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EFFECTS OF SIGMA SUBUNIT AND DNA TEMPLATE ON THE ACCESSIBILITY OF RIFAMYCIN BOUND TO RNA POLYMERASE

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Summary. Measurements of energy transfer in the rapid-diffusion limit from terbium complexes to rifamycin bound to E. coli RNA polymerase core and holoenzyme show that removal of the enzyme's sigma subunit markedly decreases the accessibility of bound rifamycin to small probe molecules in solution. Binding of holoenzyme to DNA also decreases the accessibility of enzyme-bound rifamycin. These results are consistent with the notion that rifamycin binds in a cleft on RNA polymerase which is held open by the sigma subunit, and which is involved in DNA binding. The use of D_2O buffers to improve experimental accuracy is also described.

INTRODUCTION: DNA-dependent RNA polymerase is a multisubunit enzyme which catalyzes the transcription of DNA. In procaryotes, the enzyme has two basic functional forms; core, $\beta\beta'\alpha_2$, and holoenzyme, $\beta\beta'\alpha_2\sigma$ (1). The sigma subunit has a well-defined role in transcription, greatly increasing the specificity of initiation (2).

Rifamycin specifically inhibits procaryotic RNA polymerases; it binds tightly to both core- and holoenzyme as well as to RNA polymerase:DNA complexes (3). The primary locus of the rifamycin binding site on RNA polymerase appears to be on the beta subunit (4) although affinity labeling experiments have shown that the rifamycin binding site actually lies within about 1 nm of all the subunits of the enzyme including sigma (5,6).

The accessibility of enzyme-bound rifamycin to small molecules in solution has been probed with energy transfer in the "rapid-diffusion limit," in which a freely diffusing energy donor is influenced by many possible energy acceptors during its lifetime (7). Because all mechanisms of

Abbreviations; HED3A, N-hydroxyethylethylenediaminetriacetate;
Tb-HED3A, the terbium(III) complex of HED3A.

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radiationless energy transfer depend strongly on the distance between donor and acceptor, the efficiency of energy transfer in the rapid-diffusion limit will depend on whether a macromolecule-bound energy acceptor is accessible to collisions with the energy donor. The transfer of energy from freely-diffusing luminescent terbium chelates to the red chromophore of rifamycin showed that holoenzyme-bound rifamycin is exposed to small molecules in the solvent (7).

As described below, diffusion-enhanced energy transfer experiments performed with rifamycin bound to pure core enzyme, or to the holoenzyme-DNA complex, show that while holoenzyme-bound rifamycin is freely accessible to small probe molecules in the solvent, rifamycin's accessibility is reduced by removal of the sigma subunit, or by addition of the DNA template.

MATERIALS AND METHODS

These were as previously described (7), except for the following. The THGD buffer contains 10 mM tris(hydroxymethyl)aminomethane hydrochloride, pH 7.5, 0.1 mM dithiothreitol, 0.1 mM HED3A and 5% glycerol (v/v). For experiments in D_2O buffers, stock solutions were made in 98% D_2O and stored desiccated at 4°C. No special effort was made to achieve 100% deuteration.

The method of Lowe et al. (8) was used to purify RNA polymerase from E. coli MRE 600 cells which were purchased from the Grain Processing Co. To separate core from holoenzyme, the method of Weissbach and Poonan (9) was used to prepare a single-strand DNA column. Enzyme purity was confirmed by SDS-gel electrophoresis (10). Before physical studies the enzyme was concentrated, either by stepwise elution from a DEAE-cellulose column using THGD + 0.5 M NaCl, or by using a Millipore ultrafiltration cell, to give a final enzyme concentration of 10-12 mg/ml. After concentration the enzyme was dialyzed into THGD + 0.5 M NaCl in either H_2O or D_2O. If DNA template was included, RNA polymerase and template were concentrated together and dialyzed against THGD + 0.1 M NaCl in D_2O buffers. Protein concentrations were determined by the method of Sedmak and Grossberg (11). The assay method of Burgess (12) was used to determine RNA polymerase activity.

Stock solutions of rifamycin SV were freshly prepared in the appropriate buffer. The terbium chelate stock solutions were prepared in either H_2O or D_2O and were always 50 mM Tb (III) with a 20% excess of HED3A to ensure complete binding of the metal ion. The concentrations of the rifamycin SV solutions were measured on a Gilford spectrophotometer using $\varepsilon_{445} = 14240\ cm^{-1}\ M^{-1}$ (13).

Millisecond Fluorescence Lifetime Measurements. Lifetime measurements of excited Tb donors were measured using a home-made arc-gap flash lamp as an excitation source. The lamp was pulsed at 15-25 Hz; pulse duration was $\sim 10^{-8}$ sec. The output of the excitation source was filtered with a UV band-pass filter. Terbium emission was monitored at 546 nm with a 56DVP photomultiplier tube after passing through a potassium dichromate solution filter and a 546 nm interference filter. The photomultiplier output was accumulated

Table I. Rate constants for energy transfer from Tb-HED3A to rifamycin at 20°C in THGD buffer + .5 M NaCl (for the DNA experiment, THGD + .1 M NaCl).

Acceptor	Solvent	10^{-7} $K_2(M^{-1}$ $sec^{-1})$
rifamycin	H_2O	2.5 ± 0.02
	D_2O	2.04 ± 0.6
rifamycin + holo	H_2O	1.43 ± 0.25
	D_2O	0.94 ± 0.13
rifamycin + core	H_2O	0.34 ± 0.06
	D_2O	0.28 ± 0.03
rifamycin + holo + DNA	D_2O	0.37 ± 0.05

in a Nicolet 1170 multiscaler; several thousand decays were summed. Excited state lifetimes were determined by a non-linear least squares fit of the data to a single exponential decay.

In all experiments there was a two-fold excess of RNA polymerase to rifamycin SV. Because of the limited solubility of the enzyme, only low concentrations of bound rifamycin could be studied. In some cases, the resulting change in the measured lifetime was as small as 6% (core RNA polymerase + rifamycin in H_2O buffer). To be sure that this effect was measured accurately, all experiments were performed on at least three independently prepared samples according to the following protocol. First the lifetime of the terbium chelate was measured in the presence of RNA polymerase; then the desired amount of inhibitor was added and the donor lifetime measured again. Afterward the rifamycin concentration was confirmed by visible absorbance measurements and the enzyme was assayed to confirm inhibition was occurring. In this way pipetting error (the major cause of variation in the measured lifetimes) could be corrected before calculation of the rate of energy transfer. The resulting values for the rates showed little variation, as indicated by the standard deviations in Table I.

Lifetime measurements were performed at 20°C using 50-60 µl of freshly prepared solutions. The enzyme concentrations were 20-24 µM. For the experiments involving template, the solutions contained 22 µM RNA polymerase, 0.5 mg/ml poly dAT, and 10 mM $MgCl_2$. The final concentration of rifamycin SV in each solution was 9-12 µM. The concentration of the Tb chelate was 5 mM.

Use of D_2O. For energy transfer in the rapid-diffusion limit, the rate of decay of excited terbium is $k = k_o + k_T[A] = k_r + k_{H_2O}n_{H_2O} + k_x + k_T[A]$, where k_r is the rate of photon emission, k_{H_2O} is the rate constant for de-excitation by coordinated H_2O (with n_{H_2O} the number of H_2O molecules coordinated to each terbium ion), k_x is the rate of de-excitation from other causes, and k_T is the second-order rate constant for energy transfer (with [A] the molar concentration of acceptors). For Tb-HED3A, $n_{H_2O} \cong 3$ and $k_{H_2O} \simeq 215\ s^{-1}$ (14). For Tb-HED3A in H_2O-THGD buffer, $k_o^H = \frac{1}{\tau_o^H} = 943 \pm 50\ s^{-1}$, while for Tb-HED3A in D_2O-THGD buffer, $k_o^D = \frac{1}{\tau_o^D} = 352 \pm 16\ s^{-1}$.

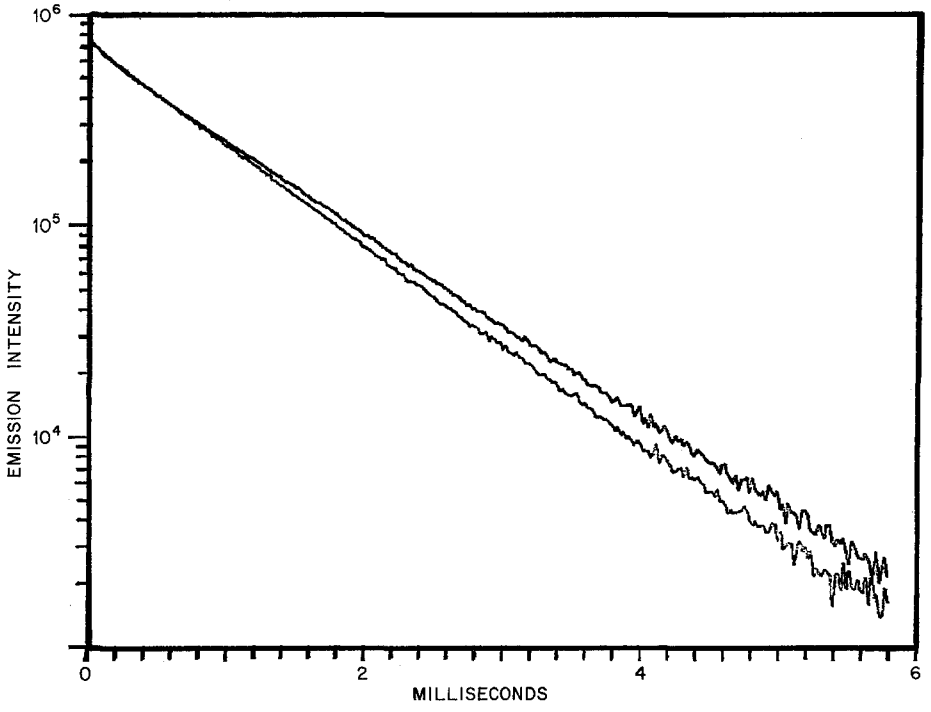

Figure 1. Semilogarithmic plot of terbium 546 nm emission intensity vs. time for 5 mM Tb-HED3A and 18 μM core RNA polymerase in H_2O-THGD buffer in the presence (lower line) and absence (upper line) of 9.4 μM rifamycin SV. This example represents the smallest effect observed. Results are summarized in Table I and Figure 2.

Since energy-transfer measurements are determined from the change in lifetime, $k_T[A] = k - k_o = \frac{1}{\tau} - \frac{1}{\tau_o}$, the results become inaccurate as $\tau \to \tau_o$. For the experiments described here $k_T[A] \approx 10^2$ sec^{-1}, which is more accurately measured as $k_T[A] \simeq 452\text{-}352$ sec^{-1} in D_2O than as $k_T[A] \simeq 1043\text{-}943$ sec^{-1} in H_2O, when experimental errors are considered.

RESULTS AND DISCUSSION

Figure 1 shows experimental Tb decays for the situation with the smallest effect due to energy transfer, the core enzyme in H_2O-THGD buffer. The results from these data are in good agreement with the more accurately measurable D_2O-THGD case, as summarized in Table I.

The most striking feature of the energy-transfer rate constants in Table I is that rifamycin bound to core enzyme is considerably less accessible than rifamycin bound to holoenzyme; removal of the sigma subunit does not expose the rifamycin site but rather has the opposite effect. This result is particularly interesting in view of previous experiments (see

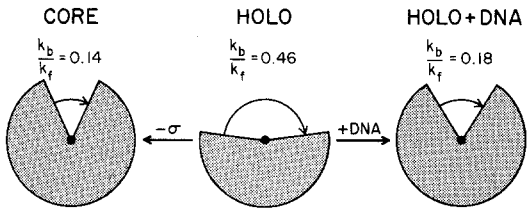

Figure 2. Accessibility of Tb-HED3A to rifamycin bound to RNA polymerase core, holo, and holo + DNA, relative to free rifamycin accessibility. Macromolecules are schematically represented as cylinders having longitudinal clefts, with rifamycin bound at the center. Tb-HED3A probes interact with bound rifamycin only when they diffuse into the clefts.

reference 7) implying that rifamycin binds in a cleft between enzyme subunits; it is consistent with the notion that the sigma subunit holds this cleft open. Because the sigma subunit is required for specific binding of RNA polymerase to promoter sites on DNA, it is natural to speculate that the cleft has something to do with DNA binding. This is consistent with the observation (Table I) that binding of DNA to holoenzyme reduces the accessibility of enzyme-bound rifamycin.

The results of these experiments are illustrated in Figure 2, where the energy-transfer rate for free rifamycin is compared to that of rifamycin bound to each macromolecular complex. The enzyme is schematically represented as a long cylinder (viewed end-on) with a longitudinal cleft, having rifamycin at the center; the cleft angle may be calculated from the ratio of energy-transfer rate constants.

Because it reveals the accessibility of chromophores bound to macromolecules, diffusion-enhanced energy transfer can provide a quantitative measure of conformational changes which would be difficult to obtain otherwise. Studies of RNA polymerase as a function of temperature, and comparison to the extensive kinetic and thermodynamic data available (e.g. 3, 15, 16) may provide further insight into the structure and functions of this multisubunit enzyme.

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