TRIPLET STATE SUBLEVEL KINETICS OF TRYPTOPHAN 54 IN THE COMPLEX OF *ESCHERICHIA COLI* SINGLE-STRANDED DNA BINDING PROTEIN WITH SINGLE-STRANDED POLY(DEOXYTHYMIDYLIC) ACID

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ABSTRACT The individual sublevel kinetics of the lowest triplet state of tryptophan 54 (Trp 54) which is highly perturbed in the complex of *Escherichia coli* single-stranded DNA binding protein (Eco SSB) with poly(deoxythymidylic) acid (poly[dT]) have been studied by optically detected magnetic resonance (ODMR) spectroscopy. The triplet sublevel decay constants of Trp 54, k_x, k_y, k_z, are 0.99, 0.072, and 0.045 s^{-1}, respectively, in the poly(dT) complex of a point-mutated Eco SSB in which Trp 88 is substituted by phenylalanine. T_x is the only radiative triplet sublevel. Negative polarity of the $T_x \leftrightarrow T_z$ and $T_x \leftrightarrow T_y$ phosphorescence-detected ODMR signals results from the steady state population pattern, $n_x > n_y, n_z$, and implies that the relations, $p_x \geq 14p_y$, and $p_x \geq 22p_z$ exist for the relative populating rates. Spin-orbit coupling between radiative singlet states and the T_x sublevel of the lowest triplet state of Trp 54 is enhanced selectively upon complexing of Eco SSB with poly(dT).

INTRODUCTION

The role of tryptophan residues in the binding of *Escherichia coli* single-stranded binding protein (Eco SSB) to poly (deoxythymidylic) acid (poly[dT]) has been studied recently by using modified proteins in which Trp 40, 54, and 88 were selectively, one residue at a time, replaced by phenylalanine (1, 2). The direct evidence obtained from fluorescence titrations and optically detected magnetic resonance (ODMR) spectroscopy indicates that among the four tryptophan residues of Eco SSB, only Trp 54 and Trp 40 are involved in stabilizing the protein-poly(dT) complex via stacking interactions. These extensive studies show that a unique interaction between Trp 54 and thymine bases in the complex leads to a pronounced red shift of the phosphorescence 0,0 band, a significant reduction of the D-parameter describing the zero field splitting (ZFS), the reversal of the polarity of the Trp 54 steady state ODMR signals, and a large decrease in the average phosphorescence lifetime (1, 2). A structural model has been proposed to attempt to explain the special properties of Trp 54 in the complex, as well as the role of Phe 60 in the binding of single-stranded DNA (3). Although the significant effects of complex formation on the phosphorescence spectrum, the ODMR spectrum, and the phosphorescence lifetime have been reported, the individual triplet state sublevel kinetic properties of Trp 54 in the Eco SSB/poly(dT) complex have not been studied.

Studies of SSB protein complexes with mercurated or brominated polynucleotides show that the triplet state kinetic properties of those tryptophans that are involved in stacking interactions with nucleotide bases change dramatically due to external heavy-atom perturbations in the binding environments (4–7). However, there has been no report on the kinetic properties of those tryptophans that undergo stacking when complexed with poly(dT); no heavy atom is involved in this case.

In this paper, the individual triplet sublevel decay constants of Trp 54 in Eco SSB, their relative radiative rate constants, and their relative populating rate constants upon binding of the protein with poly(dT) are reported for the first time. Zero field ODMR spectroscopy has been applied extensively in this study to reveal the triplet state kinetics of Trp 54. Eco SSB W88F, in which tryptophan 88 is substituted by phenylalanine, was chosen for this work. From previous work (1, 2), we know that the phosphorescence of Trp 54 as well as that of Trp 88 is red shifted to a large extent in the Eco SSB-poly(dT) complex; the Trp 40 and Trp 135 emissions remain relatively blue shifted. Therefore, it is possible to isolate the phosphorescence of Trp 54 from that of these two residues by exciting the complex at the red edge of the singlet absorption band and monitoring the emission on the red edge of the 0,0 phosphorescence emission band. Interference from Trp 88 emission is avoided by using the mutant protein, Eco SSB W88F.

Our results show that the T_x sublevel of Trp 54 is affected significantly in that its decay constant, k_x, is about four times larger than that of normal tryptophan, while the

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T_y and T_z sublevel decay kinetics are almost unaffected. In addition, the T_x populating rate, p_x, is significantly enhanced relative to p_y and p_z.

MATERIALS AND METHODS

Sample Preparation

Eco SSB W88F was prepared by site-directed oligonucleotide mutagenesis as described by Mark et al. (8). The mutant gene was subsequently cloned into a vector which allows temperature-dependent expression under regulation of the phage lambda leftward promoter. Purification of the mutant protein was done according to previously published procedures for the preparation of wild type Eco SSB (9–11) with minor variations. Specific details of the mutagenesis, purification procedures, and protein chemistry will be published elsewhere. Poly(dT) (P. L. Biochemicals, Milwaukee, WI) was used without further purification. A stock solution was prepared by dissolving poly(dT) in a low ionic strength (1 mM) cacodylate buffer, pH = 7.0, containing 0.1 mM EDTA.

The Eco SSB W88F/poly(dT) complex was prepared under the conditions described in previous papers (6, 7). The final protein concentration in the complex was 1.5×10^{-4} M. Samples contained 30% (vol/vol) glycerol (spectrophotometric grade, Aldrich Chemical Co., Inc., Milwaukee, WI) as cryosolvent.

Instrumental

The sample was transferred to a 1-mm i.d. Suprasil quartz sample tube (Heraeus-Amersil, Inc., Sayreville, NJ) and placed within a microwave slow wave copper helix terminating a coaxial transmission line. This was then immersed in liquid nitrogen or liquid helium for spectroscopic measurement. The apparatus and methods for performing phosphorescence lifetime and ODMR measurements have been described before (12, 13).

Methods

In order to obtain selectively the phosphorescence emission from Trp 54, the sample was photoexcited with a 100-W high pressure mercury arc near the red edge of the tryptophan singlet-singlet absorption band (318 nm using a 16-nm excitation bandwidth) and all lifetime and ODMR measurements were performed while monitoring the emission on the red edge of the 0, 0 emission band (418 nm). At 77 K, where rapid spin-lattice relaxation equalizes the triplet sublevel populations, the average phosphorescence lifetime was obtained from the phosphorescence decay measurement. In order to quench the spin-lattice relaxation, low temperature (1.1–1.2 K) was achieved by pumping on the liquid helium. All ODMR and low temperature phosphorescence decay measurements were done under these conditions. ODMR slow passage experiments were performed by monitoring the phosphorescence intensity with the emission monochromator set at a certain wavelength while sweeping the microwaves slowly through a pair of triplet sublevels in zero magnetic field. The microwave frequencies that match the energy differences between sublevels characterize the zero field splittings of the triplet state.

The decay constants of radiative sublevels were obtained by the ODMR fast passage transient experiment in which the microwaves are swept through a transition more rapidly than the shorter lived decay time in the transition pair (14). The measured values were corrected by extrapolation to zero optical pumping intensity.

Microwave-induced delayed phosphorescence (MIDP) measurements were performed to obtain the decay constants of the long-lived nonradiative sublevels. This technique has been described by Schmidt et al. (15). In the steady state MIDP measurements, the sample was photoexcited for 25 s, and after a short time following the opening of the emission shutter, the microwaves were swept rapidly through the $T_x \leftrightarrow T_y$ and $T_x \leftrightarrow T_z$ ODMR transitions, respectively. Non–steady-state MIDP measurements utilizing pulsed excitation times of 100 ms were performed to obtain the relative populating rates of the triplet-state sublevels.

Phosphorescence decay and microwave double-saturated decay at 1.2 K provided more information about the individual triplet-state sublevel kinetics.

All decays were deconvoluted by a nonlinear least squares Marquardt algorithm designed to minimize the chi-square of the fitting function and whose goodness-of-fit was monitored via a residuals plot.

RESULTS

Phosphorescence Spectrum and Zero Field ODMR Transitions

The phosphorescence spectrum of the Eco SSB W88F/poly(dT) complex when excited at 318 nm with 16-nm bandwidth shows a slight red shift of the 0,0 band maximum (416.4 nm) compared with that obtained by photoexciting the same sample at 295 nm with 16 nm bandwidth (415.6 nm) (1). Under our experimental conditions, the ODMR slow passage measurement gave negative polarity |D-E| and |2E| microwave transitions (Fig. 1 *A*) with frequencies of 1.60 and 2.44 GHz, respectively. D and E are the zero field splitting parameters; the transitions between sublevels are shown in the energy diagram at the top of Fig. 1. We were unable to obtain ODMR signals for the |D| + |E| transition.

Decay Constant of the T_x Sublevel and its Radiative Character

By rapidly sweeping the microwaves through the |2E| transition at the rate of 60 GHz s^{-1} during continuous optical pumping, a negative ODMR fast passage signal was obtained (Fig. 1 *B*). Computer analysis gives a single exponential fitting, which indicates that only one of the coupled sublevels is radiative. Measurements were

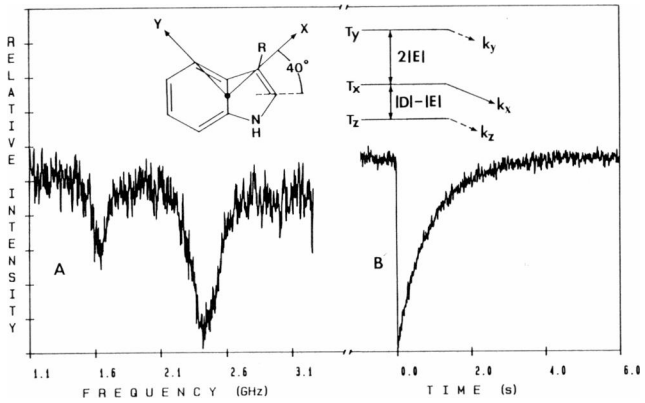

FIGURE 1 (*A*) |D-E| and |2E| transitions of Trp 54 in the Eco SSB W88F/poly(dT) complex. The microwave sweep rate was 58 MHz s^{-1}, and eight scans were averaged; (*B*) microwave rapid passage response (104 scans averaged) for the |2E| transition using a microwave sweep rate of 60 GHz s^{-1}. The excitation was at 318 nm using 16-nm band pass and the emission was monitored at 418 nm using a monochromator band pass of 3 nm. The temperature was 1.17 K. The sample was prepared in 20 mM cacodylate buffer, pH = 7.0, containing 1 mM EDTA and 30% glycerol. The final protein concentration was 1.5×10^{-4} M in monomer and poly(dT) was in 20-fold molar excess.

repeated over a range of incident light intensities by placing various neutral density filters in the excitation path. The decay constant obtained from each measurement was plotted vs. the relative excitation intensity, and the true decay constant was obtained by extrapolation to zero optical pumping intensity; this procedure gave $k_x = 0.99\ s^{-1}$. The same measurement was also made by sweeping microwaves through the |D-E| transition. Although the signal was considerably weaker, the same extrapolated decay constant was obtained within experimental error. These measurements indicate that T_x is the only radiative sublevel of Trp 54, and its total decay constant, $k_x = 0.99$ (± 0.05) s^{-1}.

It is very important to be certain that we have chosen the proper experimental conditions to selectively examine Trp 54 in this multi-tryptophan complex. A series of microwave rapid passage transient measurements were made in which the excitation and monitored emission were moved to shorter wavelengths. No significant change in the response (Fig. 1 *B*) was observed until the excitation wavelength reached 300 nm and the emission wavelength monitored reached 414.5 nm, when a positive signal response from other normally behaved tryptophans began to appear (Fig. 2). These observations demonstrate that under our experimental condition Trp 54 is examined selectively.

MIDP Measurement

MIDP responses were measured for both the $T_x \leftrightarrow T_z$ and $T_x \leftrightarrow T_y$ transitions, respectively. In our steady state MIDP measurements, using long (25-s) excitation periods, we were able to obtain the apparent decay constants k_y and k_z for the nonradiative T_y and T_z sublevels from the time dependence of the MIDP response amplitudes according to the established procedure (15, 16). An example of a steady state MIDP response is given in Fig. 3 *A*. Plots of the natural logarithm of the amplitute vs. the delay time of the microwave pulse for the $T_x \leftrightarrow T_y$ and $T_x \leftrightarrow T_z$ transitions

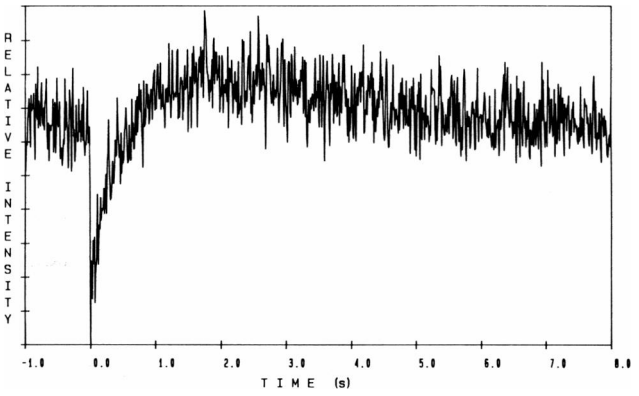

FIGURE 2 Microwave fast passage response (453 scans averaged) for the Trp |2E| transition of the Eco SSB W88F/poly(dT) complex using a microwave sweep rate of 60 GHz s^{-1}. The excitation was at 300 nm using 16-nm band pass, and the emission was monitored at 414.5 nm using a monochromator bandwidth of 3 nm.

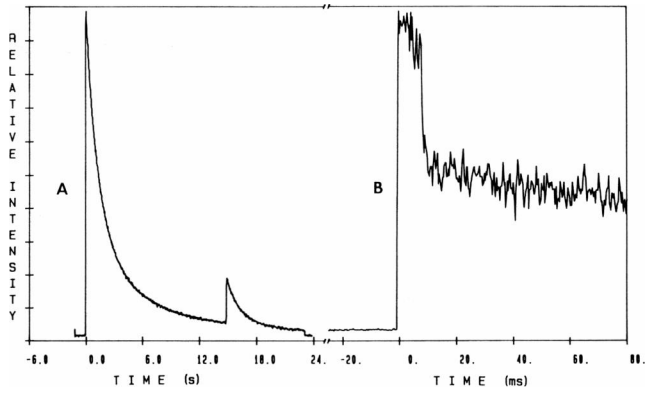

FIGURE 3 MIDP responses of Trp 54 in the Eco SSB W88F/poly(dT) complex at 1.2 K excited at 318 nm with 16-nm bandwidth and monitored at 418 nm followed by microwave rapid passage through the |D-E| transition: (*A*) with steady-state excitation of 25 s; (*B*) with pulsed excitation of 0.1 s. The other conditions are the same as those given in Fig. 1.

are shown in Fig. 4. The apparent decay constants obtained from the slopes are reported in Table I; they are equal to the actual sublevel decay constants only when the spin-lattice relaxation is negligible.

Pulsed excitation (0.1-s) MIDP measurements, in which the sample was excited for a relatively brief period ($t_{exc} \ll k_u^{-1}$, $u = x, y, z$), were made in order to obtain the "instantaneous" relative populating rates p_u. The pulsed excitation MIDP response for the $T_x \leftrightarrow T_z$ transition is shown in Fig. 3 *B*. A similar response was observed for $T_x \leftrightarrow T_y$ transition. The microwave sweep was applied with a minimum delay after closing of the excitation shutter in order to minimize the effects of spin-lattice relaxation and the decay of the sublevels to the ground state. Since T_x is the only radiative sublevel, the strong negative responses observed for both microwave-induced

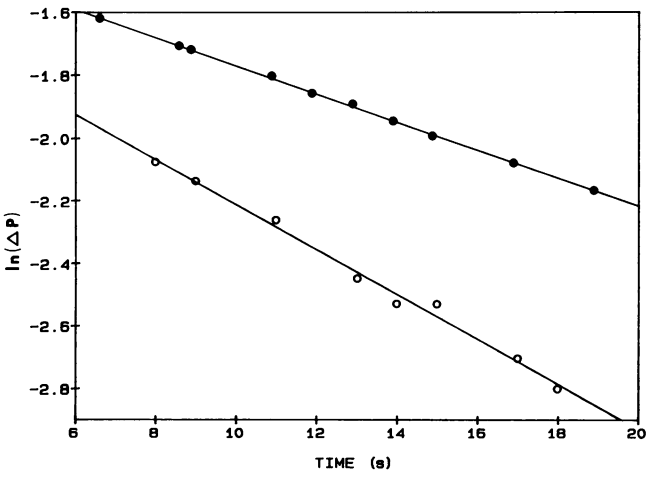

FIGURE 4 Time dependence of the natural logarithm of the MIDP response amplitudes for the |D-E| (*full circles*) and for the |2E| (*open circles*) transitions of Trp in the Eco SSB W88F/poly(dT) complex. The sample is described in the caption of Fig. 1. The microwave sweep conditions are given in the Fig. 1 *B* caption.

TABLE I
SUMMARY OF TRIPLET-STATE KINETICS OF TRP 54 IN ECO SSB W88F/POLY(DT) COMPLEX AND NORMAL TRYPTOPHANS

Sample	k_x	k_y	k_z	k_{av} (calc)‡	p_x*	p_y*	p_z*	Reference
	s^{-1}	s^{-1}	s^{-1}	s^{-1}				
SSB W88F + poly(dT)	0.99	0.072	0.045	0.369	1.0	<.07	<.04	This work
RNase T1§	0.264	0.076	0.051	0.130				13
Tryptophan‖	0.240	0.119	0.038	0.134	1.0	0.97	0.59	17

*Populating rates, p_u (u = x, y, z), are relative values.
‡k_{av}(calc) = $(k_x + k_y + k_z)/3$.
§Contains a single tryptophan. Measured at 1.2 K in 40% glycerol-phosphate buffer.
‖Measured at 1.2 K in ethylene glycol-water (1:1 vol/vol), pH 7.

transitions indicate that $p_x \gg p_y, p_z$. Analysis of heights of MIDP responses results in $p_x/p_y/p_z$ = 1.0:0.0:0.0, within experimental error. Upper limits for the relative populating rates of T_y and T_z are obtained from the observation that the slow passage steady state ODMR signals are negative for both $T_x \leftrightarrow T_z$ and $T_x \leftrightarrow T_y$ transitions. This indicates that p_y/k_y, $p_z/k_z < p_x/k_x$ even if spin lattice relaxation is not negligible.

The triplet-state sublevel kinetic information about Trp 54, obtained from this work, is summarized and compared with previous kinetic measurements on other tryptophan-containing samples in Table I.

Phosphorescence Decay and Microwave Double Saturated Decay

The results of phosphorescence lifetime measurements are presented in Table II. Two exponential components were obtained from computer analysis at 77 K, and three were obtained at 1.2 K. Comparison with previous work (Table II) shows that at 77 K the percentage of the short lifetime component increases with increasing excitation wavelength and monitored emission wavelength, confirming that Trp 54 has a shorter average lifetime than the other Trp residues contributing to the phosphorescence. At 1.2 K, because of the quenching of the spin-lattice relaxation (SLR), it is possible to deconvolute the radiative T_x sublevel lifetime of Trp 54. The 1.12-s component obtained (55% of the total intensity) is consistent with the value we obtained from the microwave rapid passage transient measurements on Trp 54. Microwave double saturation of both the $T_x \leftrightarrow T_y$ and $T_x \leftrightarrow T_z$ transitions produces a pseudo–one-level system which decays as an average of the three sublevels at 1.2 K. The shorter observed lifetime is similar to that obtained at 77 K and to the average lifetime of the three sublevels of Trp 54 (Table I). The lifetime of the long component is slightly longer at 1.2 K than that observed at 77 K.

DISCUSSION

Tryptophan 54 is one of the three Trp residues which are present in the point-mutated protein, Eco SSB W88F. The other Trps are located at position 40 and 135. Previous measurements on point-mutated Eco SSB in which Trps 40, 54, and 88 are individually replaced by phenylalanine have shown (1, 2) that in complexes with single-stranded polynucleotides, only Trp 40 and 54 undergo stacking interactions with the nucleic acid bases. In the complexes of Eco SSB with poly(dT), we found that the phosphorescence of Trp 54 is remarkably red-shifted relative to that of Trp 40 and 135, leading to the possibility that it could be excited and/or monitored exclusively using optical selection methods in the absence of the normally red-shifted Trp 88. In this work, we have shown that it is possible to obtain selectively the kinetic parameters of the Trp 54

TABLE II
PHOSPHORESCENCE DECAY AND MICROWAVE DOUBLE SATURATED DECAY OF TRYPTOPHANS IN ECO SSB W88F/POLY(DT) COMPLEX AT VARIOUS TEMPERATURES

T	λ_{ex}*	λ_{em}*	MW‡	Lifetime components§	Reference
K					
77	318.0	418.0	Off	2.59(45%), 4.77(55%)	This work
77	295.0	415.6	Off	1.70(35%), 4.80(65%)	1
1.17	318.0	418.0	Off	1.12(55%), 3.13(34%), 12.83(11%)	This work
1.17	318.0	418.0	On	2.69(45%), 5.17(55%)	This work

*Wavelengths are in nanometers.
‡Microwave double saturation was achieved by setting the central frequency to 2.02 GHz and frequency modulating over 1,500 MHz at a rate of 40 Hz. The power level incident on the sample was 500 mW.
§Decay was fit to multi-exponential components; lifetimes are given in seconds. Preexponential contributions are given in parentheses.

triplet sublevels in the complex Eco SSB W88F/poly(dT). Although it was determined in previous work (1) that the mean phosphorescence lifetime of Trp 54 is reduced upon complex formation with poly(dT), this work shows that the phosphorescence lifetime reduction originates exclusively in the T_x sublevel. The T_x sublevel lifetime is reduced by about a factor of four relative to "normal" Trp T_x sublevel lifetimes, while the T_y and T_z sublevel lifetimes are unaffected by complex formation (Table I). Furthermore, the populating pattern of the sublevels (which may be due to either intersystem crossing, triplet-triplet energy transfer sensitized by thymine, or both), is highly selective to the T_x sublevel (Fig. 3 *B*, Table I). The steady state population pattern, $n_x > n_y, n_z$, allows us to establish the upper limits for the relative populating rates, p_y and p_z, as given in Table I; p_y and p_z obtained from the MIDP measurements are zero within experimental error. This highly selective populating pattern has not been observed previously for Trp-containing systems and it is indicative of an unusual interaction. It should be noted that T_x is dominant in both the decay to the ground electronic state and in the triplet-state populating dynamics. Since both $S_1 \rightsquigarrow T_1$ intersystem crossing and radiative decay of T_1 are controlled by spin-orbit mixing of T_1 sublevels with excited singlet states (18), it appears that we are observing the introduction of a highly selective spin-orbit coupling process which induces radiative singlet character into the T_x sublevel. Although we have not made phosphorescence quantum yield measurements on Trp 54, the unusually intense ODMR signals observed for this residue in the Eco SSB/poly(dT) complex suggests strongly that the increase in k_x is largely the result of radiative decay. Because of the consistency in the highly selective sublevel populating pattern of T_1 and its radiative decay pattern, it is likely that the T_1 state is populated by intersystem crossing from S_1 rather than by triplet-triplet energy transfer from the triplet state of thymine residues. Sensitization of the Trp T_1 state by energy transfer from the triplet state of thymine also would be inconsistent with the vanishing triplet yield normally exhibited by thymine (19). The kinetic data presented in this study thus appear to be consistent with the introduction of a highly T_x-selective spin-orbit coupling route into Trp 54, which affects both $S_1 \rightsquigarrow T_1$ intersystem crossing and the radiative decay of the T_1 state. We suggest that this enhanced spin-orbit coupling process is the result of an intimate stacking interaction between Trp 54 and a neighboring thymine residue in the complex with poly(dT). Charge transfer character in the Trp 54 excited states in the complex (which is consistent with the observed [1, 2] reduction of the zero field splitting parameter, *D*, of Trp 54 in the Eco SSB/poly[dT] complex) would result in reduced symmetry of the Trp excited states by effectively eliminating the aromatic symmetry plane. This reduction in symmetry would be expected to enhance spin-orbit coupling, as previously suggested (20). It is not clear at this time, however, why the enhanced spin-orbit coupling is so selective for a single sublevel. It should be pointed out, though, that T_x is the normally radiative sublevel of unperturbed Trp, and thus the interaction in the complex could enhance a spin-orbit coupling mechanism that is already present.

If Trp 54 were the only emitting residue under our experimental conditions, we would expect to observe only a single exponential decay of the phosporescence both at 77 K when spin-lattice relaxation averages out the individual sublevel properties, and at 1.2 K under conditions of complete saturation of sublevel populations with resonant microwaves. The predicted lifetime of Trp 54 under these conditions, $0.369^{-1} = 2.69$ s (Table I), agrees with the shorter phosphorescence lifetime component observed at 1.17 K in the presence of microwave saturation (Table II). On the other hand, the longer-lived component of 5.17 s must correspond to Trp residues that have a relatively normal lifetime. This same pattern of decay lifetimes is observed at 77 K under conditions of rapid spin-lattice relaxation. The lifetimes are slightly shorter, however, reflecting a small temperature dependence of the intersystem crossing rate constants. Previous phosphorescence decay measurements (1) at 77 K were made on this system using shorter-wavelength excitation (Table II). Although the longer of the two observed lifetimes is in good agreement with that obtained in this work, the shorter lifetime component has a smaller value in the previous measurements due to the contribution of tyrosine phosphorescence which has a shorter lifetime. The extremely long wavelength excitation used in the present work avoids tyrosine excitation quite effectively. Finally, a short component of ~1.12 s is observed in the phosphorescence decay at 1.17 K in the absence of microwave saturation. This component originates from the T_x sublevel which is now effectively isolated from the remaining sublevels and exhibits a lifetime that is in good agreement with that observed for Trp 54 using MIDP (15, 16) and microwave rapid passage transient measurements during optical pumping (14). The intermediate lifetime of 3.13 s is close to that expected from the isolated T_x sublevel of "normal" Trp, while the longest lifetime component (12.8 s) represents the radiationless decay of the longer-lived, decoupled sublevels that become visible via weak residual spin-lattice relaxation to the radiative T_x sublevel. It is apparent from the phosphorescence decay measurements that under the excitation and observation conditions of our ODMR measurements a considerable fraction of the phosphorescence emission originates from Trp residues other than Trp 54, to which we assign the abnormal kinetic properties. How is it, then, that the kinetic properties measured by the transient ODMR experiments apparently correspond to a single residue, rather than a mixture of residues from different sites? Compare, for instance, Figs. 1 *B* and 2. We think that the answer must be that the spin alignment is much greater for the triplet state of Trp 54 than it is for the other Trp residues which contribute to the emission. The ODMR signal intensity is proportional to the spin alignment, while

the phosphorescence intensity is proportional to the overall population in the triplet state.

CONCLUSIONS

In complexes of Eco SSB with poly(dT), Trp 54 undergoes a particularly large perturbation of its triplet-state properties. Its phosphorescence emission undergoes a significant red shift, and the T_x sublevel becomes particularly active in radiative decay and in the populating process which is most likely by intersystem crossing from the S_1 excited state. The decay constant of T_x is increased by a factor of four. The T_y and T_z sublevels remain nonradiative; on the other hand, their decay constants are not increased by the interaction in the complex. These effects are believed to arise from an aromatic stacking interaction between Trp 54 and thymine which occurs in the complex of Eco SSB with poly(dT). Charge transfer character associated with such a stacking interaction is consistent with enhanced spin-orbit coupling interactions of Trp resulting from the reduced local symmetry. Charge transfer interactions associated with stacking have been postulated previously (1, 2) for Trp 54 in this complex based on the observed reduction of the zero field splitting D-parameter of this residue.

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