Rose Bengal: a Spectroscopic Probe for Ribonucleic Acid Polymerase†

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ABSTRACT: Interaction of Rose Bengal with RNA polymerase of *Escherichia coli* has been studied by absorption and fluorescence spectroscopy. Upon binding to RNA polymerase, the absorption maximum of Rose Bengal shifted from 546 to 561 nm, while the fluorescence emission maximum shifted from 567 to 575 nm. The protein-induced enhancement in fluorescence intensity of Rose Bengal (the quantum yield changed from 0.013 to 0.12), however, was much greater than the increase in absorbance (an 18% increase in extinction coefficient). The stoichiometry and dissociation constants of the enzyme–inhibitor complex were determined by difference-spectrophotometric and fluorimetric titrations. In both cases, a dissociation constant of 6×10^{-7} M was obtained. This value is in good agreement with the K_i value (7×10^{-7} M) calculated from kinetic studies. The number of Rose Bengal binding sites estimated on RNA polymerase is 1.3/enzyme molecule (mol wt 500,000). In order to gain insight into the nature of the Rose Bengal binding site, the effect of solvent polarity on the fluorescence properties of Rose Bengal has been investigated. It was concluded that the environment of the Rose Bengal binding site on RNA polymerase is highly nonpolar and perhaps consists of a hydrophobic pocket. Furthermore, the fluorescence of tryptophan residues of RNA polymerase was quenched on addition of Rose Bengal. Excitation spectrum of the Rose Bengal–enzyme complex indicates that this is due to energy transfer from tryptophan residues to the bound dye. The efficiency of transfer is markedly decreased by addition of DNA and NTP.[1] This could be explained by template and substrate-induced conformational changes of RNA polymerase. Although the binding of Rose Bengal markedly alters the catalytic properties of RNA polymerase, all the experimental results are consistent with a picture in which the Rose Bengal binding site is spatially distinct from the template or substrate binding sites on the enzyme.

In the preceding paper, we have reported that Rose Bengal is a potent inhibitor of *Escherichia coli* RNA polymerase, which selectively inhibits RNA chain elongation. It binds reversibly to the enzyme but does not bind to the template. Kinetic analysis suggests that the Rose Bengal binding site on the enzyme may be spatially distinct from the substrate or template sites. However, when Rose Bengal is bound to RNA polymerase, the enzyme is incapable of synthesizing RNA chains. Thus, it was of interest to further study the Rose Bengal–RNA polymerase interaction and the nature of the Rose Bengal binding sites on the enzyme.

Absorption and fluorescence spectroscopy are useful techniques for measuring the interaction between ligands and macromolecules. Spectroscopic changes due to the binding of Rose Bengal to liver alcohol dehydrogenase (Brand *et al.*, 1967) and 6-phosphogluconate dehydrogenase (Rippa and Picco, 1970) have been used to probe the active sites of these enzymes. In this paper, we show that the interaction of Rose Bengal with RNA polymerase results in alternation of both absorption and fluorescence spectra of the dye, as well as the fluorescence spectra of the enzyme. On the basis of these spectroscopic studies, we have determined some physical and chemical properties of the Rose Bengal site on RNA polymerase. Furthermore, the stoichiometry and binding constants measured by spectroscopic methods are in agreement with those obtained by kinetics described in the preceding paper (Wu and Wu, 1973)

Experimental Section

Materials. *E. coli* RNA polymerase, calf thymus DNA, poly[d(A-T)], nucleoside triphosphates, and Rose Bengal were purchased or purified as described in the preceding paper (Wu and Wu, 1973). All solvents used were Spectral Grade, and obtained from Fisher Scientific Co. The concentration of RNA polymerase was determined by measuring the absorption at 280 nm using an $A_{1\text{ cm}}^{0.1\%}$ of 0.65 (Richardson, 1966). A molecular weight of 500,000 daltons for the enzyme was used in all calculations. The concentrations of Rose Bengal was determined by a molar extinction coefficient of 9.5×10^4 cm^2 $mmol^{-1}$ at its absorption maximum of 545 nm (Brand *et al.*, 1967).

Spectrophotometric Measurements. Spectrophotometric determinations were carried out with a Cary 118C recording spectrophotometer, in a 1-cm light-path quartz cell. The cell compartment was thermostatically controlled at 25 ± 0.1° for all measurements. Difference spectra were measured in rectangular quartz tandem cells (Pyrocell Mfg. Co.) having either 1.0- or 0.44-cm path length in each chamber. Titration experiments and calculations to determine the dissociation constants were performed essentially as described by Eckfeldt *et al.* (1970), using an 0.1-mm slit width at a wavelength of 567 nm.

Fluorimetric Measurements. Fluorescence excitation and emission spectra were recorded in a Hitachi Perkin-Elmer fluorescence spectrophotometer, Model MPF-3, equipped with a corrected spectra accessory. With this accessory, excitation spectra were corrected for the wavelength dependency

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[1] Abbreviations used are: NTP, nucleoside triphosphate; RB, Rose Bengal.

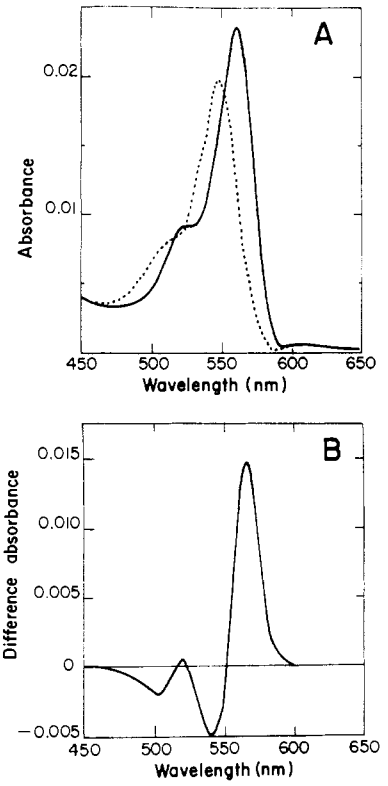

FIGURE 1: Spectral studies. (A) Absorption spectra of free and RNA polymerase bound Rose Bengal. (· · · · · · · ·) Absorption spectrum of 2×10^{-7} M Rose Bengal in 0.05 M Tris-HCl (pH 8), 0.2 M KCl, 0.1 mM dithiothreitol, and 0.1 mM EDTA (buffer A); (——) absorption spectrum of 2×10^{-7} M Rose Bengal in the same buffer containing 1×10^{-5} M RNA polymerase (with or without addition of 1 mg/ml of calf thymus DNA, 0.1 mM ATP, and 0.1 mM GTP). (B) Difference spectrum for the interaction of RNA polymerase with Rose Bengal. Absorbance of the sample cell which contained 2×10^{-7} M Rose Bengal and 1×10^{-5} M RNA polymerase in buffer A minus the absorbance of the reference cell which contained 2×10^{-7} M Rose Bengal alone in buffer A.

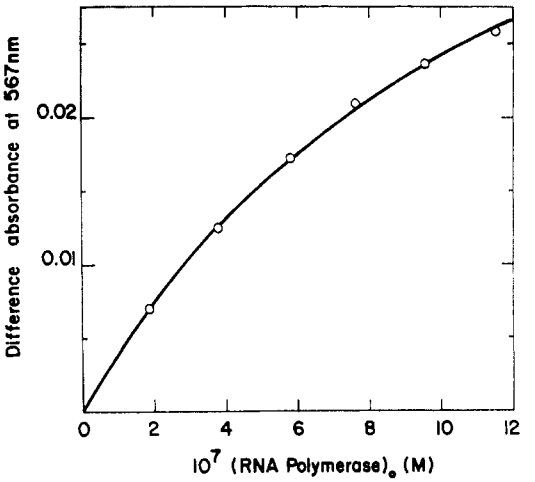

FIGURE 2: Difference spectrophotometric titration of Rose Bengal with RNA polymerase. Titrations were carried out in a Cary 118C spectrophotometer using an 0.1-mm slit width and 0.05 full-scale absorbance range. Difference absorbance at 567 nm was monitored. Five aliquots of enzyme were added to 1 ml of Rose Bengal in buffer A. Not more than three to four sequential additions to any given solution were made to minimize accumulated volumetric errors. Rose Bengal concentrations used in this experiment was 5×10^{-7} M while the enzyme concentrations ranged from 2 to 12×10^{-7} M.

of source output and excitation monochromator efficiency over a range of 250 to 600 nm. Similarly, the emission spectra were corrected for the variation with wavelength in the sensitivity of the detection system.

Quantum yield φ of a sample was calculated from the observed absorbance (A) and the area enclosed by the corrected emission spectrum using the method of Parker and Rees (1960), with a correction for the refractive index of the solvent (n).

$$\varphi_S = \varphi_R \frac{(1-10^{-A_R})}{(\text{area})_R} \frac{(\text{area})_S}{(1-10^{-A_S})} \frac{n_R^2}{n_S^2}$$

where S and R refer to sample and reference, respectively. 5-Anilinonaphthalene-1-sulfonate in ethanol was used as a reference of quantum yield 0.37 (Stryer, 1965). The areas of the corrected spectra were obtained by planimetry or by digital integration. Both methods gave identical results.

The solutions used for fluorescence studies had absorbances of less than 0.05 at excitation wavelength to obviate inner filter effect. Cylindrical quartz cells (0.5-cm light path) for fluorimetry (Hitachi) were used in a thermostatically controlled cell holder, and the temperature was maintained at $25 \pm 0.2°$.

Fluorescence titrations were carried out and analyzed by the method of Brand *et al.* (1967). A 0.005-ml aliquot of dye was added to a cuvette which contained 0.5 ml of reaction mixture. In each case, a titration took about 5 min and six to ten points were taken for each titration.

Evidence That No Significant Photooxidation Occurs During Spectroscopic Studies. Ishihama and Hurwitz (1969) found that photooxidation of RNA polymerase in the presence of Rose Bengal resulted in the loss of enzyme activity. However, this required prolonged illumination with an intensive light source. In our experiments described below, enzyme activity was routinely measured before and after spectrophotometric and fluorimetric experiments. No significant loss of enzyme activity was detected under the experimental conditions described in this paper.

Results

Absorption Spectrum of the Rose Bengal–RNA Polymerase Complex. Rose Bengal in aqueous solution possesses an absorption maximum of 546 nm with a molar extinction coefficient of 9.5×10^4 cm^2 mmol^{-1} (Figure 1). When a high concentration of RNA polymerase (10^{-5} M) was added to relatively low concentrations of Rose Bengal (2×10^{-7} M) essentially all of the Rose Bengal was bound to the enzyme. The absorption spectrum of the bound Rose Bengal is characterized by a red shift of the absorption maximum from 546 to 561 nm with an 18% increase in extinction coefficient (Figure 1A). Addition of DNA or nucleoside triphosphates did not alter the absorption spectrum of the Rose Bengal–enzyme complex.

The difference spectrum produced by the binding of Rose Bengal to RNA polymerase is demonstrated in Figure 1B. The maximum of the difference absorbance was at 567 nm. The dissociation constant of the Rose Bengal–enzyme complex was determined by difference spectrophotometric titration. In Figure 2, the difference absorbance at 567 nm was monitored as a function of the concentration of RNA polym-

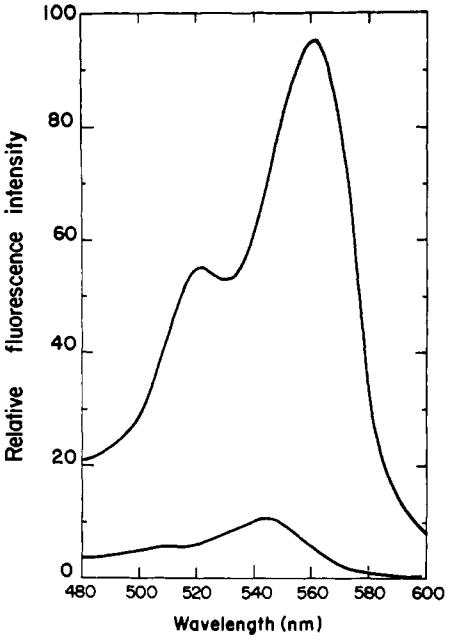

FIGURE 3: Corrected fluorescence excitation spectra of free (lower curve) and bound (upper curve) Rose Bengal. The solution contained 4×10^{-7} M Rose Bengal in buffer A, while RNA polymerase, 1×10^{-5} M, was present in the case where bound Rose Bengal was measured. The emission wavelength was held constant at 610 nm.

erase. The dissociation constant was calculated using the equation

$$K = \frac{[\bar{E}][\bar{RB}]}{[\overline{E - RB}]} = \frac{([E]_0 - \Delta a/\Delta\epsilon)([RB]_0 - \Delta a/\Delta\epsilon)}{\Delta a/\Delta\epsilon} \quad (1)$$

where $[\bar{E}]$, $[\bar{RB}]$, and $[\overline{E - RB}]$ are equilibrium concentration of enzyme, Rose Bengal, and the enzyme–Rose Bengal complex, respectively; $[E]_0$ and $[RB]_0$ are total concentration of enzyme and Rose Bengal, Δa is the measured difference absorbance at 567 nm, and $\Delta\epsilon$ is the molar difference extinction coefficient at the same wavelength. After assuming a value of $\Delta\epsilon$, K was calculated for all enzyme concentrations using the measured values of Δa. The best value of $\Delta\epsilon$ was taken to be that which minimized the per cent standard deviation of K. The dissociation constant thus obtained was 6×10^{-7} M and the best fit for $\Delta\epsilon$ was 88,000 M^{-1} cm^{-1}.

Fluorescence Spectra of the Rose Bengal–RNA Polymerase Complex. The excitation spectra of free Rose Bengal and the Rose Bengal–RNA polymerase complex are shown in Figure 3. The free Rose Bengal exhibits an excitation peak at 546 nm, and the bound Rose Bengal at 561 nm. These wavelengths are identical with that of the maxima of absorption spectra (Figure 1). However, the protein-induced enhancement in fluorescence intensity (10-fold) is much greater than the increase in absorbance (18%). This indicates the increased sensitivity of the fluorescence measurement over the absorption measurement.

A similar enhancement of fluorescence intensity was also seen in the emission spectra (Figure 4). On excitation at 500 nm, the emission maximum of the free Rose Bengal was at 567 nm. When Rose Bengal was bound to RNA polymerase, the emission maximum shifted to 575 nm, while the quantum yield of Rose Bengal increased from 0.013 to 0.12. Moreover, both excitation and emission spectra of the Rose Bengal–enzyme complex were not altered by addition of DNA and nucleoside triphosphates.

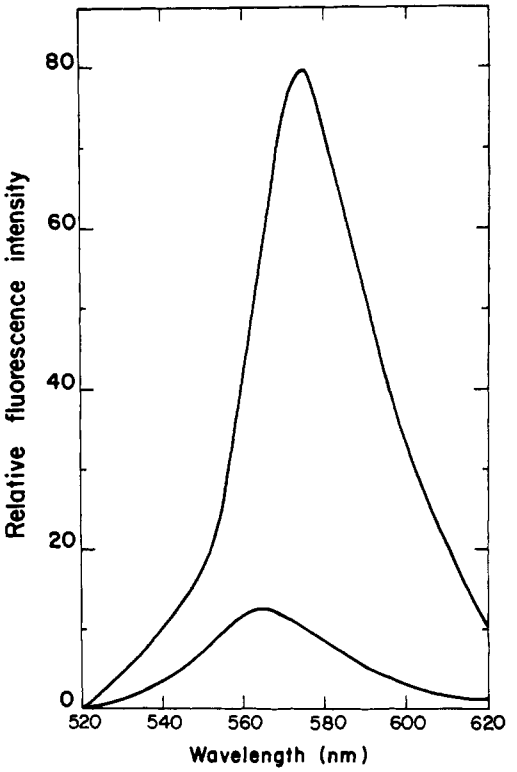

FIGURE 4: Corrected fluorescence emission spectra of free (lower curve) and bound (upper curve) Rose Bengal. Solutions were the same as in Figure 3 and the excitation wavelength was held constant at 500 nm.

The stoichiometry and dissociation constant of the enzyme–dye complex was measured by making use of the fluorescence enhancement observed when Rose Bengal was bound to RNA polymerase. The fluorescence titration of Rose Bengal with different concentrations of RNA polymerase is shown in Figure 5. The lower curve represents the titration of Rose Bengal into Tris buffer and gives the fluorescence intensity of free dye (F_f) as a function of its concentration. The upper curve shows the titration of dye into saturating concentrations of RNA polymerase (2×10^{-5} M) and gives the fluorescence intensity of bound dye (F_b). The fluorescence intensities at lower protein concentrations when both free and bound dye are in equilibrium (F) were used to calculate x, the fraction of dye bound (Brand *et al.*, 1967)

$$x = (F - F_f)/(F_b - F_f) \quad (2)$$

and the average number of dye molecule bound per molecule of enzyme $\bar{\nu}$.

$$\bar{\nu} = X[RB]_0/[E]_0 \quad (3)$$

Figure 6 shows the Scatchard (1949) plot of the results. The solid line gives the theoretical plot for 1.3 binding sites (n) and a dissociation constant of 6×10^{-7} M. The fluorescence binding data are consistent with the results obtained from absorption spectroscopy and kinetic studies.

Solvent Effect on Rose Bengal Fluorescence. In order to gain insight into the nature and mechanism of fluorescence changes observed for the Rose Bengal–RNA polymerase complex, measurements of fluorescence spectra and quantum yields of Rose Bengal were carried out in several organic solvents and organic solvent–water mixtures. The emission maximum of

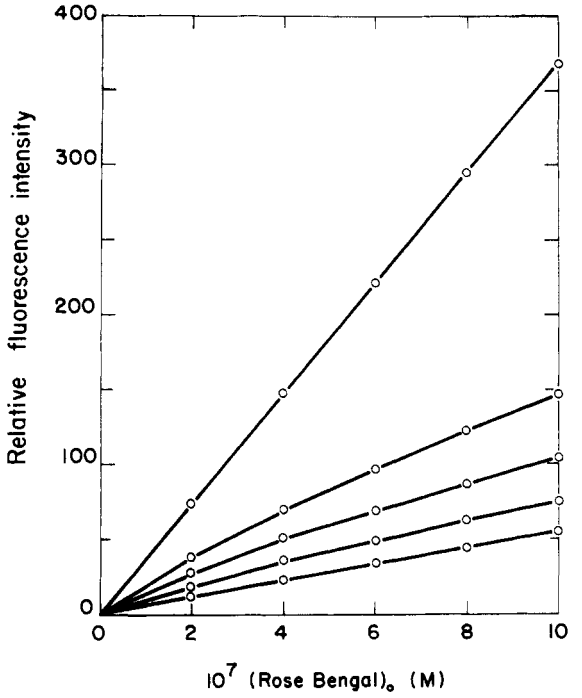

FIGURE 5: Fluorimetric titration of RNA polymerase with Rose Bengal. The solutions contained 0, 8×10^{-8}, 2×10^{-7}, 4×10^{-7}, and 2×10^{-5} M RNA polymerase (bottom to top). Aliquots (0.005 ml) of Rose Bengal were added consecutively to 0.5 ml of RNA polymerase solution in buffer A. The concentration of Rose Bengal varied from 0 to 1×10^{-6} M. The excitation was at 460 nm, while the emission was at 620 nm, and a Corning CS 3-66 filter was used before the emission monochromator.

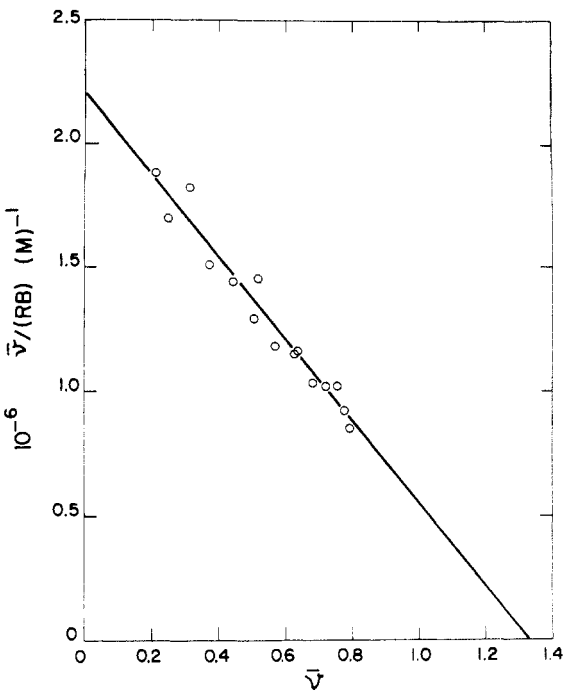

FIGURE 6: A Scatchard plot of Rose Bengal binding by RNA polymerase as determined by fluorimetric titration. Data were taken from Figure 5. $\bar{\nu}$ is the average number of Rose Bengal bound per molecule of enzyme while (RB) is the free Rose Bengal concentration.

Rose Bengal shifted toward the red and its quantum yield increased as the polarity of the solvent was decreased. As an example of such a change, the effect of increasing ethanol concentration on Rose Bengal fluorescence spectra in ethanol–buffer mixtures is shown in Figure 7.

Kosower (1958) has introduced an empirical polarity scale, the Z value, based on the transition energies of a pyridinium–iodine complex in various solvents. We have also observed a good correlation between the Z value and the fluorescence properties of Rose Bengal. As shown in Figure 8, an increase in wave number (8A) and a decrease in quantum yield (8B) could be correlated with an increasing in Z value (*i.e.*, increase in solvent polarity).

As described above, the Rose Bengal–RNA polymerase has an emission maximum of 575 nm and a quantum yield value of 0.12. This corresponds to the fluorescence properties of Rose Bengal in a solvent with a Z value of about 69. An organic solvent having the same Z value is *N,N'*-dimethylformamide. The emission maximum of Rose Bengal in *N,N'*-dimethylformamide is 576 nm, and the quantum yield is 0.13. Thus it is possible to measure the polarity of Rose Bengal binding sites without knowledge of the chemical composition of this binding site.

Quenching of RNA Polymerase Fluorescence by Rose Bengal. When excited at 280 nm, *E. coli* RNA polymerase possesses an emission maximum at 335 nm mainly due to fluorescence of tryptophan residues (Wu and Goldthwait, 1969). The fluorescence intensity at 335 nm was quenched without apparent wavelength shift on addition of Rose Bengal. The effect of different concentrations of Rose Bengal on the fluorescence intensity of the enzyme is shown in Figure 9. In the presence of DNA, the extent of fluorescence quenching by Rose Bengal was reduced. A further reduction of the quenching was observed when both DNA and nucleoside triphosphates were present.

This is a surprising finding since both DNA and nucleoside triphosphate did not influence the fluorescence of Rose Bengal. Nevertheless, the extent of fluorescence quenching can be used as a means of following the binding of Rose Bengal to RNA polymerase in the absence and presence of DNA and NTP. The decrease in fluorescence intensity at 335 nm (Δf) is related to the concentration of Rose Bengal by an expression analogous to the Lineweaver–Burk equation (Wu and Goldthwait, 1969) as follows

$$1/\Delta f = 1/\Delta F + K/\Delta F[\mathrm{RB}] \qquad (4)$$

where ΔF is the maximum fluorescence quenching and K is the dissociation constant for the Rose Bengal–RNA polymerase complex. The plot of $1/\Delta f$ *vs.* $1/[\mathrm{RB}]$ reveals that the value K is 1.3×10^{-6} M in the absence of ligands, 1.4×10^{-6} M in the presence of DNA, and 1.3×10^{-6} M in the presence of both DNA and nucleotide triphosphates (Figure 10). Thus DNA and NTP binding have no significant effect on the affinity of the enzyme for Rose Bengal.

Energy Transfer from Tryptophan Residues to Rose Bengal. The quenching of tryptophan fluorescence of RNA polymerase suggests that energy is transferred from tryptophan residues to bound Rose Bengal. The excitation spectrum of the Rose Bengal–RNA polymerase complex shows that this transfer in fact exists (Figure 11). The excitation spectrum of the enzyme–Rose Bengal complex has a shoulder at 280 nm (where nearly all of the absorption is due to the aromatic residues of the protein), while no such shoulder is observed in the excitation spectrum of Rose Bengal in the absence of enzyme.

The efficiency of transfer, T, is related to the intensity of

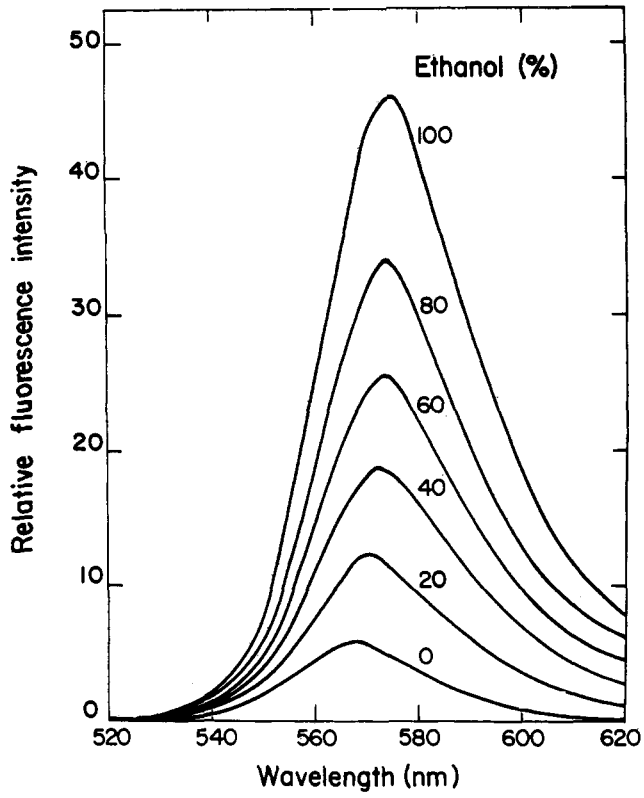

FIGURE 7: Corrected emission spectra of Rose Bengal in ethanol–buffer A mixtures. The excitation wavelength was at 500 nm, and the Rose Bengal concentration was 5×10^{-7} M. The per cent ethanol contents indicated in the figure were v/v.

tryptophan emission in the presence and absence of Rose Bengal (F and F_0, respectively) by the expression

$$T = 1 - (F/F_0) \quad (5)$$

From the emission spectrum of RNA polymerase, with excess Rose Bengal, the transfer efficiency was calculated to be 47%. This value reduced to 42% in the presence of DNA, and to 33% in the presence of both DNA and nucleoside triphosphates (calculated from ΔF in Figure 10). In principle, the transfer efficiency can also be obtained from the excitation spectrum of Rose Bengal fluorescence. However, quantitative measurement is difficult in this case due to high absorbance of the protein, DNA, and NTP in the ultraviolet region.

Discussion

Rose Bengal is an ideal spectroscopic probe for RNA polymerase because: (a) it is a specific inhibitor of RNA polymerase; (b) it absorbs and emits in a visible region far from that of the enzyme, template, and substrates; and (c) its absorption and emission spectra are very sensitive to the environment. Spectrophotometric and spectrofluorimetric measurements have revealed that Rose Bengal binds stoichiometrically and very tightly with *E. coli* RNA polymerase. The dissociation constant of the Rose Bengal–RNA polymerase complex measured by these spectroscopic studies is in good agreement with the K_i value obtained kinetically. However, the dissociation constant measured by quenching of tryptophan fluorescence is about two times greater than that obtained by other methods. This is considered to be within experimental error.

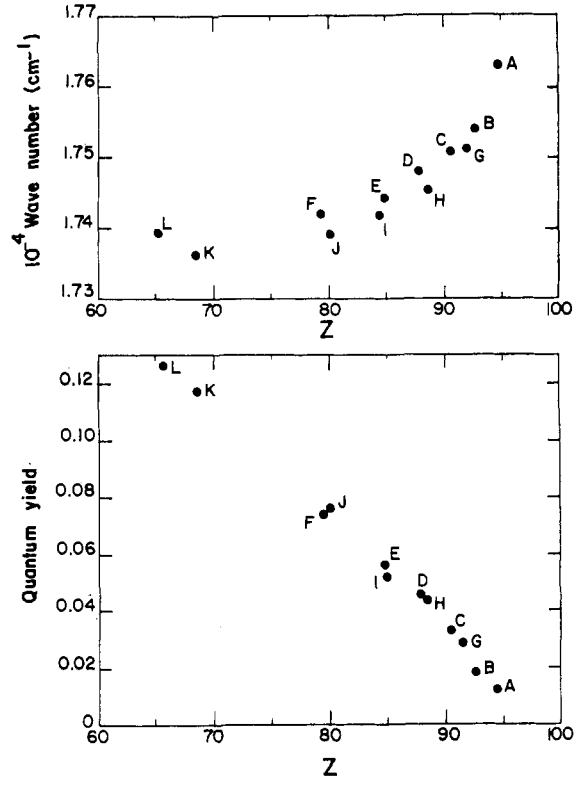

FIGURE 8: Plots of emission maximum and quantum yield of Rose Bengal *vs.* the empirical solvent polarity scale, Z (Kosower, 1958). The solvents used were A: buffer A, B: 20% ethanol, C: 40% ethanol, D: 60% ethanol, E: 80% ethanol, F: 100% ethanol, G: 20% dioxane, H: 40% dioxane, I: 60% dioxane, J: 80% dioxane, K: *N,N'*-dimethylformamide, and L: acetone. The excitation wavelength and Rose Bengal concentrations were the same as in Figure 7.

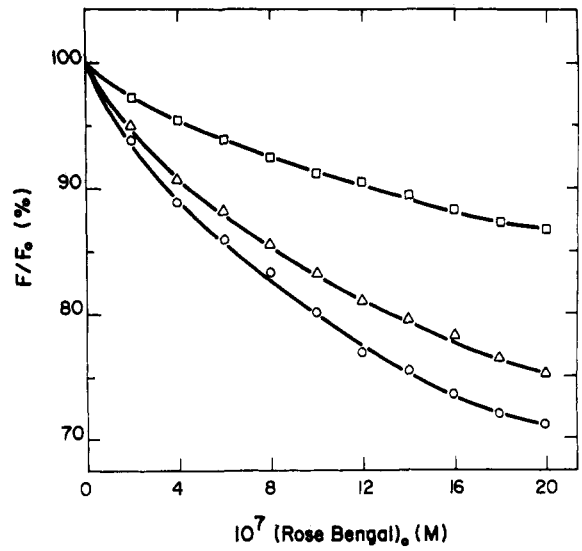

FIGURE 9: Quenching of RNA polymerase fluorescence by Rose Bengal. Studies were performed with 5×10^{-7} M RNA polymerase in buffer A containing 10 mM $MgCl_2$. The wavelength of excitation and emission were 280 and 335 nm, respectively. Aliquot (0.005 ml) of Rose Bengal was added each time to 0.5 ml of the enzyme solution. The data were corrected for both dilution and the inner filter effect. (□) Titration of enzyme alone; (△) in the presence of 0.5 mg/ml of calf thymus DNA; (○) in the presence of 0.5 mg/ml of calf thymus DNA and 0.1 mM each of ATP and GTP. F and F_0 are the fluorescence intensities at 335 nm in the presence and absence of Rose Bengal, respectively.

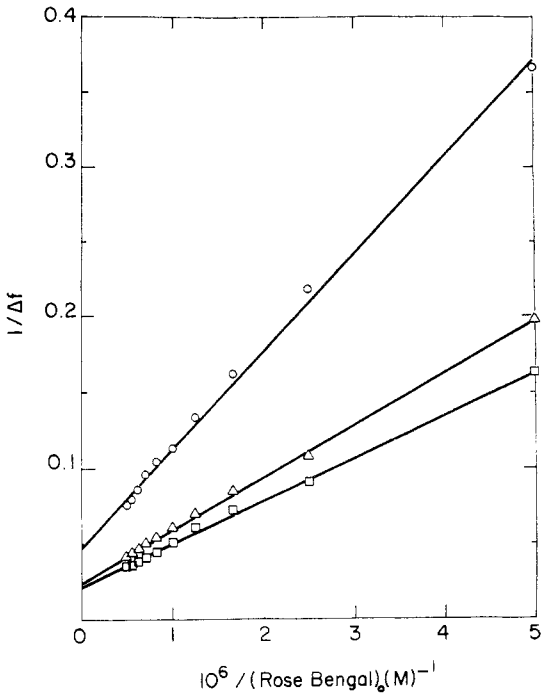

FIGURE 10: The effect of DNA and ATP on the quenching of RNA polymerase fluorescence by Rose Bengal. Data were taken from Figure 9 and plotted according to eq 4. The quenching of fluorescence, Δf, is the intensity observed in the absence of Rose Bengal minus the intensity observed in the presence of Rose Bengal. (□) Enzyme alone; (△) in the presence of 0.5 mg/ml of calf thymus DNA; and (○) in the presence of 0.5 mg/ml of calf thymus DNA and 0.1 mM each of ATP and GTP.

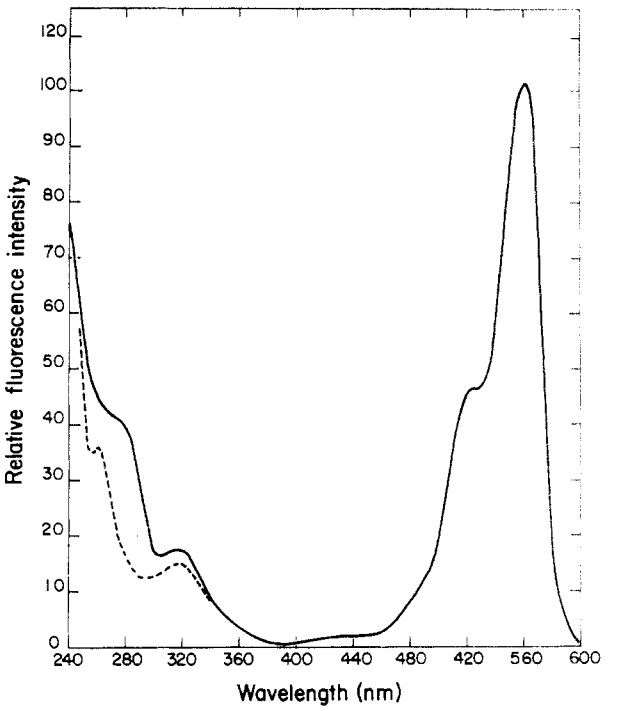

FIGURE 11: Energy transfer from tryptophan residues to the bound Rose Bengal as demonstrated by excitation spectra. The emission wavelength was at 640 nm and a Corning CS 3-69 filter was used before the emission monochromator. (——) The observed excitation spectrum of Rose Bengal–RNA polymerase complex. Rose Bengal, 5×10^{-7} M; RNA polymerase, 1×10^{-6} M. (· · · · · ·) The calculated excitation spectrum of bound Rose Bengal (5×10^{-7} M) in the absence of energy transfer.

Rippa and Picco (1970) have correlated the absorption properties of Rose Bengal to Z, the empirical solvent polarity scale of Kosower (1958). Using this correlation, they estimated the polarity of the Rose Bengal binding site of 6-phosphogluconate dehydrogenase from *Candida utilis*. When bound to the TPN binding site of 6-phosphogluconate dehydrogenase, Rose Bengal exhibited the same spectral characterization as that in a solvent having a polarity Z value of 87. Such a value is comparable to an environment similar in polarity to 60% ethanol. Rose Bengal is also a competitive inhibitor with respect to DPN binding by liver alcohol dehydrogenase (Brand *et al.*, 1967). An enhancement of dye fluorescence and a small red shift in the emission maximum were observed upon binding of the dye to the enzyme, suggesting the binding sites were hydrophobic. Similar changes are observed upon binding of Rose Bengal to RNA polymerase. There are red shifts in both absorption and fluorescence emission spectra. It can be concluded from these shifts, together with the enhancement of extinction coefficient and fluorescence quantum yield, that the environment of the Rose Bengal binding site on RNA polymerase is highly nonpolar (Z = 69, corresponding to solvent polarity of *N,N'*-dimethylformamide), and may consist of a hydrophobic pocket.

Glazer (1970) has pointed out that strong binding of dyes to proteins (characterized by a dissociation constant of 10^{-5} M or lower) takes place in hydrophobic areas. These areas often overlap with the binding sites for substrates, coenzymes, and prosthetic groups. In the present case, however, Rose Bengal does not bind to the "active site" of RNA polymerase since it does not compete with substrate or template of the enzyme. Nevertheless, since the binding of Rose Bengal to RNA polymerase does alter catalytic behavior (as presented in the preceding paper (Wu and Wu, 1973)), it is possible that Rose Bengal either binds in the immediate vicinity of a functionally important region on the enzyme or else exerts its effects in an allosteric manner.

The precise mechanism for the effect of solvent polarity on the absorption and fluorescence of Rose Bengal remains to be elucidated. A red shift of the absorption and emission maximum in nonpolar solvents is expected in those cases in which the first singlet excited-state dipole moment is smaller than that of the ground state (Lippert, 1957; McClure and Edelman, 1966). In addition to the shift in wavelength maxima, our data also give a linear relationship between fluorescence quantum yield of Rose Bengal and solvent polarity (Figure 8). A plausible explanation for the relationship of quantum yield to the emission maximum has already been given by Turner and Brand (1968).

The fluorescence of RNA polymerase is largely due to the tryptophan residues, as characterized by the excitation and emission spectra. *E. coli* RNA polymerase core enzyme contains 20 tryptophan residues/mol wt 400,000 (Burgess, 1969). The quenching of tryptophan fluorescence by Rose Bengal could be attributed to a ground-state complex (static quenching) or an excited-state encounter (dynamic quenching) of the tryptophan chromophore with the quencher. In either case, close contacts between the chromophore and the quencher are necessary (less than 5 Å). In order for these short-range processes to account for the observed quenching, at least one-half of the tryptophan residues should be clustered within 5-Å distance from the Rose Bengal binding site. (At concentrations less than 10^{-5} M, no evidence of more than a single Rose Bengal binding site exists.) Physically, this seems to be unlikely.

An important process responsible for the quenching of tryptophan fluorescence is the electronic excitation energy transfer from tryptophan residues to Rose Bengal, as shown by the excitation spectrum (Figure 11). When excited at 280 nm, an increase in fluorescence was observed upon addition of RNA polymerase to a solution of Rose Bengal. In Förster's theory of dipole–dipole energy transfer (Förster, 1947), the transfer efficiency E is related to the distance r between the donor and acceptor by

$$E = \frac{r^{-6}}{r^{-6} + R_0^{-6}} \tag{6}$$

R_0, the distance (in Å) at which the transfer efficiency is 50%, is given by

$$R_0 = (JK^2Q_0n^{-4})^{1/6}(9.79 \times 10^3) \tag{7}$$

where K^2 is the orientation factor for dipole–dipole transfer, Q_0 is the quantum yield of the donor in the absence of transfer, and n is the refractive index of the medium. J, the spectral overlap integral (in $cm^3\,M^{-1}$), is given by

$$J = \frac{\int F(\lambda)\epsilon(\lambda)\lambda^4 d\lambda}{\int F(\lambda) d\lambda} \tag{8}$$

where $F(\lambda)$ is the fluorescence intensity of the donor at wavelength λ and $\epsilon(\lambda)$ is the extinction coefficient of the energy acceptor at that wavelength. For tryptophan to Rose Bengal transfer, R_0 is calculated to be 19 Å using experimental values of $J = 6.1 \times 10^{-15}$ and $Q_0 = 0.05$, and assuming $n = 1.4$ and $K^2 = 2/3$ (random orientation of donor–acceptor pairs). Since the rate constant for energy transfer is proportional to the inverse sixth power of the distance between the energy acceptor and donor, tryptophan residues within 40-Å distance to the bound Rose Bengal will contribute significantly to the energy transfer. Thus in order to explain the observed quenching by energy transfer at least half of the tryptophan residues would have to be clustered in a region less than 40 Å away from the Rose Bengal binding site.

The most striking experimental finding is that DNA and NTP decrease the quenching of tryptophan fluorescence by Rose Bengal but have no effect on the spectroscopic properties of bound Rose Bengal. This could be explained by fluorescence quenching *via* energy transfer. All the experimental results are consistent with a picture in which the Rose Bengal binding site is spatially removed from the DNA or NTP sites. Although spectral properties of Rose Bengal are very sensitive to the environment, the binding of DNA and NTP do not change the immediate environment of the Rose Bengal binding site. This is in accord with the noncompetitive kinetics obtained for Rose Bengal inhibition of the RNA polymerase reaction (with respect to varying concentrations of NTP), and that Rose Bengal does not affect the DNA–enzyme binding reaction (Wu and Wu, 1973). It is also consistent with the finding that the dissociation constant of the Rose Bengal–enzyme complex remains the same when DNA or NTP is added. However, the binding of DNA or NTP to the enzyme induces certain conformational changes. Since the efficiency of energy transfer is dependent upon both the distance and the orientation between tryptophan residues and Rose Bengal, such conformational changes may reduce the efficiency of energy transfer. As electronic excitation energy transfer is a long-range interaction, it will reflect structural changes outside the immediate neighborhood.

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