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# Spatial Relationship of the $\sigma$ Subunit and the Rifampicin Binding Site in RNA Polymerase of *Escherichia coli*†

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ABSTRACT: $\sigma$ subunit of *Escherichia coli* RNA polymerase is known to stimulate specific RNA chain initiation. Rifampicin, an inhibitor of RNA chain initiation, binds to a single site on the $\beta$ subunit of RNA polymerase. We have used the fluorescence energy transfer technique to deduce proximity relationships of $\sigma$ subunit and the rifampicin binding site on the enzyme. Isolated $\sigma$ subunit was covalently labeled with fluorescent donors in two ways: specific labeling of a single sulfhydryl residue with *N*-(iodoacetylaminoethyl)-5-naphthylamine-1-sulfonate (1,5-I-AENS) and nonspecific labeling on the surface of the protein with dansyl chloride (Dns-Cl) adsorbed on Celite. The labeled $\sigma$ subunits were biologically active and formed a stoichiometric complex with core polymerase. The efficiency of energy transfer was obtained from the fluorescence intensity and the excited-state lifetime of the $\sigma$-labeled holoenzyme in the presence and absence of rifampicin, which served as an energy acceptor. The transfer efficiency (2%) from AENS to rifampicin placed AENS somewhere between 42 and 85 Å away from the rifampicin binding site. The rotational mobility of the donor was determined by nanosecond fluorescence depolarization spectroscopy, while the acceptor orientation was assumed to be fixed at some unknown angle. The efficiency measured for energy transfer from Dns to rifampicin was 10% in the presence of 0.2 M KCl. The distance from the surface of $\sigma$ subunit to the rifampicin binding site was calculated to be 27–38 Å for a model having a randomly distributed and oriented array of donors on the surface of a spherical $\sigma$ subunit of 31-Å radius. Our results indicate that rifampicin does not inhibit the initiation of transcription by RNA polymerase through a direct interaction with $\sigma$ subunit. In addition, energy transfer measurements under low salt conditions suggest that in RNA polymerase dimer the two rifampicin binding sites are symmetric with respect to each $\sigma$ subunit.

The $\sigma$ subunit of *Escherichia coli* RNA polymerase is known to stimulate specific initiation that yields asymmetric transcripts resembling the in vivo RNA products (Burgess et al., 1969; Travers and Burgess, 1969). Since rifampicin, a specific inhibitor of the initiation of RNA chains, has been shown to bind to a single site on RNA polymerase (Zillig et al., 1970), it is of interest to determine the spatial relationship of the rifampicin binding site and $\sigma$ subunit in RNA polymerase.

Singlet energy transfer can be used to determine proximity relationships between sites on macromolecules which have been specifically labeled with fluorescent probes (Stryer, 1968). This technique can also be used to estimate intersubunit distances in multisubunit protein complexes by means of random surface labeling (Gennis and Cantor, 1972). This paper reports the use of singlet energy transfer to measure the distances from the rifampicin binding site on RNA polymerase to the surface of $\sigma$ subunit randomly labeled with dansyl chloride (Dns-Cl[1]) and

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[1] Abbreviations used are: 1,5-I-AENS, *N*-(iodoacetylaminoethyl)-5-naphthylamine-1-sulfonate; AENS, *N*-(acetylaminoethyl)-5-naphthylamine-1-sulfonate; AENS-$\sigma$, fluorescent labeled $\sigma$ subunit; Dns-Cl, 5-dimethylamino-1-naphthalenesulfonyl chloride or dansyl chloride; Dns, dansyl group; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

to a sulfhydryl residue in the $\sigma$ subunit which has been specifically labeled with *N*-(iodoacetylaminoethyl)-5-naphthylamine-1-sulfonate (1,5-I-AENS). Energy transfer measurements from each of these two chromophores to rifampicin were performed in the labeled $\sigma$–core polymerase complex. The results indicate that rifampicin is not in direct contact with the $\sigma$ subunit. In addition, energy transfer measurements at low salt concentrations have yielded information concerning the possible geometric arrangements of the two $\sigma$ subunits in the RNA polymerase dimer.

## Materials and Methods

*Chemicals.* *N*-(Iodoacetylaminoethyl)-5-naphthylamine-1-sulfonate was synthesized by the method of Hudson and Weber (1973). [$^3$H]Iodoacetate (New England Nuclear Corp.) was used to synthesize 1,5-I-[$^3$H]AENS. Unlabeled and $^{14}$C-labeled rifampicin were gifts of Drs. R. White and L. Sylvestri of Gruppo-Lepetit Laboratories. Unlabeled ribonucleoside triphosphates were purchased from P-L Biochemicals and $^3$H-labeled ribonucleoside triphosphates from New England Nuclear Corp. [$^3$H]Dansyl chloride (30–100 cpm/pmol, New England Nuclear Corp.) adsorbed on Celite was prepared by the method of Rinderknecht (1960).

*RNA Polymerase and $\sigma$ Subunit.* RNA polymerase holoenzyme was purified from *E. coli* B as described previously (Wu and Wu, 1973). The enzyme was 98% pure and contained all subunits ($\alpha$, $\beta$, $\beta'$, and $\sigma$) as shown by sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis. Core enzyme ($\alpha_2\beta\beta'$) and $\sigma$ subunit were prepared from holoenzyme by the procedure of Berg et al. (1971). The purity of $\sigma$ subunit was estimated by electrophoresis in acrylamide gels to be 90–95%. Enzymatic activity of RNA polymerase and stimulatory effect of $\sigma$ subunit on DNA-dependent RNA synthesis by core enzyme were determined as described previously (Yarbrough and Wu, 1974).

*Fluorescent Labeling of $\sigma$ Subunit.* For labeling of isolated $\sigma$ subunit with 1,5-I-AENS, 2–50 molar excess of the dye was added to 0.5–1 mg of $\sigma$ subunit in 0.05 M Tris-HCl (pH 8), 0.2 M KCl, $10^{-4}$ M EDTA, and 5% glycerol (final volume 0.5–1 ml). The solution was incubated in the dark for 30 min at 23 °C followed by an overnight incubation at 3 °C. Unreacted reagent was removed by a Sephadex gel filtration followed by extensive dialysis.

The method of labeling with a Celite-adsorbed dye was essentially that of Rinderknecht (1962). Solutions of $\sigma$ subunit (1 ml) containing approximately 1 mg of protein were dialyzed at 3 °C against 0.02 M potassium phosphate buffer (pH 8) containing 0.05 M KCl, 1 mM EDTA, and 5% glycerol (flushed with $N_2$ to remove $O_2$ since reducing agent was absent). After dialysis, the pH of the solution was adjusted to about 10 by adding 0.1 ml of 1 M $Na_2CO_3$. An amount of Celite dye to give 50–100 molar excess of Dns-Cl to protein was added and the mixture agitated gently for 5–10 min at room temperature. The resultant slurry was centrifuged and the supernatant applied to a Sephadex G25-fine column equilibrated with 0.05 M Tris-HCl buffer (pH 8) containing 0.05 M KCl, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 5% glycerol. Elution was with the same buffer at 3 °C. Because considerable amounts of $\sigma$ subunit aggregated during the labeling reaction, labeled $\sigma$ subunit was purified by glycerol gradient (15–30% in 0.05 M Tris-HCl (pH 8), 0.05 M KCl, 0.1 mM EDTA, 0.1 mM dithiothreitol) centrifugation at 49 000 rpm for 20 h at 3 °C (SW 50.1 rotor). It was observed that the aggregation could be prevented to some extent if the sulfhydryl groups of $\sigma$ subunit were blocked by potassium tetrathionate (Yarbrough and Wu, 1974) before dye labeling. After the labeling, the blocked sulfhydryl group could be completely regenerated by treatment with reducing reagents such as dithiothreitol.

The stoichiometry of dye to $\sigma$ subunit was determined by counting radioactivity of protein-bound [$^3$H]Dns or [$^3$H]AENS and measuring $\sigma$ concentration using $A_{280nm}^{0.1\%} = 1.0$ (after correcting for the absorbance at 280 nm due to bound dye) or the method of Bücher (1947).

*Spectral Measurements.* Absorption spectra were obtained with a Cary 118C recording spectrophotometer. Fluorescence excitation and emission spectra were recorded on a Hitachi Perkin-Elmer fluorescence spectrophotometer (Model MPF-3) equipped with a corrected spectra accessory. The solutions used had absorbance of less than 0.05 at the excitation wavelength to obviate inner filter effects. Quantum yields were determined with 8-anilinonaphthalene-1-sulfonate in ethanol as a standard of quantum yield 0.37 (Stryer, 1965). All spectral measurements were carried out at 22 ± 1 °C.

*Lifetime and Time-Dependent Emission Anisotropy Measurements.* Excited-state lifetime measurements were made by the single photon counting technique (Lami et al., 1966) using an Ortec 9200 nanosecond fluorescence spectrometer. The light source was a spark-gap lamp filled with air and operating at about 40 kHz. The exciting light was filtered through a Corning 7-60 filter and polarized in the $y$ direction by a Polaroid HNB sheet polarizer. The emitted light was detected through a Kodak Wratten 65(A) filter at 90° to the incident light. For anisotropy a polarizer on the emission side was used to select the $x$ and $y$ components of the fluorescence, $F_x(t)$ and $F_y(t)$. The time dependence of the total emitted light, $S(t) = F_y(t) + 2F_x(t)$, was obtained by setting the emission polarizer at 54.7° to the $y$ direction. The fluorescence intensity as a function of time was recorded on a multichannel analyzer interfaced to a PDP-11 digital computer for data analysis.

In nanosecond fluorescence spectroscopy, the recorded fluorescence curve $S(t)$ is always distorted by the finite duration of the lamp pulse and the response time of the detecting system. The true time-dependent emission $F(t)$ is related to the recorded $S(t)$ by the convolution integral:

$$S(t) = \int_0^t L(T)F(t - T)\mathrm{d}T \qquad (1)$$

where $L(T)$ is the exciting light pulse as measured by the instrument. For a single chromophore in a homogeneous environment

$$F(t) = F_0 e^{-t/\tau} \qquad (2)$$

where $F_0$ is the fluorescence intensity immediately following an infinitely narrow pulse of excitation and $\tau$ is the excited-state lifetime of the chromophore. For more complex systems, $F(t)$ can be expressed as a sum of exponential terms. The method of moments (Isenberg and Dyson, 1969) was used to deconvolute and analyze $S(t)$ in terms of single- or double-exponential decays. The final set of parameters chosen to describe the fluorescence decay was that which yielded the smallest sum of nonweighted squares of the residuals (Yguerabide, 1972).

The time-dependent emission anisotropy, $A(t)$, defined for excitation with light polarized along the $y$ direction as (Perrin, 1934; Jablonski, 1962)

$$A(t) = \frac{F_y(t) - F_x(t)}{F_y(t) + 2F_x(t)} \qquad (3)$$

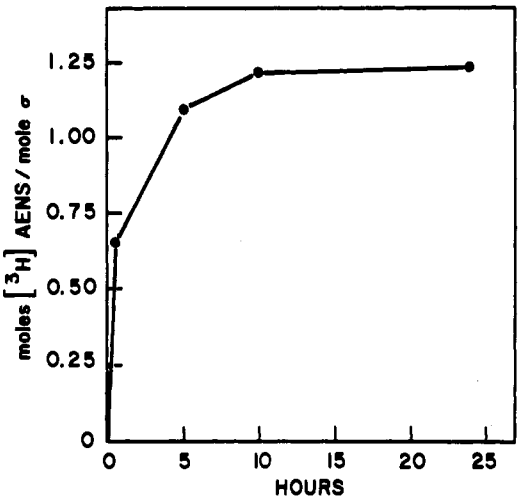


FIGURE 1. Reaction of isolated $\sigma$ subunit with 1,5-I-[$^3$H]AENS. The reaction mixture (1 ml) contained 6.3 nmol of $\sigma$ subunit and 320 nmol of 1,5-I-[$^3$H]AENS in 0.05 M Tris-HCl (pH 8), 0.05 M KCl, $10^{-4}$ M EDTA, and 5% glycerol. Incubation was for 30 min at 23 °C followed by 24 h at 3 °C. Aliquots of the sample were taken at various times and precipitated with trichloroacetic acid, and the radioactivity of pellet was counted by liquid scintillation.

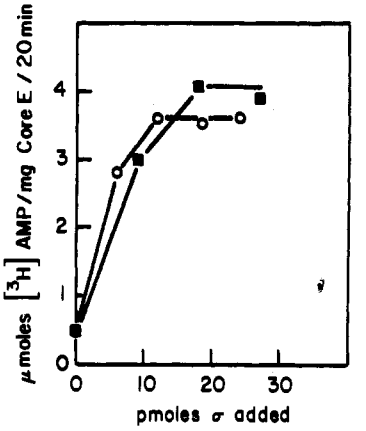


FIGURE 2: Titration of core enzyme with $\sigma$ subunit. The indicated amount of $\sigma$ subunit was added to reaction mixtures (0.25 ml) containing 6.6 pmol of core enzyme in 0.05 M Tris-HCl (pH 7.8), 0.2 M KCl, 0.01 M $MgCl_2$, 1 mM dithiothreitol, 0.4 mM each of [$^3$H]ATP (6300 cpm/nmol), GTP, UTP, and CTP, and 15 nmol of T7 DNA. Samples were incubated at 37 °C for 10 min and precipitated with trichloroacetic acid. The trichloroacetic acid precipitable counts were determined by liquid scintillation. (○) AENS-$\sigma$; (■) unlabeled $\sigma$.

was calculated by computer. For a rigid sphere, $A(t)$ decays exponentially

$$A(t) = A_f e^{-t/\phi} \quad (4)$$

$A_f$, the fundamental emission anisotropy, is affected by photoselection, which sets a theoretical upper limit of 0.4. The rotational correlation time ($\phi$) is related to the volume $V$ of the hydrated sphere, the viscosity $\eta$ of the solution, the absolute temperature $T$, and the Boltzman constant $k$ by

$$\phi = V\eta/kT \quad (5)$$

In principle, $F_y(t)$ and $F_x(t)$ must be deconvoluted to yield $A(t)$. However, deconvolution was not necessary in this study since both $\tau$ and $\phi$ are sufficiently long compared with the exciting light-pulse duration and the instrument response time.

## Results

*Specific Site Labeling of $\sigma$ Subunit with 1,5-I-AENS.* Figure 1 shows the reaction of isolated $\sigma$ subunit of *E. coli* RNA polymerase with 50 molar excess of 1,5-I-[$^3$H]AENS for 30 min at 23 °C followed by prolonged incubation at 3 °C. About 1.1 mol of AENS was incorporated per mol of $\sigma$ after 5 h incubation, and 1.2 mol per mol of $\sigma$ after 24 h. The results indicate that there are a fast reacting sulfhydryl residue and probably another slowly reacting one in $\sigma$ subunit. In fact, modification of $\sigma$ subunit with [$^{35}$S]tetrathionate showed 2 mol of sulfenyl group incorporated per mol of $\sigma$ (Yarbrough and Wu, 1974). In order to insert a single fluorescent probe into $\sigma$ subunit, the labeling was carried out with lower molar excess (two- to fivefold) of 1,5-I-AENS. Under these conditions, only 1 mol of AENS was incorporated per mol of $\sigma$ even after prolonged incubation. This preparation of the fluorescent labeled $\sigma$ subunit (AENS-$\sigma$) was used for the physical and biological studies described below. AENS-$\sigma$ was capable of stimulating RNA synthesis catalyzed by core polymerase using T7 DNA as template to about the same extent as unmodified $\sigma$. This is shown in Figure 2.

*Fluorescence Properties of AENS-$\sigma$.* The fluorescence excitation and emission spectra of AENS-$\sigma$ are given in Figure 3. The excitation and emission maxima are at 350 and 495 nm,

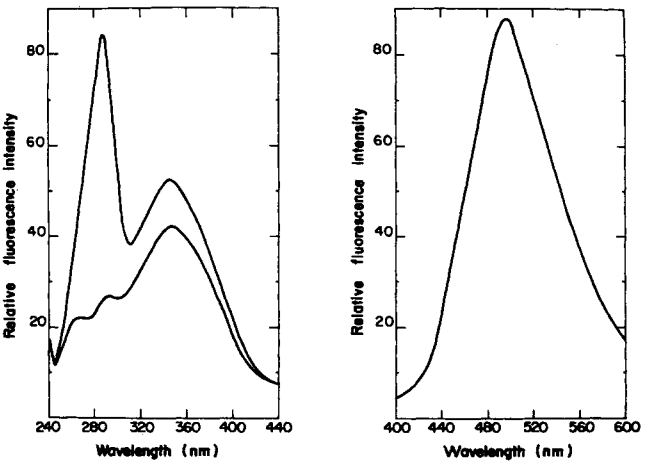


FIGURE 3: Fluorescence excitation and emission spectra (uncorrected) of AENS-$\sigma$. The solution (0.5 ml) contained 50 pmol of AENS-$\sigma$ in 0.05 M Tris-HCl (pH 8), 0.5 M KCl, 0.1 mM EDTA, and 0.1 mM dithiothreitol. (Right) The emission spectrum of AENS-$\sigma$. The excitation wavelength was 350 nm. (Left) The excitation spectra of AENS-$\sigma$. The emission wavelength was 500 nm. (Lower curve) Same solution as for emission spectrum; (upper curve) 50 pmol of core enzyme added.

respectively. The quantum yield of the bound dye was 0.12. The nanosecond emission kinetics of AENS-$\sigma$ showed a linear plot of log $S(t)$ vs. time (Figure 4) and were fitted by a single excited-state lifetime of 15 ns. In contrast, the time dependence of the logarithm of the emission anisotropy is clearly nonlinear (Figure 5). The plot of log $A(t)$ vs. time shows a fast drop of the anisotropy to 0.16 (within 10 ns) followed by a slow decay. The initial decrease in emission anisotropy could arise from the rotational freedom of the fluorescent probe or the local mobility of the binding site on $\sigma$ subunit. The slow decay, which reflects the rotational motion of $\sigma$ subunit as a whole, could be fitted with a rotational correlation time of about 100 ns.

*Interaction of AENS-$\sigma$ with Core Polymerase.* On excitation at 350 nm, addition of a molar excess of core polymerase to a solution of AENS-$\sigma$ increased the AENS fluoresence intensity by 20% and caused a 2–3-nm blue shift of the wavelength of maximum emission (data not shown). The corresponding enhancement of fluorescence intensity in the excitation spectrum (emission at 500 nm) was seen in the 350-nm

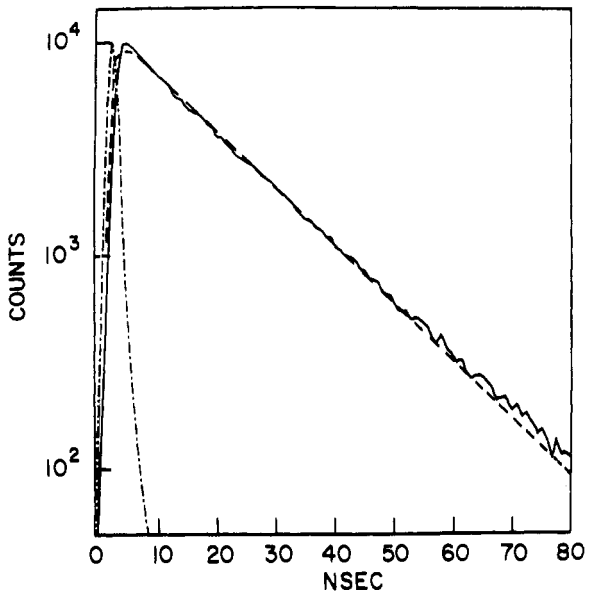


FIGURE 4: Nanosecond emission kinetics of AENS-$\sigma$. The solution contained $3 \times 10^{-7}$ M of AENS-$\sigma$ in 0.05 M Tris-HCl (pH 8), 0.5 M KCl, 0.1 mM EDTA, and 0.1 mM dithiothreitol. (---) Light pulse; (—) observed fluorescence decay; (- - -) calculated fluorescence decay with $\tau$ = 15 ns.

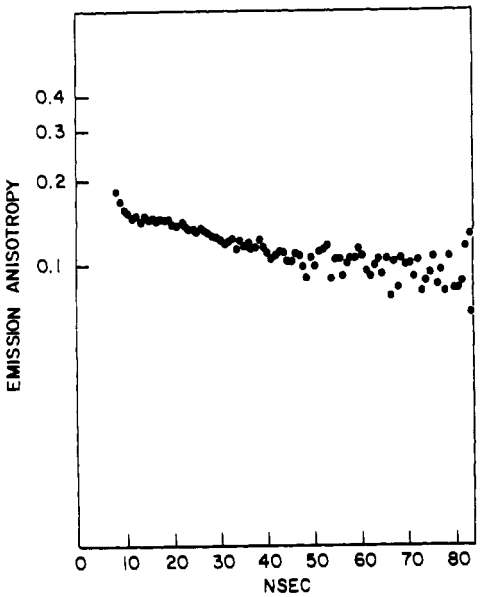


FIGURE 5: Time dependence of the emission anisotropy of AENS-$\sigma$. The solution used was the same as that in Figure 4.

band (Figure 3). These changes in AENS fluorescence are due to increased quantum yield of the probe when $\sigma$ is bound to core enzyme. An additional band peaked at 293 nm was observed in the excitation spectrum of the AENS-$\sigma$-core polymerase complex. This distinct increase in the magnitude of the excitation spectrum is due to energy transfer from tryptophan residues in core polymerase to the bound dye on $\sigma$. Changes in this signal as various amounts of core polymerase were added to 50 pmol of labeled $\sigma$ are shown in Figure 6. There is a linear increase of the fluorescence intensity up to about a 1:1 mol ratio of $\sigma$ to core enzyme and then the curve levels off. With a large excess (> tenfold) of core enzyme, however, a slow increase in fluorescence intensity with respect to addition of core enzyme was observed. This increase was probably due to some aggregation processes. Since the binding appears to be stoichiometric under the conditions described in Figure 6, an ap-

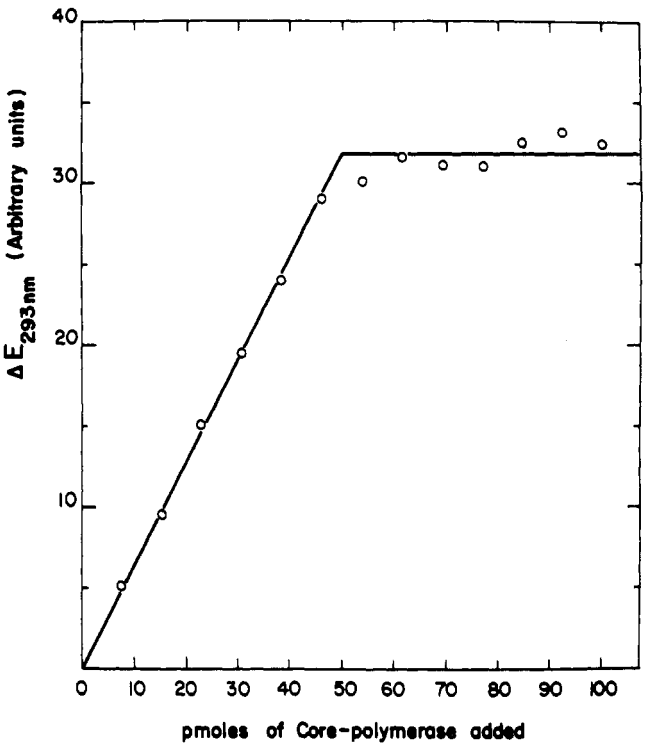


FIGURE 6: Fluorimetric titration of AENS-$\sigma$ with core enzyme. The solution contained 50 pmol of AENS-$\sigma$ in 0.05 M Tris-HCl (pH 8), 0.5 M KCl, 0.1 mM EDTA, and 0.1 mM dithiothreitol. Aliquots of core enzyme (1 mg/ml) were added consecutively to 0.5 ml of the AENS-$\sigma$ solution. Excitation was at 293 nm and emission was at 500 nm. A Corning CS 3-69 filter was placed in front of the emission monochromator.

parent dissociation constant for the $\sigma$-core enzyme interaction can be estimated to be equal to or less than $2 \times 10^{-9}$ M.

The AENS-$\sigma$-core polymerase complex has a single excited-state lifetime of 16.4 ns. A biphasic decay of emission anisotropy similar to that of AENS-$\sigma$ alone was observed for the complex. The fast drop of the emission anisotropy to 0.16 remained the same. However, the second decay was much slower with a rotational correlation time of about 500 ns, indicating the formation of a molecular complex larger than $\sigma$ subunit.

*Energy Transfer from AENS-$\sigma$ to Rifampicin on RNA Polymerase.* By means of gel filtration technique (Yarbrough and Wu, 1974), we found that the labeled $\sigma$-core polymerase complex was able to bind rifampicin to the same extent as unlabeled holoenzyme. Thus energy transfer measurements were carried out to estimate the distance between rifampicin and the AENS binding site in the AENS-$\sigma$-core polymerase complex. Such measurements were possible because rifampicin, which has an absorption maximum at 470 nm, is an ideal energy acceptor for AENS (Figure 7).

For energy transfer measurements, one usually determines the value of $R_0$ (Förster, 1966), the distance at which the transfer efficiency is 50%. $R_0$ contains all the physical information necessary to describe dipole-dipole energy transfer between the donor and acceptor except their separation:

$$R_0 = (JK^2Q_0n^{-4})^{1/6}(9.79 \times 10^3) \quad (6)$$

$J$, the spectral overlap integral, is calculated from the corrected fluorescence emission spectrum of the AENS-$\sigma$-core polymerase complex and the absorption spectrum of the rifampicin-holoenzyme complex (Figure 7). The value of $J$ thus obtained was $4.03 \times 10^{-14}$ cm$^3$ M$^{-1}$. $Q_0$, the quantum yield of the AENS-$\sigma$-core polymerase complex, was determined to be

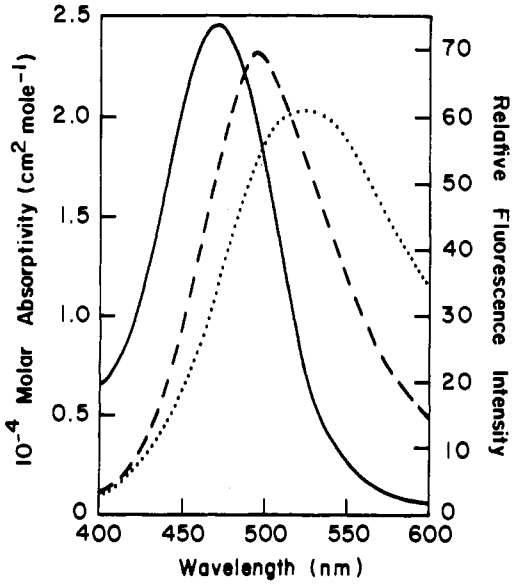


FIGURE 7: Overlap of the absorption spectrum of rifampicin with the fluorescence spectra of AENS-$\sigma$ and Dns-$\sigma$ in the rifampicin–enzyme complex. (—) Absorption spectrum of rifampicin; (- - -) corrected fluorescence emission spectrum of AENS-$\sigma$; (. . .) corrected fluorescence emission spectrum of Dns-$\sigma$.

0.13. Although $n$, the refractive index of the medium through which the energy transfer occurs, cannot be measured directly, there is relatively little uncertainty as to its value, which we assume to be 1.4. $K^2$, the orientation factor, which depends on the relative orientation of transition dipoles of each donor–acceptor pair, is often unknown as is the case here. Thus it is more convenient to use a characteristic distance $R_c = R_0/(K^2)^{1/6}$ rather than $R_0$ in order to describe the strength of interaction of a donor–acceptor pair. For energy transfer from AENS to rifampicin on the AENS-$\sigma$–core polymerase complex, $R_c$ was calculated to be 33.2 Å.

The efficiency of energy transfer, $T$, was determined from excited-state lifetime of the donor in the presence and absence of acceptor (denoted by $\tau$ and $\tau_0$, respectively):

$$T = 1 - (\tau/\tau_0) \qquad (7)$$

A transfer efficiency of $0.02 \pm 0.01$ was observed when a molar excess of rifampicin ($5 \times 10^{-7}$ M) was added to a solution of the AENS-$\sigma$–core polymerase complex. This transfer efficiency represents the averaged results of three different preparations of AENS-$\sigma$, and for each preparation several lifetime measurements were performed. In this analysis, we made the assumption that energy transfer was the only mechanism responsible for shortening of the lifetime of AENS in holoenzyme upon addition of rifampicin.

*Nonspecific Surface Labeling of $\sigma$ Subunit with Dns-Cl on Celite.* To label randomly as many amino groups as possible on the surface of $\sigma$ subunit, the reaction was carried out with Dns-Cl adsorbed on Celite in aqueous solutions of $\sigma$ subunit buffered near the $pK_a$ of the $\epsilon$-amino group of lysine. It has been found that, under these conditions, surface labeled proteins with a relatively large average number of dye molecules per protein could be obtained in short reaction time (Gennis et al., 1972). For $\sigma$ subunit, 1 to 5 Dns groups were bound per protein molecule during 5–10 min reaction at 23 °C. The preparations of Dns-$\sigma$ (unaggregated monomer) were >80% active when compared with unlabeled $\sigma$ in stimulating the transcription of T7 DNA by core enzyme.

*Energy Transfer from Dns-$\sigma$ to Rifampicin on RNA Polymerase.* The fluorescence emission spectrum of Dns-$\sigma$ is shown in Figure 7. The emission maximum is at 520 nm. Unlike that of AENS-$\sigma$, the fluorescence emission spectrum of Dns-$\sigma$ did not change upon addition of core polymerase.

From Figure 7, it can be seen that rifampicin is also a potential energy acceptor for the Dns chromophore. The value of $J$ for this donor–acceptor pair was determined to be $3.37 \times 10^{-14}$ $cm^3$ $M^{-1}$. Again assuming $n = 1.4$, a value of 40.7 Å was obtained for $R_c$.

The nanosecond emission kinetics of Dns-$\sigma$ could be fitted by two excited-state lifetimes, $\tau_1 = 4$ ns and $\tau_2 = 20$ ns, with relative amplitudes $a_1 = 0.4$ and $a_2 = 0.1$. Multiple lifetimes, which would be expected for a protein labeled with more than one chromophore, are difficult to use in energy transfer analysis. Thus the efficiency of transfer of excitation energy from Dns-$\sigma$ to rifampicin on RNA polymerase was determined from the measured quenching of Dns fluorescence of the Dns-$\sigma$–core enzyme complex in the presence of rifampicin. Addition of saturating rifampicin ($4.8 \times 10^{-7}$ M) to a solution of the Dns-$\sigma$–core enzyme complex in 0.2 M KCl resulted in a 10% decrease in fluorescence intensity. If the KCl concentration was lowered to 0.05 M, 20% quenching of fluorescence intensity was observed. No spectral shift was associated with the quenching.

## Discussion

*AENS-$\sigma$.* The isolated $\sigma$ subunit of *E. coli* RNA polymerase contains two sulfhydryl residues as determined by reaction with tetrathionate (Yarbrough and Wu, 1974). Kinetic studies of the reaction of $\sigma$ with 1,5-I-AENS, a fluorescent derivative of iodoacetamide, indicate that one sulfhydryl residue is highly reactive while the other reacts very slowly. AENS-$\sigma$, in which the reactive sulfhydryl residue has been labeled, still retains its original ability to stimulate specific RNA chain initiation. Thus this reactive sulfhydryl residue is not essential for the biological activity of $\sigma$.

The observed increase in quantum yield and small blue shift of the emission maximum of AENS-$\sigma$ upon binding to core polymerase can be explained in two ways. Core enzyme interacts directly with the chromophore by binding $\sigma$ in a region overlapping the AENS binding site. Alternatively, a conformational change in $\sigma$ is induced by binding to core enzyme, which caused increased hydrophobicity in the environment of the chromophore. The former case seems less likely since AENS-$\sigma$ binds core polymerase as tightly as unmodified $\sigma$ does.

The nanosecond fluorescence kinetics of AENS-$\sigma$ can be adequately represented by a single exponential decay (Figure 4). This is consistent with our observation that there is one AENS group bound per molecule of $\sigma$. Although the lifetime of AENS-$\sigma$ is not long enough to allow detailed analysis of the size and shape of $\sigma$ subunit, the rotational correlation times obtained for $\sigma$ and the $\sigma$–core enzyme complexes are comparable to those obtained with longer lifetime probes such as pyrenemaleimide and Dns-Cl (Yarbrough and Wu, to be published). An interesting observation in the emission anisotropy measurements of AENS-$\sigma$ and the AENS-$\sigma$–core enzyme complex is the fast decay of emission anisotropy to 0.16 within the excited-state lifetime of the chromophore (Figure 5), indicating its rapid reorientation independent of macromolecular rotation. Following an approach for the analysis of segmental motion in macromolecules detailed elsewhere (Dale and Eisinger, 1974, 1975), we have determined the dynamic depolarization factor $\langle d' \rangle_d$ for rapid reorientation of the AENS chromophore bound to $\sigma$. In the case where the ab-

sorption and emission transition moments of the chromophore coincide, $\langle d' \rangle_d = A_0/A_f = 0.16/0.4 = 0.4$, where $A_0$, the limiting value of the emission anisotropy at time zero, was obtained by extrapolating the linear portion of the logarithmic emission anisotropy plot at long times to time zero (Figure 5). Furthermore, if the transition moment of AENS is constrained to the surface of a cone of half-angle $\psi$ during its rapid reorientation, $\psi$ can be shown to be on the order of 30° as calculated from $\langle d' \rangle_d = 0.16$ using the following equation (Dale and Eisinger, 1974)

$$\langle d' \rangle_d = \left(\frac{3}{2}(\cos^2 \psi) - \frac{1}{2}\right)^2 \quad (8)$$

In the above calculation, an assumption was made that the value of $A_f$ for AENS-$\sigma$ fluorescence is 0.4. However, there is evidence that for AENS the value of $A_f$ may be as low as 0.32 (Hudson and Weber, 1973). The value of $\psi$ corresponding to $A_f = 0.32$ is 28°. Use of this value in place of 30° has little effect on the interpretation of the transfer efficiency into a distance. It should be pointed out that the measured emission anisotropy curve might be distorted in the time range comparable to the duration of lamp pulse (initial 5 ns) since it was computed from fluorescence decays convoluted with the lamp pulse. At present accurate methods for computing deconvoluted anisotropy data are not available. In any case, since we made use of values of the anisotropy at long times in our computation of $\langle d' \rangle_d$, the validity of our estimate of the rapid segmental mobility should not be affected by any convolution artifact (even if such artifact does exist, it will not broaden the range of the distance interpretation since it tends to increase falsely the emission anisotropy near zero time and thereby leads to underestimation of the degree of rapid reorientation). In order to further examine the reliability of the interpretation of segmental motion from the observed rapid decay of emission anisotropy, we have determined the steady-state emission anisotropy $\langle A \rangle$. This parameter is related to $A_0$ by (Jablonski, 1962)

$$\langle A \rangle = A_0[\phi/(\tau + \phi)] \quad (9)$$

where $\tau$ is the excited-state lifetime of the chromophore and $\phi$ is the rotational correlation time of the macromolecule. Using the experimentally measured values of $\langle A \rangle$ (0.146), $\tau$ (16.4 ns), and $\phi$ (450 ns) for the AENS-$\sigma$-core polymerase complex, a value of $A_0$ of 0.15 was obtained. This is in good agreement with the value of $A_0 = 0.16$ obtained by extrapolation of the time-dependent emission anisotropy.

A serious limitation in applying the energy transfer technique to measure intramolecular distances is the difficulty in assigning a proper value to the orientation factor of the donor–acceptor pairs. Theoretically the orientation factor can have any value between 0 and 4, thereby introducing a fundamental uncertainty into the distance determination. Time-dependent emission anisotropy measurements of transfer depolarization are potentially useful in determining the mutual orientation of the donor–acceptor pairs (Dale and Eisinger, 1974). For the energy transfer from AENS to rifampicin in the AENS-$\sigma$-core polymerase complex, such determination is not possible because the acceptor is not fluorescent. Nevertheless, the orientation freedom of the donor within its excited state lifetime allows us to average its emission dipole orientation over a cone surface of 30° half-angle when computing $K^2$ in the energy transfer calculations. Since rifampicin, the acceptor, is noncovalently attached in a very tight fashion to a single site on the enzyme (Zillig et al., 1970), we assume it to be held at some unknown fixed orientation in respect to an arbitrary direction in the enzyme, the same orientation for all molecules. (For the single, covalently attached AENS donor the same assumption applies to the axis of its cone of orientation freedom.) We obtain a maximum value of 3.0 for $K^2$ when the axis of the donor's cone of orientation freedom is parallel to and in line with the absorption transition moment of the acceptor, and a minimum value of 0.12 for $K^2$ when one of these two vectors is in line and the other perpendicular to the line separating them.

The transfer efficiency measured from the AENS donor on $\sigma$ to the rifampicin acceptor on core enzyme was $0.02 \pm 0.01$. Although this value of transfer efficiency is very low and approaches the limit of accuracy in fluorescence lifetime measurements, its validity is supported by (a) the standard deviation of the results which summarize several measurements performed with different preparations of AENS-$\sigma$, and (b) the conclusion that except for a small number of orientation geometries of the AENS-rifampicin pair, the spatial relationship of $\sigma$ and rifampicin obtained with Dns-labeled $\sigma$ requires that the AENS–rifampicin pair show a transfer efficiency of $\geq 0.01$ (see below). According to Förster theory of singlet–singlet energy transfer (Förster, 1966), the distance $R$ between the donor and the acceptor can be calculated from the transfer efficiency, $T$, using the equation

$$R = R_0\left(\frac{1}{T} - 1\right)^{1/6} \text{ or } R = R_c\left[K^2\left(\frac{1}{T} - 1\right)\right]^{1/6} \quad (10)$$

Taking $R_c = 33.2$ Å and $0.12 \leq K^2 \leq 3.0$, the distance between the labeled sulfhydryl residue on $\sigma$ and the rifampicin binding site on core polymerase ($R$) is estimated to be 42–85 Å. However, using the statistical interpretation detailed in the accompanying paper (Hillel and Wu, 1976), we have calculated that there is only a small probability ($\leq 5\%$) that $R$ is larger than 72 Å. While this large range of $R$ is of little help in locating the exact positions of these specific sites, it is consistent with the more precise spatial relationship between $\sigma$ and the rifampicin binding site on RNA polymerase obtained by energy transfer measurements using Dns-$\sigma$.

*Dns-$\sigma$.* The use of Celite-bound reactive dye in the labeling of protein was first introduced by Rinderknecht (1960, 1962). Gennis et al. (1972) subsequently applied this technique to label trypsin and trypsin inhibitor for energy transfer measurements and their results were in good agreement with calculations which assume random surface labeling of the proteins. In accord with this assumption, we have found that the distribution of dye in each subunit of RNA polymerase holoenzyme labeled with Dns-Cl on Celite is roughly proportional to the exposed surface of the subunits (Wu, unpublished data). Thus the Celite technique was utilized in order to maximize the random surface labeling of $\sigma$ subunit. The observation that the efficiency of energy transfer from Dns to rifampicin is independent of the stoichiometry of Dns bound supports the assumption of random surface labeling. Hence until the three-dimensional structure of $\sigma$ is determined by other methods, e.g., x-ray crystallography, and the exact position and orientation of the bound Dns are known, it seems reasonable to analyze the energy transfer data for Dns-$\sigma$ in terms of a model having a randomly distributed and oriented array of donors on the surface of $\sigma$ subunit. $\sigma$ subunit has a molecular weight of 100 000 (Burgess, 1969) and, assuming specific volume of 0.73 ml/g, a radius of 31 Å can be assigned to the equivalent anhydrous sphere. Measurements of fluorescence depolarization of the Dns-$\sigma$-core enzyme complex yielded a value of 0.6 for $\langle d' \rangle_d$. Thus the rotational mobility of Dns chromophore can be assigned to the surface of a cone of 24°

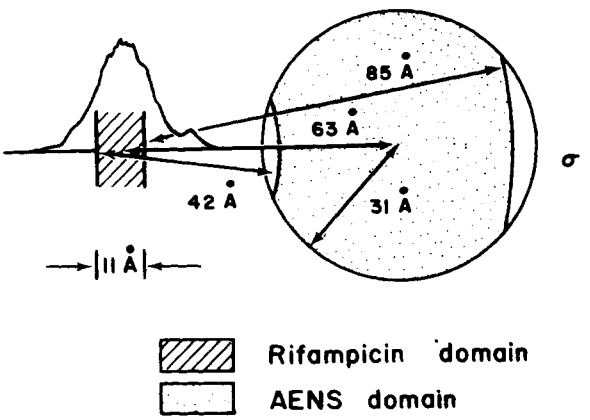


FIGURE 8: Distance relationships of $\sigma$ subunit and the rifampicin binding site in RNA polymerase according to a model which assumes $\sigma$ to be spherical. The region marked rifampicin domain was calculated using an averaging analysis of the energy transfer data. The bell-shaped curve represents the probability density function for the rifampicin-$\sigma$ separation according to a statistical interpretation of the same data (see the following paper in this issue).

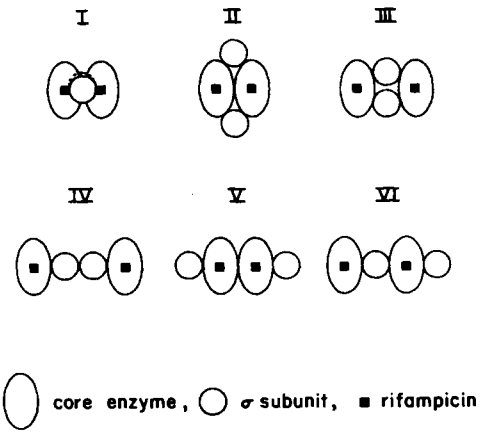


FIGURE 9: Simple models for geometric arrangements of the $\sigma$ subunits in dimeric RNA polymerase.

half angle on the average (Dale and Eisinger, 1974). Using this mobility, the 31-Å radius of $\sigma$, the value of 40.7 Å for $R_c$ of the Dns-rifampicin donor-acceptor pair and the 10 ± 1% fluorescence quenching of the Dns-$\sigma$-core enzyme complex in the presence of rifampicin, the distance from the surface of $\sigma$ subunit to the rifampicin binding site on RNA polymerase was calculated to be 32 ± 5.5 Å according to the averaging method described in the accompanying paper (Hillel and Wu, 1976).

The distance range calculated by the averaging method is not the only interpretation consistent with the experimental data. For a more critical analysis, we have also interpreted the energy transfer data of Dns-$\sigma$ using a statistical method (Hillel and Wu, 1976). The most probable range for the distance from the surface of $\sigma$ to the rifampicin binding site deduced from the statistical analysis coincides with that calculated by the averaging method. According to this model, we can further say that there is a 98% probability that the separation between the rifampicin chromophore and the closest point on the surface of spherical $\sigma$ in the holoenzyme complex is more than 15 Å. The spatial relationship of the rifampicin binding site on RNA polymerase, the $\sigma$ subunit, and the specific sulfhydryl residue on $\sigma$ labeled with AENS is illustrated schematically in Figure 8. Both the averaging and statistical analyses of the multidonor-single-acceptor system do not require knowledge of the actual number of donor molecules bound per macromolecule. What is needed is the number of potential donor sites, which we estimated to be 20 for Dns-$\sigma$. This number, which is about half of the total number of lysine residues of the $\sigma$ subunit (Fujiki and Zurek, 1975), was assumed to represent those residues on the surface of the protein available for reaction with Dns-Cl. Errors due to uncertainty in the exact number of donor sites on $\sigma$, the size of $\sigma$ subunit, and the donor emission dipole mobility are considered elsewhere (Hillel and Wu, 1976). A very important source of error in the interpretation of energy transfer from a donor array to an acceptor is the shape of the donor distribution. For the Dns-$\sigma$ system, it was assumed to be spherical. However, $\sigma$ subunit may be asymmetric. Preliminary interpretation of anisotropy decay measurements of fluorescent-labeled $\sigma$ suggested that the $\sigma$ subunit may be pictured as having the shape of an ellipsoid of revolution with an axial ratio in the range of 0.5–2 (Yarbrough and Wu, to be published). The effect of this asymmetry on the interpretation of energy transfer from Dns-$\sigma$ to rifampicin was analyzed in the accompanying paper in terms of various ellipsoidal shapes for $\sigma$. The results indicate that for axial ratios in the range of 0.5–2 there is at least a 95% probability that the distance from the surface of $\sigma$ to rifampicin is greater than 10 Å if $\sigma$ is an oblate ellipsoid. However, if $\sigma$ is a prolate ellipsoid there is an extreme case in which there is only a 90% probability that rifampicin is more than 5 Å away from the $\sigma$ surface.

There is genetic evidence suggesting that the rifampicin binding site resides on the $\beta$ subunit (Zillig et al., 1970). The radius of a spherical $\beta$ subunit is about 37 Å (although $\beta$ may be elongated); thus $\beta$ could be in contact with $\sigma$. However, the 10-Å lower limit of the distance from the rifampicin binding site to the surface of $\sigma$ suggests that there is little possibility of a direct interaction between rifampicin and $\sigma$ subunit. Therefore rifampicin inhibition of RNA chain initiation by RNA polymerase does not involve a direct rifampicin-$\sigma$ interaction. This is expected since rifampicin also interacts with core polymerase (Wehrli and Staehelin, 1969) and inhibits initiation of nonspecific transcription catalyzed by core polymerase (de Mauro et al., 1969).

RNA polymerase holoenzyme forms a dimer under conditions of low ionic strength (Richardson, 1966) and the dimer binds two molecules of rifampicin (Wehrli and Staehelin, 1969). We have observed that the efficiency of energy transfer from Dns-$\sigma$ to rifampicin bound to the enzyme increases from 10% in 0.2 M KCl to 20% in 0.05 M KCl solutions. Such increases in transfer efficiency may be due to an ionic-strength-sensitive conformational change in holoenzyme monomer that shortens the distance between the rifampicin binding site and $\sigma$. This is not very likely because no change in the fluorescence spectrum of the Dns-$\sigma$-core polymerase complex was observed by lowering the salt concentration in the absence of rifampicin. Another explanation for the doubling of the transfer efficiency at the low salt concentration is that each Dns-$\sigma$ transfers energy equally to both rifampicin acceptors in the dimeric enzyme. Under conditions of low energy transfer such as observed here, the transfer efficiency would roughly double if the number of acceptors doubled and if the distances from each donor distribution to each of the two acceptors were about equal. Several models for simple geometrical arrangements of $\sigma$ subunits in RNA polymerase dimer are given in Figure 9. In these models, core polymerase has an elongated shape as suggested by a low angle x-ray scattering study (Pilz and Kratky, 1972) and by our fluorescence polarization data (Yarbrough and Wu, to be published). Models I–III in which the two rifampicin binding sites are equidistant to each $\sigma$ subunit are consistent with our energy transfer results.

Models IV–VI can be eliminated since the rifampicin binding sites are not symmetric with respect to their distance to each $\sigma$ subunit.

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