AZOTOBACTER VINELANDII RNA POLYMERASE XI. EFFECT OF TRANSCRIPTION ON RIFAMPICIN BINDING

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SUMMARY: A 1/1 complex of RNA polymerase protomer and ^{3}H-rifampicin is formed with either free enzyme or the enzyme-d(A-T) complex. The ternary polymerase-d(A-T)/r(A-U) complex formed during transcription is unable to bind ^{3}H-rifampicin.

INTRODUCTION: Rifampicin specifically inhibits DNA-dependent RNA polymerase from a variety of bacterial sources (1). The antibiotic inhibits transcription by both holoenzyme and core forms of RNA polymerase and the site of sensitivity to rifampicin resides in the β subunit (2, 3). Inhibition of RNA polymerase by rifampicin occurs at a step prior to chain initiation and following formation of the first phosphodiester bond, the ternary Enzyme/Template/Product complex is resistant to the antibiotic (4 - 6). A question which has remained unresolved is whether rifampicin would bind to, but not inhibit, RNA polymerase engaged in RNA synthesis.

MATERIALS AND METHODS: Tris, UTP, ATP, EDTA, dithiothreitol, and mercaptoethanol were obtained from Sigma Chemical Co. The glass fiber filters (GF/C) were products of Whatman and Sephadex G-25 of Pharmacia. d(A-T) was prepared using *E. coli* DNA polymerase I (7) with dATP and dTTP obtained from Plenum Research. ^{3}H-UTP and Liquifluor were purchased from

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New England Nuclear. ^{3}H-rifampicin labeled in the methyl group of the methylamino piperazine side chain was generously donated by Dr. L.G. Silvestri of Gruppo Lepetit S.p.A., Milan.

Azotobacter RNA polymerase core enzyme was purified by a modification of the published procedure (8) with chromatography on phosphocellulose (Whatman P11) as the final step in the procedure. The polymerase was at least 95% pure as judged by SDS-acrylamide gel electrophoresis.

^{3}H-Rifampicin Binding: The gel filtration method of Wehrli et al. (9) was used to separate the ^{3}H-rifampicin-RNA polymerase complex from unbound ^{3}H-rifampicin. Incubation mixtures of 0.2 ml were resolved on a 0.8 x 16 cm column of Sephadex G-25 equilibrated and developed with the following buffer mixture: 10mM Tris-HCl, pH 7.8, 10 mM $MgSO_4$, 0.1 mM EDTA, 0.1 M $(NH_4)_2SO_4$ and 10 mM mercaptoethanol. 0.25 ml fractions were collected and 0.2 ml of each was dissolved in 4 ml of ethylene glycol monomethyl ether-Liquifluor- toluene and radioactivity determined in a Beckman LS-230 scintillation counter.

RESULTS: Formation of the ^{3}H-rifampicin-RNA polymerase complex is proportional to the amount of enzyme added (Fig. 1). Assuming a molecular weight of 400,000 daltons for the Azotobacter core polymerase protomer, the average molar ratio of ^{3}H-rifampicin to enzyme is 1.13, indicating the presence of a single binding site for rifampicin per enzyme protomer. This is in agreement with the results of Wehrli et al. (10) using the E. coli core RNA polymerase.

We have used the d(A-T)-directed synthesis of r(A-U) as a model system for these studies. As shown in Figure 2, r(A-U)

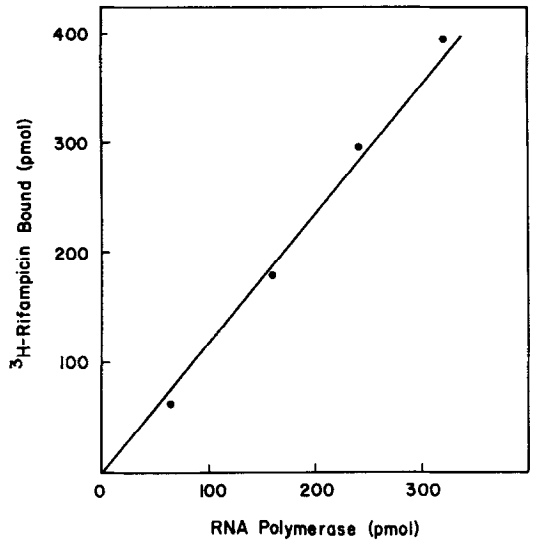

Figure 1. Relation between RNA Polymerase Concentration and ^{3}H-Rifampicin Binding. The incubation mixtures contained (final volume 0.2 ml): 20 μmol Tris-HCl, pH 7.8, 1 μmol dithiothreitol, 1 μmol $MgSO_4$ and A. vinelandii RNA polymerase core enzyme as indicated. ^{3}H-rifampicin was added at 10 times the polymerase concentration and incubated for 5 minutes at 37° prior to determining ^{3}H-rifampicin bound by gel filtration.

synthesis is rapid and ^{3}H-UMP incorporation plateaus within twenty minutes at 37° with 5 mM Mg^{++} as the divalent cation. A similar time course for r(A-U) synthesis is obtained when 5 mM Mn^{++} is used in place of Mg^{++}. Phosphodiester bond formation is completely dependent on Mg^{++} or Mn^{++} and addition of EDTA results in a block in further synthesis while maintaining the ternary enzyme$\begin{matrix}\nearrow d(A\text{-}T) \\ \searrow r(A\text{-}U)\end{matrix}$ complex. d(A-T) directed reactions containing unlabelled UTP and ATP were incubated and at various times synthesis was stopped by addition of EDTA. Following addition of ^{3}H-rifampicin and incubation for 5 minutes at 37°, the amount of antibiotic bound by polymerase was determined

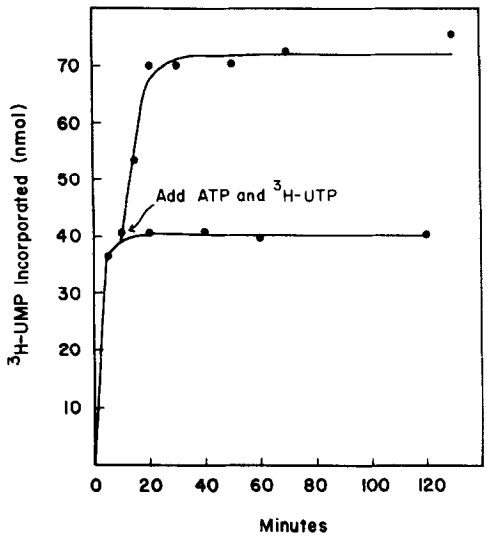

Figure 2. Time Course of r(A-U) Synthesis and Effect of Re-addition of Substrates. The reaction contained (final volume 0.1 ml): 10 μmol Tris-HCl, pH 7.8, 0.5 μmol dithiothreitol, 0.5 μmol $MgSO_4$, 25 μg d(A-T), 50 nmol ATP, 50 nmol ^{3}H-UTP ($1x10^6$ CPM/μmol) and 32 μg (80 pmol) RNA polymerase core enzyme. After 10 minutes at 37° one reaction mixture received an additional 50 nmol ATP and 50 nmol ^{3}H-UTP. The reactions were incubated at 37° and at the times indicated 10 μl aliquots were removed and added to 0.1 ml of cold 0.2 M $NaPP_i$. Following addition of 2 ml of cold 5% TCA the precipitates were collected on glass fiber filters (GF/C) and washed with 10 ml of 5% TCA.

by gel filtration (Fig. 3). The results show that with Mg^{++} as the divalent cation, a limiting ratio of 0.2 pmol ^{3}H-rifampicin/pmol enzyme is attained after 60 minutes of r(A-U) synthesis while with Mn^{++} this ratio is reached within 20 minutes. Even after 120 minutes, no further decrease in ^{3}H-rifampicin binding is noted which in part may be accounted for by inactive enzyme still able to bind rifampicin or enzyme which has terminated

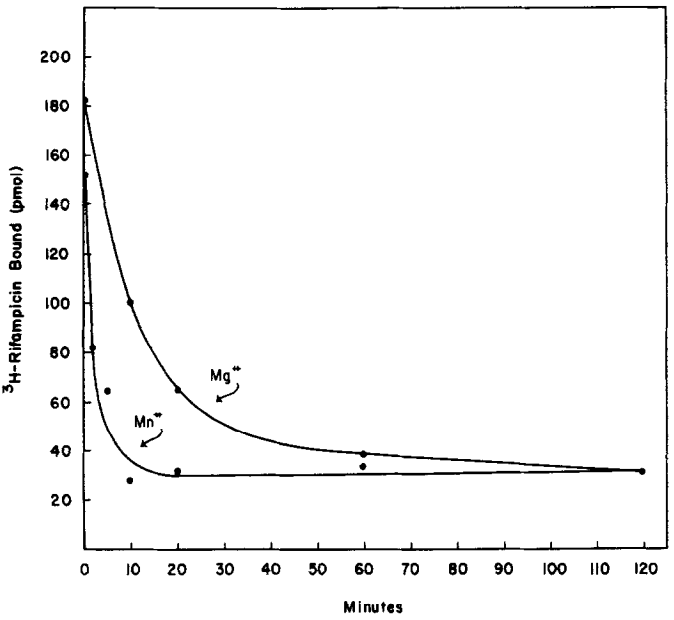

Figure 3. Binding of ^{3}H-Rifampicin in RNA Polymerase After Arrest of Transcription with EDTA. Each reaction contained (0.17 ml): 20 μmol Tris-HCl, pH 7.8, 1 μmol dithiothreitol, 1 μmol $MgSO_4$ or 1 μmol $MnSO_4$, 50 μg d(A-T), 100 nmol ATP, 100 nmol UTP and 64 μg (160 pmol) RNA polymerase core enzyme. Following incubation at 37° for the times indicated 2.5 μmol EDTA and then 600 pmol ^{3}H-rifampicin were added and each sample (0.21 ml) incubated for an additional 5 minutes at 37° prior to assaying for ^{3}H-rifampicin bound by gel filtration.

r(A-U) synthesis but has not reinitiated. The ability of Mn^{++} to apparently allow for a more rapid initiation (i.e. more rapid loss of rifampicin binding capacity) may be related to its effects on chain initiation by core enzyme in the reaction.

The ^{3}H-rifampicin binding properties of RNA polymerase are not appreciably altered by the long time of incubation or other properties of the experimental design. Incubation of enzyme

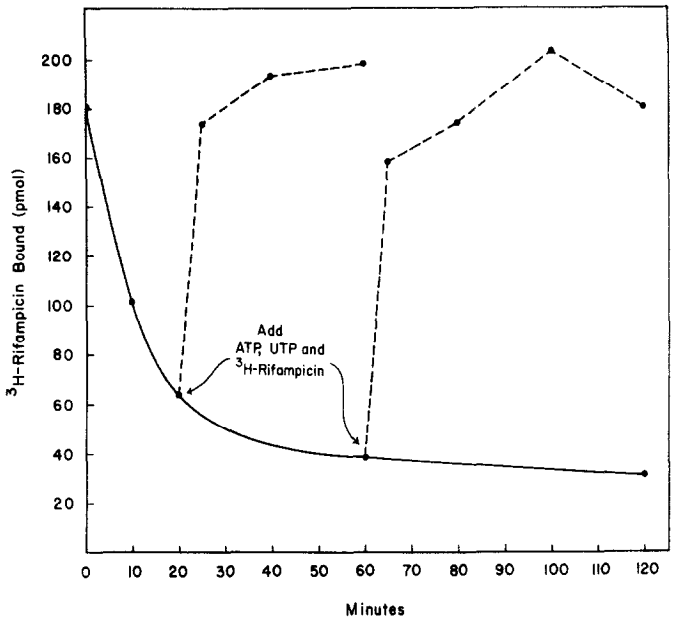

Figure 4. Binding of ^{3}H-Rifampicin to RNA Polymerase During a Second Round of r(A-U) Synthesis. The reaction mixtures were identical to those given in the legend to Figure 3 and the lower curve (o-o) shows the ^{3}H-rifampicin bound to RNA polymerase after arrest of transcription. When indicated 100 nmol ATP and 100 nmol UTP and 600 pmol ^{3}H-rifampicin were added (final volume 0.22 ml) and the incubation continued for the times indicated (o---o) after which ^{3}H-rifampicin bound was determined by gel filtration.

with rifampicin followed by addition of Mg^{++}, ATP, UTP, and d(A-T) (Table 1) shows that the inhibited enzyme-rifampicin complex does not dissociate even after an extended incubation of 120 minutes. Preincubation of enzyme with d(A-T) for 120 minutes or preincubation of core enzyme with d(A-T), ATP, UTP and EDTA followed by the assay for ^{3}H-rifampicin shows that this does not result in appreciable loss of ^{3}H-rifampicin binding. The results presented in Figure 3 can best be ex-

TABLE I

Effect of Various Incubation Conditions on ^{3}H-Rifampicin Binding

Incubation I	Incubation II	^{3}H-Rifampicin Bound (pmol)
^{3}H-Rifampicin (5 min)	d(A-T), ATP, UTP, $MgSO_4$ (120 min)	164
d(A-T) (120 min)	^{3}H-Rifampicin (5 min)	167
d(A-T), ATP, UTP, EDTA (60 min)	^{3}H-Rifampicin (5 min)	154

The reactions contained (0.2 ml final volume): 20 μmol Tris-HCl, pH 7.8, 1 μmol dithiothreitol, 64 μg (160 pmol) RNA polymerase core enzyme and where indicated: 50 μg d(A-T), 100 nmol ATP, 100 nmol UTP and 600 pmol ^{3}H-rifampicin. After incubation at 37° for the times indicated ^{3}H-rifampicin bound was determined by gel filtration.

plained as a consequence of synthesis of r(A-U) and that the nascent ternary enzyme$\begin{matrix}d(A-T)\\r(A-U)\end{matrix}$ complex is unable to bind ^{3}H-rifampicin.

The kinetics of the reaction show that r(A-U) synthesis has plateaued within 20 minutes when 80% of the input ATP and UTP has been incorporated into acid insoluble r(A-U). Re-addition of substrates allows for a further burst of r(A-U) synthesis showing that the RNA polymerase is still active (Fig. 2). Since in the d(A-T) directed reaction, termination, release of enzyme from r(A-U) and d(A-T), followed by reiniti-

ation takes place, it should be possible to demonstrate ^{3}H-rifampicin binding as a consequence of renewed transcription (i.e. termination followed by rifampicin sensitivity preceding reinitiation). The results presented in Figure 4 show that when the antibiotic is added along with the substrates ATP and UTP, a marked increase in ^{3}H-rifampicin binding is a consequence of renewed r(A-U) synthesis. This effect is presumably due to a rifampicin sensitive step following termination and preceding reinitiation of d(A-T) directed r(A-U) synthesis.

DISCUSSION: The observation that core RNA polymerase arrested in transcription is unable to bind rifampicin is consistent with the lag in inhibition displayed when the antibiotic is added to enzyme in the act of chain elongation (6). Our data suggest that the ternary enzyme$\begin{matrix}d(A-T)\\r(A-U)\end{matrix}$ complex must be disrupted, presumably during chain termination, in order for the enzyme to become available for rifampicin binding and inhibition. It is possible that the rifampicin-binding site overlaps the product terminus site (11) on the enzyme.

Conflicting results have been reported supporting the idea that rifampicin binds to RNA polymerase whether or not transcription is in progress (12). This may have come about through the addition of the labeled inhibitor to a mix containing DNA, RNA polymerase, and substrates followed by further incubation. Our results (Fig. 4) would have predicted a rapid rise in the amount of RNA polymerase-rifampicin complex under these conditions of chain termination. This effect was interpreted by Neuhoff *et al.* (12) as demonstrating the binding of rifampicin to actively transcribing enzyme.

The results reported in this paper are consonant with the accepted mechanism of rifampicin inhibition, that the sensitive

state precedes initiation and that during transcription nascent Enzyme$\begin{cases}\text{Template}\\\text{Product}\end{cases}$ complexes are neither inhibited by nor bind rifampicin.

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