Characterization of the Two Tryptophan Residues of the Lactose Repressor from *Escherichia coli* by Phosphorescence and Optical Detection of Magnetic Resonance†

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ABSTRACT: The native lactose repressor from *Escherichia coli* (Lac Rep) and two single-point mutants, W220Y and W201Y, were investigated using low-temperature phosphorescence and optical detection of magnetic resonance (ODMR) spectroscopy. Emission from two tryptophan residues was evident in the phosphorescence spectrum of native Lac Rep at 77 K. Using the single-point mutants, the triplet-state properties of tryptophans 201 and 220 were obtained independently. Trp 220 was characterized as a partially solvent-exposed residue (0,0 band centered at 409.5 nm), while tryptophan 201 exhibited the properties of a buried residue (0,0 band centered at 413.5 nm). Both single-point mutant proteins experienced changes in tryptophan triplet-state properties as a result of binding either of two inducer sugars: isopropyl β-D-thiogalactoside, a monosaccharide, or melibiose, a disaccharide. Putative singlet–singlet energy transfer from tryptophan 220 to tryptophan 201 was also investigated, but the quantitative results must be viewed with some caution.

Low-temperature phosphorescence and optical detection of magnetic resonance (ODMR)[1] have been employed previously to characterize tryptophan (Trp) microenvironments as well as Trp interactions in a variety of circumstances including protein–nucleic acid interactions (Khamis et al., 1987a,b), heavy atom perturbations with the probe CH_3Hg^{II} (Zang et al., 1988; Schlyer et al., 1992), and protein–lipid interactions (Mao et al., 1987). Detailed reviews of the biological applications of ODMR have been given by Clarke (1982), Maki (1984), and Hoff (1989).

The wavelength of the phosphorescence band origin has been shown to provide an indication of the microenvironmental surroundings of a Trp residue (Purkey & Galley, 1970; Hershberger et al., 1980). The low-temperature phosphorescence emission of a polar solvent-exposed Trp moiety tends to be blue-shifted (<409 nm) due to the impaired ability of the rigid solvent environment to stabilize an excited-state electric dipole. Conversely, the more polarizable protein interior can stabilize the larger excited dipole relative to the ground state, resulting in a phosphorescence emission that is generally red-shifted in the absence of specific polar interactions (>409 nm).

ODMR of the lowest excited triplet state is a sensitive technique that exploits the more energetic and more readily measured optical photons to monitor the lower energy microwave transitions of the triplet state. The triplet state is not degenerate in zero magnetic field due to magnetic dipole–dipole interactions that split the state into three sublevels, T_x, T_y, and T_z. These splittings are on the order of 0.1 cm^{-1} for planar aromatic molecules (Gouterman & Moffitt, 1959; van der Waals & ter Maten, 1964), and the populations usually are effectively equalized by spin lattice relaxation (SLR) at temperatures above ca. 4 K. At pumped liquid He temperatures, ca. 1.2 K, the sublevel populations can successfully achieve a state of spin alignment and are selectively probed by the use of resonant microwaves. Phosphorescence-detected ODMR signals are induced by the slow sweep of resonant microwaves through the zf transitions. They are observed only when the coupled sublevels have differing quantum yields and steady-state populations. The detailed theory of the ODMR experiment is given in three comprehensive reviews (Clarke, 1982; Maki, 1984; Hoff, 1989).

The experimentally observed zfs parameters, D and E, yield information regarding changes in electron density along the principal axes (Figure 1). Experimentally, a selective reduction of the zfs parameter, D, for example, suggests an elongation of the electron density along the z (out-of-plane) axis. This observation could result from the overlap of π electrons from a stacked aromatic residue (Tsao et al., 1989), or it could arise as the Trp encounters a more polarizable microenvironment (Hershberger et al., 1980). A simultaneous reduction of both the D and E zfs parameters would occur if an electron density expansion was relatively isotropic.

Optical characterization of Trp phosphorescence is straightforward when the emission of each chromophore is resolved. However, the situation often arises where there are multiple unresolved phosphorescent sites. In such situations, the method of wavelength-selected ODMR often can be used to identify properties of environmentally distinct Trp chromophores (von Schütz et al., 1974; Mao & Maki, 1987; Khamis et al., 1987a; Ghosh et al., 1988b). Discontinuities in a plot of resonant microwave frequencies as a function of phospho-

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[1] Abbreviations: DTT, dithiothreitol; EG, ethylene glycol; FWHH, full width at half-height; IPTG, isopropyl β-D-thiogalactoside; Lac Rep, lactose repressor protein; ODMR, optical detection of magnetic resonance; SLR, spin lattice relaxation; Trp, tryptophan; zfs, zero field splitting.

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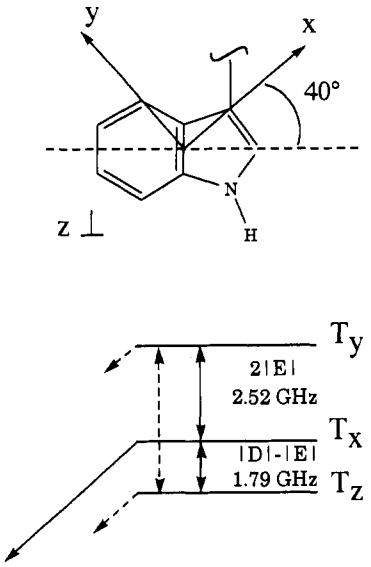

FIGURE 1: Principal spin axis system for the Trp chromophore triplet state (top) with a representation of the magnetic sublevel energy splittings (bottom). The solid arrow indicates a primarily radiative depopulating process of the associated sublevel, while the dashed arrow represents the nonradiative deactivation of the sublevel. The energy level splittings are given in terms of the zfs parameters, $|D|$ and $|E|$ while the transition frequencies are measured for the amino acid in 40% ethylene glycol–water (v/v) (Zuclich, 1970; Zuclich et al., 1973).

rescence emission wavelength monitored across the 0,0 band are indicative of distinct Trp sites.

The lactose repressor protein (Lac Rep) is a negative regulator of transcription for the lac operon structural genes. In the absence of the natural inducer allolactose, a metabolite of lactose (Jobe & Bourgeois, 1972), Lac Rep binds tightly to the lac operator with an equilibrium dissociation constant of ca. 10^{-13} M (Riggs et al., 1970). The introduction of an inducer sugar reduces the binding affinity of Lac Rep for the operator, making nonspecific DNA binding competitive and allowing the transcription of the lac structural gene (Lin & Riggs, 1975; Miller & Reznikoff, 1980). NMR studies (Zuiderweg et al., 1983; Kaptein et al., 1985) suggest that, like many DNA binding proteins, Lac Rep binds to DNA with a helix–turn–helix binding motif (Takeda et al., 1983; Pabo & Sauer, 1984; Brennan & Matthews, 1989).

The tetrameric Lac Rep (38-kDa subunits) contains eight Trp residues, two per subunit (tryptophans 201 and 220), located in the core region of the protein, separable from the helix–turn–helix N-terminal domain by proteolysis (Bayreuther et al., 1973; Sommer et al., 1978). The room-temperature fluorescence emission maxima of two suppressed amber mutants of Lac Rep suggest that Trp 220 is partially exposed to the solvent while Trp 201 is buried (Sommer et al., 1976). More recently, fluorescence quenching studies of single-point mutants with acrylamide and iodide have confirmed this assignment (Gardner & Matthews 1990). Multiple investigations indicate a change in the microenvironment of Trp 220 with the addition of the synthetic inducer, isopropyl β-D-thiogalactoside (IPTG). Studies by Laiken et al. (1972) reveal a small blue shift of the Trp 220 fluorescence maximum upon the addition of the inducer. This reported shift was confirmed by Sommer et al. (1976) using the suppressed amber Lac Rep mutants. Sommer concluded that Trp 220 is involved in the conformational changes induced by the sugar, but is not in direct contact with the IPTG. Boschelli et al. (1981), using NMR spectroscopy of fluorotryptophan-substituted repressor, also reported a modification of only the Trp 220 environment upon the addition of IPTG. More recently, however, photooxidation studies (Spodheim-Maurizot et al., 1985) and UV difference studies (Gardner & Matthews, 1990), in addition to fluorescence lifetime measurements (Royer et al., 1990), have provided evidence that both tryptophans 201 and 220 experience the perturbations effected by the inducer and suggested that contact of inducer with Trp 220 may occur.

This study employs two single-point mutants, W201Y and W220Y, and the native Lac Rep to investigate the triplet-state properties of the individual Trp residues at low temperature and their responses to two inducer sugars, IPTG, a monosaccharide, and melibiose, a disaccharide. The efficiency of singlet–singlet energy transfer from donor Trp 220 to acceptor Trp 201 is also investigated.

MATERIALS AND METHODS

Protein Preparation. Native Lac Rep and single-point mutants were purified as described by Gardner and Matthews (1990). The proteins were >95% pure as assessed by SDS gel electrophoresis, and operator binding activity was >90%. The proteins were exchanged once with potassium phosphate buffer (0.12 M, pH 7.4, 0.1 mM DTT) using Centricon-10 dialysis filters (Amicon). Ethylene glycol (Fluka, puriss.) was added (20% v/v) as a cryosolvent, and samples were used promptly after the above preparation. All samples were stored in the phosphocellulose column elution buffer at −20 °C until needed. Concentration was determined by UV absorbance using extinction coefficients from Gardner and Matthews (1990).

IPTG, a nonmetabolized inducer, (purchased from Sigma), was added to Lac Rep samples in a 100-fold per subunit excess. Samples were allowed to stand at 4 °C in the dark for 30 min. Melibiose binding to Lac Rep is significantly weaker than that of IPTG; therefore, a larger excess of melibiose per subunit (~350:1) was used. Because Lac Rep is sensitive to room-temperature exposure, the samples were incubated in the dark at 4 °C for 30 min.

Spectroscopic Measurements. Low-temperature phosphorescence spectra were obtained by placing the sample, which had been pipeted into a Suprasil quartz sample tube (1 mm i.d.), inside a copper helix that terminates a coaxial transmission line. This device was then placed into a dewar equipped with optical ports and submerged in either liquid He or N_2 to achieve the appropriate temperature. The protein sample was excited at 295 nm by a 100-W Hg arc lamp, whose emission passed through a $NiSO_4$ solution IR filter and then through a 10-cm monochromator. Emission was monitored through a 1-m monochromator (McPherson, Inc., Model 2051) at 90° to the excitation source and detected by a cooled photomultiplier tube (TE-104RF, Products for Research). All data were collected on a 4096 channel signal averager (Tracor Northern TN1550) and transferred to a DEC PRO350 microcomputer for further analysis. The low temperatures required (ca. 1.2 K) for the zf ODMR experiments were obtained by pumping on the liquid He. Microwaves were generated by a Hewlett-Packard Model 8350B microwave sweep oscillator fitted with a plug-in capable of generating microwaves from 0.1 to 20 GHz (Model 83592A). Typical microwave power was 10 mW. A rotating sector with a dead time of 0.8 ms was used to eliminate interference from Trp fluorescence. The observed ODMR transitions were corrected for rapid passage effects by extrapolating the measured values to zero sweep rate. The ODMR apparatus and methods have been described previously in greater detail (Maki et al., 1978).

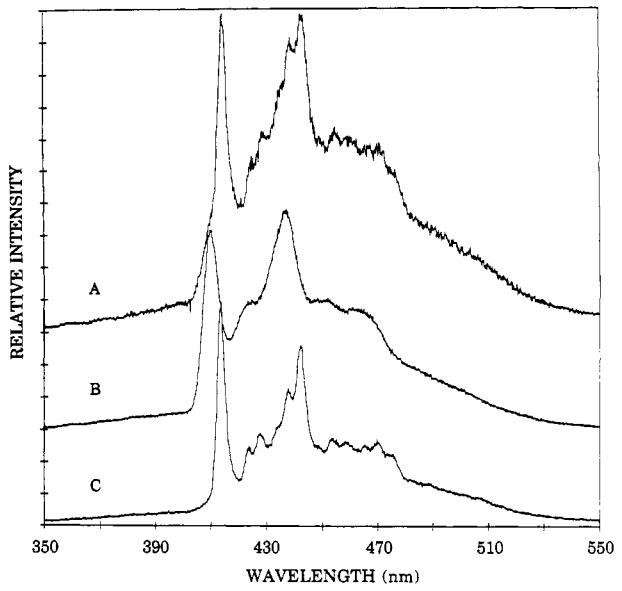

FIGURE 2: Phosphorescence of the native Lac Rep (A) and two single-point mutants, W201Y (B) and W220Y (C). Samples are dissolved in 0.12 M potassium phosphate–buffer, 0.1 mM DTT, pH 7.4. Ethylene glycol was added (20%, v/v) as a cryosolvent. Sample temperature was 77 K, while excitation was at 295 nm with 8-nm bandpass. Emission bandpass was 1.5 nm. Each sample concentration was ca. 50 μM.

Phosphorescence decays were deconvoluted using a nonlinear least-squares Marquardt algorithm designed to minimize the χ^2. Goodness of fit was determined by a residuals plot.

RESULTS AND DISCUSSION

Phosphorescence of Lac Repressor and Single-Point Mutants W220Y and W201Y. The low-temperature phosphorescence spectrum of the native Lac Rep is shown in Figure 2A. Prominent features of the phosphorescence spectrum are a very narrow 0,0 band at 414.7 nm (FWHH of 3.2 nm) and a broad sloping shoulder to the blue. The phosphorescence spectra of the two single-point mutants obtained at 77 K are shown in Figures 2B,C. The mutant W220Y, retaining Trp 201, has a highly resolved phosphorescence spectrum with a narrow 0,0 band peak at 413.5 nm (FWHH of 3.0 nm). The 0,0 band energy and corresponding vibronic structure of W220Y are invariant to changes in excitation wavelength from 280 to 305 nm. Trp 220, as found in W201Y, has a single 0,0 band centered at 409.7 nm (FWHH of 4.5 nm) when excited at 295 nm. The peak wavelength emission varies with the energy of excitation; a red shift of ca. 0.3 nm is observed upon excitation at 305 nm and a blue shift of ca. 0.9 nm is observed when excited at 280 nm. Although not as dramatic as the shifts seen for L-Trp in an aqueous glass (Galley & Purkey, 1970; Itoh & Azumi, 1973, 1975), the observed shifts are very reproducible and are an indication of the heterogeneity of the Trp 220 microenvironment, in contrast with Trp 201. A comparison of the emission characteristics of native Lac Rep with the two single-point mutants shows that the weak shoulder at ca. 410 nm originates from Trp 220, while the intense phosphorescence 0,0 band peaking at 414.7 nm is due to Trp 201 emission. The 77 K phosphorescence emission of the native Lac Rep is dominated by Trp 201, which has the characteristics of a residue buried in a polarizable microenvironment. However, the two single-point mutants, W201Y and W220Y, appear to have comparable phosphorescence intensities under similar experimental conditions. We will return to this point later. Values for the 0,0 band peak wavelengths can be found in Table I.

Studies by Purkey and Galley (1970) and Hershberger et al. (1980) qualitatively link the energy and width of the phosphorescence 0,0 band with the microenvironmental status of Trp residues. A working guideline is that buried residues exhibit 0,0 band emission maxima between 410 and 415 nm in the absence of specific polar interactions, while solvent-exposed residues characteristically have 0,0 emission maxima between 405 and 409 nm. Furthermore, solvent exposure produces a broadening of the phosphorescence bands. These guidelines have led to several successful microenvironmental assignments for the side chains of Trp residues (von Schütz et al., 1974; Ghosh et al., 1988a,b).

The highly resolved and very red-shifted origin band of Trp 201 in the native Lac Rep suggests that this residue is buried in a hydrophobic and polarizable region of the protein core. This assignment is in agreement with studies by Gardner and Matthews (1990) and Sommer et al. (1976). The band origin at 409.7 nm does not lend itself to a straightforward microenvironmental assignment, as the working cutoff for buried residues is ca. 410 nm. The considerably less resolved phosphorescence structure of Trp 220, as well as its intermediate 0,0 band wavelength, however, implies at least a partially solvent-exposed microenvironment. Sommer et al. (1976), using the fluorescence classification system established by Burstein et al. (1973), concluded that Trp 220, with a fluorescence maximum at 338 nm, is partially solvent-exposed. Quenching studies by Bandyopadhyay and Wu (1979) also led to similar conclusions.

Of special note is the ca. 1 nm blue shift between Trp 201 in the native Lac Rep and Trp 201 of the single-point mutant W220Y (Table I). This blue shift is very reproducible and well outside the error of the phosphorescence measurement. It appears that the microenvironment of Trp 201 is affected somewhat by the replacement of Trp 220 with tyrosine. Trp 201 apparently moves to a somewhat more polar or less polarizable microenvironment. The narrow line width, however, confirms that this environment is homogeneous. Difference UV data also indicate a change in the tertiary structure as a result of the substitution of Trp 220, but not Trp 201, with tyrosine (Gardner & Matthews, 1990). The effect on the phosphorescence of Trp 220 upon substitution of tyrosine for Trp 201 cannot be determined due to the very limited contribution of Trp 220 to the phosphorescence intensity of native Lac Rep.

The phosphorescence decay kinetics of Trp is generally monoexponential under conditions of efficient equalization of triplet sublevel populations by spin lattice relaxation (Longworth, 1971). Decays observed for the native Lac Rep and its mutants are biexponential, with the major contribution assigned to Trp (ca. 6 s) and a second shorter component attributed to tyrosine. Tyrosine phosphoresces weakly with a peak wavelength at ca. 400 nm and a lifetime of ca. 1–2 s. Tabulation of the 77 K Trp lifetimes obtained at the indicated wavelengths can be found in Table I.

ODMR of Lac Repressor and W201Y and W220Y Point Mutants. The triplet-state properties of native Lac Rep and the single-point mutants were investigated further by the use of ODMR. Only microwave frequencies coupling the transitions between the T_x and T_y triplet sublevels ($2|E|$) and the T_x and T_z sublevels ($|D| - |E|$) can be observed, since Trp normally has only one radiative sublevel (T_x) (refer to Figure 1 for a schematic representation of the triplet spin axes and sublevels of tryptophan). The ODMR transitions were

Table I: Triplet-State Parameters of Native Lac Rep and Single-Point Mutants W201Y and W220Y

sample	0,0 band[a,b] (nm)	lifetimes[b] (s)		$\|D\| - \|E\|$[c] (GHz)	$2\|E\|$[c] (GHz)	$\|D\|$ (GHz)	$\|E\|$ (GHz)
Lac Rep	414.7 (3.2)	5.7 (93%)	1.2 (7%)	1.60 (34)	2.72 (88)	2.96	1.36
W220Y	413.5 (3.0)	5.6 (94%)	2.5 (6%)	1.62 (44)	2.74 (82)	2.99	1.37
W201Y	409.7 (4.5)	6.5 (80%)	2.8 (20%)	1.74 (105)	2.44 (159)	2.96	1.22

[a] Values in parentheses are the FWHH. [b] Sample temperature is 77 K. [c] Slow passage sample temperature is 1.2 K. Frequencies and line widths (MHz, in parentheses), have been corrected for rapid passage effects by extrapolation to zero sweep rate.

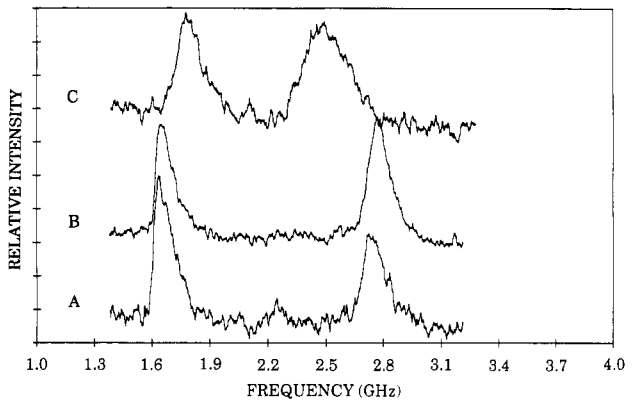

FIGURE 3: Optical response to the slow passage of resonant microwaves through the triplet sublevels at zero magnetic field: (A) native Lac Rep; (B) W220Y; (C) W201Y. The sample conditions were as described in the caption for Figure 2. Microwave sweep rates were ca. 51 MHz/s. Phosphorescence was monitored at the peak wavelength of the 0,0 band (Table I) with a 3-nm bandpass. Signal averaging was carried out for (A) 20, (B) 10, and (C) 40 scans. All responses are somewhat broadened by rapid passage effects at this sweep rate. The data in Table I were obtained by extrapolation to zero sweep rate.

obtained by monitoring the phosphorescence at the peak wavelength of the 0,0 band in zero magnetic field; peak frequencies, given in Table I, have been corrected for rapid passage effects. The $|D| - |E|$ and $2|E|$ resonance frequencies of Trp 220 are ca. 120 MHz higher and ca. 300 MHz lower, respectively, than the corresponding frequencies measured for Trp 201. An earlier study (Hershberger et al., 1980) indicates that the stabilization of the excited-state energy by a polarizable microenvironment leads to a reduction of the $|D| - |E|$ frequency and a concomitant increase in the $2|E|$ frequency of tryptophan. A polarizable microenvironment is also associated with a red shift of the phosphorescence spectrum (Purkey & Galley, 1970). The zero field ODMR transitions obtained for native Lac Rep, W201Y, and W220Y are shown in Figure 3. The lower $2|E|$ and higher $|D| - |E|$ frequencies of Trp 220 are characteristic of a relatively solvent-exposed residue (von Schütz et al., 1974). The large widths of the ODMR transitions (Figure 3) and the phosphorescence bands (Figure 2) of Trp 220 in the W201Y mutant are in accord with a relatively heterogeneous solvent-exposed environment for this residue. The relatively narrow bandwidths of Trp 201 observed in the native Lac Rep and in the W220Y mutant are consistent with the location of this residue in a buried, homogeneous microenvironment (Zang et al., 1989). The red-shifted location of the 0,0 band of Trp 201 suggests the absence of specific internal polar interactions such as those found for the single Trp 59 residue of ribonuclease T_1 from *Aspergillus oryzae* (Hershberger et al., 1980; Lam et al., 1992).

A comparison of the $|D| - |E|$ and $2|E|$ frequencies of Trp 201 in the native Lac Rep with those of W220Y reveals a slight increase of ca. 35 MHz in the $|D|$ parameter and an even smaller concomitant increase in the $|E|$ parameter that are associated with the mutation at position 220. These changes

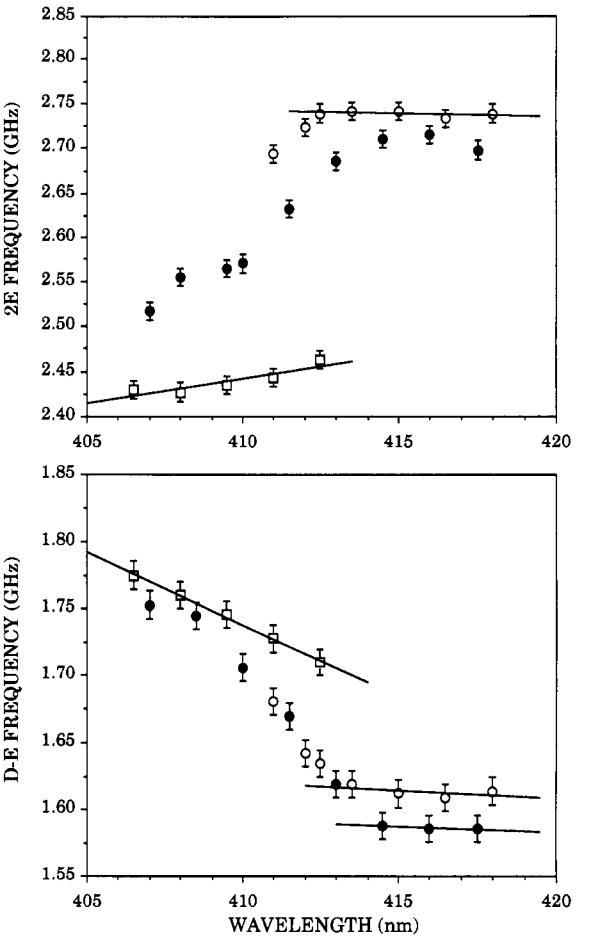

FIGURE 4: Wavelength dependence of the ODMR frequencies of native Lac Rep (●) and single-point mutants W201Y (□) and W220Y (○). Phosphorescence emission was monitored with resolution of 1.5 nm. Excitation was at 295 nm with a bandpass of 8 nm. All data have been corrected for rapid passage effects by the extrapolation of peak frequencies to zero sweep rate. The sample temperature was 1.2 K. Sample concentration was ca. 100 μM, and the sample buffer is described in the caption for Figure 2.

are reproducible and cannot be associated with a minor contribution of Trp 220 to the ODMR signal in the native Lac Rep. These changes in zfs parameters are consistent with a small overall decrease in the polarizability of the microenvironment of Trp 201 that results from replacing Trp 220 with tyrosine.

The dependence of the ODMR frequencies on emission wavelength was measured to provide a base line for comparative studies with the binding substrates. These data are plotted in Figure 4. The phenomenon of a linear dependence of ODMR frequencies as a function of phosphorescence emission wavelength monitored within the inhomogeneously broadened 0,0 band has been studied by several groups (von Schütz et al., 1974; van Egmond et al., 1975; Kwiram et al., 1978; Gradl et al., 1986). This phenomenon has been successfully modeled by a solvent-induced perturbation mechanism (van Egmond et al., 1975; Gradl et al., 1986), whereby

Table II: Triplet-State Properties of the Bound Form of Native and Single-Point Mutants of Lac Repressor

sample	0,0 band[c,d] (nm)	lifetime[c] (s)	$\|D\| - \|E\|$[e] (GHz)	$2\|E\|$[e] (GHz)	$\|D\|$ (GHz)	$\|E\|$ (GHz)
W201Y	409.7 (4.5)	6.3 (85%)	1.75	2.43	2.96	1.22
+IPTG[a]	408.9 (4.3)	6.0 (89%)	1.75	2.43	2.97	1.22
+melibiose[b]	408.4 (4.6)	6.5 (85%)	1.78	2.42	2.99	1.21
W220Y	413.5 (3.0)	5.6 (94%)	1.62	2.74	2.99	1.37
+IPTG[a]	414.5 (3.0)	5.8 (95%)	1.59	2.70	2.94	1.35
+melibiose[b]	414.5 (3.2)	5.9 (88%)	1.61	2.72	2.96	1.36

[a] Ratio of IPTG to monomer is 100:1. Protein concentration is ca. 50 μM. [b] Ratio of melibiose to monomer is 350:1. Protein concentration is ca. 50 μM. [c] Sample temperature is 77 K. [d] Values in parentheses are the FWHH of the phosphorescence 0,0 bands. [e] Slow passage sample temperature is 1.2 K. Frequencies have been corrected for rapid passage effects.

solvent electric fields induce the mixing of higher excited triplet states with the phosphorescent state. The degree to which the zfs parameters vary within the phosphorescence emission wavelength is yet another indication of the nature of the microenvironment. To date, only buried residues have been found to exhibit largely wavelength-independent zfs parameters. This behavior can be seen for the zfs frequencies of Trp 201 in both the native Lac Rep and W220Y (Figure 4), and it supports the assignment of Trp 201 as a buried residue. In contrast, Trp 220 from W201Y reveals a substantial linear dependence of both the $|D| - |E|$ and $2|E|$ ODMR frequencies vs the monitored emission wavelength, indicating a wide range of perturbations across the inhomogeneously broadened 0,0 emission maximum that are induced by the electric fields of the randomly oriented solvent dipoles.

A slight overall increase of ca. 25 MHz in both $2|E|$ and $|D| - |E|$ can be seen for W220Y relative to native Lac Rep when a comparison is made in the wavelength region (red) of native Lac Rep that is dominated by emission from Trp 201. The observed increase in both the $|D|$ and $|E|$ values indicates that, as discussed earlier, Trp 201 of W220Y is in a less polarizable and, therefore, possibly not as completely buried microenvironment as that of the native Lac Rep. This result is consistent with the ca. 1 nm blue shift observed in the W220Y phosphorescence spectrum relative to native Lac Rep discussed earlier.

As discussed previously, the characteristics of Trp 220 in the native Lac Rep are difficult to extract due to the weakness and the incompletely resolved nature of its phosphorescence emission. Thus, a comparison of the phosphorescence spectra of the native Lac Rep and W201Y does not readily reveal possible shifts in the 0,0 band of Trp 220 as a result of the substitution of Trp 201 by tyrosine. Wavelength-dependent ODMR has been successfully employed to differentiate multiple Trp sites that emit within a single unresolved 0,0 phosphorescence band (von Schütz et al., 1974). This technique was utilized to compare the triplet-state properties of Trp 220 in the native Lac Rep with those of the single-point mutant W201Y (Figure 4). A pronounced shift in ODMR frequencies is seen for the single-point mutant W201Y, which exhibits a large decrease in the $2|E|$ frequency (greater than 100 MHz) relative to native Lac Rep when a comparison is made in the blue range where Trp 220 emission dominates. The emission from Trp 220, however, is so weak in the native Lac Rep that its ODMR frequency could be influenced by the blue edge of the more strongly emitting Trp 201 0,0 band. On the other hand, the good agreement of the $|D| - |E|$ frequencies of W201Y and native Lac Rep when compared on the blue edge (Figure 4) suggests that the shift in the $2|E|$ frequency induced by the Trp 201 mutation is not an artifact. The $2|E|$ and $|D| - |E|$ transitions of Trp 220 should be affected to a similar extent by overlap with the blue edge of Trp 201 emission. We conclude, therefore, that substitution of Trp 201 with tyrosine affects the local environment of Trp 220.

Inducer Binding. There is considerable evidence in the literature that Trp 220 is present in, or is very near, the inducer binding site of Lac Rep (Sommer et al., 1976; Royer et al., 1990; Gardner & Matthews, 1990). Two inducers, IPTG and melibiose, were selected for the binding experiments. The monosaccharide, IPTG, was chosen because of its well-documented use as an inducer (Barkley et al., 1975; Müller-Hill, 1971). Difference UV studies (Gardner & Matthews, 1990) of the individual single-point mutants, W220Y and W201Y, indicate that there are more extensive interactions between the disaccharide melibiose and Trp 220 than were seen with the monosaccharide IPTG. The evidence of stronger perturbations induced by the disaccharide influenced our choice of melibiose as an inducer.

W201Y and Inducers. A blue shift of ca. 10 nm in the room-temperature fluorescence of Lac Rep upon binding of IPTG has been reported (Sommer et al., 1976; Gardner & Matthews, 1990). Although we observed this fluorescence shift as well, only very minimal changes were found in the phosphorescence 0,0 band wavelength or phosphorescence lifetime of the Trp chromophore upon binding IPTG to W201Y (Table II).

The observed phosphorescence 0,0 band of W201Y undergoes a minor blue shift of ca. 0.8 nm upon binding IPTG, with essentially no change in the structure or resolution of the spectrum. The binding of melibiose produced a somewhat larger blue shift of the phosphorescence emission (ca. 1.3 nm). Although these blue shifts are small in both complexes, they are reproducible. This blue shift of the phosphorescence suggests a small increase in the polar character of the Trp side-chain microenvironment. The slightly larger blue shifts induced by melibiose binding to W201Y could be the result of an interaction with Trp 220 through the glucose ring. Studies by Gardner and Matthews (1990) also reveal features in the difference UV spectra of W201Y complexed with melibiose that are absent in the IPTG complex. Interaction with the glucose ring is the speculated cause of a new spectral peak (Gardner & Matthews, 1990).

Slow passage ODMR measurements of the inducer-bound Lac Rep W201Y mutant also are consistent with the minor shifts observed in the phosphorescence origins. An increase in the $|D| - |E|$ frequency accompanied by a decrease in $2|E|$ is observed for residues in more polar microenvironments (Hershberger et al., 1980). These changes are seen in the $|D| - |E|$ and $2|E|$ resonance frequencies, respectively, upon binding melibiose to W201Y. Smaller shifts in zfs frequencies are seen for IPTG binding to W201Y, but these are comparable to the error of the measurement and may not be real. The ODMR line widths for the melibiose complex are also significantly narrower than those of free W201Y (data not shown). The line-width narrowing indicates that there is a reduction in the heterogeneity of the local environment of Trp 220 upon binding of the inducer, melibiose. The wavelength dependence plots for melibiose binding are included to

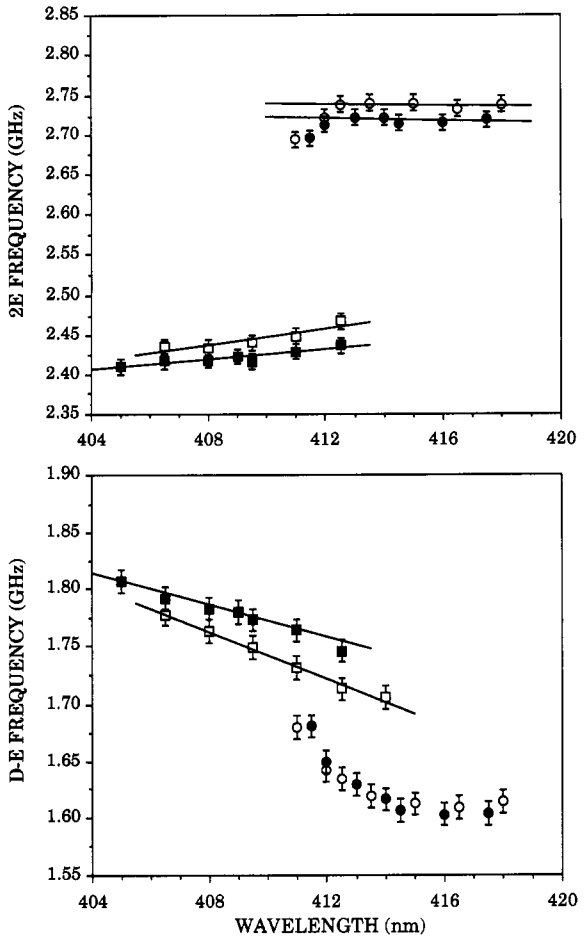

FIGURE 5: Plot of zero field ODMR frequencies as a function of emission wavelength for melibiose-bound single-point mutants: W220Y/melibiose (●); and W201Y/melibiose (■). Data for the free mutant proteins, W220Y (○) and W201Y (□), have been included for comparison. All experimental conditions are as described in the caption for Figure 4. Binding ratios are given in Table II.

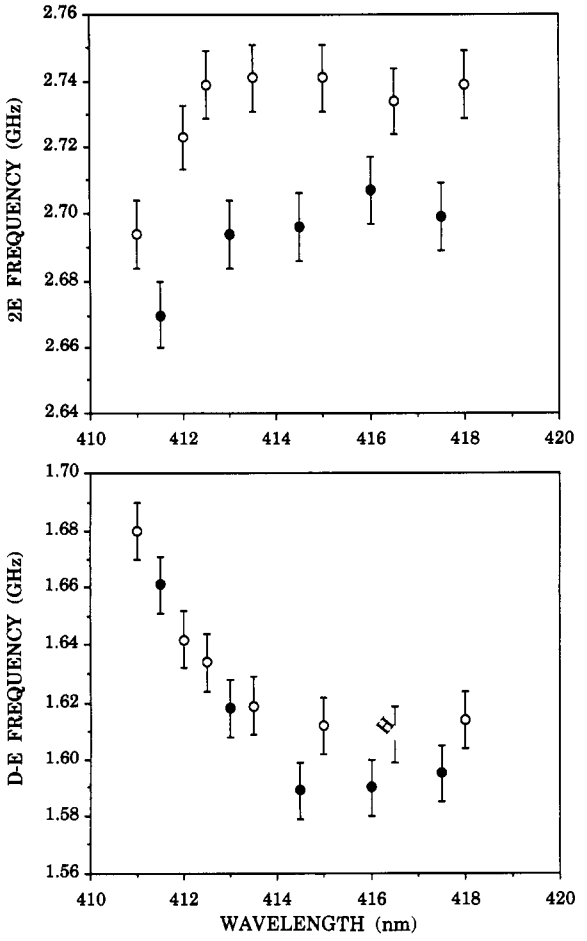

FIGURE 6: Comparison of zero field ODMR frequencies as a function of emission wavelength shown for IPTG-complexed W220Y (●) and free W220Y (○). All experimental conditions are as described in the caption for Figure 4. IPTG is present in a 100-fold excess per W220Y subunit.

demonstrate that the induced zfs shifts are consistent across the monitored emission wavelengths (Figure 5).

From the data in Table II, it appears that melibiose interacts more extensively with Trp 220 and induces more prominent changes in its local environment than does IPTG; IPTG has only a very minimal effect on the low-temperature triplet-state properties of Trp 220. The very moderate perturbations of the excited triplet state of Trp 220 by both IPTG and melibiose could indicate that, rather than direct interactions with the binding substrate, Trp 220 interacts indirectly, responding to minor local conformational changes.

W220Y and Inducers. Several groups have cited evidence that inducer binding produces effects on Trp 201 as well as local effects on Trp 220 (Spodheim-Maurizot et al., 1985; Gardner & Matthews, 1990; Royer et al., 1990). Therefore, the possibility of long-range effects of inducer binding on Trp 201 also was investigated using phosphorescence and slow passage ODMR spectroscopy. Melibiose and IPTG complexes with W220Y both consistently show a ca. 0.8 nm *red* shift of the 0,0 band relative to unbound W220Y. In contrast with the lack of local Trp 220 interactions with the inducers, we find long-range interactions with Trp 201 that appear to be more extensive for IPTG than for melibiose binding. The ODMR frequency shifts induced by melibiose binding are in the same direction, but are considerably less than those induced by IPTG (Table II). These shifts are shown in Figure 6 as a function of wavelength. ODMR bandwidths of IPTG-complexed W220Y are 10–20 MHz narrower than those of either free W220Y or melibiose-bound W220Y. The corrected line widths of IPTG-bound W220Y ($|D| - |E|$, ca. 25 MHz; $2|E|$, ca. 46 MHz) suggest a very homogeneous local environment (Purkey & Galley, 1970; Gradl et al., 1986) and are consistent only with a buried residue (Hershberger et al., 1980).

Melibiose and IPTG were bound to the native Lac Rep, but did not induce a noticeable red shift in the phosphorescence 0,0 band at 77 K. Therefore, the red shifts observed for inducer binding to W220Y may result from features characteristic of the single-point mutant but not of the native Lac Rep itself.

Energy Transfer. The discrepancy between the phosphorescence emission intensity of the native Lac Rep and that expected from the superposition of the two single point mutant spectra might indicate the depletion of Trp 220 phosphorescence intensity through energy-transfer quenching of Trp 220 by Trp 201. Singlet–singlet energy transfer between Trp residues has been observed and quantitatively estimated from the phosphorescence spectra of two similar Trp mutant systems: T4 lysozyme (Ghosh et al., 1988a, 1993) and *E. coli* Trp repressor (Burns, 1992; Eftink et al., 1993). A number of problems arise at the outset if one attempts to use a similar approach to estimate the energy transfer in Lac Rep. First, while crystals of Lac Rep have been grown, they have not been of X-ray diffraction quality (Pace et al., 1990), so that crystallographic estimates of the distances between tryptophans 220 and 201 within the monomer and between monomers are unavailable. Thus, the possibility of a triplet–

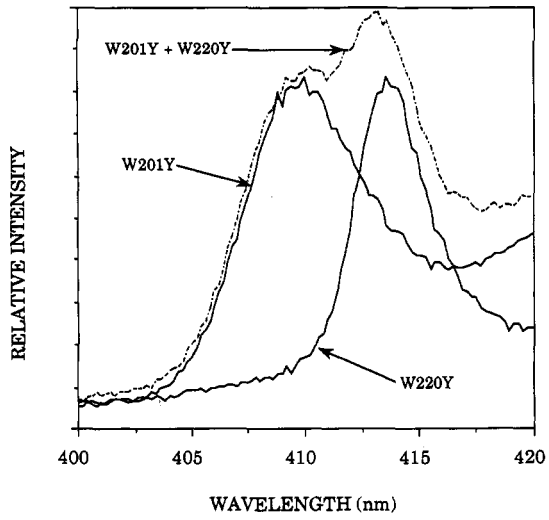

FIGURE 7: Comparison of the phosphorescence spectra of the two single-point mutants W201Y and W220Y with that of the equimolar solution of W201Y + W220Y. All sample concentrations were 30 μM. Excitation was at 305 nm with a 16 nm bandpass. Emission resolution was 1.5 nm.

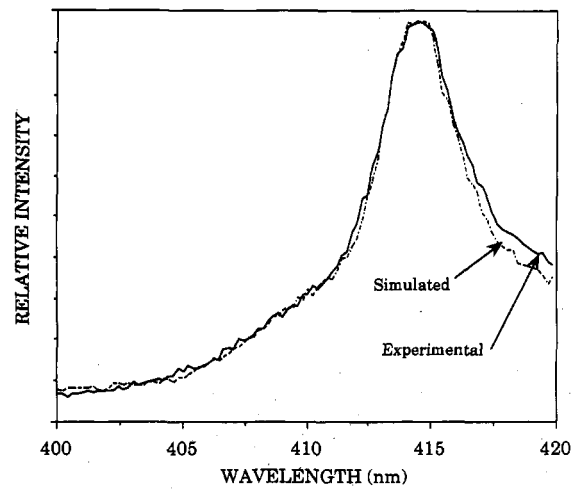

FIGURE 8: Simulation of the experimentally obtained native Lac Rep spectra in the 0,0 band region by an appropriate combination of the normalized W201Y and W220Y spectra. The simulation was achieved by summing 33 ± 2% W201Y and 100% W220Y. Experimental conditions of the native Lac Rep were identical to those described in the caption for Figure 7.

triplet energy-transfer contribution to the quenching of Trp 220 phosphorescence is uncertain. The tryptophans within the monomer may be in quite close proximity, which could explain the small effects of substrate binding on each tryptophan (Table II). A minor contribution of tyrosine to the phosphorescence makes the interpretation of wavelength-dependent kinetic analysis uncertain. Second, proper analysis (Ghosh et al., 1988a, 1993) requires that the excited triplet-state properties of the chromophores that are present must remain virtually indifferent to the replacement of a Trp with tyrosine (or other amino acid residues). As seen from the phosphorescence and ODMR characterizations, both Trp 220 and Trp 201 experience changes in their microenvironments as a result of point mutation of the other Trp residue. Possible complications of Tyr → Trp energy transfer are minimized by exciting the sample well to the red of the tyrosine absorption band.

The estimate of the energy transfer from Trp 220 to Trp 201 utilized the unique nature of the two single-point mutants. Because each mutant has only the phosphorescence intensity and emission attributes of a single isolated tryptophan, the spectra of the two mutants can be mixed in such proportions that they reproduce the native Lac Rep spectrum as closely as possible. A dilute equimolar solution of W220Y and W201Y is used to correct for intrinsic differences in the phosphorescence intensity of the two mutants that result from variations in experimental conditions. The normalized spectra are then used to simulate the spectrum of the native Lac Rep. Excitation was centered at 305 nm with a 16-nm bandpass to minimize any energy transfer from tyrosine. Sample concentrations were the same for all spectra (ca. 30 μM). This level is sufficiently low to effectively eliminate trivial inter-protein energy transfer in the equimolar solution of W201Y and W220Y. Figure 7 shows the phosphorescence of an equimolar solution of W201Y and W220Y along with those of the normalized single-point mutants. The simulated 0,0 band of the native Lac Rep was fit best by adding 33% of the W201Y spectrum to 100% of the W220Y spectrum. The fit in the 0,0 band region is shown in Figure 8. The phosphorescence intensities of the two chromophores in the simulated and equimolar spectra can be related to the efficiency of energy transfer in the following manner (Ghosh et al., 1988a, 1993):

$$\rho'_{DA}/\rho^{\circ}_{DA} = (1 - f_t)/(1 + f_a\alpha_D/\alpha_A) \quad (1)$$

where ρ'_{DA} and ρ°_{DA} are the ratios of the donor (Trp 220) to acceptor (Trp 201) phosphorescence intensities of the simulated Lac Rep spectrum and of the equimolar solution of W220Y and W201Y, respectively. The term f_t is the fraction of excited donor molecules, D, that decay through radiationless energy transfer to acceptor molecules, A. The terms α_D and α_A are the absorbances of the donor, D (Trp 220), and the acceptor, A (Trp 201). The value for $\rho'_{DA}/\rho^{\circ}_{DA}$ calculated by the spectral simulation was 0.13. The ratio α_D/α_A was estimated using published extinction coefficients (Gardner & Matthews, 1990), resulting in $f_t = 0.72$.

This analysis assumes that energy transfer takes place predominantly at the singlet level. It should be emphasized that the calculated singlet–singlet energy-transfer efficiency applies to a rigid unrelaxed local environment and will probably differ significantly for Lac Rep at ambient temperature.

One final piece of information may be gleaned from this simulation. The best fit of the normalized single-point mutants to the experimental spectrum of the native Lac Rep was obtained when the 0,0 bands of both W201Y and W220Y were red-shifted by 0.8 nm. This requirement indicates that the reduction in frequency observed for the $2|E|$ ODMR resonance of W201Y relative to the Trp 220 component of native Lac Rep is accompanied by a red shift of the 0,0 band. Such a shift is not apparent in a direct comparison of the native Lac Rep and the W201Y point mutant phosphorescence spectra.

CONCLUSIONS

The native Lac Rep has a low-temperature phosphorescence spectrum that provides evidence for a contribution from two Trp sites: a buried site with a band origin at 414.7 nm and a second less intense, and more poorly resolved, site that is partially solvent-exposed (shoulder at ca. 409.5 nm). Using low-temperature phosphorescence and slow passage ODMR to study two single-point mutants, W201Y and W220Y, we were able to confirm the microenvironmental assignments of the two Trp residues of Lac Rep. Trp 201 has been assigned to a buried, polarizable region of the protein with a 0,0 band

peak that is found at 413.7 nm in W220Y. Trp 220, on the other hand, has a broader, blue-shifted phosphorescence 0,0 band at 409.7 nm in W201Y; these are characteristics of a partially solvent-exposed residue. Zero magnetic field ODMR signals provide support for these assignments through both their frequency and their bandwidth characteristics. Comparisons of the phosphorescence and of the wavelength-dependent slow passage ODMR signals suggest that the two single-point mutants do not provide completely accurate representations of the microenvironments of the two intrinsic Trp residues of native Lac Rep.

A small blue shift was observed upon binding the inducer, melibiose, to W201Y, with an even smaller spectral blue shift associated with IPTG binding. Small shifts in the ODMR frequencies of Trp 220 were produced by melibiose binding, but any shifts induced by IPTG were not detectable within experimental error. Trp 220 has been proposed to be located at or near the substrate binding site. However, the relatively moderate perturbations of the triplet state that result from inducer binding might indicate that Trp 220 is not intimately involved with the binding interactions, but rather that the spectral shifts represent a response to structural changes, creating a somewhat more polar Trp site that is also more homogeneous. Long-range effects of inducer binding were also observed at the site of Trp 201. The ODMR line widths of W220Y narrowed significantly, and a small but reproducible shift of the phosphorescence to longer wavelengths was measured in the presence of both inducers, which indicates an increase in the polarizability of the (remote) site. A similar red shift was not observed, however, for the native Lac Rep. This absence of comparable shifts in the native protein could indicate that W220Y, whose inducer binding affinity is ca. 30-fold lower than that of native Lac Rep (Gardner & Matthews, 1990), does not accurately represent the binding features of the native Lac Rep.

The apparent differences in intrinsic Trp phosphorescence intensity of the native and the two single-point mutants were suggested to result from singlet–singlet energy transfer from Trp 220 to Trp 201. This possibility led us to attempt to quantify the energy transfer between these two Trps. The fraction of Trp 220 sites that decay by radiationless energy transfer to Trp 201 was calculated to be 0.72 in the rigid system at 77 K. This value must be viewed with caution for the following reasons: (1) The calculation assumes that the excited triplet-state properties of the native Lac Rep are accurately represented by the single-point mutants. Minor shifts in 0,0 phosphorescence origins and small variations in the zfs parameters of the native Lac Rep relative to the mutants W220Y and W201Y indicate that the Trp environments in the single-point mutants are not completely accurate representations of native Lac Rep. (2) The model assumes that triplet–triplet transfer is not a significant contributor to the phosphorescence quenching. This condition was difficult to verify by monitoring the wavelength dependence of the phosphorescence kinetics of native Lac Rep because of an intrinsic short component attributed to tyrosine.

This work demonstrates that the properties of the photoexcited triplet state as measured by phosphorescence and ODMR spectroscopy can be successfully used to characterize and detect small changes in the local environment of Trp residues in proteins. The high resolution of both the phosphorescence and ODMR spectra of Trp, relative to fluorescence, is a particularly valuable feature of the triplet state in this regard. The availability of single Trp point mutants makes possible the absolute assignment of triplet-state properties to individual Trp residues, and the quantitative evaluation of energy-transfer efficiencies between Trp sites.

REFERENCES

Bandyopadhyay, P. K., & Wu, C. W. (1979) *Arch. Biochem. Biophys. 195*, 558–564.

Barkley, M. D., Riggs, A. D., Jobe, A., & Bourgeois, S. (1975) *Biochemistry 14*, 1700–1712.

Bayreuther, K., Adler, K., Geisler, N., & Klemm, A. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3576–3580.

Boschelli, F., Jarema, M. A., & Lu, P. (1981) *J. Biol. Chem. 256*, 11595–11599.

Brennan, R. G., & Matthews, B. W. (1989) *J. Mol. Biol. 264*, 1903–1906.

Burns, L. E. (1992) Ph.D. Thesis, University of California at Davis, Davis, CA.

Burstein, E., Vedenkina, E., & Ivokova, N. (1973) *Photochem. Photobiol. 18*, 263–279.

Clarke, R. H., Ed. (1982) *Triplet State ODMR Spectroscopy*, Wiley-Interscience, New York.

Eftink, M. R., Ramsay, G. D., Burns, L. E., Maki, A. H., Mann, C. J., Matthews, C. R., & Ghiron, C. A. (1993) *Biochemistry 32*, 9189–9198.

Galley, W. C., & Purkey, R. M. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 1116–1121.

Gardner, J. A., & Matthews, K. S. (1990) *J. Biol. Chem. 265*, 21061–21067.

Ghosh, S., Zang, L.-H., & Maki, A. H. (1988a) *J. Chem. Phys. 88*, 2769–2775.

Ghosh, S., Zang, L.-H., & Maki, A. H. (1988b) *Biochemistry 27*, 7816–7820.

Ghosh, S., Zang, L.-H., & Maki, A. H. (1993) *J. Chem. Phys. 99*, 771.

Gouterman, M., & Moffitt, W. (1959) *J. Chem. Phys. 30*, 1107–1108.

Gradl, G., Friedrich, J., & Kohler, B. E. (1986) *J. Chem. Phys. 84*, 2079–2083.

Hershberger, M. V., Maki, A. H., & Galley, W. C. (1980) *Biochemistry 19*, 2204–2209.

Hoff, A. J. (1989) in *Advanced EPR with Applications in Biology and Biochemistry* (Hoff, A. J., Ed.) pp 633–684, Elsevier, Amsterdam.

Itoh, K., & Azumi, T. (1973) *Chem. Phys. Lett. 22*, 395–399.

Itoh, K., & Azumi, T. (1975) *J. Chem. Phys. 62*, 3431–3438.

Jobe, A., & Bourgeois, S. (1972) J. *Mol. Biol. 69*, 397–408.

Kaptein, R., Zuiderweg, E. R. P., Scheek, R. M., Boelens, R., & van Gunsteren, W. F. (1985) *J. Mol. Biol. 182*, 179–182.

Khamis, M. I., Casas-Finet, J. R., Maki, A. H., Murphy, J. B., & Chase, J. W. (1987a) *J. Biol. Chem. 262*, 10938–10945.

Khamis, M. I., Casas-Finet, J. R., Maki, A. H., Murphy, J. B., & Chase, J. W. (1987b) *FEBS Lett. 211*, 155–159.

Kwiram, A. L., Ross, J. B. A., & Deranleau, D. A. (1978) *Chem Phys Lett. 54*, 506–509.

Laiken, S. L., Gross, C. A., & von Hippel, P. H. (1972) *J. Mol. Biol. 66*, 143–155.

Lam, W.-C., Maki, A. H., Itoh, T., & Hakoshima, T., (1992) *Biochemistry 31*, 6756–6760.

Lin, S-Y., & Riggs, A. D. (1975) *Cell 4*, 107–111.

Longworth J. W. (1971) in *Excited States of Proteins and Nucleic Acids* (Steiner, R. F., & Weinryb, I., Eds.) pp 319–484, Plenum Press, New York.

Maki, A. H. (1984) in *Biological Magnetic Resonance* (Berliner, L. J., & Reuben, J., Eds.) Vol. 6, pp 187–294, Plenum, New York.

Maki, A. H., Svejda, P., & Huber, J. R. (1978) *Chem. Phys. 32*, 369–380.

Mao, S. Y., & Maki, A. H. (1987) *Biochemistry 26*, 3576–3582.

Mao, S. Y., Maki, A. H., & de Haas, G. H. (1987) *FEBS Lett. 211*, 83–88.

Miller, J. H., & Reznikoff, W. S., Eds. (1980) *The Operon*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Müller-Hill, B. (1971) *Angew. Chem., Int. Ed. Engl. 10*, 160–172.

Pabo, C. O., & Sauer, R. T. (1984) *Annu. Rev. Biochem. 53*, 293–321.

Pace, H. C., Lu, P., & Lewis, M. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1870–1873.

Purkey, R. M., & Galley, W. C. (1970) *Biochemistry 9*, 3569–3575.

Riggs, A. D., Suzuki, H., & Bourgeois, S. (1970) *J. Mol. Biol. 48*, 67–83.

Royer, C. A., Gardner, J. A., Beechem, J. M., Brochon, J.-C., & Matthews, K. S. (1990) *Biophys. J. 58*, 363–378.

Schlyer, B. D., Maki, A. H., & Hawrot, E. (1992) *FEBS Lett. 297*, 87–90.

Sommer, H., Lu, P., & Miller, J. H. (1976) *J. Biol. Chem. 251*, 3774–3779.

Spodheim-Maurizot, M., Charlier, M., & Helene, C. (1985) *Photochem. Photobiol. 42*, 353–359.

Takeda, Y., Ohlendorf, D. H., Anderson, W. F., & Mattews, B. W. (1983) *Science 221*, 1020–1026.

Tsao, D. H. H., Casas-Finet, J. R., Maki, A. H., & Chase, J. W. (1989) *Biophys. J. 55*, 927–936.

van der Waals, J. H., & ter Maten, G. (1964) *Mol. Phys. 8*, 301–318.

van Egmond, J., Kohler, B. E., & Chan, I. Y. (1975) *Chem. Phys. Lett. 34*, 423–426.

von Schütz, J. U., Zuclich, J., & Maki, A. H. (1974) *J. Am. Chem. Soc. 96*, 714–718.

Zang, L.-H., Ghosh, S., & Maki, A. H. (1988) *Biochemistry 27*, 7829–7825.

Zang, L.-H., Ghosh, S., & Maki, A. H. (1989) *Biochemistry 28*, 2245–2251.

Zuclich, J. J. (1970) *Chem. Phys. 52*, 3586–3591.

Zuclich, J., Schweitzer, D., & Maki, A. H. (1973) *Photochem. Photobiol. 18*, 161–168.

Zuiderweg, E. R. P., Kaptein, R., & Wüthrich, K. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 5837–5841.