Mechanism of Inhibition of *Escherichia coli* RNA polymerase by captan

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(Received 10 July 1981/Accepted 4 September 1981)

Captan (*N*-trichloromethylthiocyclohex-4-ene-1,2-dicarboximide) was shown to inhibit RNA synthesis *in vitro* catalysed by *Escherichia coli* RNA polymerase. Incorporation of [γ - 32 P]ATP and [γ - 32 P]GTP was inhibited by captan to the same extent as overall RNA synthesis. The ratio of [3 H]UTP incorporation to that of [γ - 32 P]ATP or of [γ - 32 P]GTP in control and captan-treated samples indicated that initiation was inhibited, but the length of RNA chains being synthesized was not altered by captan treatment. Limited-substrate assays in which re-initiation of RNA chains did not occur also showed that captan had no effect on the elongation reaction. Studies which measured the interaction of RNA polymerase with template DNA revealed that the binding of enzyme to DNA was inhibited by captan. Glycerol-gradient sedimentation of the captan-treated RNA polymerase indicated that the inhibition of the enzyme was irreversible and did not result in dissociation of its subunits. These data are consistent with a mechanism in which RNA polymerase activity was irreversibly altered by captan, resulting in an inability of the enzyme to bind to the template. This interaction was probably at the DNA-binding site on the polymerase and did not involve reaction of captan with the DNA template.

RNA synthesis in *Escherichia coli* is carried out by a single high-molecular-weight multi-subunit DNA-dependent RNA polymerase (EC 2.7.7.6) (Burgess, 1969*b*). This enzyme requires zinc for activity (Scrutton *et al.*, 1971; Miller *et al.*, 1979) and also contains essential thiol (Yarbrough & Wu, 1974; Krakow, 1975) and arginine groups (Srivastava & Modak, 1980). Transcription proceeds by at least five distinguishable steps: (i) formation of the open promoter conformation, (ii) binding of the enzyme to specific promoter sites on the DNA template, (iii) initiation of synthesis with either ATP or GTP, (iv) elongation of the RNA molecule and (v) termination of the chain at a specific site (Chamberlin, 1976). The elucidation of the characteristics of the enzyme molecule and the mechanism of the polymerase reaction are due in a large part to studies which employed inhibitors of that catalytic activity.

A new inhibitor, captan (*N*-trichloromethylthiocyclohex-4-ene-1,2-dicarboximide), has been shown to alter RNA synthesis *in vivo* (Gale *et al.*, 1971), and preliminary studies from our laboratory have demonstrated that *E. coli* RNA polymerase is inhibited *in vitro*. The present study examines the mechanism by which captan inhibits the synthesis of

RNA *in vitro* catalysed by *E. coli* RNA polymerase. It is shown that captan blocks the binding of the enzyme to the template, thus inhibiting initiation while having no effect on the elongation reaction.

Materials and methods

Analytical-grade captan (98.6%) was supplied by Chevron Corp. (Richmond, CA, U.S.A.). Sigma Chemical Co. was the source of ATP, GTP, CTP, UTP and highly polymerized calf thymus DNA (type I). Nitrocellulose filters (BA85) were products of Schleicher and Schuell (Keene, NH, U.S.A.). ICN Pharmaceuticals (Irvine, CA, U.S.A.) was the source of [5,6- 3 H]UTP (26 Ci/mmol), [8- 3 H]GTP (13.5 Ci/mmol), [2,8- 3 H]ATP (41.6 Ci/mmol), [γ - 32 P]GTP (25 Ci/mmol) and [γ - 32 P]ATP (25 Ci/mmol). New England Nuclear (Boston, MA, U.S.A.) supplied sodium [32 P]pyrophosphate (7.0 Ci/mmol). *E. coli* RNA polymerase (2.71 units/mg of protein) containing sigma factor [isolated by the method of Burgess & Jendrisak (1975) and showing four bands on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis] was obtained from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.). Miles Laboratories (Elkhart, IN, U.S.A.) supplied poly(dA-dT)·poly(dT-dA) ($s_{20,w}$ = 10.9 S) and poly-

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(dG)•poly(dC) ($s_{20,w} = 10.5$ S). Glass-distilled dimethyl sulphoxide was the product of Burdick and Jackson Laboratories (Muskegon, MI, U.S.A.). Bio-Rad Laboratories (Richmond, CA, U.S.A.) was the source of protein assay reagent (dye-binding assay).

RNA polymerase assays

RNA synthesis, initiation and elongation. RNA polymerase activity was assayed by a modification of the method of Hoffman & Niyogi (1973). Overall RNA-synthesis rates were measured by using an incubation mixture containing 20 mM-Tris/HCl (pH 8.0), 10 mM-MgCl₂, 0.25 mM-ATP, -CTP and -GTP, 0.25 mM-[³H]UTP (sp. radioactivity 1000 c.p.m./nmol) and 10–30 µg of calf thymus DNA in a total volume of 200 µl.

RNA chain initiation was determined by using the same incubation mixture with [γ -³²P]ATP or [γ -³²P]GTP replacing [³H]UTP as the label. Final specific radioactivity for either nucleotide was 10⁵ c.p.m./nmol.

The effect of captan on chain elongation was determined by a modification of the method of Yarbrough *et al.* (1977). This limited-substrate assay utilized an incubation mixture containing, in 0.2 ml: 2 mM-Tris/HCl (pH 8.1), 10 mM-MgCl₂, 0.25 mM-ATP, -CTP and -GTP, 2.5 mM-[³H]UTP (40 c.p.m./pmol) and 10 µg of calf thymus DNA. Reactions were initiated by addition of RNA polymerase to the incubation cocktail and inhibitors were added 5 min later.

All RNA polymerase assays were incubated at 37°C and contained 4–8 µg of *E. coli* RNA polymerase. After incubation, assays were stopped by adding 2 ml of a solution containing 10% (w/v) trichloroacetic acid and 1% sodium pyrophosphate and were filtered on GF/A glass-fibre filters. Filters were washed with 30 ml of 10% trichloroacetic acid and 10 ml of chloroform/methanol (1:1, v/v), dried and counted for radioactivity in 10 ml of toluene containing 4 g of 2,5-diphenyloxazole/litre and 0.05 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene/litre.

RNA polymerase binding

The binding of *E. coli* RNA polymerase to bacteriophage-T7 DNA was determined by the method of Jones & Berg (1966). ³H-labelled phage-T7 DNA (2500 c.p.m./µg of DNA) was incubated for 10 min at 25°C with RNA polymerase in 0.25 ml of a solution containing 10 µmol of Tris/HCl (pH 8.0) and 1 µmol of MgCl₂. Then 2 ml of an ice-cold solution of 0.01 M-Tris/HCl (pH 8.0) and 0.05 M-NaCl was added to the solution, which was filtered on a nitrocellulose filter that had been previously soaked in the Tris/salt solution, and washed with 40 ml of the same solution. Filters were

dried and counted for radioactivity as described above.

[³²P]Pyrophosphate exchange

The pyrophosphate-exchange reaction was performed by the method of Krakow & Fronk (1969). Incubations contained, in a total volume of 0.25 ml, 80 mM-Tris/HCl (pH 7.8), 4 mM-MgCl₂, 4 mM-UTP, 1 mM-sodium [³²P]pyrophosphate (2×10^4 c.p.m./nmol), 0.1 A₂₆₀ unit of poly(dA-dT)•poly(dT-dA) and 10 µg of RNA polymerase. After incubation for various times up to 8 min at 37°C the reaction was terminated by addition of 0.2 ml of 0.1 M-EDTA (pH 6.0), and 0.1 ml of 0.1 M-sodium pyrophosphate (pH 6.0). The reaction solution was mixed with 0.5 ml of a 10% (w/v) suspension of acid-washed activated charcoal in 0.01 M-sodium pyrophosphate and the mixture was filtered through a GF/A glass-fibre filter. The charcoal was washed with 40 ml of 0.01 M-sodium pyrophosphate, dried and counted for radioactivity by using a Geiger tube with a 1.4 mg/cm² window and a Nuclear-Chicago model 181A Scaler.

Glycerol-gradient sedimentation of RNA polymerase

This was done in the presence of high salt by the method of Burgess (1969a). RNA polymerase was sedimented in linear 10–30% (v/v) glycerol gradients containing 0.01 M-Tris/HCl (pH 7.9), 0.01 M-MgCl₂, 0.1 mM-EDTA and 1.0 M-KCl. Centrifugation was performed in a Spinco SW 50.1 rotor at 40000 rev./min (150000 g) for 12.5 h. Gradients were collected in 0.25 ml fractions and each sample was assayed for protein and RNA polymerase activity.

Protein assay

Protein concentrations were determined by using either the Coomassie Brilliant Blue G dye-binding assay (Bradford, 1976) with bovine γ -globulin as a protein standard, or the method of Lowry *et al.* (1951) with bovine serum albumin as a protein standard.

DNA and RNA assays

The concentrations of DNA and RNA in various preparations were determined by the diphenylamine method (Burton, 1956) and the orcinol method (Parish, 1972) respectively.

Results

That captan inhibited RNA polymerase activity is shown in Fig. 1. The inhibition was both time- and concentration-dependent. Short time periods of reactions showed little effect by captan; however, with increasing time the degree of inhibition was enhanced. The time-dependent inhibition indicated a

slow step in the mechanism; therefore, before assay RNA polymerase was preincubated for 10 min with captan in the presence of nucleoside triphosphates, but no DNA template. With preincubation, linear inhibition was observed (Fig. 2) and an ID_{50} value (dose giving 50% inhibition) of $60\mu\text{M}$ was established.

To determine the nature of the inhibition on the RNA polymerase reaction, the effects of captan on initiation and chain elongation were measured. Initiation was measured by the incorporation of either $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ or $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ into acid-insoluble material. Table 1 shows that in the presence of $100\mu\text{M}$ -captan initiation was retarded by approximately the same degree as was RNA synthesis measured by the incorporation of $[\text{H}]\text{UTP}$. These results suggest that the inhibition of RNA synthesis

takes place at the initiation step and that chain elongation is not significantly altered. The ratio of ^3H to ^{32}P , which gives a measure of the length of the RNA chain, also indicates that chain elongation is not significantly inhibited.

By using a limited-substrate assay the effect of captan on the elongation reaction was measured by a more direct method (Yarborough *et al.*, 1977). In this assay, where re-initiation does not occur, inhibition by captan was similar to that observed with rifampicin (Fig. 3). These data confirm that captan inhibits initiation, but not elongation.

The inability of captan to alter chain elongation is supported by the fact that the poly-(dA-dT)·poly(dT-dA)-dependent pyrophosphate-exchange reaction is not significantly inhibited by captan at a concentration ($500\mu\text{M}$) that would strongly inhibit the RNA polymerase activity (Fig. 4).

Fig. 1. Inhibition of *E. coli* RNA polymerase activity by captan

RNA polymerase activity was determined as described in the Materials and methods section. Captan was added to the incubation mixture before addition of the enzyme. The reaction was initiated by addition of RNA polymerase and terminated at indicated times by addition of 10% trichloroacetic acid. (A) Dimethyl sulphoxide; (B) $50\mu\text{M}$ -captan; (C) $100\mu\text{M}$ -captan; (D) $100\mu\text{M}$ -captan; (E) $500\mu\text{M}$ -captan.

Fig. 2. Effect of preincubation on captan inhibition of *E. coli* RNA polymerase

Captan was added to the incubation mixture lacking DNA; RNA polymerase was then added, followed by incubation at 37°C for 10 min. RNA synthesis was initiated by addition of DNA, and the reaction was terminated at times indicated. (a): (■) dimethyl sulphoxide; (A) $5\mu\text{M}$ -captan; (B) $10\mu\text{M}$ -captan; (C) $50\mu\text{M}$ -captan; (D) $100\mu\text{M}$ -captan; (E) $500\mu\text{M}$ -captan. (b) Percentage inhibition versus log of captan concentration.

Table 1. Effect of captan on initiation of RNA synthesis by *E. coli* RNA polymerase
For details see the Materials and methods section.

Sample	Label	Incorporation	Inhibition (%)	$^3\text{H}/^{32}\text{P}$
Control	$[\text{H}]\text{UTP}$	4.9 nmol		
+ $100\mu\text{M}$ -Captan	$[\text{H}]\text{UTP}$	2.9 nmol	40	
Control	$[\gamma\text{-}^{32}\text{P}]\text{ATP}$	2.02 pmol		2425
+ $100\mu\text{M}$ -Captan	$[\gamma\text{-}^{32}\text{P}]\text{ATP}$	1.21 pmol	40	2397
Control	$[\gamma\text{-}^{32}\text{P}]\text{GTP}$	2.62 pmol		1870
+ $100\mu\text{M}$ -Captan	$[\gamma\text{-}^{32}\text{P}]\text{GTP}$	1.55 pmol	37	1747

In order to determine if the DNA template exerted an influence on the inhibition of RNA polymerase, various DNA species were used as a template for RNA synthesis in the presence of captan. In all cases captan was inhibitory to approximately the same degree (Table 2), and in a time-dependent manner; therefore no template specificity was apparent.

The available data indicated that captan inhibited

E. coli RNA polymerase by prohibiting the initiation reaction while having little effect on elongation. Therefore studies were conducted to determine if the binding of RNA polymerase to the DNA template was affected. Control experiments showed that the binding of DNA to the filter was a direct function of the amount of added enzyme, and all assays were run in the linear region of this curve. The effect of captan on DNA binding was determined by using

Fig. 3. Effect of captan on the elongation reaction of *E. coli* RNA polymerase

A limited substrate assay ($2.5 \mu\text{M}$ -UTP) was performed as described in the Materials and methods section. Assays were initiated by addition of enzyme and were allowed to proceed for 5 min, after which various compounds were added. Arrow marks point of addition of (○) dimethyl sulphoxide, (●) $1 \mu\text{M}$ -rifampicin or (■) $100 \mu\text{M}$ -captan.

Fig. 4. Effect of captan on poly(dA-dT)·poly(dT-dA)-dependent pyrophosphate exchange by *E. coli* RNA polymerase

The pyrophosphate-exchange reaction was carried out as described in the Materials and methods section. Captan was added to the incubation mixture before addition of enzyme, and the reaction was initiated by addition of RNA polymerase. (●) Dimethyl sulphoxide; (■) $50 \mu\text{M}$ -captan.

Table 2. Influence of DNA template on inhibition of *E. coli* RNA polymerase activity by captan

When poly(dA-dT)·poly(dT-dA) was the template, only ATP and [^3H]UTP were present in the incubation mixture and when poly(dG)·poly(dC) was the template, only CTP and [^3H]GTP were present. When poly(dA-dT)·poly(dT-dA) or poly(dG)·poly(dC) was the template, acid-insoluble precipitates were collected on nitrocellulose filters. The degree of inhibition varies with incubation time and the values listed were determined at indicated incubation times. For full details see the Materials and methods section.

Template	Concn.		Incubation time (min)	Inhibition (%)
	Template ($\mu\text{g}/\text{ml}$)	Captan (μM)		
Calf thymus DNA	40	100	10	65
Phage-T7 DNA	69	100	8	26
Phage-T7 DNA	69	500	8	48
Phage-T7 DNA	69	1000	8	60
Poly(dA-dT)·poly(dT-dA)	40	100	8	40
Poly(dG)·poly(dC)	40	100	8	30

three different treatment schemes (Table 3). In the first case, when DNA and polymerase were incubated for 10 min, followed by addition of captan and further incubation for 10 min, very little inhibition was observed. In the second procedure DNA and captan were preincubated before addition of enzyme, and again little inhibition was observed. In the third case, captan was incubated with the polymerase for 10 min, followed by addition of DNA and further incubation for 10 min; a strong inhibition of binding of the polymerase to DNA was then observed. These results are consistent with a mechanism in which captan inhibits RNA synthesis by blocking the binding of RNA polymerase to the template. These data also imply that the rate constant for binding of the polymerase to the template is much larger than that for the interaction of captan with the polymerase.

The kinetics of inhibition of template binding indicate that captan reacts with the polymerase in a time-dependent manner (results not shown). Replotting these data (Fig. 5) as log (percentage of control activity remaining) versus time results in a straight line, suggesting that under our assay conditions the inhibition is a pseudo-first-order process, and the rate of inhibition depends on the concentration of inhibitor (Webb, 1963). From the slope of this curve and the relationship $\text{slope} = k_i[\text{inhibitor}]/2.303$, a rate constant of $37.5 \text{ M}^{-1} \cdot \text{s}^{-1}$ was determined. This value is much lower than the rate constant for the binding of RNA polymerase to phase-T7 DNA, $10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$, estimated by Hinkle & Chamberlin (1972). From the calculated value for

k_i and the relationship that $k_i = 0.695/\text{ID}_{50}t$, an ID_{50} value may be determined for any time t . For a 10 min incubation time, an ID_{50} value of $30.8 \mu\text{M}$ was obtained. This value compares very well with the ID_{50} value of $60 \mu\text{M}$ for the inhibition of RNA synthesis determined by preincubating the enzyme for 10 min with captan (Fig. 2). Thus captan appears to inhibit RNA synthesis solely by inhibiting the binding of the enzyme to the template.

In order to determine if captan inhibits binding by causing dissociation of the subunits of the polymerase, through either a loss of the σ subunit or dissociation of α, β or β' subunits, treated RNA polymerase was sedimented through glycerol gradients (Fig. 6). The locations of protein and of the RNA polymerase activity in the gradient were determined. The enzyme activity sedimented in the same position in treated and untreated samples, and the total amount of protein recovered in the peak of activity was the same in captan-treated and control samples (37.5 and $36 \mu\text{g}$ respectively). Thus captan at a concentration known to cause significant inhibition of polymerizing activity, does not cause the dissociation of the polymerase molecule. Furthermore, sedimentation did not cause the enzyme to regain catalytic activity. Therefore RNA polymerase under these conditions was irreversibly inhibited by captan by a mechanism that does not involve the dissociation of the enzyme complex.

Table 3. Inhibition of binding of *E. coli* RNA polymerase to phage-T7 DNA by captan: effect of order of addition of components

Operational sequences were as follows. A: ^3H -labelled phage-T7 DNA; RNA polymerase; incubate 10 min; captan; incubate 10 min. B: ^3H -labelled phage-T7 DNA; captan; incubate 10 min; RNA polymerase; incubate 10 min. C: RNA polymerase; captan; incubate 10 min; ^3H -labelled phage-T7 DNA; incubate 10 min. After the last 10 min incubation, samples were diluted and the amount of DNA binding to a nitrocellulose filter was determined.

Order of addition	Compound added ($100 \mu\text{M}$)	Label bound (c.p.m.)	Inhibition of binding (%)
A	Control	4910	7.9
	Captan	4524	
B	Control	6568	2.5
	Captan	6405	
C	Control	5069	88.0
	Captan	628

Fig. 5. Kinetics of inhibition of DNA binding of *E. coli* RNA polymerase by captan

RNA polymerase ($2.5 \mu\text{g}$) was incubated with $100 \mu\text{M}$ -captan for 10 min, followed by addition of ^3H -labelled phage-T7 DNA and further incubation for 10 min. The ability of the enzyme to bind DNA was determined by the amount of DNA bound to a nitrocellulose filter as described in the Materials and methods section. The degree of inhibition was determined by comparison with solvent (3% dimethyl sulfoxide) controls.

Fig. 6. Glycerol-gradient sedimentation of captan-treated *E. coli* RNA polymerase

RNA polymerase (40 μ g) was incubated for 5 min with either dimethyl sulphoxide (control, *b*) or 100 μ M-captan (*a*), layered on glycerol gradients (linear, 10–30%) and sedimented as described in the Materials and methods section. ■, Protein concentration; ●, RNA polymerase activity.

Discussion

The results presented show that captan inhibits RNA synthesis by preventing the binding of the enzyme to the template. A proposed mechanism involves the inhibition of enzymic activity by interaction of captan with the free enzyme, producing a modified protein which has lost its ability to form a complex with DNA. Captan appears to be unable to inhibit effectively the polymerase–template complex. Captan inhibition is not mediated through a modification of DNA template; in fact, DNA appears to protect the enzyme.

That the inhibitor acts in an irreversible way is supported by both kinetic analysis and velocity-sedimentation studies. The results obtained from sedimentation studies on captan-treated polymerase also document that the site of captan's action was the enzyme and not the template. This observation was further supported by the fact that when DNA was treated with captan before the addition of RNA polymerase, the degree of enzyme binding to template was not decreased; thus DNA was not the site of captan's action. Captan has been shown by

Anderson & Rosenkranz (1974) to interact with the DNA template; however, this interaction, if it occurs in the assays *in vitro* utilized here, does not result in inhibition of polymerase activity.

Captan interacts slowly with the polymerase. A rate constant of $37.5 \text{ M}^{-1} \cdot \text{s}^{-1}$ was determined for the inhibition of binding of RNA polymerase. This slow reaction accounts for the fact that when polymerase was added to an assay mixture containing captan, there was very little initial inhibition of the binding reaction or of polymerizing activity. The rate constant for the binding of RNA polymerase to phage-T7 DNA has been estimated to be $10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Hinkle & Chamberlin, 1972), and thus the binding of the polymerase to DNA is the favoured reaction. When the polymerase comes off the template two competing reactions must occur. One of these is the relatively fast reaction of the reassociation of the polymerase with the DNA; the other is the captan–polymerase interaction. Even though the rate constants for these two processes are greatly different, each time the polymerase leaves the template there is a finite probability that it will interact with a captan molecule. Therefore, since interaction with captan results in irreversible inhibition of the enzyme, increasing incubation time results in increasing inhibition.

The proposal that captan cannot interact with polymerase once it is bound to the template is indicated by the fact that when RNA polymerase has initiated an RNA chain before the addition of captan, the elongation reaction is not inhibited. Further, the pyrophosphate-exchange reaction, which involves the formation of phosphodiester bonds followed by pyrophosphorolysis, is not inhibited by captan. This latter reaction may be thought of as an idling reaction, and once the polymerase is on the template the reaction continues without net synthesis of RNA and without the enzyme leaving the template. Finally, preincubation studies showed that the complex between RNA polymerase and phage-T7 DNA was stable in the presence of captan.

The concentration of captan required to give 50% inhibition of polymerase activity is approx. 60 μ M. The value is intermediate in the ID_{50} range for other inhibitors of RNA polymerases, such as iodoacetamide (330 μ M; Yarbrough & Wu, 1974), Rose Bengal (1.4 μ M; Wu & Wu, 1973), phosphonoacetic acid (100 μ M; Allaudeen & Bertino, 1978), aurintricarboxylic acid (20 μ M; Blumenthal & Landers, 1973) and phenylglyoxal (100 μ M; Srivastava & Modak, 1980).

Captan has been shown to react with thiol groups on a number of proteins (Lukens & Sisler, 1958), and in many systems the toxicity of captan has been attributed to its ability to act as a nucleophile by attacking thiol and amino groups (Richmond &

Somers, 1970). *E. coli* RNA polymerase is known to contain 32 thiol residues on the core enzyme (Burgess, 1969*b*). Some of these groups are thought to be required for template recognition and specific initiation (Yarbrough & Wu, 1974), whereas others are important for the structural integrity of the enzyme (Krakow, 1975). Thiol-modifying agents were shown to cause a loss of template binding ability and of structural integrity. Since captan is known to react with thiol groups, its ability to prevent the binding of enzyme to template may involve a modification of thiol groups on the enzyme. However, since captan at 100 μ M does not cause a loss of the quaternary structure in the enzyme (as evidenced by complete recovery of protein in only one peak, which corresponded to the active protein, i.e. no dissociation occurred), not all thiol groups on the enzyme are attacked by captan.

Captan is a newly described inhibitor of RNA polymerase which prevents the formation of template-enzyme complex. The inhibition of polymerase-DNA complex-formation appears to be a general phenomenon for nucleic acid polymerases, since a similar inhibition has also been reported for a variety of DNA polymerases (Dillwith & Lewis, 1979).

This is a contribution of the Nevada Agricultural Experiment Station, Journal Series No. 527.

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