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Differential Effects of Captan on DNA Polymerase and Ribonuclease H Activities of Avian Myeloblastosis Virus Reverse Transcriptase†

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ABSTRACT: Captan was used as an inhibitor of avian myeloblastosis virus reverse transcriptase to study the polymerase and RNase H catalytic activities. With purified enzyme, RNase H activity was 10-fold more sensitive to captan than was either the DNA-dependent or RNA-dependent DNA polymerase activity. Inhibition of the RNA-dependent polymerase activity could be prevented by dTTP. Conversely, inhibition of this polymerase activity was enhanced by template/primer. The calculated K_{dTTP} of the uninhibited reaction was 5.6 μM. Kinetic studies allow for the proposition of a model for the interaction of captan with the polymerase active center. RNase H activity showed a sigmoidal relationship between activity and substrate concentration. Nuclease activity decreased in V_{max} with no change in the Hill coefficient in the presence of captan. Addition of dithiothreitol to the incubation cocktail prevented inhibition by captan of both RNA-dependent polymerase and RNase H activities, suggesting that the (trichloromethyl)thio moiety of captan is involved in the inhibitory action. Captan inhibition suggests the presence of essential amino residues in both polymerase and RNase H active centers.

Reverse transcriptases are unique enzymes. They are capable of polymerizing DNA from an RNA template (Baltimore, 1970; Temin & Mizutani, 1970) or from a DNA template (Verma, 1977). Many also have a ribonuclease H activity that is used to processively degrade, in either a 5′ → 3′ or a 3′ → 5′ direction, the RNA strand of an RNA–DNA hybrid (Perbal, 1984; Molling et al., 1971). AMV[1] reverse transcriptase is composed of two structurally related polypeptides, an α subunit of approximately 65 kDa and a β subunit of 95 kDa assembled into an $\alpha\beta$ holoenzyme (Grandgenett et al., 1973). This dimer is thought to be generated by proteolytic cleavage of a minor, less active $\beta\beta$ precursor (Gibson & Verma, 1974). It has been reported that the polymerizing activity is associated with the β subunit whereas both polymerase and ribonuclease H activities are observed in connection with the α subunit (Grandgenett et al., 1973). Little is known concerning the relationship of the active sites to each other or to the enzyme as a whole, although both polymerase and ribonuclease H activities arise from a single gene product (Verma et al., 1974). Whether RNase H and polymerase activities reside in different areas of the enzyme or have overlapping active centers has been a subject of debate (Jacob, 1983). Another area of active research is the identification of amino acid residues that are important to catalysis.

Captan is a known inhibitor of RNA and DNA polymerases (Dillwith & Lewis, 1980; Dillwith & Lewis, 1982a; Dillwith & Lewis, 1982b). Captan has also proved to be an inhibitor of AMV reverse transcriptase. In this study, we present evidence that captan preferentially inhibited RNase H as compared to polymerase function, indicating that DNA polymerase and RNase H activities have independent active sites. In this way captan is acting unlike other reported chemical modifiers of this enzyme (Gorecki & Panet, 1978; Brewer & Wells, 1974; Srivastava et al., 1981). Inhibition data also support the hypothesis that lysine is involved both in the dTTP binding

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[1] Abbreviations: EDTA, (ethylenedinitrilo)tetraacetic acid; DTT, dithiothreitol; BSA, bovine serum albumin; NEM, *N*-ethylmaleimide; captan, *N*-[(trichloromethyl)thio]-4-cyclohexene-1,2-dicarboximide; AMV, avian myeloblastosis virus; TCA, trichloroacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Me_2SO, dimethyl sulfoxide.

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site of the polymerase function and in the RNase H active center.

Experimental Procedures

Materials

AMV reverse transcriptase was purchased from Bethesda Research Laboratories, Inc., Gaithersburg, MD. *Escherichia coli* RNA polymerase was from Worthington Enzymes, Freehold, NJ. Poly(A) was purchased from Miles Laboratories, Inc., Elkhart, IN. Oligo(dT)$_{12-18}$, RNA (*Torula* yeast, type VI) and poly(dA) were obtained from Sigma Chemical Co., St. Louis, MO. Poly(dT) was from Collaborative Research, Inc., Waltham, MA. ICN Corp., Irvine, CA supplied [^{3}H]dTTP (12–52 Ci/mM) and [^{3}H]ATP (30 Ci/mM). GF/A and GF/B glass fiber filters were purchased from Whatman Ltd., England, U.K. Captan was a generous gift of Chevron Corp., Richmond, CA.

Methods

Preparation of Poly(A)–Oligo(dT)$_{12-18}$ and Poly(dA)–Oligo(dT)$_{12-18}$. Poly(A), oligo(dT)$_{12-18}$, and poly(dA) were diluted to 1 μg/μL solutions in 10 mM Tris/HCl (pH 7.2) containing 0.15 M NaCl. Poly(A)–oligo(dT)$_{12-18}$ or poly(dA)–oligo(dT)$_{12-18}$ was prepared by mixing equimolar concentrations of each corresponding component. The solutions were heated to 70 °C for 5 min and allowed to slowly cool for 8 h at room temperature.

[^{3}H]Poly(rA-dT) Synthesis. A modified version of the RNA polymerase activity protocol outlined by Burgess (1969) was used to synthesize [^{3}H]poly(rA)–poly(dT). A total of 0.80 mM (330 μCi) of [^{3}H]ATP and 0.15 mg of poly(dT) was combined in the presence of 50 units of RNA polymerase. The mixture was allowed to react for 1 h at 37 °C in a buffer containing 0.04 M Tris-HCl (pH 7.9), 0.01 M $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT, 0.15 M KCl, and 0.5 mg/mL BSA (1-mL reaction volume). The resulting polynucleotide was phenol-extracted, ethanol-precipitated, and dried under vacuum. The specific activity was determined to be 4.26×10^5 cpm/μg of [^{3}H]poly(rA)–poly(dT).

Assay of Reverse Transcriptase Polymerase Activity. Unless otherwise indicated, polymerization assays were performed at 37 °C in a total volume of 50 μL containing 6 units of enzyme, 0.5 μg of either poly(A)–oligo(dT)$_{12-18}$ or poly(dA)–oligo(dT)$_{12-18}$, 50 mM Tris-HCl (pH 8.0), 40 mM KCl, 10 mM $MgCl_2$, 200 μM dTTP, and 2 μCi of [^{3}H]dTTP. These represent saturating substrate and template/primer concentrations. Captan solutions for all enzyme assays were prepared with Me_2SO as solvent, and both captan-treated and control reactions contained a final concentration of 1% Me_2SO. In all studies, captan was added immediately prior to addition of enzyme. Enzyme was added last, to start the reaction. Reactions were stopped by addition of 1 mL of 20% TCA after 20 min of incubation and placed on ice for 30 min. Acid precipitates were collected by vacuum on GF/A glass fiber filters, washed sequentially with 10% TCA, 5% TCA, and 95% ethanol, and then dried. The acid-insoluble radioactivity was quantified by standard liquid scintillation techniques.

Assay of AMV Reverse Transcriptase Ribonuclease H Activity. A modification of the method of Srivastava et al. (1981) was employed to assay for AMV reverse transcriptase RNase H activity. Unless otherwise indicated, an incubation cocktail of 80 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 100 mM KCl, and 0.1 μg of [^{3}H]poly(rA)–poly(dT)/unit of enzyme was allowed to react at 37 °C for 15 min. These represent saturating substrate conditions. Aliquots of 100 μL were taken at times 0 and 30 min and delivered into 1 mL of cold 20% TCA. After overnight precipitation at 4 °C, samples were filtered onto GF/B glass fiber filters and washed with 20 mL of cold 10% TCA followed by 40 mL of cold 5% TCA and 10 mL of cold 95% ethanol. The filters were dried thoroughly, and radioactivity of the insoluble material was determined by liquid scintillation techniques.

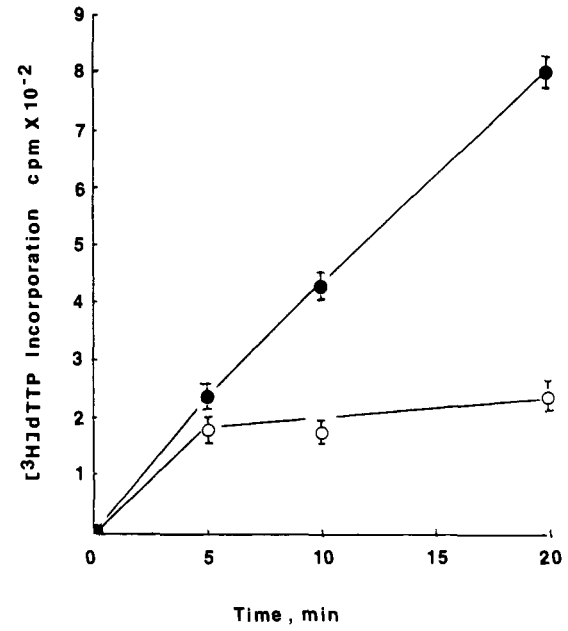


FIGURE 1: Effect of captan on polymerase activity with time. Polymerase activity was assayed in the presence (○) or absence (●) of 1 mM captan with poly(rA)–oligo(dT) as template/primer, as described under Methods. Each point represents three replicates, with standard deviation as indicated by error bars.

Results

Although captan is an inhibitor of eucaryotic and procaryotic polymerases, no retroviral enzymes have been previously tested for sensitivity to this compound. Therefore, the effects of captan on both polymerase and RNase H functions of AMV reverse transcriptase were examined. Captan-treated reverse transcriptase showed little change in polymerizing activity for 5 min after treatment (Figure 1). However, after 10 min of reaction the rate of polymerization was inhibited 90% with poly(rA)–oligo(dT)$_{12-18}$ as template/primer. Similar results were seen with poly(dA)–oligo(dT)$_{12-18}$ (data not shown). Since the polymerizing activities reacted comparably on both the RNA and DNA templates and since both types of polymerization are thought to be functions of the same catalytic site (Smoler et al., 1971), all subsequent polymerization studies were performed with poly(rA)–oligo(dT)$_{12-18}$.

Ribonuclease H activity was also inhibited by 1 mM captan (Figure 2) when [^{3}H]poly(rA)–poly(dT) was used as substrate. Unlike polymerase activity, RNase H activity exhibited no lag time between addition of the inhibitor and decreased activity. This implies different mechanisms of inhibition for RNase H and polymerase activities.

The extent of inhibition of polymerizing and nuclease activities was approximately the same at 1 mM captan concentration. However, when polymerase and RNase H were compared as a function of captan concentration, the sensitivities of the two activities to captan were distinctly different (Figure 3). For example, polymerization was inhibited 50% at 500 μM captan, whereas RNase H activity showed 50% inhibition at a captan concentration of 50 μM. This represents a 10-fold greater sensitivity of RNase H to captan under these assay conditions. Furthermore, at certain concentrations of

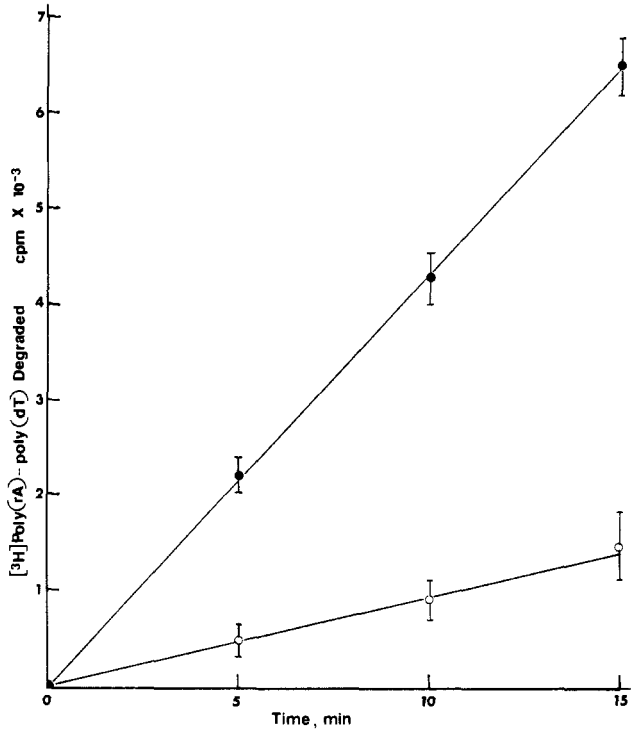


FIGURE 2: Effect of captan on RNase H activity with time. RNase H activity was assayed with (○) or without (●) 1 mM captan with [^{3}H]poly(rA)–poly(dT) as described under Methods. Each point represents the mean of two separate assays, each executed with duplicate samples. Standard deviations are indicated by error bars.

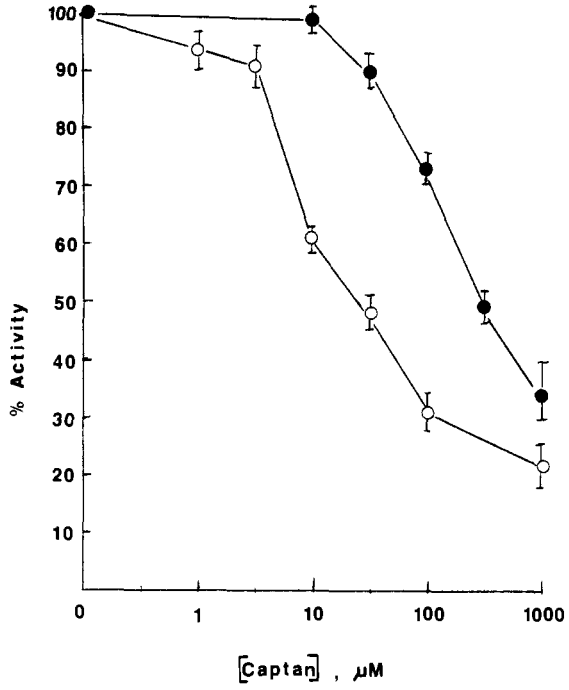


FIGURE 3: Effect of captan concentration on polymerase and exonuclease activities. The RNase H activity (○) and the RNA-dependent DNA polymerizing activity (●) were assayed independently, in the presence of varying concentrations of captan, as described under Methods. In each case, captan was added immediately before enzyme was added to start the reaction. Each point represents the mean of two samples in a representative assay. Error bars indicate standard deviation.

captan it was possible to inhibit the RNase H activity with little change in polymerizing activity.

Both activities of AMV reverse transcriptase are extremely temperature sensitive. Although preincubation studies in-

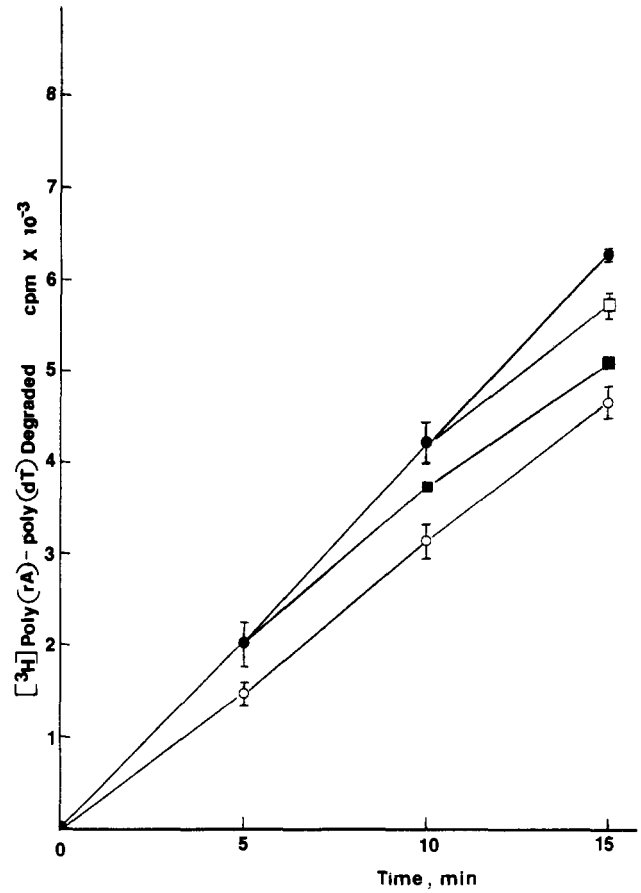


FIGURE 4: Inhibition of RNase H by captan. Captan (10 μM final concentration) was added at 0 (○), 5 (■), and 10 min (□) after initiation of the reaction. The control reaction (●) contained no captan. This reaction progress curve for the RNase H activity is represented by the mean of duplicate samples for each point. Error bars indicate standard deviation.

volving this enzyme with various inhibitors have been accomplished by others (Gorecki & Panet, 1978), the necessity of removing all compounds from the reaction mixture that might interact with captan and that also stabilize the enzyme (i.e., both sulfhydryl reagents such as DTT and mercaptoethanol and proteins such as BSA) led to rapid inactivation of the AMV reverse transcriptase at room temperature. Thus, we tested both polymerase and RNase H activities for sensitivity to captan by adding captan at various times while the reactions were in progress. This was an attempt to determine whether some component of the incubation cocktails could protect polymerase activity and/or RNase H activity from inhibition by captan. Captan (1 mM), data not shown, added at various time points during the course of the polymerization reaction exhibited the same response as was observed when it was added prior to the initiation of the reaction (see Figure 1). The ribonuclease H activity was also inhibited when captan was added during the course of the reaction (Figure 4). Thus, the inhibition of either RNase H or polymerization by captan was independent of the time of addition of the inhibitor.

The (trichloromethyl)thio moiety [–SC(Cl)$_3$] of captan has been previously implicated as the active part of the molecule (Dillwith & Lewis, 1980); therefore, the influence of DTT on captan inhibition was tested. If addition of DTT could protect either of the enzymatic activities from inhibition by captan, it would suggest that the (trichloromethyl)thio group was the moiety interacting with AMV reverse transcriptase. Figure 5 shows that inhibition of polymerization by captan (1 mM) could be prevented by the addition of 10 mM DTT to the

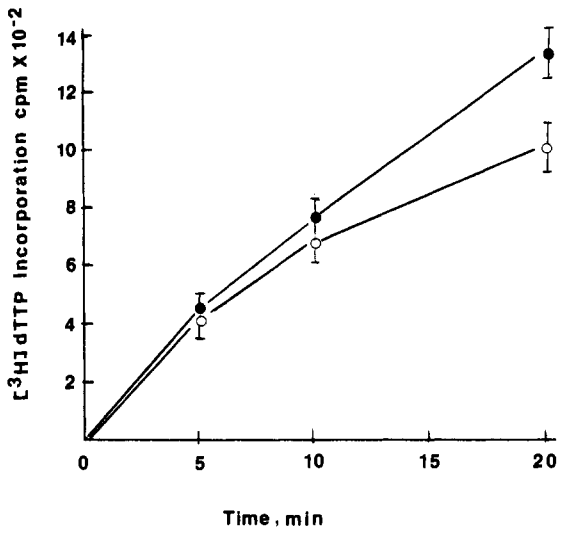


FIGURE 5: Protection of polymerase activity from inhibition by captan with DTT: 10 mM DTT (●) or 10 mM DTT and 1 mM captan (○) were added at 0 min. Captan was added immediately after the addition of DTT and immediately before addition of enzyme. Reactions were started as described under Methods. Each point represents the mean of three samples. Error bars represent standard deviation.

Table I: Effects of DTT on Captan Inhibition of RNase H Activity

addition	time of addition (min)	rate (counts released/min)	activity (%)
none	0	24.5 ± 3	100
DTT[a]	0	29.9 ± 2	122
captan[b]	0	11.9 ± 4	49
captan and DTT	0	29.1 ± 3	119
captan	0	11.8 ± 3[c]	48
followed by DTT	10	22.5 ± 2[d]	92

[a]DTT = 10 mM in all cases. [b]Captan = 100 μM in all cases. [c]Rate before addition of DTT (over 0–10-min time period). [d]Rate after addition of DTT (over 10–30-min time period).

incubation cocktail prior to the addition of enzyme (compare to Figure 1). Protection by DTT from inhibition of polymerase activity was essentially complete for 10 min, after which only a small degree of inhibition was observed (10%) after 20 min. The inhibition of RNase H activity by captan was also blocked by the addition of 10 mM DTT (Table I). Prevention of captan-mediated inhibition by addition of DTT implies that the $-SC(Cl)_3$ of captan is interacting with the thiol groups on DTT and not with the reverse transcriptase. Since DTT prevented inhibition of both polymerase and RNase H activities by captan, it is further implied that the $-SC(Cl)_3$ moiety of captan rather than the cyclohexenedicarboximide moiety is the interactive part of the captan molecule at both active sites.

Furthermore, addition of 10 mM DTT after the reaction had proceeded for 10 min in the presence of 100 μM captan relieved inhibition of RNase H activity (Table I). This reversal of inhibition can be explained if DTT is doing two things: First, it must inactivate the captan that is free in solution. This was shown in Table I to occur. Second, DTT must disrupt any existing captan–enzyme bond. This is necessary only if captan is an irreversible inhibitor.

Captan was originally shown to be an irreversible inhibitor of DNA polymerase I and *E. coli* RNA polymerase by Dillwith and Lewis (1982a,b). In order to determine if captan binds irreversibly to this enzyme, AMV reverse transcriptase treated with 50 μM captan was sedimented in a glycerol gradient to separate free captan from enzyme. Fractions from

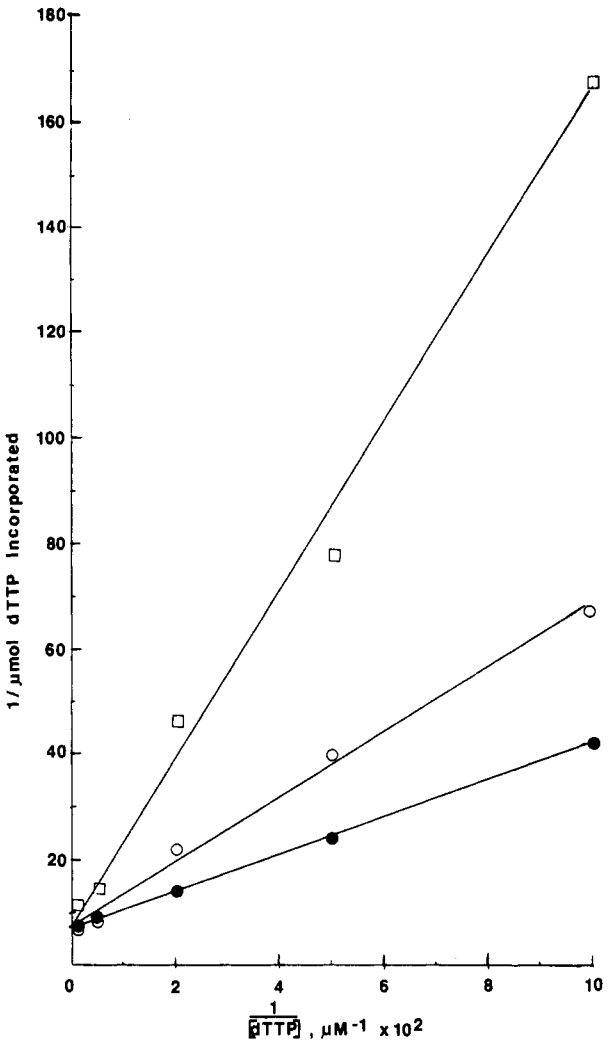


FIGURE 6: Lineweaver–Burk analysis of polymerase activity with varying dTTP in the presence of captan. Rates were determined by the total radioactivity, incorporated over a 20-min reaction period. Polymerization was assayed in the presence of varying amounts of dTTP and 0.5 μg of template/primer and 0 (●), 50 (○), or 500 μM (□) captan.

the gradient were assayed for both polymerase and RNase H activities, and captan inhibited both activities, 20% and 80%, respectively. If captan interaction was freely reversible, no inhibition should have been observed with the gradient-fractionated enzyme. Therefore, captan is an irreversible inhibitor of both RNase H and polymerase activities.

Double-reciprocal plots of the effect of captan on varying concentrations of dTTP show that this substrate protects completely against captan inhibition of polymerase activity (Figure 6). The K_{dTTP} for polymerization was calculated to be 5.6 μM under standard reaction conditions. A Lineweaver–Burk plot of polymerase activity where template/primer was varied at fixed captan concentration shows parallel lines (Figure 7). Therefore, the presence of template/primer enhances inhibition of polymerase activity by captan.

Sigmoidal curves were obtained when activity vs. substrate concentration data were plotted for the RNase H activity (Figure 8). Hofstee analysis of these data (Dixon & Webb, 1979) showed positive cooperativity and yielded a change in V_{max} from 0.45 pg of AMP released/min in the uninhibited reaction to 0.09 pg of AMP released/min in the captan-treated samples (data not shown). A subsequent Hill plot (Purich, 1983) showed that although captan altered the V_{max} of the RNase H activity, the Hill coefficient (1.3 ± 0.2) remained

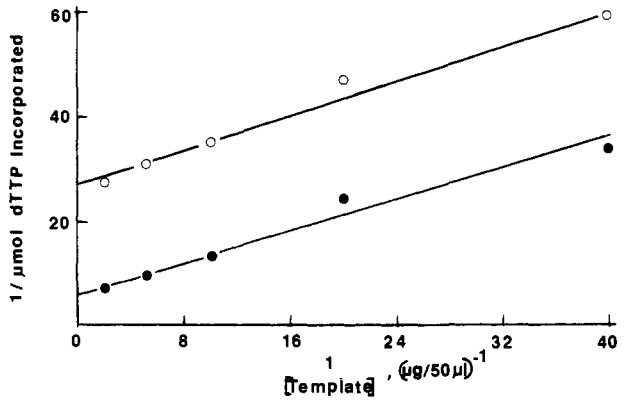


FIGURE 7: Lineweaver–Burk analysis of polymerase activity with varying poly(rA)–oligo(dT) in the presence of captan. Rates were determined by the total radioactivity incorporated over a 20-min reaction period. Polymerase activity was assayed in the presence of 200 μM dTTP, with (○) or without (●) 500 μM captan.

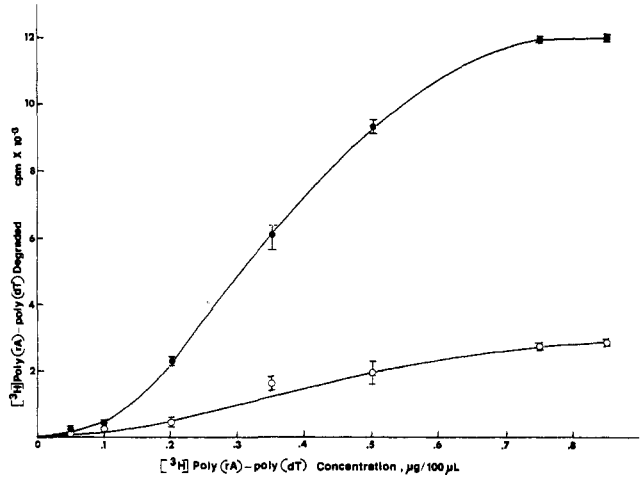


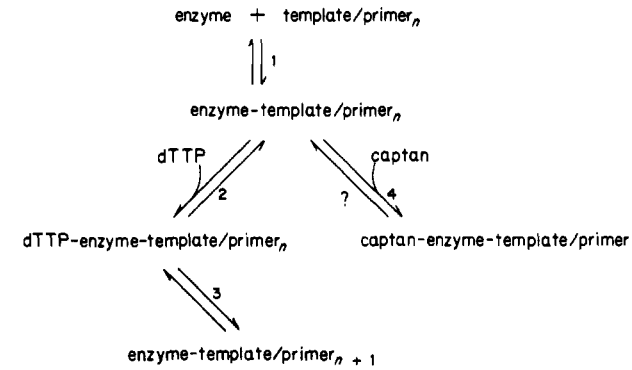
FIGURE 8: Effect of captan on RNase H activity. RNase H was assayed in the presence of varying amounts of [^{3}H]poly(rA)–poly(dT), with (○) or without (●) 1 mM captan. Each point represents the mean of duplicate samples from a representative assay.

constant; therefore, the degree of cooperativity was not altered by captan.

DISCUSSION

The structure–function relationship between AMV reverse transcriptase polymerase and RNase H activities has been explored with site-specific chemical modifications (Gorecki & Panet, 1978; Gorecki et al., 1978; Modak, 1976; Brewer & Wells, 1974; Wright & Brown, 1984; Smith & Gallo, 1974; Srivastava & Mukund, 1982). In one report, the polymerase active site was shown to be sensitive to hydrophobic sulfhydryl reagents such as 5,5′-dithiobis(2-nitrobenzoic acid) and *p*-(hydroxymercuri)benzoate but was also shown to be resistant to sulfhydryl reagents with hydrophilic properties. This implies a cysteine residue in a hydrophobic environment at the polymerase active site. In the same study, RNase H activity was resistant to sulfhydryl-specific reagents with either hydrophobic or hydrophilic properties but was sensitive to NEM, although less so than was the polymerase activity (Gorecki & Panet, 1978). This type of selective inactivation of DNA polymerase and RNase H implies that these activities occur at separate sites. Further evidence that the sites are separate comes from the observation that separate amino acids are involved with the inhibition by NEM for each activity. Differential inhibition of polymerase and exonuclease activities in the presence of captan also occurs. In this case, RNase H has a 10-fold

Scheme I



greater sensitivity to captan than does the polymerase activity. These data add support to the hypothesis that RNase H and polymerase activities occur at separate sites.

Two amino acid residues have been previously identified as important to the AMV reverse transcriptase polymerase activity. A sulfhydryl is thought to be located in or near the template/primer binding site since incubation of the enzyme in the presence of polynucleotides, such as poly(A)–oligo(dT) or chicken liver tRNA, resulted in partial protection of polymerase activity against NEM (Gorecki & Panet, 1978). Inhibition of polymerization by pyridoxal phosphate has also been reported (Modak, 1976). This latter reagent functions through a Schiff base formation between the aldehyde group of the pyridoxal phosphate and an ϵ-amino group of a lysine in the active site. The lysine is probably located in the triphosphate binding site of the enzyme because addition of dTTP was shown to block polymerase inhibition by pyridoxal phosphate.

Captan is known to react with both amino and sulfhydryl groups in proteins (Siegel, 1970; Lukens & Sisler, 1958; Dunbar & Rogers, 1966; Siegel & Sisler, 1968). Both of these residues have been identified as participants in the catalytic functions of reverse transcriptase, with a lysine amino group associated with the substrate binding site of the polymerase activity and a cystine sulfhydryl associated with the template/primer binding site of the polymerase activity. Since dTTP acts to protect against captan inhibition, we hypothesize that captan interacts with the ϵ-amino group of lysine in the substrate binding site. The cysteine in the template/primer binding site does not seem to be directly involved in the interaction of captan with either the polymerase or RNase H activities because binding of template/primer does not protect from inhibition.

Analysis of the double-reciprocal plots of polymerization data (Figure 6) suggests that binding of the template/primer occurs in the polymerase catalytic site prior to the binding of captan. Thus, we propose the model shown in Scheme I for the interaction of captan with the polymerase site of AMV reverse transcriptase, where (1) is formation of the binary complex, (2) is formation of the tertiary complex, (3) is normal polymerization, and (4) is inhibition by captan.

RNase H has a greater sensitivity to captan than does the polymerase activity. This preferential inhibition of RNase H activity by captan is of interest since to date only low pH (Gorecki & Panet, 1978) or salts such as NaF and KCl (Brewer & Wells, 1974) have been shown to preferentially inhibit RNase H activity. Since RNase H activity is insensitive to sulfhydryl-specific reagents, this suggests there is no sensitive cysteine in this active center. However, as shown in this study RNase H activity is sensitive to captan and, as previously shown, to NEM (Gorecki & Panet, 1978), both of which also

interact with primary amines; this implies that a captan-sensitive amino group is in the RNase H active center.

In this study, we presented evidence that AMV reverse transcriptase has separate catalytic sites for its polymerase and nuclease activities and that lysine residues are implied to have a major role in captan inhibition of these two active centers. It has recently been determined that RNase H and DNA polymerase activities arise from a single gene product and that the domains of primary sequence that constitute the two activities are separate (Gradgenett et al., 1985). The RNase H activity is located near the NH_2 terminus of the β subunit, and the polymerase-containing segment is in the center of the polypeptide chain. Even though primary sequence data suggest that the two activities are separate, a three-dimensional structure for the enzyme has yet to be determined so the possibility of overlapping sites cannot be ruled out.

Regardless of the physical structure of the enzyme, since captan preferentially inhibits the RNase H activity over polymerase activity, it is a potentially useful tool for synthesizing cDNA without concomitant degradation of the RNA template. The differential inhibition of RNase H and polymerase activites may turn out to be useful in in vitro studies as an aid to better understand the relationship between the activities of AMV reverse transcriptase and RNA tumor virus expression, as well as to more effectively use AMV reverse transcriptase in molecular biological techniques.

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Registry No. DTT, 3483-12-3; dTTP, 365-08-2; RNase H, 9050-76-4; captan, 133-06-2; reverse transcriptase, 9068-38-6; L-lysine, 56-87-1.

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