THE INHIBITION OF NUCLEIC ACID-BINDING PROTEINS BY AURINTRICARBOXYLIC ACID

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Summary: Qβ replicase, Escherichia coli RNA polymerase, and T7 RNA polymerase are inhibited by low concentrations of the dye aurintricarboxylic acid (ATA). In each case initiation by the enzyme was preferentially inhibited. The elongation of initiated polynucleotide chains by Qβ replicase was insensitive to ATA in the range of concentrations required to inhibit initiation. Treatment of Qβ replicase, RNA polymerase and lac repressor with ATA prevented enzyme-mediated binding of the templates to nitrocellulose filters. We propose that the inhibitor combines with the template binding site of these proteins to prevent initiation.

The triphenylmethane dye aurintricarboxylic acid (ATA) has been shown to inhibit both the initiation and elongation of protein synthesis in vitro (1, 2, 3). The dye (at low concentrations) inhibits initiation by preventing mRNA binding to ribosomes (1, 4, 5). ATA has also been shown to inhibit protein synthesis Elongation Factor EF-Ts activity in vitro (6).

Since both EF-Tu and EF-Ts form part of the Qβ replicase complex and are required for activity (7), we tested this enzyme for sensitivity to ATA. We have found that the initiation but not elongation reaction of Qβ replicase is very sensitive to this dye. Subsequent testing of other template-utilizing enzymes yielded similar results. The mode of action of the drug appears to be the same as for the inhibition of initiation of protein synthesis.

RESULTS

Inhibition of Qβ Replicase: The inhibition of Qβ replicase activity by increasing concentrations of ATA is shown in fig. 1. If the drug is added before the template [poly(UC)], the enzyme is quite sensitive, but if the enzyme

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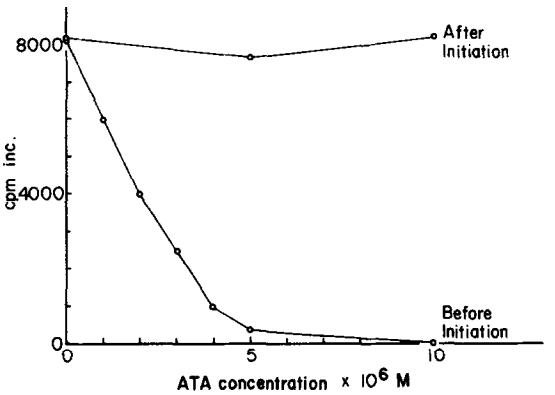

FIG. 1. Inhibition of Qβ Replicase by ATA. Qβ replicase (5 μg/ml) was added to a reaction mix containing 0.05 M Tris Cl, pH 7.5; 0.01 M magnesium acetate, 0.1 mM DTT; 0.2 mM GTP; 2 mM phosphoenolpyruvate; 20% glycerol; and 15 μg/ml pyruvate kinase. ATA was added to this solution at various concentrations (for the experiment measuring effects on initiation). The tubes were incubated for two minutes at 0°C; poly(UC) 50 μg/ml was added and this mixture was incubated for two minutes at 30°C to initiate the chains. At this point the ATA was added to the other tubes (for the experiment measuring effects on elongation); they were incubated 2 minutes at 0°C; and [^{3}H]-ATP was added to 0.2 M to allow elongation. The tubes were incubated for one minute at 30°C, chilled and precipitated, filtered, washed, and counted according to Kamen (11). Qβ replicase was prepared by the method of Kamen (11). ATA ("Aluminon" grade) was purchased from Fisher.

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is allowed to initiate by incubation with template and GTP before the ATA and H^3-ATP are added, no effect of the dye is seen. All assays were performed for short times to circumvent effects of reinitiation. We conclude that the initiation reaction is sensitive but the elongation reaction is not.

Inhibition of Ts Activity and Poly(G) Synthesis: Since the concentrations of ATA required to inhibit EF-Ts activity were reported to be considerably higher than those shown in fig. 1, we performed an experiment to determine if the inhibition of replicase initiation resulted from the inhibition of EF-Ts. Intact Qβ replicase serves as a source of EF-Ts activity for the EF-Ts catalyzed exchange of GDP in EF-Tu-GDP (8). Although the Qβ replicase supplies an equivalent amount of EF-Tu to the EF-Ts assay, that amount is negligible with respect to exogenous EF-Tu-GDP added. The data in Table 1 show that the concen-

TABLE 1

Inhibition of EF-Ts Activity and Poly(G) Synthesis by Treatment of Qβ Replicase with ATA

Activity Measured	Enzyme Concentration	ATA Concentration	Molar Ratio ATA/Enzyme	% Inhibition
EF-Ts	0.1	10	10,000	71
	0.1	2	2,000	14
poly(G) synthesis	0.5	2	400	100
	1.0	2	200	69
	3.0	2	70	32

EF-Ts activity was assayed as described by Landers et al. (8). Assays were checked to make sure they were within the linear range. EF-Tu-GDP used in the EF-Ts assays was prepared by the method of Miller and Weissbach (12). Poly(G) synthesis was assayed according to Kamen (11).

tration of ATA required to inhibit the EF-Ts activity of replicase is approximately 50 times greater than that required to inhibit poly(G) synthesis on a poly(C) template. We conclude that the inhibition of poly(G) synthesis is not a consequence of the interaction between EF-Ts and ATA.

Interaction of ATA with Qβ Replicase: It can also be noted in Table 1 that the ratio of ATA concentration to enzyme concentration determines the amount of inhibition observed even though the concentration of ATA is more than two orders of magnitude greater than the concentration of enzyme. This data is plotted in fig. 2A. If ATA were the true inhibitor, variations in the amount of enzyme present would not be expected to affect the proportion inhibited at a given ATA concentration. Thus it seems that ATA itself is not the true inhibitor. Preliminary analytical thin-layer chromatography of our batch of ATA on polyamide and silica gel plates revealed the presence of numerous minor

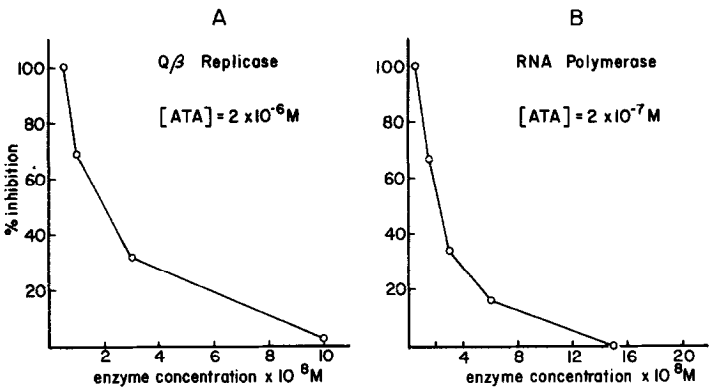

FIG. 2. Inhibition of Varying Concentrations of Qβ Replicase and RNA Polymerase by ATA. A. Inhibition by 2 x 10^{-6} M ATA of the concentrations of Qβ replicase shown was assayed according to Kamen (11). B. Inhibition by 2 x 10^{-7} M ATA of E. coli RNA polymerase was assayed using E. coli DNA (35 μg/ml) as template according to Travers et al. (13).

components. Others have also noted that commercial and individually synthesized batches of ATA are actually a mixture of compounds (1, 2).

When ATA is mixed with Qβ replicase and the mixture passed through a nitrocellulose filter, the red color (presumably the ATA) is retained on the filter, as is the true inhibitor. The bulk of the ultraviolet-absorbing material, judged by A_{230}, however, passes through. The filtrate does not inhibit replicase. Neither the red color nor the true inhibitor binds to the filter in the absence of replicase.

Using the observation that ATA binds to bovine serum albumin (BSA) (9), we found that the inhibition of replicase by ATA was at least partially reversible. Replicase was incubated with sufficient ATA to completely inhibit activity, then before assaying, enough BSA was added to bind the ATA present. The control consisted of the same amounts of ATA and BSA incubated together before being added to the assay. Under these conditions, 37% of the poly(G) polymerase activity was restored after two minutes at 0^{o} in the presence of BSA.

Inhibition of Other Enzymes: We have also investigated the effect of ATA on two DNA-dependent RNA polymerases. Both were found to be inhibited (Table 2).

TABLE 2

Inhibition of RNA Polymerases by ATA

Enzyme	CPM Incorporated		
	No ATA	ATA Added	
		Before Initiation	After Initiation
Qβ Replicase	1734	0	1027
E. coli RNA Polymerase	4647	0	1342
T7 RNA Polymerase	1334	0	418

Qβ replicase was assayed on poly(A,C) as described in the caption to fig. 1 except sufficient time was allowed to permit reinitiation (10 minutes, 30°C). Elongation was begun with the addition of [^{3}H]-UTP. E. coli RNA polymerase (2×10^{-8} M) was assayed by the method of Travers et al. (13) using E. coli DNA (35 μg/ml) as template. The ATA was added thirty seconds after adding the template to the reaction mix in the "After Initiation" experiment. The reaction was incubated for ten minutes at 37°C before TCA precipitation. T7 RNA polymerase (2 μg/ml) was added to a mixture containing 0.02 M Tris Cl pH 7.5, 0.01 M $MgCl_2$, 0.01 M 2-mercaptoethanol, 0.15 mM ATP, 0.15 mM GTP, 0.15 mM CTP, 1 mM $NaPO_4$, 30 μg/ml pyruvate kinase, 1 mM phosphoenol pyruvate, and 1 μg/ml rifampicin. For the "Before Initiation" experiment ATA was added before the T7 DNA and the mixture was incubated five minutes at 0°C. T7 DNA (1 μg/ml) was then added and the mixture incubated for five minutes at 37°C before ATA was added to the "After Initiation" tubes. [^{3}H]-UTP was added to 0.2 mM to allow elongation to proceed. All tubes were incubated for ten minutes at 37°C. In all experiments ATA was present at 2×10^{-5} M.

When ATA was added before template to E. coli DNA-dependent RNA polymerase (provided by C. Hering) or T7 phage DNA-dependent RNA polymerase (supplied by W. Summers) the enzymes were inhibited. When the ATA was added after initiation the enzyme was not as severely inhibited. If the inhibition seen in the latter case is due to inhibition of subsequent rounds of synthesis, the ratio of the last column to the first (Table 2) is a measure of the enzyme's ability

to reinitiate under the conditions used. In other experiments (not shown) β-galactosidase and EF-Tu-GDP binding activity were not inhibited by 10^{-4} M ATA.

Fig. 2B shows that E. coli RNA polymerase is also inhibited to varying degrees by a constant concentration of ATA depending on the enzyme concentration. The fact that a 14-fold lower inhibitor concentration is required to inhibit RNA polymerase than is required to inhibit Qβ replicase suggests that there may be more than one inhibiting substance present in the ATA mixture, or that Qβ replicase must bind more than one molecule of the inhibitor in order to be inactivated.

Template Binding: One feature common to these three enzymes, affected by ATA, is the catalysis of template-directed ribonucleotide polymerization. The observation that the drug inhibits initiation of protein synthesis by blocking template binding to ribosomes led us to examine its effects on the ability of the RNA polymerases to bind radioactively labeled template to nitrocellulose filters. Table 3 shows that binding of [^{3}H]-poly(C) to Qβ replicase and [^{32}P]-lac DNA fragments (supplied by W. Gilbert) to RNA polymerase are inhibited by ATA at approximately the same ATA/enzyme ratios required to inhibit their activities.

The E. coli lac repressor has been shown to bind fragments of DNA containing the lac operator to nitrocellulose filters (10). We found that lac repressor (a gift of C. Hering) is unable to bind the [^{32}P]-lac operator DNA fragments to nitrocellulose filters in the presence of ATA. The specificity of lac DNA binding in the absence of ATA was verified by its sensitivity to 2×10^{-3} M βD-isopropyl thiogalactoside (not shown).

DISCUSSION

We have shown that ATA (or a contaminant of the commercial ATA preparation) combines with Qβ replicase to prevent initiation on artificial templates.

TABLE 3

Enzyme	Enzyme Concentration x 10^8	ATA Concentration x 10^6	Molar Ratio ATA/Enzyme	CPM Bound -ATA	CPM Bound +ATA
Qβ Replicase	0.5	2	400	1748	0
	5		40	1295	1260
RNA Polymerase	3	2	67	5572	0
		0.2	7	5572	5411
Lac Repressor	1	2	200	5118	0
		0.2	20	5118	821

Qβ replicase was mixed with ATA at the given concentrations in a buffer containing 0.05 M Tris Cl pH 7.5, 0.01 M magnesium acetate, 2 mM EDTA, and 0.1 mM DTT. Five μl [^{3}H]-poly(C) was added and the mixture incubated for five minutes at 22°C. The mixtures were filtered through 6 mm Schleicher and Schuell B6 nitrocellulose filters, washed with 0.2 ml of the same buffer, dried and counted in toluene-Omnifluor. E. coli RNA polymerase and lac repressor were mixed with ATA at the given concentrations in the buffer described above (plus 0.01 M KCl). Two μl of [^{32}P]-lac DNA fragments were added and the mixture incubated and filtered as above.

The range of inhibitor/enzyme ratios required to inhibit activity correlates well with the ratios required to inhibit the binding of template to the enzyme. It seems probable, therefore, that ATA inhibits Qβ replicase by preventing template binding. Once initiation has occurred the addition of ATA has no inhibitory effect on the subsequent incorporation of nucleotides into RNA.

E. coli RNA polymerase is more sensitive to ATA than is Qβ replicase. Initiation of this enzyme is preferentially inhibited (although we have not rigorously shown that elongation is not also inhibited), and loss of template binding again correlates well with loss of activity. Initiation of T7 RNA

polymerase is also preferentially inhibited. Lac operator DNA-binding by lac repressor is quite sensitive to ATA as well.

Since we have found that template binding to both DNA-binding and RNA-binding proteins is inhibited by ATA, and since it has also been shown that template binding to ribosomes is inhibited by ATA (1), we predict that most if not all nucleic-acid-binding proteins will be found to be sensitive to this dye. ATA can therefore be viewed as a useful tool for distinguishing between initiation and elongation by enzymes that replicate, transcribe or translate nucleic acid templates. In a separate communication (8) we have used ATA to demonstrate that the two lightest subunits of Qβ replicase (protein synthesis elongation factors EF-Tu and EF-Ts) are required for the initiation reaction but not for the elongation reaction.

The question of whether ATA itself is the actual inhibitor awaits further experimentation.

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