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Optically Detected Magnetic Resonance Study of Tyrosine Residues in Point-Mutated Bacteriophage T4 Lysozyme†

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ABSTRACT: Two spectroscopically distinct types of tyrosine (Tyr) residues in triply point mutated bacteriophage T4 lysozyme, which contains no tryptophan (Trp), have been detected by optical detection of triplet-state magnetic resonance (ODMR) spectroscopy. Their triplet states are characterized by similar E but different D values. The Tyr site which exhibits the lower D value and has the red-shifted phosphorescence origin is quenched by energy transfer to Trp and has D and E values comparable to previously studied Tyr residues. The blue-shifted Tyr site, which is not quenched by Trp, exhibits a larger D value than has been found previously. Calculation of energy-transfer efficiencies of Tyr–Trp pairs based on the crystal structure of the native enzyme provides a possible assignment of Tyr sites to the two different spectral types.

Phosphorescence studies of Tyr residues reveal that their microenvironments in proteins cannot be resolved because of the broad spectra (Longworth, 1971). The zero-field splittings (ZFS)[1] of the lowest triplet states of the tyrosinate anion, the CH_3Hg–tyrosine complex, and Tyr itself have been measured by ODMR spectroscopy (Co et al., 1974; Hershberger & Maki, 1980; Zuclich et al., 1973). However, ODMR study of Tyr in proteins has been reported only in bacterial blue copper azurin, where a single set of ZFS parameters was obtained for the two Tyr residues (Ugurbil et al., 1977a).

Wild-type bacteriophage T4 lysozyme (T4 lysozyme) contains three Trp residues at positions 126, 138, and 158 and six Tyr residues at positions 18, 24, 25, 88, 139, and 161. Triplet-state properties of Trp residues in T4 lysozyme and its various point mutants in which one or two of the Trp residues are substituted by Tyr residues, respectively, have been investigated by low-temperature phosphorescence and ODMR spectroscopy (Ghosh et al., 1988). In this paper, we report our measurements on the triplet state of Tyr residues in wild-type T4 lysozyme and in some mutants, including the one containing nine Tyr residues but no Trp. We have been able to detect two sets of ODMR transitions from this mutant, providing the first example of the recognition of distinct Tyr environments using ODMR. However, the disappearance of the signals of one of the Tyr sites from the native enzyme and from most of the mutants containing Trp residues suggests that the phosphorescence originating from this Tyr residue is selectively quenched by energy transfer to Trp. (For simplicity of terminology, we will refer to a spectroscopically distinct group of residues in the singular, even though the group may contain more than one residue.) On the other hand, the other Tyr residue is not quenched by Trp. The unquenched residue has an unusually high D value. Comparison of the phosphorescence spectra shows that the origin of the quenched Tyr is red-shifted relative to that of the unquenched one.

Materials and Methods

T4 lysozyme and various mutants were generous gifts from L. McIntosh, Institute of Molecular Biology, Eugene, OR. In our description of the protein, the Trp residues are represented by W and Tyr by Y. Thus, WWW = Trp-126, Trp-138, and Trp-158 (wild type), while YYY = Tyr-126, Tyr-138, and Tyr-158 (non-Trp-containing mutant). The other mutants used in this study, which are missing some of the Trp residues,

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[1] Abbreviations: D and E, triplet-state zero-field splitting parameters; DTT, dithiothreitol; ODMR, optical detection of triplet-state magnetic resonance; ZFS, zero-field splitting(s).

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Table I: Zero-Field ODMR Transition Frequencies and ZFS Parameters of Tyr Residues in Bacteriophage T4 Lysozyme and Its Mutants[a]

sample[b]	$\|D\| - \|E\|$ (GHz)	$2\|E\|$ (GHz)	$\|D\| + \|E\|$ (GHz)	$\|D\|$ (GHz)	$\|E\|$ (GHz)
YYY	2.22 (R), 2.43 (B)	3.48	5.59 (R), 5.81 (B)	3.90 (R), 4.12 (B)	1.74
WYY	2.3 (R), 2.43 (B)	3.48	5.6 (R), 5.82 (B)	3.9 (R), 4.13 (B)	1.74
YWY	2.44	3.50	5.82	4.13	1.76
YYW	2.47	3.52	5.81	4.14	1.76
WYW	2.45	3.52	5.81	4.13	1.76
WWW	2.46	3.54	5.84	4.15	1.77
azurin A[c]	2.23 (−)	3.51	5.63	3.93	1.71
azurin B[c]	2.12 (−)	3.54	5.65	3.89	1.77
L-tyrosine[d]	2.20 (−)		5.59	3.90	1.70

[a] (R) and (B) represent the phosphorescence red- and blue-shifted sites, respectively. Signals are an increase in phosphorescence intensity unless it is labeled by (−) to indicate a decrease in phosphorescence intensity. [b] Lysozyme samples were excited at 285 nm. Emission was monitored at 380.0 nm with a 3-nm bandwidth for WYY, YWY, YYW, WYW, and WWW; for YYY, the emission was monitored at 362.0 nm to obtain the $|D| - |E|$ and $2|E|$ signals and at 415 nm to obtain the $|D| + |E|$ signal. [c] Data from Ugurbil et al. (1977a). [d] Data from Zuclich et al. (1973).

are YWY, WYY, YYW, and WYW. The proteins were stored in 100 mM sodium phosphate buffer containing 500 mM NaCl and 0.01% NaN_3. Dithiothreitol (DTT) was added to each protein solution before the spectroscopic measurements to give a final concentration of ~1 mM in order to prevent the oxidation of cysteine residues. The protein solution was then mixed with 30% glycerol (by volume). The final protein concentration was about 2×10^{-4} M with pH 6.5.

The apparatus and the experimental procedures for low-temperature phosphorescence and ODMR slow-passage measurements in zero magnetic field have been described elsewhere (Ghosh et al., 1984).

Results and Discussion

Phosphorescence. The phosphorescence spectra of YYY and WWW are shown in Figure 1. The Tyr spectral shape and emission wavelength of WWW are similar to those of YYY, although the full spectrum cannot be obtained due to the overlap with Trp phosphorescence which originates at ~400 nm. However, comparison of the blue edge of the two spectra (see the inset in Figure 1) reveals not only that the vibronic structure of the wild-type protein is better resolved but also that its spectrum is blue-shifted by about 4 nm. The Tyr phosphorescence spectra of WYY, YWY, and YYW are each blue-shifted to some extent relative to YYY, but not to the same extent as WWW. The changes observed in the Trp-containing proteins are not unexpected, since Tyr phosphorescence is known to be quenched by energy transfer to Trp. In fact, the ODMR study, which is discussed below, shows that the red-shifted Tyr residues are selectively quenched in the Trp-containing enzymes. This is consistent with the changes observed in the phosphorescence (Figure 1).

Just as with azurin (Ugurbil et al., 1977a), the phosphorescence decay of YYY observed at 77 K was found to be nonexponential. Any attempt to fit the phosphorescence decay with a single exponential produced very poor agreement with the data. The decay could be fit with two components of 1.10 s (45%) and 2.60 s (55%). The results were independent of the wavelength which was monitored. Therefore, the two lifetimes cannot be assigned simply to two environmental types of Tyr residues. Similar complex decay kinetics have been observed even in Tyr itself (Siegel, 1979) and are not fully understood.

Zero-Field ODMR Transitions of YYY. In order to determine whether the zero-field splittings (ZFS) of the Tyr residues vary with their local environment, we carried out slow-passage ODMR measurements on the mutant enzyme YYY. The most striking observation was that at certain monitored wavelengths, a single-peaked $2|E|$ transition, but double-peaked $|D| - |E|$ and $|D| + |E|$ transitions, all of positive polarity, was observed. An energy diagram of the lowest triplet

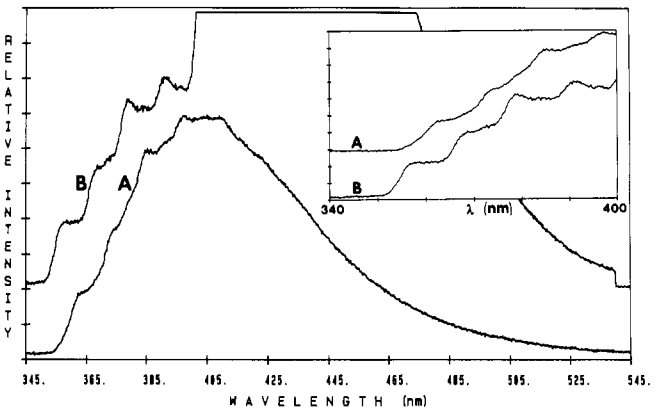

FIGURE 1: Phosphorescence spectra of (A) YYY (2×10^{-4} M) and (B) WWW (2×10^{-4} M). Excitation was at 285 nm with 16-nm band-pass, and the emission was collected with 3-nm band-pass slits. The temperature was 4.2 K, and the samples were prepared in 100 mM sodium phosphate buffer, pH 6.5, containing 500 mM NaCl, 0.01% NaN_3, 1 mM DTT, and 30% glycerol.

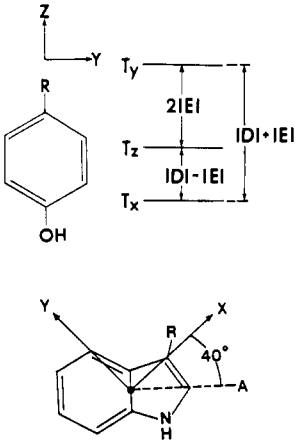

FIGURE 2: Triplet-state energy diagram of tyrosine and principal axis conventions of tyrosine and tryptophan in zero field. D and E are the zero-field splitting parameters.

state of Tyr which defines the ZFS parameters, D and E, is shown in Figure 2. The ODMR results are presented in Table I.

In order to characterize further the Tyr sites in YYY, wavelength-selected ODMR measurements were performed by monitoring the phosphorescence at different wavelengths between the blue edge of the phosphorescence spectrum and the shoulder of the 0,0 band using a narrow-detection bandwidth. The $|D| - |E|$ and $2|E|$ ODMR signals are shown in Figure 3, while the $|D| + |E|$ signals are shown in Figure 4. These spectra reveal clearly that by monitoring the emission at the blue edge of the phosphorescence, single $|D| - |E|$ and

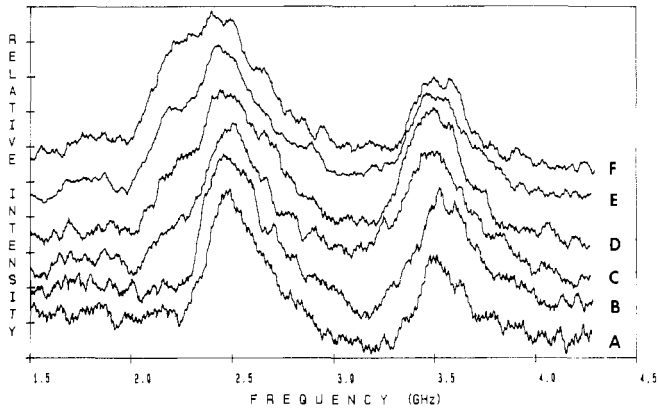

FIGURE 3: Wavelength-selected ODMR slow-passage signals of $|D| - |E|$ and $2|E|$ transitions of tyrosines in YYY. Excitation was at 285 nm with 16-nm band-pass. The emission wavelengths monitored (with 1.5-nm band-pass) are (A) 355.8, (B) 357.4, (C) 359.0, (D) 360.6, (E) 362.2, and (F) 363.8 nm. The microwave sweep rate was 167 MHz/s, and 100 scans were averaged for each spectrum. The sample temperature was 1.2 K.

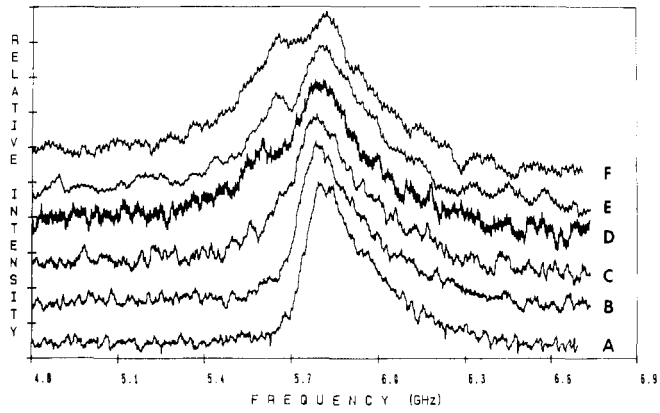

FIGURE 4: Wavelength-selected ODMR slow-passage signals of the $|D| + |E|$ transition of tyrosines in YYY. The emission wavelengths increase from 355.8 (A) to 363.8 nm (F). The conditions are the same as described in the Figure 3 caption.

$|D| + |E|$ signals are observed. However, as the monitored wavelength is shifted to the red, a shoulder begins to appear on the lower frequency edge of the $|D| - |E|$ and $|D| + |E|$ signals, while the $2|E|$ signal is shifted somewhat to a lower frequency. The frequency differences between the two $|D| - |E|$ and two $|D| + |E|$ peaks are similar. This behavior shows that we have resolved two distinct Tyr sites which have similar E values but quite different D values. The Tyr site having the blue-shifted phosphorescence exhibits the higher D value. The behavior of the $2|E|$ transition indicates that the red-shifted site has a somewhat lower E value than the blue-shifted site.

Fluorescence studies have shown that both tyrosines in azurin A occupy hydrophobic sites (Ugurbil & Bersohn, 1977), while pK_a studies have suggested that the Tyr residues in azurin B are buried as well (Ugurbil et al., 1977b). We find that the D and E values originating from the red-shifted site in YYY are similar to those observed in azurins A and B. However, Tyr in ethylene glycol–H_2O, which provides a solvent-exposed environment, also has D and E values which are similar to those observed in the azurins (Zuclich et al., 1973). The implication is that solvent-exposed residues do not produce very different ZFS than those found for Tyr buried in hydrophobic regions of a protein. Therefore, we cannot say much about the local environment of the red-shifted Tyr in YYY. On the other hand, the blue-shifted site most likely originates from an environmentally unusual Tyr residue.

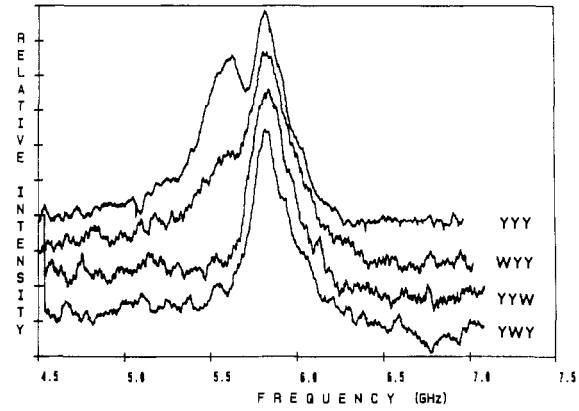

FIGURE 5: ODMR $|D| + |E|$ transitions of YYY (50 scans), WYY (20 scans), YWY (26 scans), and YYW (24 scans). Emissions were monitored at 390 nm for all of the enzymes with 3-nm slits. The sample preparations of WYY, YWY, and YYW are the same as those of YYY and WWW as described in Figure 1. Other conditions are the same as mentioned in Figure 3.

ODMR Transitions of Wild-Type and Mutated Proteins. To further identify the two environmentally distinct sites, we performed ODMR slow-passage measurements of WWW, WYW, WYY, YWY, and YYW, utilizing similar experimental conditions. The results are presented in Table I. Also, wavelength-selected ODMR measurements were conducted on WWW by monitoring different wavelengths from the blue edge of WWW phosphorescence to the shoulder of the 0,0 band, as we did for YYY. Independent of the wavelength monitored, only one set of ODMR signals was observed. The frequencies correspond to the blue-shifted site. The ODMR signals corresponding to the red-shifted site are partially or completely quenched in all of the tryptophan-containing proteins. The results are consistent with our phosphorescence measurements, in which the YYY origin is red-shifted compared with tryptophan-containing enzymes. It is peculiar, however, that none of these enzymes shows a significant weakening or quenching of ODMR signals of the blue-shifted residue no matter which of the three tyrosines residues is replaced by the normally occurring Trp.

As shown in Figure 5, the $|D| + |E|$ signal originating from the red-shifted site is completely quenched when a Trp residue appears at position 138 or 158 (YWY or YYW), and it is partially quenched when the Trp residue appears at position 126 (WYY). In order to search for an explanation for less efficient energy-transfer quenching by Trp-126 relative to the other Trp residues, we calculated the energy-transfer efficiencies of all Tyr–Trp pairs in WWW.

According to Turoverov et al. (1985), the efficiency of nonradiative resonance energy transfer between donor and acceptor molecules separated by a distance r can be evaluated as

$$\epsilon = [1 + 2(r/R_0)^6/3\kappa^2]^{-1}$$

where κ^2 is the orientation factor and R_0 is the critical distance at which, under the condition of fast Brownian rotation, energy-transfer efficiency would be 50%. The corresponding Förster equation (Förster, 1960) can be easily obtained by taking $\kappa^2 = \overline{\kappa^2} = {}^2/_3$. In the wild-type T4 lysozyme, we calculated r and κ^2 by using crystal structure data (Remington et al., 1978). In our calculation, r is the distance between the center of the phenol ring of Tyr and the center of the $C_{\delta 2}$–$C_{\epsilon 2}$ bond of Trp. When calculating κ^2 for a Tyr–Trp pair, we assumed that the transition oscillator of the Tyr residue is directed along the z axis (Figure 2) (Prabhumirash et al.,

Table II: Calculated Geometric Parameters and Energy-Transfer Efficiencies of Tyr–Trp Pairs in Wild-Type Bacteriophage T4 Lysozyme

donor–acceptor (Tyr–Trp)	r (Å)	κ^2_x[a]	κ^2_y[a]	ϵ_x[b]	ϵ_y[b]
18–126	32.64	0.084	0.997	0.0005–0.0012	0.0059–0.014
24–126	32.04	0.00915	0.0216	6.1×10^{-5}–1.4×10^{-4}	1.4×10^{-4}–3.4×10^{-4}
25–126	38.33	0.489	1.266	0.0011–0.0026	0.0029–0.0068
88–126	14.72	0.563	0.0859	0.32–0.52	0.058–0.13
139–126	17.77	0.519	0.523	0.11–0.22	0.11–0.22
161–126	16.20	0.00196	1.343	7.8×10^{-4}–2×10^{-3}	0.35–0.56
18–138	18.83	0.0821	2.385	0.013–0.031	0.28–0.48
24–138	16.62	0.152	0.854	0.050–0.11	0.23–0.41
25–138	23.23	0.113	1.840	0.0052–0.012	0.078–0.17
88–138	17.59	0.113	0.630	0.027–0.061	0.13–0.27
139–138	7.654	0.346	1.084	0.93–0.97	0.97–0.99
161–138	11.85	2.914	0.639	0.88–0.95	0.63–0.80
18–158	23.80	0.692	2.577	0.027–0.061	0.093–0.19
24–158	28.57	0.339	1.02	0.0045–0.011	0.016–0.036
25–158	31.17	1.500	1.575	0.011–0.025	0.011–0.026
88–158	14.06	2.329	0.126	0.69–0.84	0.11–0.22
139–158	19.96	0.925	0.0244	0.096–0.20	0.0028–0.0065
161–158	6.525	0.428	3.529	0.98–0.99	0.997–1.0

[a] Labels x and y indicate that the transition dipole moment of Trp is taken along the x and y molecular axes, respectively, for κ^2 calculations. [b] Energy-transfer efficiencies corresponding to two transition dipole moment directions, respectively. The limits of ϵ correspond to R_0 = 13 and 15 Å.

1983). An early study shows that the ^1L_a oscillator is responsible for the long-wavelength absorption band of the Trp residue (Weber, 1960). More recent studies of indole show, however, that the ^1L_b of indole in the gas phase is the lowest excited electronic state and that the transition moment lies ±(45° ± 5°) with respect to the A axis or roughly along either the x or the y axis of Trp as shown in Figure 2 (Yamamoto & Tanaka, 1972; Evleth, 1970; Philips & Levy, 1986). A recent study (Ghosh et al., 1988) of Trp–Trp nonradiative energy transfer in T4 lysozyme has suggested that the effective transition oscillator for this process lies close to x axis (^1L_b). Therefore, we calculated κ^2 with the oscillator direction taken as both the x axis and the y axis for the Trp residues. The R_0 was assumed to be in the range 13–15 Å (Steinberg, 1971). The calculated results are given in Table II. The following discussion assumes that the effective Trp oscillator has x polarization.

The calculations of ϵ_x show that only Tyr-18, -24, and -25 have a very low energy-transfer efficiency to any of the three Trp residues in WWW. The blue-shifted site is predicted to contain a contribution from at least one of these residues, since the blue-shifted phosphorescence is not quenched in WWW. On the other hand, a rather high energy-transfer efficiency (ϵ_x) to Trp-138 and Trp-158, but a much lower efficiency to Trp-126 (Table II), suggests that Tyr-161 may be one of the red-shifted residues. Furthermore, even if the ^1L_a (y axis) oscillator of the Trp residue also participates in the energy transfer, the quenching of Tyr-18, -24, and -25 by any of the Trp residues is relatively inefficient, and the transfer efficiencies of Tyr-161 to Trp-158, -138, and -126 also show the order of $\epsilon_y(158), \epsilon_y(138) > \epsilon_y(126)$. The crystal structure of T4 lysozyme (Remington et al., 1978) shows that Tyr-18, -24, and -25 are located in the amino-terminal lobe which contains no Trp and that all are solvent exposed. Among all Tyr and Trp residues, only these three residues are located on the extended β-sheet region. Such a location of Tyr may be responsible for the unusual ZFS. Although the red-shifted phosphorescence observed in YYY cannot be due to Tyr-126, a site which is normally occupied by Trp, because its ODMR signal is still observed in WYY (Figure 5), we have not yet ruled out that it may originate from Tyr-138 and/or Tyr-158. The fact that its ODMR signal is not seen in YYW or YWY (Figure 5) implies that Trp at position 138 or 158 would quench a Tyr at position 158 or 138, respectively. According to the crystal structure of the wild-type enzyme (Remington et al., 1978), however, the aromatic indole rings of Trp residues located at these sites are separated by about 16.5 Å. This distance is somewhat beyond our assumed range of energy-transfer quenching of Tyr by Trp, making it unlikely that the red-shifted phosphorescence can be assigned to Tyr at any of the mutated positions. It is assumed in this analysis that the phenol ring is located near the indole ring position in the mutated enzyme.

In summary, our investigation of Tyr residues occurring in triply point mutated T4 lysozyme, YYY, reveals that the heterogeneity of Tyr residues can be resolved by using ODMR spectroscopy. Two spectroscopic sites characterized by similar E but different D values were observed. Comparison with previous studies of different Tyr systems indicates that the YYY site having the red-shifted phosphorescence which gives a lower D value probably originates from normal Tyr residues. The higher D value obtained from the blue-shifted site has not been observed for Tyr before. ODMR studies of wild-type and other mutated proteins containing Trp show that the red-shifted site can be selectively quenched by energy transfer to Trp residues. The preliminary assignment of one or more of Tyr-18, -24, and -25 to the blue site and of Tyr-161 to the red site was based on calculations of energy-transfer efficiencies in combination with the experimental observations.

Although there have been many examples of the sensitivity of Trp ODMR properties to the changes in the local environment, this has never been observed previously for Tyr. This work, therefore, demonstrates that the ZFS of Tyr are sensitive to differences in local environment, and thus suggests the usefulness of another more ubiquitous ODMR probe residue for the investigation of protein structure.

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Comparative Triplet-State Properties of the Three Tryptophan Residues in Bacteriophage T4 Lysozyme and in the Enzyme Complex with Methylmercury(II)†

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ABSTRACT: Triplet-state energies, zero-field splittings (ZFS), and total decay rate constants of the individual triplet-state sublevels of the tryptophan (Trp) residues located at positions 126, 138, and 158 in bacteriophage T4 lysozyme have been determined by using low-temperature phosphorescence and optical detection of magnetic resonance spectroscopy in zero applied magnetic field. An investigation of spectral and kinetic properties of individual Trp residues was facilitated by measurements on point-mutated proteins containing two Trp → Tyr substitutions. We find that the phosphorescence lifetime of the buried Trp-138 is considerably shorter than those of the solvent-exposed Trp residues. CH_3Hg^{II} binding to cysteine residues in T4 lysozyme selectively perturbs the triplet state of Trp-158 by means of an external heavy-atom effect. In contrast with the previous observation of selective x-sublevel perturbation in the Trp–CH_3Hg complex, the radiative character of the z sublevel (z is the out-of-plane axis) is selectively enhanced due to the heavy-atom perturbation of Trp-158. The observed pattern of radiative and total sublevel decay constants of the perturbed Trp is attributed to a special orientation of the Hg atom with respect to the indole plane.

Bacteriophage T4 lysozyme is an interesting enzyme for studies related to protein structure and function for several reasons. It has been well characterized biochemically, and various mutated enzymes which differ from the wild-type enzyme in thermodynamic stability and in intrinsic catalytic activity have been studied (Ellwell & Schellman, 1975, 1977, 1979; Schellman et al., 1981). The crystal structures of the wild-type enzyme and of several mutants have been determined at 0.17-nm resolution (Matthews & Remington, 1974; Remington et al., 1978; Weaver & Matthews, 1987). The overall structure of the wild-type T4 lysozyme (18 700 daltons) is roughly ellipsoidal with a diameter of about 3 nm and a length of about 5 nm. It has a bilobal structure with the active site located at the conjunction of the two domains. The enzyme has 164 amino acid residues that include 3 Trp residues located at positions 126, 138, and 158 and 2 cysteine (Cys) residues at positions 54 and 97. The crystal structure (Matthews & Remington, 1974; Remington et al., 1978; Weaver & Matthews, 1987) shows that Trp-126 and -158 are at the protein surface and that Trp-138 is a buried residue located near the active site of the protein. Recently, Hudson and co-workers (Hudson et al., 1986; Harris et al., 1986) have studied T4 lysozyme and its several mutants using fluorescence anisotropy decay to probe the Trp environments as well as the perturbation of Trp environments resulting from point mutations.

Optical detection of magnetic resonance (ODMR)[1] of the triplet state is a useful technique to probe Trp and Tyr residues in proteins (Maki, 1984; Davis & Maki, 1984) and to investigate protein–nucleic acid interactions (Khamis et al., 1987) as well as protein–lipid interactions (Mao et al., 1985, 1986, 1987). Our recent investigation (Ghosh et al., 1988) of long-range nonradiative singlet–singlet energy transfer among the Trp residues in T4 lysozyme was carried out by utilizing low-temperature phosphorescence and ODMR methods. That study showed that Trp-126 transfers energy efficiently and

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[1] Abbreviations: D and E, triplet-state zero-field splitting parameters; DTT, dithiothreitol; MIDAS, molecular interactive display and simulation; MIDP, microwave-induced delayed phosphorescence; ODMR, optical detection of triplet-state magnetic resonance; ZFS, zero-field splitting(s).

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