CHARACTERIZATION OF T7 SPECIFIC RNA POLYMERASE. III. INHIBITION BY DERIVATIVES OF RIFAMYCIN SV.

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Derivatives of rifamycin SV which contain a variety of substituents at C-4 of the napthoquinone ring are active inhibitors of T7 specific RNA polymerase. Several of the drugs appear to inhibit RNA chain initiation in preference to RNA chain elongation. Studies of the kinetics of T7 RNA synthesis in the presence of one such derivative suggest that there are several large transcription units of differing sizes in the late region of T7 DNA. Other rifamycin SV derivatives inhibit both chain initiation and chain elongation suggesting that there may be 2 sites on T7 RNA polymerase at which rifamycin derivatives can act.

Growth of T7 bacteriophage in the bacterial cell involves the participation of two separate DNA dependent RNA polymerases. The host cell RNA polymerase is required for the transcription of regions of the T7 genome needed immediately after infection (1), while a T7 specific RNA polymerase (gene 1 protein) is essential for efficient transcription of the remainder of the genome (2). The T7 RNA polymerase differs from the bacterial enzyme in its size and subunit structure, and is not inhibited by drugs such as rifamycin, streptovaricin or streptolydigin (3) which are potent inhibitors of the bacterial RNA polymerase (4). While this difference has been useful in the identification and isolation of T7 RNA polymerase, the availability of inhibitors analogous to rifamycin would be of great utility in the study of the structure of T7 polymerase and its reaction sequence. In this communication we report the inhibition of T7 RNA polymerase by several derivatives of rifamycin SV.

METHODS AND MATERIALS: The procedures employed for the purification and assay of T7 RNA polymerase have been described elsewhere (2). The T7 RNA polymerase preparation employed (fraction F) had a specific activity of 64,000 units/mg. The rifamycin SV derivatives employed were the generous gifts of Dr. L.

Silvestri of Gruppo Lepetit S.p.A., Milan, Italy. The nomenclature used is that of Gruppo Lepetit; exact chemical structures are given in the review by Riva and Silvestri (4). Drugs were prepared for use as concentrated solutions in dimethylformamide. In all experiments 5 µl of dimethylformamide with or without drug was added to the standard T7 RNA polymerase assay (final volume, 100 µl). This concentration of dimethylformamide does not measurably affect the rate of T7 RNA synthesis.

RESULTS: A number of derivatives of rifamycin SV are found to inhibit T7 RNA synthesis when added to a standard T7 RNA polymerase reaction prior to the addition of enzyme (Table I). All of the derivatives tested give complete inhibition of T7 RNA synthesis by *E. coli* RNA polymerase at concentrations below 10^{-5} M; however, inhibition of the phage specific enzyme requires higher concentrations of the drugs. The amount of T7 RNA polymerase activity decreases as the drug concentration is increased for each of the active rifamycin derivatives until complete inhibition is obtained at a drug concentration of from 2 to 5 x 10^{-4} M. The T7 RNA polymerase is not affected by these concentrations of rifamycin, streptovaricin or streptolydigin (3).

If it is assumed that the rifamycin derivatives inhibit the T7 RNA polymerase by binding to a single site on the enzyme in a simple noncompetitive manner and that all forms of the enzyme have equal affinities for the drug, an apparent value of an inhibitory constant can be estimated from the concentration of drug needed to give 50% inhibition of T7 RNA synthesis. These values are shown in Table I. The actual curves obtained for inhibition of T7 polymerase activity as a function of drug concentration for 3 of the active rifamycin SV derivatives (Fig. 1, solid lines) do not fit those predicted for simple, noncompetitive inhibition at a single site on the enzyme (dashed lines) In particular the drugs are less inhibitory at low concentrations and more inhibitory at high concentrations. The complexity of the reactions involved in RNA synthesis makes it difficult to analyze the kinetics of inhibition more exactly without a great deal of further data. However, the experimental curves

Table 1. Summary of Inhibition of T7 Polymerase by Rifamycin SV Derivatives.

Experiments were done exactly as in Fig. 1 to determine $C_{50\%}$, the concentration of each derivative needed to give 50% inhibition of T7 RNA polymerase when added before enzyme in a standard assay. Figures in parenthesis represent concentrations in µg/ml. The column *% inhibition of chain elongation assay* shows the result obtained in experiments identical to those in Fig. 2 when a final concentration of 500 µg/ml of drug was added 15 sec after chain initiation. Note that 87.5% inhibition in such an experiment corresponds to 100% inhibition of chain elongation *i.e.* no further synthesis after 15 sec.

Drug	$C_{50\%}$	% Inhibition of chain elongation assay
AF/DNFI	4.4×10^{-5}M (40)	88
AF/013	5.3×10^{-5}M	80
AF/05	6.6×10^{-5}M	86
AF/015	7.0×10^{-5}M	47
AF/01	8.9×10^{-5}M	55
AF/ABDP	9.7×10^{-5}M (90)	23
AF/BO	1.6×10^{-4}M	
PR 19	2.6×10^{-4}M (225)	23
AF/AP	No inhibition at 5×10^{-4}M	

appear to be somewhat cooperative in nature as would be expected, for example, if inhibition required the binding of 2 molecules of inhibitor to each T7 RNA polymerase molecule.

The concentrations of rifamycin SV derivatives required to give 50% inhibition (10^{-4} - 10^{-5} M) are very much greater than the concentration of T7 RNA polymerase in the reaction (10^{-8} M). Hence, the affinity of these analogues for T7 RNA polymerase is much lower than the affinity of rifamycin for the bacterial RNA polymerase. This lower affinity carries with it the implication that formation of the rifamycin derivative-T7 polymerase complex is readily and rapidly reversible.

Preliminary experiments showed that the sensitivity of T7 RNA synthesis by

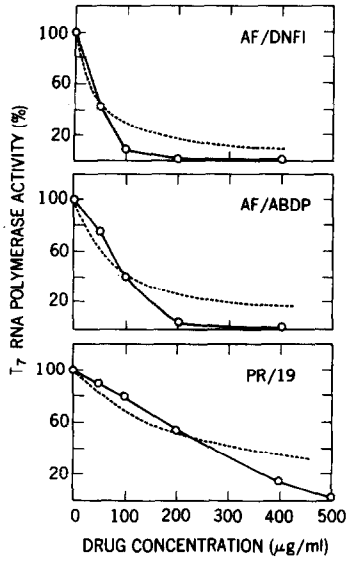

Fig. 1.

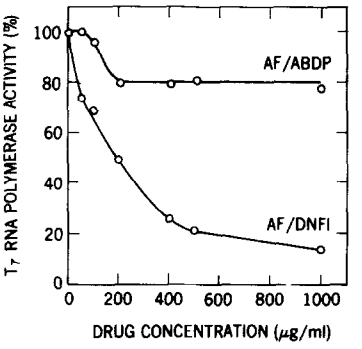

Fig. 2.

Fig. 1 Inhibition of T7 RNA polymerase by rifamycins AF/DNFI, AF/ABDP and PR19. Experiments were carried out in the standard 100 µl T7 RNA polymerase assay solution (2). Drugs and all substrates were added to the mixture prior to the addition of T7 RNA polymerase (the rifamycin SV derivatives are soluble in this mixture up to at least 500 µg/ml the highest concentrations employed in our experiments). Each reaction contained 0.28 µg of T7 RNA polymerase fraction F. In the absence of drug 1.5 nmoles of labeled AMP were incorporated into T7 RNA in a 5 min reaction.

Fig. 2 Inhibition of T7 RNA polymerase by rifamycins AF/DNFI and AF/ABDP added after chain initiation. Experiments were carried out in the standard T7 RNA polymerase assay solution. However, the concentration of CTP was reduced to 2.5×10^{-6} M and the total reaction time allowed was reduced to 2 min. Drugs were added to the reaction 15 sec after RNA synthesis had been initiated. Each reaction contained 0.28 µg of T7 RNA polymerase. In the absence of drug from 5.1 to 5.7 pmoles of labeled CMP were incorporated.

T7 RNA polymerase was greatly reduced if the drugs were added to the reaction 15 sec after the initiation of RNA synthesis. This suggested that the rifamycin SV derivatives acting on the phage enzyme might selectively inhibit RNA chain initiation and hence mimic the effect of rifamycin on the bacterial RNA polymerase. To study this possibility a modified assay procedure was employed to minimize RNA chain termination and reinitiation; in the standard assay procedure each T7 RNA polymerase molecule initiates and terminates several RNA chains (5). In the modified assay the concentration of CTP was reduced to

2.5×10^{-6} M and the incubation time was reduced to 2 min. Under these conditions RNA chains are still rapidly initiated (5), but the RNA chain growth rate is reduced about 15 fold, giving a final rate of about 7-10 nucleotides per sec. Hence, in a 2 min assay period, only RNA chains synthesized on rather short ($3-4 \times 10^5$ daltons) transcription units will have been completed. In this modified assay the amount of nucleotide incorporation should reflect primarily the number of active RNA polymerase molecules and their chain growth rate.

Using the modified assay procedure, it became apparent that several of the rifamycin SV derivatives inhibit RNA chain elongation as well as chain initiation (Fig. 2). Thus, with AF/DNFI, for example, the addition of 500 μg/ml of the drug 15 sec after the initiation of RNA synthesis, completely inhibits further synthesis by the enzyme. In contrast the addition of AF/ABDP after the onset of T7 RNA synthesis gave only a slight inhibition of activity in the modified assay at a drug concentration of 200 μg/ml and the inhibitory effect was not increased by the addition of increasing amounts of this drug up to 1 mg/ml (Fig. 2). Thus, rifamycin AF/ABDP appears to inhibit T7 RNA chain initiation selectively as compared to chain elongation.

The actual reduction of the rate of T7 RNA synthesis in the modified assay when rifamycin AF/ABDP is added at 15 sec is about 20%. We have considered two possible sources of this inhibition. First, the drug may inhibit RNA chain elongation partially, that is, the growing complex may bind rifamycin AF/ABDP and be reduced in its rate of chain growth by 20%. Alternatively, there may be transcription units on T7 DNA which are very short, on the order of 4×10^5 daltons, in which case the 20% inhibition might reflect simply the inhibition of reinitiation of RNA synthesis by T7 polymerase molecules acting on these transcription units. We currently favor the latter possibility; previous studies employing UV irradiated T7 DNA as a selective inhibitor of T7 RNA chain initiation showed a similar reduction in the rate of synthesis when the inhibitor was added after initiation of synthesis (3). In addition, prelim-

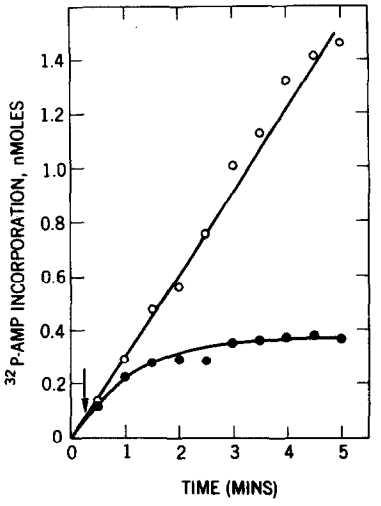

Fig. 3 Kinetics of T7 RNA synthesis in the presence and absence of rifamycin AF/ABDP. Experiments were carried out in the standard T7 RNA polymerase assay solution. The reaction was initiated by the addition of 0.28 µg of T7 RNA polymerase and after 15 sec 5 µl of dimethyl formamide containing 40 µg of rifamycin AF/ABDP were added. Each time point represents a separate reaction.

inary studies of the molecular weight of the RNA products transcribed by T7 RNA polymerase in the in vitro system indicates that several discrete products are found with molecular weights below 10^6 daltons (M. Golomb, unpublished obs).

The effect of adding high concentrations of the different rifamycin derivatives, to the reaction 15 sec after the initiation of synthesis is summarized in Table I. Those derivatives which inhibit synthesis by about 20% are considered selective inhibitors of chain initiation while derivatives which give substantially greater amounts of inhibition must inhibit RNA chain elongation as well.

If rifamycin AF/ABDP is able to selectively inhibit T7 RNA chain initiation, then the kinetics of T7 RNA synthesis in a standard T7 RNA polymerase assay to which excess drug has been added after chain initiation should reveal the length of the longer transcription units read by T7 RNA polymerase on the T7 genome. The results obtained in such an experiment (Fig. 3) are similar to those reported earlier using the UV-irradiated T7 DNA inhibitor (3): there is a marked reduction in the rate of synthesis after about 60 sec and synthesis is

completely stopped by 2.5-3 min. Assuming a chain growth rate of 100 nucleotides/sec (5), the experiment suggests that there are at least 2 classes of large transcription units on the T7 genome, one class of mw about 2-3 x 10^6 and one class of about 5 x 10^6. As the entire late region of T7 DNA is transcribed in these experiments and represents a total of about 10 x 10^6 daltons, the simplest arrangement of unique transcription units would be 2 units of 2-3 x 10^6 and one of 5 x 10^6.

DISCUSSION: Derivatives of rifamycin have been found to inhibit a large variety of different RNA and DNA polymerases as well as the viral reverse transcriptase enzymes (4). Hence, the finding that certain of these derivatives are active against the T7 specific RNA polymerase is not surprising. The ubiquitous sensitivity of template directed polymerases to rifamycin derivatives makes it attractive to suppose that they possess some common structural feature which renders them sensitive to this class of inhibitors. Furthermore, because the different polymerases obtained from bacterial, phage and eucaryotic systems probably have quite different evolutionary origins, it seems likely that this structural feature is essential to the function of a template directed enzyme. However, studies of the mechanism of inhibition and of the locus at which rifamycin analogues act pose a paradox. In the most extensively studied instance, rifamycin interacts with the bacterial RNA polymerase to selectively block RNA chain initiation; the rate of RNA chain elongation is essentially unaltered by rifamycin (4). Similarly, in the case of the eucaryotic cell RNA polymerases (6) and in the current instance with the T7 phage RNA polymerase, certain rifamycin derivatives selectively inhibit RNA chain initiation. Surprisingly, however, other rifamycin derivatives inhibit RNA chain growth by T7 RNA polymerase, and in recent experiments it has been found the rifamycin AF/DNFI inhibits RNA chain growth by *E. coli* RNA polymerase (G. Rhoades, unpublished expts.). In addition, certain rifamycin derivatives inhibit DNA polymerases, which are unable to initiate polynucleotide chains under normal assay conditions and hence carry out only chain elongation (7).

Thus, for different rifamycin derivatives either polynucleotide chain initiation or polynucleotide chain growth can be affected. This suggests that there may be 2 kinds of sites on DNA directed RNA polymerases which can potentially interact with rifamycin or its derivatives: one, which affects chain initiation, the other, chain elongation. Since rifamycin blocks the binding of the initial purine nucleoside triphosphate to the bacterial RNA polymerase (8) these 2 sites may be correlated with the binding sites for the nucleoside triphosphates required for chain initiation and chain elongation respectively; a common feature of all template directed polymerases is their ability to bind and to utilize the complementary nucleoside triphosphates. For template directed polymerases of differing origins, the fine structure of the proteins in the region of the nucleotide binding sites may vary considerably. Thus, these different polymerases could show considerable differences with respect to which rifamycin derivatives would be inhibitory. This model would explain the sensitivity of DNA polymerases to rifamycin derivatives since these enzymes possess a binding site for nucleoside triphosphates used in chain elongation although they probably lack the second nucleotide binding site used in chain initiation which is found on the DNA directed RNA polymerases.

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