 95% ethanol. The precipitates were collected by centrifugation and dried under vacuum. Samples were heated to 90° for 1.5 min in a solution containing 80% deionized formamide, 0.5x TBE and 1x bromophenol blue and xylene cyanol loading buffer before being loaded onto the gel. Fluorography was done by the methods of Bonner and Laskey [15] and Laskey and Mills [16].

Captan binding. The interaction of [^{14}C]captan with RNA polymerase was measured under various conditions of preincubation of T_7 DNA, NTP*

* Abbreviation: NTP, nucleoside triphosphate.

substrates and/or captan. Those conditions are outlined in the legends of the corresponding figures. Regardless of the preincubation conditions, the binding studies employed a solution containing 8 μg of *E. coli* RNA polymerase holoenzyme and 15 μg of [^{14}C]captan (56 mCi/mmol) in a total volume of 30 μL. Binding took place during a 1-min incubation on ice. After incubation, 5 μL of a dye solution containing 0.015% bromophenol blue, 6% sodium dodecyl sulfate (SDS) and 5% glycerol was added and the solution was heated in boiling water for 5 min. This solution was electrophoresed in a 7% polyacrylamide gel containing 1% SDS. Gel preparation and electrophoresis were carried out as described by Smith [17]. After electrophoresis, the gel was stained with Coomassie Brilliant Blue and dried under vacuum. Autoradiography was performed with Kodak XAR-5 film. The intensities of the labeled protein bands on the developed film were determined by scanning with a spectrodensitometric scanner (EC910).

RESULTS

RNA synthesis carried out *in vitro* by *E. coli* RNA polymerase was inhibited by captan in a concentration-dependent manner (Fig. 1). T_7 DNA which has natural promoters for *E. coli* RNA polymerase was used as template and it was the last component added to the incubation solution. Captan was reported previously to be inhibitory to *in vitro* RNA synthesis with calf-thymus DNA as template [11]. In that report *E. coli* RNA polymerase was the last component added to the incubation solution. Thus, the enzyme was not preincubated with captan. Also, higher concentrations of [^{3}H]UTP were used in the previous study. The importance of contrasting these conditions will become apparent in the following experiments.

The earlier study [11] showed that captan inhibited the binding of T_7 DNA to *E. coli* RNA polymerase and preincubation of the enzyme with T_7 DNA protected against captan-mediated inhibition of DNA and polymerase association. Therefore, it was believed that the DNA binding site on the enzyme was at least one of the target sites involved in captan inhibition. To determine if the DNA binding site was the only target, preincubation experiments were carried out (Fig. 2). Pretreatment of the enzyme with either T_7 DNA or NTP substrates alone did not protect the enzymatic activity from captan inhibition. Preincubation of the enzyme with both T_7 DNA and NTP substrates caused significant protection of its activity from captan inhibition (65%) when RNA synthesis proceeded for 15 min at 37°. This observation indicates that both the DNA and NTP binding sites were involved in captan inhibition and the availability of either the DNA or NTP binding site for captan interaction was enough to cause the inhibition. Since RNA synthesis proceeded for 15 min, several rounds of transcription were completed. Thus, preincubation of the enzyme with both the DNA template and NTP substrates did not provide 100% protection against captan inhibition. Enzyme samples preincubated with T_7 DNA at 0°, which allowed only the association of DNA and polymerase to occur, or at 25°, which permitted open promoter–polymerase complex to form [18], were equally inhibited by captan (data not shown). Thus, captan did not seem to affect the close-to-open promoter complex transition.

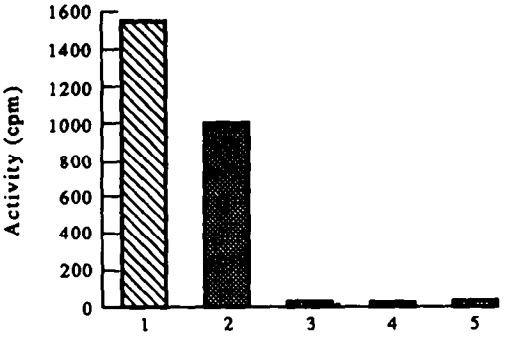

Fig. 2. Protection from inhibition by captan by combined action of DNA and NTPs. *E. coli* RNA polymerase activity was assayed in the presence of 70 μM captan with a [^{3}H]-ATP concentration of 0.04 mM and CTP, GTP and UTP of 0.4 mM each. Reactions proceeded at 37° for 15 min. The sequences of addition of DNA, NTPs and captan to the reaction solution containing RNA polymerase were as follows: (1) DNA + NTPs, no captan; (2) (DNA + NTPs) 1st, captan 2nd; (3) captan 1st, (DNA + NTPs) 2nd; (4) DNA 1st, captan 2nd, NTPs 3rd; and (5) NTPs 1st, captan 2nd, DNA 3rd. The length of time between the addition of captan and the addition of the other component(s) was 2 min (on ice).

Table 1. Effect of preincubation and UTP concentration on IC_{50} values

Preincubation	[UTP] (mM)	IC_{50} (μM)
DNA + NTPs*	0.04	41
DNA + NTPs†	0.01	14
NTPs, 1st; Captan, 2nd†	0.04	16
DNA, 1st; Captan, 2nd†	0.04	14

RNA synthesis was initiated by the addition of remaining components. Concentrations of DNA and NTPs and preincubation conditions are reported in Materials and Methods and the legend to Fig. 1.

* IC_{50} value was taken from Fig. 1.

† IC_{50} value was taken from data not shown.

Effects of preincubation and UTP concentration on the IC_{50} values of the captan-mediated inhibition are summarized in Table 1. Captan was less toxic to the enzyme sample which had both its DNA and NTP binding sites preoccupied by DNA and NTPs. Captan was equally toxic to the enzyme samples with either the DNA or NTP binding site open for captan interaction. The fact that the enzyme preincubated with T_7 DNA and a low concentration of UTP (0.01 mM) was more sensitive to captan inhibition than that which was preincubated with same amount of T_7 DNA and a higher concentration of UTP (0.04 mM) strongly implied that UTP

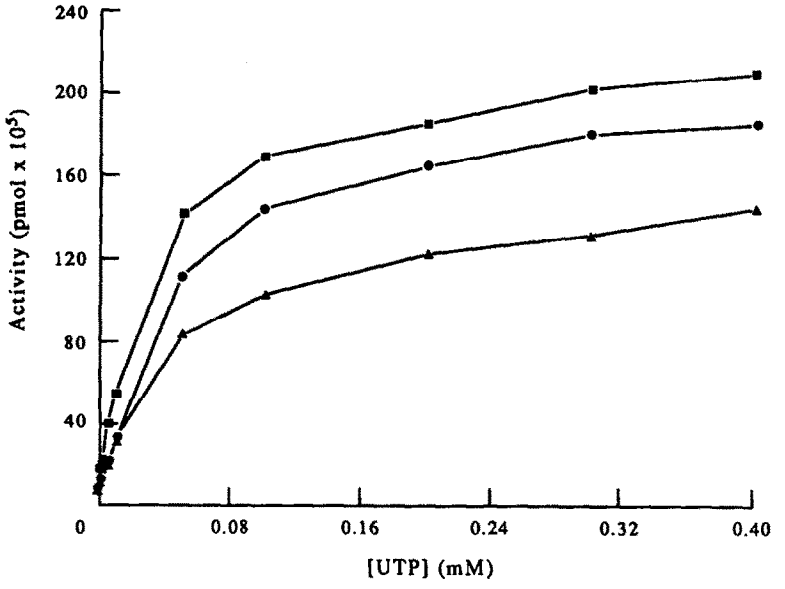

Fig. 3. Inhibition of *E. coli* RNA polymerase activity as a function of UTP concentration and preincubation with T_7 DNA. Captan (33 μM) was added to the incubation solutions containing RNA polymerase and all four NTPs (on ice for 2 min) before (▲) or after (●) the addition of T_7 DNA. Controls were assayed in the absence of captan (■). The final concentrations of ATP, CTP and GTP were 0.4 mM. The final concentrations of [^{3}H]UTP are as indicated. RNA synthesis proceeded for 20 min at 37°.

concentration may play an important role in captan action.

To determine the effect of UTP concentration on the inhibition caused by captan, *E. coli* RNA polymerase activity was assayed in the presence of a fixed concentration of captan under various UTP concentrations ranging from 0.33 μM to 0.4 mM. The concentrations of ATP, CTP and GTP were 0.4 mM each. The results (Fig. 3) show that at levels of UTP below 10.3 μM, captan caused the same degree of inhibition of RNA polymerase activity whether RNA polymerase was preincubated with template DNA or not. Furthermore, the inhibition caused by captan could be overcome partially by the increase of UTP concentration. At levels of UTP higher than 10.3 μM, preincubation of the enzyme with T_7 DNA partially protected its activity from captan inhibition. The protection was increased with preincubation with both DNA and a higher UTP concentration. A Lineweaver–Burk plot at UTP levels below 10.3 μM presented in Fig. 4A shows that captan behaved as a competitive-like inhibitor against UTP, indicating that the UTP binding site on the enzyme was involved in captan interaction. A Lineweaver–Burk plot of the higher range of UTP concentrations (Fig. 4B) shows a mixed inhibition pattern with both K_m and V_{max} being changed by the captan action, suggesting the involvement of both DNA and UTP binding sites on the enzyme in captan interaction. The effects of preincubation and UTP concentration on the apparent K_i values are summarized in Table 2.

E. coli RNA polymerase has four subunits with specific functions for RNA synthesis. To find out to which subunit(s) captan binds, ^{14}C-labeled captan was incubated with *E. coli* RNA polymerase

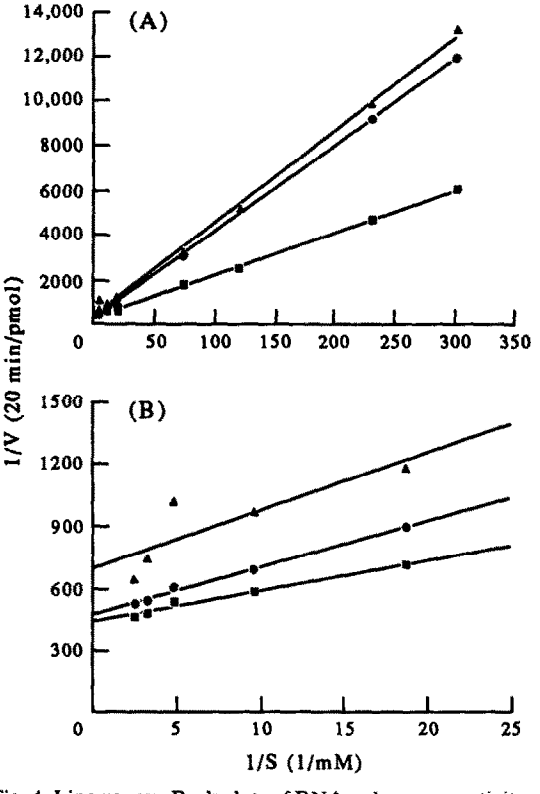

Fig. 4. Lineweaver–Burk plots of RNA polymerase activity vs UTP concentration. The curves presented in Fig. 3 were replotted as Lineweaver–Burk plots at lower and higher UTP levels. Symbols indicating different preincubation conditions and controls are the same as in Fig. 3. (A) UTP levels between 0.33 and 10.3 μM. (B) UTP levels between 10.3 μM and 0.4 mM.

Table 2. Effect of preincubation and UTP concentration on apparent K_i values

Preincubation	[UTP] (mM)	Apparent K_i (μM)
DNA + NTPs*	5.0×10^{-2}–3.0×10^{-1}	61
NTPs, 1st; Captan, 2nd†	3.3×10^{-4}–5.0×10^{-2}	29
NTPs, 1st; Captan, 2nd*	5.0×10^{-2}–3.0×10^{-1}	33
DNA + NTPs†	3.3×10^{-4}–5.0×10^{-2}	21

RNA synthesis was initiated by the addition of remaining components. The concentrations of DNA and NTPs and preincubation conditions are reported in Materials and Methods and the corresponding figure legends.

* Apparent K_i value was calculated from Fig. 4A.

† Apparent K_i value was calculated from Fig. 4B.

holoenzyme and assayed for radioactive captan-bound subunits. When the enzyme–captan complex was subjected to SDS–PAGE and autoradiographic analysis, the data showed that captan bound to all the subunits (Fig. 5). Results obtained from spectro-densitometric scanning of the labeled subunit bands revealed that 70% of the label migrated with the β' and β subunits, 17% with the σ subunit and 13% with the α subunit. The β' and β subunits are believed to possess the DNA and NTP binding sites, respectively. These two subunits migrate too similarly to distinguish between the amount of radioactivity on each subunit. However, that radioactivity migrated with both subunits supports the

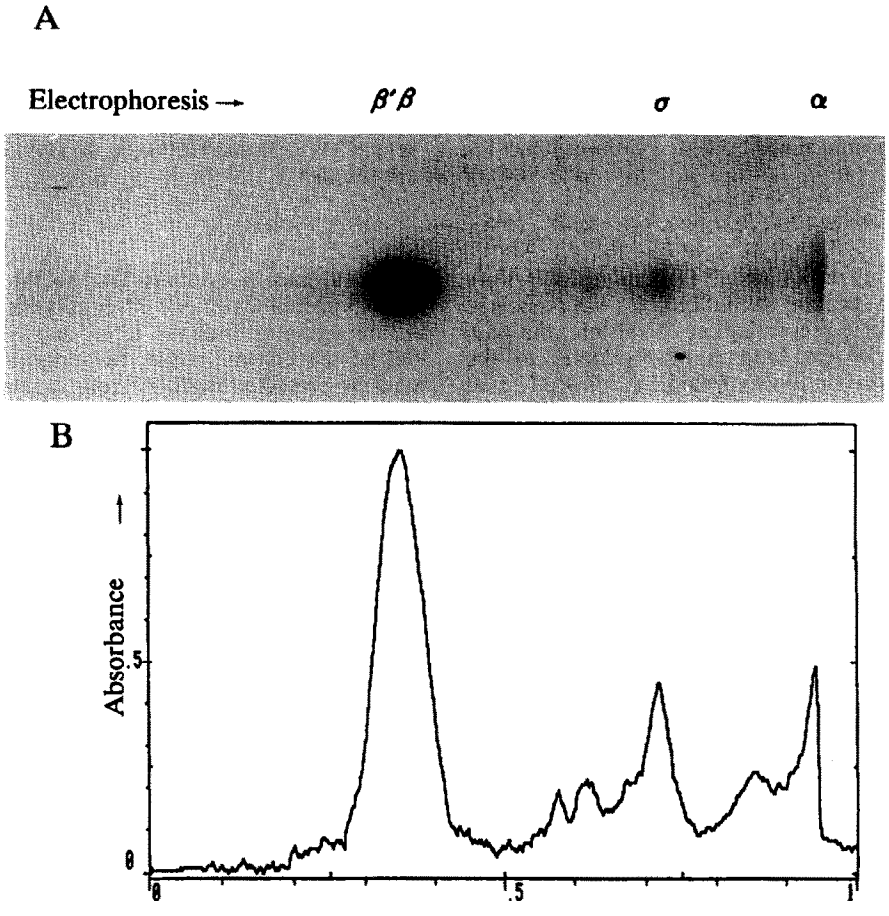

Fig. 5. Binding of [^{14}C]captan to the subunits of *E. coli* RNA polymerase. *E. coli* RNA polymerase holoenzyme was incubated with [^{14}C]captan (166 μM) on ice for 2 min. Electrophoretic separation of the [^{14}C]captan-bound subunits and autoradiography were done as described in Materials and Methods. A 7% SDS–PAGE gel was used. The subunits of RNA polymerase were identified by comparison of their rates of migration to those of standard proteins. (A) Autoradiography. (B) Spectrodensitometric scanning of the bands shown in A.

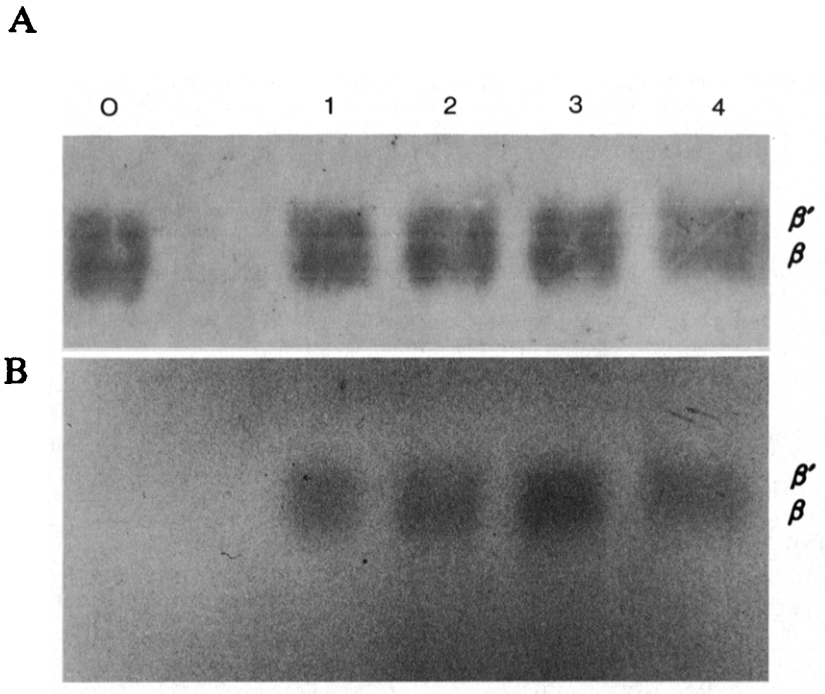

Fig. 6. Effect of preincubation on the binding of [^{14}C]captan to the β and β' subunits. (A) Coomassie Blue staining of β and β' subunits. (B) Autoradiography. Sequential addition of DNA, NTPs and [^{14}C]-captan to RNA polymerase prior to SDS–PAGE are as follows: (0) no captan; (1) captan 1st, (DNA + NTPs) 2nd; (2) DNA 1st, captan 2nd, NTPs 3rd; (3) NTPs 1st, captan 2nd, DNA 3rd; and (4) (DNA + NTPs) 1st, captan 2nd. In all cases, the captan concentration was 100 μM. The T_7 DNA concentration was 2.5 mM, and the concentrations of ATP, CTP, GTP and UTP were 100 mM each. A 5% SDS–PAGE gel was used.

idea that both DNA and NTP binding sites were involved in captan inhibition. It was reported previously that preincubation of the enzyme with captan blocks the binding of T_7 DNA to the enzyme [11], but it was found in this study that preincubation of the enzyme with high concentrations of DNA and NTPs did not prevent all the binding of [^{14}C]captan to the β' and β subunits (Fig. 6). This indicates that captan interacts with sites other than the DNA and NTP binding sites. As shown in previous data (Fig. 2), the binding of captan to those other sites did not affect the ability of the enzyme to synthesize RNA. Therefore, the nonspecific binding of captan to the polymerase was not investigated further.

DISCUSSION

Captan inhibited RNA synthesis in a concentration-dependent manner when *E. coli* RNA polymerase used T_7 DNA as template. The concentration of captan required to give 50% inhibition of the polymerase activity was approximately 16 μM. This was significantly lower than the IC_{50} value of 60 μM obtained in a previous study in which calf thymus DNA (which does not contain natural promoters for this polymerase) was used as template [11]. Thus, even nonspecific binding of polymerase to DNA is blocked by captan, but to a lesser degree.

Several lines of evidence lead to the conclusion that captan inhibits *E. coli* RNA polymerase activity by interacting at both the DNA and NTP binding sites of the enzyme. First, the enzymatic activity could be protected partially from captan inhibition only by preincubation with both the DNA template and NTP substrates. Preincubation with DNA or NTP substrates alone did not provide such protection. Second, kinetic analysis showed that under low UTP concentrations captan behaved as a competitive-like inhibitor against UTP, whereas under higher UTP concentrations the inhibition pattern was mixed. Third, ^{14}C-labeled captan bound to both the β and β' subunits of the enzyme. These two subunits possess the DNA and NTP binding sites, respectively.

The binding of radioactive captan to *E. coli* RNA polymerase appears to be more than a general, nonspecific interaction. When a number of proteins were tested for captan binding, including carbonic anhydrase, alcohol dehydrogenase, egg albumin, bovine albumin, phosphorylase B, β-galactosidase, *E. coli* RNA polymerase and *E. coli* DNA polymerase I, only *E. coli* RNA polymerase and *E. coli* DNA polymerase I were found to interact with captan (see Ref. 8, Figs. 5 and 6, and unpublished data). These observations are consistent with the fact that captan is known to be inhibitory to a number of DNA and RNA polymerases [3–9, 11], but not inhibitory to other DNA-requiring enzymes, including a wide variety of nucleases (EcoR I, Hind

III, BamH I, Xho I and Hae I, exodeoxyribonuclease Exo III, nuclease S1, pancreatic DNase I and pancreatic RNase) [19]. In this group, it was only those enzymes which bind DNA as template that also interacted with captan. These results are consistent with the idea that the catalytically active centers in *E. coli* DNA polymerase I and RNA polymerase are targets of captan interaction and that the active sites of these two enzymes may possess some structural similarities.

Allison *et al.* [20] reported that there is a weak homology between amino acid residues 666–695 which constitute a part of the DNA binding domain in DNA polymerase I and residues 350–380 of the β' subunit of the *E. coli* RNA polymerase. Furthermore, Darst and coworkers [21] found by using electron crystallography that there is a structure in RNA polymerase similar to the DNA binding cleft observed in DNA polymerase I [22]. The similarities between the two enzymes in DNA binding function, amino acid sequence and structure in the DNA binding regions may contribute to their similar accessibilities to captan binding and similar sensitivities to captan inhibition.

Captan can react with thiol groups on some proteins [23]. *E. coli* RNA polymerase is known to contain 32 thiol groups on the core enzyme [24], and these constituents may be at least partially involved in captan interaction. But, the interaction seems more complex than simply the interaction with thiol groups because: (i) it was found in this study that captan did not bind to alcohol dehydrogenase and β-galactosidase, both of which contain thiol groups; and (ii) captan inhibits *E. coli* DNA polymerase I activity by binding only to the DNA binding site which does not contain the sole thiol group of the enzyme [7, 25]. In addition to the thiol group, some other amino acid residues located in the polymerase activity center may also be attacked by captan. The trichloromethylthio group of captan is believed to be responsible for the toxicity of captan to the DNA polymerase β activity in isolated bovine nuclei [3]. This group is a strong electrophile; thus, negatively charged amino acid residues in the protein are possible targets for attack by captan. Recently, it was found that the amino acid residues Lys^{1065}, His^{1237} and Lys^{886} are located in the β subunit and form a part of the RNA polymerase activity center [26, 27]. It is possible that these amino acids are involved in captan action.

The observations that (i) the β subunit is believed to be responsible for the correct termination of RNA transcription [2, 28], (ii) captan binds to a site(s) other than the NTP binding site on the β subunit, and (iii) an earlier report showed that captan stimulated *in vivo* RNA synthesis in toluenized *E. coli* cells and that the increase of RNA synthesis was due to the increase of the lengths of RNA products, rather than an increase in the number of RNA transcripts [10], lead to the assumption that the binding of captan to the β subunit at loci different from the DNA or NTP binding sites may cause the enzyme to disregard the correct termination sites. This would result in the increase of the lengths of RNA transcripts. Investigations testing this possibility are currently underway.

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