Journal of Fluorescence, Vol. 4, No. 2, 1994

Steady-State and Time-Resolved Phosphorescence of Wild-Type and Modified Bacteriophage λ *c*I Repressors

Aaron K. Sato,[1] Eric R. Bitten,[1] Donald F. Senear,[2] J. B. Alexander Ross,[3] and Kenneth W. Rousslang[1,4]

Received October 7, 1993; revised April 9, 1994; accepted April 21, 1994

We have measured the steady-state phosphorescence and decay times of wild-type λ *c*I repressor and compared it with that of a modified λ *c*I repressor in which >95% of the tryptophans were replaced with 5-hydroxy-L-tryptophan (5-OHTrp). The wild-type and 5-OHTrp-λ *c*I repressors are spectroscopically distinct such that we can selectively excite the 5-OHTrp-λ *c*I even in the presence of a 15-fold molar excess of *N*-acetyltryptophanamide (NATrpA). The phosphorescence band of wild-type λ *c*I is red-shifted by 3 nm relative to NATrpA, characteristic of buried tryptophan. Similarly, the phosphorescence of 5-OHTrp-λ *c*I repressor is red-shifted relative to the model, 5-OHTrp, showing that according to the phosphorescence, the modified repressor is structurally indistinguishable from the native repressor. While the phosphorescence decay of both NATrpA and 5-OHTrp are single exponentials, the decay of both wild-type and 5-OHTrp-λ *c*I repressors is complex, requiring three decay components whose fractional contributions to the phosphorescence are the same for both repressors. Because the 5-OHTrp phosphorescence can be excited at wavelengths outside the absorbance range of tryptophan and DNA, a protein spectrally enhanced with this emitter will aid the investigations of protein–protein or protein–DNA interactions.

KEY WORDS: λ *c*I repressor; phosphorescence; triplet lifetimes; protein luminescence; 5-hydroxy-L-tryptophan.

INTRODUCTION

Triplet state investigations of proteins have been almost exclusively limited to the use of tyrosine and, more commonly, tryptophan [4,15]. Often, however, a protein will contain more than one tryptophan which leads to ambiguity in the interpretation of the protein phosphorescence [8,10,12]. The same considerations also limit the use of intrinsic tryptophan fluorescence in the study of structure and function of proteins. Consequently, many investigators have employed extrinsic dansyl or bis-ANS fluorescent probes to investigate protein conformation and motional dynamics. Recently, several workers have replaced specific tryptophan residues by incorporating 5-hydroxytryptophan (5-OHTrp) into proteins expressed in *Escherichia Coli* with >95% replacement [2,9]. These spectrally enhanced proteins (SEPs) take advantage of the fact that the tryptophan derivative has absorbance and fluorescence that is easily distinguishable from the native protein. 5-OHTrp has a red-shifted absorption spectrum relative to tryptophan, permitting selective excitation. In particular, Ross and co-workers [9] used fluorescence emission and decay times to demonstrate that the 5-OHTrp containing bacteriophage λ *c*I repressor exhibits spectral features dis-

[1] Department of Chemistry, University of Puget Sound, 1500 North Warner, Tacoma, Washington 98416.
[2] Department of Molecular Biology and Biochemistry, University of California, Irvine, California 92717.
[3] Department of Biochemistry, Mt. Sinai School of Medicine, One Gustave L. Levy Place, New York, New York 10029.
[4] To whom correspondence should be addressed.

1053-0509/94/0600-0195$07.00/0

tinct from those of the wild-type protein. Since this SEP is functionally equivalent to its parent native protein [9], it should prove to be a useful spectroscopic probe in investigations of molecular protein–protein or protein–DNA interactions.

In this paper, we report the steady-state phosphorescence emission and decay times of wild-type and 5-OHTrp-λ *c*I repressors. The active form of λ repressor is a dimer that binds the operator sites on viral DNA. The primary sequence of amino acids in λ repressor is known and the crystal structure of the N-terminal domain (1–92) has been determined [3]. The N-terminal domain makes all the necessary contacts with the operator region leading to gene regulation. However, little is known about the C-terminal domain (93–236) of this protein. Of the 134 amino acids in the C-terminal domain, there are three tryptophans, located at residues 129, 142, and 230. These six tryptophan moieties and their 5-OHTrp derivatives in the dimer serve as internal spectroscopic probes for phosphorescence and allow the chemical microenvironments of these tryptophans to be determined, leading to a better understanding of the three dimensional structure of the protein.

MATERIALS AND METHODS

Chemicals. Samples of 5-OHTrp λ repressor were prepared as described by Ross *et al.* [9]. In addition, we also prepared a modified λ repressor (Y88C) in which tyrosine-88 is replaced with cysteine [14]. This mutation site is identified as the best possible position for adding a disulfide bridge to cross-link the monomers and increase dimer stability [6]. The samples were initially prepared in Tris buffer, but since phosphorescence measurements were conducted at 77 K, and because the pH of the Tris buffer changes dramatically as a function of temperature, the aliquots of the stock solutions were dialyzed against a 10 m*M* phosphate and 100 m*M* KCl buffer at pH 7.5 using microdialysis. To prepare a sample for spectroscopy, a small sample of the dialyzed aliquot was typically diluted to 1.4 μ*M* protein with buffer and then to 1.0 μ*M* with spectroscopic grade glycerol from Kodak. The glycerol is added to ensure that the sample, when frozen, is a clear glass. The protein stock solutions were stored in a freezer at −78.6°C until they were needed and the dialyzed aliquots were stored at 5°C.

Samples of *N*-acetyl-L-tryptophan-amide (NATrpA) and 5-hydroxytryptophan were prepared by dissolving them in the same buffer used for the protein to obtain an optical density of ~0.1 at 297 and 315 nm, respectively, in a 0.3-cm quartz cell. NATrpA was obtained from Accurate Chemical and Scientific Corporation (Westbury, NY) and 5-hydroxy-L-tryptophan (>99%) was obtained from Aldrich Chemical Company, Inc., and used without further purification.

To determine whether unmodified tryptophan contributed to the SEP phosphorescence, we also prepared 5-OHTrp λ repressor in a solution of 15-fold molar excess NATrpA. The NATrpA solution was prepared at a concentration 15 times greater than the 5-OH-tryptophan in the modified protein assuming six 5-OH-tryptophans per dimer.

Spectroscopy. A conventional right-angle configuration was utilized for measuring both the steady-state and the time-resolved phosphorescence of a sample and has been described in detail elsewhere [7]. The excitation and emission bandpass varied with the sample that was being irradiated but was generally between 1 and 3 nm. The detected signal was collected and processed by a 486/DX2-66 EISA personal computer equipped with a 100-kHz, 12-bit A/D converter, which was also used to control the spectrometer. Time resolution for the decay experiments was 25 μs. All spectra were acquired with the sample immersed in liquid nitrogen. Phosphorescence was signal averaged to improve the signal-to-noise ratio. Decay curves were analyzed using a least-squares program based on the Marquardt algorithm [5]. Satisfactory fits were judged by weighted residuals, autocorrelation of the residuals, and reduced chi-square values, χ^2_ν (see Fig. 1). The function that describes the decay intensity, $I(t)$, as a function of time is

$$I(t) = \sum_{i=1}^{n} \alpha_i \exp(-t/\tau_i)$$

where each of the n components is described by its normalized amplitude, α_i, and lifetime, τ_i. In addition, we report the mean lifetime, $\langle\tau\rangle$, for the total decay,

$$\langle\tau\rangle = \frac{\sum_i \alpha_i \tau_i^2}{\sum_i \alpha_i \tau_i}$$

RESULTS

Steady-State and Time-Resolved Phosphorescence of Models. For comparison purposes, we measured the steady-state and time-resolved phosphorescence of the model compounds, NATrpA and 5-OHTrp. The steady-state phosphorescence spectra of *N*-acetyltryptophanamide (NATrpA) and 5-hydroxytryptophan (5-OHTrp)

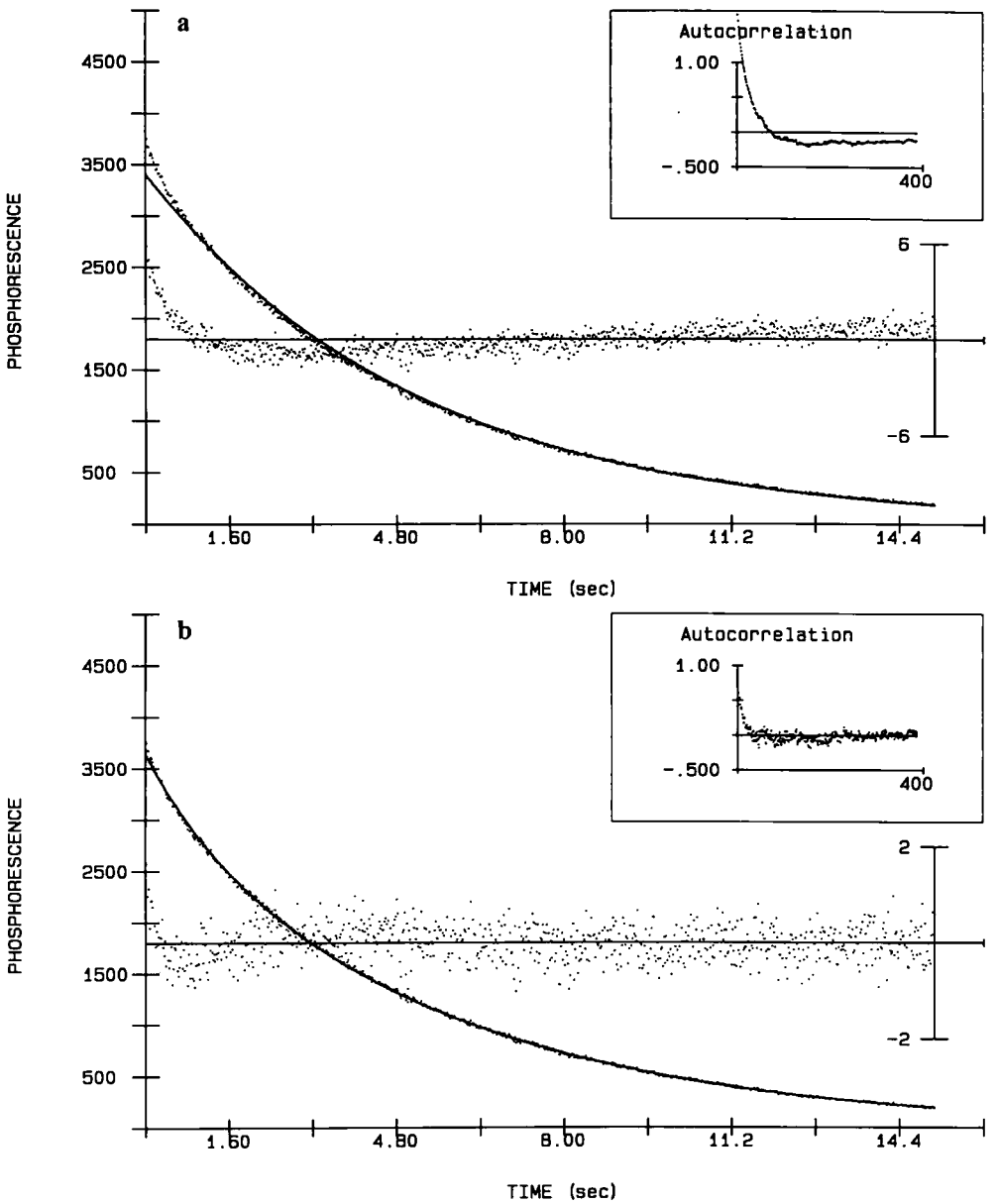

Fig. 1. Phosphorescence decay fit to a single (a), double (b), and triple exponential (c). (Example shown is that of wild-type λ *c*I repressor.) Residuals are on the same horizontal scale as the decay. The fitted function (——) is superimposed upon the original data set (····). Excitation and emission bandwidths were 3 nm, unless otherwise noted. Exciting light was extinguished prior to data acquisition.

were taken with excitation at 297.0 and 315.0 nm, respectively. The wavelengths for the 0–0 bands, $\lambda_{0\text{-}0}$, and the emission maxima, λ_{max}, are summarized in Table I. The 5-OHTrp results compare favorably with the room-temperature phosphorescence reported by Haustein *et al.* [1]. In the lifetime experiments, the models were excited at the same wavelenths as above and emissions were observed at their respective 0–0 bands. The phosphorescence decays of NATrpA and 5-OHTrp models were adequately described by single-exponential time constants of 6.6 and 4.9 s, respectively, as reported in Table II.

Steady-State and Time-Resolved Phosphorescence of Wild-Type and Y88C λ cI Repressor. The steady-state spectra of wild-type and Y88C repressors were acquired with excitation at 297.0 nm. The two are spectroscopically indistinguishable as shown in Table I. The 0–0 band of the emission from tryptophan in both repressors

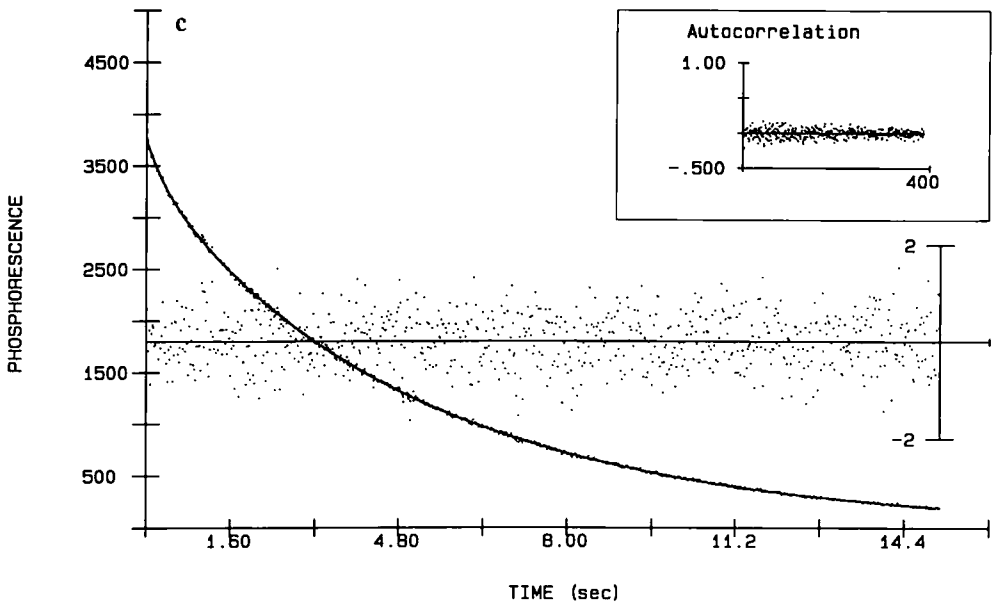

Fig. 1. Continued.

Table I. Model and Protein Steady-State Phosphorescence Results[a]

Sample	λ_{ex}(nm)	λ_{0-0}(nm)	λ_{max}(nm)
NATrpA	297	404.6	431.2
Wild type	297	407.4	434.0
Y88C	297	407.8	435.4
5-OHTrp	315	424.8[b]	441.4
SEP	315	429.2[b]	443.6

[a]Data were obtained at 77 K, with an excitation bandpass of 3 nm and an emission bandpass of 1 nm. Model concentrations were $10^{-5}M$ and protein concentrations were $10^{-6}M$ in 7:3 buffer–glycerol. Estimated precision of emission maxima is ±0.5 nm.
[b]Represents the most clearly resolved vibronic band aside from the maximum.

is red-shifted by about 3 nm relative to NATrpA, which is evident by inspection of Fig. 2. The decay constants associated with the wild-type and Y88C repressors are given in Table II. In both cases, three components were necessary to produce satisfactory fits to the data (see Materials and Methods), with time constants of 5.3, 1.3, and 0.20 s for the wild-type and the same values within experimental error for Y88C.

Steady-State and Time-Resolved Phosphorescence of SEP. The SEP protein samples were irradiated at 315 nm to excite 5-OHTrp selectively independent of tryptophan and tyrosine. An overlay of the phosphorescence spectrum of the 5-OHTrp model with that of the SEP reveals a 3-nm red shift of the phosphorescence maximum (Fig. 3). Similar to the native and Y88C mutant proteins, SEP λ repressor phosphorescence decay is best described by a triple exponential (Table II). An overlay of the phosphorescence spectra of SEP and SEP with 15-fold molar excess of NATrpA is shown in Fig. 4.

DISCUSSION

Since the 0–0 phosphorescence band of wild-type λ reprerssor is red-shifted 3 nm relative to that of free NATrpA in solution, and since previous work shows that such an observation is evidence that the internal probe resides within the interior of the protein, we conclude that the emitting tryptophans in λ repressor are either in the interior of the wild-type dimer or at least protected from solvent [11,13]. This corroborates fluorescence work reported by Ross *et al.* [9]. By comparison, the phosphorescence envelope of 5-OHTrp-λ repressor is also red-shifted relative to that of the 5-OHTrp model. Because it is difficult to resolve clearly any particular vibronic band of 5-OHTrp, we used a 3-nm shift in the phosphorescence maximum as our criterion for a corresponding red shift. Since 5-OHTrp-λ repressor retains its structural and functional integrity, our results imply that the microenvironment of the 5-OHTrp residues is comparable to that of the tryptophans in the native protein.

Whereas the phosphorescence decay of NATrpA is

Table II. Model and Protein Time-Resolved Phosphorescence Results[a]

Sample	λ_{ex}(nm)	λ_{em}(nm)	α_1	τ_1(s)	α_2	τ_2(s)	α_3	τ_3(s)	$\langle\tau\rangle$(s)
NATrpA	297	404.0	1.00	6.6					6.6
Wild type	297	407.4	0.66	5.3	0.20	1.1	0.14	0.20	4.6
Y88C	297	406.0	0.68	5.5	0.16	1.3	0.16	0.24	5.2
5-OHTrp	315	415.6	1.00	4.9					4.9
SEP	315	415.8	0.56	3.9	0.25	0.85	0.19	0.19	3.6

[a]Data were obtained at 77 K, with an excitation bandpass of 3 nm and an emission bandpass of 1 nm. Model concentrations were 10^{-5} *M* and protein concentrations were 10^{-6} *M* in 7:3 buffer–glycerol. Amplitudes, α_i, are normalized to a sum of 1.0.

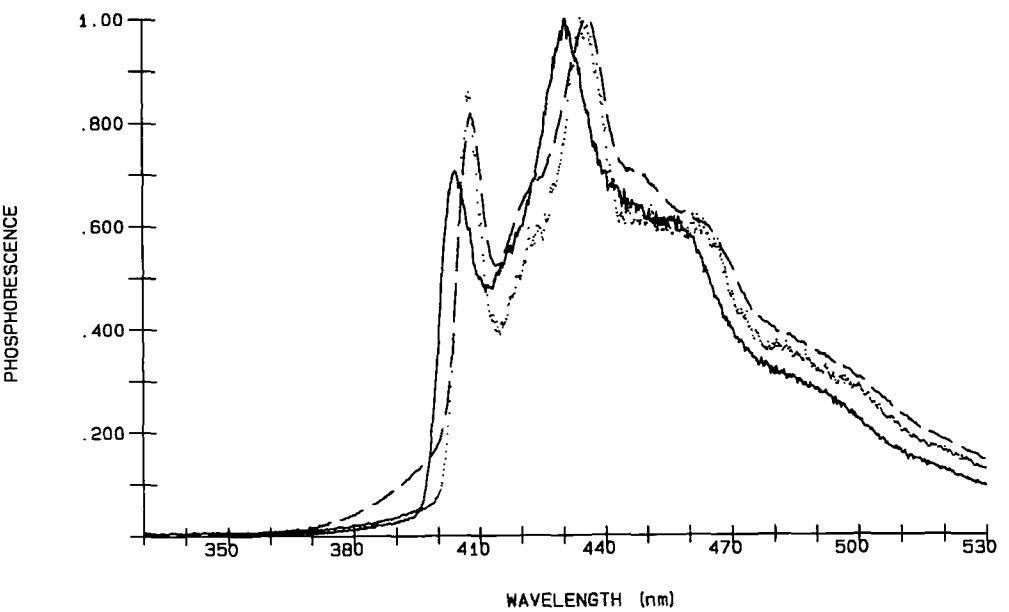

Fig. 2. Peak normalized phosphorescence spectra excited at 297 nm of NATrpA (——), λ *cI* repressor (····), and Y88C-λ *cI* repressor (----). The concentration of NATrpA was 10 μ*M*, and concentrations of wild-type and Y88C λ *cI* repressors were 1 μ*M* in monomer. All samples were in phosphate buffer at pH 7.5. Excitation and emission bandwidths were 3 nm, unless otherwise noted.

composed of a single-exponential component, the emission decay of the wild type consists of the superposition of three exponentials. While it is possible that each component belongs to one of the three tryptophans in the monomer, the multiexponential decay of the repressor proteins could describe either complex phosphorescence decay of a single residue, assuming that the other two are nonphosphorescent, or decay from more than one residue. If the number of time constants represents the number of stable tryptophan conformers in the protein sample at 77 K, without selective modification of each tryptophan, it is impossible to distinguish a single chromophore in multiple conformational environments from several chromophores in their own unique microenvironment.

The origin of the shortest-lived (~0.2 s) of the three decay components in the protein samples is unclear. The decay time in general are independent of the excitation wavelength. That the decay of NATA is monoexponential indicates that there are no detectable luminescent impurities in the buffer/glycerol solvent. Because little is known about the structure of the C-terminal domain of λ repressor where the tryptophan residues reside, it is difficult to speculate about the nature of the quenching process that produces the unusually short 0.2-s decay component.

As mentioned earlier, Y88C is a covalent dimer and thus all spectra of this sample were presumed to represent the dimer. A comparison of the wild-type and Y88C steady-state phosphorescence spectra showed that

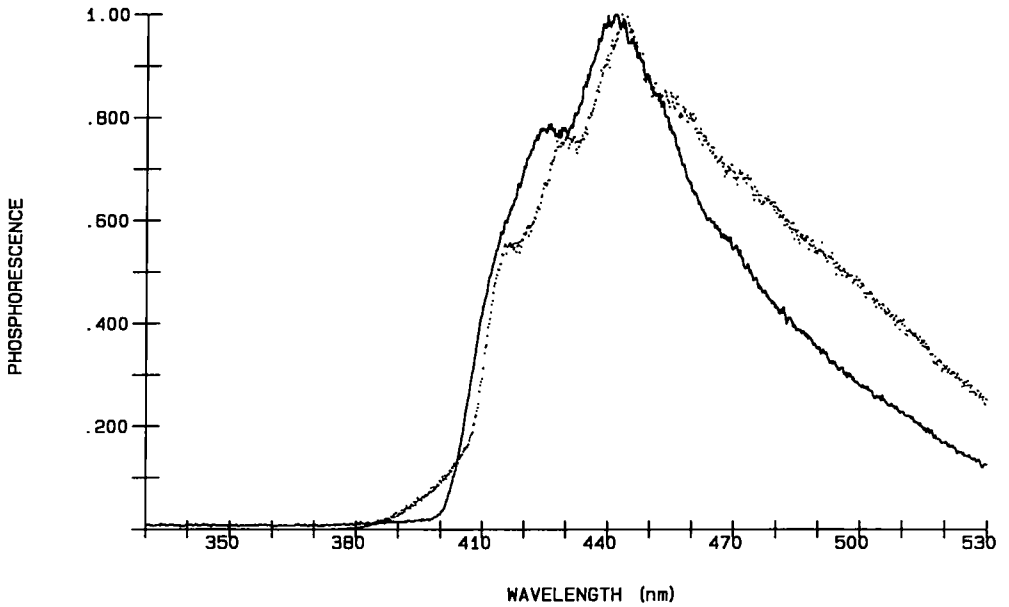

Fig. 3. Peak normalized phosphorescence spectra excited at 315 nm of 5-OHTrp (——), and 5-OHTrp-λ *c*I repressor (····). The concentration of 5-OHTrp was 100 μ*M*, and the concentration of 5-OHTrp-λ *c*I repressor was 1 μ*M* in monomer. All other experimental conditions were the same as for Fig. 2.

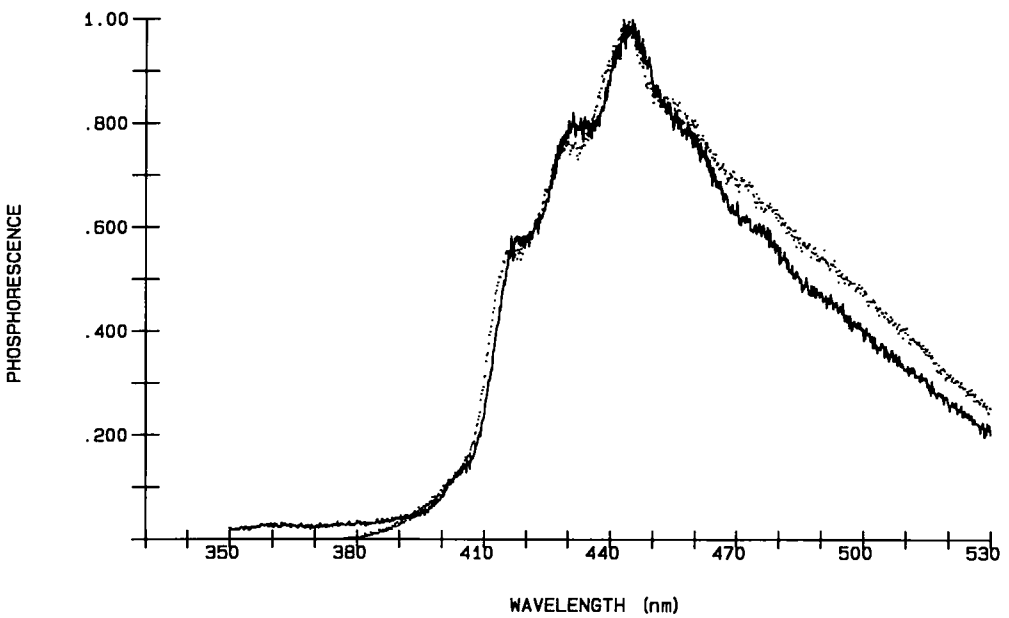

Fig. 4. Peak normalized phosphorescence spectra of 5-OHTrp-λ *c*I repressor alone (····) and with a 15 M excess of NATrpA (——). Experimental conditions were the same as for Fig. 2.

the λ_{0-0} and λ_{max} bands for both samples were the same within experimental error (Fig. 2 and Table I). In the lifetime analysis, both samples were fit to the sum of three exponential terms with identical time constants and normalized amplitudes within experimental error (Table II). These results suggest that the Y88C tryptophans are

in similar microenvironments to the wild-type tryptophans.

Similar to the results with NATrpA and wild-type, the 5-OHTrp model and SEP triplet-state decays are composed of one and three exponentials, respectively (Table II). In addition, the fractional contributions of the three components in SEP are distributed in nearly the same way as in wild-type and Y88C repressors.

Because there is 95% replacement of tryptophan with 5-OHTrp in the SEP, the possibility arises that free tryptophan contributes to both the steady-state and the time-resolved phosphorescence. To verify that the SEP emission is free of pho

ACKNOWLEDGMENTS

The authors are grateful for support from the M.J. Murdock Charitable Trust and the Northwest Area Foundation of Research Corporation.

REFERENCES

1. C. Haustein, W. D. Savage, C. F. Ishak, and R. T. Pflaum (1989) *Talanta* **36,** 1065–1068.
2. C. W. V. Hogue, I. Rasquinha, A. G. Szabo, and J. P. MacManus (1992) *FEBS Lett.* **310,** 269–272.