MECHANISM OF ETHIDIUM BROMIDE INHIBITION OF RNA POLYMERASE

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Summary: The interaction of RNA polymerase with DNA prior to the initiation of RNA synthesis involves the formation of a specific complex (I) which is in equilibrium with the so-called rapidly starting (RS) complex. Ethidium bromide, which is an effective inhibitor of RNA synthesis, has no effect on either the rate of transformation of the I complex to the RS complex or the rate of transformation of the RS complex to the initiation complex. Rather this intercalating inhibitor, which modifies the conformation of the template, apparently acts by limiting the number of enzyme molecules which form I complexes.

Ethidium bromide (3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide), which is an effective antitrypanosomal drug and a strong inhibitor of RNA polymerase, may act by interfering with the template activity of DNA (1). The inhibitor forms complexes with DNA which consist of ethidium bromide molecules intercalated between base pairs of the template (2).

Inhibition of RNA synthesis has been reported to result primarily from ethidium bromide interference with the formation of a specific complex between E. coli DNA and RNA polymerase (3). Recently, however, the sequential formation of three specific complexes between T7 DNA and the enzyme has been demonstrated (4). RNA polymerase initially recognizes some specific site on the template and binds to it giving the first specific complex (I). This I complex is in equilibrium with the so-called readily starting (RS) complex. From this latter state rapid initiation of RNA chains takes place. The transition between the I to the RS complex apparently involves localized separation of DNA strands during which the enzyme is repositioned around or within the strand opening (5).

In view of these findings we have re-examined the mechanism of inhibition of RNA synthesis by ethidium bromide in an attempt to identify the primary

site and the mode of action of this inhibitor. To this end we measured the rate of RNA synthesis in the presence of rifampicin.

Results and Discussion

Rifampicin has a pronounced inhibitory effect on RNA synthesis but it has no such effect if it is added to an RNA synthesizing system after the initiation complex is formed. As a result of these properties it can be shown (4) that the slope of the line obtained by plotting RNA polymerase activity expressed as V/V' vs rifampicin concentration is proportional to the ratio of the rate constant of rifampicin attack on the binary RNA polymerase-DNA complex over the apparent first order rate constant for RNA chain initiation. In this expression V stands for the rate of RNA synthesis in the absence of rifampicin and V' is the same rate in the presence of rifampicin. Furthermore, the intercept on the V/V' axis provides a measure of the number of RS complexes which are being transformed to the rifampicin resistant ternary complex (4).

Assuming that the rate of rifampicin attack on the complex is not altered in the presence of ethidium bromide, the near identity of slopes for the inhibited and uninhibited reactions (Fig. 1) indicates that the rate constant for enzyme molecules which are transformed from the RS complex to the initiation complex is not appreciably affected by the presence of ethidium bromide. However, the number of enzyme molecules which participate in this step is substantially decreased in the presence of this inhibitor as indicated by the V/V' intercept. At a ratio of bound ethidium bromide to DNA phosphate (r) of 0.035 (Fig. 1) the value of this intercept for the inhibited reaction is about one half of that obtained in the absence of the inhibitor indicating that the number of enzyme molecules which are able to initiate RNA chains is substantially decreased. This decrease may result from either interference of the inhibitor with the formation of the RS complex or alternatively the I complex, which is formed in the preceeding step. A distinction between these two alternatives can be made however if the number of RNA polymerase

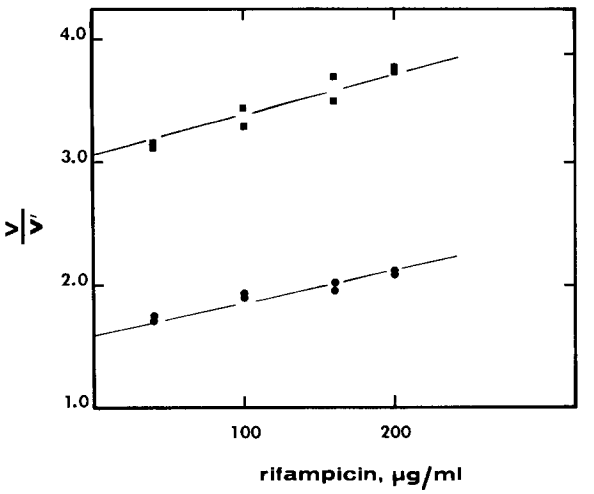

Figure 1. The rate of RNA synthesis in the absence of rifampicin (V=8.4 x 10^3 cpm) divided by the rate of synthesis (V') in the presence of: (a) rifampicin —●— and (b) both rifampicin and ethidium bromide (r of 0.035) —■— . DNA dependent RNA polymerase (Sigma) was assayed as previously reported (4). DNA (50µM final concentration) isolated from T_2 coliphage (Miles Laboratories), as previously described (13), ethidium bromide (Calbiochem), the assay buffer and deionized water were adjusted to a volume of 78µl and cooled to 0°C for about 10 min. The enzyme (2mg, 800 units/mg protein when T_2 DNA is used as a template) was then added in 2µl of assay buffer. After pre-incubation (7 min. at 30°C) the reaction was initiated by the rapid addition of a solution (20µl) containing GTP, CTP, UTP (0.4mM) and 8- [^{3}H] -ATP (0.25 µCi/nmole) and appropriate amounts of rifampicin (Sigma). The mixtures were incubated at 30°C for 1 min. 30 sec. and the reaction was terminated by the sequential addition of 0.5ml sodium pyrophosphate (0.1M)-ATP (0.002M) and 0.5ml trichloroacetic acid (11%)-KCl (1.0M). The precipitate was collected on a Whatman GM/C filter and washed extensively and sequentially with 5% trichloroacetic acid and 95% ethanol. The dried filter was counted under 0.4% 2,5-diphenyloxazole in toluene.

molecules which are involved in the transformation of the I complex to the RS complex is measured.

A plot of activity vs time of pre-incubation at 30°C of binary DNA-RNA polymerase complexes formed at 0°C (I complexes) and subsequently initiated at 30°C with a mixture of nucleotides either with or without rifampicin is shown in Fig. 2. The slope of the obtained exponential curve provides a measure of the rate constant of the transformation of the I to the RS complex (5). The effect of ethidium bromide, at an r value of 0.035, on the dependence of activity on time of pre-incubation for synthesis initiated with either a

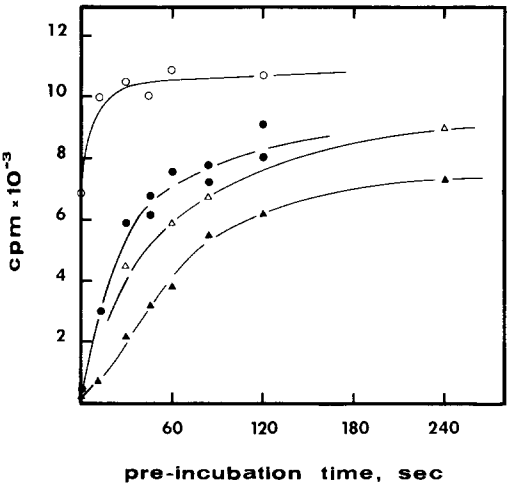

Figure 2. The rate of RNA synthesis as a function of pre-incubation time. The procedure described in the legend to Fig. 1 was used except that the DNA concentration was 90 μM and 2 μg of enzyme was used. Following the addition of the enzyme the assay mixture was first kept at 0°C for 30 min. and subsequently was transferred to a bath maintained at 30°C for the indicated pre-incubation time. The reaction was initiated with a solution (20 μl) containing 0.4 M each of GTP, CTP, UTP and 8-[^{3}H]-ATP (0.25 μCi/nmole) and, whenever appropriate, rifampicin (30 μg/ml). RNA synthesis in the absence of both rifampicin and ethidium —○—; in the presence of rifampicin only —●— ; in the presence of ethidium bromide only (r=0.035) △ ; in the presence of both rifampicin and ethidium bromide —▲—.

mixture of nucleotide substrates or a mixture of these substrates plus rifampicin is also shown in Fig. 2.

In every instance a generally similar dependence of activity (expressed as cpm of UMP incorporated into RNA) on time of pre-incubation is obtained suggesting that the rate of transformation of the I complex to the RS complex is not affected by the presence of ethidium bromide. It is also apparent that in the presence of both ethidium and rifampicin incorporation of UMP is much lower than that obtained in the presence of rifampicin. This provides additional evidence that ethidium bromide limits the number of enzyme molecules which can form rifampicin-resistant ternary initiation complexes.

Since the kinetics of transformation of the I complex to the RS complex follow a first order relationship the apparent half-life of the complex can be

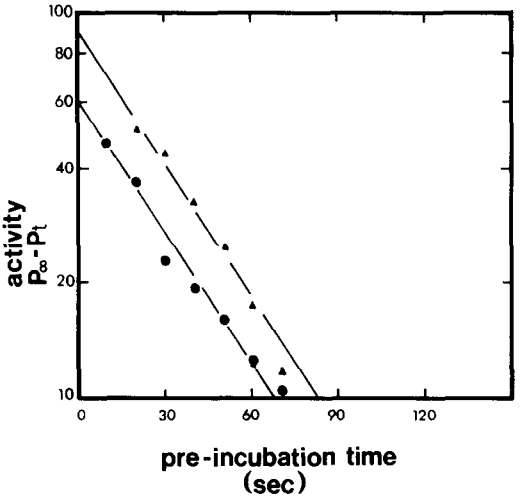

Figure 3. The dependence of the logarithm of the rate of RNA synthesis at infinite pre-incubation time (P_∞) minus the rate at any time (P_t) vs pre-incubation time (t). Enzymic rate (%) is calculated as cpm incorporated in 90 sec. of reaction in the presence of rifampicin divided by the cpm incorporated in its absence. Rate of synthesis in the absence of ethidium bromide —●— ; in the presence of ethidium bromide (r=0.035) —▲— .

estimated by plotting the logarithm of $P_\infty - P_t$ vs pre-incubation time (Fig. 3), where P_∞ is a measure of UMP incorporation at infinite pre-incubation time and P_t is the same parameter at any time (5). Both P_∞ and P_t are expressed as per cent of cpm incorporated in the presence of rifampicin divided by cpm in its absence. The same slope and similar intercepts are obtained in both the presence and the absence of ethidium bromide indicating similar half-lives for the I complex under these two sets of conditions. Clearly then ethidium bromide has no appreciable effect on the rate of transformation of the I complex to the RS complex.

In summary, ethidium bromide appears to limit the number of RNA polymerase molecules which are able to form initiation complexes. The inhibitor however appears to have no effect on the rate of transformation of the I complex to the RS complex or the rate of transformation of the RS complex to the initiation complex. These findings indicate indirectly that ethidium bromide inhibits RNA synthesis by reducing the number of RNA polymerase molecules which are

able to form I complexes. If this were not the case, it would be difficult to explain how the number of RNA polymerase molecules reaching the initiation complex via the RS complex are reduced in spite of the fact that ethidium has no appreciable effect on the rate of conversion of the I complex to the RS complex or the subsequent conversion of the RS complex.

Thus, it appears that ethidium bromide inhibits RNA polymerase as a result of modification of template sites with which the enzyme interacts to form I complexes. It may be assumed that the intercalated inhibitor distorts these sites in a manner interfering with the productive binding of the enzyme. This is not at all surprising in view of the substantial changes in conformation which accompany the binding of ethidium bromide to DNA as reflected by the results of circular dichroism (7,8) sedimantation (9) and recent X-ray diffraction (10) studies of DNA-ethidium complexes.

The exact structural requirements for effective recognition and productive binding between RNA polymerase and the promoter region in DNA are not well established. However, the effects of specific ring substituents on the intercalating properties of the phenanthridinium ring and the changes in DNA conformation which these substituents bring about, are currently been elucidated (11,12). Therefore, the systematic study of phenanthridinium derivatives with appropriate ring substituents may provide information regarding, at least, the extent to which changes in template conformation may modify the ability of the template to interact with RNA polymerase and direct RNA synthesis.

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