Molecular Mechanism of the Rifampicin–RNA Polymerase Interaction†

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ABSTRACT: Equilibrium and kinetic studies of the interaction of rifampicin with RNA polymerase of *Escherichia coli* were performed by exploiting the quenching of intrinsic fluorescence of the protein by the drug. Fluorimetric titrations show that rifampicin binds stoichiometrically to the core and holoenzyme with an apparent K_d of $\leq 3 \times 10^{-9}$ M. Neither the addition of template nor the formation of the initiation complex in the presence of dinucleotide and nucleoside triphosphate prevents the rifampicin-enzyme interaction. Although the equilibrium binding constant for the rifampicin–RNA polymerase complex is about the same for the core and holoenzyme and the holoenzyme–T7 DNA complex, stopped-flow studies indicate that the rates at which rifampicin interacts with these enzyme forms are different. In all three cases, the kinetic data can be interpreted in terms of a mechanism in which the rapid bimolecular binding of rifampicin to RNA polymerase is followed by a relatively slow isomerization of the drug–enzyme complex:

$$E + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ER \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ER'$$

While the values of dissociation constant, $K_1 = (k_{-1}/k_1)$, for the first binary complex (ER) are similar, the rate constant for the forward isomerization, k_2, decreases in the order of core enzyme > holoenzyme > the holoenzyme–T7 DNA complex. The fact that this order is parallel to the relative rates of inactivation of the enzymes and the enzyme–DNA complex suggests that the inactivation may be due to the rifampicin-induced isomerization (conformational change) of the enzyme. This is supported by our observation that an enzyme complex which is in the process of elongating RNA chains can still bind rifampicin, although the enzyme activity is not inhibited by such binding. The values of overall binding constants calculated from the kinetic parameters, $1\text{–}2 \times 10^{-9}$ M, are in good agreement with the values of the apparent K_d obtained from fluorimetric titrations and K_i determined by enzymatic assays. In addition, the observations that the formation of an initiation complex leads to a significant but not complete rifampicin-resistant RNA synthesis and the recent finding that rifampicin only partly inhibits the formation of the first phosphodiester bond in an abortive initiation of RNA chains are consistent with our kinetic mechanism, i.e., the existence of two forms of the rifampicin–RNA polymerase complex, only one of which is able to initiate the RNA chains.

The antibiotic rifamycin inhibits bacterial RNA synthesis (Hartmann et al. 1967) by binding to the DNA-dependent RNA polymerase (Wehrli et al., 1968; di Mauro et al., 1969; Riva and Sylvestri, 1972). The interaction of RNA polymerase with rifamycin has been extensively studied: (1) Rifamycin binds tightly to a single site on both the holo and core enzyme by a noncovalent interaction (di Mauro et al., 1969; Wehrli and Staehelin, 1969); however, there are indications that another weak binding site may exist (Riva et al., 1972; Fietta and Sylvestri, 1975). (2) Genetic evidence suggests that the rifamycin binding site resides in the β subunit (Heil and Zillig, 1970). This has been confirmed by affinity labeling of core polymerase with an alkylating derivative of rifamycin SV (Stender et al., 1975). (3) Although rifamycin interferes with the binding of purine nucleotides to the enzyme (Wu and Goldthwait, 1969a), covalent attachment of a fluorescent nucleotide analogue to one of the substrate sites, the initiation site, does not affect the binding of rifamycin. In addition, energy transfer measurement indicates that the initiation and rifamycin binding sites are not adjacent to each other (Wu and Wu, 1974a). (4) Rifamycin binds to both free enzyme and the enzyme–DNA complex. There are conflicting reports as to whether rifamycin binds to the enzyme–DNA–RNA ternary complex (Neuhoff et al., 1971; Lill and Hartmann, 1973; Eilen and Krakow, 1973).

Since RNA synthesis becomes resistant to rifamycin once RNA chain initiation has been accomplished (Sippel and Hartmann, 1968), the antibiotic must act to block one of the earlier steps in RNA synthesis. Earlier studies have shown that rifamycin does not affect the binding of RNA polymerase to DNA (Umezawa et al., 1968), thus it seems likely that the drug acts to prevent RNA chain initiation. However, Johnston and McClure (1976) found recently that rifamycin has little effect on the formation of the first phosphodiester bond in abortive initiation of in vitro RNA synthesis, indicating that it inhibits at some step(s) after the formation of the first phosphodiester bond.

Hinkle et al. (1972) have suggested that the relative resistance of the RNA polymerase–DNA complex to attack by rifamycin is due to a kinetic phenomenon, namely the rapid rate of RNA chain initiation by this complex when the drug is added together with the nucleoside triphosphates and the slower rate of the drug binding to the DNA–enzyme complex than that of free enzyme. Assuming a simple kinetic competition between nucleoside triphosphates and rifamycin for binding at RNA polymerase–promotor complex, Mangel and Chamberlin (1974) have published methods for measuring the rate of extent of in vitro RNA chain initiation from T7 DNA. Such assays have some serious drawbacks. If the chain initiation is not directly competitive with the binding of rifamycin then the loss of RNA polymerase activity will not reflect the

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rate of attack by rifamycin on the DNA-enzyme complex. Moreover, the rate of attack by rifamycin was measured indirectly by the inactivation of RNA polymerase (Hinkle et al., 1972), which is not necessarily the same as the rate of rifamycin binding to the enzyme. The inactivation of RNA polymerase could occur due to the bimolecular binding of rifamycin to the enzyme, or alternatively, due to other processes such as a conformational change of the enzyme subsequent to the binding process, which converts the enzyme into an inactive form. In addition, the initiation rates measured with this rifamycin challenge technique is further complicated by the fact that the drug allows the formation of the first phosphodiester bond by RNA polymerase (Johnston and McClure, 1976). In view of the above complications, the mechanism of actions of rifamycin warranted further investigation.

In this paper, we report kinetic and equilibrium studies of the interaction of rifampicin, a most commonly used rifamycin derivative, with RNA polymerase of *E. coli*. The interaction was monitored using the quenching of intrinsic fluorescence of the protein by rifampicin (Wu and Goldthwait, 1969b). The results show that although the dissociation constant for the rifampicin–RNA polymerase complex is about the same for the holo- and core enzyme preparation, the rates of rifampicin binding to RNA polymerase are in the order of core enzyme > holoenzyme > the holoenzyme–DNA complex. In addition, the kinetic data are consistent with a mechanism in which a rapid bimolecular binding of rifampicin to RNA polymerase is followed by a relatively slow isomerization of the complex. Comparison of the rate constants derived from our kinetic analysis with that obtained from the inactivation of RNA polymerase by rifampicin suggests that the inactivation is caused by the isomerization and not the binding step. This is in agreement with our finding that the holoenzyme-T7 DNA complex, which is in the process of synthesizing RNA, binds rifampicin almost as well as free enzyme.

Materials and Methods

RNA polymerase was purified from *E. coli* B cells, as described previously (Wu and Wu, 1973). Core polymerase and the σ subunit were prepared by the methods of Berg et al. (1971). Holoenzyme with saturating amounts of σ was obtained by adding an excess of isolated σ subunit to the enzyme, followed by removal of free σ via glycerol-gradient centrifugation. The purity of enzyme was more than 97% as judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Unlabeled and [^{14}C]rifampicin were gifts of Dr. R. White and L. Sylvestri of Gruppo-Lepetit. [γ-^{32}P]ATP and [^{3}H]GTP were products of New England Nuclear Corp. Unlabeled nucleoside triphosphates were purchased from Schwarz/Mann. All other biochemicals were the highest available reagent grade.

RNA polymerase assays were performed as described previously (Wu and Wu, 1973). RNA chain initiation was determined by the incorporation of [γ-^{32}P]ATP into 5′-termini of RNA products (Maitra and Hurwitz, 1965). Reaction mixtures (0.1 ml) for [γ-^{32}P]ATP incorporation contained: 0.05 M Tris[1] (pH 8.0), 0.05 M KCl, 10^{-2} M $MgCl_2$, 10^{-3} dithiothreitol, 10^{-4} M EDTA, 2×10^{-4} M each of [γ-^{32}P]ATP (1560 cpm/pmol), GTP, and CTP, 2×10^{-6} M UTP, 3 pmol of holoenzyme, 0.38 pmole of T7 DNA, and 6×10^{-7} M rifampicin, as indicated. After 5 min of incubation of enzyme, DNA, and the four nucleoside triphosphates at 23 °C, rifampicin was added and the incubation continued for an additional 15 min; samples were then processed, as described previously. The average efficiency of initiation was calculated assuming that the ratio of ATP/GTP termini is 2/1 with T7 DNA as template (Chamberlin and Ring, 1972).

The effect of rifampicin on the kinetics of the RNA polymerase reaction was studied as follows. Holoenzyme was diluted to a final concentration of 2.4×10^{-9} M in 0.05 M Tris (pH 7.9), 0.2 M KCl, 10^{-2} M $MgCl_2$, 10^{-3} M dithiothreitol, either in the presence or absence of 2.4×10^{-9} M rifampicin. After 5 min at 23 °C, 0.25-ml aliquots were taken and 32 nmol of T7 DNA were added to each aliquot. UTP, ATP, and CTP were then added to give a final concentration of 2×10^{-4} M along with the indicated amount of [^{3}H]GTP (10 cpm/pmol). Following a 5-min incubation at 37 °C, the acid-insoluble radioactivity was determined by liquid scintillation counting.

Stoichiometry of rifampicin binding to RNA polymerase was determined by both gel filtration and equilibrium dialysis. In gel filtration studies, a two-fold molar excess of [^{14}C]rifampicin (40 cpm/pmol) was added to an enzyme solution. After incubation at 37 °C for 2 min, the sample was passed through a Sephadex G-75 column. Two peaks of radioactivity were observed; one eluted in the void volume and contained protein-bound rifampicin, while the second contained free ligand. Equilibrium dialysis was performed as described by Wu and Goldthwait (1969a). Twenty microliters of enzyme solution ($0.5–1 \times 10^{-5}$ M in 0.05 M Tris, pH 8, 0.2 M KCl, 0.1 mM dithiothreitol, 0.1 mM EDTA) was added to the sample compartment. An equal volume of [^{14}C]rifampicin (22 cpm/pmol, $1–5 \times 10^{-5}$ M in the same buffer) was added to the other compartment and the dialysis was carried out for 3 h at 23 °C with gentle shaking. After equilibration 5-μl aliquots were removed and counted in Bray's solution. Under the conditions used here, the loss of rifampicin due to possible adsorption on the membrane or dialysis cell was less than 5% of the initial concentration added. Protein concentrations were determined both before and after binding by the method of Bücher (1947) and by the method of Lowry et al. (1951), as standardized by Berg et al. (1971) for RNA polymerase.

Steady-state fluorescence measurements were made using a Perkin-Elmer MPF-3 fluorescence spectrophotometer equipped with a corrected spectra attachment. Temperature was maintained by a thermostated cell holder connected to a Lauda-K2R circulation bath. All spectral measurements were performed at 23 ± 0.1 °C. Absorbances of all the samples were below 0.05 to obviate inner filter effect.

The stopped-flow apparatus with fluorescence detection was constructed in this laboratory and has been described before (Wu and Wu, 1974b). The dead time of the apparatus was about 2 ms. The light source was a Hanovia 200W Xe–Hg arc lamp. The excitation wavelength was selected at 290 nm by a Baush and Lomb high intensity uv monochromator. The fluorescence emission at right angle was passed through Corning 0-52 and 7-51 filters to remove exciting light. The kinetic information was recorded on a Tektronic 543 storage oscilloscope and transferred on line to a PDP-11 digital computer through a Biomation 802 transient recorder. Relaxation times were calculated by a nonlinear least-squares analysis of the data. Unless noted otherwise, each relaxation time represents the mean value obtained from at least five experiments.

Results and Data Analysis

Fluorimetric Studies of Rifampicin Binding to E. coli RNA Polymerase. The corrected fluorescence emission spectrum of RNA polymerase core enzyme is shown in Figure 1. The

[1] Abbreviations used are: EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

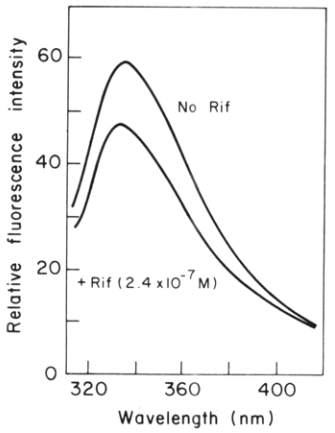

FIGURE 1: Corrected fluorescence emission spectra of RNA polymerase core enzyme in the presence and absence of rifampicin. The concentration of core enzyme was 3×10^{-8} M in 0.05 M Tris (pH 8.0), 0.2 M KCl, 10^{-2} M $MgCl_2$, 5×10^{-4} M dithiothreitol, and 10^{-4} M EDTA. The temperature was 23 °C. Excitation was at 290 nm. The upper curve is the corrected fluorescence emission spectrum of core enzyme alone; the lower curve is the corrected fluorescence emission spectrum of the core enzyme in the presence of 2.4×10^{-7} M rifampicin.

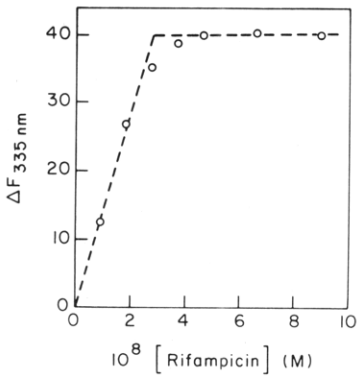

FIGURE 2: Fluorimetric titration of core enzyme with rifampicin. The solution was as described in Figure 1. Rifampicin concentrations were varied from 1×10^{-8} to 9×10^{-8} M. ΔF represents the decrease in relative fluorescence intensity of core enzyme at 335 nm.

emission maximum at 335 nm is characteristic of intrinsic tryptophan fluorescence observed with other proteins. Addition of saturating concentration (2.4×10^{-7} M) of rifampicin produced a 22% decrease in the fluorescence intensity with a small (2–3 nm) blue shift in emission maximum. The effect of different concentrations of rifampicin on the fluorescence intensity of core polymerase is shown in Figure 2. As one can see, it is a typical stoichiometric binding, and from the titration curve an upper limit of the apparent K_d of 3×10^{-9} M for the rifampicin–core enzyme complex was estimated. A fluorimetric titration of the holoenzyme with rifampicin yielded a similar apparent K_d and a 18% maximum quenching of fluorescence intensity. Both the degree of fluorescence quenching and the value of the apparent K_d were the same, whether the titration was carried out under high salt (≥0.2 M KCl) or low salt (≤0.05 M KCl) conditions. No fluorescence quenching by rifampicin was observed for the isolated σ subunit.

Effects of Template and Nucleotides on Rifampicin Binding. The effects of templates and nucleotides on the fluorescence quenching of RNA polymerase by rifampicin are given in Table I. When poly[d(A-T)] was added to a solution of core polymerase, a small decrease in fluorescence intensity of the enzyme was observed (3–4%, data not shown). Further

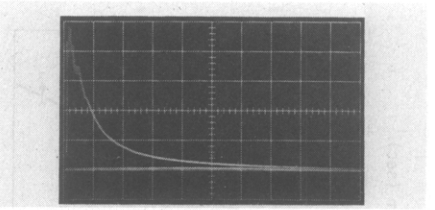

FIGURE 3: Oscilloscope trace of the kinetics of rifampicin binding to the holoenzyme. The enzyme concentration was 3×10^{-8} M in 0.05 M Tris (pH 8.0), 0.2 M KCl, 10^{-2} M $MgCl_2$, 5×10^{-4} M dithiothreitol, and 10^{-4} M EDTA. The rifampicin concentration was 6×10^{-7} M. Measurements were made at 23 °C. The ordinate is the fluorescence change at 340 nm (arbitrary units) and the abscissa is time (2 s/division).

TABLE I: Effects of Template and Nucleotides on Rifampicin Quenching of RNA Polymerase Fluorescence.[a]

Conditions	Fluorescence Quenching by Rifampicin (%)
Core E	22
Core E + poly[d(A-T)]	13
Holo E	18
Holo E + T7 DNA	12
Holo E + poly[d(A-T)]	14
Holo E + poly[d(A-T)] + ApU	14
Holo E + poly[d(A-T)] + ApU + ATP	14
σ subunit	0

[a] The concentrations used were: enzyme or σ subunit, $2–4 \times 10^{-8}$ M; poly[d(A-T)], 2×10^{-5} M; ApU, 1.4×10^{-4} M; ATP, 10^{-4} M; and rifampicin, 6×10^{-7} M. The molar ratio of enzyme to T7 DNA was 8. The excitation wavelength was 295 nm and the fluorescence quenching was measured at 340 nm. All experiments were performed at 23 °C.

addition of rifampicin to the core enzyme–poly[d(A-T)] complex resulted in a 13% decrease in fluorescence intensity, indicating that binding of the template did not prevent the interaction of enzyme with rifampicin. A similar degree of quenching was observed for rifampicin binding to the T7 DNA–holoenzyme complex.

With poly[d(A-T)] as template, the presence of the dinucleotide, ApU, and ATP should lead to the formation of an "initiation complex" resistant to inactivation by rifampicin (So and Downey, 1970). Since the inhibitor produced a 14% fluorescence quenching of the holopolymerase–poly[d(A-T)] complex both in the absence and presence of ApU and ATP (Table I), it is evident that the complex was capable of interacting with rifampicin.

Kinetics of RNA Polymerase Interaction with Rifampicin. Stopped-flow technique was used to study the kinetics of interactions between rifampicin and RNA polymerase. RNA polymerase preparations usually contain both the holo and core enzymes. When such a preparation was rapidly mixed with an excess amount of rifampicin and the fluorescence change at 340 nm monitored, a biphasic curve was observed (not shown). However, if the holoenzyme preparation saturated with isolated σ subunit was mixed with an excess of rifampicin, a single exponential decay curve of the fluorescence change was seen. A typical oscilloscope trace of such change is shown in Figure 3. It should be noted that the total extent of quenching observed kinetically was about 12%, while the steady-state measurement under the same conditions was about 18%. This suggests that

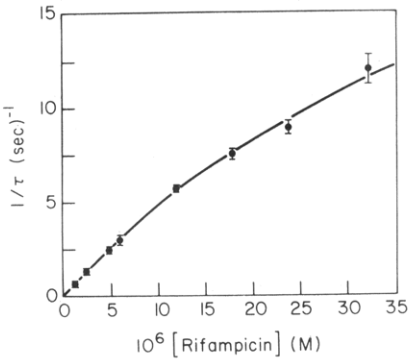

FIGURE 4: Concentration dependence of $1/\tau$ for rifampicin binding to the holoenzyme. Data were obtained and analyzed as described in the Materials and Methods section. The solutions were the same as in Figure 3 except that the rifampicin concentration was varied. The solid line is a theoretical curve calculated according to eq 3 using the kinetic parameters in Table II.

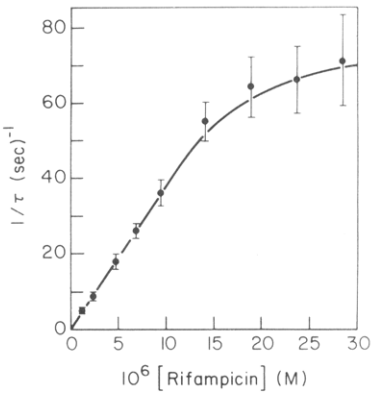

FIGURE 5: Concentration dependence of $1/\tau$ for rifampicin binding to the core enzyme. Conditions were as described in the legend to Figure 4. The solid line is a theoretical curve calculated according to eq. 3 using the kinetic parameters in Table II.

a fast process beyond the time resolution of the instrument (2 ms) might have occurred.

The dependence of the reciprocal relaxation time, $1/\tau$, of the observed single exponential fluorescence change for the holoenzyme on the concentration of rifampicin is shown in Figure 4. The nonlinearity of the concentration dependence indicates that the binding observed is not a simple bimolecular reaction. Such behavior, however, is consistent with a mechanism in which the bimolecular binding is followed by a slow unimolecular isomerization of the binary complex:

$$E + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ER \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ER' \qquad (1)$$

where E is the enzyme, R is rifampicin, ER and ER′ are two isomers of the enzyme–rifampicin complex, and k_i's are the rate constants. According to this mechanism, the relaxation time associated with the unimolecular isomerization process can be expressed as:

$$\frac{1}{\tau} = k_{-2} + \frac{k_2}{1 + \dfrac{k_{-1}/k_1}{[\bar{E}] + [\bar{R}]}} \qquad (2)$$

where $[\bar{E}]$ and $[\bar{R}]$ are the equilibrium concentrations of free enzyme and rifampicin, respectively. In the presence of excess amounts of rifampicin, $[\bar{R}] \gg [\bar{E}]$, and $[\bar{R}]$ is approximately equal to $[R]_0$, the initial concentration of rifampicin. Thus eq 2 can be written as:

$$\frac{1}{\tau} = k_{-2} + k_2/(1 + K_1/[R]_0) \qquad (3)$$

where $K_1 = k_{-1}/k_1$. When $[R]_0 \rightarrow 0$, $1/\tau \rightarrow k_{-2}$. It can be seen from Figure 4 that k_{-2} is very small. Under this situation eq 3 can be rearranged to give

$$\tau \simeq \frac{1}{k_2} + \frac{K_1}{k_2}\frac{1}{[R]_0} \qquad (4)$$

Thus, by a linear least-squares analysis of the plot of τ vs. $1/[R]_0$, k_2 and K_1 were determined to be 33 s^{-1} and 6.0×10^{-5} M, respectively. Using these values in a nonlinear least-squares analysis of the kinetic data (Figure 4) according to eq 3, a value of 1×10^{-3} M was obtained for k_{-2}.

The overall binding constant for the rifampicin–RNA polymerase interaction (K_0) according to the two-step mechanism (eq 1) can be shown as:

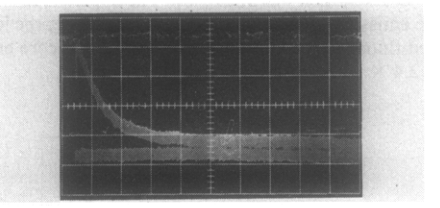

FIGURE 6: Oscilloscope trace of the kinetics of rifampicin binding to the holoenzyme–T7 DNA complex. Enzyme concentration was 1.4×10^{-8} M; DNA concentration was 1.8×10^{-9} M; rifampicin concentration was 6×10^{-7} M, and the buffer was 0.05 M Tris (pH 8.0), 0.05 M KCl, 10^{-2} M $MgCl_2$, 5×10^{-4} M dithiothreitol, 10^{-4} M EDTA. The ordinate is fluorescence change at 340 nm (arbitrary units) and the abscissa is time (1 s/division).

$$K_0 = \frac{(\bar{E})(\bar{R})}{(\overline{ER}) + (\overline{ER}')} = K_1K_2/(1 + K_2) \qquad (5)$$

where $(\overline{ER})$ and $(\overline{ER}')$ are the equilibrium concentrations of the two isomerized rifampicin–enzyme complexes. Since K_2 $(= k_{-2}/k_2)$ is much smaller than 1 (or $k_{-2} \ll k_2$), K_0 is approximately equal to K_1K_2, and was calculated to be 1.8×10^{-9} M from the kinetic parameters for binding of rifampicin to the holoenzyme.

As observed with the holoenzyme, the fluorescence change following rapid mixing of core polymerase with an excess amount of rifampicin showed a single exponential decay. The concentration dependence of $1/\tau$ for the core enzyme–rifampicin interaction was nonlinear (Figure 5) and could be fitted to eq 3. Using the procedures described above for analysis of the holoenzyme data, values of K_1, k_2, and k_{-2} were obtained to be 4.1×10^{-5} M, 190 s^{-1}, 5×10^{-3} s^{-1}, respectively. Similar kinetic studies were carried out with the holoenzyme–T7 DNA complex. Although the signal to noise ratio was relatively low, the fluorescence change also appeared to be a single exponential decay (Figure 6) and the concentration dependence of $1/\tau$ was clearly nonlinear (Figure 7). Therefore, the two-step mechanism described by eq 1 is also applicable to the interaction of rifampicin with the holoenzyme–T7 DNA complex. The values for K_1, k_2, and k_{-2} for such an interaction are 6.8×10^{-6} M, 3.7 s^{-1}, and 1×10^{-3} s^{-1}, respectively. The kinetic and equilibrium parameters for the rifampicin–RNA polymerase interactions are summarized in Table II.

Stoichiometry of Rifampicin Binding at High Concentrations. To elucidate the reaction mechanism by kinetic analysis

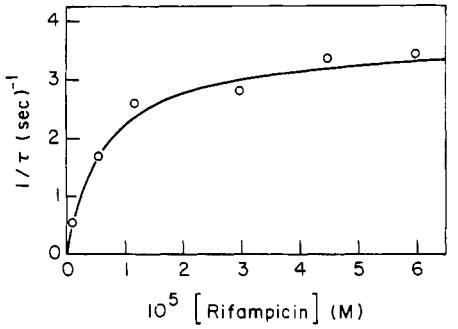

FIGURE 7: Concentration dependence of $1/\tau$ for rifampicin binding to the holoenzyme-T7 DNA complex. Conditions were as described in the legend to Figure 6. Each data point represents the average of 2-3 determinations.

requires that concentrations of reactants be varied as widely as possible. In the kinetic experiments described above, high concentrations of rifampicin (up to 5×10^{-5} M) were used. Since it has been reported that a second rifampicin binding site exists on RNA polymerase (Riva et al., 1972; Fietta and Sylvestri, 1975), a question may be raised whether our kinetic results can be influenced by the binding of a second rifampicin molecule at higher concentrations. We have, therefore, determined the stoichiometry of binding of [^{14}C]rifampicin to the enzyme using both equilibrium dialysis and gel filtrations. The results obtained by these two techniques did not differ appreciably. At the rifampicin concentration of 5×10^{-7} M, we found that the number of moles of rifampicin bound per mole of the core enzyme, the holoenzyme, and the holoenzyme-T7 DNA complex were 0.95 ± 0.15, 1.23 ± 0.12, and 1.22 ± 0.12, respectively. Increasing the rifampicin concentration to 5×10^{-5} M did not significantly alter the stoichiometry of the binding. Under the latter conditions, the isolated σ subunit bound less than 0.06 mol of rifampicin/mole of protein.

Effect of Rifampicin on the Kinetics of RNA Polymerase Reaction. Although the kinetics of RNA polymerase reaction is rather complicated, it has been shown that when three nucleoside triphosphates were present in excess and the concentration of the fourth was varied, normal Michaelis-Menten kinetics was observed (Anthony et al., 1969; Downey and So, 1970). The resulting double-reciprocal plot becomes linear under these conditions and thus can be used to study inhibitor effects by kinetic analysis. Using such analysis, rifampicin behaves as a noncompetitive inhibitor with respect to GTP binding to the enzyme (data not shown). Assuming that the inhibition is noncompetitive, as suggested by the data, a K_i value of 1.6×10^{-9} M was obtained for rifampicin, in good agreement with the value of overall binding constant (1.8×10^{-9} M) for the interaction of rifampicin with the holoenzyme-T7 DNA complex determined by fluorescence stopped-flow measurements. The apparent K_m for GTP was 4.4×10^{-5} M.

Effect of Rifampicin on the Initiation Complex. As mentioned before, So and Downey (1970) found that incubation of the enzyme-poly[d(A-T)] complex with ApU and ATP resulted in the formation of a rifampicin-resistant complex. In their studies, rifampicin was added simultaneously with the missing nucleotide (UTP). Hinkle et al. (1972) pointed out, however, that if rifampicin and nucleoside triphosphates are added simultaneously, the resistance of the enzyme is determined by the relative rates of rifampicin inactivation of enzyme and RNA chain initiation. To obviate such a kinetic effect, we

TABLE II: Kinetic and Equilibrium Parameters for Interaction of RNA Polymerase with Rifampicin.[a]

Parameters	Core E	Holo E	Holo E + T7 DNA
k_2/K_1 (M^{-1} s^{-1})	4.6×10^6	5.5×10^5	5.5×10^5
K_1 (M)	4.1×10^{-5}	6.0×10^{-5}	6.8×10^{-6}
k_2 (s^{-1})	190	33	3.7
k_{-2} (s^{-1})	5.0×10^{-3}	1.0×10^{-3}	1.0×10^{-3}
K_2	2.6×10^{-5}	3.0×10^{-5}	2.7×10^{-4}
K_0 (M)	1.1×10^{-9}	1.8×10^{-9}	1.8×10^{-9}
K_d (M)	$\leq 3 \times 10^{-9}$	$\leq 3 \times 10^{-9}$	$\leq 3 \times 10^{-9}$
K_i (M)			1.6×10^{-9}

[a] The overall binding constant, K_0 was calculated from the kinetic parameters according to eq. 5. The apparent K_d's were measured by fluorimetric titrations (Figure 2). K_i for rifampicin inactivation of RNA polymerase using T7 DNA as template was determined as described in the text.

TABLE III: Effect of Nucleotides and Dinucleotides on Rifampicin-Resistant RNA Synthesis.[a]

Incubations			pmol of [^{3}H]AMP	
I	II	III	Incorporated	% Control
ATP + ApU	—	UTP	8580	100
Rif	—	ApU + ATP + UTP	180	3
ATP	Rif	ApU + UTP	960	11
ATP + ApU	Rif	UTP	3140	37
ATP + ApU	—	UTP (2×10^{-5} M)	1900	100
ATP + ApU	Rif	UTP (2×10^{-5} M)	900	47

[a] Reaction mixture (0.25 ml) contained: 0.05 M Tris (pH 7.9), 0.05 M KCl, 10^{-2} M $MgCl_2$, 10^{-3} M dithiothreitol, 40 nmol of poly[d(A-T)], and 6 pmol of holoenzyme. Samples were incubated with compounds listed in column I for 2 min at 37 °C. Following the addition of rifampicin (II), samples were incubated for 2 min at 37 °C after which compounds listed in column III were added and the incubation continued for another 5 min at 37 °C. Concentrations of the compounds used were 1.2×10^{-6} M for rifampicin, 2×10^{-4} M each for ApU, ATP, and UTP except as otherwise indicated. Acid-insoluble radioactivity was determined by liquid scintillation counting.

have examined the effect of rifampicin on the preinitiated enzyme complex. As shown in Table III preincubation of the enzyme-poly[d(A-T)] complex with ATP alone yields 11% rifampicin-resistant synthesis. Preincubation with both ApU and ATP results in 37% rifampicin-resistant synthesis; this value increases to 47% when the UTP concentration is decreased tenfold to retard chain elongation.

Effect of Rifampicin on the Elongation Complex. If one nucleoside triphosphate is present at limiting concentrations, the elongation of RNA chains occurs at a much slower rate (Anthony et al., 1969; Rhodes and Chamberlin, 1974). Under these conditions, there is no significant reinitiation of RNA chains and thus the properties of the elongation complex can be studied. Such a system is shown in Figure 8. The incorporation of [^{3}H]GMP into RNA with T7 DNA as template was almost linear with respect to time for at least 20 min when the UTP concentration was limiting (2×10^{-6} M). Addition of

TABLE IV: Binding of [^{14}C]Rifampicin to Holoenzyme, Enzyme–DNA Complex, and Elongation Complex.[a]

Conditions	mol of [^{14}C]Rifampicin/mol of Enzyme
Holo E	1.23 ± 0.12
Holo E + T7 DNA	1.22 ± 0.12
Holo E + T7 DNA + 4 NTP's (elongation complex)	1.07 ± 0.18

[a] Reaction mixtures (1 ml) containing 30 pmol of enzyme in 0.05 M Tris (pH 8), 0.05 M KCl, 10^{-2} M $MgCl_2$, 10^{-3} M dithiothreitol, and 10^{-4} M EDTA were incubated, where indicated, with 3.8 pmol of T7 DNA in the presence or absence of 2×10^{-4} M each of ATP, GTP, and CTP; and 2×10^{-6} M UTP for 5 min at 37 °C. After 5 min, [^{14}C]rifampicin (40 cpm/pmol) was added to a final concentration of 6×10^{-7} M and incubated for 2 min. Immediately following this the samples were passed through a Sephadex G-75 column equilibrated with the above buffer. The radioactivity eluting in the void volume was determined by scintillation counting.

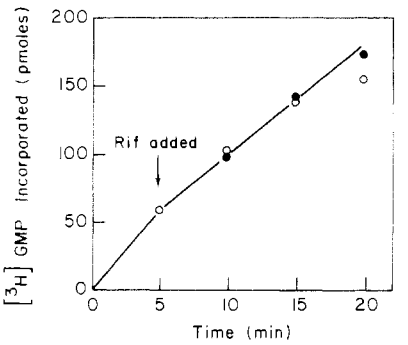

FIGURE 8: Effects of rifampicin on kinetics of [^{3}H]GMP incorporation at limiting UTP concentration. Reaction mixtures (0.25 ml) contained: 0.05 M Tris (pH 7.8), 0.05 M KCl, 10^{-2} M $MgCl_2$, 10^{-3} M dithiothreitol, 2×10^{-4} M each of ATP, CTP, and [^{3}H]GTP (10 cpm/pmol), 2×10^{-6} M UTP, 0.44 pmol of T7 DNA, and 3 pmol of the σ-saturated holoenzyme. Following 5 min of incubation at 37 °C, rifampicin was added to a final concentration of 9.6×10^{-7} M and the incubation continued for the time period indicated. (●), rifampicin added at 5 min; (○), no rifampicin.

rifampicin 5 min after synthesis had started did not inhibit nucleotide incorporation. The relative efficiency of chain initiation under these conditions was determined by measuring [γ-^{32}P]ATP incorporation into 5′-termini of RNA chains (Maitra and Hurwitz, 1965). If rifampicin was added at 5 min, 0.44 mol of [γ-^{32}P]ATP was incorporated/mole of enzyme as compared to 0.40 mol without addition of rifampicin. Thus, rifampicin did not affect the chain initiation at 5 min after the reaction started; presumably all active enzyme molecules had initiated RNA chains by then and were in the process of elongating the chains. There appeared to be no significant reinitiation during this period, and assuming the ratio of ATP/GTP to initiate RNA chains is 2/1 with T7 DNA (Chamberlin and Ring, 1972), about 60–66% of RNA polymerase molecules were actively participating in RNA synthesis under these conditions.

Since the elongation complex described above is resistant to rifampicin, we examined whether such a complex binds rifampicin. Table IV shows that both the holoenzyme and the holoenzyme–T7 DNA complex bind about 1 mol of [^{14}C]rifampicin/mol of enzyme. This value is not significantly altered for the elongation complex. Since about 60% of RNA polymerase was actively engaged in RNA synthesis under these conditions, one would expect less than 0.4 mol of rifampicin bound/mole of enzyme if the elongation complex did not bind rifampicin. Thus, an enzyme complex which is elongating RNA chains can still bind rifampicin, although the enzyme activity is not inhibited by such binding.

Discussion

By means of fluorimetric titration we have demonstrated that rifampicin binds tightly to both the holo and core enzyme of *E. coli* RNA polymerase with an apparent K_d of $\leq 3 \times 10^{-9}$ M. Thus, the presence of σ subunit does not significantly alter the interaction of rifampicin with RNA polymerase. This is not surprising, since rifampicin inhibits in vitro RNA synthesis catalyzed by either holo or core polymerase (di Mauro et al., 1969) and agrees with our previous observations that the rifampicin binding site is at least 10 Å away from the surface of σ subunit based on energy transfer measurements (Wu et al., 1976). Recently, however, Stender et al. (1975) found that an alkyl derivative of rifamycin SV reacts with the σ, as well as the β subunit in the holoenzyme. Since alkylating reagents may attack sulfhydryl groups without discrimination,[2] the modification of σ could be nonspecific. That this may be the case is suggested by our observations that the stoichiometry of rifampicin binding to both the core and holoenzyme is close to 1:1 even at high concentrations of the drug and that the isolated σ subunit does not bind significant amounts of rifampicin. The values of apparent K_d for the rifampicin–holoenzyme complex and the degree of fluorescence quenching observed under low salt ($\leq$0.05 M KCl) or high salt ($\geq$0.2 M KCl) conditions were essentially the same. RNA polymerase holoenzyme is known to exist as a monomer in the presence of high salt and as a dimer in the presence of low salt (Richardson, 1969; Berg and Chamberlin, 1970). Our results indicate that the rifampicin binding sites are exposed when RNA polymerase exists as a dimer. These observations are in accord with the sedimentation analysis of Wehrli and Staehelin (1969) which showed that the monomeric form of RNA polymerase binds one molecule of rifampicin, while the dimer binds two molecules.

RNA polymerase isolated from mutants of *E. coli* resistant to rifampicin inhibition has been shown to contain an altered β subunit (Heil and Zillig, 1970). Since RNA polymerase covalently labeled with a fluorescent nucleotide analogue at the initiation site on the β subunit still binds rifampicin (Wu and Wu, 1974a), the rifampicin binding site does not overlap with the initiation site on the enzyme. In fact, fluorescence energy-transfer measurements indicate that these two sites are about 37 Å apart (Wu and Wu, 1974a). This is consistent with the present observation that fluorescence quenching of RNA polymerase by rifampicin is not altered by addition of nucleotides and that rifampicin is a noncompetitive inhibitor with respect to nucleoside triphosphate. These results also agree with the finding of Mangel and Chamberlin (1974) that the presence of ATP does not affect the rate of inactivation of RNA polymerase by rifampicin.

If the binding of rifampicin to RNA polymerase is a simple bimolecular process, the apparent pseudo-first-order rate constant for binding ($1/\tau$) should exhibit a linear dependence on the rifampicin concentration. However, this is not the case. The value of $1/\tau$ increases with increasing rifampicin concentration and starts to level off when the rifampicin concentration is beyond 10^{-5} M. Such a kinetic behavior can be in-

[2] We have reported that one sulfhydryl group of σ reacts readily with a hydrophobic iodoacetamide derivative, *N*-(iodoacetamidoethyl)-5-naphthylamine-1-sulfonate (Wu et al., 1976).

terpreted by a two-step mechanism (eq 1) in which a rate-limiting isomerization follows a rapid bimolecular binding. The fluorescence change observed in the kinetic studies, which accounts for most part of the total quenching measured by the steady-state method, is associated with the isomerization step. The bimolecular step, on the other hand, is presumably too rapid to be detected by the stopped-flow technique.

The kinetic parameters for the two-step mechanism are summarized in Table II. The dissociation constant K_1 ($= k_{-1}/k_1$) is roughly the same for the core and holoenzyme but about tenfold less for the holoenzyme-T7 DNA complex. Physically, this means that the presence of the σ subunit or DNA does not prevent the formation of the first enzyme-rifampicin binary complex (ER). In contrast, the rate for the forward step in the isomerization, k_2, decreases in the order of core enzyme > holoenzyme > the holoenzyme-T7 DNA complex. Since the rate constant k_{-2} is extremely small compared to k_2, the isomerization process is practically irreversible. The values of K_0 calculated from the kinetic parameters are $1\text{-}2 \times 10^{-9}$ M for core enzyme, holoenzyme, and the holoenzyme-T7 DNA complex, in good agreement with the values of apparent K_d obtained from fluorimetric titrations and K_i determined by enzymatic assays.

Hinkle et al. (1972) found that core polymerase is inactivated more rapidly by rifampicin than the holoenzyme which in turn is inactivated more rapidly than the holoenzyme-T7 DNA complex. The fact that this order of inactivation is parallel to the relative rates of isomerization (k_2) of the rifampicin-enzyme and rifampicin-enzyme-DNA complex suggests that the inactivation may be due to the rifampicin-induced isomerization (or conformational change) of the enzyme. This contention is further supported by the agreement between our kinetic parameters (Table II) and the kinetic data of Hinkle et al. on the inactivation of RNA polymerase by rifampicin (1972). In the presence of a tenfold molar excess of rifampicin, the inactivation kinetics was pseudo-first order. Assuming that the inactivation was due to a simple bimolecular binding, an apparent second-order rate constant could be calculated. (According to our mechanism, this is, of course, not a true second-order rate constant but represents k_2/K_1). The value of the apparent second-order rate constant for inactivation of the holoenzyme by rifampicin (4.4×10^5 M^{-1} s^{-1}) obtained by Hinkle et al. (1972) agrees well with our value of k_2/K_1 (5.5×10^5 M^{-1} s^{-1}) for the rifampicin-holoenzyme interaction. However, this value is not consistent with the observed rate constant for the rifampicin-enzyme-DNA complex. For inactivation of the holoenzyme-T7 DNA complex by rifampicin the apparent second-order rate constant was calculated to be 3.5×10^3 M^{-1} s^{-1} (Hinkle et al., 1972), whereas the value of k_2/K_1 for the interaction of the holoenzyme-T7 DNA complex with rifampicin does not differ from that of the holoenzyme alone. It is possible that this marked difference is due to the temperature used in our experiments (25 °C) which differed from that used by Hinkle et al. (1972) (37 °C). Alternatively, the isomerization of the rifampicin-holoenzyme-T7 DNA complex we observed is not the one responsible for inactivation; inactivation could be due to an even slower conformational transition of the complex which is not detectable spectroscopically. In any case, the inactivation is not due simply to the bimolecular binding which occurs much faster. It should be pointed out that the "bimolecular rate constant" (3.5×10^3 M^{-1} s^{-1}) for the holoenzyme-T7 DNA complex obtained indirectly from inactivation kinetics is most likely not a true bimolecular rate constant. This value is too low in comparison with the known values of bimolecular rate constants for protein-ligand interactions (10^6-10^8 M^{-1} s^{-1}) (Hammes and Schimmel, 1970).

If the inactivation of RNA polymerase is indeed due to a conformational change of the enzyme induced by rifampicin, rather than the binding process itself, it is conceivable that there might be conditions under which the enzyme could bind rifampicin and not suffer inactivation. We have found that the holoenzyme-T7 DNA elongation complex fulfilled such conditions. The elongation complex obtained by limiting the concentration of one of the four nucleoside triphosphates bound rifampicin almost as well as free holoenzyme or the holoenzyme-T7 DNA complex (Table IV). Under the same conditions used for binding studies, rifampicin did not inhibit RNA synthesis for at least 15 min (Figure 8). Thus, an enzyme molecule actively engaged in elongation of RNA chains can still bind rifampicin. Our results are consistent with those of Neuhoff et al. (1971) but differ from those of Lill and Hartmann (1973) and Eilen and Krakow (1973), who found that the elongation complex bound only 20-25% as much rifampicin as the enzyme-DNA complex. The reasons for this discrepancy are not clear. Since we have demonstrated that there was no significant reinitiation of RNA chain, the observed binding of rifampicin to the complex cannot be attributed to enzyme release and reinitiation, as suggested by Eilen and Krakow (1973). Perhaps a possible explanation for the less than stoichiometric binding of rifampicin by the elongation complex is due to dissociation of rifampicin from the complex during the separation process. The time required for 50% exchange of [^{14}C]rifampicin from the holoenzyme-rifampicin complex is approximately 30 min (Yarbrough and Wu, unpublished observation). In our binding studies, the rifampicin-enzyme-DNA elongation complex was separated within 7 min after rifampicin was added. Delay in separation process could result in considerable dissociation of rifampicin from the complex.

The nature of the rifampicin-resistant enzyme complex is not known. Several reports suggest that RNA chain initiation and perhaps the formation of at least two phosphodiester bonds are necessary for RNA polymerase to become rifampicin resistant (Sippel et al., 1968; Hinkle et al., 1972; So and Downey, 1970). Consonant with the observations of So and Downey (1970) we have found that when poly[d(A-T)] was used as template, ATP provided little protection against rifampicin inactivation; in contrast, preincubation of the enzyme with ATP and ApU resulted in a partial but significant rifampicin-resistant RNA synthesis. Since ApU can act as a primer and yield an initiation complex in the presence of ATP (Downey and So, 1970; Downey et al., 1971), these results indicate that phosphodiester bond formation is necessary but not sufficient for the production of a rifampicin-resistant initiation complex. This is consistent with the finding (Johnston and McClure, 1976) that rifampicin only partly inhibits the synthesis of dinucleoside tetraphosphate by RNA polymerase. These partial inhibition or protection data can be interpreted based on our kinetic mechanism: the existence of two RNA polymerase-rifampicin complexes, only one of which is able to bind the initiating triphosphates and catalyze the phosphodiester bond formation.

Knowledge concerning the kinetics of RNA chain initiation is important in elucidating the molecular mechanism of gene transcription. Although RNA chain initiation has been extensively studied by following the incorporation of γ-^{32}P-labeled ribonucleoside triphosphates, the rate of RNA chain initiation is too rapid to be measured directly. When RNA polymerase-T7 DNA complexes are simultaneously challenged with a mixture of rifampicin and the four ribonucleoside

triphosphates, two reactions occur: (a) inactivation of the enzyme by rifampicin, and (b) initiation of RNA chains which results in the formation of the rifampicin-resistant complex (Hinkle et al., 1972). Assuming that these two reactions are irreversible and competing with each other, Mangel and Chamberlin (1974) have attempted to measure the intrinsic rate of RNA chain initiation using the second-order rate constant for rifampicin attack on the holoenzyme–T7 DNA complex indirectly obtained, as discussed before. Since the inactivation of the enzyme by rifampicin is apparently not due to the bimolecular binding but rather due to an isomerization step following it, the use of the "second-order" rate constant for rifampicin inhibition is obviously oversimplified. Furthermore, RNA chain initiation, which converts the enzyme–DNA complex to a rifampicin-resistant form, is rather complicated and requires at least (a) the binding of two ribonucleoside triphosphates to the enzyme–DNA complex (second order) and (b) the formation of first phosphodiester bond (first order). Possibly other steps such as substrate-induced conformational change of the enzyme and translocation of the enzyme on the template are also involved. The rates at which these reactions occur are presently unknown. However, in view of the complexity and rapidity of the initiation processes, the elucidation of the kinetic mechanism of RNA chain initiation will require direct measurements by fast kinetic techniques.

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