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Binding of Captan to DNA Polymerase I from *Escherichia coli* and the Concomitant Effect on 5′→3′ Exonuclease Activity†

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ABSTRACT: Captan (*N*-[(trichloromethyl)thio]-4-cyclohexene-1,2-dicarboximide) was shown to bind to DNA polymerase I from *Escherichia coli*. The ratio of [^{14}C]captan bound to DNA pol I was 1:1 as measured by filter binding studies and sucrose gradient analysis. Preincubation of enzyme with polynucleotide prevented the binding of captan, but preincubation of enzyme with dGTP did not. Conversely, when the enzyme was preincubated with captan, neither polynucleotide nor dGTP binding was blocked. The modification of the enzyme by captan was described by an irreversible second-order rate process with a rate of 68 ± 0.7 M^{-1} s^{-1}. The interaction of captan with DNA pol I altered each of the three catalytic functions. The 3′→5′ exonuclease and polymerase activities were inhibited, and the 5′→3′ exonuclease activity was enhanced. In order to study the 5′→3′ exonuclease activity more closely, [^{3}H]hpBR322 (DNA–[^{3}H]RNA hybrid) was prepared from pBR322 plasmid DNA and used as a specific substrate for 5′→3′ exonuclease activity. When either DNA pol I or polynucleotide was preincubated with 100 μM captan, 5′→3′ exonuclease activity exhibited a doubling of reaction rate as compared to the untreated sample. When 100 μM captan was added to the reaction in progress, 5′→3′ exonuclease activity was enhanced to 150% of the control value. Collectively, these data support the hypothesis that captan acts on DNA pol I by irreversibly binding in the template–primer binding site associated with polymerase and 3′→5′ exonuclease activities. It is also shown that the chemical reaction between DNA pol I and a single captan molecule proceeds through a Michaelis complex. The final, irreversible step results in inhibited polymerase and 3′→5′ exonuclease activities as well as enhanced 5′→3′ exonuclease activity.

Kinetic studies of procaryotic, eucaryotic, and viral polymerases have been employed to examine the inhibitory effects of captan (*N*-[(trichloromethyl)thio]-4-cyclohexene-1,2-dicarboximide), an inhibitor which acts on a variety of enzyme systems including nucleic acid synthesis (Lewis & Brown, 1978; Gale et al., 1971; Martin & Lewis, 1979). Although the mechanism of captan inhibition of polymerases has been investigated, efforts have mainly focused on the polymerase activity of the enzymes (Dillwith & Lewis, 1982a,b; Freeman-Wittig & Lewis, 1986). Of the DNA polymerases, DNA polymerase I from *Escherichia coli* has been examined extensively (Dillwith & Lewis, 1982a; Freeman-Wittig & Lewis, 1986). In one report, it was noted that captan caused irreversible loss of DNA pol I polymerase activity which was accounted for by captan–protein interactions. Kinetic studies showed that DNA binding by enzyme and/or initiation of polymerization was inhibited by captan but that elongation of DNA was not. The template–primer binding site was shown to be the locus of captan interaction, and DNA served as a protection against captan's action (Dillwith & Lewis, 1982a).

A subsequent investigation of the effects of captan on DNA pol I revealed that the 3′→5′ exonuclease activity which shares the polynucleotide binding site with the polymerase activity was inhibited in the presence of captan and substantiated that

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DNA and captan competed for the same site on the polymerase (Freeman-Wittig & Lewis, 1986). Since the two activities share the single polynucleotide binding site, protection of both functions by DNA might be expected (Kornberg, 1980). In addition to the polymerase and 3′→5′ exonuclease activities, DNA pol I has a locus for 5′→3′ polynucleotide degradation which is physically and kinetically distinct from the other two activities (Burtlag et al., 1969; Klenow & Henningsen, 1970). In the presence of captan, 5′→3′ exonuclease activity was enhanced in a manner which appeared to be related to DNA concentration (Freeman-Wittig & Lewis, 1986).

In this investigation, the physical interaction between DNA pol I and captan is explored. The apparent relationship between polynucleotide concentration and captan-mediated alteration of the three activities is explained. These findings are related to what is already known concerning the kinetic relationships between polymerase and 3′→5′ exonuclease activities and to how the captan–pol I interaction alters 5′→3′ exonuclease activity.

Materials and Methods

Materials were provided by the following sources: DNA polymerase I, Klenow fragment, and RNA polymerase were supplied by Cooper Biomedical, Freehold, NJ. *Eco*RI and enzyme-grade sucrose were purchased from Sigma Chemical Co., St. Louis, MO. DE81 filter paper was a product of Whatman, United Kingdom. [^{3}H]Deoxyguanosine (10 Ci/mmol), [^{3}H]deoxyadenosine (17 Ci/mmol), [^{3}H]deoxycytidine (25 Ci/mmol), [^{3}H]thymidine (50 Ci/mmol), [^{3}H]ATP (30 Ci/mmol), [^{3}H]GTP (13.5 Ci/mmol), and [^{3}H]UTP (23 Ci/mmol) were synthesized by ICN Corp., Irvine, CA. [α-^{32}P]dGTP (25 Ci/mmol) was a product of New England Nuclear, Boston, MA. [^{14}C]Captan (*N*-[(trichloro[^{14}C]-methyl)thio]-4-cyclohexene, 0.185 mCi/mg) was a generous gift of the Stauffer Chemical Co., Farmington, CT, and unlabeled captan was a gift of the Chevron Corp., Richmond, CA. *Escherichia coli* Thy$^-$ strain KL161 (CGSC 4207) was obtained from the *E. coli* Genetic Stock Center, Yale University, New Haven, CT. Dr. Berch E. Henry, Department of Microbiology, University of Nevada–Reno, provided the pBR322.

Plasmid Transformation of E. coli. KL161 was grown in thymidine-supplemented medium and transformed by the standard transformation protocol of Hanahan (1985) using BR322 plasmid (pBR322). Colony transformation was tested with both ampicillin and tetracycline.

Synthesis of [^{3}H]hpBR322. Plasmid BR322 was cleaved by combining 1 μg of DNA for every 50 units of *Eco*RI in a buffer containing 100 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 50 mM NaCl for 4 h at 37 °C. Linear, duplex DNA was denatured by boiling for 5 min, vortexing for 30 s, and cooling rapidly by placing on ice. This single-stranded DNA was then employed as a template for RNA polymerase from *E. coli* using a modified version of the protocol outlined by Burgess (1969). A total of 200 μCi (0.15 mM) each of [^{3}H]GTP, [^{3}H]ATP, and [^{3}H]UTP was combined with 0.15 mM CTP in the presence of 50 units of RNA polymerase and allowed to react for 1.5 h at 37 °C in a 1-mL reaction volume. The resulting polynucleotide was phenol extracted, ethanol precipitated, and dried under vacuum. The specific activity was determined to be (5.5–6.5) × 10^3 cpm/g.

DNA Polymerase I Polymerizing Activity. Polymerase activity was assayed by the method of Dillwith and Lewis (1982a), except pBR322 DNA was substituted for the activated calf thymus DNA. DMSO (1% final concentration) was used as the solvent for captan.

DNA Polymerase I 3′→5′ Exonuclease Activity. The 3′→5′ exonuclease activity was assayed for its ability to degrade heat-denatured [^{3}H]pBR322 DNA according to the method previously published (Freeman-Wittig & Lewis, 1986). DMSO (1% final concentration) was used as a solvent for captan.

DNA Polymerase I 5′→3′ Exonuclease Activity. The 5′→3′ exonuclease activity was assayed according to the method of Freeman-Wittig and Lewis (1986) for its ability to degrade [^{3}H]hpBR322. Activity was assayed in the presence of varying amounts of captan dissolved in DMSO (1% final concentration).

Filter Binding Assays. Preincubations were executed at 0 °C at 2-min intervals in the presence of various compounds, as described in the tables. To measure captan binding, [^{14}C]captan was used, whereas when dGTP binding was explored [α-^{32}P]dGTP was employed. Excess captan was destroyed by the addition of 100 mM 2-mercaptoethanol. Samples were spotted on Whatman DE81 filter paper and washed 4 times for 5 min with cold 5% (v/v) 2-mercaptoethanol in 0.3 M ammonium formate (pH 8.0), followed by three washes with cold 95% ethanol. The samples were then dried, and the amount of radiolabel present was quantitated by liquid scintillation chromatography. The final DMSO concentration was 1% in all samples.

DNA Binding Assays (Sucrose Gradients). A modification of the method outlined by Englund et al. (1969) was used. Continuous 5-mL gradients of 5–20% buffered sucrose [50 mM HEPES (pH 7.4), 5 mM $MgCl_2$, and 10 mM KCl] were centrifuged at 2 °C for 4.5 h. Gradients were fractionated into 20 tubes using the drop method. The amounts of protein, DNA, and radiolabel present in each fraction were determined by A_{280}, A_{260}, and liquid scintillation techniques, respectively.

Determination of Rate Constants. Buffered holoenzyme was treated on ice with 10–1000 μM [^{14}C]captan, and aliquots were taken every 15 s for up to 3 min. Reactions were terminated with 100 mM 2-mercaptoethanol and spotted onto DE81 filter paper, washed, and quantitated as described above under filter binding assays. The pseudo-first-order rate constant (k_0') was determined from the integrated rate equation (see Results). The apparent second-order rate constant (k_0) was calculated as the slope of a k_0' vs [captan] plot. K_D was determined by extrapolation using the minitab statistics package to analyze plots of $1/k_0$ vs 1/[captan].

Results

[^{14}C]Captan Binds to the DNA Binding Site. Although it had been previously reported that captan irreversibly altered the polymerase and exonuclease activities of DNA polymerase I from *E. coli*, the mechanism by which the changes took place was not understood. For example, either captan could have bound irreversibly to DNA pol I and thereby blocked the polymerase and 3′→5′ active sites or the compound could have caused a partial denaturation of the polypeptide by its binding or by some other chemical means (Dillwith & Lewis, 1982a; Freeman-Wittig & Lewis, 1986). In this study, [^{14}C] captan, labeled on the trichloromethyl carbon, was used to probe for the binding of captan to DNA pol I. In filter binding assays, enzyme which was preincubated with captan alone was shown to irreversibly bind one molecule of captan for each molecule of DNA pol I present (Table I). The locus of the protein–ligand interaction was probed using hpBR322 as a ligand for the polynucleotide binding site and dGTP as a ligand for the nucleotide triphosphate binding site. It was observed that preincubation of DNA pol I with hpBR322, either in the

Table I: Effects of Preincubation on Captan Binding to DNA Pol I[a]

first incubation	second incubation	molecules of captan bound per molecule of enzyme
none	[^{14}C]captan	1.1 ± 0.1
hpBR322	[^{14}C]captan	0.0 ● 0.2
dGTP	[^{14}C]captan	1.1 ● 0.2
dGTP + DNA	[^{14}C]captan	0.1 ± 0.1

[a] DNA pol I (2.5 polymerase units) was incubated with either 200 μg of hpBR322 or 150 μM dGTP or both, followed by incubation with 1 mM [^{14}C]captan. All incubations were carried out for 2 min on ice. Captan was dissolved in DMSO, and all reactions contained a final concentration of 1% DMSO. Captan incubations were followed by additions of 100 mM 2-mercaptoethanol to quench the reaction.

absence or in the presence of dGTP, prevented the binding of captan to enzyme. Identical studies which employed calf thymus DNA as a competitor for the DNA binding site yielded similar results (data not shown). It was also seen that dGTP alone could not prevent captan binding.

Since preincubation of DNA pol I with polynucleotide prevented captan from binding, captan-modified DNA pol I may have been unable to bind polynucleotide. To test this possibility, the binding of DNA to the enzyme–captan complex was assayed by sucrose gradient centrifugation techniques. [^{14}C]Captan-treated DNA pol I and [^{3}H]hpBR322 were each loaded on separate sucrose gradients, centrifuged, and fractionated (Figure 1, panel A). Overlapping peaks of radioactivity and protein indicated that a labeled adduct of captan bound irreversibly to DNA pol I. Calculations verified a 1:1 ratio of [^{14}C]label to protein. Enzyme and polynucleotide samples were distinctly separable on the 5–20% gradients. When DNA pol I was preincubated with [^{14}C]captan followed by incubation with [^{3}H]hpBR322 before sucrose gradient centrifugation, peaks of both free enzyme and free polynucleotide appeared in the expected fractions (Figure 1, panel B). However, a complex which was heavier than either unbound polynucleotide or free enzyme also appeared. The new peak was identified as a complex of [^{14}C]captan-labeled protein and [^{3}H]hpBR322. This indicated that preincubation of DNA pol I with captan does not prevent the enzyme from binding to DNA. If DNA pol I was preincubated with [^{3}H]hpBR322 before incubation with [^{14}C]captan, no ^{14}C appears in the heavy peak (Figure 1, panel C). This verifies that the enzyme–polynucleotide complex is resistant to interaction with captan.

In a separate experiment (data not shown), radioactive captan was preincubated with pol I and subsequently subjected to SDS gel electrophoresis. Autoradiographic analysis revealed that the radioactivity migrated with the pol I, indicating a possible covalent interaction between the inhibitor and enzyme.

Captan binding could not be prevented by the preincubation of enzyme with dGTP, as seen in Table I. However, the possibility that captan could prevent dGTP binding as a method of preventing polymerization still remained. In order to determine if captan had any effect on dGTP binding, enzyme was preincubated with captan before incubation with [α-^{32}P]dGTP. Filter binding studies showed that, whether or not polynucleotide was present, preincubation with captan still allowed dGTP to bind to DNA pol I in a ratio of 1:1.

Kinetics of DNA Pol I Modification by Captan. With the information that captan bound to the polynucleotide binding site of DNA pol I in a 1:1 ratio, it was possible to do kinetic studies on the interaction between captan and DNA pol I. At a minimum, the reaction between captan (C) and enzyme (E) may be presented as an irreversible biomolecular reaction:

$$C + E \rightarrow E^*$$

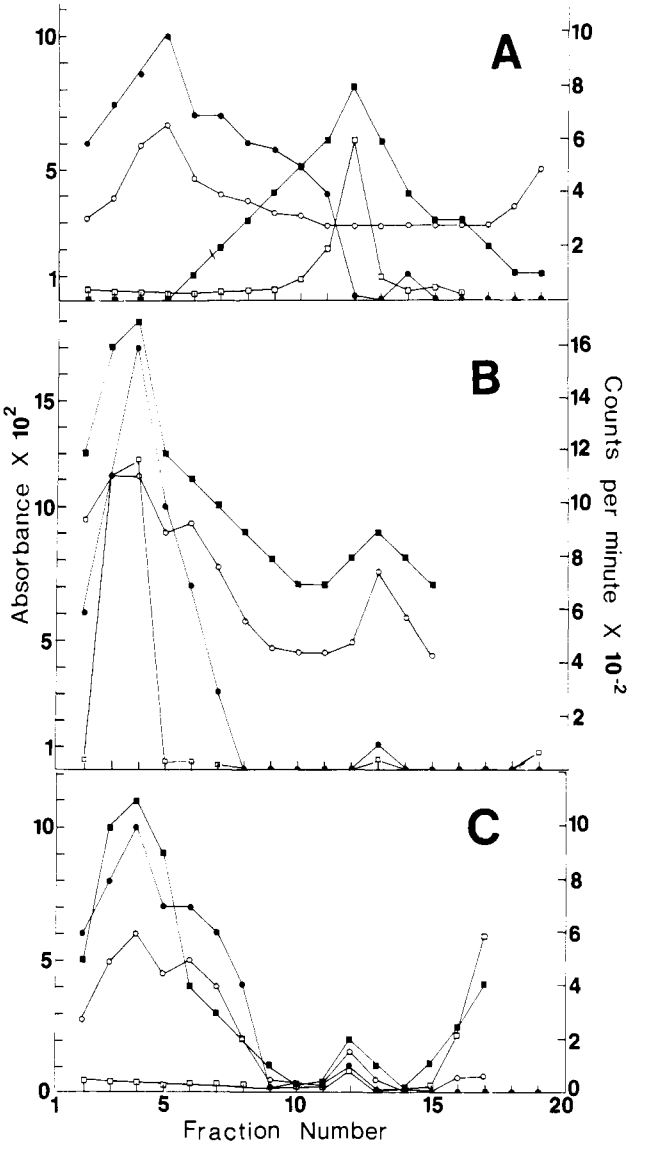

FIGURE 1: Sucrose gradient analysis of [^{3}H]hpBR322 and [^{14}C]captan binding to DNA pol I. Buffered sucrose gradients (5–20%) were loaded with samples of DNA pol I which were preincubated with either [^{14}C]captan (1 mM) or [^{3}H]hpBR322 (200 μg) or both. Fractions were collected from the bottom of each gradient. (Panel A) DNA pol I preincubated with [^{14}C]captan prior to centrifugation: peaks of protein are as shown by A_{280} (■) and ^{14}C (□). [^{3}H]hpBR322 was centrifugted on a separate gradient, but graphed for comparison: DNA peaks are as shown by A_{260} (●) and ^{3}H (○). (Panel B) DNA pol I preincubated with [^{14}C]captan followed by incubation with [^{3}H]-hpBR322 before sucrose gradient analysis. A_{280} (■), A_{260} (●), ^{3}H (○), and ^{14}C (□) of each fraction were measured. (Panel C) DNA pol I preincubated with [^{3}H]hpBR322 followed by incubation with [^{14}C]captan before sucrose gradient analysis. A_{280} (■), A_{260} (●), ^{3}H (○), and ^{14}C (□) were measured in each fraction.

where E* is the irreversibly modified enzyme. Because captan is in large molar excess (>10^2, Figure 2), the reaction can be described by a pseudo-first-order rate expression:

$$-d[E]/dt = k_0[E][C] = k_0'[E]$$

This is verified in Figure 2 where the data fit the integrated rate expression for over 90% of the reaction at the higher captan concentrations. The pseudo-first-order rate constants (k_0') obtained from the slopes were plotted vs captan concentration to obtain the second-order rate constant (k_0). The derived second-order rate constants were remarkably consistent with an average (±1 standard deviation) of 68 ± 0.7 M^{-1} s^{-1}.

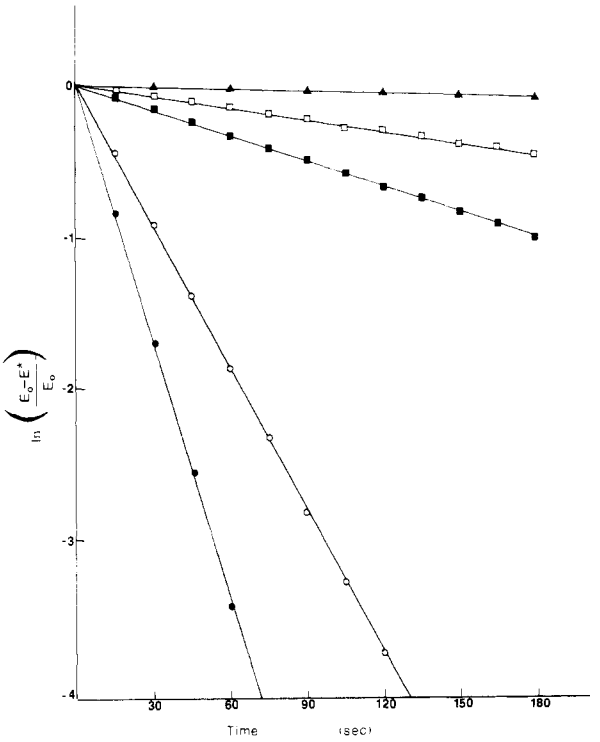

FIGURE 2: Determination of k': $\ln [([E_0] - [E^*])/[E_0]]$ vs time. DNA pol I (9.8×10^{-8} M) was incubated with 1 mM (●), 0.5 mM (○), 0.1 mM (■), 0.05 mM (□), or 0.01 mM (▲) captan. Reactions were stopped at 15-s intervals by addition of 100 mM 2-mercaptoethanol. Samples were spotted on DE81 filter paper and washed as referenced under Materials and Methods.

A likely mechanism of captan modification is the formation of a noncovalent, binary enzyme–captan complex followed by the chemical modification of the enzyme by the bound captan. The proposed mechanism can be diagrammed as

$$C + E \underset{k_2}{\overset{k_1}{\rightleftharpoons}} CE \xrightarrow{k_3} E^*$$

where E is free enzyme, CE is the binary complex of unmodified enzyme and captan, and E* is covalently modified enzyme.

The kinetic expression for the irreversible modification would be

$$d[E^*]/dt = k_3[CE]$$

The quantity [E*] is measured in the assay; the difference between the initial enzyme concentration ($[E_0]$) and [E*] will be equal to the total unmodified enzyme ($[E_u]$). Therefore

$$[E_u] = [E_0] + [CE]$$

From steady-state arguments, i.e., [captan] ≫ [enzyme] and the definition that

$$K_D = [E_0][C]/[CE]$$

it can shown that

$$[CE] = [E_u][C]/(K_D + [C])$$

and

$$d[E^*]/dt = \frac{k_3[C]}{K_D + [C]}[E_u]$$

It is evident that $k_3[C]/(K_D + [C])$ is the psuedo-first-order rate constant measured in Figure 2. According to this model, the kinetic order in captan should approach zero as captan concentration approaches infinity. However, saturation was not observed at the highest captan concentrations that could be achieved due to the limited captan solubility. A plot of $1/k_0'$ vs $1/[C]$ yielded estimates of k_3 (0.1 ± 0.1 s^{-1}) and K_D ($.002 \pm 0.002$ M). Since [captan] ≪ K_D, the expression

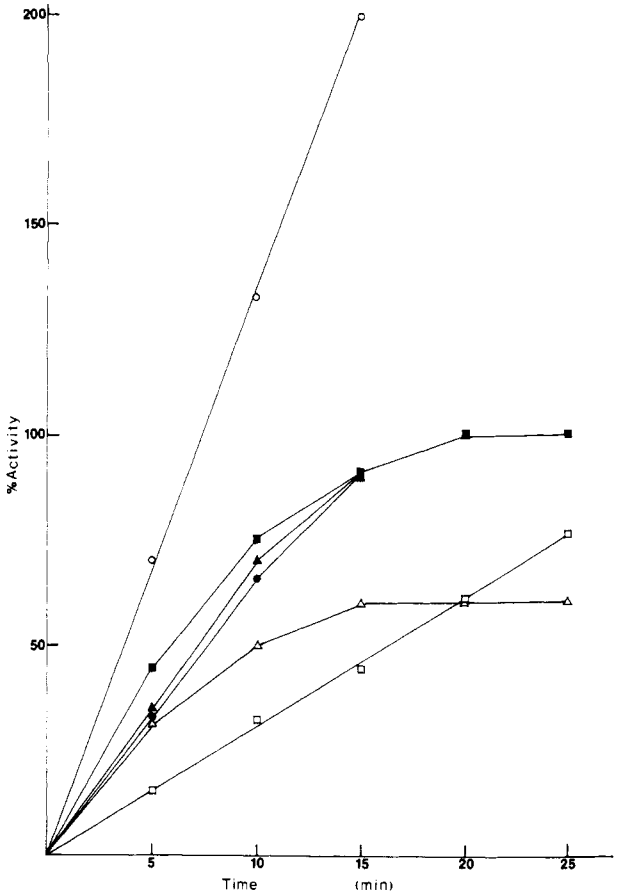

FIGURE 3: Comparative effects of captan on the three catalytic activities of DNA pol I. Polymerase function was tested in the presence (△) or absence (▲) of 100 μM captan. 3′→5′ exonuclease activity was monitored in the presence (□) or absence (■) of 100 μM captan. 5′→3′ exonuclease activity was assayed in the presence (○) or absence (●) of 100 μM captan.

$d[E^*]/dt = k_3[C][E_u]/(K_D + [C])$ reduces to $d[E^*]/dt = k_3[C][E_u]/K_D$, and the apparent second-order rate constant (68 ± 0.7 M^{-1} s^{-1}) becomes k_3/K_D.

Comparative Effects of Captan Binding on the Three Catalytic Activities of DNA Pol I. When DNA pol I was preincubated with captan under the same conditions as used for the filter binding assay, the polymerase and 3′→5′ exonuclease activities were inhibited. The corresponding 5′→3′ exonuclease activity was enhanced in the presence of captan (Figure 3). These results agree with previous studies (Dillwith & Lewis, 1982a; Freeman-Wittig & Lewis, 1986). Assurance that [^{3}H]hpBR322 served as a substrate for only the 5′→3′ exonuclease activity and not the 3′→5′ exonuclease activity was critical to this study. Therefore, Klenow fragment, a modified DNA pol I which carries active 3′→5′ exonuclease but not 5′→3′ activity, was tested with the substrate. Klenow fragment did not degrade the hybrid polynucleotide but holoenzyme did (Figure 4), showing that only the 5′→3′ exonuclease will degrade the hybrid substrate.

A doubling of 5′→3′ exonuclease activity was observed when DNA pol I was preincubated with 100 μM captan (Table III). This enhancement could be prevented if the enzyme was preincubated with hpBR322 before it was incubated with captan (Table III). Increased 5′→3′ exonuclease activity corresponded to conditions under which captan bound to DNA pol I (see Figure 1 and Table I for comparison). When [^{3}H]hpBR322 was preincubated with captan before enzyme was added, the extent of enhancement of 5′→3′ exonuclease activity at a substrate concentration of 20 μg/100 μL was 220% of the untreated sample (Figure 5, panel A). Approx-

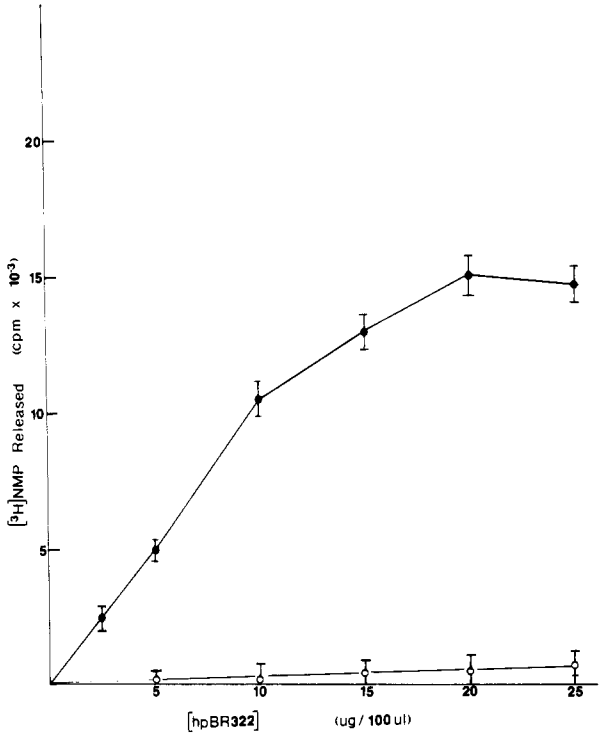

FIGURE 4: Activity of the exonuclease functions of DNA pol I on [^{3}H]hpBR322 using (○) Klenow fragment and (●) holoenzyme.

Table II: Effect of Captan on dGTP Binding[a]

first addition	second addition	molecules of dGTP bound per molecule of enzyme
none	none	1.1 ± 0.1
none	captan	0.9 ± 0.2
hpBR322	none	1.0 ± 0.1
hpBR322	captan	1.0 ± 0.2

[a] DNA pol I (2.5 polymerase units) was incubated with or without 200 μg of hpBR322 followed by incubation with or without 1 mM captan. All samples contained a final addition of 150 M [α-^{32}P]dGTP followed by spotting onto DE81 filter paper as described under Materials and Methods. All incubations were for 2 min on ice. All reactions contained a final concentration of 1% DMSO.

Table III: Effects of DNA and Captan Preincubation on 5′→3′ Exonuclease Activity[a]

first addition	second addition	5′→3′ exonuclease activity (1%)
none	captan	216 ± 10
DNA	none	100 ± 9
DNA	captan	93 ± 11

[a] DNA pol I (0.5 polymerase unit) was preincubated either in the absence or in the presence of 200 μg of hpBR322 for 2 min on ice, followed by treatment with 100 μM captan also for 2 min on ice. All preincubations contained 1% DMSO (final concentration) and were terminated with 100 mM 2-mercaptoethanol before incubation for 10 min at 37 °C.

imately the same degree of enhancement was observed when DNA pol I was preincubated with 100 μM captan at the same substrate concentration before the reaction was initiated with [^{3}H]hpBR322 (Figure 5, panel B).

In order to determine whether captan could interact with DNA pol I while 5′→3′ exonucleolysis was in progress, captan was added 5 min after the 5′→3′ reaction had already begun (Figure 6). The captan (100 μM) treated reaction showed an enhancement of 150% of the untreated reaction 10 min after captan addition, less than that when captan and polynucleotide were added simultaneously at time zero (200% activity after 10 min of exposure to captan). Addition of captan to the 5′→3′ exonuclease reaction 10 min after the initiation of the reaction also showed enhancement of 5′→3′ exonuclease activity. The rate of degradation of the captan-treated reactions did not change from that of the untreated

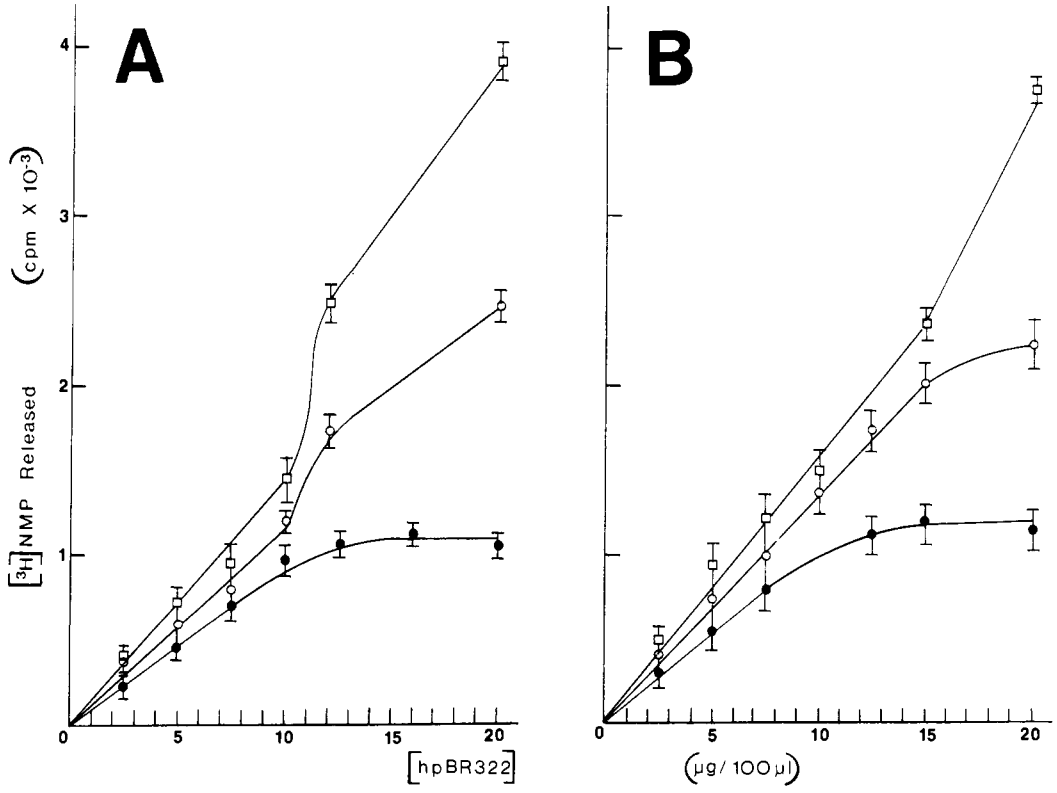

FIGURE 5: Activity of the 5′→3′ exonuclease in the presence of captan. (Panel A) Varying concentrations of [^{3}H]hpBR322 were preincubated on ice for 5 min with 100 μM (○) or 1 mM (■) captan or with 1% DMSO final solution (●). DNA pol I was subsequently added and the reaction continued at 37 °C for 10 min. (Panel B) DNA pol I was preincubated with 1 mM (□) or 100 μM (○) captan or with 1% DMSO (●). [^{3}H]hpBR322 was then added and the reaction mixture incubated at 37 °C for 10 min.

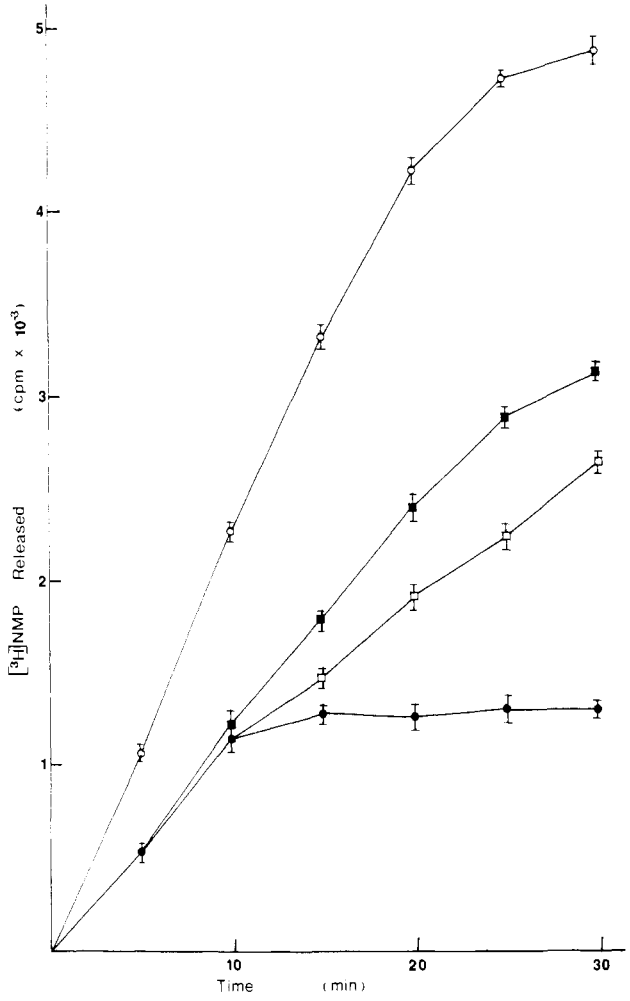

FIGURE 6: Effects of captan on the 5′→3′ exonuclease activity in progress. Captan (100 μM) was added to the DNA pol I reaction mixture at 0 (○), 5 (■), or 10 min (□) after initiation of the 5′→3′ exonuclease reaction. The untreated sample (●) contained 1% DMSO added at 0 min.

reaction. However, the extent of the 5′→3′ exonuclease reaction of captan-treated enzyme was greater.

In order to determine whether deoxynucleotide had any effect on 5′→3′ exonuclease activity, enzyme was preincubated with dGTP before degradation was initiated. A doubling of 5′→3′ exonuclease activity was observed when DNA pol I was preincubated with 100 μM captan or 150 μM dGTP. If DNA pol I was preincubated sequentially with captan and dGTP, there was no additional change in the activity. The observed doubling of 5′→3′ exonuclease activity was independent of the order of preincubation with dGTP and captan (Table IV). If DNA pol I was preincubated with hpBR322 alone before addition of 100 μM captan, enhancement was not observed (Table III), but simultaneous preincubation of enzyme with both DNA and dGTP before addition of captan resulted in a doubling of 5′→3′ exonuclease activity (Table IV).

Table IV: Effects of dGTP and Captan Preincubation on 5′→3′ Exonuclease Activity[a]

first addition	second addition	activity (1%)
none	dGTP	188 ± 8
captan	dGTP	198 ± 8
dGTP	captan	187 ± 7
dGTP + hpBR322	captan	201 ± 8
hpBR322	DMSO	100 ± 8
none	captan	202 ± 12

[a] DNA pol I (0.5 polymerase unit) was preincubated with captan (100 μM), dGTP (15 μM), or hpBR322 (200 μg) for 2 min on ice before incubation with the second addition, either dGTP (15 μM) or captan (100 μM), also on ice for 2 min. All preincubations contained a final concentration of 1% DMSO. All reactions were treated with 100 mM 2-mercaptoethanol before incubation for 10 min at 37 °C.

Discussion

Captan is an inhibitor of polymerase activities from eucaroytic, procaryotic, and viral sources (Dillwith & Lewis, 1980, 1982a,c; Freeman-Wittig & Lewis, 1986; Freeman-Wittig et al., 1986). In this study, DNA polymerase I from *E. coli* was used for more detailed studies of the captan–enzyme interaction and the concomitant effect on enzyme activity.

Both filter binding studies and sucrose gradient analyses showed that irreverisible binding of captan to DNA pol I occurred in a ratio of 1:1. Competition studies showed that the reaction with the [^{14}C]captan moiety was blocked by DNA (only a single polynucleotide binding site is known to exist on DNA pol I) and that captan binding was unaffected by dGTP. The binding of captan to DNA pol I was directly in proportion to the inhibition of polymerase and 3′→5′ exonuclease activities previously reported (Freeman-Wittig & Lewis, 1986). Since this and previous studies showed that DNA could serve as a protection against the interaction of captan, it was concluded that the interaction of captan in the polynucleotide binding site is responsible for the the irreversible inhibition of both 3′→5′ exonuclease and polymerase activities of DNA pol I.

This is the first report of a compound which reacts with a specific site in the template-primer binding locus and thereby causes an alteration in the physically distinct 5′→3′ exonuclease activity. Although other studies have used site-specific modification to elucidate the catalytic relationships between the polymerase and 3′→5′ exonuclease activity, the 5′→3′ exonuclease activity was not examined (Que et al., 1979; Stern et al., 1980; Harza et al., 1984).

When captan was tested for its ability to prevent substrate binding, it was shown that [α-^{32}P]dGTP binding was completely unaffected by captan. It was also shown that, despite verification that the [^{14}C]captan adduct had bound to DNA pol I, polynucleotide binding could still occur. Captan was shown by substrate protection studies to be bound in a locus physically related to the polynucleotide binding site of DNA pol I. Since there is only one polynucleotide binding site (Kornberg, 1980), captan modification does not inhibit by preventing polynucleotide binding per se. One explanation is that there are at least two compartments in the polynucleotide binding site. One compartment is known to serve the polymerase and 3′→5′ exonuclease activities and is blocked by bound captan. Captan does not block DNA binding to the second compartment, and the function of this second locus is presently unknown. Mizrahi et al. (1986) have shown that DNA sequences upstream from the 3′ terminus are important for the binding of DNA to the polymerase site. Perhaps it is this area of DNA which binds to DNA pol I in the presence of captan.

Since it was established that captan bound to DNA pol I in a 1:1 ratio even at high [captan]:[enzyme] ratios and that the binding could be monitored via the ^{14}C radiolabel, meaningful kinetic analysis can be made. A second-order rate constant of 68 ± 0.7 M^{-1} s^{-1} was determined; the reaction appeared to be first order with respect to both protein and captan. It appears that the reaction rate is quite slow. The second-order rate constant for the DNA pol I–captan interaction is similar to the second-order rate constant reported for the reaction between captan and hydroxide ion [(5.7 ± 0.4) × 10^2 M^{-1} s^{-1} at 28 °C, estimated to be 30 M^{-1} s^{-1} at 0 °C; Wolfe et al., 1976].

It is reasonable to assume a mechanism of action which

involves a noncovalently bound captan–enzyme intermediate which precedes the formation of the irreversibly bound captan, but it cannot be proven with the present data. Due to solubility, captan concentration was limited to 1 mM, and, therefore, the enzyme cannot be saturated if K_D is large (>1 mM). Values obtained were reasonable for a mechanism which includes an enzyme–captan intermediate, in which the modifying reagent has a low affinity for the enzyme. The estimated modification rate ($k_3 = 0.1\ s^{-1}$) is faster than the pseudo-first-order rate of the reaction of captan with water ($1.8 \times 10^{-5}\ s^{-1}$; Wolf et al., 1976) but considerably less than one would have predicted if a group as reactive as hydroxide were in close proximity to the bound captan.

In this study, we have identified a different aspect of the polynucleotide binding site. Although DNA binding can block modification by captan, the modification does not prevent the binding of polynucleotide to DNA pol I. Therefore, we propose that the polynucleotide binding site may have two compartments, one of which serves the polymerase and 3′→5′ exonuclease activities and can be blocked by captan. The other compartment can still bind polynucleotide even after DNA pol I has been treated with captan.

Registry No. DNA polymerase, 9012-90-2; 5′→3′ exonuclease, 79121-99-6; 3′→5′ exonuclease, 79393-91-2; captan, 133-06-2.

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Determination of RNA–Protein Contacts Using Thiophosphate Substitutions†

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ABSTRACT: The binding of the bacteriophage R17 coat protein to its RNA binding site is an example of a specific RNA–protein interaction. Extensive analysis has revealed that the binding is dependent upon a unique hairpin structure that contains four essential single-stranded nucleotides. Additional specificity is thought to be due to four or five ionic contacts between the protein and phosphates on the RNA. Transcription of synthetic DNA with T7 RNA polymerase, using one of the nucleoside 5′-*O*-(1-thiotriphosphates) [NTP(αS)s], allows the synthesis of RNAs specifically substituted with thiophosphates. Eleven sequence variants of the R17 coat protein binding site were synthesized with different NTP(αS)s and tested for coat protein binding to deduce positions of thiophosphates that alter the binding affinity. Of the twenty-one phosphate positions in the molecule, two were found to decrease the K_a 3-fold when substituted with a thiophosphate, one position decreased the K_a 10-fold, and one position increased the K_a 10-fold. Substitution of any of the other 17 positions with thiophosphates does not alter the K_a. The four positions that alter the K_a are located in a uniquely structured region of the RNA, and it is postulated that these thiophosphates affect binding because they contact coat protein directly.

One of the best characterized RNA–protein interactions is the binding of the bacteriophage R17 coat protein to the translational initiation region of the replicase gene, resulting in the repression of translation (Barnardi & Spahr, 1972). This interaction has been extensively characterized by using synthetic bacteriophage RNA fragments that contain the −17 to +4 positions of the replicase gene (Figure 1). The changes in the affinity of the coat protein with more than 100 synthetic sequence variants have allowed the sequence and structural requirements of binding to be determined (Carey et al., 1983a; Romaniuk et al., 1987; Wu & Uhlenbeck, 1987). The coat

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