Phosphorescence and Optically Detected Magnetic Resonance Measurements of the 2′AMP and 2′GMP Complexes of a Mutant Ribonuclease T_1 (Y45W) in Solution: Correlation with X-ray Crystal Structures†

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ABSTRACT: Phosphorescence and ODMR measurements have been made on ribonuclease T_1 (RNase T_1), the mutated enzyme RNase T_1 (Y45W), and their complexes with 2′GMP and 2′AMP. It is not possible to observe the phosphorescence of Trp45 in RNase T_1 (Y45W). Only that of the naturally occurring Trp59 is seen. The binding of 2′GMP to wild-type RNase T_1 produces only a minor red shift in the phosphorescence and no change in the ODMR spectrum of Trp59. However, a new tryptophan 0,0-band is found 8.2 nm to the red of the Trp59 0,0-band in the 2′GMP complex of the mutated RNase T_1 (Y45W). Wavelength-selected ODMR measurements reveal that the red-shifted emission induced by 2′GMP binding, assigned to Trp45, occurs from a residue with significantly different zero-field splittings than those of Trp59, a buried residue subject to local polar interactions. The phosphorescence red shift and the zero-field splitting parameters demonstrate that Trp45 is located in a polarizable environment in the 2′GMP complex. In contrast with 2′GMP, binding of 2′AMP to RNase T_1 (Y45W) induces no observable phosphorescence emission from Trp45, but leads only to a minor red shift in the phosphorescence origin of Trp59 in both the mutated and wild-type enzyme. The lack of resolved phosphorescence emission from Trp45 in RNase T_1 (Y45W) implies that the emission of this residue is quenched in the uncomplexed enzyme. We conclude that local conformational changes that occur upon binding 2′GMP remove quenching residues from the vicinity of Trp45, restoring its luminescence. The binding of 2′AMP, on the other hand, must produce different or no large conformational changes in the vicinity of Trp45 since its phosphorescence is not restored. These spectroscopic results are in excellent accord with recent X-ray crystal structure determinations of the 2′GMP and 2′AMP complexes of RNase T_1 (Y45W), suggesting that glutamic acid 46 interactions with Trp45 may be responsible for quenching.

Ribonuclease T_1 (RNase T_1)[1] is a monomeric enzyme isolated from *Aspergillus oryzae* that cleaves single-stranded RNA with great specificity at the 3′ position of guanosine. RNase T_1 contains 104 amino acids that include a single tryptophan residue at position 59. The structure of the enzyme complexed with a specific substrate analog, 2′GMP, has been solved using X-ray diffraction (Arni et al., 1988). Trp59 is found to be buried in the interior of the enzyme in contact with an occluded water molecule, while the tyrosine at position 45 is located at the solvent-exposed surface of the 2′GMP recognition site in partial contact with guanine. The distance between Tyr45 and guanine, as well as their relative orientations, however, are less than ideal for aromatic stacking interactions (Arni et al., 1988). Photo-CIDNP spectroscopy (Nagai et al., 1985) has shown previously that Tyr45 is involved in interactions with the guanine ring upon 2′GMP binding. Also, the phenolic side chain mobility is suggested to be restricted when 2′GMP is bound. Two-dimensional NMR spectroscopy (Shimada & Inagaki, 1990) has shown that Tyr45 moves to a different chemical environment when 2′GMP is bound.

In this study, we employ a mutated RNase T_1 that has a tryptophan residue substituted for Tyr45 by site-directed oligonucleotide mutagenesis (Hakoshima et al., 1991a). Thermal denaturation and circular dichroism measurements (Nishikawa et al., 1988) indicate that the wild-type enzyme and RNase T_1 (Y45W) have a similar conformation. Furthermore, enzymatic activity is found to be even somewhat enhanced by the Y45W mutation (Nishikawa et al., 1988). Recent X-ray diffraction measurements (Hakoshima et al., 1991b) have shown that 2′GMP binds to wild-type RNase T_1 and to the Y45W mutant in a similar manner. In order to learn about environmental perturbations induced in the vicinity of the aromatic residue at position 45, we have employed low-temperature phosphorescence and optical detection of triplet-state magnetic resonance (ODMR) to study the effect of substrate analog binding on the tryptophan residue at position 45 of the mutated enzyme, RNase T_1 (Y45W). The phosphorescence spectrum of tryptophan, as well as the zero-field splittings (zfs) of the phosphorescent triplet state, are sensitive to changes in the local environment (Hershberger et al., 1980; Kwiram, 1982). Comparisons are made of the phosphorescence spectrum, 0,0-band energies, and the changes that occur upon binding of 2′GMP and 2′AMP to the wild-type RNase T_1 and to the mutant enzyme RNase T_1 (Y45W). Triplet-state parameters *D* and *E* and ODMR line widths of the tryptophans are compared to characterize further their local environments. These spectroscopic measurements are interpreted in light of recent X-ray crystal structures (Hakoshima et al., 1992) of the 2′AMP and 2′GMP complexes

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[1] Abbreviations: AMP, adenosine monophosphate; GMP, guanosine monophosphate; ODMR, optically detected magnetic resonance; photo-CIDNP, photochemically-induced dynamic nuclear polarization; RNase T_1, ribonuclease T_1; zfs, zero-field splittings.

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of RNase T_1 (Y45W).

Tryptophan has been widely used as a probe in fluorescence and phosphorescence studies of proteins and their interactions with ligands. When tryptophan is promoted to an electronically excited state, it relaxes radiationlessly to the lowest singlet state. At this point, it can undergo intersystem crossing into the triplet manifold and end up in the lowest triplet state, which decays to the ground state, emitting phosphorescence. The two unpaired electrons in the triplet state interact via magnetic dipole–dipole interactions because each electron possesses a magnetic dipole resulting from its spin. This interaction splits the energy level of the triplet state into three sublevels even in the absence of a magnetic field. This splitting is affected by the local environment of the tryptophan and therefore provides an additional level of information on its surroundings. ODMR spectroscopy probes these splittings and provides information on the kinetics of the three sublevels. Since ODMR spectroscopy uses tryptophan as a probe, it is suitable for studying native proteins and their interactions with ligands. In this study, the splittings between the zero-field magnetic sublevels are measured by sweeping the microwave frequency through the zero-field resonances. This induces population shifts between sublevels that are detected by monitoring the phosphorescence intensity. The experiment is feasible at low temperature when the sublevel populations are isolated from each other. The ODMR method has been described in detail (Maki, 1984; Hoff, 1989).

MATERIALS AND METHODS

RNase T_1 was obtained from Sigma. RNase T_1 (Y45W) was produced as described earlier (Hakoshima et al., 1991a; Nishikawa et al., 1988). Enzymes were dissolved in aqueous 10 mM phosphate buffer, pH 7, containing 0.2 mM EDTA, to which was added 20% (v/v) ethylene glycol as cryosolvent for spectroscopic measurements. Enzyme concentration was ca. 0.2 mM. Enzyme complexes with 2′GMP (Sigma) and 2′AMP (ICN) were formed by mixing aqueous solutions of enzyme and nucleotide and incubating for 1 h. These nucleotides were present in a 1:1 molar ratio and in a 25-fold molar excess, respectively, and we estimate that the enzyme was at least 80% complexed in these solutions based on the measured K_d's (Hakoshima et al., 1992). Nucleotide binding to the enzyme also was carried out in 50 mM Tris-HCl and 0.2 mM EDTA buffer (pH 7.1) for comparison. Samples, ca. 10 μL, were contained in a 1-mm i.d. Suprasil quartz sample tube which was mounted in a helical slow-wave structure of copper wire which terminated a rigid stainless steel coaxial microwave transmission line. The latter was immersed in a liquid helium dewar with quartz optical windows for phosphorescence and ODMR measurements. Phosphorescence spectra were obtained at ca. 77 K, while ODMR was measured at ca. 1.2 K, the temperature of pumped liquid helium. Slow-passage ODMR spectra were corrected for rapid passage effects by making measurements over a range of microwave sweep rates sweeping from low to high frequencies and extrapolating the peak frequencies to zero sweep rate. Details of the ODMR spectrometer have been described previously (Tsao et al., 1991).

RESULTS

The phosphorescence spectra of wild-type RNase T_1 and its complex with 2′GMP are compared in Figure 1. The well-resolved spectra are very similar, revealing only a minor red shift (0.6 nm) of the 0,0-band maximum of the Trp59 phosphorescence upon binding of 2′GMP. The 0,0-band

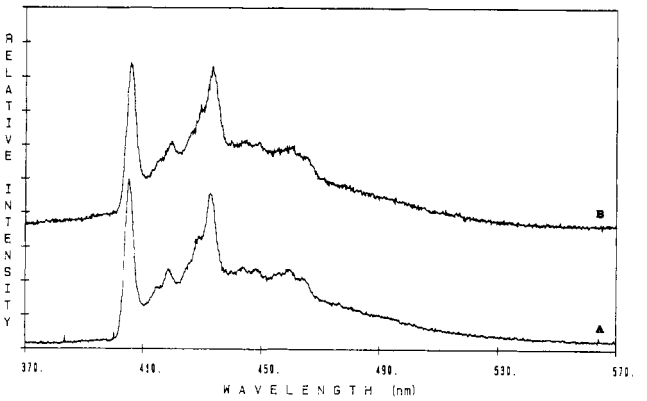

FIGURE 1: Phosphorescence spectra of (A) RNase T_1 and (B) RNase T_1 + 2′GMP. See text for details.

Table I: Triplet-State Spectroscopic Parameters of Tryptophan in Various Samples

sample[a]	$\lambda_{0,0}$[b] (nm)	D[c] (GHz)	E[c] (GHz)	$D-E$[c] (GHz)
RNase T_1	404.9	3.02	1.24	1.78
RNase T_1 + 2′GMP	405.5	3.01	1.24	1.77
RNase T_1 (Y45W)	405.1	3.02	1.24	1.78
RNase T_1 (Y45W) + 2′GMP	405.5 413.7	3.03 2.98	1.26 1.29	1.77 1.69
RNase T_1 (Y45W) + 2′AMP	405.5			
somatostatin[d]	408.9	2.98	1.25	1.73
N-acetyltryptophanamide[d]	406.8	2.98	1.21	1.77

[a] RNase T_1 samples are in 10 mM phosphate buffer, pH 7, containing 0.2 mM EDTA and 20% (v/v) ethylene glycol. Excitation wavelength is 305 nm (16-nm band-pass) for RNase T_1 samples and 297 nm (3-nm band-pass) for others. [b] 0,0-band maximum from phosphorescence at 77 K. [c] Measurements made monitoring 0,0-band maximum with 2-nm band-pass at $T = 1.2$ K. Estimated accuracy is ±0.01 GHz. [d] Data taken from Deranleau et al. (1978).

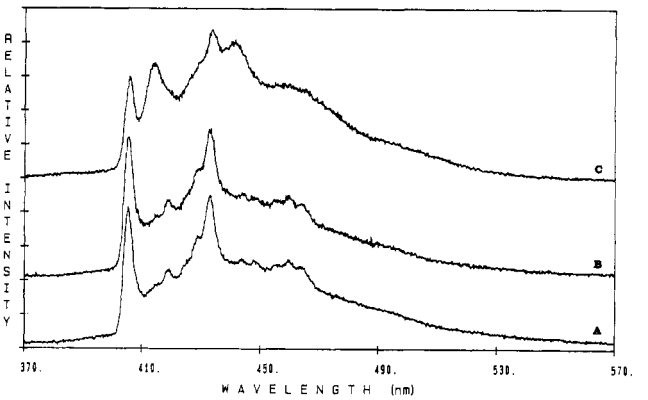

FIGURE 2: Phosphorescence spectra of (A) RNase T_1 (Y45W), (B) RNase T_1 (Y45W) + 2′AMP, and (C) RNase T_1 (Y45W) + 2′GMP. See text for details.

wavelengths are given in Table I. The phosphorescence spectrum of RNase T_1 (Y45W) is shown in Figure 2A. The 0,0-band maximum occurs at effectively the same wavelength as that of the wild-type enzyme (Table I), and the spectrum is very similar to that of the wild type. Figure 2C shows the phosphorescence spectrum of RNase T_1 (Y45W) complexed with 2′GMP. In contrast with the wild-type enzyme, in which only a minor red shift of the Trp59 0,0-band was produced, we now observe, in addition to a similar red shift, a new major band in the phosphorescence spectrum that peaks at 413.7 nm. We assign this band, which is shifted by 8.2 nm to the red of the Trp59 0,0-band, as the 0,0 of Trp45. The Trp45 band is considerably broader than that of Trp59, indicating

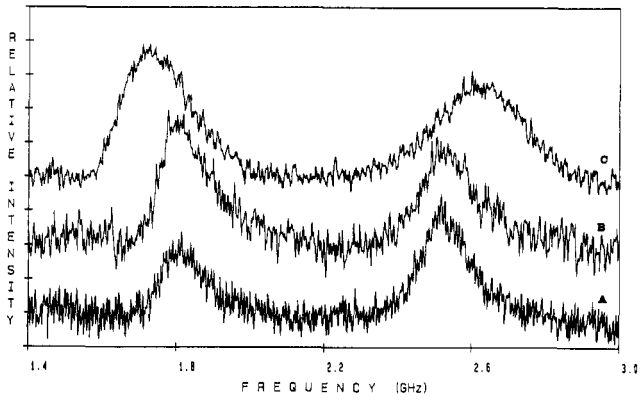

FIGURE 3: Slow-passage ODMR spectra of (A) RNase T_1 (Y45W) and (B and C) RNase T_1 (Y45W) + 2'GMP. Emission was monitored at (A) 405.1 nm, (B) 405.5 nm, and (C) 413.7 nm using 3-nm bandwidth. Sweep rate of microwaves was 53.3 MHz s^{-1}. Signal at lower frequency is $D - E$, while the other is $2E$.

a more heterogeneous (and therefore probably solvent-exposed) local environment. The assignment of the red-shifted band to a distinct tryptophan residue is confirmed by wavelength-selected ODMR experiments. The ODMR spectrum of RNase T_1 (Y45W) was measured, monitoring the phosphorescence at the 0,0-band peak wavelength using narrow-band (ca. 2-nm bandwidth) monochromator slits. The spectrum is shown in Figure 3A. Similar measurements were made on the complex of RNase T_1 (Y45W) with 2'GMP, monitoring the emission at the 0,0-band peaks that we have assigned to Trp59 (blue) and Trp45 (red), respectively. The spectra, consisting of the normally observable $D - E$ and $2E$ zero-field transitions of tryptophan, are compared in panels B and C of Figure 3. The zfs D and E parameters obtained from these spectra are presented in Table I. The significant differences observed for the zfs parameters confirm that these emission bands originate from distinct tryptophan residues. We believe that the ODMR observed at 413.7 nm for the 2'GMP complex of RNase T_1 (Y45W) is largely representative of Trp45 since the intensity of Trp59 at this wavelength is ~25% of the total emission. Also included for comparison in Table I are the results of similar measurements on wild-type RNase T_1 and its complex with 2'GMP, as well as measurements made on somatostatin and *N*-acetyltryptophanamide (Deranleau et al., 1978) that contain typical solvent-exposed tryptophan residues. The D value for tryptophan 45 in the complex of RNase T_1 (Y45W) and 2'GMP does not differ significantly from those of solvent-exposed tryptophans but the E value is significantly larger.

The phosphorescence spectrum of RNase T_1 (Y45W) complexed with 2'AMP is seen in Figure 2B. The spectrum resembles that of the uncomplexed enzyme; only a minor red shift of the 405.1-nm 0,0-band (ca. 0.4 nm) occurs on binding of the nucleotide. An analogous minor shift also is produced by the binding of 2'GMP to both wild-type and mutated enzymes (see Table I). Most obvious is the lack of a new 0,0-band in the phosphorescence spectrum, such as that produced by the binding of 2'GMP (Figure 2C). The binding of these nucleotides to the enzyme in Tris buffer gave phosphorescence spectra that were identical to those observed in phosphate buffer.

DISCUSSION

Comparison of the spectrum of RNase T_1 (Figure 1A) with that of the mutated enzyme, RNase T_1 (Y45W) (Figure 2A), leads to the conclusion that the substitution of Trp45 for tyrosine does not contribute significantly to the phosphorescence. Since this point mutation is known to preserve enzymatic activity (Nishikawa et al., 1988) and to have minimal effects on enzyme conformation based on circular dichroism and thermal denaturation studies (Nishikawa et al., 1988), Trp45 should be located on the exterior surface of the nucleotide-binding site (as is Tyr45 in the wild-type enzyme). Such a location for Trp45 has been confirmed recently (Hakoshima et al., 1991b, 1992) in the X-ray crystal structures of the 2'GMP and 2'AMP complexes of the mutated RNase T_1 (Y45W). Trp45 is thus solvent-exposed to a large degree and in the free enzyme it should have a phosphorescence 0,0-band wavelength maximum near that of somatostatin, for example (Table I). The 0,0-band would be expected to be rather broad due to the local heterogeneity that results from solvent exposure (Kwiram, 1982). If Trp45 emitted reasonably intense phosphorescence, it should be clearly resolved from that of the unusually blue-shifted and narrow phosphorescence 0,0-band of Trp59. Trp59, although buried, is in a polar environment that produces its blue shift (Hershberger et al., 1980). The polar interaction appears to be due to an interior water molecule that is hydrogen bonded to Trp59 (Arni et al., 1988). The most reasonable conclusion is that the excited state of Trp45 is quenched (probably at the singlet level) in the free mutated enzyme. The emergence of phosphorescence from Trp45 when 2'GMP is bound indicates that the quenching process is no longer as effective in the complex. Since no significant change of the phosphorescence spectrum was detected upon binding of 2'AMP, the guanine-recognition site should not be occupied by the adenine base in this complex. A different binding mode is observed in the crystal structure of RNase T_1 (Y45W) complexed with 2'AMP (Hakoshima et al., 1991b, 1992). In this crystal, the adenine base binds to His92, which is a large distance from the guanine-binding site, the latter being occupied by water molecules.

Comparison of the crystal structures of RNase T_1 (Y45W) complexed with 2'AMP and 2'GMP could provide clues to a reasonable quenching mechanism of Trp45 in the free mutant enzyme and the manner in which the quenching is removed by formation of the complex with 2'GMP. Quenchers of tryptophan excited states include carboxyl groups, ammonium

Table II: Atomic Distances between Potential Tryptophan Quenchers and the Side Chain of Trp45 Observed in Crystals of the Mutant RNase T_1 (Y45W) Complexed with 2'AMP and 2'GMP[a]

		complex with 2'AMP		complex with 2'GMP	
residue	atom	atom[b]	distance (Å)	atom[b]	distance (Å)
Asn 43	O	Cδ1	*3.6*	Cδ1	*4.2*
	Oδ1	Cδ1	7.2	Nε1	*4.7*
	Nδ2	Cδ1	6.7	Cζ2	*3.3*
Asn 44	O	Cγ	5.0	Cγ	*4.9*
	Oδ1	Cγ	6.8	Cγ	7.5
	Nδ2	Cγ	8.2	Cδ1	8.3
Trp 45	O	Cε3	*3.3*	Cε3	6.4
Glu 46	Oε1	Cζ2	5.5	Cζ2	8.2
	Oε1	Cη2	5.4	Cη2	7.7
	Oε2	Cζ2	*4.0*	Cζ2	7.6
	Oε2	Cε2	*4.4*	Cε2	6.7
	Oε2	Cη2	*4.4*	Cη2	7.4
Asn 98	O	Cζ2	6.8	Cζ3	6.4
	Oδ1	Nε1	8.4	Cζ3	8.9
	Nδ2	Cζ2	6.8	Cζ3	9.0
Asn 99	O	Cζ2	7.7	Cε2	9.0
	Oδ1	Cζ2	*3.4*	Cε2	6.5
	Nδ2	Cζ2	*4.9*	Cε2	8.1

[a] Calculated from the coordinates of Hakoshima et al., (1992). [b] Atoms of the indole ring of Trp45. Distances shorter than 5 Å are in italics.

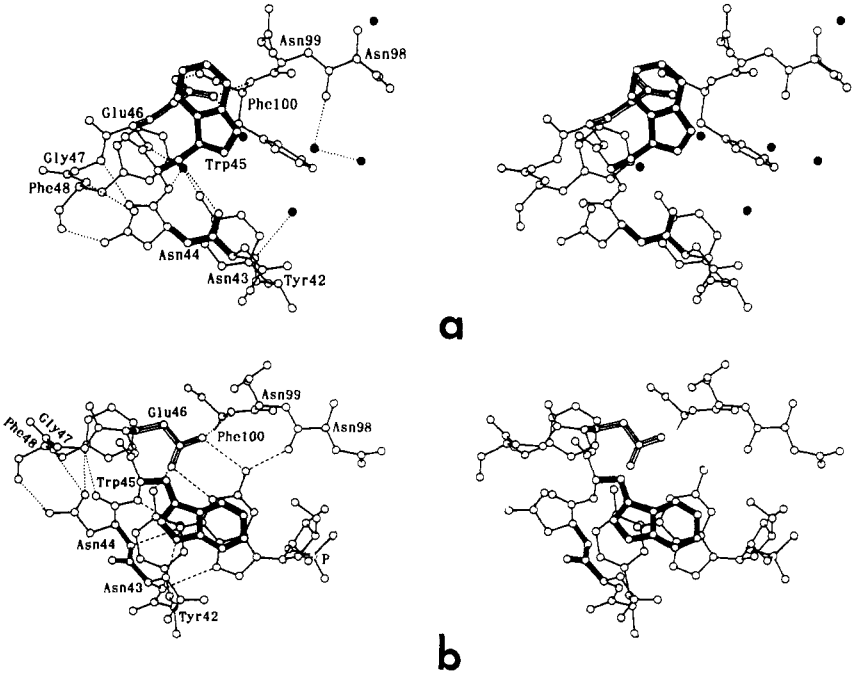

FIGURE 4: Stereoviews of the guanine-recognition sites in the crystals of the mutant RNase T_1 (Y45W) complexed with (a) 2'AMP and (b) 2'GMP (Hakoshima et al., 1992). The peptide bond between Asn43 and Asn44 and the side chain of Trp45 are drawn in heavy lines. The side chain of Glu46 is drawn in triple lines. These groups display significant conformational differences between the two complexes. Hydrogen bonds between the enzyme and the nucleotide are shown with broken lines while others are shown with dotted lines. Water molecules are represented as filled circles.

groups, disulfides, and histidines (Longworth, 1971). In Table II, we list atomic distances between potential quenching residues and the indole ring of Trp45 in the crystal structures of RNase T_1 (Y45W) complexed with 2'AMP and 2'GMP (Hakoshima et al., 1992). Views of the guanine-recognition sites of these complexes are shown in Figure 4. One of the major differences in these structures is the side-chain conformations of Trp45. The side-chain torsion angles, (χ_1,χ_2), of Trp45 are (65°, –97°) and (–64°,–31°) in the complexes with 2'AMP and 2'GMP, respectively. These changes involve almost the complete flipping of the indole ring. The other conformational changes observed are a significant reorientation of the peptide bond between Asn43 and Asn44 and a major displacement of the side chain of Glu46. These conformational characteristics of the recognition site are in good correspondence with those of the wild-type enzyme. The guanine-recognition site of the mutant RNase T_1 (Y45W) complexed with 2'AMP exhibits strong homology to that of the wild-type enzyme containing a bound vanadate ion (Kostrewa et al., 1989), in which guanine is absent at the recognition site. Furthermore, no essential differences between the binding of 2'GMP to the Y45W mutant and to the wild-type enzyme are detected.

The indole ring of Trp45 in the crystal of the complex with 2'AMP lies over the side chain of Glu46 and exhibits several contacts with oxygen atoms of the carboxyl group. On the other hand, in the complex with 2'GMP, the contacts between the indole ring of Trp45 and the carboxyl oxygen atoms of Glu46 are no longer observed. According to the crystal structure, Glu46 now forms hydrogen bonds with the guanine ring of the bound 2'GMP in the complex. It is, therefore, most likely that Glu46 is the primary quencher of Trp45 in the free mutant enzyme and also in the complex with 2'AMP. The carboxyl group of Glu46 is seen to make two hydrogen bonds with the peptide backbone of Phe100 in the crystal structure of the 2'AMP complex (Figure 4a) which would largely neutralize the negative charge on the group. It has been shown that an uncharged carboxylic acid group is an effective quencher of the tryptophan singlet state and that close proximity of the carboxyl group to the tryptophan is required for quenching (Fasman et al., 1966). Some of the other polar groups, the peptide backbone carbonyl groups of Asn43 and Trp45 and the side-chain carbonyl group of Asn99, are located near the edge of the indole ring in the complex with 2'AMP, but these groups are less effective quenchers.

In the crystal structure of RNase T_1 (Y45W) complexed with 2'GMP, the overlap of the indole ring of Trp45 with the guanine base seems to be more plausible than that between the phenolic ring of Tyr45 and the guanine base in the wild-type enzyme complexed with 2'GMP, although the indole ring is not ideally situated relative to the guanine base to undergo stacking interactions involving π–π orbital overlap. The dihedral angle between the indole and guanine rings is 15° and the closest contacts between their atoms have distances ranging from 3.9 to 4.9 Å, indicating that the overlap is quite loose compared with the ideal stacking interaction distance (3.4 Å). It is well-known that stacking interactions involving tryptophan typically lead to a specific reduction of the value of D as a result of delocalization of the electron density of the triplet state along the stacking axis (Tsao et al., 1989; Co & Maki, 1978). Since no ODMR or phosphorescence can be observed from Trp45 in the free enzyme or in the 2'AMP complex, it is not possible to use the D value as a criterion of stacking interactions with guanine in the 2'GMP complex. The D value, however, is typical of unstacked Trp residues (Deranleau et al., 1978).

The broad phosphorescence and ODMR bandwidths of Trp45 indicate that the side chain is subjected to a less homogeneous microenvironment than that of Trp59. The inhomogeneous broadening of the Trp45 transitions probably is due mainly to its solvent exposure on the side opposite to that facing the guanine ring. Although the indole ring of Trp45 lies over the guanine base relatively loosely, the local environment of Trp45 would become more polarizable on the

average than that of a completely solvent-exposed environment because of the nearby guanine residue, thus producing a phosphorescence origin that is red-shifted relative to a totally solvent-exposed residue (Table I). A relatively polarizable environment also is suggested by the observation of a small value of $D - E$ for Trp45 in the complex with 2′GMP relative to solvent-exposed tryptophan. The magnitude of $D - E$ is known to decrease as the local environment becomes more polarizable (Hershberger et al., 1980; Kwiram, 1982). Comparison with data from somatostatin and *N*-acetyltryptophanamide (Table I) suggests that the overall polarizability of the Trp45 environment in the 2′GMP complex is larger than that of solvent-exposed tryptophan.

The phosphorescence and ODMR measurements reported here are very consistent with the X-ray structures referred to above. The spectroscopic data show that conformational changes of the recognition site occur upon binding of 2′GMP, though no significant changes are detected upon binding of 2′AMP. The conformational changes involve large-scale displacements of the side chains of Trp45 and Glu46. We conclude that the conformational changes that are revealed in the X-ray structures (Figure 4) are largely responsible for the unquenching of Trp45 upon 2′GMP binding and the appearance of a new red-shifted phosphorescence origin. The present data also indicate that the π–π interactions between Trp45 and the guanine base are not typical of aromatic stacking interactions that result from ideal overlap of the rings.

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