CONGO RED INHIBITION OF INITIATION BY RAT LIVER DNA-DEPENDENT RNA POLYMERASE

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SUMMARY

DNA-dependent RNA polymerases A and B were isolated from rat liver. The effect of Congo Red was measured on the capacity of these enzymes to incorporate UMP-^{3}H in reconstituted systems using heterologous DNA as template. It was found that there was a differential sensitivity of the two enzymes to the Congo Red; A was inhibited to 50% by 16 micromolar dye whereas B required only 2 micromolar. This effect was independent of added protein (BSA) and identical conditions for maximal incorporation were found for the inhibited and uninhibited enzymes. Time course studies in which the order and the time of addition of the DNA and dye were varied suggested that the loss of activity was related to the preferential binding of the dye to the template reading site. Experiments with gamma labeled ATP revealed that both enzymes did indeed initiate during the course of the incubation, and that Congo Red promptly prevented further initiation upon addition to B. The effect on enzyme A was different in that parallel but slightly reduced initiation curves were found.

INTRODUCTION

Congo Red has been employed as a microbiological stain and as an indicator. More recently Krakow demonstrated that this sulfonated arylazo compound is bound to bacterial DNA-dependent RNA polymerase, preventing RNA formation, and that it alters the migration of the polymerase molecule in gel electrophoresis (1). We tested its effects on mammalian liver polymerase and found it also inhibited UMP-^{3}H incorporation, preferentially affecting the nucleoplasmic enzyme. It appears to bind to the DNA template site and prevent initiation. The effect on the nucleolar enzyme was significantly less marked. The following is a report of our findings.

METHODS AND MATERIAL

Rat liver DNA-dependent RNA polymerase was prepared essentially by the method of Roeder and Rutter (2). The enzyme was adsorbed onto DEAE Sephadex, washed with 0.025 M ammonium sulfate in 50 mM tris pH 8.0, 5 mM $MgCl_2$, 0.1 mM EDTA, 5 mM dithiothreitol, 25% (v/v) glycerol (TGMED), and eluted with 0.025 -

0.5 M $(NH_4)_2SO_4$ TGMED. The fractions containing peaks of enzyme activity were pooled separately, and either frozen immediately in liquid nitrogen or dialyzed against 0.025 M ammonium sulfate in TGMED, and stored at -70° C. Congo Red was obtained from Allied Chemical Corp. and purified by repeated recrystallizations. ATP, GTP, CTP, and UTP were purchased from P-L Biochemicals Inc. Rat liver DNA was prepared from isolated nuclei (3) essentially by the method of Marmur (4). Calf thymus DNA and salmon sperm DNA were purchased from Worthington Biochem. Corp., Freehold, N. J. Solutions of the DNA were prepared in 0.01 M tris pH 8.0, and maintained in ice until used. Denaturization was carried out in a boiling water bath for 10 minutes followed by rapid cooling in ice. An increase in absorption at 260 mμ was of the order of 30%. UTP-^{3}H was purchased from Amersham-Searle, at the specific activity 1 C/mM. Gamma labeled ATP-^{32}P was purchased from New England Nuclear at a specific activity of 18.6 C/mM. Incorporation of radioactively labeled nucleotides was carried out in total volumes of 0.08, 0.25, and 0.40 ml, as indicated in the accompanying Tables and Figures. The order of addition is indicated in Figures and Legends. Congo Red was prepared as a 4 mM stock solution in water and stored frozen; dilutions were prepared in 0.01 M tris pH 8.0.

Incubations were carried out at 37° C for up to 2 hours. At appropriate times, reactions were terminated and radioactivity assayed by either of two methods. In some incubations 50 μl samples were pipetted onto No. 1, 2.4 cm filter paper discs. These were dropped immediately into 10% trichloroacetic acid containing 1% sodium pyrophosphate, or into 0.4 M perchloric acid containing 1% sodium phosphate. The discs were washed repeatedly in 5% trichloroacetic acid or 0.25 M perchloric acid until no more radioactivity appeared in the bathing fluid (usually 4 times). The discs were subsequently washed in alcohol, in a mixture of alcohol and ether (equal volumes), finally in ether, and dried. Those labeled with tritium were counted in toluene containing 0.1% 2,5-diphenyloxazole and 0.05% 1,4-bis-(4-methyl-5-phenyloxazole)-benzene. The samples

labeled with ^{32}P were counted by measuring Chernakov radiation in water. Alternatively, 50 µl samples were pipetted into an ice-cold aqueous solution containing 500 µg of RNA in 500 µl. To this 0.5 ml of 0.8 M PCA containing 1% sodium phosphate was immediately added. Precipitates were permitted to form for 10 minutes, collected by centrifugation at 1,000 x g, and washed by resuspension and recentrifugation in 0.25 M PCA repeatedly. Finally, precipitates were dissolved in 0.3 M KOH, and radioactivity of ^{32}P was measured by Chernakov radiation in water. The efficiency of ^{32}P counting under these circumstances was 30% and was linear in the range used. Efficiency for tritium on the disc method was 3%.

RESULTS AND DISCUSSION

The addition of Congo Red to incubations reduced nucleolar or nucleoplasmic enzyme dependent UMP-^{3}H incorporation, however, the sensitivity of the two enzymes differed for 50% inhibition. Enzyme A required about 16 µM Congo Red whereas B required only about 2 µM (Table 1). Addition of bovine serum albumin did not alter the degree of inhibition. Optimal conditions for incorporation for control or Congo Red inhibited enzymes were identical. Alteration of ionic strength by changing the ammonium sulfate concentration did not alter Congo Red effect. Utilization of native or denatured DNA did not change the qualitative effect. The order of addition of DNA and Congo Red was important. Pre-incubation of the enzyme with Congo Red prevented incorporation of UMP upon addition of DNA to the B enzyme (Fig. 1). Addition of DNA prior to Congo Red was associated with a brief burst of RNA formation for 6 to 10 minutes with a subsequent, nearly complete inhibition of further UMP incorporation. Addition of Congo Red subsequent to the beginning of incubation was associated with a persistence of labeling for 6 to 8 minutes followed by inhibition of further radioactivity increase (Fig. 1). Since this suggested that Congo Red may be bound to the template reading site preventing further initiation, direct assays for initiation were carried out utilizing gamma labeled ^{32}P-ATP. Figures 2 and 3 show that enzymes A and B do initiate during

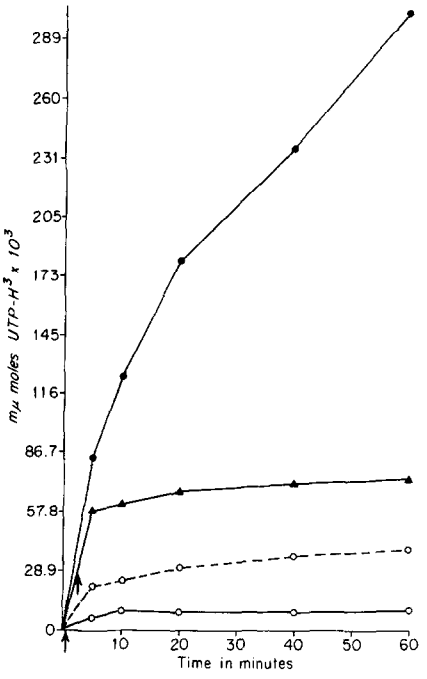

Fig. 1.

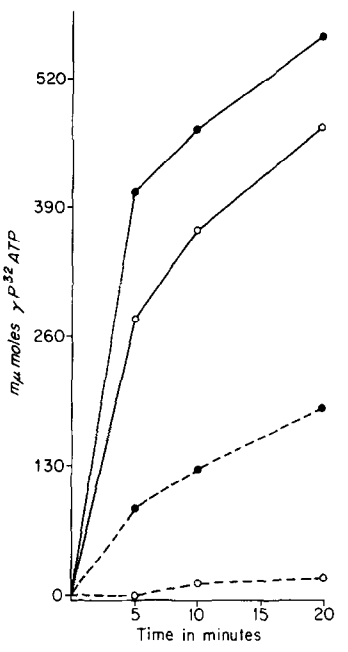

Fig. 2.

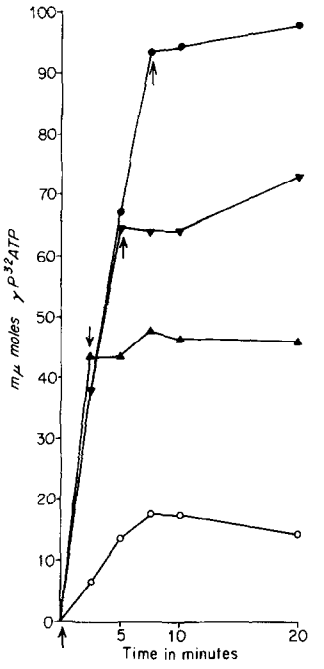

Fig. 3.

Figure 1

Incorporation of ^{3}H-UMP into TCA precipitable RNA by Enzyme B was measured in a total volume of 0.4 mls as indicated in Table 1. To separate incubation flasks, Congo Red was added to a final concentration of 5 μM before DNA (0—●), or after DNA (0----0) and then incubation started. Congo Red was also added following 2.5 minutes of incubation (▲—▲). No Congo Red was added to one set of incubation tubes (●—●). Samples (50 μl) were taken at 0, 5, 10, 15, 20, 40 and 60 minutes and placed on filter paper discs as indicated. Similar experiments were also conducted by adding Congo Red at 5, 7.5, 10, 20 and 30 minutes. These also were associated with a 6-10 minute lag prior to cessation of incorporation. Each experiment was done in duplicate. Arrows indicate time of Congo Red addition.

Figure 2

The capacity of enzymes A and B to initiate RNA chain formation was measured by using gamma labeled ^{32}P-ATP in an incubation systems described in Table 2. Congo Red was added to 5 μM, prior to addition of DNA, total volumes for the initiation experiments were 0.4 ml. Samples (50 μl) were taken at 0, 5, 10 and 20 minutes, added to carrier RNA, precipitated in 0.4 M PCA with 1% sodium phosphate, washed repeatedly in 0.25 M PCA, dissolved in 0.3 M KOH, and diluted with water. ^{32}P was counted by Chernakov radiation. (●—●) represents γ^{32}-ATP incorporation by Polymerase A, (0——0) represents γ^{32}P incorporation in the presence of 5 μM Congo Red. (●---●) represents initiation by Polymerase B without Congo Red, and (0---0) in the presence of 5 μM Congo Red.

Figure 3

The effect of different times of Congo Red addition on chain initiation was measured using gamma labeled ^{32}P-ATP as indicated in Table 2. Congo Red at 5 μM final concentration was added after DNA at 0 time (0—0), 2.5 min (▲—▲), 5 min (▼——▼) and 7.5 min (●—●). Incorporation into RNA was measured as indicated in Table 2. Arrows indicate time of Congo Red addition.

TABLE 1

Inhibition of DNA-Dependent RNA Polymerase by Congo Red[1]

Conc. of Congo Red (μM)	Enzyme A (Radioactivity CPM/mgm Protein)	% of Control	Enzyme B (Radioactivity CPM/mgm Protein)	% of Control
0	111,000	--	1,650,000	--
5	89,100	80%	313,000	18.9%
14	62,900	56%	93,800	5.6%
26	13,500	12%	71,500	4.3%

1) Enzymes were eluted from DEAE Sephadex columns as previously described. The peaks of enzyme activity were pooled. dialyzed briefly against 0.025 M $(NH_4)_2SO_4$ in 50 mM tris pH 8.0, 5 mM $MgCl_2$, 0.1 mM DTT, 5 mM EDTA Na_2 and 25% (v/v) glycerol. Assay of UMP-^{3}H incorporation was carried out in a total volume of 80 μl containing a final concentration of 0.7 mM ATP, GTP, and CTP, 6 μM UTP-^{3}H (specific activity 1 Ci/mMole), 50 mM KCl, 0.25 mM mercaptoethanol, 3.0 mM $MnCl_2$, 0.06 mM EDTA Na, 93 mM Tris-HCl (pH 7.9 at 20° C), 150 μgm/ml denatured salmon sperm DNA, 10% (v/v) glycerol. Congo Red was added after the DNA, zero time samples were removed and incubation was carried out for 30 min. at 37° C. At 0 and 30 minutes, 50 μl samples were pipetted onto Whatman No. 1, 2.4 cm diameter filter paper discs. The discs were immediately placed in 10% TCA containing 1% sodium pyrophosphate, washed 3 times with 5% TCA, alcohol, alcohol and ether, and finally with ether. The dried discs were placed in toluene - PPO, dimethyl POPOP fluid and counted in a liquid scintillation spectrophotometer. Protein content of the enzyme was measured by the Bramhall method (5).

the 20 minute period of incubation, and in fact initiation continues, but with a decreasing rate up to 60 minutes, the longest time examined. Enzyme A appears to initiate more than enzyme B, even though the total RNA formation measured as ^{3}H-UMP incorporation is greater with the B enzyme (Table 2).

Addition of Congo Red to enzyme B stops gamma labeled ATP incorporation immediately (Fig. 3). The persistence of UMP incorporation by this enzyme subsequent to Congo Red addition for 6 to 8 minutes, in view of the immediate cessation of gamma labeled ATP incorporation suggests that the Congo Red is able to compete favorably for the DNA binding site upon completion of the RNA

TABLE 2

Comparison of γ-^{32}P-ATP labeling (Initiation) and UMP-^{3}H Incorporation (Elongation) by Enzymes A and B[1]

Enzyme	γ-^{32}P-ATP Incorporation	UMP-^{3}H Incorporation	Ratio
	(mμMoles/10 minutes/mgm Protein)		^{3}H-UMP/γ-^{32}P-ATP
A	30.8	9,371	304
B	45.9	67,700	1480

1) Enzymes were prepared as indicated in Table 1. Incubations were carried out in a total volume of 400 µl, with the same composition as in Table 1 save that UTP, GTP and CTP were 0.7 mM, γ-^{32}P-ATP (specific activity 18.6 C/mMole) was 7 µM. At zero time and 10 minutes 50 µl samples were removed, added to 500 µl of a 1 mgm/ml rat liver ribosomal RNA solution to which 0.5 ml of 0.8 M PCA with 1% sodium phosphate was added. The precipitates were washed with 0.25 M PCA until no more activity appeared in the supernatant, were then dissolved in 0.3 M KOH, diluted to 10 mls with H_2O and counted by Chernakov radiation. Correction for quenching and conversion to m Moles incorporated was carried out by measuring both ^{32}P and ^{3}H standards under identical conditions. Proteins were measured by the Bramhall technique (5).

chain. This fact and the observation that the Congo Red inhibition cannot be reversed by addition of DNA in marked excess suggest that the Congo Red is bound more firmly than DNA to the enzyme.

Enzyme A responds differently to Congo Red than B. It requires a greater concentration of dye than B for similar inhibition (Table 1), and initiation is reduced to a much lesser degree than B. The preparation of A employed is not as purified as B (6). To test for non-specific protein binding by non-enzyme components, BSA was added, without changing the results. The roughly parallel nature of the initiation curves suggests that a form of A (7) may be sensitive to Congo Red and its participation blocked by Congo Red, leaving a large quantity of resistant material.

These experiments indicate that Congo Red may be a valuable tool in the analysis of the binding of DNA to the mammalian polymerase molecule, and for

studying the mechanisms of initiation of RNA formation. Furthermore, the differential capacity of the A enzyme to apparently incorporate more ATP (representing initiation) whereas forming less RNA (UMP-^{3}H incorporation) compared to the B enzyme suggests either the initiation site for the two molecules may have a different nucleotide composition near the 5' end, or the enzymes recognize differing template loci, or that the chain formation in the two systems may be rather different. The second possibility is supported by the different products formed (8,9,10), but does not exclude the other two possibilities. These areas require further investigative study.

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