Vol. 92, No. 1, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1980 Pages 38-45

DETERGENT INDUCED INHIBITION OF EUKARYOTIC RNA POLYMERASE B ACTIVITY AND AMANITIN BINDING

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Received October 18,1979

Summary:

We studied the effect of detergents on the binding of amanitin to RNA polymerase and on enzymatic activity. SDoS, Sarkosyl and deoxycholate were most inhibitory. Cholate and non-ionic detergents were less inhibitory. Evidence is presented that Sarkosyl inhibits chain elongation. The inhibition of amanitin binding was most influenced by the hydrophilicity of the detergent.

Introduction:

Since detergents are widely used to solubilize RNA-polymerase containing DNA-protein complexes from virus infected cells (1-11), we studied the effect of several commonly used detergent agents on enzyme activity and on the binding of amanitin to the polymerase molecule. Radioactive derivatives of α-amanitin have been used in amatoxin binding assays to detect eukaryotic RNA-polymerase B (12-16); in these experiments it was used as a measure of the structural integrity of the RNA polymerase molecule. In this paper, we show that anionic detergents (sodium dodecyl sulfate and Sarkosyl NL97) inhibit amanitin binding and RNA-polymerase activity significantly. Non-ionic detergents (Triton X-100 and Nonidet P40) and steroid agents (sodium cholate and sodium deoxycholate) inhibited amanitin binding to a lesser degree. Triton X-100 and sodium cholate had the least inhibitory effect on enzyme activity. In addition, we present evidence that Sarkosyl strongly inhibits chain elongation by purified RNA-polymerase; when a crude nuclear preparation was studied, the degree of inhibition was less.

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Materials and Methods:

Amatoxin Binding Assay, separation by glycerol gradient centrifugation

O-methyl-{3H}-demethyl-γ-amanitin ({3H}-amanitin) was synthesized by a previously described method (16, 17). To demonstrate binding of the {3H}-amanitin, a modification of the method of Cochet-Meilhac et al. (14, 15) was used. Aliquots of purified wheat germ enzyme (Miles) (1.63×10^{-6} μmoles) were incubated with 10 μl {3H}-amanitin, (9.13×10^{-6} μmoles ca. 2.0 Ci/mmole) in 500 μl binding buffer, (80 mM Tris HCl pH 7.9, 0.1 mM EDTA, 0.1 mM dithiothreitol, 150 mM $(NH_4)_2SO_4$, 0.2 mg/ml bovine serum albumin, 0.4 mg/ml rabbit gamma globulin, and 30% v/v glycerol). Mixtures were incubated for 18 hours at 4°C. Free and bound {3H}-amanitin were then separated on 5 ml 30-40% glycerol gradients (in binding buffer) which were centrifuged at 50,000 rpm for 16 hours at 5°C in a Spinco SW50.1 rotor. Gradient fractions (approximately 0.25 ml) were counted in an aqueous toluene based scintillation solution.

Preparation of 3T3 nuclei

3T3 cells (ATCC-CCL92) grown in Medium 199 with 10% fetal bovine serum were subcultured at three day intervals. Nuclei were prepared according to the procedure of Wilhelm et al. (11). Following disruption of the cell sheet with ATV (8 gm/l NaCl; 0.4 gm/l KCl; 1 gm/l dextrose; 0.58 gm/l $NaHCO_3$; 0.5 gm/l trypsin (Difco 1/250); 0.2 M disodium EDTA), cells were washed twice in Dulbecco's phosphate buffered saline, then resuspended in TITE buffer (20 mM Tris HCl pH 7.03, 5°C, 140 mM NaCl, 0.05% Triton X-100 and 2 mM EDTA) at a concentration of 3×10^6 cells/ml. After incubation for 10 min. at 0°C, the cells were broken with the tight fitting pestle of a Dounce homogenizer (4-6 strokes). After centrifugation for 10 min. at 1000 x g, the pellet was resuspended in TEAD buffer (20 mM Tris HCl pH 7.9, 37°C, 2 mM EDTA, 50 mM $(NH_4)_2SO_4$, 1 mM dithiothreitol) at a concentration of 1.5×10^7 nuclei/ml.

RNA polymerase assay

RNA polymerase reactions were carried out in 0.25 ml buffer containing 200 mM $(NH_4)_2SO_4$, 80 mM Tris HCl (pH 7.9 at 37°C), 4.4 mM $MnCl_2$, 0.05 mM dithiothreitol, 1 mM ATP, GTP, and CTP and 0.1 mM UTP (final concentration). Each reaction contained 0.25 μCi {3H}UTP (18-20 Ci/mM). Experiments using purified wheat germ RNA polymerase contained 1-4 μg protein per reaction and 3 mg calf thymus DNA. After incubation at 37°C for 10 minutes, reactions were stopped by the addition of 2 ml ice cold solution A (5% w/v trichloroacetic acid, 0.025 M sodium pyrophosphate). Samples were held on ice for 15 min. and, after vortexing, the precipitates were collected on Whatman GF/C filters. Each filter was washed with 50 ml solution A, 50 ml solution B (2% trichloroacetic acid, 0.01 M sodium pyrophosphate) and 10 ml absolute ethanol. Samples were counted in a nonaqueous toluene based scintillation solution. When crude 3T3 nuclear homogenates were used, the final reaction volume was 0.6 ml. Samples containing less than 300 μl of a crude nuclear suspension were adjusted with an appropriate amount of TEAD as were control reactions in which purified RNA polymerase was used.

Results:

The effect of six detergents on the binding of {3H}-amanitin to purified wheat germ RNA-polymerase was studied at a 1% (w/v) detergent concentration. Figure 1 shows the data from such an experiment. In controls, 65% of the

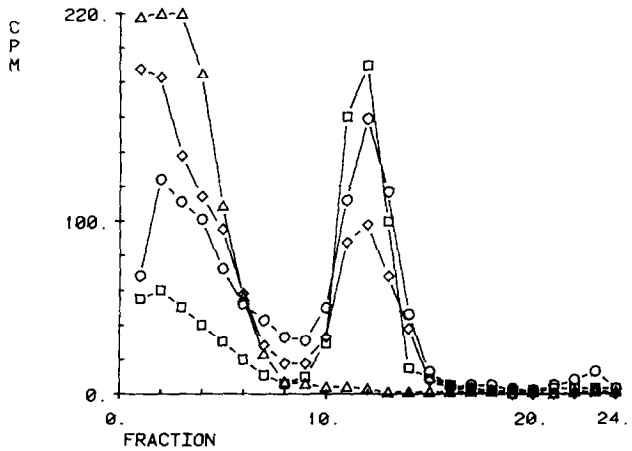

Figure 1. After amanitin binding (18 hours at 4°C) samples were layered on 30 to 40% preformed glycerol gradients in binding buffer and were centrifuged at 50,000 rpm for 16 hours at 5°C in a Spinco SW50.1 rotor. Fractions were collected and were counted in an aqueous scintillation cocktail. Control (□); Sarkosyl/SDoS (Δ); deoxycholate (◇); NP40/Triton X-100/ cholate (○).

radioactivity sedimented to the center of the gradient as an uniform peak. The presence of cholate, NP40 or Triton inhibited binding to a similar degree and the results are plotted as a single curve for clarity. For the same reason, data from Sarkosyl and SDoS containing reactions are plotted together. Table 1 summarizes the actual data obtained from these gradients. In our

Table I.

Effect of Detergents on Amanitin Binding by Wheat Germ RNA Polymerase Glycerol Gradient Analysis

Detergent	Peak I (Unbound)	Peak II (Bound)	% Bound	% Control
None (Control)	272	518	65.6	100
Sarkosyl NL-97	889	29	3.2	4.9
Sodium dodecyl sulfate	1181	10	0.9	1.4
Sodium deoxycholate	822	344	29.5	45.0
Sodium cholate	600	437	42.1	64.2
Nonidet P40	661	584	46.9	71.5
Triton X-100	650	577	46.2	70.4

experiments, since the glycerol gradients did not contain any unlabeled amanitin, some dissociation of the amanitin-enzyme complex undoubtedly occurred in spite of the very low K_d of this interaction (14, 15, 16). Therefore, percent binding was corrected for that observed in detergent-free control gradients (last column). Cholate, NP40 and Triton inhibited binding by 35.8, 28.5, and 29.6% respectively. Deoxycholate resulted in 55% reduction of binding; SDoS and Sarkosyl resulted in a > 95% reduction of binding.

Sarkosyl NL97, Triton X-100, cholate, deoxycholate and NP40 were compared with respect to their ability to inhibit RNA polymerase activity (Figure 2). Sarkosyl and deoxycholate inhibited activity significantly at concentrations as low as 0.005%, with virtually complete inhibition of activity at concentrations of 0.025% to 0.05%. Triton and cholate were the least inhibitory agents, producing a linear decrease in enzyme activity with increasing detergent concentration. NP40 was intermediate in effect at concentrations less than 0.125% (w/v), but almost completely inhibited activity at higher concentrations.

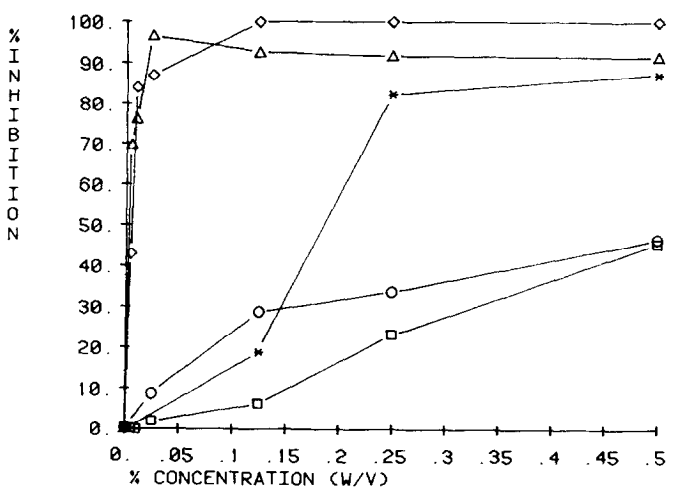

Figure 2. The detergent was added in the indicated concentrations to RNA-polymerase reaction mixture. The percent inhibition of activity as a function of detergent concentration is plotted. Sarkosyl (Δ); Triton X-100 (□); cholate (O); deoxycholate (◇); NP40 (*).

Table II.

Effect of Sarkosyl NL-97 on Wheat Germ RNA-Polymerase Activity

Time of addition	No Sarkosyl	Sarkosyl 0.25% (w/v)
Zero time after 5 min. Incubation	1276	29 (2.3)
Zero time	1766	20 (1.6)
5 min. after start of reaction	1844	876 (48)

Complete RNA polymerase reaction mixtures were incubated for 10 minutes at 37°C. "Preincubated" samples were incubated for 5 minutes at 37°C under conditions which would allow initiation, but not chain elongation (i.e., an incomplete reaction mixture lacking UTP). To these samples, UTP and Sarkosyl were added simultaneously at "zero time". Samples were then incubated for an additional 10 minutes. Values are acid precipitable counts per minute. Blank values (samples without added enzyme) of 70 to 120 counts have been substracted. Numbers in parentheses indicate percent of controls without Sarkosyl.

In Table 2 the effect of Sarkosyl addition at different times during the RNA-polymerase reaction is shown. When 0.25% Sarkosyl was added at 5 minutes, enzyme activity was reduced by 52%. If on the other hand, Sarkosyl was added at the beginning of the reaction, the incorporation of ^{3}H-UTP was reduced by > 98%. To test whether the step of initiation or that of elongation was inhibited by Sarkosyl, the reaction mixture was preincubated for 5 minutes at 37°C in the absence of detergent and UTP. At that time both were added and the mixture was incubated for an additional 10 minutes. Under these conditions, virtually no UTP incorporation could be demonstrated.

To determine whether this inhibitory effect also occurred in crude RNA-polymerase preparations, a homogenate of 3T3 nuclei was prepared (Table 3). While purified wheat germ RNA-polymerase activity was reduced by 89%, the crude nuclear preparation was reduced only 35-55% by Sarkosyl.

Discussion:

The protein binding of a labelled amatoxin derivative is a sensitive method to detect eukaryotic RNA-polymerase B in cell extracts (14, 15, 16).

Table III.

Effect of Sarkosyl NL-97 on RNA-Polymerase Activity of 3T3 Nuclear Homogenates

RNA polymerase	No Sarkosyl	Sarkosyl 0.25% (w/v)
100 µl nuclear homogenate	12,272	5,572 (45.4)
200 µl nuclear homogenate	23,856	11,915 (50.0)
300 µl nuclear homogenate	22,773	14,351 (63.0)
Purified wheat germ enzyme	70,262	7,475 (10.6)

Sarkosyl was added at the start of incubation. Numbers are acid precipitable counts per minute. Values in parentheses are percents of control values without Sarkosyl.

We showed that amatoxin binding was strongly inhibited by SDoS and Sarkosyl to a lesser extent by sodium deoxycholate and sodium cholate, and least by NP40 and Triton X-100.

The degree of inhibition of amatoxin binding is directly proportional to a power of the HLB (hydrophile-lypophile balance) for Nonidet P40 (13.1), Triton X-100 (13.5), sodium deoxycholate (16), sodium cholate (18) and sodium dodecyl sulfate (40) for which a published HLB number was available (18, 19, 20) and for Sarkosyl (27) for which the HLB was calculated (20) ($r^2 = 0.94$). The effect of cholate was slightly less than predicted but it was the only agent used at a concentration less than its critical micellar concentration. This suggests that the amatoxin binding domain of the polymerase molecule is relatively hydrophylic. The interpretation of this correlation needs further confirmation. The inhibition of enzyme activity can not be correlated with this property.

Although initiation may also be inhibited, Sarkosyl inhibits chain elongation by purified wheat germ RNA-polymerase. Crude enzyme preparations are less susceptible to inhibition by Sarkosyl presumably because of the presence of non-specifically protective contaminating proteins or membrane structures.

Our data for Triton, deoxycholate and SDoS are in agreement with that predicted from studies of the binding of these detergents to proteins (18). The strongly denaturing effect of SDoS is presumed to be due to its cooperative mode of binding to proteins (19). With regard to their effect on enzyme activity, Triton and cholate were the mildest agents. However, in other experiments we performed, Triton was the most effective agent of this pair in extracting viral nucleoprotein complexes (data not shown).

While it might appear that our data contradict those of other workers (3, 4, 7, 22) who reported that Sarkosyl "activates" RNA polymerase, we found that the degree of inhibition caused by Sarkosyl was inversely proportional to the amount of crude nuclear extract added. Thus, the data are actually in excellent agreement particularly where others have studied purified RNA polymerase and DNA template (22). The apparent stimulation seen by workers using crude enzyme preparations was felt to arise from the removal of proteins bound to the DNA template rendering greater access to the polymerase molecule. The enzyme activity of the RNA polymerase was undoubtedly impaired by the Sarkosyl in these experiments. Recently, Saragosti et al. (22) showed that Sarkosyl decreased the inhibition of RNA synthesis caused by heparin, an acidic macromolecule which may behave like an acidic nuclear protein in this system.

Current studies of transcriptionally active chromatin have attempted to use more intact, native systems. Since the inclusion of denaturing substances into these systems should be minimized, the studies reported in this paper may help in the selection of an appropriate solubilizing agent.

Acknowledgments:

This research was supported by the Veterans Administration Research Service. We wish to thank Dr. Richard Consigli for critical review of the manuscript. We also wish to thank Ms. Donna Chambers for excellent secretarial assistance.

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