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Diffusion-Enhanced Energy Transfer Shows Accessibility of Ribonucleic Acid Polymerase Inhibitor Binding Sites†

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ABSTRACT: Rifamycin and Cibacron Blue F3GA are powerful inhibitors of *Escherichia coli* deoxyribonucleic acid (DNA) dependent ribonucleic acid (RNA) polymerase. In addition, both inhibitors strongly absorb visible light, making them suitable for use as acceptors in energy-transfer experiments. Transfer of energy to these acceptors from *freely diffusing* energy donors with long excited-state lifetimes ($\approx 10^{-3}$ s) depends strongly on whether donor and acceptor can make direct intermolecular contact. We observe that the rate constant for energy transfer from a small terbium chelate to enzyme-bound rifamycin is 1×10^7 M^{-1} s^{-1}, which is about *half* as large as the rate constant observed for *free* rifamycin in solution. This relatively small change upon binding indicates that enzyme-bound rifamycin is highly accessible to small molecules in the solvent. In the case of Cibacron Blue, under conditions where ~90% of this inhibitor is bound to RNA polymerase, the small amount of unbound inhibitor accounts for practically all of the observed energy transfer. This implies that enzyme-bound Cibacron Blue is relatively inaccessible to energy donors in the solution. The dependence of energy transfer on the accessibility of the acceptor is illustrated by using simple geometric models. Synthesis of a stable, electrically neutral terbium chelate which can be efficiently excited with UV radiation is also described.

Deoxyribonucleic acid (DNA)[1] dependent RNA polymerase (EC 2.7.7.6) from *Escherichia coli* is a complex enzyme composed of five subunits, $\beta'\beta\alpha_2\sigma$ (Burgess & Jendrisak, 1975), with a composite molecular weight of 4.8×10^5. The synthesis of RNA on a DNA template by this enzyme is strongly inhibited by the antibiotic rifamycin, whose binding site is thought to be on the β subunit of the enzyme (Heil & Zillig, 1970). Affinity-labeling studies indicate, however, that the bound inhibitor is within ~1 nm of nearly all the subunits (Stender et al., 1975; Rice & Meares, 1978). The rifamycin binding site on RNA polymerase appears to lie in the path of the growing RNA chain, since only di- and trinucleotides may be formed by the enzyme in the presence of rifamycin (McClure & Cech, 1978; McClure, 1980).

Another RNA polymerase inhibitor is the dye Cibacron Blue, which is thought to bind to the "dinucleotide fold" of a variety of nucleotide-binding enzymes (Thompson et al., 1975). Evidently, Cibacron Blue and rifamycin have separate binding sites on RNA polymerase (Kumar & Krakow, 1977; L. S. Rice, unpublished observations).

As part of understanding the mechanism of their effect on RNA polymerase, it is important to know whether these inhibitors lie on the outer surface of the enzyme or buried among the subunits. Since both inhibitors absorb visible light, a means of doing this was suggested to us by the experiments of Thomas

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[1] Abbreviations used: HED3A, *N*-(2-hydroxyethyl)ethylenediaminetriacetic acid; Tb-HED3A, the terbium(III) chelate of HED3A; Bzl-HED3A, (*S*)-*N*-[2-[bis(carboxymethyl)amino]-3-phenylpropyl]-*N*-(2-hydroxyethyl)glycine (see Figure 2); Tb-Bzl-HED3A, terbium(III) chelate of Bzl-HED3A (see Figure 2); DNA, deoxyribonucleic acid; RNA, ribonucleic acid; DEAE, diethylaminoethyl.

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et al. (1978) involving excitation energy transfer between *freely diffusing* energy donors and chromophoric energy acceptors in solution. When the average excited donor is influenced by many potential acceptors during its lifetime, the interpretation of the observed rate of energy transfer can be very simple. In this "rapid-diffusion" limit, all energy donors are in equivalent environments because, on the long time scale of the experiment ($\approx 10^{-3}$ s for terbium complexes), each donor samples a quasi-equilibrium distribution of chromophores in solution around it. Because all mechanisms of radiationless energy transfer depend strongly on the distance between donor and acceptor (Dexter, 1953), the efficiency of energy transfer from a small, freely diffusing donor to a macromolecule-bound chromophore will depend on whether the chromophore is accessible to collisions with the donor. Here we describe the use of two electrically neutral chelates of terbium(III) as long-lived energy donors to probe the accessibility of enzyme-bound rifamycin or Cibacron Blue to small molecules in solution.

Theory

The transfer of electronic excitation from one molecular species to another a fixed distance away has been thoroughly studied, both experimentally and theoretically (Förster, 1948; Dexter, 1953; Stryer, 1978). A few workers have also investigated the opposite extreme: energy transfer in the rapid-diffusion limit (complete-mixing limit), where the average energy donor encounters many possible energy acceptors during its lifetime (Galanin, 1960; Shakverdov & Bodunov, 1973; Thomas et al., 1978). The mechanism of energy transfer from a donor to a fixed acceptor a few nanometers away can be described by the interaction of the transition dipoles of donor and acceptor (Förster, 1948; Stryer & Haugland, 1967). In contrast, if an excited donor can undergo collisions with energy acceptors, energy transfer may be dominated by the electron-exchange mechanism (C. F. Meares, S. M. Yeh, and L. Stryer, unpublished results). This process depends on overlap of the electron clouds of donor and acceptor (Dexter, 1953).

In any case, energy transfer in the rapid-diffusion limit obeys pseudo-first-order kinetics: after a pulse of exciting light, the population of excited donors decays exponentially with time and the rate constant for this decay is linearly related to the concentration of acceptors. Thus

$$-\frac{d[Tb^*]}{dt} = \frac{1}{\tau}[Tb^*] = \left(\frac{1}{\tau_0} + k_2[A]\right)[Tb^*] \qquad (1)$$

where [Tb*] is the molar concentration of excited donors, τ is the observed donor lifetime in the presence of acceptor, τ_0 is the lifetime in the absence of acceptor, [A] is the molar concentration of acceptors, and k_2 is the observed second-order rate constant for energy transfer.

All donors in the system will have the same value of k_2 *only if* each excited donor is influenced by many potential acceptors during its lifetime. This condition is assured if $[6(D_A + D_D)\tau]^{1/2} \gg s$, where D_A and D_D are the diffusion coefficients of acceptor and donor and s is the average distance between acceptors. By use of the results of Chandrasekhar (1943), $s = (6.5 \times 10^{-10})[A]^{-1/3}$ m. Therefore, for a small, spherical donor in aqueous solution at 293 K with $D_D \cong 5 \times 10^{-10}$ m^2/s and $\tau \cong 10^{-3}$ s, the rapid-diffusion limit will be reached if $[A]^{1/3} \gg 4 \times 10^{-4}$, independent of the size or shape of the acceptor. Concentrations of acceptor $> 10^{-6}$ M satisfy this condition adequately.

For *spherical* donors and acceptors (of radius R_D and R_A, respectively), the dipole–dipole contribution to the second-order rate constant for energy transfer is calculated by integrating over all possible separations r:

$$k_{df} = \frac{10^{-24}N}{\tau_0}\int_{R_A+R_D}^{\infty}\left(\frac{R_0^6}{r^6}\right)4\pi r^2\,dr = \frac{2.523R_0^6}{\tau_0(R_A + R_D)^3}\ M^{-1}\ s^{-1} \qquad (2)$$

where all distances are in nanometers, R_0 is the distance at which energy transfer efficiency is 50%, and $N = 6.023 \times 10^{23}$ mol^{-1}. R_0 is calculated from the expression

$$R_0 = 979(JQ_0\kappa^2 n^{-4})^{1/6}\ \text{nm} \qquad (3)$$

$$J = \frac{\int_0^{\infty} F(\lambda)\epsilon(\lambda)\lambda^4\,d\lambda}{\int_0^{\infty} F(\lambda)\,d\lambda} \qquad (4)$$

in which J is the spectral overlap integral, $F(\lambda)$ is the relative fluorescence intensity of the donor at the wavelength λ, and $\epsilon(\lambda)$ is the molar extinction coefficient of the acceptor at the same wavelength. Q_0 is the quantum yield of the donor in the absence of the acceptor, κ^2 is the orientation factor between the dipoles, and n is the refractive index of the intervening medium.

By comparing an observed k_2 to k_{df} calculated from eq 2, the importance of the dipolar mechanism to a particular experimental situation may be determined. For example, we have found that for energy transfer between terbium chelates and transition metal chelates in solution, the dipolar mechanism could only account for a few percent of the observed k_2 (C. F. Meares, S. M. Yeh, and L. Stryer, unpublished results); this implies that the exchange mechanism provides the major route for energy transfer in those systems.

An explicit equation analogous to eq 2 is not available for the exchange mechanism, but this process is expected to have exponential dependence upon distance so that the rate constant is proportional to

$$\int_{R_A+R_D}^{\infty} e^{-2r/L}r^2\,dr \qquad (5)$$

where r is the distance between donor and acceptor and L is the average effective Bohr radius of donor and acceptor (Dexter, 1953). This mechanism has a very sharp distance dependence; it drops off much more rapidly than the dipole–dipole mechanism, so that energy transfer by exchange practically requires *direct contact* between the donor and acceptor.

For a description of energy transfer involving a chromophore bound to a macromolecule, it must be recognized that the steric features of the macromolecule can hinder or even prevent a freely diffusing energy donor from colliding with the chromophore. If the rate of energy transfer is dominated by the exchange interaction, it will depend directly on the fraction of surface area of the acceptor chromophore which is accessible to the donor. If energy transfer is dominated by the dipolar interaction, which declines more gently with distance, the rate of energy transfer will still be affected significantly by the accessibility of donor to acceptor.

Two simple physical models serve to illustrate the effect of accessibility on energy transfer. In the first model (Figure 1a) the bound chromophore is located in a deep cleft in a macromolecule, such that for either mechanism energy transfer from donors located outside the cleft will be insignificant. In this case, if the rate of energy transfer to the bound chro-

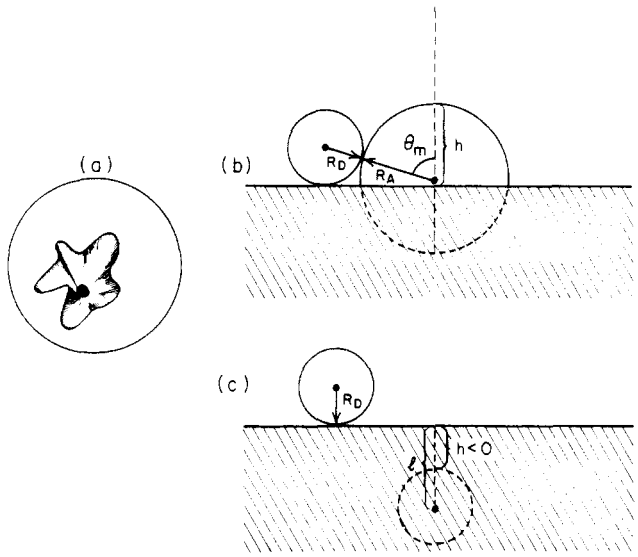


FIGURE 1: Simple models for enzyme-bound energy acceptors. (a) Acceptor in a deep cleft with accessible solid angle Ω. (b) Acceptor on a flat surface, partially exposed to collisions with donor. Maximum value of angle θ for which direct donor–acceptor contact is possible is denoted θ_m. (c) Acceptor totally buried beneath surface. This corresponds to negative values of h in Figure 4.

mophore is compared with energy transfer to an otherwise identical but *unbound* chromophore, a measure of the solid angle (Ω) subtended by the cleft will be obtained. That is

$$k_{bound}/k_{free} \approx \Omega/4\pi \quad (6)$$

Equation 6 involves the assumption that donor and acceptor have spherical electron clouds and isotropic transition moments. It provides a useful, simple approximation but is not expected to be quantitatively accurate.

Figure 1b,c shows the second simple model, a spherical chromophore buried to various degrees in a flat surface. Here the critical parameter is the distance h, which represents the height that the chromophore extends above the surface. In Figure 1b direct contact between donor and acceptor is possible, and the rate of energy transfer includes contributions from both the exchange and dipole–dipole mechanisms.

For the exchange mechanism, the rate constant for energy transfer to the bound chromophore, k_{xb}, must be expressed in terms of that for the free chromophore, k_{xf}. In Figure 1b, where $0 \leq h \leq 2R_A$, the rate constant for the exchange mechanism is

$$k_{xb} = k_{xf}\left(\frac{1}{2}\int_0^{\theta_m} \sin\theta\, d\theta\right) = k_{xf}\left[\frac{h}{2(R_a + R_D)}\right] M^{-1}\,s^{-1} \quad (7)$$

For the dipolar mechanism when $0 \leq h \leq 2R_A$, it is shown under Appendix that

$$k_{db} = \frac{0.9461R_0^6}{\tau_0(R_A + R_D)^3}\left(\frac{1}{3} + \frac{h}{R_A + R_D}\right) M^{-1}\,s^{-1} \quad (8)$$

with all distances in nanometers.

When the chromophore is completely beneath the surface (Figure 1c), only the dipolar mechanism is operative, and, as shown in the Appendix, the rate constant is given by

$$k_{db} = \frac{0.3154R_0^6}{\tau_0(R_D + R_A - h)^3} M^{-1}\,s^{-1} = \frac{0.3154R_0^6}{\tau_0(R_D + l)^3} M^{-1}\,s^{-1} \quad (9)$$

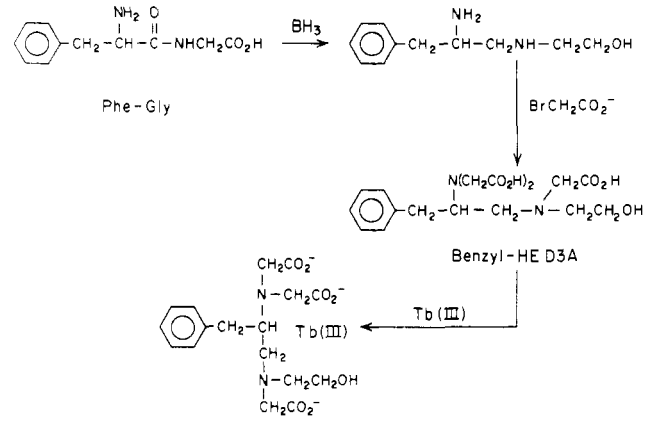


FIGURE 2: Synthesis of terbium-Bzl-HED3A starting from L-phenylalanylglycine. Chelated Tb(III) may be excited to the fluorescent $^5D_4$ state either directly (by using intense 488-nm light) or indirectly, via absorption of UV radiation by the aromatic ring.

with all distances in nanometers, where l is the distance from the surface to the center of the acceptor. Equations 8 and 9 may be compared with the dipolar rate constant for energy transfer to an *unbound* acceptor given by eq 2 above.

Materials and Methods

N-(2-Hydroxyethyl)ethylenediaminetriacetic acid (HED3A) was purchased from Aldrich Chemical Co. Terbium chloride was from Alfa Ventron. Cibacron Blue F3GA was from Pierce Chemical Co. and was recrystallized twice from methanol. Rifamycin SV came from Warf Institute, Inc. DEAE-cellulose was purchased from Whatman, and AG50 ion-exchange resin came from Bio-Rad Laboratories. *E. coli* MRE 600 cells were purchased from Grain Processing Co.

Buffer. There is only one buffer system used throughout, which is designated THGD and contains 10 mM tris(hydroxymethyl)aminomethane hydrochloride, pH 7.5, 0.1 mM dithiothreitol, 0.1 mM HED3A, 5% glycerol (v/v), and 0.5 M NaCl.

Stock solutions of rifamycin SV and Cibacron Blue F3GA were freshly prepared in the THGD buffer. The terbium chelate stock solutions always contained 50 mM Tb(III), with a 20% excess of the chelating agent to ensure complete binding of the metal ion. Concentrations of rifamycin SV and Cibacron Blue were measured spectroscopically by using ϵ_{445} = 14240 cm^{-1} M^{-1} for rifamycin SV (Sensi et al., 1961) and ϵ_{610} = 13600 cm^{-1} M^{-1} for Cibacron Blue (Kumar & Krakow, 1977).

Synthesis of (S)-N-(2-[Bis(carboxymethyl)amino]-3-phenylpropyl)-N-(2-hydroxyethyl)glycine (Bzl-HED3A, Figure 2). This compound was prepared in two ways. The more direct procedure was borane reduction of the amide and carboxyl groups of L-phenylalanylglycine to yield (*S*)-2-[(2-amino-3-phenylpropyl)amino]ethanol, followed by reaction with 3 equiv of bromoacetate to yield the product. The less expensive procedure was conversion of phenylalanine to the 2-hydroxyethylamide by using classical methods (Chambers & Carpenter, 1955), followed by borane reduction of the amide group and reaction with bromoacetate.

Reduction of L-Phenylalanylglycine. This compound (4.05 mmol, Sigma Chemical Co.) was dissolved in a mixture of 50 mL of tetrahydrofuran (which had been dried over Linde 3A molecular sieves) and 100 mL of 1,2-dimethoxyethane (also dried over molecular sieves). The solution was cooled on ice and flushed with N_2, after which 37 mmol of BH_3 (as 1 M solution in tetrahydrofuran) was added slowly (45 min) with stirring. The reaction mixture was then refluxed for 5 h under

anhydrous conditions (Brown et al., 1970). Following this, the reaction mixture was cooled on ice and 50 mL of dry CH_3OH was added to the unstoppered vessel. The solution then was saturated with HCl(g), refluxed 1 h, and evaporated to dryness under reduced pressure. The resulting solid was purified by elution from a 1 × 40 cm Bio-Rad AG50 (NH_4^+ form) column with a gradient of 0–6 M NH_4OH. The product, (*S*)-2-[(2-amino-3-phenylpropyl)amino]ethanol, elutes roughly between 2 and 4 M NH_4OH, depending on conditions. Absorbance peak fractions were pooled and lyophilized; yield was 60%. The product has $R_f = 0.68$ upon thin-layer chromatography on E. Merck Silica Gel 60 F_{254} in a solvent composed of 4 volumes 95% ethanol to 1 volume 6.5 M NH_3(aq).

Reduction of Phenylalanyl(2-hydroxyethyl)amide. This starting material was prepared by reaction of 30.3 mmol of L-phenylalanine methyl ester hydrochloride (Chambers & Carpenter, 1955) with excess ethanolamine (91 mmol) in 25 mL of dry CH_3OH for 3 days at room temperature, and purified on the Bio-Rad AG50 (NH_4^+) column with a gradient of 0–2.0 M NH_4OH. This secondary amide (24.2 mmol) was dissolved in 100 mL of dry tetrahydrofuran, cooled on ice, and flushed with N_2. Over a 1.5-h period, 169 mmol of BH_3 (1 M solution in tetrahydrofuran) was added to the cold solution. The solution then was treated exactly as in the reduction of phenylalanylglycine (above).

Carboxymethylation. The resulting (*S*)-2-[(2-amino-3-phenylpropyl)amino]ethanol prepared by either route was treated with bromoacetate and worked up according to the procedure of Yeh et al. (1979) to give pure (*S*)-*N*-(2-[bis-(carboxymethyl)amino]-3-phenylpropyl)-*N*-(2-hydroxyethyl)glycine. The structure of this compound (Bzl-HED3A) is illustrated in Figure 2.

Purification of RNA Polymerase. The method of Burgess & Jendrisak (1975) was used to purify *E. coli* RNA polymerase from *E. coli* MRE 600 cells purchased from Grain Processing Co. Before physical studies, the enzyme was concentrated, either by stepwise elution from DEAE-cellulose with THGD buffer or by using a Millipore ultrafiltration cell, to give a final enzyme concentration of 10–12 mg/mL. The assay method of Burgess (1969) was used to determine RNA polymerase activity, and protein concentrations were determined by using either dye binding (Sedmak & Grossberg, 1977) or $E_{280}^{1\%} = 6.2$ cm^{-1} (Burgess & Jendrisak, 1975).

Millisecond Fluorescence Lifetime Measurements. Lifetimes of excited terbium donors were measured with the instrument described by Thomas et al. (1978). Experiments were performed either by using the 488-nm radiation of the argon laser to directly excite the $^7F_6 \rightarrow {}^5D_4$ terbium transition or by using 257-nm radiation (frequency-doubled 514 nm) to excite the aromatic ring of the Tb-Bzl-HED3A chelate, which then transfers energy to raise the adjacent terbium ion to the $^5D_4$ level (see Figure 2). Terbium emission was monitored at 546 nm; several hundred decays were accumulated, and the excited-state lifetime was determined by a least-squares fit of the digitized data to a single exponential function plus horizontal base line (Thomas et al., 1978). Experiments were performed at 20 °C on freshly prepared solutions containing RNA polymerase (24–27 μM), terbium chelate (1–1.5 mM), and rifamycin SV or Cibacron Blue (12–14 μM) in a final volume of 50–60 μL in THGD buffer. Each solution was saved to be assayed later for enzyme activity and final concentration of added inhibitor.

Because of limited solubility of the enzyme, only low concentrations of bound inhibitor (≈10^{-5} M) could be studied.

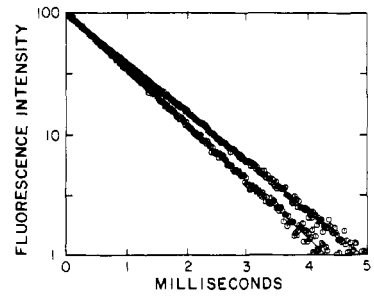


FIGURE 3: Semilogarithmic plot showing fluorescence decay of terbium-HED3A in the presence and absence of 9 μM rifamycin SV.

The resulting energy transfer changed the donor lifetime by as little as 6% in the worst case (RNA polymerase plus Cibacron Blue). To be sure that this effect was measured accurately, we performed all experiments on three independently prepared samples according to the following protocol. First, the lifetime of the terbium donor in the presence of RNA polymerase was measured; then the desired amount of inhibitor was added and the donor lifetime measured again. Afterward, the inhibitor concentration was confirmed by a visible absorbance measurement and the enzyme was assayed to confirm the expected amount of inhibition (enzyme was always present in 2-fold excess over inhibitor). In this way, pipetting error (the major cause of the variations in measured lifetimes) could be corrected before calculation of the rate constant for energy transfer. The resulting values for the rate constants showed remarkably little variation, as indicated by the standard deviation values given in Table I.

It is important to consider the possibility that the donor binds to the protein. If this occurs, the resulting change in the environment of terbium would be expected to cause a significant change in the lifetime (Yeh & Meares, 1980); in all cases the observed lifetime of terbium chelates in the presence of RNA polymerase was within 7% of the value in buffer alone. Because the concentration of donors is 100 times larger than the concentration of acceptors (1.5 vs. 0.015 mM), it is unlikely that the experimental results would reflect any binding of donor by acceptor.

Another important possibility is that chelated terbium will bind chloride or other ions in the solution and thus acquire a net electric charge. Since this would be accompanied by a change in the ligands about terbium it would also lead to a difference between the lifetime of chelated terbium in the buffer and in water (Yeh & Meares, 1980); measured lifetimes were the same within 3%.

Results

Typical experimental data showing the effect of rifamycin on the fluorescence decay of chelated terbium(III) are plotted in Figure 3. The second-order rate constants for energy transfer, which are collected in Table I, were determined from terbium fluorescence lifetime measurements in the presence and absence of energy acceptors by using eq 1. The use of the electrically neutral Tb(III) chelates should eliminate complications due to electrostatic interactions with charged residues on the enzyme. As described elsewhere (Yeh & Meares, 1980), chelated terbium may be electronically excited either *directly*, with 488-nm light, or *indirectly*, via an adjacent aromatic group which absorbs ultraviolet light. Because of the very small absorptivity of terbium ($\epsilon_{488} \simeq 0.05$ M^{-1} cm^{-1}), only a laser can provide 488-nm light of the intensity required for direct excitation. As shown in Table I, energy transfer from directly excited Tb-HED3A to various acceptors occurs at rates generally in agreement with those observed by using

Table I: Second-Order Rate Constants for Energy Transfer

donor	acceptor	obsd rate constant[a] (M^{-1} s^{-1})	dipolar rate constant[b] (M^{-1} s^{-1})
Tb-Bzl-HED3A	rifamycin	$(1.9 \pm 0.2) \times 10^7$	0.1×10^7
	rifamycin + RNA polymerase	$(0.9 \pm 0.2) \times 10^7$	
	Cibacron Blue	$(4.5 \pm 0.5) \times 10^7$	0.8×10^7
	Cibacron + RNA polymerase	ND[c]	
Tb-HED3A	rifamycin	$(2.5 \pm 0.02) \times 10^7$	0.1×10^7
	rifamycin + RNA polymerase	$(1.1 \pm 0.2) \times 10^7$	
	Cibacron Blue	$(9.9 \pm 0.1) \times 10^7$	0.8×10^7
	Cibacron + RNA polymerase	ND	

[a] All experiments were performed at 20 °C in THGD buffer at pH 7.5. In each case, standard deviations were calculated from results on three independently prepared samples. Due to incomplete binding, the rate constant for enzyme-bound Cibacron could not be measured satisfactorily. [b] Calculated by using eq 2 and a value for $R_A + R_D$ of 1.06 nm. [c] ND = not determined.

indirectly excited Tb-Bzl-HED3A.

Complete binding of rifamycin to excess RNA polymerase at 10^{-5} M is assured by the very high association constant of this complex, $K_s = 3 \times 10^9$ M^{-1} (Bähr et al., 1976; Wehrli et al., 1976). The association constant for binding of Cibacron Blue to RNA polymerase was determined to be $K_s = 1.0 \times 10^6$ M^{-1} at 298 K, pH 7.5, in THGD buffer, by measuring the decrease in enzyme fluorescence caused by addition of Cibacron Blue. For the terbium energy transfer experiments, the total enzyme concentration typically was 20 μM and the total cibacron concentration 10 μM; thus 8–9% of the Cibacron was not bound by the enzyme. The presence of unbound inhibitor obscured the (much smaller) contribution of bound Cibacron to energy transfer, as discussed below.

The critical distance for dipole–dipole energy transfer, R_0, was calculated for each acceptor from eq 3 and 4. The terbium quantum yield in the absence of acceptor was determined to be $Q_0 = 0.2 \pm 0.05$ (Yeh & Meares, 1980), and the refractive index of the buffer was $n = 1.33$. The spectral overlap integral J was 1.5×10^{-14} cm^3 M^{-1} for rifamycin SV and 8.9×10^{-14} cm^3 M^{-1} for Cibacron Blue; absorption spectra were found to be in satisfactory agreement with those published elsewhere (Sensi et al., 1961; Kumar & Krakow, 1977). The dynamically averaged value $^2/_3$ was used for the orientation factor κ^2, leading to R_0 values of 2.87 nm for terbium to rifamycin SV and 3.87 nm for terbium to Cibacron Blue.

Discussion

The second-order rate constants for energy transfer given in Table I are all much smaller than the diffusion-limited value 3×10^9 M^{-1} s^{-1} appropriate for these conditions, which implies that the average donor encounters many possible acceptors during its lifetime. Also, the rate constants observed for both free chromophores and for enzyme-bound rifamycin are much larger than would be expected for a pure dipole–dipole energy transfer mechanism. This is best seen by comparison of the observed rate constants with values calculated from eq 2, with the approximation that donors and acceptors are spheres. A space-filling model of the donor is approximately spherical, with radius 0.4 ± 0.1 nm, and from the molecular volume of rifamycin (Brufani et al., 1974), the radius of an equivalent sphere should be 0.7 nm; therefore the distance of closest approach should be no less than 1 nm. According to eq 2, $k_{df} \leq 1.3 \times 10^6$ M^{-1} s^{-1} for terbium to rifamycin and $k_{df} \leq 8 \times 10^6$ M^{-1} s^{-1} for terbium to Cibacron Blue. Since these values are 1 order of magnitude smaller than the observed values, we conclude that in each case energy transfer occurs almost entirely by the exchange mechanism. This is not unexpected, since other recent work has indicated that the exchange mechanism can dominate energy transfer in cases where direct collisions between donor and acceptor are possible (C. F. Meares, S. M. Yeh, and L. Stryer, unpublished results). The fact that the exchange interaction depends on direct donor–acceptor contact makes these experiments very sensitive to whether a bound chromophore is exposed to small molecules in the surrounding medium or buried within the protein. This is illustrated in Figure 4, where the experimental values of k_f for rifamycin and Cibacron Blue have been used with eq 7–9 to show how the exposure of an energy acceptor above a flat surface will affect the rate constant for energy transfer, k_b. It is particularly interesting to note that the numerical value of R_0 becomes important only when an acceptor is completely buried beneath the surface.

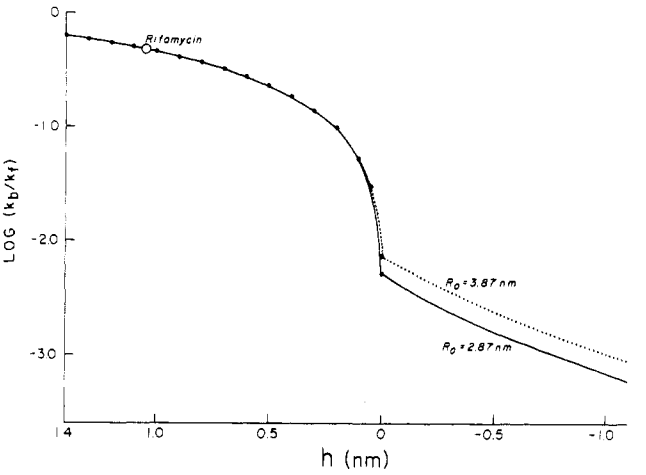
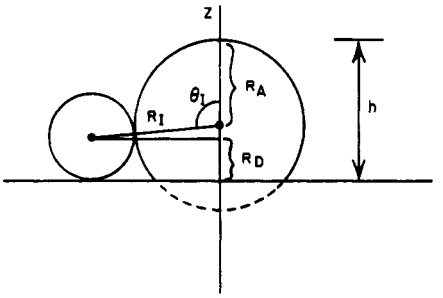


FIGURE 4: Semilogarithmic plot of k_b/k_f, the ratio of rate constants for energy transfer to enzyme-bound vs. unbound inhibitors, as a function of the distance of extension h of each bound inhibitor above a flat surface, inferred from experimental data for rifamycin and Cibacron Blue as explained in the text. Dotted line is for Cibacron Blue (R_0 = 3.87 nm); solid line is for rifamycin (R_0 = 2.87 nm). The large open circle indicates the value of log (k_b/k_f) observed experimentally for rifamycin bound to RNA polymerase. Present data do not permit the value of log (k_b/k_f) for bound Cibacron to be located with certainty but do imply that log (k_b/k_f) < −1.

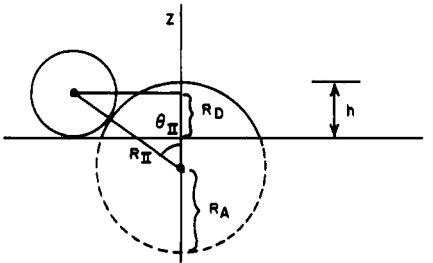
For the terbium to Cibacron energy-transfer experiments, 8–9% of the Cibacron was not bound by the enzyme. This could not be avoided, since raising the concentration of enzyme could lead to aggregation, while lower concentrations of Cibacron could not be measured spectrophotometrically. Both enzyme-bound and unbound Cibacron will contribute to energy transfer, in proportion to the concentration of each and depending on the accessibility of the bound Cibacron to donor molecules in solution. The observed energy transfer from the terbium chelates to Cibacron in the presence of RNA polymerase was 9–15% of that observed in the absence of enzyme; since the concentration of unbound Cibacron in the presence of RNA polymerase was 8–9% of the Cibacron concentration in the absence of enzyme, most of the observed energy transfer is due to the presence of unbound Cibacron. This implies that (in contrast to rifamycin) enzyme-bound Cibacron is buried inside the RNA polymerase complex, practically inaccessible to the donor molecules in solution.

As shown in Table I, the rate constant for energy transfer to rifamycin is reduced by a factor of ~2 when rifamycin is quantitatively bound to RNA polymerase. This relatively



CASE I

FIGURE A1: Bound acceptor extends farther above surface than $R_A + R_D$. Angle θ_I is the maximum value of the acceptor-centered polar angle θ permitted when donor and acceptor can touch. Distance R_I is the *maximum* donor–acceptor separation for a given value of θ (when $\theta = \theta_I$, then $R_I = R_A + R_D$; otherwise, $R_I > R_A + R_D$ and when $\theta \leq \pi/2$, $R_I = \infty$).

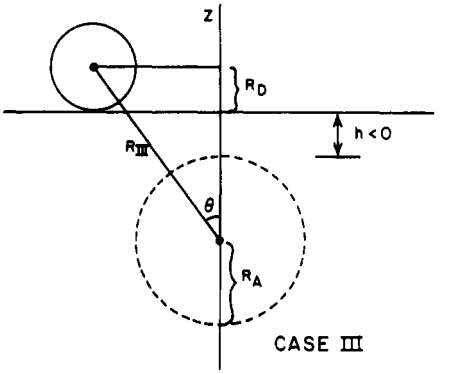


CASE II

FIGURE A2: Bound acceptor extends above surface a distance $\leq R_A + R_D$. Angle θ_{II} is the maximum value of the acceptor-centered polar angle θ for which donor and acceptor can touch. Distance R_{II} is the *minimum* donor–acceptor separation for a given value of θ (when $\theta \leq \theta_{II}$, $R_{II} = R_A + R_D$; otherwise, $R_{II} > R_A + R_D$.

small change can only be interpreted as indicating that bound rifamycin is still directly accessible to terbium chelates in solution; for the model of Figure 1a, $\Omega/4\pi \simeq {}^1/_2$, while for the model of Figure 1b, $h \simeq R_A + R_D$. The fact that a large number of rifamycin derivatives having bulky substituents at the 3 position are excellent inhibitors of RNA polymerase is consistent with bound rifamycin being in an exposed position (Sensi, 1975). The topography of the rifamycin binding site has been previously studied by Stender & Scheit (1977), who used antibodies to the dinitrophenyl hapten which was attached to rifamycin by spacer groups of various lengths. These antibody probe experiments led to the conclusion that bound rifamycin was 1.4–1.9 nm below the surface of the enzyme. Affinity-labeling studies by Stender et al. (1975) and by us (Rice & Meares, 1978) indicate that several different subunits of the enzyme are adjacent to the rifamycin binding site. Taken together, all the experiments suggest that bound rifamycin lies in a cleft between subunits, with the minimum cleft diameter being much greater than the 0.8-nm diameter of a terbium chelate but less than the 4-nm width of the binding portion of an IgG molecule.

In the future, experimental study of how energy transfer to enzyme-bound inhibitors is affected by removal of the σ subunit or addition of DNA template, nucleotide substrates, or other inhibitors should add to our understanding of the structure of RNA polymerase. The experimental approach described here should also be useful in the study of other systems, such as heme proteins or Cibacron-binding enzymes (Stellwagen, 1977), in which it is important to know whether the chromophore is accessible or buried. Conversely, it should be possible to study metal-binding sites [such as calcium-binding sites, since terbium(III) readily substitutes for Ca^{2+}] by observing energy transfer from a bound lanthanide to freely diffusing energy acceptor molecules in solution.



CASE III

FIGURE A3: Bound acceptor is buried beneath surface. Distance R_{III} is the *minimum* separation between donor and acceptor for a given value of θ. Distance from edge of acceptor to surface is given by negative h.

Acknowledgments

Professor Lubert Stryer introduced us to diffusion-enhanced energy transfer, provided many helpful discussions during the course of this work, and generously permitted these experiments to be performed in his laboratory. Dr. David Thomas and William Carlsen provided useful advice on many occasions. David Sherman synthesized the Bzl-HED3A.

Appendix

Derivation of Equations 8 and 9. Equation 8 may be derived in two segments. As shown in Figure A1, case I is for $h \geq R_A + R_D$ while as shown in Figure A2, case II is for $h \leq R_A + R_D$. First we consider case I, for which

$$k_{db}{}^I = \frac{0.6023R_0{}^6}{\tau_0}\left(\int_0^{2\pi} d\phi \int_0^{\pi/2} \sin\theta\, d\theta \int_{R_A+R_D}^{\infty} \frac{r^2\, dr}{r^6} + \int_0^{2\pi} d\phi \int_{\pi/2}^{\theta_I} \sin\theta\, d\theta \int_{R_A+R_D}^{R_I} \frac{r^2\, dr}{r^6}\right) \quad (A1)$$

where the second term encompasses energy transfer from all positions where the center of the acceptor is farther from the surface than is the center of the donor. From Figure A1, for $\pi/2 \leq \theta \leq \theta_I$

$$-\cos\theta = \cos(\pi - \theta) = \frac{h - (R_D + R_A)}{R_I}$$

where R_I is the maximum donor–acceptor distance for a given value of θ, and

$$-\cos\theta_I = \cos(\pi - \theta_I) = \frac{h - (R_D + R_A)}{R_D + R_A} = \frac{h}{R_D + R_A} - 1$$

where θ_I is the maximum value of θ permitted when donor and acceptor can touch. Because the problem has cylindrical symmetry, integration over ϕ just yields 2π, leaving for case I

$$k_{db}{}^I = \frac{2\pi(0.6023)R_0{}^6}{\tau_0}\left\{\int_0^{\pi/2} \sin\theta\, d\theta \left[\frac{1}{3(R_A + R_D)^3}\right] + \int_{\pi/2}^{\theta_I} \sin\theta\, d\theta \left[\frac{1}{3(R_A + R_D)^3} - \frac{1}{3R_I{}^3}\right]\right\} \quad (A2)$$

$$k_{db}^{I} = \frac{2\pi(0.6023)R_0^6}{3\tau_0}\left[\int_0^{\theta_I}\frac{\sin\theta\,d\theta}{(R_A+R_D)^3} - \int_{\pi/2}^{\theta_I}\frac{\cos^3\theta\sin\theta\,d\theta}{(R_D+R_A-h)^3}\right] \quad (A3)$$

Now we leave this for a moment to consider case II, for which $h \leq R_A + R_D$

$$k_{db}^{II} = \frac{0.6023R_0^6}{\tau_0}\left(\int_0^{2\pi}d\phi\int_0^{\theta_{II}}\sin\theta\,d\theta\int_{R_A+R_D}^{\infty}\frac{r^2\,dr}{r^6} + \int_0^{2\pi}d\phi\int_{\theta_{II}}^{\pi/2}\sin\theta\,d\theta\int_{R_{II}}^{\infty}\frac{r^2\,dr}{r^6}\right) \quad (A4)$$

where the second term encompasses energy transfer from all values of θ for which donor and acceptor cannot touch. As shown in Figure A2

$$\cos\theta = \frac{R_A + R_D - h}{R_{II}}$$

where R_{II} is the minimum donor–acceptor distance for a given value of θ, and

$$\cos\theta_{II} = \frac{R_A + R_D - h}{R_A + R_D} = 1 - \frac{h}{R_A + R_D}$$

where θ_{II} is the maximum value of θ at which donor and acceptor can touch. Now, again integrating over ϕ and r

$$k_{db}^{II} = \frac{2\pi(0.6023)R_0^6}{\tau_0}\left\{\int_0^{\theta_{II}}\sin\theta\,d\theta\left[\frac{1}{3(R_A+R_D)^3}\right] + \int_{\theta_{II}}^{\pi/2}\sin\theta\,d\theta\left(\frac{1}{3R_{II}^3}\right)\right\} \quad (A5)$$

$$k_{db}^{II} = \frac{2\pi(0.6023)R_0^6}{3\tau_0}\left[\int_0^{\theta_{II}}\frac{\sin\theta\,d\theta}{(R_A+R_D)^3} + \int_{\theta_{II}}^{\pi/2}\frac{\cos^3\theta\sin\theta\,d\theta}{(R_A+R_D-h)^3}\right] \quad (A6)$$

$$= \frac{2\pi(0.6023)R_0^6}{3\tau_0}\left[\int_0^{\theta_{II}}\frac{\sin\theta\,d\theta}{(R_A+R_D)^3} - \int_{\pi/2}^{\theta_{II}}\frac{\cos^3\theta\sin\theta\,d\theta}{(R_A+R_D-h)^3}\right] \quad (A7)$$

Note that this is identical in form with eq A3, so that we can now proceed to solve both equations as

$$k_{db} = \frac{2\pi(0.6023)R_0^6}{3\tau_0}\left\{\frac{1}{(R_A+R_D)^3}\left[1-\left(1-\frac{h}{R_A+R_D}\right)\right] + \frac{1}{(R_A+R_D-h)^3}\left[1/4\left(\frac{R_A+R_D-h}{R_A+R_D}\right)^4\right]\right\} \quad (A8)$$

which reduces to eq 8 in the text

$$k_{db} = \frac{0.9461R_0^6}{\tau_0(R_A+R_D)^3}\left(1/3 + \frac{h}{R_A+R_D}\right)\ M^{-1}\ s^{-1} \quad (A9)$$

When the acceptor is completely below the surface, $h < 0$ as shown in Figure A3, and the distance of closest approach of donor to acceptor is $R_{III} = (R_D + R_A - h)/\cos\theta$. For this case

$$k_{db} = \frac{0.6023R_0^6}{\tau_0}\int_0^{2\pi}d\phi\int_0^{\pi/2}\sin\theta\,d\theta\int_{R_{III}}^{\infty}\frac{r^2\,dr}{r^6} \quad (A10)$$

$$k_{db} = \frac{2\pi(0.6023)R_0^6}{3\tau_0(R_D+R_A-h)^3}\int_0^{\pi/2}\cos^3\theta\sin\theta\,d\theta \quad (A11)$$

$$k_{db} = \frac{0.3154R_0^6}{\tau_0(R_D+R_A-h)^3} = \frac{0.3154R_0^6}{\tau_0(R_D+l)^3}\ M^{-1}\ s^{-1} \quad (A12)$$

where the distance from the surface to the center of the acceptor is $l = R_A - h$.

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Glucose Oxidase Contains a Disubstituted Phosphorus Residue. Phosphorus-31 Nuclear Magnetic Resonance Studies of the Flavin and Nonflavin Phosphate Residues†

Thomas L. James, Dale E. Edmondson,* and Mazhar Husain

ABSTRACT: Glucose oxidase from *Aspergillus niger* was studied by ^{31}P NMR. In addition to the two resonances from its flavin adenine dinucleotide (FAD) coenzyme, a resonance from a phosphorus residue covalently bound to the enzyme was also observed. The spectra indicate that the two subunits of glucose oxidase are identical with regard to the single FAD per subunit. Comparison of the ^{31}P NMR spectra of free FAD with that of native glucose oxidase reveals a substantial shift in one of the resonances of the pyrophosphate linkage upon binding to the enzyme. No changes in conformation are indicated for either the FAD pyrophosphate or the covalent phosphorus with change in oxidation state of the flavin ring of FAD among oxidized, semiquinone, and fully reduced species. Formation of a sulfite adduct also leaves the resonances unaltered. The unpaired electron on the FAD in the semiquinone form results in differential broadening of the ^{31}P NMR resonances of the FAD pyrophosphate, indicating that one phosphorus nucleus is nearer to the flavin ring than the other. A lack of broadening in the ^{31}P NMR resonance of the covalent phosphorus for the semiquinone species of glucose oxidase indicates that the covalent phosphorus is remote from the flavin. Addition of paramagnetic Mn(II) to the enzyme solution results in broadening of the ^{31}P NMR resonance assigned to the covalent phosphorus residue, showing that it is near the surface of the enzyme. Mn(II) addition does not perceptibly broaden the ^{31}P NMR resonances of the bound FAD, demonstrating that the pyrophosphate moiety if buried in the enzyme. Glucose oxidase is a glycoprotein. Evidence that the covalent phosphorus moiety is bound to the protein, and not to the carbohydrate moiety, is due to (a) no phosphorus release from the enzyme with periodate treatment and (b) separation of all glycopeptides from the phosphopeptide on a Sephadex G-25 column following Pronase digestion of the enzyme. The observed independence of the ^{31}P NMR chemical shift of the covalently bound phosphorus in the holoprotein and in the phosphopeptide with change in pH from 4.7 to 8.2 shows it to be disubstituted. The implication is that a phospho bridge exists, linking two amino acid residues in the polypeptide chain of the enzyme much as disulfide links in proteins modify the secondary structure.

Glucose oxidase from *Aspergillus niger* is a dimeric enzyme which catalyzes the reaction:

$$\text{D-glucose} + O_2 \rightarrow \text{D-gluconic acid} + H_2O_2$$

Each subunit of the enzyme is ~70 000 daltons and contains ~16% (w/w) carbohydrate as well as 1 mol of firmly bound FAD[1] (Pazur, 1966).

In a previous report (Edmondson & James, 1979), we noted that several flavoproteins contain covalently bound phosphate residues, in addition to those of the flavin coenzyme. Glucose oxidase contains 1 mol of noncoenzyme phosphorus/subunit (Swoboda & Massey, 1966). We have previously demonstrated by using ^{31}P NMR that a covalently bound phosphate in flavodoxin from *Azotobacter vinelandii* is disubstituted, apparently forming a phosphodiester linkage between two hydroxyl amino acids in the protein (Edmondson & James, 1979). The present investigation is designed to provide information on the structure and environment of the covalent phosphorus residue as well as the flavin phosphates and to monitor any alterations of the environment that may occur on changes in oxidation–reduction level of the enzyme.

As demonstrated in this paper by using ^{31}P NMR, the covalently bound phosphorus residue in glucose oxidase is a disubstituted phosphate attached to the protein moiety near the protein surface but remote from the flavin. ^{31}P NMR results on the FAD coenzyme also show that binding of FAD to glucose oxidase changes the conformation of the pyrophosphate moiety. When bound to the enzyme, the pyrophosphate moiety is not exposed on the surface of the enzyme.

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[1] Abbreviations used: FAD, flavin adenine dinucleotide; AMP, adenosine 5′-phosphate; FMN, flavin mononucleotide; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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